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2022-05-25

Ghini , V , Abuja , P M , Polasek , O , Kozera , L , Laiho , P , Anton , G , Zins , M , Klovins , J , Metspalu , A , Wichmann , H-E , Gieger , C , Luchinat , C , Zatloukal , K & Turano , P 2022 , ' Impact of the pre-examination phase on multicenter metabolomic studies ' , New Biotechnology , vol. 68 , pp. 37-47 . https://doi.org/10.1016/j.nbt.2022.01.006

http://hdl.handle.net/10138/342678
https://doi.org/10.1016/j.nbt.2022.01.006

New BIOTECHNOLOGY 68 (2022) 37–47

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Full length Article

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ARTICLE INFO

Keywords:
Metabolomics
Pre-analytical procedures
NMR
Biobanks

ABSTRACT

The development of metabolomics in clinical applications has been limited by the lack of validation in large multicenter studies. Large population cohorts and their biobanks are a valuable resource for acquiring insights into molecular disease mechanisms. Nevertheless, most of their collections are not tailored for metabolomics and have been created without specific attention to the pre-analytical requirements for high-quality metabolome assessment. Thus, comparing samples obtained by different pre-analytical procedures remains a major challenge. Here, ^{1}H NMR-based analyses are used to demonstrate how human serum and plasma samples collected with different operating procedures within several large European cohort studies from the Biobanking and Biomolecular Resources Infrastructure – Large Prospective Cohorts (BBMRI-LPC) consortium can be easily revealed by supervised multivariate statistical analyses at the initial stages of the process, to avoid biases in the downstream analysis. The inter-biobank differences are discussed in terms of deviations from the validated CEN/TS 16945:2016 / ISO 23118:2021 norms. It clearly emerges that biobanks must adhere to the evidence-based guidelines in order to support wider-scale application of metabolomics in biomedicine, and that NMR spectroscopy is informative in comparing the quality of different sample sources in multi cohort/center studies.

Introduction

The human metabolome is the consequence of interplay between individual genetic and non-genetic factors, such as the individual genome sequence, epigenetic modifications, microbiome, nutrition and physical activity. Consequently, the metabolome is the molecular imprint closest to individual health or disease status. Advancements in key technologies to analyze the metabolome (*i.e.*, mass spectrometry

Abbreviations: BMI, Body mass index; CA, Canonical Analysis; CEN, European Committee for Standardization; CPMG, Carr-Purcell-Meiboom-Gill; DA, Discriminant Analysis; EDTA, Ethylenediaminetetraacetic acid; HMDB, Human Metabolome Database; ISO, International Organization for Standardization; MS, Mass Spectrometry; NMR, Nuclear Magnetic Resonance; NOESY, Nucleal Overhauser Effect Spectroscopy; OPLS, Orthogonal Projections to Latent Structures; PCA, Principal Component Analysis; PLS, Partial Least squares; RF, Random Forest; SOPs, Standard Operating Procedures; TMSP, Sodium Trimethylsilyl Propionate; TS, Technical Specifications.

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https://doi.org/10.1016/j.nbt.2022.01.006
Received 27 May 2021; Received in revised form 17 January 2022; Accepted 19 January 2022
Available online 20 January 2022

and NMR spectroscopy) as well as increased bioinformatics capabilities have markedly improved the opportunities and relevance of metabolomics studies, which now often are combined with other omics investigations including genomic studies [1–10]. The growing importance of metabolomics is exemplified by the EU funded research project Biobanking and Biomolecular Resources Infrastructure – Large Prospective Cohorts (BBMRI-LPC) involving 21 multicenter cohorts from 10 countries, providing access to samples and associated data from 3,378,310 participants for multi-omics studies [11].

While standardization of pre-analytical factors is strongly advisable for analytical data integration and data reproducibility, there is a wealth of samples stored in biobanks that have not been collected specifically for metabolomics and that might be of great interest, especially to establish and validate metabolomic biomarkers in large multicenter studies. The question arises as to whether or not these samples are suitable for metabolomics and to what extent data from different cohorts can be integrated and jointly analyzed [12,13]. In the literature, there are examples of metabolomic studies of biofluids that have highlighted problems related to the use of samples collected in multiple centers that adopt different operating procedures [14,15]. The impact of pre-analytical procedures on the accuracy of downstream metabolomics analysis has been already addressed in a number of studies [16–20].The typical pre-analytical workflow for serum and plasma is reported in Fig. 1; sample quality is intimately related to its pre-analytical history.

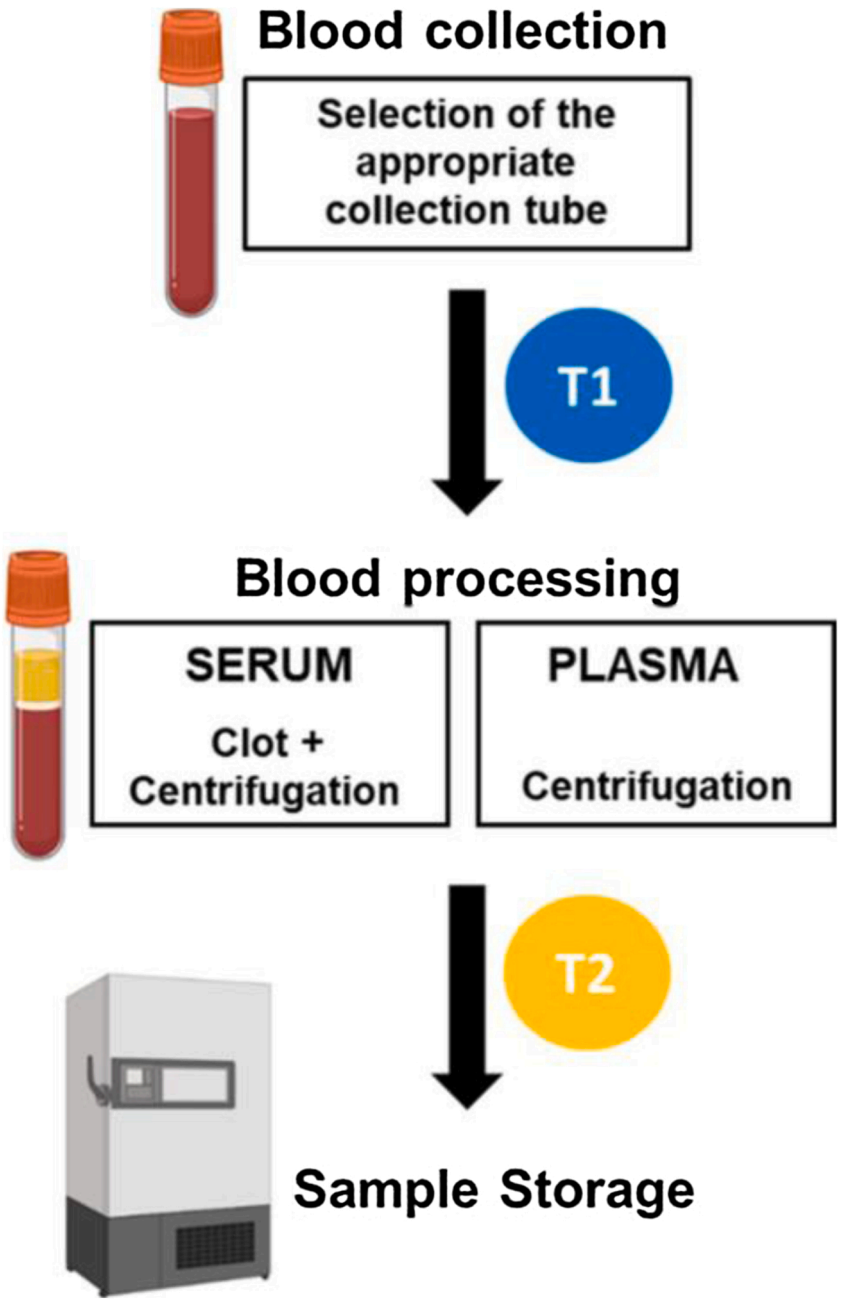

Fig. 1. Workflow of the pre-analytical phase of serum and plasma. The blue and yellow circles indicate the delays between sample collection and processing (T1) and between sample processing and storage (T2), of the pre-analytical phase that have been shown to be extremely important for the final sample quality. Created in BioRender.com (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

By simulating a large variety of pre-analytical treatments and evaluating their impact on the sample metabolome of serum and plasma, evidence-based standard operating procedures (SOPs) covering the pre-analytical phase have been developed. A relevant example is the activity of the EU-funded projects SPIDIA (FP7, https://cordis.europa.eu/project/id/222916) and SPIDIA4P (H2020, https://cordis.europa.eu/project/id/733112), which had as a main outcome a series of CEN Technical Specifications (CEN/TS). These specifications have now been translated into ISO Standards for "In-Vitro Diagnostic examinations - Specifications for Pre-examination Processes" (some already published, some others still under development) [21]. Coordinated initiatives on biobanks, such as those organized by the European Research Infrastructure Consortium BBMRI-ERIC (https://www.bbmri-eric.eu/) play a critical role in disseminating such standards and promoting their adoption [22].

Here, the aim was to test the suitability of samples collected from different existing large population cohorts from 8 different European biobanks participating in BBMRI-LPC; the samples were not initially intended to be used for metabolomics studies. Each cohort and biobank were asked to provide a selection of samples from healthy subjects according to common inclusion criteria. The resulting 234 plasma and 150 serum samples were then analyzed following a standard NMR workflow [26–28]. NMR-based metabolomics has the advantage of being fast, highly reproducible, and requiring only minimal further sample handling, although only the most abundant (>1 μM) metabolites can be measured.It is also intrinsically quantitative and perfectly suited for untargeted fingerprinting, where all metabolites above the detection limit are observed simultaneously, independently of their chemical nature. The NMR spectra acquired on serum or plasma samples were initially evaluated in terms of unsupervised and supervised multivariate approaches, which revealed differences among biobanks that largely exceeded the inter-individual differences within the same biobank. It was demonstrated that major contributions to these differences come from different concentrations in metabolites known to be affected by pre-analytical treatment. It was possible to establish correlations between unusual levels of these metabolites and differences in SOPs adopted by the various biobanks. In summary, the previous existing detailed analysis of the influence of pre-examination procedures on metabolome stability is here used *a posteriori* to interpret the observed differences in concentration for some sensitive metabolites. Thus it is shown what the main pitfalls of multicenter studies are, using samples collected/handled/stored by procedures that deviate from standard protocols for metabolomics, and provide a key to the interpretation of the differences that can be observed as a function of the collection center.

Materials and methods

Population

Samples were provided by biobanks of the BMMRI-LPC consortium located in seven European countries (Austria, Croatia, Finland, Germany, France, Latvia, Estonia). Each participating cohort and biobank provided serum and plasma samples from healthy volunteers, as summarized in Table 1. Inclusion criteria were: self-reportedly healthy (*i.e.* no evidence of acute or chronic disease), no medication other than contraceptives, age between 18 and 60 years, body mass index (BMI) between 18 and 30, no intensive training in the 2 weeks preceding blood donation. Each participating biobank was contractually obligated to have appropriate ethics approval and Informed Consent from each donor according to national requirements.

NMR sample preparation

Frozen serum and plasma samples were thawed at room temperature and shaken before use, then were prepared according to SOPs [26,29]. A total of 300 μL of a sodium phosphate buffer (70 mM Na_2HPO_4; 20 % (v/v) ^2H_2O; 6.1 mM NaN_3; 4,6 sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TMSP); the pH was adjusted to the final value of 7.4 using 1 M HCl) was added to 300 μL of each sample, and the mixture was homogenized by vortexing for 30 s. A total of 450 μL of this mixture was transferred into a 4.25 mm NMR tube (Bruker BioSpin srl, Rheinstetten, Germany)) for the analysis.

NMR spectra acquisition

All the NMR analyses were conducted at CERM, the Center of Magnetic Resonance of the University of Florence, Italy. One-dimensional ^1H NMR spectra for all samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm CPTCI ^1H-^{13}C-^{31}P and ^2H-decoupling cryoprobe including a z axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probehead, for temperature equilibration (310 K for serum samples). According to standard practices [29], two monodimensional ^1H NMR spectra were acquired with water peak suppression and different pulse sequences that allowed the selective observation of different molecular components: 1) a standard NOESY pulse sequence, using 32 scans, 98,304 data points, a spectral width of 18,028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 0.01 s; this pulse sequence was designed to obtain a spectrum in which both signals of metabolites and high molecular weight molecules (lipids and lipoproteins) are visible; 2) a standard spin

Table 1
List of the biobanks participating in the study; the type and number of samples received, and the main demographical features of the healthy donors are provided.

BIOBANK	COLLECTION DATE	N°	M	F	Age (mean)	BMI (mean)	NMR
BB1	2008–2009-2012–2013	30	15	15	42.7 ± 11.4	22.5 ± 2.2	EDTA-plasma
BB2	2013	30	8	22	39.8 ± 12.1	24.0 ± 3.8	EDTA-plasma Serum
BB3	2007	30	15	15	40.4 ± 7.6	24.5 ± 3.1	EDTA-plasma Serum
BB4	2012	24	5	19	32.1 ± 10.3	22.2 ± 3.3	CITRATE-plasma (Ficoll)
BB5	NA	30	15	15	38.4 ± 2.3	24.4 ± 2.1	EDTA-plasma Serum
BB6	2001–2002-2003	30	15	15	54.5 ± 3.6	23.4 ± 2.7	EDTA-plasma Serum
BB7	2008–2009	30	4	26	36.6 ± 9.0	24.1 ± 3.2	EDTA-plasma Serum
BB8	2007-2008–2009-2010-2011	30	15	15	29.0 ± 10.5	22.0 ± 1.8	EDTA-plasma

echo Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence, with 32 scans, 73,728 data points, a spectral width of 12019 Hz and a relaxation delay of 4 s; this sequence was used for the selective detection of low molecular weight metabolites, suppressing signals arising from macromolecules.

Processing of the NMR spectra

Free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line broadening factor before applying Fourier transformation. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (anomeric glucose doublet at 5.24 ppm) using TopSpin 3.5pl5 (Bruker Biospin srl).

Statistical analysis of the NMR data

Various kinds of multivariate statistical techniques were applied on bucketed NMR spectra using R 3.0.2 in house scripts. Bucketing is a means of reducing the number of total variables and compensating for small shifts in the signals, making the analysis more robust and reproducible. In this respect, each 1D spectrum in the range 0.2–10.00 ppm was segmented into 0.02 ppm chemical shift buckets and the corresponding spectral areas were integrated using AMIX software (version 3.8.4, Bruker BioSpin). Regions between 4.5 and 6.0 ppm containing residual water signal were removed. The total spectral area was calculated on the remaining bins and total area normalization was carried out on the data prior to pattern recognition.

Principal Component Analysis (PCA) was used to obtain a preliminary outlook of the data (visualization in a reduced space, clusters detection, screening for outliers). Random Forest (RF) algorithm, Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA), PCA combined with canonical analysis (PCA-CA) and Partial Least Squares (PLS)-CA, were used to increase the supervised separation of the analyzed groups [30].

The global accuracy for OPLS-DA, PCA-CA and PLS-CA classifications were assessed by means of a Montecarlo cross-validation scheme. Accordingly, each dataset was randomly divided into a training set (90 % of the data) and a test set (10 % of the data). The training set was used to build the model, whereas the test set was used to validate its discriminant and predictive power; this operation was repeated 500 times. For each model, the resultant confusion matrix was reported, and its discrimination accuracy, specificity and sensitivity were estimated according to standard definitions.

Each classification model was also validated using permutation tests; the permutations were repeated 100 times and the resulting p-value was calculated.

Univariate statistical techniques were applied to evaluate significant differences in metabolite levels. For this objective, 19 metabolites, for which peaks in the spectra were well defined and resolved, were assigned (Suppl. Table S1). Signal identification was performed using a library of NMR spectra of pure organic compounds, public databases (such as HMBD, Human Metabolic Database, and SDBS, Spectra Database for Organic Compounds) storing reference NMR spectra of metabolites, spiking NMR experiments and literature data [31,32]. The relative concentrations of the various metabolites were calculated by integrating the corresponding signals in a defined spectral range [33].

The non-parametric Kruskal-Wallis test was used for the determination of the meaningful metabolites. False discovery rate (FDR) correction was applied using the Benjamini & Hochberg method [34]: an adjusted p-value of 0.05 was considered statistically significant.

For the metabolites with FDR adjusted p-value below 0.05, the Kruskal-Wallis test was followed by Dunn post-hoc analysis [35] to determine which biobanks differ to each other. Again, FDR correction was applied and an adjusted p-value < 0.05 was considered statistically significant.

Results and discussion

Eight European biobanks provided samples from healthy donors, as detailed in Table 1. Samples were not collected specifically for metabolomics and each biobank operates according to internal procedures that vary among centers. The procedures adopted by the 8 different biobanks participating in this study are summarized in Table 2, where they are compared with the requirements of the technical specification CEN/TS 16945:2016 / ISO 23118:2021 [23,24].

The ^{1}H NMR spectra of plasma and serum samples were acquired according to standard procedures [26,27]. The resulting spectra were initially analysed with a fingerprinting approach, where the entire spectrum is considered as a whole, independently from signal assignment. Data reduction was performed *via* a bucketing procedure (see Material and Method section), and then multivariate unsupervised (PCA) and supervised (RF, OPLS-DA, PCA-CA, PLS-CA) statistical techniques were applied to obtain sample classification. Different statistical methods were used to assess that the obtained results were significant and method independent. Subsequently, spectral profiling was obtained based on the assignment and univariate statistical analysis on 19 metabolites, which could be unambiguously assigned and accurately integrated in all the spectra of EDTA- or citrate-plasma and serum samples (Suppl. Table S1). The results of the univariate analyses permitted the quantification of the metabolite levels in each sample. The results were plotted to reveal significant differences in metabolites levels among different biobanks.

Plasma samples

A blood derivative that is frequently stored in biobanks is plasma. For its preparation, the pre-analytical guidelines must include the selection of the best anticoagulants, since they can interfere with the sample composition [36]. The most frequently used are EDTA and citrate; nevertheless, heparin, sodium fluoride and potassium oxalate are also in common use.

Seven out of the 8 biobanks provided 30 EDTA-plasma samples (BB1, BB2, BB3, BB5, BB6, BB7, BB8); BB4 provided 24 citrate-plasma samples. In the case of NMR, the spectra of EDTA and citrate plasma are dominated by the intense signals of the anti-coagulant agents. Accordingly, the PCA score plot in Fig. 2A clearly shows that citrate-plasma samples of BB4 cluster separately from the EDTA-samples from the other 7 biobanks.

Among the EDTA-plasma samples, BB7 and BB8 were also significantly different from the others (Fig. 2A). EDTA introduces several signals in the NMR spectra; among them, the singlets at 3.61 ppm and 3.21 ppm are the most intense and their chemical shifts are strongly pH dependent (see below for the origin of the different pH). The NMR chemical shifts of these signals in the spectra of BB7 and BB8 are different from the same signals in the spectra of the other biobanks and they contributed to the observed clustering.

In order to remove the specific effects of the anticoagulant for the following supervised analyses of the NMR data, (i) the citrate plasma samples were excluded, and (ii) the spectral buckets containing the EDTA signals were not taken into account. Supervised multivariate statistical analysis was then used to determine how the NMR spectra of EDTA-plasma were influenced by the different procedures even after removing the confounding contributions from the EDTA signals. Using different models (Suppl. Table S2), all samples could be almost perfectly discriminated (discrimination accuracy ≥ 90 %, p-value $= 0.01$) on the basis of the biobank where they were collected and stored, Fig. 2C. The results are essentially independent of the multivariate method used, demonstrating that the imprint due to the biobank is extremely strong. This approach revealed that the NMR spectra of samples collected by different biobanks differ in the concentration of some metabolites.

Consistently, the levels of the 19 most abundant metabolites present in all the spectra significantly exceeded the FDR in at least one biobank,

Table 2
Operating Procedures adopted from each biobank and comparison with the requirements of the CEN/TS 16945:2016 / ISO 23118:2021.

Biobank	Blood Collection Tube		T1	Processing		T2	Storage
	Serum	Plasma		Serum	Plasma		
CEN/TS 16945: 2016 / ISO 23118:2021	Anticoagulant free tubes with no other additives	Anticoagulant tubes with no other additives	Max. of 30 min at RT	Allow the blood to clot from 30–60 min at RT Centrifugation at 1500 g for 10 min at RT	Centrifugation at 820 g for 10 min at 4 °C	Immediate freezing	< -70 ° C
BB1	–	EDTA-tubes (brand not specified)	Max. of 30 min (T not specified)	–	Centrifugation at 2000 g for 15 min at 4 °C	Not specified	−70 ° C
BB2	Tubes with clot activator (brand not specified)	EDTA-tubes (brand not specified)	Max. of 4 h (T not specified)	Allow the blood to clot from 30–60 min (T not specified) Centrifugation 2000 g for 15 min (T not specified)	Centrifugation 2000 g for 15 min (T not specified)	Not specified	−80 ° C
BB3	Tubes with separation gel and clotting activator (VACUETTE)	EDTA-tubes (VACUETTE)	Max. of 60 min at RT	Allow the blood to clot from 30–60 min (T not specified) Centrifugation 2200 g for 11 min at 20–22 ° C	Centrifugation 2200 g for 11 min at 20–22 ° C	Not specified	−20 ° C
BB4	–	Cell preparation tube with sodium citrate (BD)	Max. of 120 min at RT	–	Centrifugation 1500 g for 20 min at RT	Not specified	Short time at -20 ° C then transfer at -80 ° C
BB5	Tubes with separation gel and clotting activator (SARSTED)	EDTA-tubes (SARSTED)	Max. of 30 min at RT	Allow the blood to clot from 30–45 min (T??) Centrifugation 2000 g for 10 min at 15 ° C	*Centrifugation 2000 g for 10 min at 15 ° C*	Put the tubes in ice water	−80 ° C
BB6	Tubes with separation gel and clotting activator (BD)	EDTA-K3 tubes (BD)	Max. of 2 h (T not specified)	Allow the blood to clot from 30 min at 20–25 °C centrifugation (not after one hour from the sampling) at 1000 g for 10 min (T not specified)	Centrifugation (in the hour following sampling) at 1000 g for 10 min (T not specified)	dry ice at -80 ° C	in liquid nitrogen at - 196 ° C
BB7	Tubes with separation gel and clotting activator (BD)	EDTA tubes (BD)	Max. of 72 h at 4 °C	Allow the blood to clot from 30 min at RT Centrifugation at 3220 g for 15 min at 4 °C	Centrifugation at 3220 g for 15 min at 4 °C	Not specified	short time at-20 ° C then transfer to at -70 ° C
BB8	–	K2 EDTA tubes (brand not specified)	Max. of 72 h at 4 °C	–	Centrifugation at 800 g for 15 min at 20 ° C	storage at +4 – 8 ° C then transfer to the storage straws within 2 h	storage at −20 ° C for 2 h, and at -70 ° C for overnight. Long term storage into a liquid nitrogen storage vessel at 170°-196 ° C

as summarized in Fig. 2E. Of these, the main differences came from biobank BB4. As mentioned above, BB4 provided citrate-plasma instead of EDTA-plasma, and it is known that the absolute levels of metabolites in plasma are strongly affected by the choice of the anticoagulant [36, 37]. Nevertheless, significant differences were also observed among the other biobanks and in particular for BB7 and BB8.

Serum samples

Five biobanks also provided 30 serum samples: BB2, BB3, BB5, BB6, and BB7 (Table 1). In general, for ^{1}H NMR metabolomics serum is preferred to plasma due to the absence of anticoagulant signals. Analogously to the plasmas, samples provided by the various biobanks were compared. Unsupervised PCA analyses were used to have an initial overview of the dataset, Fig. 2B.

The score plots of Fig. 2B show that the samples provided by BB7 were clearly different from all the others. Using different supervised models (Suppl. Table S2), an almost perfect discrimination was obtained (accuracy > 96 %, p-value = 0.01) among the samples from the different biobanks, Fig. 2D. Also in this case, the discriminatory power was high and independent of the statistical method applied.

As for plasma, the levels of 9 out of 19 most abundant metabolites present in all the spectra significantly exceeded the FDR in at least one biobank, as summarized in Fig. 2E.

Correlation between metabolite levels and SOPs

The concentrations of the metabolites responsible for the clustering as a function of the biobank of origin were compared (Fig. 3). An attempt was made to interpret the possible origin of the observed differences between biobanks. This study dealt with samples from quite homogeneous cohorts of healthy donors in terms of age, sex and BMI (Table 1). The two main differences can be traced back to the geographical origin of donors and the operating procedures adopted by the biobanks. It has already been established by dedicated studies that the former is not a relevant confounding factor, at least at the European level. For example, data resulting from the FP7 PATHWAY-27 project demonstrated that blood serum/plasma composition is strictly regulated and, although the individual metabolome can be partially modulated by diet interventions [38–40], regional dietary habits do not cause strong discrimination among individuals [41]. Thus, at least within Europe, there are no significant differences in the metabolomics profiles of blood of healthy adults attributable to the country of origin. On the other hand, existing literature related to the effect of pre-examination procedures suggests that some of the SOPs of Table 2 are potentially able to influence the concentrations of a number of metabolites [16,17,19,20,25]. Here, a strict correlation could be established between the concentration of these molecules and the pre-analytical parameters already identified as critical for sample integrity/stability, as detailed below.

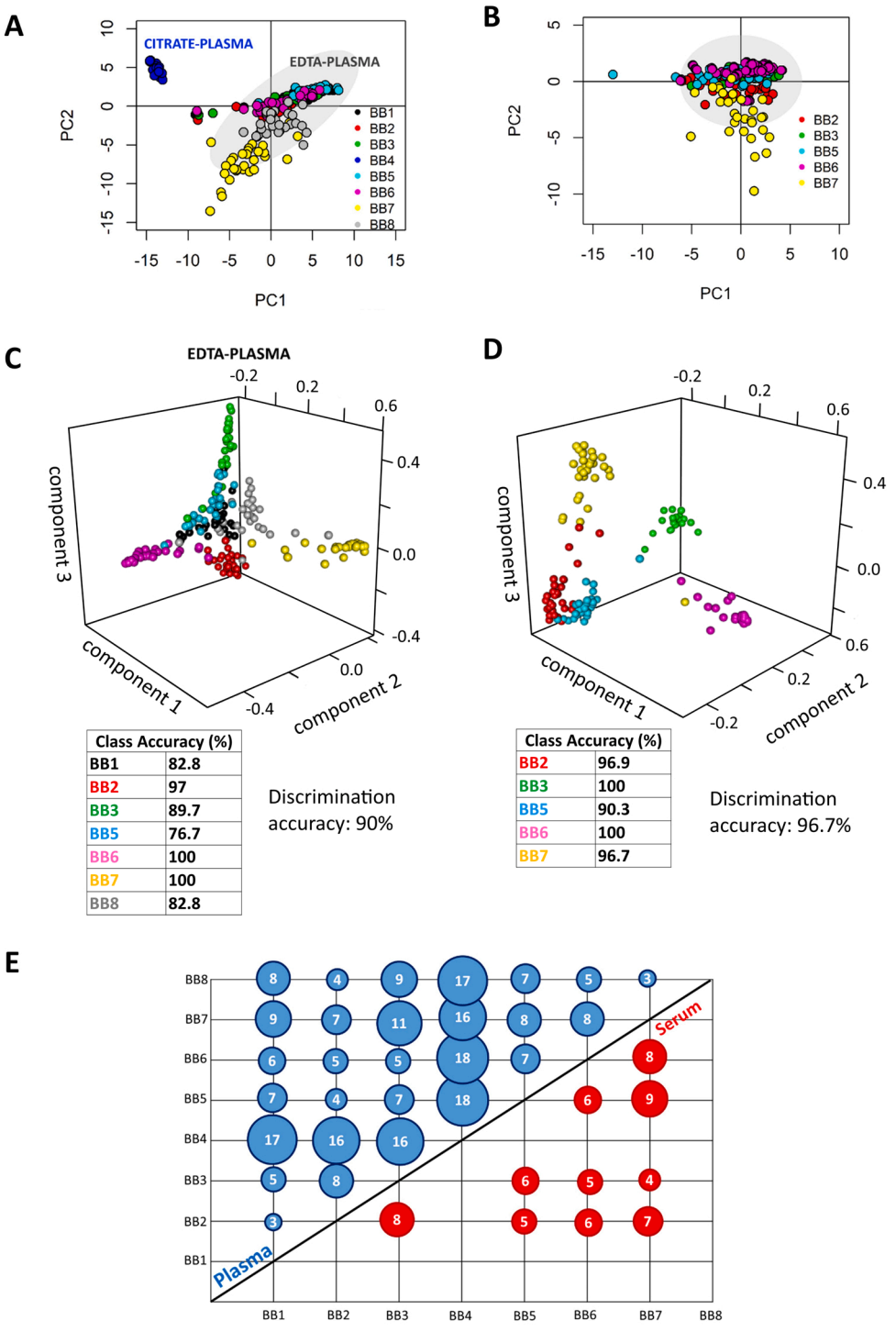

Fig. 2. Discrimination among samples collected from different biobanks in the BBMRI-LPC consortium. (A-B) PCA analysis of (A) plasma and (B) serum spectra. (C-D) RF analysis of (C) EDTA-plasma and (D) serum spectra. Color code: Black, BB1; red, BB2; green, BB3; blue, BB4; cyan, BB5; magenta, BB6, yellow, BB7; grey, BB8. (E) Overview of univariate analysis results. Number of metabolites with significantly different levels among biobanks (p-value < 0.05 after FDR correction) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Processing Time, T1

The presence of cellular activity during the time between blood collection and processing to obtain plasma or serum, T1, has been proposed as a major source of alterations. In particular, *in vitro* erythrocyte activity changes the levels of important metabolites, such as glucose and lactate [16,17,19,42]. Higher levels of glycerol-3-phosphate (G3P) and 3-phosphoglycerate (3-PGA) are also indicative of long blood incubation before processing [19]. It is known, indeed, that erythrocytes, when removed from the circulation, exhibit severe disturbances of glycolytic flow, with accumulation not only of lactate, but also of several upstream metabolic intermediates such as G3P and 3-PGA [43]. Similarly, arginine decreases, probably because of degradation by erythrocyte arginase activity [20]. Taurine increase during T1 indicates prolonged blood coagulation and can be related to platelet activity [20]. The above metabolites often play key roles in the signature of some diseases, particularly in cancer where low glucose and high lactate are indicators of enhanced glycolysis characteristic of the Warburg effect [44]. Therefore, it is very important that their plasma and serum levels

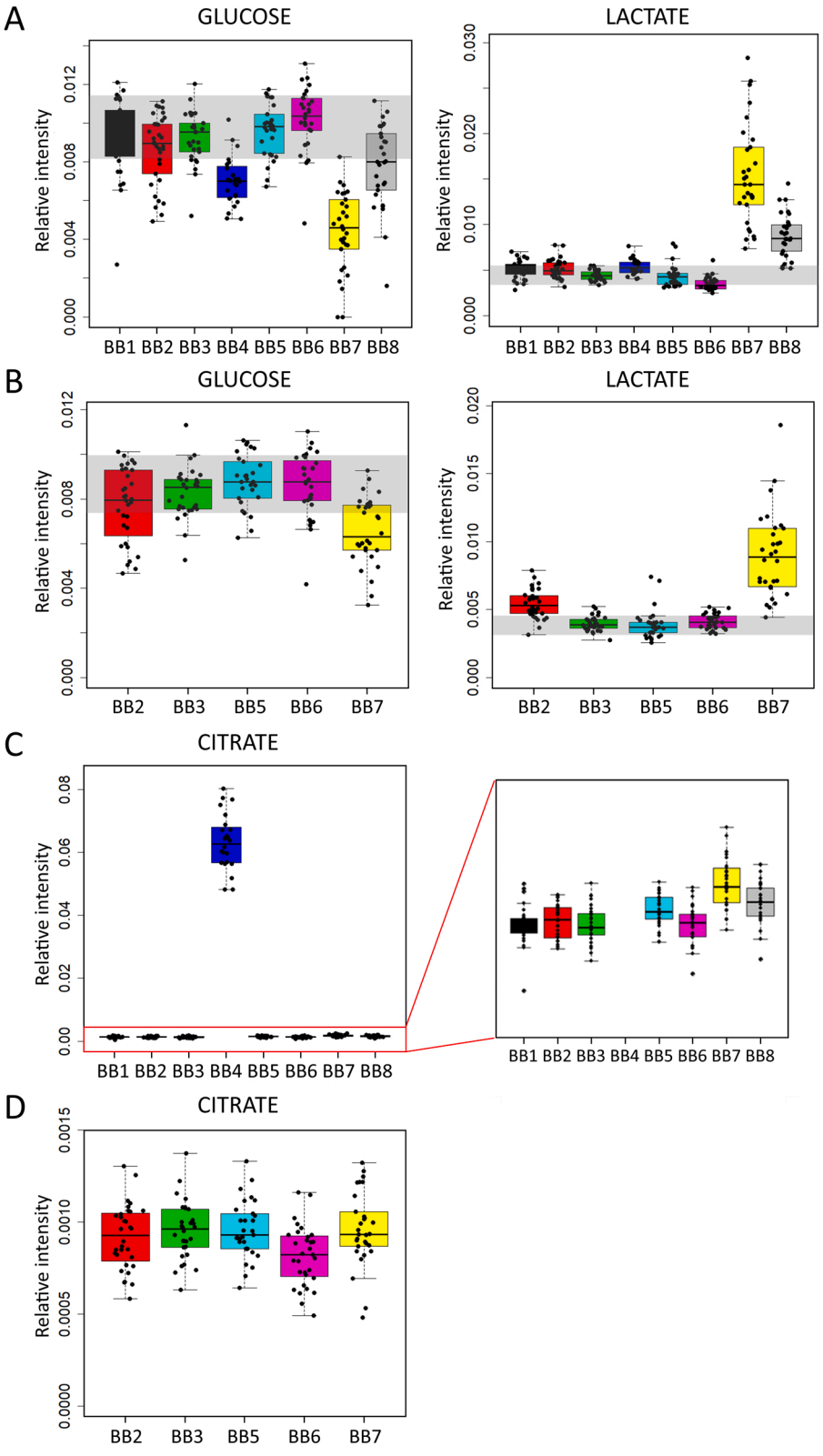

Fig. 3. Correlation between metabolite levels and SOPs: impact of T1 and T2. Box plots of the lactate and glucose levels in (A) plasma and (B) serum samples, given as the relative intensity of the main NMR signal. Box plots of the citrate levels in (C) plasma and (D) serum samples. Black: BB1; red: BB2; green: BB3; blue: BB4; cyan: BB5; magenta: BB6, yellow: BB7; grey: BB8. The shaded area in panels A and B represents the median value ± MAD calculated using the levels of glucose and lactate calculated respectively in EDTA-plasma and serum samples from biobanks that respect in their SOPs the restraint of T1 $\leq$ 120 min (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

represent, as far as possible, the *in vivo* levels. To minimize alterations, serum and plasma preparation should be initiated as soon as possible (within 30 min) after blood collection. Once the cellular component is removed by centrifugation, the concentrations of these metabolites remain stable over time [16,17,20].

In the SOPs adopted by BB1, BB2, BB3, BB4, BB5 and BB6, the processing phase must be initiated 30 min-1 h after blood collection, with a maximum allowed time of 4 h for BB2. In contrast, the SOPs of BB7 and

BB8 indicate that the pre-processing phase can last up to 72 h after blood collection. Glucose and lactate levels wre measured in both plasma and serum from all biobanks, whereas G3P, 3-PGA, arginine and taurine concentrations were below NMR detection. Lactate and glucose levels of samples BB7 and BB8 were significantly altered compared to those from other biobanks (Fig. 3A-B), suggesting that delayed blood processing negatively affected plasma quality.

As discussed above, a number of signals in the EDTA-plasma samples of BB7 and BB8 show pH-dependent chemical shifts. These alterations are interpretable in terms of an acidification due to the accumulation of lactic acid and contribute to the discrimination in multivariate analyses based on the whole spectra.

Processing Time, T2

After blood processing, the resulting serum or plasma should be immediately frozen at -80 ° C (or below), otherwise the samples will undergo changes due to redox reactions. As already demonstrated, proline and citrate (together with lipoproteins) are the most affected components [16,17,27,45]. Similar effects are observed as a consequence of repeated freeze/thaw cycles. The main feature diagnostic for sample damage associated with improper handling during T2 – as well as for multiple rounds of freezing and thawing - is a decreased level of citrate. It has been reported that in samples maintained at 4−6 ° C for up to 12 h, citrate NMR signals disappear completely [16]. Here, in all the NMR spectra, there were measurable levels of citrate (Fig. 3C-D). Endogenous citrate levels of BB4 spectra were not further considered because the high levels of the citrate from the anticoagulant prevented the evaluation of the endogenous one.

Table 2 shows that phase T2 is not well regulated by the various SOPs. As a consequence, especially for plasma, quite variable levels of citrate were observed within each biobank and between the different biobanks (Fig. 3C, inset, and Fig. 3D).

Recent mass spectrometry (MS) studies have also reported cystine, lysophosphatidylcholine and ribose as sensitive indicators of prolonged (24 h) storage at room temperature (unfortunately, no time course of the changes was reported) [20]. The increase in ribose during prolonged storage of serum/plasma at elevated temperature has been attributed to the activity of NAD^+ nucleosidase and nucleotidase [20]. Cysteine and lysophosphatidylcholine remained below detection in the present NMR data while the concentration levels of ribose were difficult to analyze due to the very low signal intensities.

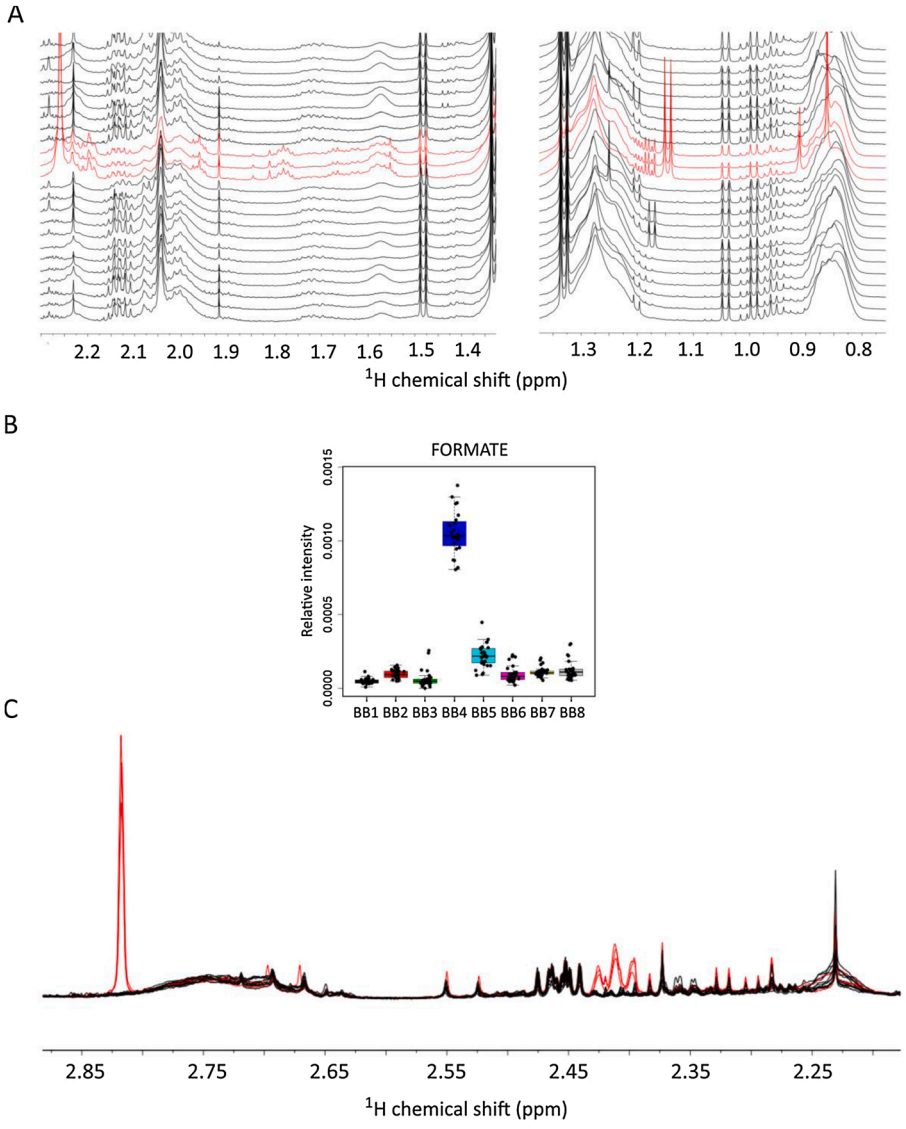

Fig. 4. Correlation between metabolite levels and SOPs: impact of collection tubes. (A) Representative CPMG ^{1}H-NMR spectra of plasma samples. BB4 spectra (red) contain several signals not present in any of the other spectra. (B) Box plots of the formate levels in plasma samples. Colors as in Fig. 3. (C) Representative CPMG ^{1}H-NMR spectra of serum samples. BB3 spectra (red) contain a few signals not present in any of the other spectra (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Selection of the collection tubes

Upstream of all measurements, is the choice of the appropriate collection tube. These may contain additional additives, such as anticoagulants (EDTA, sodium citrate, heparin) or polymer gels (gel separator) that facilitate the separation of plasma/serum from the cellular component [46]. Here, a number of differences emerged that contributed to the clustering of samples from the different biobanks and originated from the use of different selection tubes.

BB4 provided citrate-plasma samples; these samples, besides being characterized by very high citrate levels, also show several other resonances in their NMR spectra not observed in any of the other plasma spectra, Fig. 4A. The levels of glucose also appeared to be affected by the presence of this anticoagulant, Fig. 3A. These samples were collected using BD Vacutainer® CPT™, a fully closed system for separation of mononuclear cells from whole blood, where cell separation is carried out in the primary blood collection tube. CPT™ is a sterile blood collection tube containing buffered sodium citrate anticoagulant and Ficoll polymer gel. The use of Ficoll strongly alters the metabolomic profiles of plasma samples, as previously reported [25]. Additionally, plasma samples from these tubes with the Ficoll-Hypaque solution showed unusually high levels of formate, as evident from the untargeted NMR approach, Fig. 4B. The source of formate contaminationwas the additive contained in the tube, as here demonstrated by recording the NMR spectrum of a reference sample produced by applying the manufacturer's preparation method to a buffer/physiological solution in place of plasma.

EDTA-plasma samples from biobank BB6 showed a set of NMR signals in the range 2.85–2.95 ppm and 3.3–3.4 ppm. Notably, this biobank is the only one which declared the use of K3EDTA tubes. However, in the absence of further information we cannot unequivocally establish the origin of these signals.

Serum tubes contain silica particles (clot activator), which activate clotting. Some types also contain a gel separator. From a visual inspection, we could assess that the NMR spectra of the BB3 samples showed a few signals that were not present in any of the other spectra, Fig. 4C. Additionally, these signals were not present in the plasma spectra of the same subjects, suggesting that they originated from the gel separator in the collection tube. The origin of these signals was definitely identified as due to the gel separator, by testing the contamination of buffer introduced into empty tubes of the same type.

Conclusions

Here an outcome is described that is often encountered in multicenter metabolomics studies. The use of supervised multivariate analysis revealed differences in the samples provided by different centers; a situation where the inter-individual variations within the same center are significantly smaller than those among subjects recruited at different centers should always call for a careful analysis of possible confounding factors. It was demonstrated that among these factors, a major contribution comes from the pre-analytical history of the samples. Within the analyzed sets, the availability of the pre-analytical procedures adopted by each biobank permitted a detailed comparison of the deviations with respect to the standard procedures that have been developed to limit drifts from the *in vivo* metabolome. In this respect, some molecules could be used to highlight non-conformities at specific steps of the pre-examination pipeline.

While CEN/ISO standards and related literature provide hints on the design of the best-performing SOPs, to guarantee an optimal preservation of the *in vivo* metabolome of biosamples, the present findings offer practical guidance for assessing the quality and comparability of samples selected from non-homogeneously collected cohorts.

The data generated also show the need to test whether integrated metabolomics data analysis is feasible with samples collected, handled, and stored at different centers [47]. Whenever samples collected outside controlled and validated SOPs are used, an initial check of possible discrimination in terms of biobank/collection center should be performed. If differences exist, their possible origin should be carefully investigated and the metabolites removed from the analysis for which-their levels can be affected by pre-analytical treatments. Quality control is essential to capture the knowledge on diseases contained in biosamples and should be considered as a requirement to ensure that analytical results are reproducible and compliant with FAIR data principles, and to minimize the risk that biomarkers discovered in such studies fail in later development [48]. From the point of view of the analytical platform, this study shows that ^{1}H NMR, used as a simple fingerprinting approach, is a sensitive tool to reveal metabolomics sample heterogeneity induced by the different SOPs adopted by the various cohort studies and biobanks. Given its intrinsically untargeted nature, NMR metabolomics can also reveal the presence of unexpected contaminants, and is therefore particularly appropriate for the (preliminary) examination of the suitability of collection tubes and other plasticware or stabilization reagents employed during the pre-analytical phase. Needless to say, the described changes in metabolite levels as a function of the processing times T1 and T2, and temperatures thereof, are methodology-independent, and therefore detectable also by MS. NMR has the advantage of being fast and highly reproducible. Nevertheless, given the higher sensitivity of MS, the panel of molecules affected by pre-analytical treatments will be larger than that here described [16,17,19,20,25].

In this context, biobanks interested in metabolomics should consider the use of the available ISO standards to ensure high quality data are collected on their samples; even more so, if they aim to include collection of samples for metabolomics among the activities for which they wish to be accredited ISO 20387:2018 [49,50]. It is worth noting that BBMRI-ERIC is actively promoting the use of the pre-analytical CEN and ISO standards developed by the SPIDIA/SPIDIA4P projects; this effort is expected to improve awareness of the pre-examination pitfalls within the biobanking community. The present article, which directly involves a number of European biobanks, may represent a further contribution.

Data availability

Data will be made available on request.

Funding

AM was supported by Gentransmed (Project No. 2014-2020.4.01.15-0012). This project has received support from the EU Horizon 2020 research and innovation programme under grant SPIDIA4P (agreement No 733,112) and former FP7 projects: BBMRI-LPC (GA no. 313010313010) and SPIDIA (agreement No 222,916). In the course of this study, VG was initially supported by an AIRC/FIRC fellowship for Italy and then by a post-doctoral fellowship funded by Regione Toscana via the COMETA project.

Author contributions

PT, KZ, CL and PMA conceived this study; VG performed the metabolomic analyses; PT and VG interpreted the NMR results. OP, LK, PL, GA, MZ, JK, and AM provided biobank samples and anonymised patient data. PT, VG and CL drafted the initial manuscript. KZ, PMA, CG and EW contributed to the manuscript writing, which all authors subsequently revised. All authors approved the final version of the work.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

PT, VG and CL acknowledge the support and the use of resources of

Instruct-ERIC, a Landmark ESFRI project, and specifically the CERM/CIRMMP Italy Centre.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2022.01.006.

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