Considerations for peripheral blood transport and storage during large-scale multicentre metabolome research

We read with interest the multiomic studies by Kong *et al* and Chen e*t al* examining gut microbiome–metabolome interactions and potential diagnostic and classification biomarkers in colorectal cancer.[1 2] Large-scale, multicentre, multisample, longitudinal studies are imperative to understand complex relationships between the metabolome and digestive diseases.

Analysis of matched blood and stool can advance biomarker development and aid mechanistic exploration, providing samples are collected and stored appropriately. Buffered kits are commercially available for stable transfer of stool samples to storage.[3] However, stabilisation of metabolites from blood requires freezing that may be confounded by transport and storage variables including time to whole blood centrifugation, time to freezing and freezing temperature. The gold standard of immediate sample separation and freezing[4 5] must be carefully balanced with pragmatic protocols, needed to facilitate standardised, cost-effective collection by busy clinical research facilities across multiple recruiting sites.

In preparation for the CD-metaRESPONSE precision medicine multicentre study (www.ibd-response.co.uk), we undertook this research to assess the impact of sample collection, shipping and storage on circulating metabolites. We collected whole blood in lithium heparin tubes from five non-fasting adult participants. Ten conditions were tested to model varying storage times of whole blood at 4°C from collection to central lab centrifugation, subsequent storage time of plasma at 4°C before freezing and the impact of long-term storage at −20°C or −80°C. We performed metabolomics using both liquid chromatography–mass spectrometry (LC-MS) and nuclear magnetic resonance

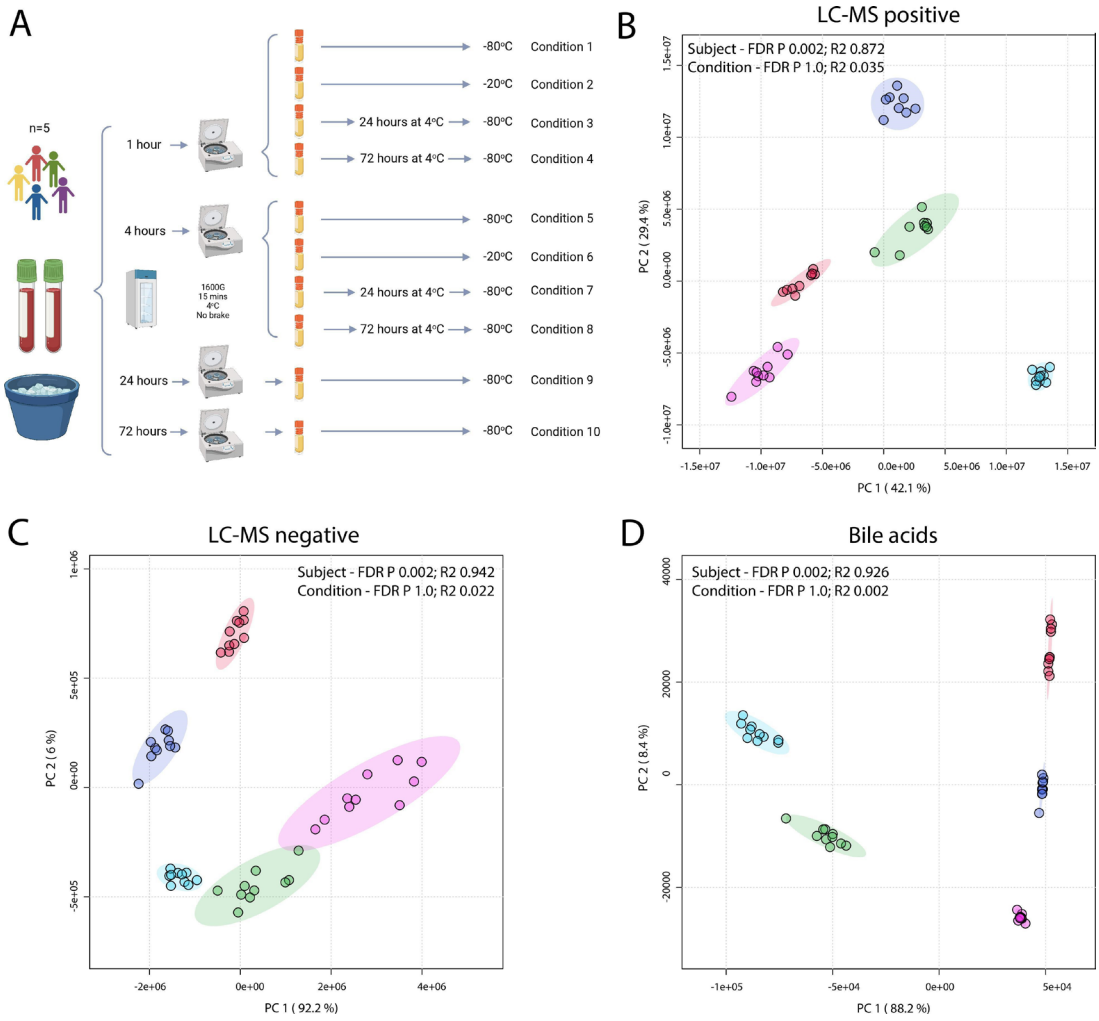

Figure 1 Study design and liquid chromatography–mass spectrometry (LC-MS) derived datasets. (A) Schematic study design showing the ten storage conditions underlying the research to model blood collection and plasma processing/storage in the context of large scale multicentre metabolomic analysis: Whole blood storage time at 4°C (conditions 1/5/9/10) for between 1 and 72 hours prior to centrifugation and immediate plasma storage at −80°C; plasma storage time for 24 or 72 hours at 4°C prior to −80°C freezing from whole blood that had been stored at 4°C for 1 hour and 4 hours prior to centrifugation (conditions 3/4 and 7/8); plasma freezing temperature at −20°C or −80°C immediately after centrifugation of blood stored for 1 hour or 4 hours at 4°C (conditions 1/2 and 5/6). A total of five participants were included. (B) Principal components analysis (PCA) ordination showing LC-MS positive mode ionisation metabolomic profiles. (C) PCA ordination showing LC-MS negative ionisation mode metabolomic profiles. (D) PCA ordination showing LC-MS bile acid metabolomic profiles. Samples coloured by subject number. 'adonis' ('vegan' R package) was used to generate estimated effect size (R2) and false discovery rate (FDR) adjusted p values (1000 permutations).

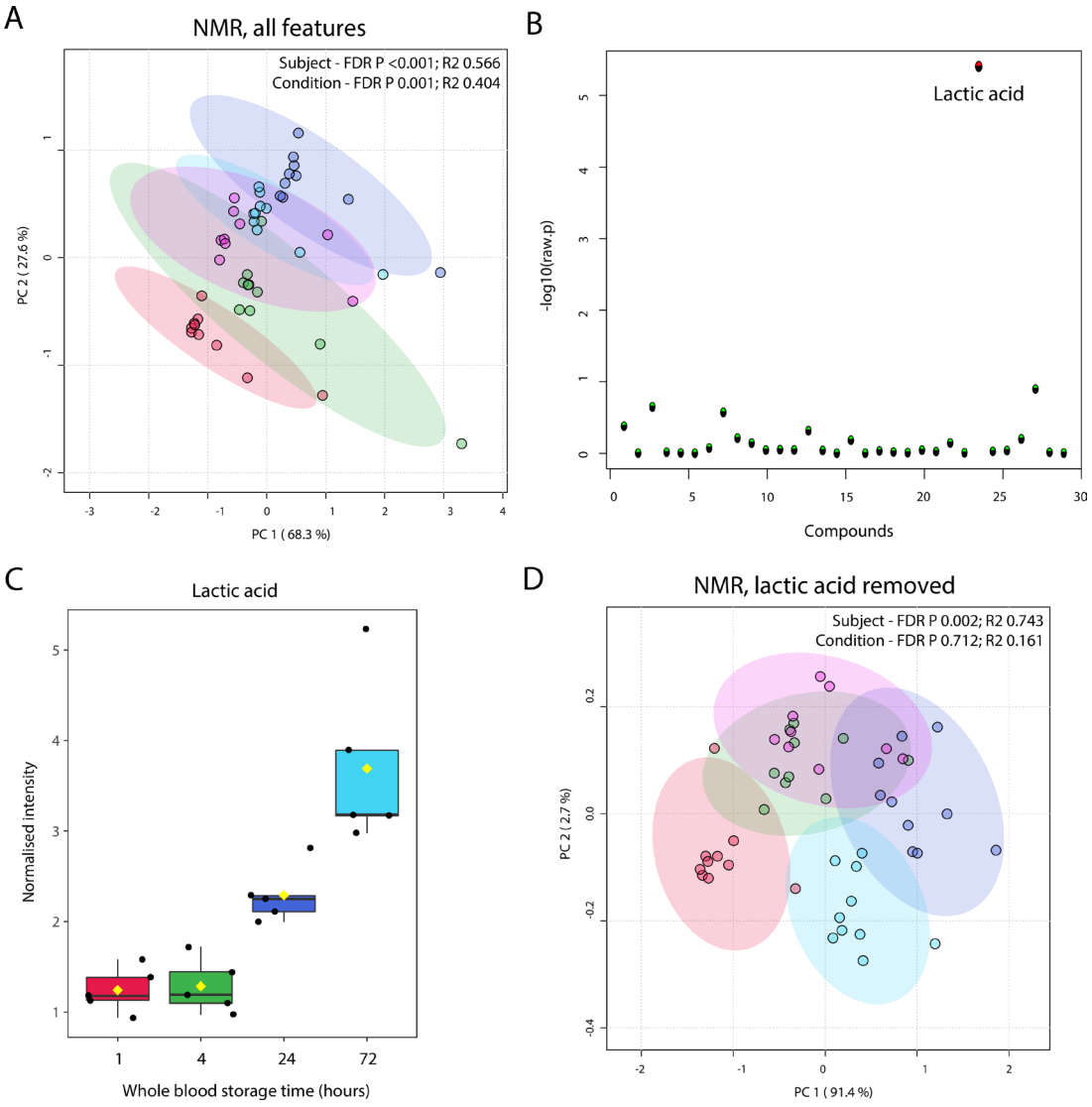

Figure 2 Proton nuclear magnetic resonance (NMR) dataset. (A) Principal components analysis (PCA) ordination showing original NMR profiles before removal of any features. (B,C) Stratified analysis of whole blood storage time from 1 to 72 hours (conditions 1, 5, 9 and 10); (B) significance plot showing lactic acid as the only significant feature; and (C) corresponding box plot of lactic acid. (B) PCA ordination showing NMR profiles after removal of lactic acid. Samples coloured by subject number. 'adonis' ('vegan' R package) was used to generate estimated effect size (R2) and false discovery rate (FDR) adjusted p values (1000 permutations) for metabolite profiles in (A) and (D).

(NMR) spectrometry to capture a broad range of metabolite classes, including lipids and bile acids by reversed-phase chromatography (RPC) LC-MS method, and small molecule metabolites by NMR.[6–8] Raw data were preprocessed and quality assessed, as previously described,[9] to generate global profiling datasets and targeted extraction of predefined metabolite panels.[8 10] Analyses were perfomed in MetaboAnalyst V.5.0 and R. For metabolite profile analysis, 'adonis' was used to generate estimated effect size (R2) and false discovery rate (FDR) adjusted p values (1000 permutations). For single metabolite analysis, p values are based on Kruskal-Wallis with Fishers least significant difference (LSD) post-hoc test.

Analysis was first performed on all 10 conditions to determine if metabolite profiles were driven by subject, storage time of whole blood or plasma at 4°C or final freezing temperature (each experimental condition detailed in figure 1A). LC-MS metabolite profiles clustered by subject (all p=0.002), irrespective of whole blood or plasma storage time or freezing temperature (figure 1B–D). Further, the variation in conditions was not significantly associated with positive or negative mode LC-MS (including bile acids). Analysis of whole blood storage time found all metabolites detected by LC-MS to be stable with the exception of lysophosphatidylserine (18:0/0:0) and lysophosphatidic acid (22:6/0:0) (both p<0.001), which were more abundant at 72 hours (negative mode only). Interindividual differences were maintained up to 72 hours at 4°C. No single LC-MS metabolite was linked to plasma storage time or freezing temperature.

Small molecule NMR metabolite profiles similarly clustered by subject (p<0.001; figure 2A); however also by condition (storage time/temperature) (p=0.001). Further analysis of different whole blood storage times showed lactic acid was significantly higher after 24 and 72 hours at 4°C (p<0.001; figure 2B,C). No other small molecule metabolite was correlated with plasma storage time or freezing temperature. Following bioinformatic removal of lactic acid, the

NMR dataset continued to cluster significantly by subject (p=0.002) and was no longer significantly associated with whole blood or plasma storage time or freezing temperature (figure 2D). Bioinformatic removal of lactic acid may be necessary to overcome confounding by sample storage time in large scale studies.

Our data provide reassurance regarding variations in blood sample handling and storage required to allow collection of plasma in the context of pragmatic multicentre large-scale clinical studies. These results form a basis for designing multicentre metabolomic biomarker studies.

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