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Sodium fluoride preserves blood metabolite integrity for biomarker discovery in large-scale, multi-site metabolomics investigations†

Wenzheng Xiong, [a,b] Daniel C. Anthony,[b] Suzie Anthony,[c] Thi Bao Tien Ho, [b] Edouard Louis,[d] Jack Satsangi[e] and Daniel E. Radford-Smith *[a,b]

Background: Metabolite profiling of blood by nuclear magnetic resonance (NMR) is invaluable to clinical biomarker discovery. To ensure robustness, biomarkers require validation in large cohorts and across multiple centres. However, collection procedures are known to impact on the stability of biofluids that may, in turn, degrade biomarker signals. We trialled three blood collection tubes with the aim of solving technical challenges due to preanalytical variation in blood metabolite levels that are common in cohort studies. *Methods*: We first investigated global NMR-based metabolite variability between biobanks, including the large-scale UK Biobank and TwinsUK biobank of the general UK population, and more targeted biobanks derived from multicentre clinical trials relating to inflammatory bowel disease. We then compared the blood metabolome of 12 healthy adult volunteers when collected into either sodium fluoride/potassium oxalate, lithium heparin, or serum blood tubes using different pre-processing parameters. *Results*: Preanalytical variation in the method of blood collection strongly influences metabolite composition within and between biobanks. This variability can largely be attributed to glucose and lactate. In the healthy control cohort, the fluoride oxalate collection tube prevented fluctuation in glucose and lactate levels for 24 hours at either 4 °C or room temperature (20 °C). *Conclusions*: Blood collection into a fluoride oxalate collection tube appears to preserve the blood metabolome with delayed processing up to 24 hours at 4 °C. This method may be considered as an alternative when rapid processing is not feasible.

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Introduction

Metabolite profiling of serum and plasma is emerging as an invaluable tool spanning multiple domains of medical research.[1] Nuclear magnetic resonance (NMR)-based metabolomics techniques involve the simultaneous profiling of many metabolites (small molecular intermediates) in a biofluid or other medium.[2] Inexpensive, high-throughput, and highly reproducible, global metabolite quantification by NMR is now being employed at the frontier of medicine, ranging from population-wide health profiling[3,4] to predicting diagnosis,[5] progression,[6] and response to treatment[7] in a spectrum of diseases.

Clinical biomarkers require validation in large cohorts across multiple centres.[8] NMR-based metabolomics requires minimal sample preparation compared to other metabolomics techniques (*i.e.* mass spectrometry methods).[9] Nevertheless, centrifugation speed and time,[10] incubation temperature,[11–13] storage time at −20 °C and −80 °C,[13,14] the type of collection tube,[15,16] and the number of freeze–thaw cycles[11,14,17,18] are just some of the pre-analytical variables known to strongly influence metabolite composition in blood. While many of these variables may be able to be well-controlled within a given study, the time between blood collection and erythrocyte separation by centrifugation and the time to sample storage at −80 °C present unique challenges due to the pragmatic requirements of large-scale, multi-centre clinical trials from which clinical biomarkers may be robustly identified.[13,19,20] These two factors have been shown to drastically influence the relative abundance of metabolites involved in glycolysis.[10–12,14,18,21–26] To date, it has not been investigated

[a]*Department of Chemistry, University of Oxford, Oxford, UK. E-mail: daniel.radford-smith@some.ox.ac.uk*
[b]*Department of Pharmacology, Medical Sciences Division, University of Oxford, Oxford, UK*
[c]*Department of Radiology, Oxford University Hospitals NHS Foundation Trust, Oxford, UK*
[d]*Department of Gastroenterology, University Hospital CHU of Liège, Liège, Belgium*
[e]*Translational Gastroenterology Unit, Nuffield Department of Experimental Medicine, University of Oxford, Oxford, UK*

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whether the type of blood collection tube used affects metabolite integrity due to a delay in centrifugation and/or sample measurement. Moreover, conflicting evidence exists as to whether other metabolites not directly involved in glycolysis, such as lipoproteins, are affected by time to centrifugation.[14,21] In this study we aimed to systematically evaluate and compare the relative efficacy of sodium fluoride/potassium oxalate, lithium heparin, and additive-free serum blood tubes in preserving metabolite integrity for up to 24-hours before centrifugation, and up to 48-hours before sample measurement after NMR sample preparation. We expected that sodium fluoride/potassium oxalate blood tubes, which inhibit glycolysis, preserve the metabolic state at the time of blood collection more effectively than the widely used lithium heparin or serum blood vacutainers.

Ideally, standard operating procedures (SOPs) are, prospectively, put in place in advance of longitudinal, multicentre research projects to reduce pre-analytical variability in metabolite levels within and between biobanks. In practice, this may be difficult to achieve depending on the scale and resources of individual collection sites. Previous studies have demonstrated conflicting efficacy of keeping blood tubes refrigerated at 4 °C prior to centrifugation, aliquoting, and freezing on the variability in metabolite levels.[13,18,26] Moreover, a secure cold chain may not be feasible in all sites, and the level of compliance to SOPs is unclear and is often left unreported. Most importantly, all previous studies addressing these pre-analytical variables treated all samples identically when varying time to erythrocyte separation and time at room temperature (RT). Therefore, it is largely unsurprising that, in at least one analytical dimension, inter-individual differences are maintained. In practice, this identical treatment of individual differences does not occur; samples may be processed at any time between, for example, 0 and upwards of 24 hours.

Guidelines on how to prepare samples and observations on the effects of delayed erythrocyte separation are insufficient to advance methods of reducing biomarker variability for NMR. Similarly, *z*-scoring of metabolite concentrations to facilitate comparisons across cohorts[3,27] impedes the discovery of healthy and disease-associated metabolite ranges that may be clinically relevant. Practical solutions to stably preserve blood metabolite levels are required, and clear reporting guidelines are required to explain potential non-biological between-sample variability. In this study, we first investigate global NMR-based metabolite variability between biobanks, including the large-scale UK Biobank and TwinsUK biobank of the general UK population, and more targeted biobanks derived from multicentre clinical trials relating to IBD. We then compared the blood metabolome of 12 healthy adult volunteers when collected into either sodium fluoride/potassium oxalate, lithium heparin, or serum blood tubes using different pre-processing parameters, with the aim of determining the optimal tube type for preserving an individual's blood metabolomic fingerprint with significantly delayed sample processing of up to 24 hours.

Methods

Cohorts

UK Biobank. The UK Biobank is a general population cohort recruited from 22 assessment centres across England, Scotland, and Wales between 2006 and 2010. It comprises 502 411 individuals of middle and old age (mean 56.5 years, range 37–73 years). Written, informed consent was provided by all participants. The UK Biobank has generic ethical approval from the Northwest Multi-centre Research Ethics Committee (ref. 11/NW/03820). The current study is registered under the approved research ID 95409. At the time of this study, NMR metabolomic data was available on ~120 000 participants. Individuals were typically non-fasting prior to blood sampling (median fasting time 3 hours, standard deviation 2.4 hours). Whole blood was collected into spray-coated K_2EDTA tubes and immediately centrifuged at 2500*g* for 10 minutes at 4 °C. Plasma was not aliquoted. Rather, tubes were transported to a central storage facility at 4 °C for aliquoting and archiving at −80 °C (mean time from venepuncture to freezing 24 hours, standard deviation 2.5 hours).[28] Acquisition of NMR metabolite data was performed by Nightingale Health Ltd and is described in detail elsewhere.[4]

TwinsUK. TwinsUK is a volunteer twins cohort in the United Kingdom.[29] Generic ethic approval was obtained from Guys & St Thomas' Trust ethics committee. At the time of this study, NMR metabolomic data was available on ~2000 female individuals across up to three timepoints (total samples 4830) collected between 1996 and 2014. The mean age at blood sampling was 59.4 years (range 34–88 years). Samples were collected after a minimum fast of 6 hours into serum separator blood tubes (BDVacutainer®SST™). The tubes were then allowed to rest after 3× inversions for 40 minutes at 4 °C. Samples were centrifuged for 10 minutes at 1439*g*. Serum was collected immediately and stored at −45 °C.[30] Acquisition of NMR metabolite data was performed by Nightingale Health Ltd and is described elsewhere.[27]

STORI. The STORI trial (2006–2009) investigated the risk of relapse in Crohn's disease after cessation of infliximab in patients who had achieved prolonged remission on combination therapy: infliximab alongside antimetabolites.[31] We included in our study patients with a baseline serum aliquot available for analysis (n = 97).

SPARE. The SPARE trial (2015–2021) sought to evaluate three treatment approaches for Crohn's disease patients in steroid-free remission due to combined infliximab and immunosuppressant therapy: maintaining the combination, continuing only the immunosuppressant, or continuing only infliximab.[32] We include here a subgroup of patients from the SPARE trial who had been randomised to infliximab withdrawal and had a baseline serum aliquot available for analysis (n = 63).

In both STORI and SPARE, biomarkers associated with relapse was a pre-defined secondary endpoint. The standard operating procedure for serum collection and processing is provided in the ESI.† Briefly, blood was collected into BD Vacutainer SST II Advance Tubes (BD 367958), allowed to clot,

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and centrifuged at room temperature for 10 minutes at 3000*g*. The target for clotting time at room temperature was between 0.5 and 2 h, and the target time-to-freezing of serum aliquots was within 4 hours of the blood draw. Serum aliquots were then stored at −80 °C. All patients provided informed consent and ethical approval was obtained. All patients had been treated with a combination therapy of infliximab (IFX) and anti-metabolite >8 months and had been in sustained steroid-free remission >6 months. Patients were not fasted prior to blood collection.

Healthy volunteers and controlled variation of pre-analytical parameters. 12 healthy volunteers were recruited from Oxford, UK (mean age 30.3 years, range 23–53 years). Blood was collected under informed written consent. Local ethical approval was obtained from the University of Oxford Medical Sciences Interdivisional Research Ethics Committee.

Blood was collected into BD Vacutainer blood tubes® under the conditions described in Table 1. Vacutainer tubes were centrifuged initially at 300*g* for 10 minutes at 4 °C. After the supernatant (either serum or plasma) was carefully collected into a new tube, the sample was again centrifuged at 5000*g* for 10 minutes at 4 °C and the supernatant collected into a new tube and stored at −80 °C prior to sample preparation.

NMR spectroscopy

NMR spectroscopy and global metabolite quantification from serum and plasma was performed in-house for the SPARE, STORI, and healthy volunteer cohorts. On the day of NMR spectroscopy, serum or plasma samples were thawed at room temperature, mixed by pipetting, and combined with 75 mM sodium phosphate buffer prepared in D_2O (pH 7.4) as a 1 : 5 ratio of serum to buffer (total volume 600 μL) in a 5 mm borosilicate glass NMR tube (Norell 502-7).

^{1}H NMR spectra were acquired at 298 K using a 700 MHz Bruker AVIII spectrometer operating at 16.4 T equipped with a ^{1}H [^{13}C/^{15}N] TCI cryoprobe (Department of Chemistry, University of Oxford). 1D NOESY and CPMG experiments were conducted on each sample, and CPMG spectra were used for downstream analysis. Pooled plasma samples were spread throughout the run of the SPARE and STORI samples to monitor technical variation.

NMR Spectra were phased, baseline corrected (using a fifth-degree polynomial), and referenced to the lactate-CH_3 doublet resonance at δ = 1.33 ppm in Topspin 4.1.4 (Bruker). Spectra were exported to ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc.) for data-preprocessing. The NMR spectra were segmented into 100 integral regions using a manual binning approach, with exclusion of noise and water signals. The integration of metabolite peaks was performed judiciously to minimize overlapping and aligned with literature assignments. Resonances were assigned to metabolites using a combination of literature values and 2D spectra of human plasma and serum, leading to the confident identification of 39 metabolites. Spectral regions with overlapped metabolites were annotated in the assignment using a '/' and '[ ]' notation. The absolute integral values were subject to the main statistical analysis unless stated otherwise. Additional statistical analyses using probabilistic quotient normalisation (PQN)[33] and sum normalisation were included in the ESI.† In the healthy control cohort, lactate and glucose levels were also calculated based on the absolute mmol concentration using a standard curve.

Statistical analysis

Statistical analysis was performed in R 4.1.2 and GraphPad Prism 9. After pareto scaling of the absolute integral values, multivariate principal component analysis (PCA) was performed using the ropls package to visualise and compare individual metabolite profiles in an unsupervised fashion.[34]

Post-hoc analysis of TwinsUK, STORI, and SPARE NMR data was limited to univariate *t*-testing of glucose and lactate metabolite levels, based on inspection of the corresponding PCA loadings plots. UK Biobank NMR metabolite data were further correlated against time of day of blood collection, fasting time, and delay in sample measurement using Pearson's correlation coefficient. In the healthy control cohort, individual metabolites (39 identified metabolites) were subject to repeated measures two-way ANOVA with Šidák *post hoc* test,[35] and Benjamini–Hochberg method was used for p value adjustment to control the false discovery rate across all metabolites. Adjusted two-tailed p values (q values) ≤ 0.05 were considered statistically significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Table 1 Summary of pre-analytical parameters in the healthy volunteer cohort. n = 12 per condition

Tube	Time to centrifugation (hours)	Delay between sample preparation and measurement (hours)
Serum (Red, BD 367837)	0.5 RT	0 (RT), 12 (4 °C), 24 (4 °C), 48 (4 °C)
	24 RT	0 (RT)
	24 4 °C	0 (RT)
Lithium Heparin Plasma (Green, BD 367885)	0.5 RT	0 (RT), 12 (4 °C), 24 (4 °C), 48 (4 °C)
	24 RT	0 (RT)
	24 4 °C	0 (RT)
Fluoride/Oxalate Plasma (Grey, BD 368921)	0.5 RT	0 (RT), 12 (4 °C), 24 (4 °C), 48 (4 °C)
	24 RT	0 (RT)
	24 4 °C	0 (RT)

Results

Lactate and glucose drive metabolite variation between and within biobanks

NMR metabolite data was collated between the UK Biobank and TwinsUK cohorts. As the TwinsUK cohort is predominately female sex, a female cohort from the UK Biobank (n = 4830) was randomly sampled for comparison. Principal component analysis revealed greater variation in the metabolome within

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the UK Biobank cohort compared to the TwinsUK cohort along the first principal component, as well as systematic variation between the two biobanks along the same component (Fig. 1A). Inspection of the corresponding loadings plot revealed the key role of lactate in driving this variation along the first principal component, and glucose in driving within-biobank variation along the second principal component (Fig. 1B).

Lactate levels were increased in the UK Biobank cohort (mean 3.7) relative to the TwinsUK cohort (mean 1.37; mean difference 2.36 [Welch's *t*-test 95%CI 2.33–2.40], Fig. 1C). Glucose levels were decreased in the UK Biobank cohort (mean 3.48) relative to the TwinsUK cohort (mean 3.76; mean difference 0.29 [Welch's *t*-test 95%CI 0.26–0.31], Fig. 1D). Using available metadata pertaining to the UK Biobank, we identified systematic, linear variation in plasma lactate

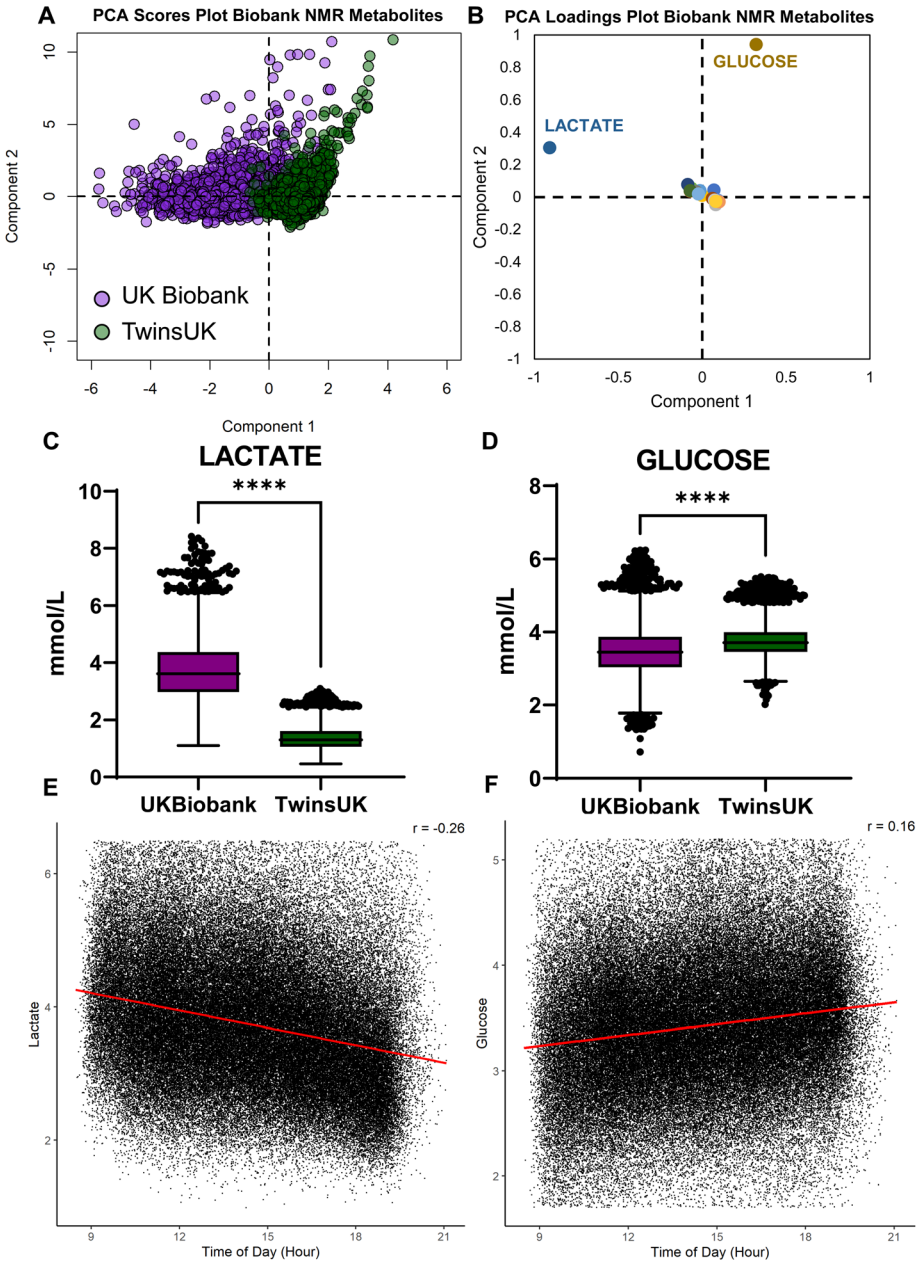

Fig. 1 Lactate and glucose levels drive variation in the blood metabolome within and between biobanks. PCA plot (A) and corresponding loadings plot (B) of TwinsUK and sampled female UK Biobank participants with blood metabolomics data. Boxplots comparing lactate (C) and glucose (D) levels in participants between the UK Biobank and TwinsUK study indicate systematically elevated lactate levels and decreased glucose levels in the UK Biobank, both $p < 0.0001$. Pearson correlation between time of day of sample collection and lactate (E) or glucose (F) concentration in the UK Biobank samples, both $p < 0.0001$. Samples collected earlier in the day likely had a delayed processing time as compared to samples collected later in the day.

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(Pearson's r = −0.26, n = 118 021, Fig. 1E) and glucose (Pearson's r = 0.16, n = 118 021, Fig. 1F) levels according to the time of sample collection. This may correspond to a systematic delay between sample collection and centrifugation according to queued collected samples.

As a second example, and on a smaller scale, lactate and glucose were also found to drive serum metabolome variation between clinical biobank data pertaining to the SPARE and STORI cohorts (Fig. 2). Here, data acquisition and relative metabolite quantification were performed in-house. The PCA scores plot (Fig. 2A) and corresponding loadings plot (Fig. 2B) illustrate systematic variation in lactate and glucose levels along the second principal component. Glucose levels were decreased in the STORI cohort (mean 0.0058) relative to the SPARE cohort (mean 0.011; mean difference 0.0055 [Welch's t-test 95%CI 0.0046–0.63]). Lactate levels were increased in the STORI cohort (mean 0.13) relative to the SPARE cohort (mean 0.053; mean difference 0.073 [Welch's t-test 95%CI 0.064–0.082]). These differences are visualised in the median NMR spectrum of each cohort (Fig. 2E).

Effects of delayed sample measurement on absolute plasma and serum metabolite concentrations

UK Biobank metabolomic metadata was used to determine the effect of time between sample preparation and measurement on plasma metabolite levels. Approximately 25% of samples were run within 12 hours of sample preparation, 63% within 24 hours, and 97% within 48 hours (Fig. 3A). The top six metabolites had a Pearson correlation coefficient >0.1 (p < 0.0001) and are shown in Fig. 3B–G. Plasma histidine concentration showed a strong negative correlation with time to sample preparation (Pearson's r = −0.36, Fig. 3B). Glycine (r =

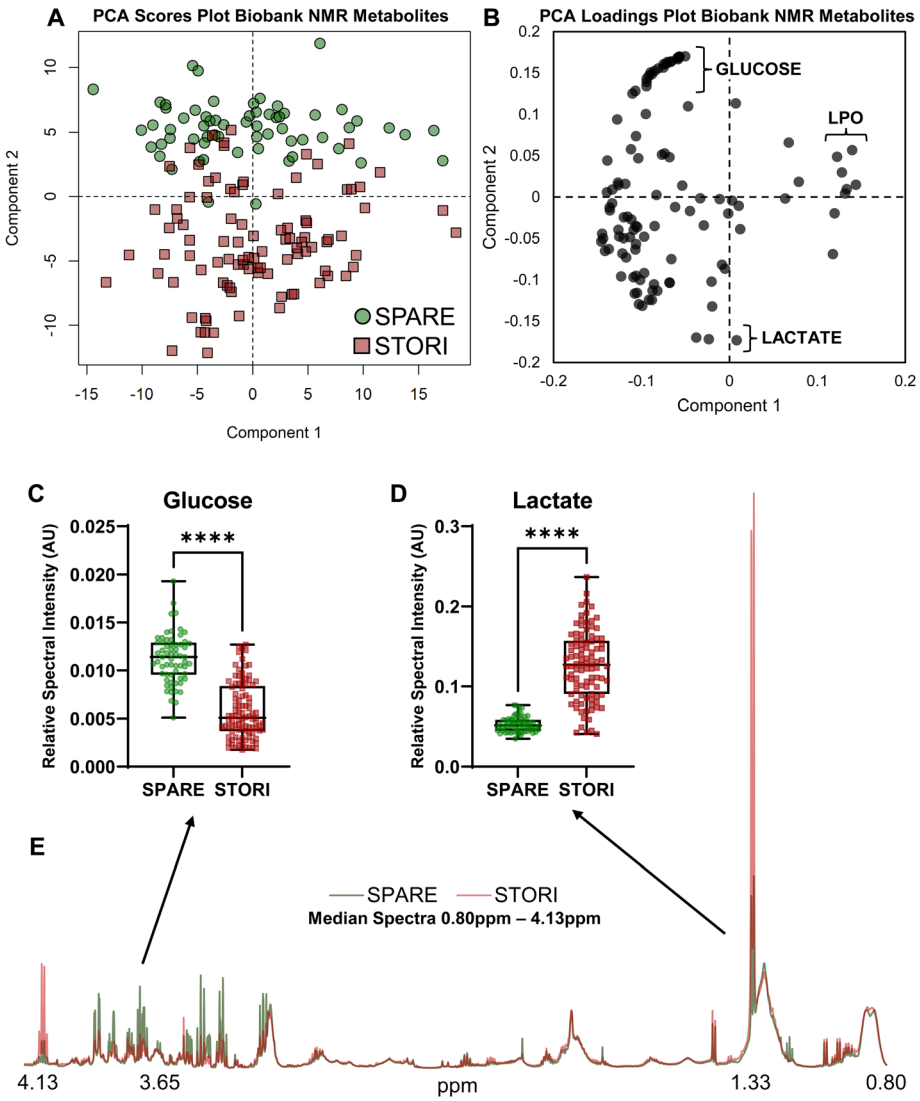

Fig. 2 Lactate and glucose levels contribute to blood metabolite variation between discovery and validation cohorts in clinical trials. PCA plot (A) and corresponding loadings plot (B) of the SPARE and STORI participant baseline serum metabolomics data. Variation between cohorts is driven by serum glucose (C) and lactate (D) resonances, both p < 0.0001. Median NMR spectrum of SPARE and STORI samples (E).

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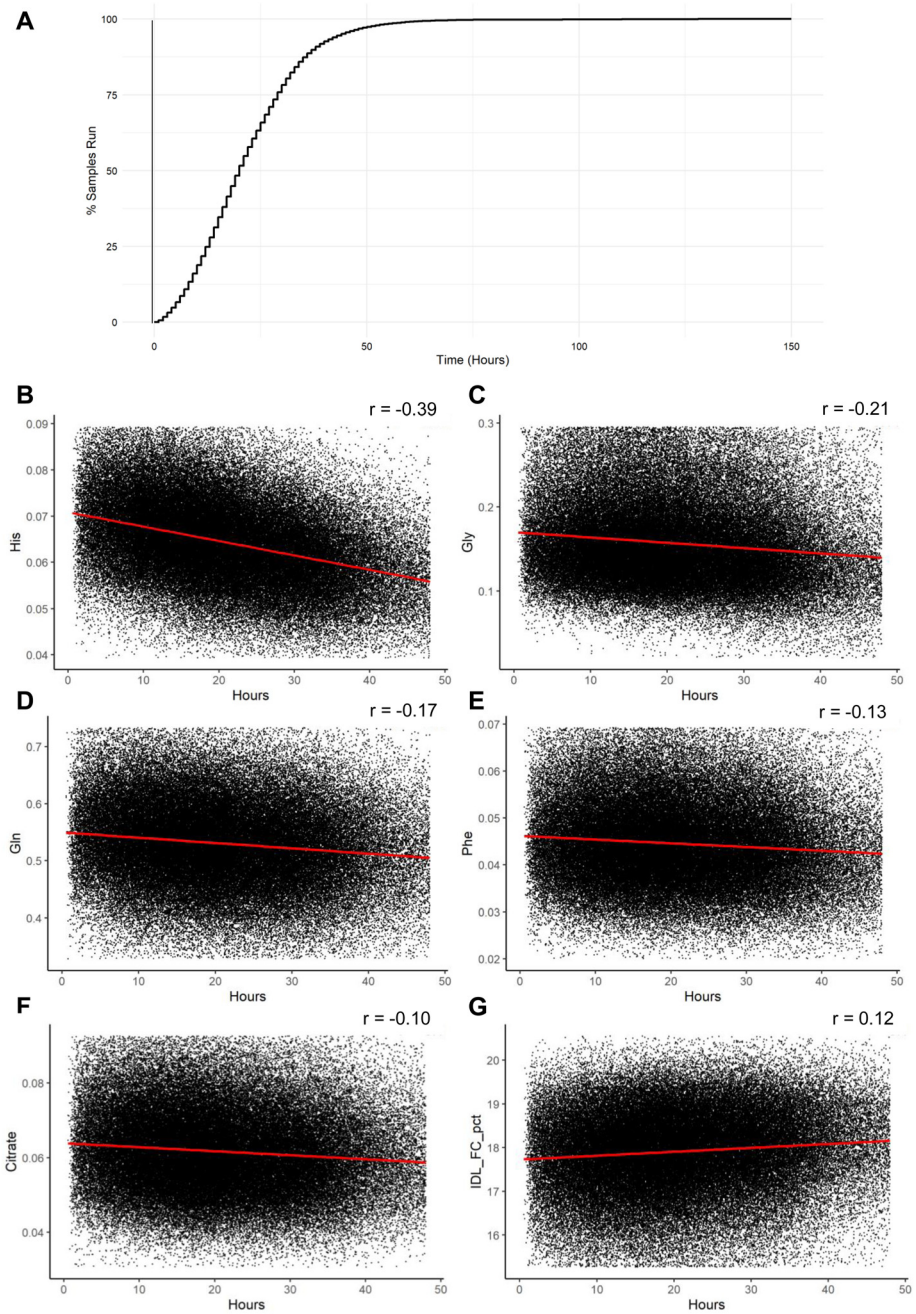

Fig. 3 Histidine is the metabolite predominately affected by delayed sample measurement in the UK Biobank. Line graph indicating the percentage of plasma samples analysed by NMR once prepared for analysis, within a given timeframe (A). Pearson correlation between time to sample measurement by NMR and histidine (B), glycine (C), glutamine (D), phenylalanine (E), citrate (F), and IDL_FC_pct (G), all $p < 0.0001$.

−0.14), glutamine ($r = -0.13$), phenylalanine ($r = -0.09$), citrate ($r = -0.10$) were also negative correlated with time to sample preparation, while free cholesterol in intermediate density lipoprotein ($r = 0.10$) was positive correlated (Fig. 3C–G, respectively).

In the healthy control cohort ($n = 12$ per condition), we found changes in the metabolome as a result of delayed sample measurement (Fig. 4). Consistent with data from the UK Biobank, the effect of delayed sample measurement was small relative to interindividual variation (Fig. 4A, B, and S1†). Most samples shifted in the second component in the PCA scores plot, driving by decreased levels of glucose, high-density lipoprotein (HDL) and glycerophosocholine (Fig. 4C and D). The stability of the serum/plasma metabolome was maintained even after a 12-hour delay in NMR measurement, as demonstrated by minimal changes in metabolite levels (Fig. 4C). However, when LH Plasma samples were subjected to a 24-hour delay in measurement, a significant decrease was

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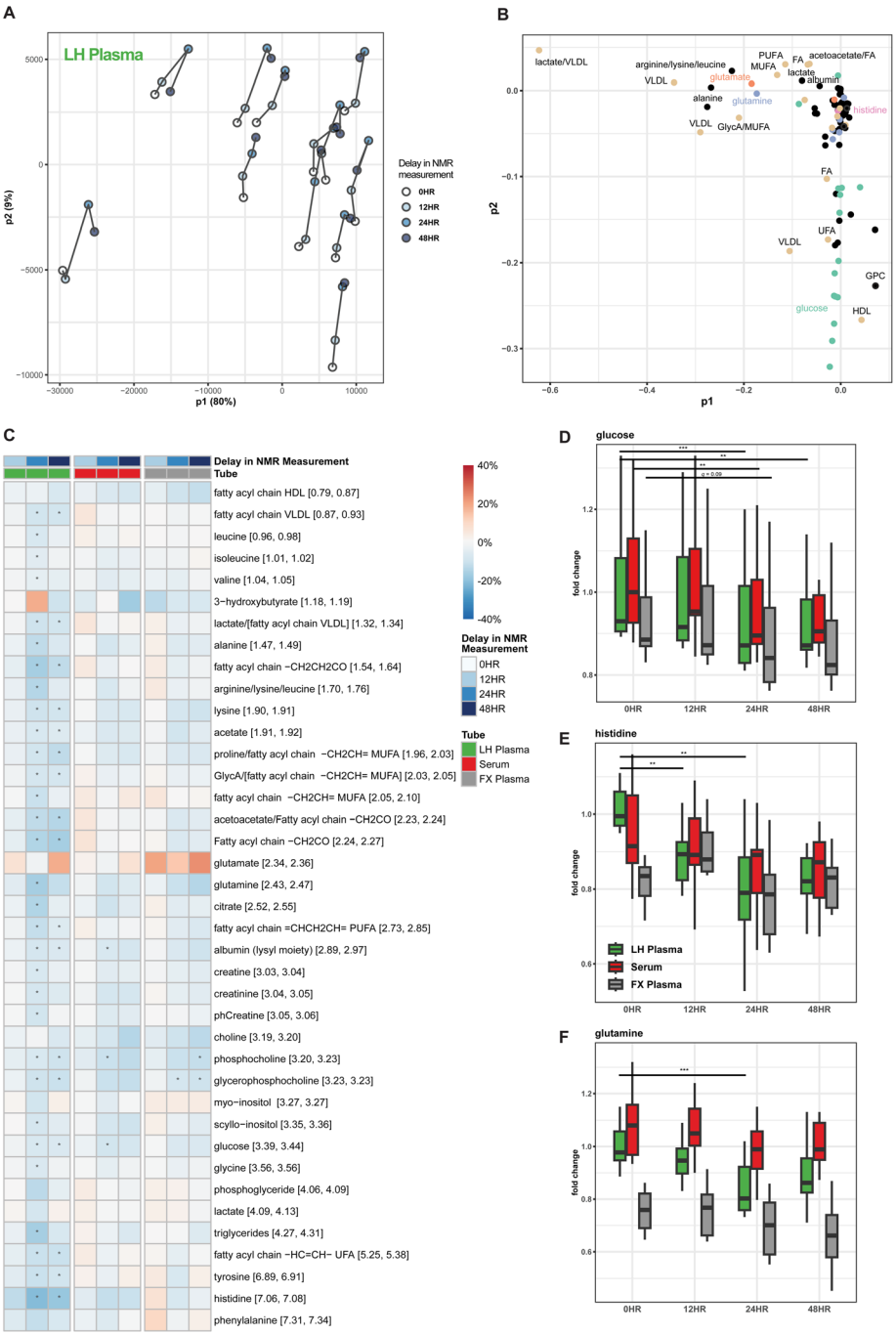

Fig. 4 Variation in the metabolome due to delayed sample measurement is small compared to interindividual variation in the healthy control cohort. PCA scores plot (A) demonstrating the effect of delayed NMR measurement on LH plasma samples. Each line connects samples from the same individual. Loadings plot (B) illustrating the metabolites that contribute to the variation observed in principal component 1 and 2. Heatmap (C) displaying percentage changes in metabolite levels resulting from varied delays in NMR measurement across three types of plasma/serum. The numbers in square brackets represent the corresponding spectral region boundaries in parts per million (ppm). Percentage changes calculated from absolute integral values and compared to the optimum processing condition. Significant differences in means of metabolites were represented by * (q < 0.05). Boxplots presenting the effect of delayed NMR measurement on levels of histidine (D), glutamine (E), and glutamate (F). "/" indicates the mentioned metabolites are overlapped in the spectral region. Metabolite names in square brackets refers to non-dominant overlapping metabolites also found in that spectral region.

observed in 31 out of 39 metabolites. Notably, it was observed that LH Plasma samples started forming precipitates after 12 hours, and all samples exhibited precipitates after 24 hours. In contrast, serum and FX plasma demonstrated better stability than LH Plasma after a 24-hour and even 48-hour delay in measurement as indicated by changes in per-

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centages of most metabolites below 10% and significant alterations observed in only a few metabolites (Table S1†). The majority of metabolites showed a decreasing trend resulting from the delay in measurement, except for glutamate, which tended to increase over time, particularly in FX plasma (Fig. 4C). Albumin, phosphocholine, glycerophosphocholine and glucose levels significantly decrease after 24 hours in at least two types of plasma/serum samples. Aligning with our observations in the UK Biobank dataset, histidine and glutamine levels tend to decrease over time, especially after 24 hours in LH Plasma (Fig. 4E and F, and Table S1†).

Metabolite differences by tube type using optimal processing parameters in the healthy control cohort

We next investigated how abundances of metabolites differed between BD Vacutainers under optimal processing conditions. Absolute metabolite concentrations of LH plasma (green) and serum (red) tubes were comparable (Fig. S2, and Table S2†). In contrast, the total integral of the spectra (spectral intensity) from FX plasma (grey) was reduced in both the NOESY and CPMG experiments due to the decline in albumin and lipoprotein levels in FX plasma (Fig. S2†). When using a PQN or sum-normalised approach to adjust for the total integral between samples and make FX plasma comparable, few differences remained between tube types (Table S2†). Branched chain amino acids (BCAAs, including leucine, isoleucine, valine) were increased in FX tubes relative to LH plasma and serum. And 3-hydroxybutyrate levels increased in FX plasma compared to LH plasma and serum, potentially influenced by variations in the lipoprotein line shape (Fig. S2†).

Evaluation of a fluoride/oxalate additive to preserve inter-individual metabolite variation using realistic timepoints

Lastly, we investigated whether the combination of sodium fluoride and potassium oxalate in a BD Vacutainer could mitigate against preanalytical variation in the individual blood metabolome. As expected, 24-hours at either RT or 4 °C resulted in an increase in lactate and a decrease in glucose when stored in either LH plasma or serum tubes (Fig. 5A–D, and Fig. S3†). When LH plasma samples were centrifuged after a 24-hour delay at 4 °C, the lactate levels increased 34% and the glucose levels decreased 18% (Table S2†). The alterations were similar for the serum samples, and further exacerbated when the samples were left at RT prior to centrifugation. Conversely, FX tubes effectively prevented anaerobic glycolysis for at least 24-hours at RT. Only 7% decrease was observed in glucose levels when FX plasma samples were centrifuged after a 24-hour delay at 4 °C. It should be noted that as demonstrated previously, glucose levels decreased 6% in FX plasma (q = 0.096, two-way ANOVA, sidak *post hoc*, FDR correction across metabolites, Table S1†) after 24 hours delay in NMR measurement at 4 °C. Therefore, the decrease of glucose in FX tubes was likely due to general degradation not due to anaerobic glycolysis.

Moreover, the combination of using FX tubes and refrigerating the blood tube during the pre-processing interval effectively prevented metabolite variation between an optimal, 30-minute pre-centrifugation delay and the 24-hour delay (Fig. 5A and E). Without refrigeration, glutamate and glutamine levels changed dramatically after 24 hours at RT (Table S2†).

Discussion

Blood is the main biofluid utilised in epidemiological studies utilising metabolomics to predict disease onset, severity, and treatment response. We hypothesised that fluoride oxalate vacutainer blood collection tubes would thus be a more appropriate blood tube for metabolome studies. To test this hypothesis, we systematically investigated the effect of preanalytical variation in blood sample handling in lithium heparin, fluoride oxalate, and serum blood tubes. To our knowledge, we demonstrate for the first time that the use of FX plasma tubes in combination with refrigeration at 4 °C negates metabolome variation due to preanalytical factors. This is important, as lactate and glucose are established biomarkers for the early diagnosis of cancer,[36,37] and may prove important to the diagnosis and prediction of other diseases when integrated into multivariate blood metabolite algorithms.[3] Our findings suggest that fluoride oxalate tubes could be beneficial in metabolomic studies where there is variability in blood processing times, pending further validation with a broader range of metabolites and larger sample sizes.

Our initial investigations revealed that much of the variation in metabolite levels within and between blood biobank cohorts could be attributed to variations in glycolysis metabolites. This variation is attributed to residual anaerobic glycolysis occurring when erythrocyte separation from serum or plasma is delayed. Delayed sample processing due to a high volume of samples being processed (UK Biobank) or collection methods where the rapid processing of bloods was not prioritised (STORI) led to increased lactate and decreased glucose resonances compared to cohorts where rapid blood processing was prioritised (TwinsUK and SPARE cohorts). As normal lactate levels are <2 mmol,[38] the absolute concentrations reported in the UK Biobank are not suitable for clinical translation.

Sodium fluoride and potassium oxalate act in concert to inhibit glycolysis in blood samples.[39] Specifically, sodium fluoride inhibits the enzyme enolase, which catalyses the conversion of 2-phosphoglycerate (2-PG) into phosphoenolpyruvate (PEP), the penultimate step in glycolysis. Potassium oxalate precipitates calcium ions to inhibit the clotting cascade. Fluoride oxalate tubes, as expected, showed baseline differences in amino acid levels and glycolytic metabolites compared to serum.[15] Consistent with other studies, the metabolite profile of serum and lithium heparin plasma were comparable,[15] and behaved similarly in response to delayed erythrocyte separation.[21]

Previous studies have documented the effects of delayed erythrocyte separation on metabolite levels as quantified by

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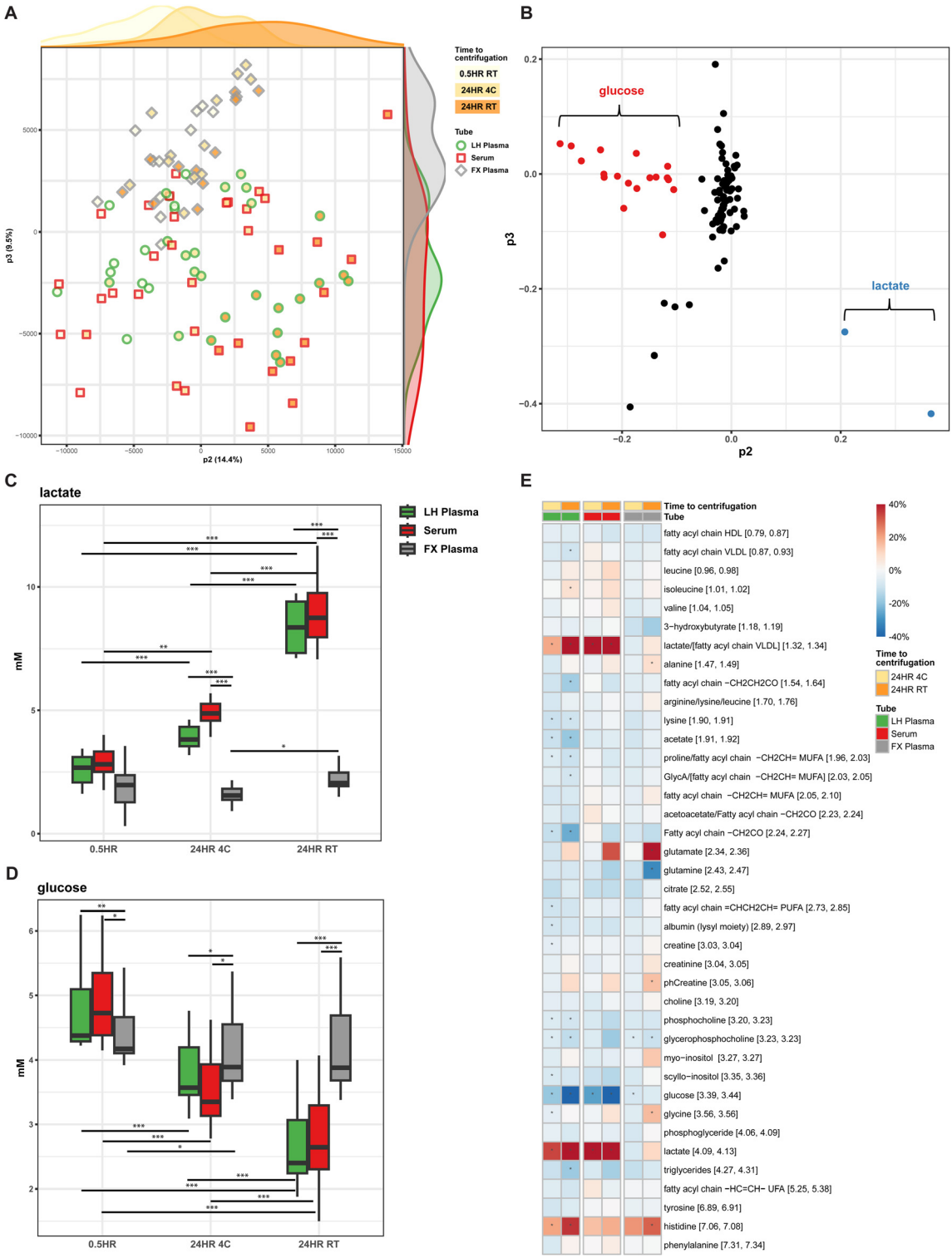

Fig. 5 Fluoride oxalate blood collection tubes prevent changes to the metabolome for at least 24 hours when stored at 4 °C. PCA scores plot (A) demonstrating the influence of different pre-processing conditions on samples, specifically observed in PC2. Loadings plot (B) of the PCA highlighting glucose and lactate as contributors to the variation observed in PC2. Boxplots showing increased lactate levels (C) and decreased glucose levels (D) in delayed pre-processing conditions for LH plasma and serum, but not in FX plasma. Heatmap (E) presenting percentage changes in metabolite levels resulting from varied pre-processing conditions across three types of plasma/serum. The numbers in square brackets represent the corresponding spectral region boundaries in parts per million (ppm). Percentage changes calculated from absolute integral values and compared to the optimum pre-processing condition. Significant differences in means of metabolites were represented by * ($q < 0.05$). "/" indicates the mentioned metabolites are overlapped in the spectral region. Metabolite names in square brackets refers to non-dominant overlapping metabolites also found in that spectral region.

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NMR-based metabolomics techniques.[10,12–14,17,19,21–24,40] Consistent with our own study, lactate and glucose are typically most affected. However, in contrast to other reports,[18] we also clearly show in both the UK Biobank and healthy volunteer data that residual glycolytic metabolism does occur (in the absence of an inhibitor of glycolysis) even when blood is stored at 4 °C prior to centrifugation.

After separation of serum or plasma from erythrocytes, metabolite variation is minimal. In the UK Biobank cohort, only histidine showed a significant effect of delay in sample measurement ($r = -0.39$) compared to interindividual biological variation. Other metabolites, while significantly correlated with measurement delay, showed smaller effect sizes between 0.01 and 0.21 which are less relevant relative to the observed interindividual variation. In our healthy volunteer study, we also investigated a delay in sample measurement over a similar timeframe, for up to 48 hours at 4 °C. Again, we found minimal variation in metabolite levels, consistent with the UK Biobank data and early UK Biobank pilot studies that tested delays of up to 24 hours at 4 °C.[41] Such variation is suitable to be corrected by previously described statistical techniques.[42]

We also provide suggestions for reporting preanalytical variation to aid in the synergy of future metabolomic studies. The reporting guideline consists of key information relating to the individual, the pre-processing variables, and the post-processing variables that may differ between samples in a single study (Table 2). The guidelines do not include variables that should be controlled for within a study and ideally between studies, for example, long-term storage temperature of frozen samples, and NMR spectra acquisition parameters. We have shown that fasting time can strongly influence the concentration of certain metabolites, primarily ketone bodies (Fig. S4†). Recording the time of blood collection, centrifugation, and storage temperatures allows studies to record SOP compliance, for which data is sorely lacking in biomarker research, and may also help identify mistreated samples. The UK Biobank has implemented pipelines that allow for automated reporting of key information. For example, barcoded blood tubes were used that, when scanned, allowed for automated logging of sample collection and processing.[43]

We recognise the limitations of this study. When comparing the UK Biobank and TwinsUK metabolomics datasets, we limited the comparison to non-lipid data (amino acids and energy metabolites) due to non-overlapping metabolite data and uncertainty in how these values were derived between the two cohorts. While our study provided insights using NMR-based metabolite profiling, which focused on metabolites commonly assayed by this technique, we acknowledge that the scope was limited with respect to the range of metabolites analysed. Future research could extend these findings by incorporating a broader spectrum of metabolomic analyses, including mass spectrometry and advanced lipoprotein assays, as well as labile metabolites detectable by NMR such as coenzymes and antioxidants,[44] to provide a more comprehensive understanding of preanalytical variability across different platforms.

In the healthy control cohort, blood storage in fluoride oxalate tubes resulted in a slight negative bias in glucose levels under optimal processing conditions, which is consistent with previous reports.[45] This bias did not show variation over time. As we suggest that the fluoride oxalate tube type should be implemented between and across studies, this small, systematic difference will not affect metabolome variability. However, the presence of this negative bias may affect the reported prevalence of fasting hyperglycaemia, which may be relevant for the screening of diabetes mellitus in the general population. Studies have also shown that both sodium fluoride[46] and potassium oxalate[47] have the potential to cause haemolysis after prolonged standing time at room temperature. We considered other, more effective methods of inhibiting glycolysis, such as citrate-buffered tubes,[46] which are also less prone to haemolysing samples over time. However, these additives are known to interfere with the NMR spectrum and the concentrations of neighbouring metabolites.[15] In our study, haemolysis was detected only in fluoride oxalate tubes left at room temperature, and not at 4 °C. Nevertheless, we encourage the discovery of novel additives that inhibit major metabolic pathways in blood under anaerobic conditions and in the absence of haemolysis.

Table 2 Key reporting parameters for blood metabolomic profiling by NMR in cohort studies

Variable	Reported by
Time since last meal (hours)	Participant
Time of blood collection	Phlebotomist
Storage temperature pre-centrifugation	
Time centrifuged	Blood processing technician
Temperature during centrifugation	
Time frozen	
Time of NMR sample preparation	NMR technician
Storage temperature pre-data acquisition	
Time of data acquisition	

Conclusions

Pre-analytical variation in blood sample collection cause variation within and between biobank biomarker studies. We trialled three blood collection tubes with the aim of solving technical challenges due to preanalytical variation in blood metabolite levels that are common in cohort studies. We found that a combination of refrigeration at 4 °C and the use of a fluoride/oxalate additive prevented changes in metabolite levels, as compared to optimal processing parameters, for at least 24 hours prior to erythrocyte separation. We also showed that the effect of delayed sample measurement on the stability of quantified metabolites was small, but dependent on the type of blood collection tube used at the pre-processing stage. Using this information, we devised a short list of key reporting guidelines that could be implemented in future cohort studies to improve the reproducibility of blood metabolite signatures as biomarkers of health and disease.

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Data availability

Metabolomics data have been deposited to the EMBL-EBI MetaboLights database[48] (**https://doi.org/10.1093/nar/gkz1019**, with the identifier MTBLS8861).

Author contributions

Conceptualisation – DCA & DRS; data curation – WX & DRS; formal analysis – WX & DRS; investigation – JS, EL, SA, & DRS; methodology – DRS, SA, WX, & HTBT; supervision – DCA & DRS; writing draft – WX & DRS; writing review and editing – WX, DCA, SA, HTBT, EL, JS, & DRS.

Conflicts of interest

There are no conflicts to declare.

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