

Review

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Plasma proteomics: considerations for preanalytical variability; a systematic review with narrative synthesis

<https://doi.org/10.1515/cclm-2026-0330>

Received March 4, 2026; accepted May 9, 2026;

published online June 8, 2026

Abstract

Background: The plasma proteome (PP) is a dynamic system subject to pathology-associated changes and a focus for novel disease biomarker discovery. Disease-related PP research assumes protein concentrations in test specimens accurately reflect the *in vivo* milieu. However, measures to maintain the physicochemical integrity of the proteome before assay are often rudimentary, poorly described, or lacking standardisation in published studies. Contrastingly, in laboratory medicine, there is an expectation that errors in the so-called “preanalytical phase” (PAP) that impact patient results are understood, monitored, and mitigated against, while also being well described in research publications. There is therefore scope for good practice from laboratory medicine to inform PP research workflows. This review considers factors in the PAP which may impact the validity of PP results.

Content: A systematic review was conducted per PRISMA guidelines, limited to English-language peer-reviewed studies (2014–2024). Candidate studies were imported, screened, and managed using Covidence systematic review software.

Summary: 15 eligible studies were reviewed, covering many relevant processes. 11 studies reported statistically significant differences in PP due to factors in the PAP. Temperature and time-to-processing were the most commonly reported

factors affecting the PP, with significant effects reported in 8 studies.

Outlook: PAP variability can significantly affect results in PP studies. Careful consideration of the effect of each stage of the PAP is needed when working with the PP. In multicenter studies, pre-defined and research question-specific sample processing workflows are essential for reducing PAP variability, which helps ensure the validity of PP studies.

Keywords: plasma proteome; preanalytical; variability; proteomics

Introduction

Clinical potential of the plasma proteome

The term “proteome”, a compound of “protein” and “genome”, was coined by Wilkins in 1994 [1]. In vertebrates, the dynamic, complex system of peptides and proteins found in an individual’s plasma, including those encoded by the host genome and those derived from surrounding cells and tissues, is termed their “plasma proteome” (PP) [2].

The human PP holds enormous potential to revolutionise diagnosis, prognosis, and therapeutic monitoring of disease [3]. An individual PP reflects physiology at a given time point and, because it responds to many stimuli, can change depending on the physiological or pathological state. The study of PP differences in individuals with distinct physiological or disease states is termed “differential proteomics” [4], and it is this branch of proteomics that can aid in the discovery of novel biomarkers for disease diagnosis and monitoring.

The preanalytical phase in laboratory medicine and research settings

The total testing process (TTP) concept in laboratory medicine holds that the generation of any result comprises many

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steps, grouped into the preanalytical phase (PAP), the analytical phase, and the post-analytical phase [5].

Processes in the PAP concern test ordering, sample collection, specimen transport, and sample preparation before analysis, and involve staff from many disciplines, including scientists, clinicians, phlebotomists, and laboratory medicine professionals [6]. Given the involvement of multiple staff groups, including non-scientific staff, it is perhaps unsurprising that the PAP has been identified as the source of up to 68 % of all errors across the TTP [7]. Minimising errors in the PAP is, therefore, crucial to reduce direct or compound errors later in the TTP and ensure reliable clinical outcomes. Negative downstream patient effects of PAP errors include repeat phlebotomy, delays in laboratory reporting, or the release of spurious results [8].

PAP testing in laboratory medicine is informed by best-practice guidelines from international bodies and by the need to meet the relevant requirements of national accreditation bodies [7, 9]. The Working Group for the Pre-analytical Phase (WG-PRE) of the European Federation for Clinical Chemistry and Laboratory Medicine (EFLM) has endeavoured to improve and harmonise practice regarding the PAP across the laboratory medicine community [10]. Analytes measured in routine clinical practice, including some proteins, are known to be susceptible to interference or to have very short stability limits. Stringent PAP processes are required to ensure accurate and reliable results for such measurands. In research settings, one may also consider that there is a PAP, with errors that might compromise the validity of analytical results and increase the risk of missing significant effects or observing spurious effects [11]. In PP research, the stability of proteins, which may serve as novel biomarkers, is often unknown, and there are limited guidelines for harmonising and standardising PAP procedures [12]. Given that the laboratory medicine community has, over several decades, advocated for error reduction in the PAP, there is an opportunity to apply best practice from this specialism to research settings, including biomarker discovery in the PP. Considering that blood plasma is one of the most widely used specimen types in laboratory medicine, many error-mitigating processes in the PAP, such as those around phlebotomy and specimen handling, are designed to maintain the integrity of analytes in this matrix, which is identical to that used in PP studies. Such processes have long been subject to rigorous inspection and accreditation under ISO 15189, and are hence considered robust [13, 14].

Therefore, given the existing knowledge on quality control and management of the PAP in laboratory medicine, and the shared components of the PAP across both routine service provision and research, the clinical laboratory

serves as a valuable model for PAP requirements in PP research (Figure 1).

For proteomic studies, the authors have identified the following key areas in the PAP which require careful documentation and standardisation, particularly in multicenter studies.

- (1) Plasma anticoagulant type/formulation.
- (2) Pre-centrifugation delay. The time elapsed between sample collection and the separation of whole blood into its desired fractions.
- (3) Centrifugation settings. Including: time, temperature, G force, use of a brake, and multiple centrifugations.
- (4) Post-centrifugation storage delay. The time between sample centrifugation and long-term storage before analysis.
- (5) Long-term storage pre-analysis.

This systematic review aims to explore recent studies which have addressed these critical steps in the PAP, highlighting their impact on sample integrity and downstream analyses.

Methods

Criteria for consideration in the review

Given the myriad factors in the PAP that might affect analyses of the PP, the inclusion criteria for publications in this review were intentionally broad. Studies meeting the following criteria were extracted and analysed:

- Focused in whole, or in part, on quantitation or characterisation of the plasma proteome, regardless of the analytical technique used.
- Examined, in whole or in part, PP changes due to PAP variability.
- Were performed in human-derived samples.
- Contained samples from healthy controls and individuals with known pathology; for this review, only information on healthy controls was extracted.
- Published initially in English.
- Published within the 10 years 2014–2024 inclusive.

Search methods

This systematic review was conducted in accordance with PRISMA guidelines [15] (Figure 2). A comprehensive search strategy was developed using key terms such as “Preanalytical Variability”, “Plasma Proteome”, “Delayed Processing”, and “Blood”, along with the previously described inclusion criteria. Searches were performed on PubMed, Embase, and

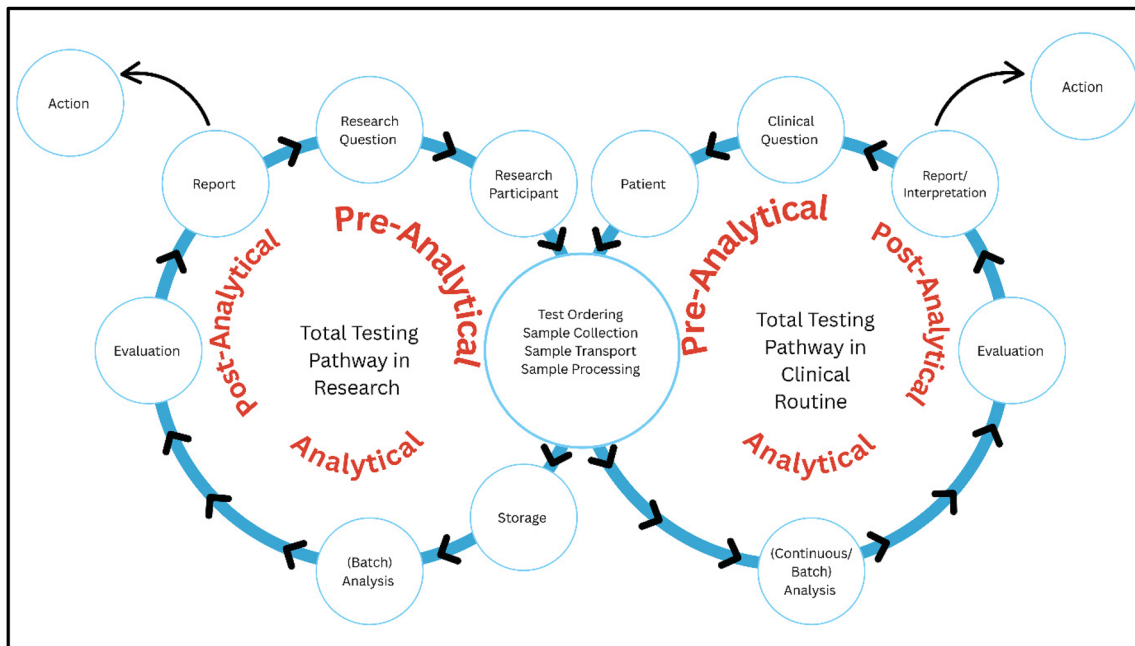


Figure 1: Shared components of the total testing pathway in research and routine clinical laboratory medicine.

Scopus databases on 19th March 2025, using database-specific strings (Supplementary Table 1, Search Strings).

Data collection and analysis

Papers returned from the database searches were imported into the systematic review software, Covidence, for management and further analysis [16]. Initial screening and data extraction were completed independently by two reviewers (CV&AS), with any conflicts resolved by a third independent reviewer (DM). Abstract screening was conducted in accordance with the inclusion criteria. Two researchers (CV&AS) developed the data extraction template. The included studies were assessed for bias using the Cochrane Risk of Bias-2 (ROB-2) assessment tool [17].

The GRADE framework was applied to assess the certainty of evidence for each preanalytical variable [18] (Supplementary Table 2, GRADE Assessment). Each of the first four key PAP variables was assessed across the five GRADE domains. Inconsistency was assessed by whether findings across studies were directionally consistent. Risk of bias was assessed as previously outlined. If one or more studies for the PAP variable being assessed by GRADE contained a high risk of bias, the certainty of evidence was downgraded. Where publication bias assessment was not possible, the bias score was marked as uncertain, and the GRADE rating remained unchanged. Final certainty ratings were classified as high, moderate, low, or very low. Combined effects of PAP variables,

e.g. anticoagulation type and pre-centrifugation delay in combination, were not assessed using the GRADE criteria.

Included studies that examined a pre-centrifugation delay and thus analyte stability were assessed against the Criteria for Reporting Stability Studies (CRESS) [19]. The CRESS criteria are a set of 20 criteria published by the EFLM-WG-PRE. These criteria assess the reproducibility and considerations for reporting stability studies. Weighted letter grading provides a score from A to D, where an achieved letter grade of A indicates “Best in Class” and a D grade indicates a “Fail”. Assessing the quality of published studies against the CRESS criteria may allow for broad commentary on the current standard for PP stability studies.

Results are presented according to the sequence in which PAP variables are typically encountered in laboratory workflows. Beginning with the anticoagulant tube type, followed by pre-centrifugation delay, centrifugation settings, post-centrifugation delays, and finally long-term storage. Studies examining the combined effects of PAP variables are reported under the first relevant PAP category. This structure provides a clear workflow-aligned framework for interpreting the evidence.

Systematic review registration

This systematic review was prospectively registered on the systematic review registry PROSPERO, ID: CRD420251014879.

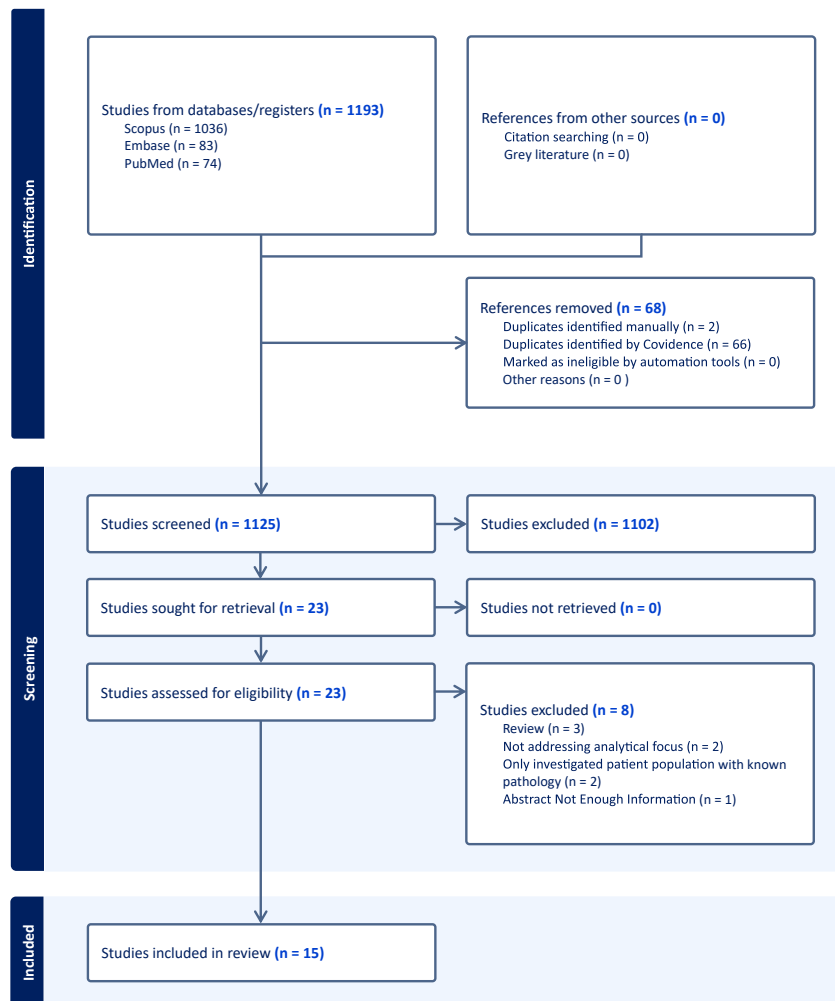


Figure 2: PRISMA flow diagram for the selection of studies.

Results

Literature search results

A total of $n=1,193$ results were returned when the search string was entered into the Scopus, PubMed, and Embase databases. Duplicates ($n=68$) were consolidated before screening. Following the inclusion criteria outlined above, $n=23$ articles were successfully retained. Three review articles were identified from the search; they include studies outside of the scope of the time period considered and serve as valuable resources for assessing whether findings have changed in more recent publications. Five studies were excluded since they did not address the analytical focus of the review ($n=2$), involved only a patient population with known pathology ($n=2$), or were conference abstracts that lacked sufficient information. Finally, $n=15$ studies remained for detailed analysis. The general characteristics of the included studies are shown in Table 1.

Overall, the retained studies varied considerably in sample size, analytical platform, and the preanalytical variables examined. Most studies assessed preanalytical variability by comparing groups and reporting statistical significance based on p-values. One study showed a high risk of bias, with limitations relating to missing data and outcome reporting (Figure 3) [20]. Based on the CRESS analysis, one study received an “A” weighted letter grade, while the remaining studies received a “B” grade (Supplementary Table 3, CRESS Assessment).

The most common analytical method in the identified studies was LC-MS/MS, while the most widely used immunoassay was Proximity Extension Assay. Studies generally assessed preanalytical variability by comparing groups and reporting statistical significance based on p-values.

Anticoagulant type/formulation

Four studies investigated the influence of the anticoagulant type used in the PAP on the plasma proteome (Table 2).

Table 1: Study characteristics.

Author and year	Title	Gender	Age range, years	Fasting status	Number of donors	Study design	Statistical test(s) ^a	Analysis technique
Ammerlaan et al. [21]	Method validation for preparing serum and plasma samples from human blood for downstream proteomic, metabolomic, and circulating nucleic acid-based applications.	Not stated	Not stated	Not stated	1	Randomised control trial	Paired <i>t</i> -test, one-way ANOVA with post-hoc correction	LC-MS
Cao et al. [22]	An integrated analysis of metabolites, peptides, and inflammation biomarkers for assessment of pre-analytical variability of human plasma.	Male and female	24–64	Yes	20	Randomised control trial	Repeated-measures ANOVA (Latin square design), with multiplicity correction	LC-MS/MS
Daniels et al. [23]	Stability of the human plasma proteome to pre-analytical variability as assessed by an aptamer-based approach.	Male and female	18–65	Yes	20	Randomised control trial	Repeated-measure ANOVA with post-hoc correction, with BH-FDR correction	SOMAscan assay version 1.3k
Gegner et al. [24]	Preanalytical processing of plasma and serum samples for combined proteome and metabolome analysis.	Male	22–37	Not stated	6	Randomised control trial	t-statistics and moderated F-statistics with BH-FDR correction (LIMMA analysis)	Mass spectrometry
Halvey et al. [25]	Variable blood processing procedures contribute to plasma proteomic variability.	Not stated	Not stated	Not stated	1–4	Randomised control trial	t-statistics and moderated F-statistics with BH-FDR correction (LIMMA analysis)	LC-MS/MS
Haslam et al. [26]	Stability and reproducibility of proteomic profiles in epidemiological studies: comparing the Olink and SOMAscan platforms.	Male and female	24–57	Yes	14	Randomised control trial	Intraclass correlation coefficients and Spearman's <i>r</i>	Olink and SOMAscan7K version 4.1
Hassis et al. [27]	Evaluating the effects of preanalytical variables on the stability of the human plasma proteome.	Male and female	22–39	Not stated	20	Randomised control trial	Linear regression, with multiplicity correction (BH)	LC-MS
Huang et al. [28]	Assessing the preanalytical variability of plasma and cerebrospinal fluid processing and its effects on inflammation-related protein biomarkers.	Male and female	23–57	Not stated	3	Randomised control trial	Paired student's <i>t</i> -test	Proximity extension assay
Ilies et al. [29]	Impact of blood sample collection methods on blood protein profiling studies.	Male and female	24–29	Not stated	6	Randomised control trial	Descriptive, Pearson correlation	LC-MS/MS
Kim et al. [30]	Stability and reproducibility of proteomic profiles measured with an aptamer-based platform.	Male and female	24–57	Yes	12	Randomised control trial	Intraclass correlation coefficients and Spearman's <i>r</i>	SOMAscan assay version 1.3k
Lee et al. [31]	Impact of whole-blood processing conditions on plasma and serum concentrations of cytokines.	Not stated	Not stated	Not stated	88	Randomised control trial	Repeated measure ANOVA, and paired 2-tailed <i>t</i> -test	Milliplex map human cytokine/chemokine magnetic bead panel kit-immunology milliplex assay

Table 1: (continued)

Author and year	Title	Gender	Age range, years	Fasting status	Number of donors	Study design	Statistical test(s) ^a	Analysis technique
Mateos et al. [20]	Multicentric study of the effect of preanalytical variables in the quality of plasma samples stored in biobanks using different complementary proteomic methods.	Male and female	Not stated	Not stated	100	Randomised control trial	Descriptive, ANOVA or <i>t</i> -test	SPE MALDI-TOF, DIGE, shotgun (iTRAQ, nLC MALDI TOF/TOF) and targeted LC MS/MS proteomic techniques
Nambu et al. [32]	Effects of differences in preanalytical processing on blood protein profiles determined with SWATH-MS.	Not stated	Not stated	Not stated	3	Randomised control trial	Student's <i>t</i> -test	SWATH-MS analysis
Roskopf et al. [33]	The preanalytical processing of blood samples for detecting biomarkers on protein microarrays.	Female	Not stated	Not stated	4	Randomised control trial	Descriptive, permutation <i>t</i> -test with random variance ($p < 0.005$ and $p < 0.01$)	Protein microarray
Shen et al. [34]	Strong impact on plasma protein profiles by pre-centrifugation delay but not by repeated freeze-thaw cycles, as analysed using multiplex proximity extension assays.	Male and female	24–62	Not stated	16	Randomised control trial	Pearson, Spearman, repeated-measures ANOVA, multiplicity correction by bonferroni.	Multiplex proximity extension assay

^aSignificant p -value < 0.05 unless otherwise stated. ANOVA, analysis of variance; BH, Benjamini-Hochberg; DIGE, differential gel electrophoresis; FDR, false discovery rate; iTRAQ, isobaric tags for relative and absolute quantitation; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; MALDI TOF/TOF, matrix-assisted laser desorption/ionisation tandem time-of-flight; nLC, nanoflow liquid chromatography; SPE, solid phase extraction; SWATH-MS, sequential window acquisition of all theoretical mass spectra.

Plasma proteome differences due to anticoagulation differences alone

Evidently, studies examining the effects of anticoagulant tube type as the main difference on the PP tried to ensure that all other PAP variables remained constant and that samples were processed and placed in long-term storage immediately after collection.

Halvey et al. [21] demonstrated statistically significant participant-specific differences in 36 proteins when comparing PP observed with different anticoagulants (EDTA vs. heparin). The majority of proteins were observed to have lower concentrations in the heparin-treated tubes than in the EDTA-containing tubes.

Ilies et al. [22] observed that different anticoagulant tube types used in the PAP can yield distinct protein profiles, specific to each tube type. They found that sets of 61, 46, and 37 proteins are unique to EDTA, sodium heparin, and sodium citrate PPs, respectively. That same study showed that a core set of 187 blood proteins was common to PPs across all anticoagulant tube types in the population examined.

Based on the synthesis of results and applying the GRADE framework for evaluating the certainty of evidence, there is a moderate (+++) certainty of evidence that the anticoagulant tube type used in the PAP has an impact on the observed PP.

Combined anticoagulation and pre-centrifugation delay differences

Haslam et al. [23] highlighted the critical impact of pre-analytical factors on downstream analyses. Their study identified that certain protein classes showed greater observed stability with specific anticoagulants, and that this observed stability was also influenced by the analytical platform utilised. When performing analyses on the Olink platform, an antibody-based proximity extension assay, they demonstrated that hormones exhibit greater observed stability in heparin-treated tubes than in EDTA-treated tubes. Transcription factors and transferases display better stability in EDTA plasma samples when measured on the same platform and subjected to identical pre-centrifugation

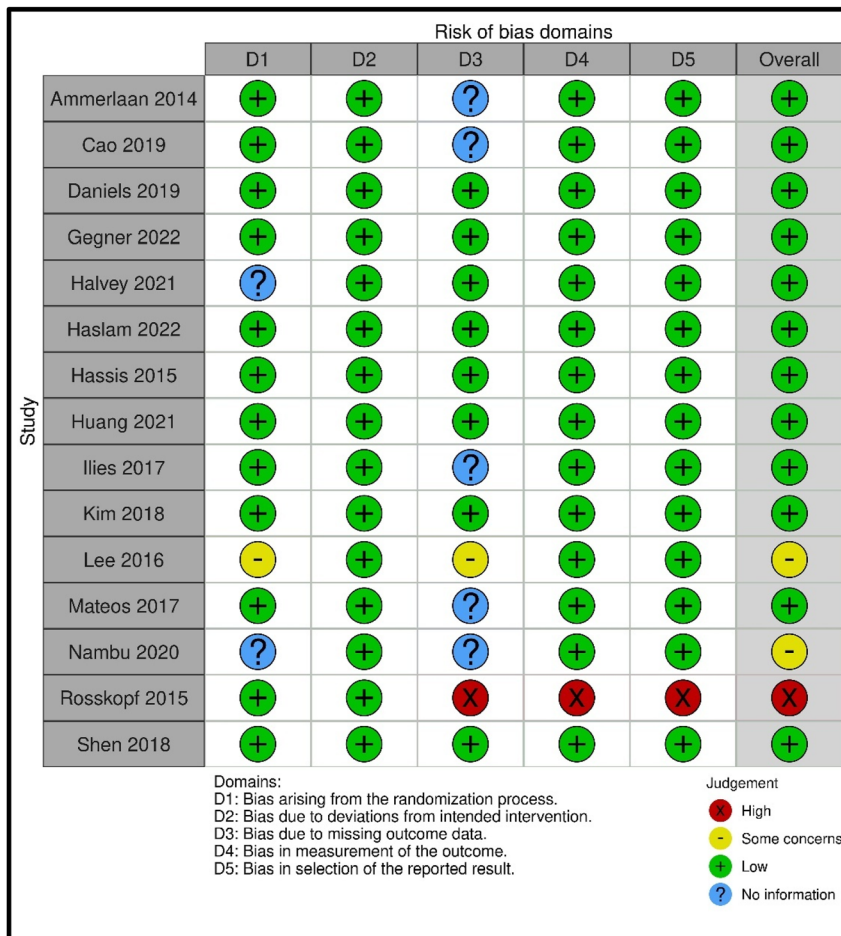


Figure 3: Risk of Bias Scores for identified studies assessed by the Risk of Bias-2 (ROB-2) assessment tool.

Table 2: Comparison of anticoagulant tube type in the plasma proteome studies.

Author	Anticoagulant	Findings
Halvey et al. [25]	K ₂ -EDTA vs. lithium heparin	36 protein abundance differences observed. Most protein concentrations are lower with lithium heparin than with EDTA.
Haslam et al. [26]	EDTA vs. heparin	The stability of protein classes differs between the anticoagulant tube types used.
Ilies et al. [29]	EDTA vs. sodium heparin vs. sodium citrate	Unique proteins were identified based on the anticoagulant used, with a core set of 187 proteins common to all three.
Kim et al. [30]	EDTA VS. sodium heparin	Protein concentrations were lower with sodium heparin than with EDTA.

delays. Interestingly, under the same experimental conditions, an aptamer-based assay on the SOMAscan 7k platform showed that overall protein stability was greater in heparin-

treated tubes than in EDTA-treated tubes. This was particularly noticeable when delays before centrifugation extended to 48 h. The proportion of proteins showing greater observed stability under these PAP conditions varied substantially by protein class when measured on the Olink platform. In contrast, observed stability under the same PAP conditions was more comparable between protein classes when measured on the SOMAscan 7k platform.

In an earlier study, using the same anticoagulants, Kim et al. [24] investigated the effects of anticoagulant type and pre-centrifugation delay using an older version of the SOMAscan assay, the SOMAscan 1.3k platform. In contrast, their study demonstrated that overall protein stability was better in EDTA samples than in heparinised samples. However, both studies agreed that stability varied across protein classes and subclasses, with hormones generally more stable than kinases. The differences in findings across platform versions are acknowledged in Haslam et al. [23]. However, the absolute number of proteins demonstrating good stability in each protein class is higher across all matrices and categories for the newer SOMAscan 7k platform.

Combined anticoagulation and temperature differences

Halvey et al. [21] demonstrated a significant interaction between anticoagulant tube type and temperature. Nevertheless, this interaction effect influences the PP to a significantly lesser extent compared to the effects of temperature and time to centrifugation, as assessed by Halvey et al. [21] through the comparison of p-values.

Pre-centrifugation delay

Several studies examined alterations in the PP due to pre-centrifugation delays (Table 3) [21, 23–33]. The studied temperatures mimic those encountered routinely in both clinical and research settings, cooler temperatures (often 0 °C or 4 °C) and warmer temperatures (room temperature or 37 °C). Unfortunately, neither Kim nor Haslam et al. [23, 24] provided information on the temperature to which their samples were subjected during their pre-centrifugation delay experiments. Given that the temperature samples are exposed to during a pre-centrifugation delay may influence the observed changes in the PP, this limited our ability to present the results of these studies in this section. Studies have been grouped according to the temperatures to which samples were exposed during the pre-centrifugation delay.

Effects of pre-centrifugation delays at 0 °C and room temperature

Both Cao et al. and Daniels et al. [25, 33] examined a pre-centrifugation delay of 6 h at 0 °C and at room temperature. However, the former group examined changes at the peptide level, while the latter examined changes in protein abundance under the specified conditions. At the peptide level, there was a greater number of peptides affected by a pre-centrifugation delay of 6 h at room temperature. At the protein level, the opposite was observed: more statistically significant alterations occurred at 0 °C for the same pre-centrifugation delay. Although results differ slightly between the research groups, both identified that pre-centrifugation delays significantly alter complement C4b, with both studies demonstrating increased levels.

Effects of pre-centrifugation delays at 4 °C and warmer temperatures

Both Halvey et al. and Shen et al. [21, 32] observed that the PP was more prone to change when delayed centrifugation was performed at room temperature. Using protein

Table 3: Summary of the impact of pre-centrifugation delay on plasma proteomic analysis.

Author	Temperature, °C	Maximum time delay, hours	Findings
Ammerlaan et al. [21]	2–8 or room temperature	2	Statistically significant differences in micro-particle counts were observed with delays in centrifugation. However, this did not have a statistically significant impact on proteomic analysis.
Cao et al. [22]	0 on wet ice or room temperature	24	The storage of whole blood for up to 24 h resulted in significant changes in peptide levels and inflammatory biomarkers.
Daniels et al. [23]	0 on wet ice or room temperature	6	Changes in protein abundance were observed in delayed processing samples compared with those processed immediately.
Halvey et al. [25]	4 or room temperature	96	As a primary effect, the time before centrifugation yielded the greatest number of statistically significant differences.
Haslam et al. [26]	No information provided	48	Reduced protein stability was observed as the time before centrifugation increased.
Hassis et al. [27]	Room temperature or 37	96	Pre-centrifugation delays of 96 h had profound effects on plasma protein abundance.
Huang et al. [28]	Room temperature	72	Measured proteins show mainly increased concentrations with pre-centrifugation time delays.
Kim et al. [30]	No information provided	48	Reduced protein stability was observed as the time before centrifugation increased.
Lee et al. [31]	Room temperature	24	Significant changes in cytokine concentration measurements were observed when centrifugation was delayed.
Mateos et al. [20]	4 or room temperature	24	No detectable significant changes were

Table 3: (continued)

Author	Temperature, °C	Maximum time delay, hours	Findings
			observed as a result of delays in blood processing.
Nambu et al. [32]	0–5 or room temperature	8	Five proteins displayed significantly higher expression after subjection to delays in processing at room temperature.
Shen et al. [34]	4 or 22	36	Extended storage of whole blood before centrifugation can alter plasma protein profiles.

concentrations measured after a 1-h delay at 4 °C as a reference, the latter team observed proteome preservation for up to 8 h at 4 °C, with 5 % of proteins surpassing their reference threshold. In comparison, proteome preservation was observed for 1 h at 22 °C, with 6 % of proteins breaking their reference threshold. Nambu et al. [31] made similar observations between samples kept at 0–5 °C and room temperature. More proteins showed statistically significant changes over time in uncentrifuged samples at room temperature than at refrigerated temperatures. This apparent temperature-dependent exacerbation of protein alterations further supports the observations of Hassis et al. [29] They studied the effects of a pre-centrifugation delay at room temperature compared to 37 °C. At room temperature, 41 statistically significant alterations were observed, compared to 83 at the higher temperature, for the same pre-centrifugation delay.

Of the papers that studied pre-centrifugation delays at these temperatures, two studies [27, 28] concluded that there were no statistically significant changes in the PP. Mateos et al. [28] demonstrated no statistically significant changes in plasma proteins when samples were subjected to pre-centrifugation delays of 4 h at room temperature and 24 h at 4 °C.

Ammerlaan et al. [27] reported no statistically significant difference in results when a 2-h pre-centrifugation delay was used at 4 °C or at room temperature for proteomic analysis. The authors acknowledge that their small sample size ($n=3$) limits the conclusions, and negative results should be interpreted with caution.

Synthesising the results on the effects of a pre-centrifugation delay on the PP and applying the GRADE

framework to evaluate the certainty of evidence, it can be concluded that the evidence is of moderate certainty. A pre-centrifugation delay in the PAP will likely affect the PP.

Centrifugation settings

Many studies directly investigated whether variability in the centrifugation step of blood processing contributed to alterations observed in the PP (Table 4) [25, 29, 33, 34].

Gegner et al. [34] initially utilised two different g-forces ($2,000 \times g$ and $4,000 \times g$) in their study and investigated whether statistically significant differences in plasma proteins occur at different centrifugal forces. They concluded that there were no significant effects due to differences in RCFs.

Hassis et al. [29] employed two different published blood-processing Standard Operating Procedures (SOPs) in their study. One of which entailed a single spin for plasma processing [35]. The other employed a double-spin process, in which blood was first processed to plasma, and then the plasma was aliquoted and spun a second time at a higher speed [36]. They concluded that changes observed due to a pre-centrifugation delay and elevated temperature during that delay could not be distinguished solely from differences

Table 4: Impact of centrifugation settings.

Author	Centrifugation settings			Findings
	<i>g</i>	Time, min	Temperature, °C	
Cao et al. [22]	2,000 or 1,300	20	Room temperature	No statistically significant observations were noted in protein measurements.
Daniels et al. [23]	2,500 or 1,300	15	Room temperature	Statistical significance in protein abundance was observed.
Gegner et al. [24]	2,000 or 4,000	10	No information provided	Could not attribute any significant changes in the plasma proteome between centrifugation settings.
Hassis et al. [27]	Double spin 1st 1,500 2nd 2,000 or single spin 1,500	Double spin 15 15 or single spin 20	4	Changes observed in the plasma proteome could not be distinguished based on centrifugation settings.

in the centrifugation protocol. Indicating that differences in centrifugation settings alone are not sufficient to cause statistically significant differences in the PP.

Cao et al. and Daniels et al. [25, 33] both defined $1,300 \times g$ as a low RCF for plasma processing. Upon processing plasma at this speed and comparing results with their control speed of $2,500 \times g$, the former group observed no statistically significant differences in the proteins they investigated. In contrast, the latter group observed statistical differences in protein abundance when a lower centrifugal force was applied. Daniels et al. [25] analysed 1,305 proteins, while Cao et al. [33] identified 15 unique proteins associated with the peptides analysed. Investigating these conflicting results further by evaluating the proteins these studies had in common revealed that neither group found statistical significance for the same proteins. The significant difference in the number of proteins studied between the groups can account for the reported differences in their findings.

Based on the synthesis of results and the GRADE framework for evaluating the certainty of evidence, there is moderate (+++) certainty that centrifugation settings alone did not contribute to observed PP differences. However, the authors strongly recommend using uniform centrifugation settings in proteomics research.

Post-centrifugation storage delay

Three studies [29, 33, 34] investigated the effects of a post-centrifugation delay. Two studies investigated the effects of delayed storage at 24 h only, while one study investigated the effects at different time points.

The studies [29, 33] that examined the effects of delayed plasma storage for 24 h only, one examined changes at 4 °C and at room temperature [33]. While the other examined effects at room temperature only [29]. Both studies observed statistically significant alterations in plasma proteins.

Gegner et al. [34] investigated the effects of delayed plasma storage at different time intervals, providing valuable information on the stability limits when a delay is unavoidable. Their study demonstrated that rapid handling of plasma at 4 °C, with storage for less than 2 h, offers the best preservation of plasma proteins.

Based on the evidence, a post-processing delay is likely to affect the observed PP; therefore, in the GRADE assessment, it has been assigned a moderate (+++) level of certainty of evidence. However, for the certainty of our recommendation for plasma proteomics, we have assigned a strong certainty of evidence to our recommendation.

Long-term storage pre-analysis

Most studies included in this systematic review stored samples at -80 °C before analysis [20–22, 25, 26, 28, 29, 31–34]. While some did not report their storage temperature [27], one study stored samples at -70 °C [30].

Discussion

Anticoagulant type/formulation and its effect on the observed PP

Different anticoagulant formulations are used across routine services and research, including sodium citrate, potassium salts of EDTA, lithium/sodium heparin, and fluoride/oxalate. The studies identified in this review collectively examined only three routinely encountered anticoagulant formulations. These anticoagulants prevent clotting through different mechanisms, including calcium chelation by citrate and EDTA, and thrombin inhibition by heparin, which may contribute to differences in the observed PP [37].

EDTA is considered a more inert anticoagulant than heparin. EDTA also inhibits divalent cation-dependent proteases, thereby offering proteins some protection from proteolytic processes. Therefore, standardised blood collection protocols generally use EDTA as the preferred anticoagulant for plasma collection [35]. Based on our findings, we recommend that, when designing the preanalytical phase of a clinical research study, the downstream analytical requirements – such as the analysis platform and analyte of interest – be considered when selecting the appropriate anticoagulant tube type, as each has its caveats. Additional complexity arises from different formulations of EDTA anticoagulant tube types being manufactured, with choices available in salt forms containing EDTA complexed with either 2 (K_2) or 3 (K_3) potassium molecules. Therefore, when EDTA is selected as the anticoagulant, we recommend that multicenter studies utilise tubes from the same manufacturer and containing the same potassium formulation whenever feasible.

While the exact analytical platform that will be used to generate results may not always be known, or may change due to technological advancements, following the study design stage. We recommend choosing K_2 -EDTA due to its protection against proteolysis.

Inconsistencies in sample handling can propagate through the analytical pipeline, affecting data quality, biomarker discovery, and clinical utility. Our findings

highlight variability in observed PP stability, depending on both the anticoagulant tube type and the analytical platform utilised, underscoring the need for preanalytical protocols tailored to downstream analyses.

Pre-centrifugation delay and its effect on the observed PP

The vast majority of papers included in this review examined and identified pre-centrifugation delay as a statistically significant factor influencing qualitative and quantitative analyses of the PP [21, 25, 26, 29–33]. It is recommended that wholeblood plasma be centrifuged as soon as phlebotomy is complete. However, if a pre-centrifugation delay cannot be avoided, it is recommended that samples be stored at 4 °C for no longer than 4 h. Our findings are consistent with previously published recommendations on preanalytical sample processing. It is also recommended that pre-centrifugation delays be meticulously documented and taken into account during data analysis and results interpretation.

Many studies within the scope of this review discussed inter-individual variation among PPs and how subject-specific PP alterations can occur within samples subject to the same PAP conditions. Cao et al. [33] reported that 4 of 20 participant samples showed distinct alterations in plasma fibrinogen concentrations under the same PAP conditions compared with other participant samples. Interestingly, the study by Mateos et al. [28], which pooled samples from 100 individuals prior to analysis, found no statistically significant changes in the plasma proteins examined. Pooling in this way does not allow researchers to account for subject-specific changes in PP concentrations under the same PAP conditions and may obscure patient-specific effects. Therefore, through their pooling of samples, Mateos et al. may have masked changes in the PP due to a pre-centrifugation delay.

The effect of centrifugation settings on the observed PP

Studies considering the effect of centrifugation protocols suggest that, at least for the parameter settings examined, they do not have a significant influence on PP compared with other preanalytical variables.

Confusion can arise from inconsistent terminology for describing centrifugation settings, and the use of different centrifuge models or rotors across laboratories can increase the risk of errors [38]. Importantly, disparities between theoretical understanding and practical application of

centrifugation principles may also further contribute to variability between study sites [39]. In the absence of standardised terminology, identical centrifugation protocols may be interpreted and executed differently in practice.

Therefore, the authors strongly recommend that centrifugation settings be consistent across multicenter studies. Through employing standardised terminology and centrifugation settings, researchers can focus on controlling other areas of the PAP that have been highlighted as having a greater impact on the PP. One approach to ensuring uniform centrifugation settings across multiple study sites would be to use blood collection tubes from the same manufacturer and adhere to that manufacturer's recommended centrifugation settings.

Post-centrifugation storage delay and its effect on the PP

All studies identified in this review agreed that delays in long-term storage of plasma lead to statistically significant alterations in components of the PP. Gegner et al. [34] reported that rapid handling of plasma at 4 °C, with storage within 2 h, offers the best preservation of the plasma proteins examined in their study. Their findings are in agreement with published guidelines on the processing and storage of plasma samples prior to analysis. The Human Proteome Organization Plasma Proteome Project (HUPO-PPP) proposes that a common practical compromise is to process and aliquot plasma samples within 1–2 h of collection, with storage at –80 °C [40].

Prolonged plasma contact with the red cell layer after centrifugation has been documented as a source of variability in routine biochemical tests [41, 42]. Delays in aliquoting and storing plasma may allow for the active and passive movement of analytes between cellular components and the plasma fraction, leading to variability in the PP. Importantly, PP integrity may also be compromised independently of red cell contact, reflecting the intrinsic instability limits of the component proteins, and underscoring the need to minimise post-centrifugation delays regardless of cellular contamination. Therefore, the authors have placed a strong certainty of evidence in our recommendation, which also aligns with previously published guidelines, thereby enhancing its credibility.

Long-term storage pre-analysis

According to the HUPO-PPP, samples should ideally be snap-frozen and stored in liquid nitrogen; this procedure was

followed by two studies identified in this review [23, 24]. However, HUPO-PPP acknowledge this storage method is impractical for most and agrees that a suitable compromise is storage at -80°C with minimal temperature fluctuations. The Early Detection Research Network, a National Cancer Institute initiative, was funded to discover and validate systemic biomarkers. They are a multinational consortium that designs SOPs for the processing of plasma samples. They agreed with Rai et al. [40] of the HUPO-PPP that, if liquid nitrogen was unavailable for sample storage, -80°C was suitable for sample preservation [35]. With almost all studies storing samples at -80°C , there is a clear understanding of the currently accepted long-term storage conditions required for sample preservation.

How strategies in the PAP of clinical laboratory medicine may help manage the PAP in plasma proteomics research

Clinical laboratories often perform measurements for analytes with short stability limits and have processes to ensure the accuracy and validity of these test results, upon which clinical decisions may be made. In recent years, accreditation bodies have placed greater emphasis on medical laboratories controlling and mitigating errors in the PAP. To meet ISO15189 standards, clinical laboratories have striven to reduce PAP errors, demonstrating a clear understanding of the PAP in this discipline and a desire for continuous improvement [43, 44]. The authors suggest, therefore, that processes required to control errors in the PAP are better progressed and more widely used in laboratory medicine than in clinical research, and that knowledge from the former field could inform the latter, including in research relating to the PP. Some key differences, however, must be considered. In laboratory medicine, routine measurement of clinically relevant proteins involves assays and procedures tailored to the PAP considerations of specific proteins, which are often designed and refined over decades. Given the heterogeneity and vast protein repertoire in the PP, and the fact that much research involves identifying proteins of interest whose susceptibility to PAP processes may not be known, it is not easy to make recommendations that are universally applicable to all components of the PP. However, the authors suggest adopting certain key principles.

It is likely that some of these proteins are sensitive to, or require, preanalytical conditions similar to those observed for analytes with known reduced stability, as in clinical

routine. Without robust PAP conditions or consideration of all factors that can influence the PAP in PP research, the discovery of novel candidate biomarkers of pathology may be hindered. A lack of PAP reporting or consideration in PP research may also lead to the false discovery or reduced reproducibility of findings, ultimately impeding biomarker discovery in PP research. Drawing on knowledge of the PAP from laboratory medicine, this review has gathered evidence on the impact of PAP variables on the PP and highlighted the importance of controlling for these variables in PP research.

Previous reviews and existing guidance

Three reviews [45–47] were identified in this systematic review. These provide valuable insight into previously identified pitfalls in the PAP. Collectively, the previously conducted reviews identified several major influencing factors on the PAP concerning the PP, including blood collection methods, processing delays, centrifugation settings, storage delays, and temperature. We have attempted to consolidate the observed differences in the PP due to preanalytical variability across analytical platforms, thereby guiding researchers in the field and setting this review apart from others. To the best of the authors' knowledge, this systematic review is the first to comprehensively evaluate the effects of preanalytical variables on the plasma proteome.

There is an understanding of the factors that can influence variability at the analytical stage of plasma proteomics. However, the importance of the PAP is, perhaps, less well appreciated. In a worst-case scenario, an uncontrolled PAP increases the likelihood of erroneous biomarker identification and potentially leads to significant pathology markers remaining undiscovered. While many studies fail to report PAP conditions, this does not suggest these studies are of poor quality, or that they did not, by design or otherwise, have stable PAP processes. Rather, it reduces study reproducibility and perhaps betrays a lack of appreciation of the importance of PAP conditions in PP research. To illustrate this issue, a targeted PubMed search conducted in the last quarter of 2024 identified 97 published articles that directly contained the words "Plasma Proteome". Of these, 13 papers focused on biomarker discovery in humans, with only 5 giving detailed accounts of their preanalytical processes and conditions.

The Minimum Information About a Proteomics Experiment (MIAPE) guidelines are a HUPO initiative being

developed to improve proteomics research by standardising data reporting and enhancing data quality. It is not a single standard but rather a collection of modules catering to different aspects of proteomics research [48]. Currently, MIAPE modules tend to focus on the minimum information required for analyser-specific analysis replication. Unfortunately, there is also limited awareness of the MIAPE guidelines, and they can be complex to understand and implement, especially without prior exposure [49]. The development of a PAP MIAPE module, along with increased awareness within the PP research field, may benefit proteomic study reproducibility and define minimum information reporting standards for the PAP in proteome research.

Notably, Lippi et al., on behalf of the EFLM-WG-PRE, have proposed recommendations for preventing pre-analytical diagnostic errors in clinical trials (PREDICT) [50]. While such guidance provides an essential framework for high-quality clinical research, it does not systematically synthesise evidence specific to PP analyses, where protein-specific stability, analytical sensitivity, and workflow-dependent effects may differentially influence outcomes. The present systematic review, therefore, aims to complement existing guidance by focusing specifically on evidence relevant to PP research.

Importance for future translation into clinical care

Major collaborations among research laboratories, diagnostic laboratories, different clinical disciplines, and clinicians are necessary for the successful implementation of a novel biomarker into routine clinical chemistry services. From a clinical perspective, a new test must provide additional or replace information from an already existing test while also improving clinical outcomes. From a laboratory operational perspective, it must be easily integrated into existing workflows, meaning there must be a clear understanding of the requirements for preanalytical handling. Novel biomarkers must also demonstrate sufficient pre-analytical stability to be integrated into the clinical laboratory workload. Investigations into the preanalytical requirements for a novel biomarker should be conducted as early as possible during the evaluation stage [51]. These investigations should include those directly involved in clinical laboratory medicine, as there is a crucial understanding of the importance of stringent preanalytical controls in this profession [5].

The EFLM-WG-PRE was established by the EFLM in 2013 to improve the quality of the PAP in Europe [10]. Since their foundation, the working group has published a set of criteria for evaluating stability studies, known as the CRESS criteria. The authors recommend using the CRESS criteria in the design of proteomic stability studies, as they enable standardised reporting and allow other laboratories to assess whether an analyte's stability criteria are met in their healthcare environments [52]. The studies included in this review scored well on the CRESS criteria, indicating that, as stability studies, they were well designed and their findings may be readily transferable to an institution's healthcare setting.

While many studies may satisfy requirements for pre-analytical stability, additional factors commonly measured in the clinical routine laboratory setting must also be considered to meet acceptability criteria. For instance, hemolysis, icterus and lipemia (HIL) are routine acceptability criteria in clinical chemistry, and affected samples may be rejected due to compromised analytical reliability. Analytical investigations to assess the interference effects of HIL are also essential before a novel biomarker can be fully adopted into clinical routine.

Gaps and limitations

Notable limitations identified by the authors for this systematic review are the limited number of eligible articles identified in the last 10 years. Studies included in this review variously examined overall changes in the PP and alterations in specific proteins in response to PAP variations. However, due to heterogeneity in study design and reporting, a quantitative synthesis was not feasible, and our ability to apply the GRADE framework to assess the certainty of evidence was reduced. This limited our ability to specify protein-specific PAP conditions. Instead, the authors make recommendations to protect the integrity of the proteins that might be considered the least stable within the PP when undertaking proteomic discovery work. Small sample sizes in some studies also contribute to the limitations of this systematic review. However, even with the relatively small number of studies identified, this highlights the need for more robust investigation and reporting of preanalytical conditions in PP research, especially in biomarker discovery. The available studies have demonstrated statistically significant alterations in the PP due to the preanalytical conditions encountered during the research PAP.

Table 5: Summary of recommendations.

Preanalytical variable	Additional relevant information	Summary of findings	Recommendation	Best practice for proteomics	Certainty of evidence in recommendation (GRADE)
Anticoagulant type	Blood-tube anticoagulants considered in the literature reviewed were: EDTA (K ₂ and K ₃ salts); Lithium Heparin; Sodium Heparin; and Sodium citrate.	A unique set of detectable proteins has been observed based on anticoagulant type, with a core set of 187 detectable in all anticoagulant types examined. Different proteins have shown varying stability depending on the anticoagulant used. Structural proteins have shown greater stability in EDTA tubes, while hormones have shown greater stability in heparin-treated tubes. EDTA is considered a more inert anticoagulant than others and provides proteins with some protection against proteolytic processes. Pre-centrifugation delay had the most pronounced effect on the observed plasma proteome. A synergistic effect between time and temperature was observed during the pre-centrifugation delay, with higher temperatures greatly reducing proteome stability. Some preservation at refrigerated temperatures was documented; however, as the pre-centrifugation delay time increased, proteome instability became apparent. Differences in centrifugation settings alone did not contribute to the differences observed in the plasma proteome.	When possible, the anticoagulant tube type should be selected with downstream analyses in mind. Tube types should be standardised across multicenter studies. Extra vigilance is advised when using EDTA, as different formulations are available. K ₃ -EDTA is not recommended for use in proteomic research. The additional potassium ion can increase blood osmolality, resulting in hyperosmolality. K ₃ -EDTA is also available in liquid formulations, which may lead to dilutional effects. Ideally, there should be no delay between sample collection and centrifugation for proteomic research. However, in instances where delays are unavoidable, samples should be stored refrigerated at 4 °C until centrifugation can take place. Centrifugation should occur within no later than 4 h post-collection.	K ₂ -EDTA	+++ Moderate
Pre-centrifugation delay				Immediate centrifugation of samples.	+++ Moderate
Centrifugation settings	Centrifugation settings considered in the literature reviewed ranged from 1,300 to 4,000g.		Uniform centrifugation settings should be implemented in multicenter studies. This can be achieved by using the same tube type from the same manufacturer and following the manufacturer's guidelines.	Uniform centrifugation settings.	+++ Strong
Post-centrifugation delay		Statistically significant differences in the plasma proteome are observed when post-processing delays occur.	Ideally, plasma should be aliquoted and stored immediately at –80 °C after processing. If post-processing delays are unavoidable, plasma aliquots should be stored refrigerated at 4 °C and then stored at –80 °C within 1–2 h.	Immediate storage of plasma aliquots at –80 °C.	+++ Strong

Conclusions and general recommendations

The PP has strong potential to revolutionise biomarker discovery for disease diagnosis and therapeutic monitoring. However, without controlling for preanalytical variability in sample processing, biomarker discovery could be compromised. Because large sample sizes are typically required for novel biomarker discovery, multicenter sites for sample acquisition are common. Based on this systematic review, SOPs tailored to the clinical research question should be provided to all study centres participating in the study. These SOPs should consider how best to protect proteins and ensure analytical validity while also being cognizant of the different facilities, staff, and infrastructure available at each site. They should also ensure uniform exposure at all sites to PAP variables, to a degree that will not invalidate or compromise PP research results.

Temperature and time appear to have the most pronounced adverse effects on the PP. In line with previously published guidelines, it is recommended to minimise pre-processing delays to no longer than 4 h, with the sample stored at 4 °C for the duration of the delay. Documentation of deviations from this is needed and should be considered in the data analysis stage if conflicting results arise. The anticoagulant type used should remain constant between study sites and be pre-determined based on the analytical platform to be utilised, highlighting the need for careful consideration of the analysis from the study design stage onward. Centrifugation settings did not appear to influence PP in the current studies. However, the authors recommend uniformity in centrifugation settings across study centres. Post-processing storage delays should not exceed 2 h. If delays before long-term storage are unavoidable, storage at 4 °C appears to offer the best preservation of the PP.

Through highlighting the significant effects of pre-analytical variations on the PP, it is anticipated that more consideration will be given to standardising the PAP for biomarker discovery through proteome research (Table 5).

Research ethics: Not applicable.

Informed consent: Not applicable.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Use of Large Language Models, AI and Machine Learning Tools: None declared.

Conflict of interest: The authors state no conflict of interest.

Research funding: This work has been supported in part by a grant from the Cerebral Palsy Foundation and Research Ireland (grant number 22/SPP/11074).

Data availability: Not applicable.

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Supplementary Material: This article contains supplementary material (<https://doi.org/10.1515/cclm-2026-0330>).