Effect of repeated freeze-thaw cycles on NMR measured lipoproteins and metabolites in biofluids

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ABSTRACT

Metabolic profiling of biofluids by Nuclear Magnetic Resonance (NMR) spectroscopy serves as an important tool in disease characterization, and its accuracy largely depends on the quality of samples. We aimed to explore possible effects of repeated freeze-thaw cycles (FTCs) on concentrations of lipoprotein parameters in serum and metabolite concentrations in serum and urine samples. After 1–5 FTCs, serum and urine samples (n=20) were analyzed by NMR spectroscopy and 112 lipoprotein parameters, 20 serum and 35 urine metabolites were quantified by a commercial analytical platform. Principal component analysis showed no systematic changes related to FTCs, and samples from the same donor were closely clustered, showing a higher between-subject variation than within-subject variation. The coefficients of variation were small (medians of 4.3%, 11.0%, and 4.9% for lipoprotein parameters, and serum and urine metabolites, respectively). Minor, but significant accumulated freeze-thaw effects were observed for 32 lipoprotein parameters and one serum metabolite (acetic acid) when comparing FTC1 to further FTCs. Remaining lipoprotein and metabolite concentrations showed no significant change. In conclusion, five FTCs did not significantly alter the concentrations of urine metabolites, and introduced only minor changes to serum lipoprotein parameters and metabolites evaluated by the NMR-based platform.

KEYWORDS: NMR analysis, freeze-thaw cycle, quantification, metabolite, lipoprotein parameter

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INTRODUCTION

Metabolomics has become an important tool in medical research, and involves analytical approaches with the capability to detect a wide range of metabolites in biofluids and tissues.[1] One of the main analytical approaches for metabolic characterization is proton nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy is high-throughput, has high reproducibility, and requires minor sample preparation, thus having a great potential in human population studies.[2, 3]

The quality and reproducibility of NMR analysis can be affected by the sample quality which is influenced by factors resulting from pre-analytical processes, including sample collection, storage, and preparation.[4-6] For example, chemical degradation processes, such as oxidation and decomposition of chemically unstable compounds, can severely influence sample composition. Biobanks are organized to collect, store, and distribute samples of human tissue and biofluids for a variety of clinical research purposes.[4] Blood plasma, serum, and urine are commonly available biofluids in biobanks, and are usually stored in –80 ℃ freezers or in liquid nitrogen tanks (–196 ℃) prior to analysis. Standard operating procedures for pre-analytical handling of blood and urine samples for biobank metabolomic studies have been published.[6, 7] Samples are recommended to be analyzed right after collection or to be stored at –80 ℃ (blood samples) or in liquid nitrogen/liquid nitrogen vapour (urine samples) until further analysis.[7] In practice, however, avoiding repeated thawing and refreezing of aliquots of biobank samples can be challenging.

Based on multivariate analysis of serum NMR spectra, a previous study showed that one FTC caused relatively small differences in the contents of lipids, alanine, glucose, and lactate.[8] Other studies have reported that serum or plasma composition was altered by multiple FTCs.[9, 10] However, these studies included a small number of subjects and did not report quantitative concentrations. While high reproducibility of NMR-measured urine samples from rats were observed for up to eight FTCs,[11] no corresponding data are available from human urine samples.

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Lipoproteins consisting of lipids and apolipoproteins, are important constituents of lipid fractions and their functions are to transfer water-insoluble lipids within bloodstream. They have been identified as the primary drivers of atherosclerotic processes for several decades. However, recent data have suggested that, compared to lipoprotein particle numbers, measuring the total amount of cholesterol in each major lipoprotein fraction has less predictive power for cardiovascular risk in patients with metabolic disorders such as obesity and diabetes mellitus.[12, 13] Improved characterization of lipoprotein subclass composition may help not only to understand the pathophysiology of atherosclerosis and diabetic dyslipidemia, but also to develop and monitor novel diet and drug therapies.[14] In addition, several epidemiological studies have indicated possible correlations between cholesterol and lipoprotein levels and risks for several cancers (such as breast cancer and prostate cancer).[15-17]

Ultracentrifugation-based lipoprotein quantification is time-consuming and labour-intensive, and NMR spectroscopy serves as an alternative rapid method for quantifying lipoproteins from plasma and serum samples. NMR spectroscopy allows for the measurement of lipoprotein subclasses, giving their lipid and apolipoprotein concentrations, as well as particle numbers and sizes.[18-20] A recently released commercial lipoprotein subclass analysis, based on an NMR-based metabolomics platform, can simultaneously quantify 112 lipoprotein parameters and 26 small molecular weight metabolites in blood.[21] In addition, an NMR-based quantification method for 50 urine metabolites has been established. While a previous study explored the effect of multiple FTC on lipoprotein particle numbers,[22] the effects of FTCs on NMR-measured concentrations of lipids and apolipoproteins in lipoprotein subclasses have not yet been reported.

In this study, we investigated the effect of repeated FTCs prior to NMR analysis on concentrations of serum lipoprotein parameters and metabolite concentrations in serum and urine samples. The aim was to gain insight on the extent that FTCs can affect the composition of these biological samples so as to avoid misinterpretation of findings arising from sample handling.

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EXPERIMENTAL SECTION

Sample collection and experimental design

Non-fasting serum and spot-urine samples were obtained from two sets of twenty anonymized healthy female and male adult donors. Blood samples were collected into serum tubes with no additives from the Blood bank, St. Olavs University Hospital, and left to coagulate for approximately 1 hour, before centrifugation (3000 RPM, 10 minutes). Urine samples were kept refrigerated (4 ℃) after collection, and were transferred to aliquots within 3 hours. Each serum and urine sample was divided into five aliquots on ice and stored at –80 ℃. The five aliquots of each sample were subjected to 1–5 FTCs before NMR analyses. For each FTC, serum and urine samples were thawed at room temperature for approximately 1 and 2 hours, respectively, and refrozen at –80 ℃ for approximately 24 hours. After the FTCs, the samples were stored in –80 ℃ until NMR analysis. The study was approved by the data protection officer at The Norwegian University of Science and Technology (NTNU). According to communication with the Regional Committee for Medical and Health Research Ethics in Central Norway, this quality control study using completely anonymized samples from healthy volunteers could be performed without formal ethical approval.

NMR analysis and data pre-processing

After thawing at room temperature, 150 µl of serum was mixed with 150 µl of buffer (D2O with 0.075 mM Na2HPO4, 5 mM NaN3, 3.5 mM TSP, pH 7.4). Thawed urine samples were centrifuged at 12121 g at 4 ℃ for 5 minutes, and 540 µL of supernatant was mixed with 60 µL of buffer (pH 7.4 1.5 mM KH2PO4 in D2O, 0.1 % TSP). Serum and urine mixtures were transferred to 3 mm and 5 mm NMR tubes, respectively. To assess the reproducibility of the NMR method and to compare with the variability of the FTC samples, four sets of quality control (QC) samples were prepared from pooled serum or urine samples: serum QC set 1 and set 2 (QC1 and QC2), and urine QC1 and QC2. Each set of QC consisted of five samples. NMR analysis was carried out on a Bruker Avance III Ultrashield Plus

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600 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), equipped with a 5 mm QCI Cryoprobe. Sample handling and data acquisition was automatically performed using SampleJet sample changer and Icon-NMR on Topspin 3.5 (Bruker BioSpin). NMR spectra were recorded using a 1D NOESY pulse sequence (noesygppr1d), with irradiation (25 Hz) on the water resonance during relaxation delay (4 s) and mixing time (10 ms). The urine spectra were recorded at 300 K, with 64k data points and 20 ppm spectral width. The serum spectra were recorded at 310 K using 96k data points and 30 ppm spectral width. For both biofluids, 32 scans were recorded and the FIDs were Fourier-transformed after 0.3 Hz line broadening to 128k real data points. For urine, 2D JRES spectra (jresgpprqf) were also recorded, using 2 scans, water presaturation (25 Hz) during relaxation delay (2 s), 8k direct and 40 indirect data points with 10026.7 Hz and 78 Hz spectral width respectively and 12820.51 μs delay incrementation.

Lipoprotein parameter analysis and metabolite quantification

Lipoprotein parameter analysis was automatically performed using the commercial Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISATM) method from Bruker BioSpin.[21] This method provides the concentrations of lipids (cholesterol (CH), free cholesterol (FC), triglycerides (TG), and phospholipids (PL)) in serum, and in four main lipoprotein classes: very low-, intermediate-, low- and high density lipoproteins (VLDL, IDL, LDL, and HDL, respectively) as well as 15 subclasses (VLDL 1–5, LDL 1–6, and HDL 1–4). Simultaneously, it quantifies the concentrations of apolipoproteins (Apo-A1, Apo-A2, and Apo-B) in serum, two main classes (HDL and LDL) and 10 subclasses (HDL 1–4 and LDL 1–6). In addition, the model gives 12 calculated parameters, including ratios of LDL-CH/HDL-CH and Apo-B/Apo-A1, and 10 particle numbers (particle numbers of total serum, VLDL, IDL, LDL, and LDL 1–6). In total, this yields 112 quantitative lipoprotein parameters (Table S1). The density ranges of different lipoprotein subclasses are listed in Table S1.

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Twenty-six (26) serum and fifty (50) urine metabolite concentrations were automatically quantified by use of Bruker B.I. Quant-PSTM and Bruker B.I.Quant-URTM methods, respectively, based on algorithms developed for fitting predefined proton signals.[21] By automatic quantification applying LODs as threshold for quantification, concentrations were not reported for several of the metabolites. We thus disregarded LODs in the quantification of these samples. Metabolites with concentrations > 0 for more than 30% of serum or urine samples were classified as quantifiable, and were included in the analysis.

Multivariate modelling and statistical analysis

The effect of repeated FTCs on the serum and urine samples was evaluated using both multivariate and univariate statistical analyses. Zero values were replaced by half of the lowest detected value of the corresponding metabolite or lipoprotein. Principal component analysis (PCA)[23] was carried out to visually assess variation in the metabolic profiles within and between donors. PCA is an unsupervised dimension-reduction technique, which makes is possible to visualize the majority of the variance in the data, projected onto a lower-dimensional space. Each point on the PCA scores plot represents one sample. PCA was performed in Matlab R2017b[24] using the PLS-toolbox version 8.6.2.[25]

Coefficients of variation (CV) and intra-class correlations (ICC) were calculated to evaluate the reproducibility of quantified lipoprotein parameters and metabolites, given repeated FTCs.[26] CVs were calculated for each lipoprotein/metabolite parameter, across all FTCs of a donor separately, to determine their relative extent of variation (within donor variation). ICCs were calculated for each lipoprotein/metabolite parameter, across all FTCs and donors to evaluate the reproducibility. For each lipoprotein parameter and metabolite separately, Wilcoxon signed-rank tests were used to test if the lipoprotein or metabolite levels between two consecutive FTCs were significantly different. To test for accumulated effects of FTCs, Wilcoxon signed-rank tests were used to compare lipoprotein and metabolite levels of FTC2–5 samples with FTC1. P-values were adjusted using the Benjamini-Hochberg

procedure[27] with significance considered for corrected p-values ≤ 0.05. Percentage changes were calculated for individual lipoprotein parameters and metabolites to obtain a visual representation of the changes in their levels between the FTCs. All statistical analyses were carried out in R.[28] The nmle package[29] was used for calculating the ICCs.

RESULTS

Concentrations of lipoprotein parameters and metabolites in serum and urine samples

Figure 1 shows the five FTC serum and urine spectra obtained from two representative donors. Variations within the same donor among FTCs are clearly much lower compared to the variations across different donors. We successfully quantified 112 lipoprotein parameters and 20 serum metabolites from the serum NMR spectra (Table S1 and S2), and 35 urine metabolites from the urine NMR spectra (Table S3).

Effect of freeze-thaw cycles on lipoprotein parameter concentrations

The impact of repeated FTCs on concentrations of lipoprotein parameters in serum samples was visualized by PCA (**Figure 2**B and **2**C). No systematic variation due to repeated FTCs was apparent. The score plot (**Figure 2**B) shows that most of the samples with different FTCs were well grouped according to donors, and the variations within five FTCs were lower than those between different donors. From FTC4 to FTC5, samples from two donors (number 13 and 16) had larger spread in the PCA scores than the QCs. However, these changes were not in the same direction, indicating that these variations are not caused by FTCs, but rather represent variations from sample preparation.

As shown in **Figure 2**A, CVs of lipoprotein parameters in samples thawed and refrozen 1–5 times were comparable to those of the QCs. Median CV for the lipoprotein parameters were between 1.1-16.8%, with a median of 4.3% across all parameters. Only 4/112 lipoprotein parameters had median CVs > 15% (IDCH, IDFC, V2FC, and V4FC). The ranges of CVs for quality control sets QC1 and QC2 were 0.0-

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82.5% (median: 5.0%) and 0.6-27.9% (median: 6.0%), respectively. ICCs of FTC samples are presented in Figure S1A. ICC was larger than 0.8 for most of the parameters (94 of 105), giving a mean ICC of 0.91 across all lipoprotein parameters.

Wilcoxon signed-rank tests were used to test whether the differences in concentrations between each pair of subsequent FTCs were significant. Most of the lipoprotein parameters did not differ significantly between any of the consecutive FTCs (Table S4). Five VLDL-related lipoprotein parameters had significantly different concentrations between FTC3 and FTC4: VLCH, VLFC, V3FC, V4CH and V1CH (adjusted p-values = 0.015, 0.015, 0.038, 0.031, and 0.003, respectively) (exemplified by V4CH in **Figure 2**D). However, relative changes in median concentrations for these five lipoprotein parameters were low (range: 2.93%–6.88% increase in concentration).

Although few significant changes in the lipoprotein levels were found between consecutive FTCs, some significant accumulated effects of FTCs were observed (Table S4). This is exemplified by V4FC in **Figure 2**D. Thirty-two lipoprotein parameters showed accumulated effects when comparing FTC1 to FTC5, of which significant accumulated effects were detectable from FTC3 for 22 parameters, from FTC4 for 8 parameters and from FTC5 for 2 parameters. This resulted in decreased concentrations for 10 parameters and increased concentrations for 22 parameters when compared to levels of FTC1 samples.

Effect of freeze-thaw cycles on metabolite concentrations in serum samples

No systematic effects of 1–5 FTCs on serum metabolite concentrations were observed as visualized by PCA (**Figure 3**A). With the exception of samples from donor 13 and 16, all samples were clustered according to the corresponding donors and the variances of samples in each donor were lower than or of the same order of magnitude than those across different donors. As shown in **Figure 3**B, median CVs of all the 20 metabolites were between 1.7-67.2%, which a median of 11.0% across all metabolites. 13/20 metabolites had median CV < 15%, and 18/20 had median CV < 25%. Among all the 20 quantified

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metabolites, trimethylamine N-oxide had the highest CV (median 67.2%). The ranges of CVs for QC1 and QC2 samples were 3.2-51.9% (median 10.4%) and 4.2-79.5% (median 11.1%). ICCs of all serum metabolites were between 0.3 and 0.9, with a mean of 0.7 (Figure S1B). Results of PCA and ICC together show a small overall variation in the data, indicating that this cohort of healthy donors represents a homogeneous group.

Wilcoxon singed-rank tests showed that the concentrations of the 20 serum metabolites did not significantly differ between two consecutive FTCs (Table S5). The concentration of serum acetic acid, was significantly increased between FTC1 and FTC5 (adjusted p value = 0.01), while no significant accumulated effects of FTCs were found for other serum metabolites (Table S5).

Effect of freeze-thaw cycles on metabolite concentrations in urine samples

We found that 1–5 FTCs had no systematic effect on the metabolite concentrations in urine samples, as shown by PCA (**Figure 3**C). Samples from the same donors were clustered and within donor variations were lower compared to between donor variations. **Figure 3**D demonstrates that the median CVs were between 1.3-76.1%, with a median of 4.9% across all metabolites. 28/35 metabolites had median CV < 15%. The CVs of creatine (median 76.1%) were higher compared to other metabolites. CVs of the two sets of quality control samples varied from 0.7-223.6% (median: 3.5%) and from 0.4-157.6% (median: 2.4%), respectively. ICCs of all urine metabolites were high (ICC > 0.9 for 33 of 35 metabolites) (Figure S1C).

Based on Wilcoxon signed-ranked tests, no significant differences between two consecutive FTCs on the metabolite concentrations were found (Table S6). No accumulated effects of FTCs on the metabolite levels were found (Table S6).

DISCUSSION

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In this study we examined the impact of repeated FTCs on the absolute concentrations of lipoprotein parameters and metabolites determined by NMR spectroscopy of human biofluids. We found that up to five repeated FTCs induced minimal changes in the measured concentrations of serum and urine metabolites and lipoproteins.

NMR-based quantitative analysis has been applied widely to evaluate the association of lipoproteins with cardiovascular diseases, diabetic dyslipidemia and cancer.[20, 30, 31] To the best of our knowledge, we are the first to report the effects of repeated FTCs on NMR-measured concentrations of lipids and apolipoproteins in lipoprotein subclasses. Using a commercial analytical platform for automatic quantification of lipoproteins and their subclasses, we found that systematic changes of lipoprotein parameter concentrations did not appear for up to five repeated FTCs. While the concentrations of five VLDL-derived parameters were significantly changed between FTC 3 and 4, the relative changes in concentration values were low. The remaining 107 lipoprotein parameters did not change between consecutive FTCs. However, minor, significant accumulated effects of FTCs were observed for 32 lipoprotein parameters, occurring after either FTC3, FTC4 or FTC5. The median CV across all the parameters was 4.3%, showing a high reproducibility for most lipoprotein parameters and little variation of samples with multiple FTCs. Median CV for total cholesterol (TPCH), LDL cholesterol (LDCH) and HDL cholesterol (HDCH) was 2.0%, 3.0%, 2.1%, respectively. This is in the range of the recommendations of the National Cholesterol Education Program (NCEP) Laboratory Standardization Panel (CV <3.0% for cholesterol, CV <4.0% for LDL cholesterol, and CV <5.0% for HDL cholesterol, respectively).[32-35]

Our findings are consistent with previous studies on clinically measured cholesterol levels, showing that FTCs had either no significant effects, or only minor effects (less than day to day variations) on serum or plasma LDL and HDL concentrations.[22, 36, 37] Conversely, compared to fresh samples, freezing prior to lipoprotein fractionation via density gradient ultracentrifugation was shown to cause large variations (up to 37%) in concentrations of HDL and LDL cholesterol, and VLDL free fatty acids in serum.[38] This

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is in line with the certification protocols for determining total cholesterol, HDL and LDL cholesterol, which recommend to use fresh samples for the reference method, and to use frozen samples with caution.[32-34] However, in large cohorts such as biobank studies it is not feasible to analyze fresh serum, thus our study is limited to the comparison of lipoprotein concentrations in frozen samples with different FTCs. Moreover, in our study, lipoprotein particle numbers did not significantly change (LDL, and subclasses of LDL-2-6) or slightly increased (VLDL, IDL and LDL-1) after multiple FTCs. Similarly, a recent study demonstrated that three or more FTCs, when thawing plasma samples in a cold room, caused significant, but relatively small, changes in lipoprotein sizing (a shift of HDL particle size from large to small, decrease in large LDL and increase in IDL), and no changes in the proteome.[22]

No significant changes in urine metabolite concentrations were observed with up to five FTCs, while only acetic acid changed significantly within small-molecule serum metabolites. Compared to urine metabolites, the spread of donors in the PCA scores plot was lower for serum metabolites, possibly reflecting high homogeneity of this cohort of healthy donors. The coefficients of variation for some of the serum metabolites were high. These high CVs could result from mean concentration values close to zero, in which case CV is not an appropriate measure of reproducibility. However, the range of ICCs of serum metabolites was also high (from 0.3 to 0.9), eight of which were below 0.7. This indicates a lower reproducibility for some of the serum metabolites, such as trimethylamine-N-oxide and acetoacetic acid.

Unlike our findings, previous NMR-based studies have reported changes in plasma composition or relative quantitative levels of metabolites by either two or multiple FTCs.[8-10] Based on three subjects, Pinto et al.[9] reported that the effects of repeated freezing and thawing on plasma metabolites appeared after four and five cycles, but not within the three first cycles. Upon five consecutive FTCs, alterations consisted of decreased lipids and acetone, and increased choline phospholipid, alanine, glucose, and pyruvate. Similarly, compared to one FTC, five or ten FTCs were shown to have a visible impact on the metabolic profile of serum samples, and the levels of several small molecular metabolites were decreased, including choline, glycerol, methanol, ethanol, and proline.[10] Four of these mentioned Values in red font are corrected in the proof version of the article.

metabolites were analyzed in our study. Among them, acetone and alanine displayed non-significant changes across five FTCs, and pyruvate and ethanol were undetectable. Moreover, Teahan et al.[8] detected minor spectral differences in samples with only one additional FTC. However, their freeze-thaw conditions are different compared to ours and the others: they thawed and re-froze sample mixtures consisting of fresh sera and saline in NMR tubes at –40 ℃ prior to NMR analysis, while we subjected serum samples more in accordance with a biobank setting. It cannot be ruled out that dilution with saline might change the stability of serum. To assess possible differences in the stability of serum and plasma metabolites, studies should be performed to compare the effects of FTCs in serum and plasma samples under the same experimental conditions

The effect of FTCs has also been evaluated by mass spectrometry-based analysis, reporting small changes in metabolic profiles in blood samples. Up to four FTCs only affected the EDTA-plasma metabolome slightly, but increased the individual variability.[39] By use of a targeted LC-MS approach, Breier et al. discovered that, compared to those in fresh-frozen serum samples, all 159 investigated metabolites, except methionine sulfoxide, maintained stable concentrations in samples with two FTCs.[40] After three FTCs, eleven metabolites had significantly decreased concentrations, among which the amino acids isoleucine, tryptophan, and valine. Similarly, Anton et al. found quite stable metabolite concentrations with up to four FTCs compared to fresh-frozen samples.[41] Among a total of 163 measured metabolites, only five amino acids including glycine, methionine, phenylalanine, tryptophan, and tyrosine had slightly, but not significantly increased concentrations with increased numbers of FTCs. Most metabolite concentrations were stable in serum samples subjected to two FTCs.

We found that urine metabolite concentrations did not significantly differ between consecutive FTCs, and that no systematic changes appeared after five FTCs. Our findings are consistent with data reported from NMR analysis of rat urine samples, which showed that up to five FTCs had no influence on single metabolite concentrations or metabolomics classification approach.[11] In contrast, data from metabolic profiling of human urine samples measured by a LC-MS/MS-method showed that three FTCs, but not

one or two cycles, significantly increased concentrations of two metabolites (propionylcarnitine and hexose), when compared to samples immediately frozen at –80 ℃.[42] However, these two metabolites were not quantified in our study.

CONCLUSIONS

In conclusion, evaluated by the use of a commercially available NMR-based platform, quantification of lipoprotein parameters and metabolites were reproducible even with five repeated FTCs. No significant effects on concentrations of small-molecule metabolites in urine samples were observed. Minor accumulated changes were observed in concentrations of 32/112 lipoprotein parameters and 1/20 metabolites in serum samples. Although significant, the variation was still within the recommended NCEP guidelines for total cholesterol, LDL cholesterol and HDL cholesterol. Taken together, our results show that using this platform clearly allows for utilizing samples with different FTCs for quantification of metabolites and lipoprotein parameters in the same study.

ASSOCIATED CONTENT

Supporting Information.

The supporting information is available free of charge.

Figure S1, Intraclass correlation coefficients for lipoproteins (A), serum (B) and urine (C) metabolites. Table S1, List of measured lipoprotein parameters. Table S2, List of measured serum metabolites. Table S3, List of measured urine metabolites. Table S4, Effects of FTCs on lipoprotein parameter concentrations. Table S5, Effects of FTCs on serum metabolite concentrations. Table S6, Effects of FTCs on urine metabolite concentrations) (pdf)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡ F.W. and J.D. contributed equally as first authors, and T.F.B. and G.F.G. contributed equally as last authors to this work.

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Notes

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ABBREVIATIONS

NMR, nuclear magnetic resonance; FTC, freeze-thaw cycle; QC, quality control; PCA, principal component analysis, CV, coefficients of variation; ICC, intra-class correlations.

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Figures and Legends

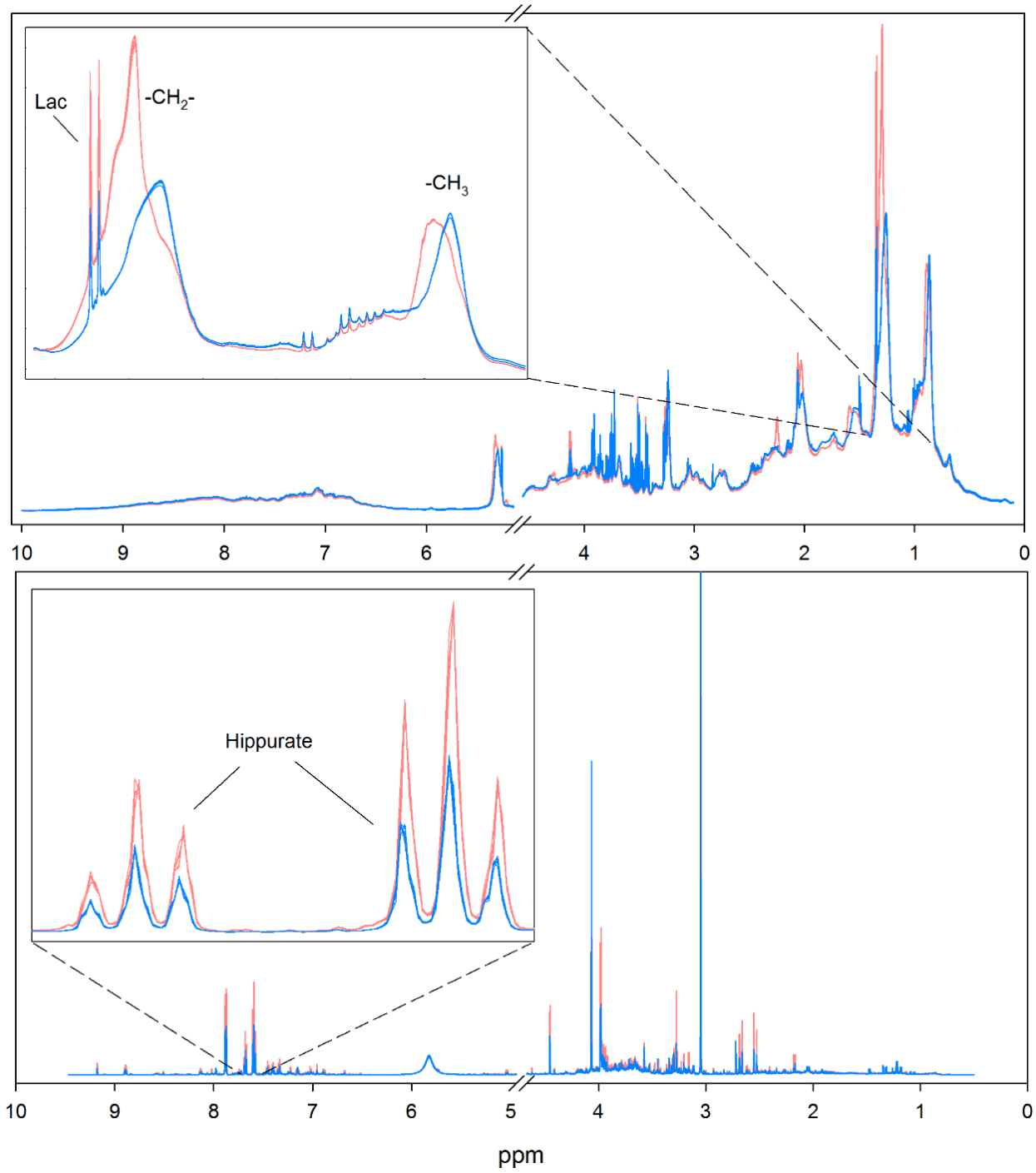

Figure 1. NMR-spectra of serum and urine samples. The figure shows serum (upper panel) and urine (lower panel) spectra from two representative donors, colored in orange and blue. All five spectra, one from each FTC are plotted. For the serum spectra, the area in focus shows part of the spectral region where the lipoprotein signals appear. Spectral position of the -CH2- and -CH3 signals reflect the lipoprotein particle size.[43] The area in focus for the urine spectra shows signals from hippurate as an example.

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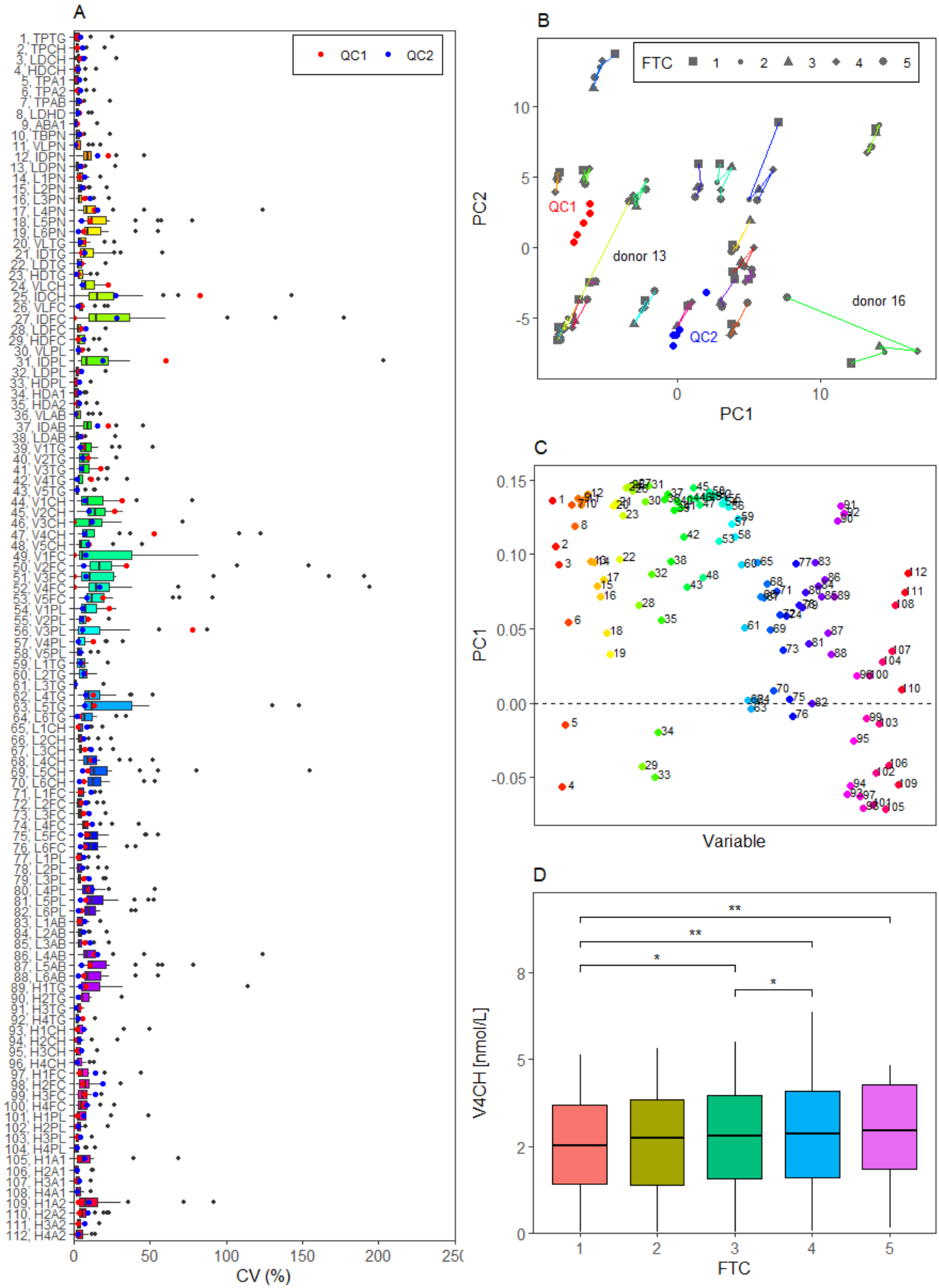

Figure 2. Effects of five FTCs on concentrations of 112 lipoprotein parameters in serum samples. A, Box-plots of coefficients of variation for all lipoprotein parameters. Median values, interquartile

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range and outliers are presented. Values from quality control samples (QC1 (red) and QC2 (blue)) are shown for comparison. • represents outliers. B, PCA scores plot. Samples from the same donor are connected by lines of different colors. C, Loadings of PC1 from PCA. Numbers represent each lipoprotein parameter, as shown in A. Colors for loadings are similar as those for corresponding coefficients of variation in A. D, V4CH concentrations of samples with different numbers of FTCs. Significance tested by Wilcoxon signed-rank tests. *: adjusted p-value ≤ 0.05; **: adjusted p-value ≤ 0.01.

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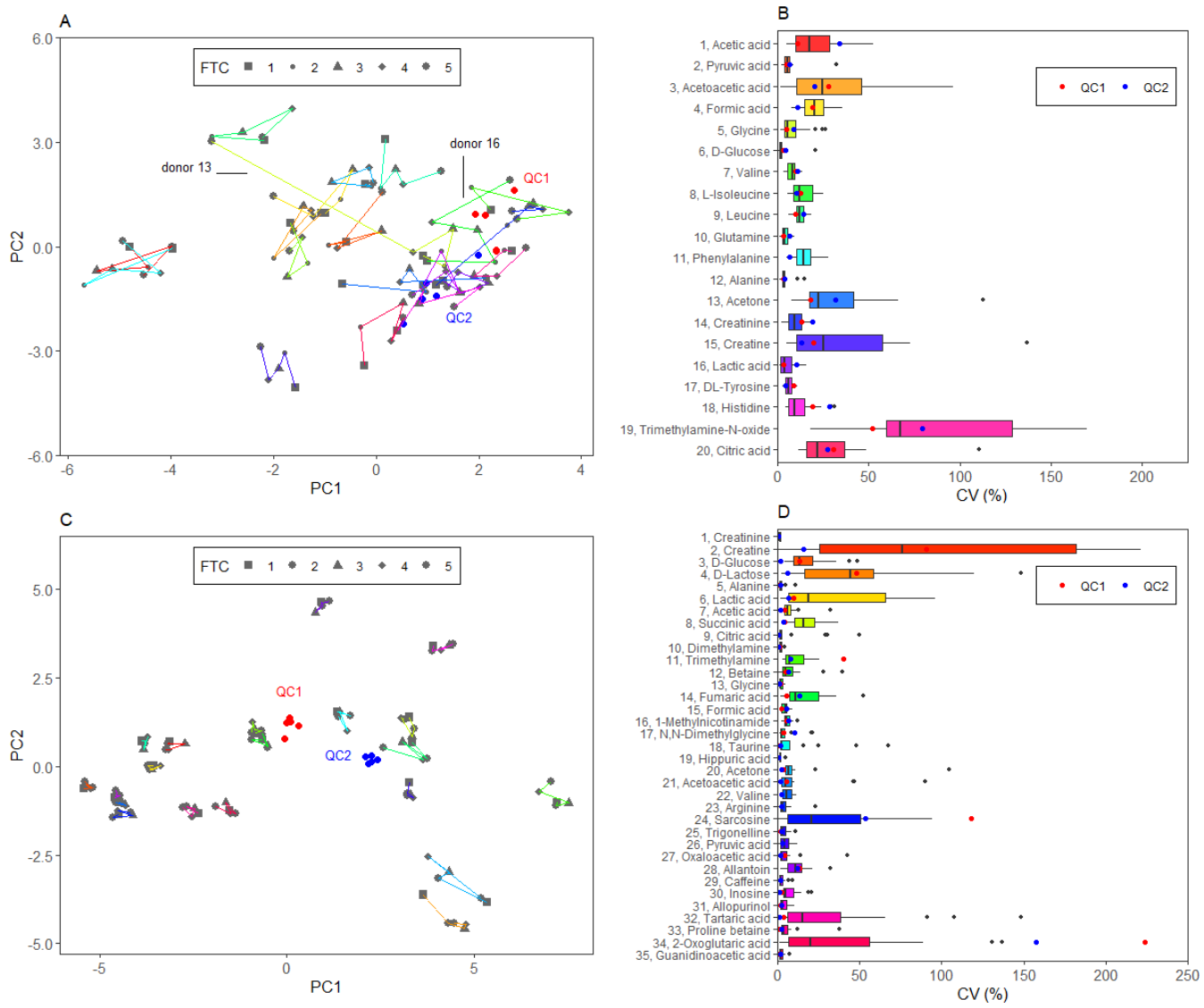

Figure 3. Effects of five FTCs on serum and urine metabolite concentrations. A,C) PCA scores plots for serum (A) and urine (C) metabolites. Samples from the same donor are connected by lines of different colors. Values from quality control samples (QC1 (red) and QC2 (blue)) are shown for comparison. B,D) Box-plots of the coefficients of variation for serum (B) and urine (D) metabolites. Median values, interquartile ranges, and outliers are presented.

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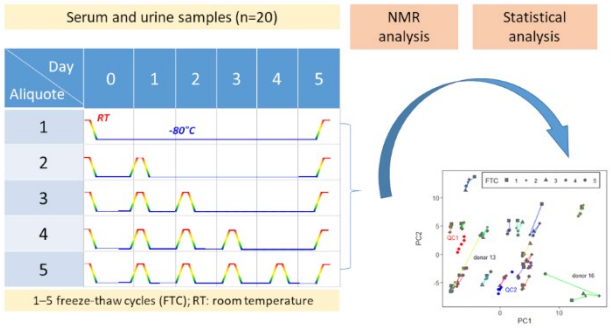
Table of Contents Artwork



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