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Relevance of pre-analytical factors in multiomics: Toward a standardized blood processing protocol

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ABSTRACT

To implement multiomic studies successfully, there is a need to overcome challenges in steps ranging from study design to data integration. As blood is the preferred matrix for sampling in such studies, we review how pre-analytical factors affect genomics, transcriptomics, proteomics, and metabolomics and propose a harmonized blood processing protocol. Plasma is preferred, as clotting of serum may cause contamination from lysed cells. Transcriptomics is highly sensitive to platelet contamination, making platelet-poor plasma ideal. Processing delays and room-temperature storage compromise the stability of several analytes classes. To ensure comparability, the Standard PREanalytical Code (SPREC) should document all phases of sample handling. We recommend collecting blood in K_2EDTA tubes and separating plasma via two centrifugations (1600×*g* and 16,000×*g*, 10 min

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at 4 °C). Samples should be checked for hemolysis, icterus, and lipemia and then stored at −80 °C [SPREC: PL2.PED.A1.C.J.A.D]. Following this standardized protocol or documenting deviations from it can improve multiomic reproducibility.

1. Introduction

To implement multiomic studies successfully, there is a need to overcome challenges across the entire pipeline, from study design to data integration [1]. Although multiomic approaches better capture the complexity of biological systems, they remain highly sensitive to pre-analytical variability, which can compromise reproducibility, accuracy, and ultimately clinical translation. This includes not only the physiological variables but also aspects related to sample manipulation. The former includes non-modifiable patient-related factors such as age, sex, and chronic medical conditions and modifiable factors such as drugs and lifestyle factors that must be controlled during study design [2]. The latter encompasses all variables related to sample collection, handling, and transportation to the site of processing and storage that influence the identification and/or quantification of the many biomolecules in samples. These variables should thus be defined *a priori*.

Blood samples, mostly venous ones, are routinely used for multiomic studies as their use allows the combination of a minimally invasive collection method with the opportunity to obtain a comprehensive overview of the status of an individual at a single point in time. For this reason, we focus here on venous blood, henceforth referred to simply as blood. Blood sampling also offers the possibility of performing repeated sampling rather easily, allowing time- or treatment-dependent data to be obtained, potentially with a high degree of reproducibility. However, while repeated sampling can be highly reproducible within a single laboratory, achieving consistency across multicenter studies or between independent investigations remains far more challenging, with the harmonization of pre-analytical practices representing one of the greatest obstacles in the field of multiomics. Each omic approach, from genomics to metabolomics, has its own particularities with regard to sample processing. There is an emerging need to standardize protocols for each omic method to improve interstudy comparisons and allow the identification of pan-omics benchmarking analytes. To enable an even greater level of integration, it would be beneficial to coordinate these standardization efforts across omics disciplines and to ultimately support the paradigm of "single sample–single protocol–multiomic screening" by finding a compatible baseline valid across all omics approaches.

In this regard, initiatives like the Standard PREanalytical Code (SPREC) project have considerable merit as they facilitate the in-depth and precise documentation of approaches and methods and encourage the transparent reporting of critical pre-analytical parameters, which are otherwise rarely described comprehensively and accurately. SPREC, currently in its fourth version, is a seven-letter codification system that describes relevant pre-analytical variables. For fluid samples, for instance, it includes the type of sample (including serum, or single- or double-spun plasma), type of primary container (e.g., potassium EDTA, sodium citrate, or lithium heparin), pre-centrifugation handling time and conditions (e.g., time and temperature), centrifugation steps and conditions (e.g., centrifugal force *g*, temperature, time, braking on/off, number of cycles), post-centrifugation conditions (time and temperature), and long-term storage conditions (e.g., container type, volume, temperature) [3]. The widespread use of SPREC is encouraged to simplify the reporting of sample processing, facilitate protocol reproduction, and promote research standardization.

Undertaking multiomic studies with blood samples implies the need to control, record, and standardize the aforementioned pre-analytical variables as much as possible to ensure high standards. Maximizing the sample quality for a cohort enhances the potential for collaborative work across multiple institutions and can elevate its scientific impact. "Pre-analytical" is defined here as all steps prior to the omics-specific sample preparation, with the main focus on blood processing parameters. Therefore, we begin by briefly discussing factors before the blood-processing to consider when designing a multiomics study based on blood samples (Section 2). We subsequently present a literature review on the effect of pre-analytical factors across different omic disciplines (Section 3), following the SPREC initiative framework. Finally, given the lack of standardized protocols or methods, we propose a consensus blood processing protocol for multiomics analysis based on integrative synthesis of evidence (Section 4).

2. General considerations for study design

A detailed protocol is the foundation of a successful clinical study. It should provide clear instructions for clinicians, nurses, and researchers involved in sample collection and handling, ensuring standardized procedures that promote uniformity in data collection, analysis, and interpretation. This consistency is vital for the reliability and validity of the study results and allows other researchers to replicate the study or build on its findings. There are several recommendations and guidelines available on which factors to consider for the design of studies in which biospecimens are collected [4–6]. Omics approaches monitoring fold changes of endogenous molecules are particularly sensitive to errors or bias introduced in the pre-analytical phase.

Numerous factors—from participant recruitment to sample storage—need to be well described in the standard operating procedure (SOP) for the project, including how to handle and report deviations from the protocol. Benchmarking omics studies aimed at evaluating and improving the laboratory analytical process often fail to achieve desired benchmarks due to inherent pre-analytical limitations [7], including errors in patient and sample identification, blood collection, sample handling, transportation, and storage. Thus, in addition to the time of blood collection, time of first centrifugation, assessment of sample quality (e.g., presence of hemolysis or lipemia), and time delay until the storage of each blood component, aliquots should also be recorded. The SOP should also define the number of aliquots, sample coding, volumes, and storage tubes with detailed records of the locations of samples within the freezer. Automation of laboratory workflows can help improve traceability with documented chain-of-custody and protocol deviations.

Three main measures for mitigating variability need to be considered during study design. Fixed variables, such as biological sex, age, and season of the year, cannot be modified and should thus be recorded. Modifiable variables such as smoking, diurnal variation, and fasting status are known to influence the levels of analytes in blood and should preferably be controlled rather than just recorded [8–10]. For subjects who have suffered a recent infection, have recently been vaccinated, or are pregnant, it is recommended that blood collection be postponed [11–13]. If modifiable variables are to be controlled, clear instructions on this should be given to the research participants in advance, verbally and in writing, and adherence to such instructions should be assessed before blood sampling, such as in terms of maintaining normal physical activity and dietary habits and avoiding strenuous exercise [14–16]. Obviously, if the study involves the collection of samples after acute events such as myocardial infarction, control of these factors is not possible. A summary of the variables that should be considered, grouped by mitigation measures to minimize their impact, is presented in Fig. 1.

Fixed and modifiable variables can be captured in a questionnaire that may include questions aimed at extracting data on known risk factors and confounders of the condition under study. Since some blood

parameters fluctuate with the circadian rhythm, the time of sample collection should be consistent and recorded [17,18]. Certain blood parameters are also affected by the subject's position during blood sampling (i.e., supine or sitting) [19]. Blood is preferably collected from participants who are resting, that is, have been in a supine position for at least 10 min, with the tourniquet applied for no longer than 1 min [20]. The order of blood collection tubes and the number of inversions should follow the recommendations of the Clinical and Laboratory Standards Institute (CLSI; https://clsi.org/about/blog/order-of-blood-draw-tubes-and-additives/).

2.1. Benchmarking

The benchmarking of pre-analytical errors and failures to conform to the set protocol offers various benefits for quality control in omics studies. These include ensuring reproducibility and reliability, improving data quality and integrity, optimizing experimental protocols, facilitating comparative studies, and guaranteeing that observed differences reflect actual biological variation rather than technical artifacts. A body of evidence demonstrates that pre-analytical errors for benchmarking should be grouped into two major categories: errors concerning specimen identification (e.g., unlabeled, misidentified, mismatched, and inadequately labeled specimens) and sample-related problems (e.g., hemolyzed, clotted, and icteric/lipemic samples, incorrectly filled containers and improperly transported and stored samples) [21]. To mitigate problems caused by errors in the first category, standardized recording and subsequent correction protocols should be implemented. Decreasing the number of pre-analytical errors of the second category can be achieved with modern laboratory information management systems by logging sampling, preprocessing, and storage steps as well as through precision biobanking [22]. In some cases, benchmarking can be achieved through known biochemical assays [23], such as the hemolysis, icterus, and lipemia (HIL) index [23]. Among these three variables, hemolysis can be quantified by methods such as optical spectroscopy or through the quantification of microRNAs (miRNAs) [24]; these techniques are preferred because they provide quantitative and recordable data. In terms of lipemia, this affects not only lipidomics but also non-coding RNAs (ncRNAs), since lipoproteins can act as carriers of particular miRNAs. The excessive amounts of lipid particles found in lipemia can be removed from serum and plasma via a second centrifugation step (10,000×*g* for 15 min) [25]. Exposure to high temperatures and the duration of pre-centrifugation storage can also be objectively determined by measuring the metabolites inosine and hypoxanthine [26], although these are themselves unstable in serum [27]. At present, there is no list of transcripts or proteins to uniformly predict an error type or combination of errors or to decipher pre-analytical outliers in omics studies, but certain bioinformatic and artificial intelligence applications may help detect these anomalies [7,23] (https://github.com/itmat/OmicsBenchmarkReport).

3. The impact of pre-analytical factors across different omics

This section deals with the importance of pre-analytical factors for the quality of results obtained in the different omics fields. All of these disciplines share the feature that the targets are not predefined, and quality can be measured by multiple variables that lead to contradictory conclusions on which pre-analytical parameters are best. Quality measures can be as diverse as analytical signals (e.g., chromatographic peak shape), sensitivity (e.g., number of targets detected), bias, and variance. As a further complication, targets within each omics field can be highly heterogeneous, and what is evaluated in one part of the omics chemical space may not extrapolate to the whole. For this reason, this review focuses on comprehensive analyses covering wide chemical spaces. Because blood-derived products (plasma/serum) are far more widely used than whole blood (e.g., dry blood spots), we focus on such products here.

SPRECs are used in this review to code variables extracted from original research papers that evaluate the impact of pre-analytical

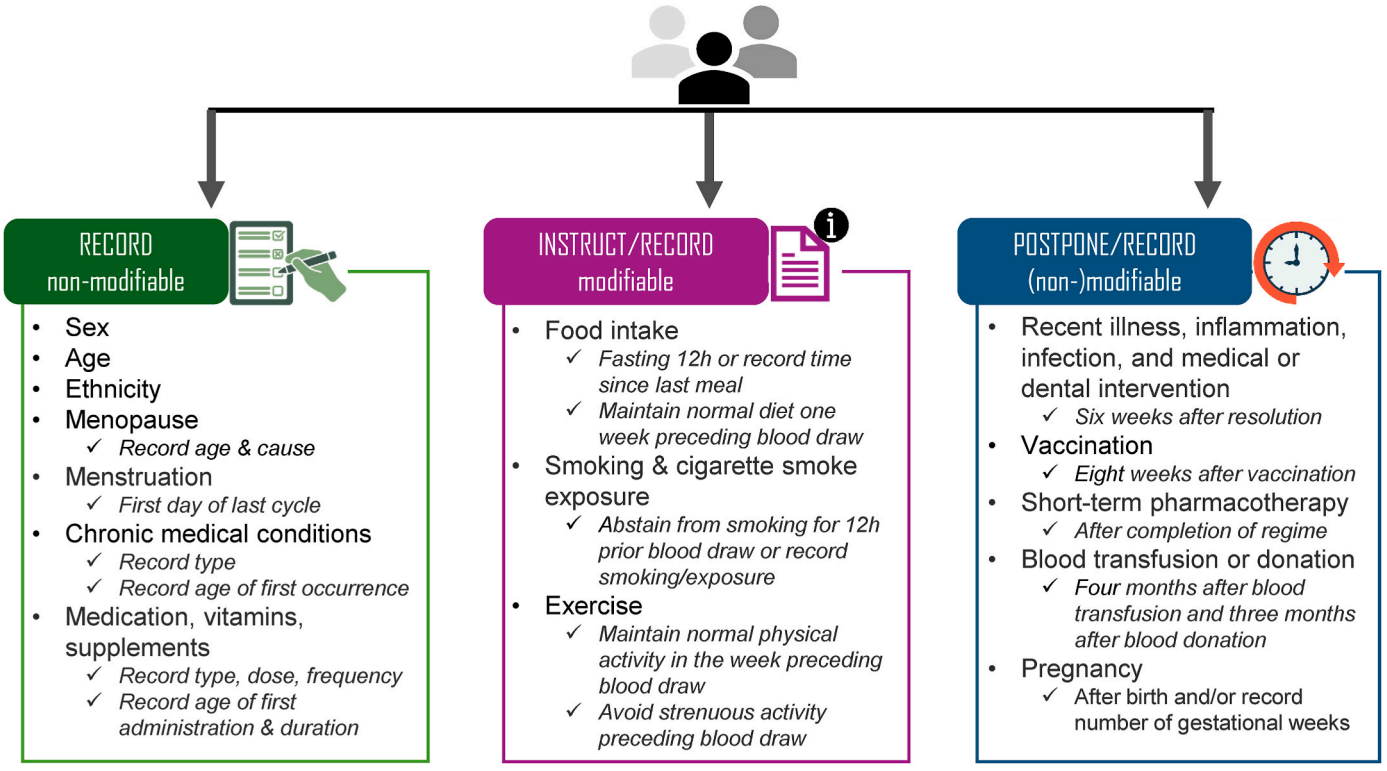

Fig. 1. Management of pre-analytical factors before blood sampling. Summary of variables affecting omics data accompanied by proposed mitigation measures either by controlling or by recording non-modifiable variables.

factors on one or more omics. We apply this coding system, despite it not being used in the original research articles, to enhance the systematic nature of the review and allow the comparison of data across different disciplines. This approach involves making certain assumptions. These include i) that temporary storage "on ice packs" involved a temperature range of 2–10 °C, ii) that centrifugation was performed at room temperature (RT) when no mention of the temperature is made, iii) that variables are classified into the most conservative SPREC category when there is an overlap, iv) the default use of brakes when this is not specified, and v) the use of 0.5–2 mL polypropylene tubes when only long-term storage at −80 °C is indicated. The fact that such assumptions are necessary already highlights the importance of researchers using systems such as SPREC to ensure transparency, traceability, and reproducibility. The latest version of SPREC (4.0) [3] has more variables than earlier versions, although some settings are still missing.

3.1. Genomics

DNA in blood primarily originates from two sources: genomic DNA (gDNA) and cell-free DNA (cfDNA). Various pre-analytical factors affect gDNA and cfDNA differently, and the choice of which of them to analyze determines the type of collection tube required. Notably, cells, and therefore gDNA, are considered contaminants of plasma/serum in transcriptomics, proteomics, and metabolomics.

Genomic DNA is generally isolated from the buffy coat layer or whole blood depending on the isolation protocol. Blood in discard tubes can also be repurposed for isolating DNA. Since DNA from the buffy coat mostly originates from leukocytes, the yield of DNA can vary in smokers and cancer patients, for example [28]. Collecting gDNA from the buffy coat layer is convenient in protocols in which samples are needed for multiple purposes.

EDTA or citrated tubes are preferred for the isolation of DNA from peripheral blood leukocytes. Blood collection tubes with clot activators must be avoided. To isolate cfDNA, both serum and plasma can be used. Although a higher yield can be obtained from serum, the risk of contamination with gDNA is greater in this case due to the lysis of cells that become entrapped during clotting [29]. As such, plasma is recommended, and cfDNA can be isolated after two sessions of centrifugation, first at 1600×*g* for 10 min and then at 16,000×*g* for 10 min, both at 4 °C, and within 4 h of blood sampling [SPREC: PL2.PED.C.D.J.A.A] [30]. A two-step centrifugation protocol (low-speed followed by high-speed) is also recommended by the International Society of Liquid Biopsy for the analysis of circulating tumor DNA [31]. To avoid degradation by nucleases, cfDNA should be isolated immediately. Otherwise, plasma can be stored for up to 3 h at 4 °C, or split into smaller aliquots that can be frozen at −20 °C or −80 °C for long-term storage, avoiding multiple freeze/thaw cycles [SPREC: PL2.PED.E/F.X.X.E.A] [30,32]. Hemolysis can have an impact on cfDNA [33], but it does not affect gDNA isolated from leukocytes, although protocol modifications may be required to remove hemoglobin and erythrocytic debris [30]. It is recommended that hemolytic, icteric, and opaque plasma samples not be used for cfDNA analyses [30].

gDNA is ideally isolated as soon as possible or within a few weeks, although it can be isolated from whole blood stored at −20 °C even after many years [34]. Freeze/thaw cycles of whole blood should be avoided since this can affect DNA integrity [35]. Conversely, repeat freeze/-thawing has been reported to have minimal influence on the quality, concentration, or purity of frozen DNA [36]. Extracted gDNA can be stored in the long term at 4 °C in Tris-EDTA (TE) buffer at pH 7.4, which ensures that nucleic acids avoid enzymatic degradation [37,38], or frozen in TE buffer at either −20 °C or −70 °C [39,40]. Low-DNA-binding polypropylene or polyethylene storage tubes of appropriate sizes based on the volume of the DNA samples should be used for long-term storage, ideally closed with push caps to avoid evaporation. For high-throughput sequencing, DNA dissolved in water or low TE buffer at pH 8.0 is preferred, but this is not suitable for long-term storage [41].

3.2. Transcriptomics

Recent technological advances in next-generation sequencing have enabled a comprehensive exploration of the transcriptome, covering both coding and non-coding transcripts. NcRNAs, which make up the majority of circulating RNA, include a diverse range of molecules, such as miRNAs, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). Members of this family have emerged as promising biomarkers for various diseases due to their regulatory roles. Among ncRNAs, miRNAs are the most extensively studied, whereas lncRNAs and circRNAs remain less explored [42,43]. However, despite numerous ncRNAs being proposed as diagnostic and prognostic biomarkers, there has been little success in their validation due to inconsistencies in pre-analytical protocols and low expression levels.

One major pre-analytical concern in the analysis of circulating RNAs is the choice of primary container and the risk of contamination during sample processing. Platelets, as a reservoir of megakaryocyte-derived RNAs, are a major source of plasma RNAs, alongside residual blood cells [44,45]. In fact, high similarity between platelet-rich plasma and platelet miRNA profiles has been reported [46]. To reduce the contribution from platelets, plasma samples should be used in the form of platelet-poor plasma (PPP). In accordance with the recommendations of the International Society on Thrombosis and Haemostasis [47], PPP is prepared with two centrifugation steps, the first lasting 15 min at 2500×*g* and RT and the second lasting 15 min at 2500×*g* and RT. The extra step removes additional cellular nucleic acids attached to cell debris and minimizes the contamination of DNA and RNA derived from damaged blood cells [SPREC: PL2.SCI.A.A.A.A.A] [48]. The European Committee for Standardization (CEN) protocol, which is actually for obtaining cfDNA, can be applied for circulating RNAs, although high-speed centrifugation may remove RNA carried by extracellular vesicles [49]. Therefore, the approach most widely used for obtaining PPP for circulating RNAs is as follows: first centrifugation at 2000×*g* for 10 min at 4 °C in a horizontal rotor (swingout head) and then repetition of this but at 10,000×*g* [SPREC: PL2.ZZZ.B1.D.H.A.A] [50].

The choice of blood collection tubes and anticoagulant significantly influences the outcomes of RNA analysis. Heparin is not recommended for the analysis of RNA in circulation since it interferes with downstream reactions [51]. Samples with EDTA [SPREC: PL2.PED.A1.A.A.X.A], which is the most commonly used anticoagulant for the analysis of circulating RNA, exhibit slightly lower levels of platelet-derived miRNAs than tubes with other anticoagulants [SPREC: PL2.SCI/ZZZ/ACD.A1.A.A.X.A] [52]. In the extensive exRNAQC study, which evaluated 10 different collection tubes, it was demonstrated that most tubes with RNA preservatives performed worse than those without, suggesting that they should be avoided [53]. After evaluating a large number of containers with different pre-centrifugation delays [SPREC: SER.CAT.A1/C/I.Z.Z.A.A and PL2.PED/SCI.A1/C/I.Z.Z.A.A, three centrifugations at RT: 20 min at 400×*g*, 10 min at 800×*g*, and 15 min at 2500×*g*] with different mRNA and miRNA kits, the authors recommended using citrate tubes for extracellular RNA analysis, and processing of samples within 4 h of blood sampling. The choice of technical parameters influences which pre-analytical conditions are preferred, and these choices are further shaped by the analytical panel used [53].

Pre-processing time and sample stability are also relevant variables for technical variance and bias. For example, circulating RNAs in processed, biobanked plasma samples exhibit remarkable stability, with one report stating that they maintained integrity for over 17 years at ultra-low temperatures, even after multiple freeze/thaw cycles [54].

Hemolysis is a critical variable in plasma transcriptome analysis. Various miRNAs, such as hsa-miR-23a-3p and hsa-miR-451a, serve as hemolysis markers, with their ratio correlating with hemoglobin levels as measured spectrophotometrically at 414 nm [55]. A commonly applied hemolysis cut-off is dCt >7. However, the expression of miRNAs

and lncRNAs can be affected by even low or mild hemolysis [56,57]. Lipemia also introduces variability since various disease-associated miRNAs are actually carried by lipoproteins. For example, miR-146a is bound to low-density lipoproteins and miR-223 to high-density lipoproteins, but both of these are markers of inflammation [58].

In summary, the use of properly prepared PPP ensures that expression levels of circulating RNAs are unaffected by platelets and hemolysis, making it the optimal sample type for accurate and reproducible transcriptomic measurements [57].

3.3. Proteomics and peptidomics

Proteomic analysis involves qualitative and quantitative characterization of the proteins present in a biological sample. For blood proteomics, the choice of the blood-derived product, that is, serum or plasma, is extremely important as these yield different protein profiles [59]. Serum is generated by the clotting of blood, which involves the activation of proteases of the coagulation cascade that promote protein degradation and are difficult to control, resulting in significant heterogeneity. This adds to the complexities arising from the action of proteases activated during sample collection, processing, and storage, regardless of whether plasma or serum samples are used. In addition, some proteins bind to, or are entrapped by, blood clots in an uncontrolled and variable manner [60], which makes the comparison of serum proteomes across laboratories (e.g., in multicenter studies) difficult and can reduce reproducibility in long-term studies. Given these processes, if serum samples are used, the loss of certain proteins and the occurrence of additional proteome modifications are inevitable. West-Nielsen et al. [61] characterized the effects of sample handling, including clotting conditions, storage temperature, storage time, and freeze/thaw cycles, on mass spectrometry (MS)-based proteomics of human serum. They found that the timing and temperature (RT vs. 4 °C) of coagulation affect the results, with better separation being achieved for samples processed at RT within 2 h.

Even the use of different primary containers (heparin vs. EDTA vs. citrate) to obtain plasma can give rise to different MS profiles [59]. For plasma collection, potassium EDTA tubes are often preferred to minimize artifactual proteome modifications [62]. For analyses dedicated to hemostasis, it is important to use citrate tubes, but one must account for potential dilution effects [59].

Apart from the additives present in the primary collection vessel, pre-centrifugation delays and storage temperature can affect the stability of plasma proteins. For instance, at least 90 % of plasma proteins display a CV of <20 % with a pre-centrifugation delay of 30 min at RT [SPREC: PL1.SED.A1.C.N.X.A] or 8 h at 0–5 °C [SPREC: PL1.SED.F.C.N.X.A] [63]. Kaisar et al. reported that the majority of plasma proteins (~95 %) remain stable with a pre-centrifugation delay of up to 48 h at RT [SPREC: PL1.EDG.K.B.N.X.A] [64], although such long delays should be avoided as they may result in the loss of critical data on low-abundance proteins (such as those involved in signaling) and affect the presence and level of post-translational modifications (PTMs). A larger (N = 100 donors) study applied a shorter limit of 24 h, with no significant protein changes detected between samples stored for time periods of 4 h [SPREC: PL2.PED.C.D.D.C.S] and 24 h [SPREC: PL2.PED.J.D.D.C.S]. However, at longer storage times, increased hemolysis was observed [65], which should be avoided as it results in changes in the protein profile [59]. Specifically, the levels of alpha- and beta-hemoglobin doubled in samples kept for 4 h before centrifugation [SPREC: PL1.SED.D.D.N.X.A] compared with the levels for those kept for 1 h [SPREC: PL1.SED.B.D.N.X.A] [63]. It has also been reported that plasma collected in potassium EDTA can be centrifuged at 2000×*g* [SPREC: PL1.PED.X.B.N.X.A] or 4000×*g* for 10 min [SPREC: PL1.PED.X.E.N.X.A], with no differences being reported between these two settings [66].

For both plasma and serum samples, samples should be kept at −80 °C (or lower temperatures) for long-term storage [SPREC: PL1.SED.F.F.N.A.D]. Under such conditions, samples can remain useable for >10 years. In a recent study, samples stored sequentially from 1998 until 2003 were analyzed, and no differences in the proteomic profile were detected across this period when quantifying ~1300 proteins. Moreover, the CV remained below 5 % [67].

It has been reported that the stability of the serum and plasma proteomes was not affected by up to three freeze/thaw cycles, as long as the thawed serum was kept on ice [SPREC: SER.CAT.A.D.N.N.A] [68] or thawed plasma was left for <1 h at RT [SPREC: PL2.PED.C.D.D.C.S] [65]. Nevertheless, if possible, such cycles should be limited to just a single one, in order to avoid activation of processing enzymes and proteases, loss of unstable PTMs, formation of protein aggregates, and potential decreases in the concentrations of low-abundance species to below the detection limit.

Peptidomics focuses on the study of naturally occurring peptides, the low-molecular-mass component of the proteome [69,70]. Peptides can diffuse passively through endothelial barriers, and at higher rates through damaged endothelial cell layers. This makes the peptidome of plasma/serum an invaluable source of disease-related information, with substantial promise for clinical applications, particularly in atherosclerotic cardiovascular disease [71,72].

The choice between plasma or serum is again important in this context, and despite serum samples being commonly used for such analyses, the Human Proteome Organization (HUPO) recommends the use of PPP to minimize the interference of platelet-derived peptides during analysis [SPREC: PL2.PED.X.D.D.C] [60]. Even different plasma (EDTA, citrate, heparin) and serum collection tubes (silica-coated with a gel separator or plain tubes with no additives) can result in changes in the peptide profile [62]. The addition of protease inhibitors to plasma EDTA tubes immediately after blood sampling [SPREC: PL1.PED + PIX.A1.Z.N.B.A] has been shown to improve plasma stability and is recommended to avoid the formation of additional peptides. However, beyond adding complexity to pre-processing, protection against additional proteolysis can be short-lived, with this practically disappearing if the delay after centrifugation exceeds 8 h [SPREC: PL1.PED + PIX.A1.Z.N.F.A]. Thus, it has been recommended that protease inhibitors not be added for clinical peptidomics [73], and delays of this length should be avoided [74].

Pre-centrifugation delays can affect serum and plasma differently, with temperature during this period having more of an effect on plasma. Blood plasma tubes should be kept at RT, as low temperatures can activate platelets and leukocytes, leading to the release of peptides and enzymes [75]. For serum, a clotting time of 2 h (at RT or 4 °C) is recommended [SPREC: SER.SST.A.Z.D.A.A] for the best recovery of MS peaks, and this factor can be more critical than storage conditions (time and temperature) in terms of ensuring reproducibility [76].

Another source of variability in plasma peptidomics stems from the so-called plasma degradome (peptide products resulting from unintended enzymatic and physicochemical degradation of proteins), although it should also be noted that a significant number of the peptides present in plasma may arise from the degradation of cell/tissue proteins (e.g., as a result of disease [74]) that subsequently efflux into blood). While the former source is problematic and a confounding factor, cell- or tissue-derived peptides present in blood can be highly valuable sources of information on the presence or severity of disease. The latter peptides are attracting increasing attention as potential clinical biomarkers. Although the majority of plasma proteins (~95 %) remain stable for a pre-centrifugation delay as long as 48 h at RT [SPREC: PL1.EDG.K.B.N.X.A], the generation of peptides from the remaining 5 % of susceptible proteins can introduce bias in peptide biomarker studies [64]. Therefore, ideally, blood should be centrifuged as soon as possible after sampling to obtain plasma, after the anticoagulant has been carefully mixed. A second centrifugation at 2500×*g* for 15 min at RT [SPREC: PL2.PED.A1.B.B.X.A or PL2.SCI.A1.B.B.X.A] is also recommended to obtain PPP, which is fundamental for minimizing interference from platelet-derived peptides [75]. Data obtained to date suggest that the storage of serum at −20 °C results in changes in MS peaks after as little as 3 months, but this does not occur upon storage either at −80 °C or in

liquid nitrogen for up to 12 months, regardless of the thawing method [77]. Nonetheless, there is a clear need for more research on the effects of long-term storage on the plasma/serum peptidome.

3.4. Metabolomics and lipidomics

Metabolomics involves the analysis of low-molecular-mass endogenous metabolites in biological fluids. The composition of the metabolome fluctuates depending on sampling time, health status, and drug use, among others, and it spans a broad chemical space. A key performance measure of any metabolomics protocol is its ability to distinguish meaningful biological variation from bias and random variation, making the considerations discussed in Section 2 particularly relevant here.

Pre-analytical factors significantly influence metabolomics results, a topic extensively reviewed by Chen et al. [78]. Both serum and plasma are widely used in metabolomics, and studies have shown differences based on the primary containers used [79]. In general, metabolites measured in serum show higher sensitivity than in plasma [78]. The generation of serum relies on inherent clotting factors that also serve as therapeutic targets (e.g., in cardiovascular diseases [80]). This may introduce bias in serum samples from patients receiving drugs that affect the blood clotting process. For example, Hagn et al. found that, while metabolite results from serum [SPREC: SER.SST.A1.A.N.B.A] and plasma [SPREC: PL1.PED.A1.A.N.B.A] correlate well, only plasma analyses could reliably detect the impact of acetylsalicylic acid (an inhibitor of cyclooxygenase 2, which affects platelet activation) on metabolites and lipids *in vivo* [81]. Meanwhile, high concentrations of additives in plasma containers can lead to analytical challenges. For example, issues were observed with the analysis of citric acid and its derivatives in citrate tubes [SPREC: PL1.SCI.A1.A.N.X.D] and polar metabolites in EDTA tubes [SPREC: PL1.PED.A1/B1.C.N.X.D], compared with the case for serum [SPREC: SER.SST.A1/B1.C.N.X.D] [82]. The same study found that the pre-centrifugation temperature of serum affects its relative metabolite composition, which is not the case for plasma [82]. The presence of lithium heparin may introduce high background and/or lithium adducts with soft ionization techniques relevant for MS [83,84]. A comprehensive comparison of double-spun and single-spun plasma, utilizing three untargeted metabolomics methods, did not show significantly different results for any of the metabolites analyzed [SPREC: PL1.HEP.X.B.N.B.A and PL2.HEP.X.B.Z.B.A] [85].

Many studies have explored the impact of sample stability during different stages of processing and storage [78]. While some metabolite groups are more prone to degradation, many key human metabolites in serum and plasma remain stable under standard processing and shipping conditions [86]. For example, an evaluation of the impact of an extended pre-centrifugation time showed that, out of 159 metabolites, 140 were stable for up to 24 h on cool packs before centrifugation [SPREC: SER.SST.A1/D/F/.J.A.N.X.Z], while the corresponding number in plasma was 145 [SPREC: PL1.PED.A1/D/F/J.A.N.X.Z] [86]. A small number of freeze/thaw cycles does not appear to significantly affect the metabolome [27,78,86]. Generally, storage at RT at any stage of the process should be avoided [78,87]. Kamlage et al. investigated the impact of hemolysis on plasma metabolomics and found that 18 % and 30 % of the measured metabolites, including lipids, were changed in a significant manner in grade 1 and grade 2 hemolyzed samples [SPREC: PL1.PED.X.C.N.X.A] [87]. The use of hemolyzed plasma will thus introduce bias in metabolomics and lipidomics [84,87].

Lipidomics, which focuses on the analysis of lipids and lipid-like small organic molecules, is sometimes considered a separate omics field and sometimes a subset of metabolomics. The lipid profile appears to be less affected by the type of primary containers used, although some differences have been reported [78,82,88,89], and both serum and plasma are commonly used in clinical lipidomics. Pre-analytical factors can influence both the quantity and quality of lipid extracts, but lipids are particularly sensitive to temperature, light, and artifactual oxidation. Hahnefeld et al. compared two types of plasma coagulants and tested various pre- and post-centrifugation delays [SPREC: PL1.PED/-CIF.A1/B1/C/D/I/J.C.N.Z.A and PL1.PED/CIF.B1.C.N.A/B/C/D/E/F/G/H.A]. They concluded that plasma must be stored on ice and centrifuged within 2 h, and the centrifuged plasma should be frozen within 2 h [88], as was also concluded in other studies [82]. A similar study conducted by the "preanalytics interest group" in the Lipidomics Standards Initiative concluded that plasma samples should be cooled at once and then kept cold, and plasma should be separated within 4 h. While faster and colder processing is generally better, the effects of temperature are more significant than those of time [90].

4. Toward a unified and consensual multiomics sample processing protocol

Single omic studies are often difficult to replicate, which is in part due to the underreporting of sample preparation conditions. Furthermore, the use of different processing protocols is a major source of analytical variation that jeopardizes reproducibility and, ultimately, stalls clinical translation. In the setting of multiomics, this problem is exacerbated by the integration of various omics disciplines in which samples tend to be treated differently. However, there is a theoretical advantage in using the same sample, prepared under the same processing conditions, to run multiomics experiments, as this would reduce variation due to technical artifacts and bring biological variation to the fore.

Liquid biopsies such as blood, which is readily available and can be collected in a minimally invasive manner, are an obvious choice for conducting multiomics studies. Therefore, after reviewing the influence of different pre-analytical factors in all main omics fields, we aimed to propose a unified protocol for blood processing to conduct multiomics on the same sample (Fig. 2). The recommended protocol is intended to deliver the best compromise between classical omics approaches, as supported by the literature reviewed above. If highly specific or particularly sensitive omics analyses are planned, it may be appropriate to consider alternative blood-processing protocols. In these cases, deviations should be reported. In any case, given common laboratory constraints (such as the impossibility of processing samples immediately after obtaining them), alternative steps are suggested to minimize pre-analytical variance. We also pinpoint the major protocol checkpoints such as evaluating hemolysis to control sample quality and take the necessary action if shortcomings are identified.

The first point to address is the choice of the blood-derived product that offers the best compatibility with all omics. The overall recommendation is plasma rather than serum, for which there are several reasons. First, plasma can, in theory, be processed immediately, minimizing the loss of unstable molecules. Second, obtaining serum implies activation of the coagulation cascade, platelet degranulation, and the lysis of some clot-entrapped leukocytes and erythrocytes, with the subsequent release of contaminant DNA, RNA, proteins, and metabolites. Intrinsic coagulation factors will be disrupted if the donor has consumed anticoagulants. Third, for the same reason, circulating molecules may be degraded due to the release of enzymes (DNases, RNases, proteases, metabolic enzymes) and the activation of coagulation proteases. Nevertheless, we also stress that serum might be an appropriate alternative in specific cases. One example of this is in metabolomics, where a high concentration of plasma coagulants can cause severe matrix interference [78]. Before collecting blood into an EDTA tube, it is strongly recommended that a discard tube be used to prevent tissue factor activation. gDNA should be directly extracted from the discard tube; otherwise, it can be collected from the buffy coat after the first centrifugation.

As described in Section 3, plasma can be obtained with different anticoagulants. However, collection tubes with different chemistries (EDTA, heparin, and citrate) protect specific molecule groups while interfering with others, meaning that there is no optimal solution. Heparin is not advised because it interferes with transcriptomics and

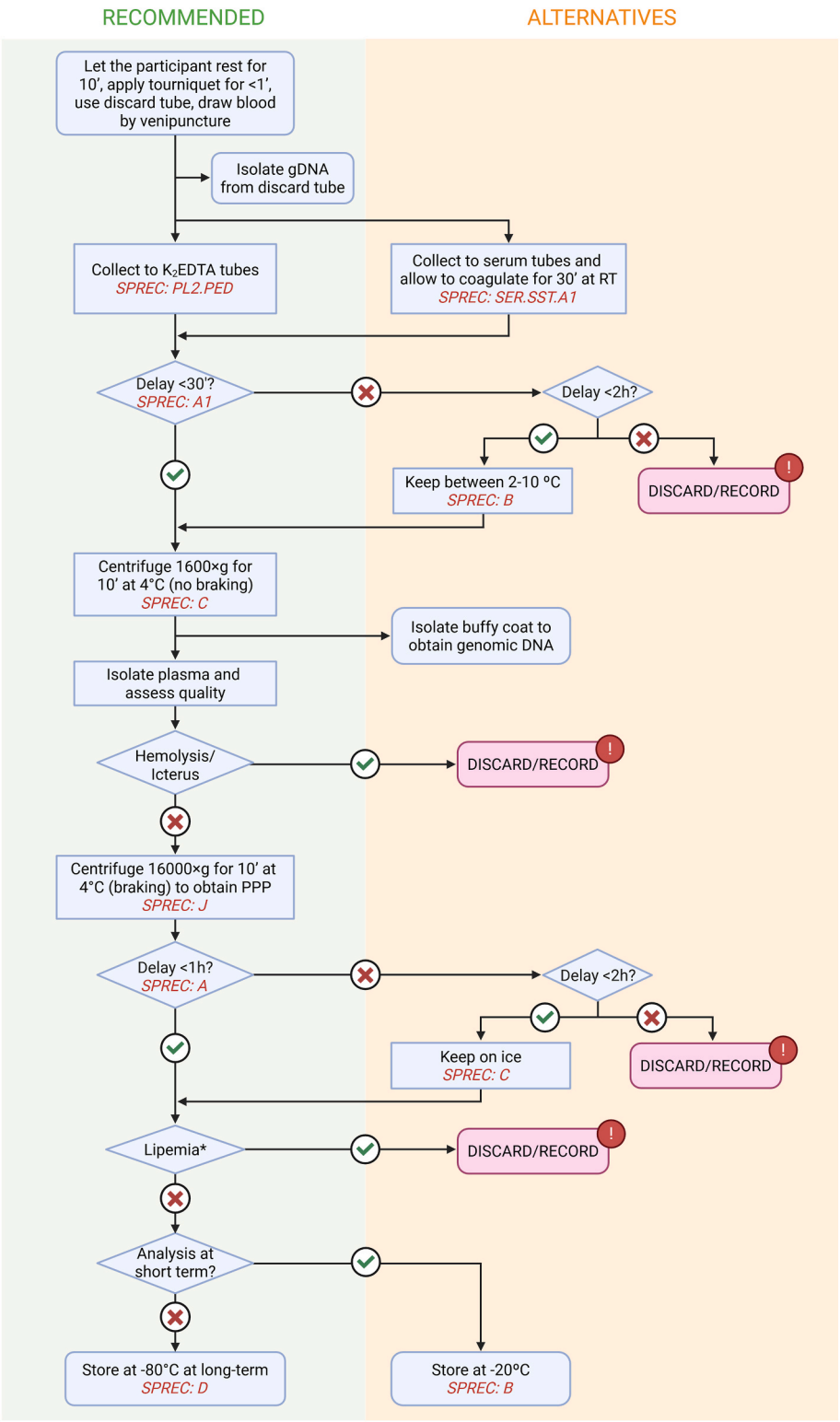

Fig. 2. Unified blood processing protocol for multiomics studies. *Lipemia can be minimized with the second centrifugation to obtain PPP. For samples assigned as lipemic in the first centrifugation round, discarding is not recommended before the second round of centrifugation in which lipemia is reassessed.
Abbreviations: EDTA: ethylenediaminetetraacetic acid; HIL: hemolysis, icterus, and lipemia; PPP: platelet-poor plasma; RT: room temperature; SPREC: Standard PREanalytical Code. Created with BioRender.

may also lead to ion suppression and induce matrix effects in MS, disturbing proteomic and metabolomic profiling. Citrate is less problematic (and has even been recommended for blood RNA analysis), but it also induces a dilution effect and may act as a confounding factor for metabolomic data on the citric acid cycle. EDTA also has its limitations and may interfere with the analysis of some polar metabolites, but our review shows that it offers the greatest pan-omic compatibility among the three standard anticoagulants and should thus be the first choice. Notably, there is insufficient data to draw any definitive conclusions about the best EDTA salts for use in multiomics studies, and the effects of

different salts should be addressed in future work. For now, we follow the Biorepositories and Biospecimen Research Branch of the NIH protocols for cfDNA [91] and miRNA analyses [92], and HUPO for proteomics [60], which opt for K_2 formulations.

Depending on the study design, one may aim to characterize a specific omic subspace. Adding additives to blood tubes is sometimes seen as advantageous for preserving certain molecules and their modifications, such as protease inhibitors for peptidomics. However, the effects of including these substances are not well studied across the omics fields. Therefore, we recommend shortening the pre-centrifugation delay to mitigate any impacts on the peptidome. If opting for an alternative route, the deviation from the standard protocol should be recorded.

Ideally, blood should be processed immediately to avoid the degradation of specific analytes. However, in practice, this is often not possible due to the time for transit between the collection site and the laboratory, and the collection of different samples simultaneously, among other factors. If the pre-centrifugation delay is expected to exceed 2 h, it may be advantageous to keep blood between 2 and 10 °C. The research team should plan this beforehand and carefully assess whether the risk of unplanned delays outweighs the risk of inducing leukocyte/platelet activation caused by cooling of the samples after blood sampling. In any case, if the processing delay extends over 2 h (1st checkpoint), the sample should be discarded or, at least, the delay should be recorded.

For a multiomics protocol, we recommend the use of double-spun plasma, given the well-documented interference of platelets in transcriptomics. Introducing a freezing step between the two centrifugation cycles is discouraged, as freeze–thawing platelet-rich plasma after the first centrifugation has been shown to induce the release of platelet-derived microparticles, that interferes with transcriptomic and peptidomic analyses [93]. The presented studies will often have slightly different centrifugation times and speeds depending on the protocol used and the centrifuge available. Following the reconciliation of different studies and organizations, we recommend a first round of centrifugation at 1600×*g* for 10 min at 4 °C, followed by a second round at 16,000×*g* for 10 min at 4 °C. Centrifugation of blood serves to sediment particulates (cells, cellular debris, platelets, etc.) from solution. Particles in fluid accelerate until the friction equals the net force of gravity. As presented by Chandler [93], Stoke's law is used to estimate sedimentation rate due to centrifugation including the sedimentation of particles in blood. Larger particles will have a higher sedimentation velocity, which explains why methods sensitive to interference by platelets, as the smallest blood cells, require more centrifugation. The terminal sedimentation rate can be expressed as a linear relationship between centrifugation time and acceleration. Thus, variations in these settings are possible, as long as Stoke's law is respected. For instance, when the centrifuge's maximal speed is 12,000×*g*, we recommend extending the centrifugation to exactly 13 min and 20 s (16,000 × 10/12,000 = 13.3 min).

After the first centrifugation, plasma should be carefully inspected (2nd checkpoint), and the levels of hemolysis, icterus, and lipemia (HIL index) should be determined spectrophotometrically. There is currently no standardized system to report the HIL index, but there are guidelines for its assessment (CLSI C56). Generally, samples with a high HIL index should be discarded or, at the very least, recorded. The highest acceptable HIL index should be defined *a priori* during protocol design. Notably, the high speed of the second centrifugation may be sufficient to clear the excess lipids from plasma. Therefore, the lipemia index should be reappraised after this step, before discarding or recording the sample. Additionally, for multiomics studies including miRNA analysis, it is recommended to evaluate the ratio of miR-23a-3p to miR451a and to exclude samples with a delta Cq of 7–8 or higher [94].

Once the PPP is obtained, samples should be aliquoted immediately or, if this is not possible, maintained on ice (2–10 °C) for a maximum of 1 h. The 3rd checkpoint is the time elapsed between the second centrifugation and freezing. While DNA can withstand longer delays, RNA, proteins, and metabolites are degraded more rapidly. Thus, when the post-centrifugation delay extends over 1 h, samples should be discarded or the deviation registered. Finally, samples should be stored at −80 °C in cryotubes, unless exclusively short-term analysis is envisioned, in which case samples can be maintained at −20 °C.

There is a variable level of evidence to support the proposed steps in this consensus blood processing protocol. The selection of plasma over serum, double-spun over single-spun plasma, and removal of hemolytic samples are well supported in the literature of at least one of the evaluated omics fields, while they cause limited problems for the others. However, with regard to the exact coagulant used, specific centrifugation settings, and stability with different pre- and post-analytical delays, there are greater contradictions between and within different omics protocols in the literature. Nonetheless, comparative evaluations across omics fields suggest that consistency and standardization outweigh the influence of minor adjustments to specific preanalytical parameters. Here, in addition to the available evidence in the literature, we relied on the expertise of the co-authors to find the best steps that are acceptable for all omics fields.

5. Limitations

The proposed protocol is the product of an integrative synthesis of evidence across different blood-processing parameters, and the exact protocol has not been empirically tested. A single blood-processing workflow cannot be optimal for every omics technology and analyte class. Our recommendation (K_2EDTA plasma, with PPP obtained by a second spin) represents a compromise that prioritizes broad compatibility and multicenter harmonization, while there are some limitations. Centrifugation conditions designed to reduce platelet carryover may reduce components associated with particle-associated or vesicular fractions, which can be relevant for vesicle-focused analyses. For work related to the work on blood extracellular vesicle research, we refer to the work of MIBlood-EV [95]. On the other hand, while PPP reduces platelet count and platelet-derived microparticles, residual platelets remain [93]. This unified protocol should be followed as a robust default for broad multi-omics profiling, but not as a universal pre-analytical solution for all analytes. If particularly sensitive or unstable analytes are to be analyzed other processing protocols could be needed. The addition of protease inhibitors may be beneficial in degradomic analyses or when examining circulating peptides derived from diseased tissues, where high levels of activated proteases and their resulting peptide products may be present [74]. EDTA improves compatibility for several omics layers but may complicate the measurement of certain metabolite subclasses [78]. Omics-specific alternative protocols and/or matrices should be considered, and any deviation should be explicitly reported (e.g., using SPREC) to preserve interpretability and cross-study comparability.

The SPREC system was used for protocols, although these codes were not presented in the original papers and certain assumptions were made when details were missing (such as type of tubes used for long-term storage at −80 °C). Based on our analysis, the assumptions should not change the overall conclusion. However, the SPRECs we present should thus be interpreted with caution, and reproduction of reviewed procedures should follow instructions in original papers.

The protocol should yield a sample with minimal pre-analytical variance and bias, making it suitable for major omics analyses; however, it is not easy to comply with current routine clinical practice. We are aware that adhering to this protocol in large-scale, multicentric sampling is challenging. There is strong evidence supporting the recommendation of double-spun plasma and the avoidance of long pre-, inter-, and post-centrifugation delays, despite the practical difficulties involved. In the cited literature, information on centrifugation braking was often missing. Disabling braking can extend centrifugation time by up to threefold (with greater effects at higher maximum RPM and in a centrifuge-dependent manner) and introduces variability in the total

centrifugation–time product. Conversely, the use of braking may disrupt pellet integrity. As a compromise between reproducibility and practical feasibility, the proposed protocol applies no braking during the first centrifugation and controlled braking during the second centrifugation.

6. Final comments/remarks

The need to standardize pre-analytical experimental procedures to ensure data reliability, reproducibility, and translatability to industrial development for final clinical application has long been well established, but unfortunately in many instances it is still not properly addressed in clinical research. The pre-analytical phase of a clinical study needs to be implemented in accordance with robust protocols and with the proper recording of a series of related information. Omics approaches may provide a large amount of data, thus accurately describing specific pathophysiological processes and related pharmacological effects [1,96]. Several pre-analytical pipelines have been proposed, mostly focusing on a single omic level, although their practical application still needs to be improved. A further level of complexity is posed by the rapidly growing field of multiomics, with the need to integrate different layers of information to address the intricacy of human pathophysiology. In this regard, there is an urgent, unmet need for a standardized experimental protocol for the pre-analytical management of blood samples. Here, such a protocol is proposed, starting from the collection of venous blood and proceeding to the preparation of PPP, its handling, and its storage for further multiomics analysis. This protocol may not completely remove every source of variability but is expected at least to improve the output of research laboratories. In fact, other pre-analytical variables were not covered here, such as blood draw strategy (participant position, tourniquet application, tube inversion), exposure to light, humidity levels, the use of different storage materials (e.g., polypropylene vs. cryotubes), storage atmosphere (oxygen vs. nitrogen), and freezing method (slow vs. snap freezing), so further research is needed. A ring trial to further identify and address the remaining sources of inter-laboratory variability could be useful to further refine the protocol [43]. Finally, this standardized protocol is expected to contribute to increasing the adoption of research findings by end-users, industry, healthcare providers, and patients.

CRediT authorship contribution statement

Fábio Trindade: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Miron Sopić:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Michael J. Davies:** Writing – review & editing, Writing – original draft, Methodology. **Christos Tsatsanis:** Writing – review & editing, Writing – original draft, Methodology. **Florence Pinet:** Writing – review & editing, Writing – original draft, Methodology. **Helena Beatriz Ferreira:** Writing – review & editing, Writing – original draft, Methodology. **Jelena Munjas:** Writing – review & editing, Writing – original draft, Methodology. **Karine R. Mayilyan:** Writing – review & editing, Writing – original draft, Methodology. **Melissa Marie Formosa:** Writing – review & editing, Writing – original draft, Visualization, Methodology. **Ritienne Attard:** Writing – review & editing, Writing – original draft, Methodology. **Rosienne Farrugia:** Writing – review & editing, Writing – original draft, Visualization, Methodology. **Rui Vitorino:** Writing – review & editing, Writing – original draft, Methodology. **Soliman Khatib:** Writing – review & editing, Writing – original draft, Methodology. **Stephanie Bezzina Wettinger:** Writing – review & editing, Writing – original draft, Methodology. **Susana Novella:** Writing – review & editing, Writing – original draft, Methodology. **Vít Kosek:** Writing – review & editing, Writing – original draft, Methodology. **Yahya Sohrabi:** Writing – review & editing, Writing – original draft, Methodology. **Paolo Magni:** Writing – review & editing, Writing – original draft, Methodology. **Yvan Devaux:** Writing – review & editing, Writing – original draft, Methodology. **David de Gonzalo-Calvo:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Marie Mardal:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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