Pre-analytical considerations in biomarker research: focus on cardiovascular disease

Abstract: Clinical biomarker research is growing at a fast pace, particularly in the cardiovascular field, due to the demanding requirement to provide personalized precision medicine. The lack of a distinct molecular signature for each cardiovascular derangement results in a one-size-fits-all diagnostic and therapeutic approach, which may partially explain suboptimal outcomes in heterogeneous cardiovascular diseases (e.g., heart failure with preserved ejection fraction). A multidimensional approach using different biomarkers is quickly evolving, but it is necessary to consider pre-analytical variables, those to which a biological sample is subject before being analyzed, namely sample collection, handling, processing, and storage. Pre-analytical errors can induce systematic bias and imprecision, which may compromise research results, and are easy to avoid with an adequate study design. Academic clinicians and investigators must be aware of the basic considerations for biospecimen management and essential pre-analytical recommendations as lynchpin for biological material to provide efficient and valid data.

Keywords: biomarker; blood handling; hemolysis; plasma; serum.

Introduction

Clinical use of biomarkers is increasing rapidly, and biomarkers have become key for screening, diagnosis, prognosis, and management in cardiovascular diseases. In the case of heart failure, the cardiac response to acute and chronic injury is characterized by a complex series of transcriptional, signaling, structural, electrophysiological, and functional alterations ultimately leading to myocardial remodeling. This variety of pathophysiological processes makes it difficult for a single biomarker to define the stage of a disease, and multi-marker approaches using different biomarkers are strongly needed. However, before thinking which biomarkers may better define a cardiac disorder it is necessary to focus on the origin of the biological material, the state of which will be decisive for the results obtained. The contribution of biospecimens for research is a generous and voluntary action to advance scientific discovery and disease management; therefore, researchers must use this biological material in the most correct and efficient way possible.

The establishment of a study protocol, which includes traceability between laboratory and clinical personnel, is crucial for obtaining reliable results in cardiovascular biomarker research. This protocol must summarize the study objectives and the biospecimen life cycle (Figure 1). The Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines are an important resource for improving the
quality of scientific biomarker research [1]. It is recommended that at least collection, handling, processing, and storage of the biospecimens shall be defined [2]. Another interesting tool is the Standard PREanalytical Code (SPREC), a code system that allows to identify and to record the impact that pre-analytical variables have on biospecimens integrity during collection, processing and storage [3].

Studying circulating blood biomarkers by different approaches may allow a molecular understanding of the myocardial status, as if a liquid biopsy were performed (Figure 2). Cardiologists and investigators need to be introduced to basic considerations and recommendations for the management of biospecimens, since imperfect biospecimen handling or processing may introduce systematic bias and lead to reporting incorrect biomarker conclusions.

We will review the importance of understanding the pre-analytical variables of blood biospecimens with a cardiovascular approach. First, we will point out the important stages of the pre-analytical process. Then, we will review these variables, emphasizing those biomarkers approved by European and American clinical guidelines, used in clinical practices, and emerging biomarkers that are being investigated in large cohorts stored at low temperatures.

**Pre-analytical variables**

**Blood collection**

Mistakes in sample collection procedures are the most frequent failures in the pre-analytical phase [4–6]. Any defect during the collection process can alter the analysis of blood-derived samples. In 1982, Calam and Cooper defined an order for blood collection to avoid cross-contamination of additives [7]. The current guidelines have slightly modified these recommendations, but basically indicate that it is preferable to collect in tubes without additive first [8].

**Hemolysis**

Hemolysis is the most frequent pre-analytical problem that may interfere with biomarker analysis in blood-derived specimens [9, 10]. It subsumes 40–70% of all pre-analytical confounds [11]. The hemolytic process involves rupturing the erythrocyte membrane and releasing some of its contents, such as hemoglobin, potassium, or lactate dehydrogenase. Less than 2% of biological samples with hemolysis are due to in vivo hemolysis or endogenous causes [12, 13], whereas in vitro hemolysis may occur during the collection, handling, processing, transportation, or storage process [13, 14]. Hemolysis may interfere with biochemistry results through additive, spectral, chemical, and dilutional mechanisms [13] (Figure 3). The presence of hemolysis in the sample can be detected visually when the free hemoglobin concentration is 0.2–0.3 g/L; however, the cut-off of at which free hemoglobin can cause interference clinically in sample analysis has been defined by some authors as 0.5 g/L [15]. The interference caused by hemolysis in classical cardiac biomarkers has been extensively studied [16–20].

The IFCC Committee on Cardiac Biomarkers has compiled information from existing tests for troponins and natriuretic peptides (NPs) and designed a series of informative tables to help solve problems of discordant analytical results due to hemolysis [21]. In any case, it is important to reference the assay inserts of each immunoassay technique
and to carry out a literature search in order to review the latest specific interference studies. In the event that there is no literature associated with the biomarker to be studied, it would be best not to work with hemolyzed samples.

**Anticoagulants**

The appropriate anticoagulant must be selected when plasma samples are used in analytical processes. Different types of anticoagulants prevent clot formation through several mechanisms, and their choice depends on the type of study to be carried out. The anticoagulant may affect the measurement of small molecules [22], metabolic [23] and lipidomic profiles [24], and other clinical parameters [25]. Generally, the most widely used anticoagulants are ethylenediaminetetraacetic acid (EDTA), heparin, and citrate (Table 1). Typically, EDTA- or heparin-plasma are the sample matrix used for clinical chemistry and immunochemistry testing; however, Demonte et al. described that citrate-plasma could be used on exceptional occasions if there is no other option by applying a correction factor [26]. EDTA and citrate inhibit coagulation via chelate formation with ion-dependent enzymes. In contrast, heparin accelerates the inhibition of Xa factor by antithrombin III, preventing fibrinogen formation from fibrin [27, 28]. Lithium, sodium, and ammonium salts are used in conjunction with heparin as an anticoagulant. Heparin may interact with several proteins [29], interfere in the antigen-antibody union [30], and interfere with liquid chromatography/mass spectrometry (LC-MS/MS) techniques. Therefore, heparin-plasma is not recommended for peptide or proteomic analysis. Also, Glinge et al. do not recommend the use of heparin-plasma when measuring miRNAs [31]. In contrast, the use of heparin-plasma is highly recommended for metabolomics studies using nuclear magnetic resonance (NMR) spectroscopy or different mass spectrometry assays [32–34].
Table 1: Principal additives, sample matrix, assay test, and matrix usage reference examples.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Sample matrix</th>
<th>Test</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>None/clot activator</td>
<td>Serum</td>
<td>Clinical chemistry, immunochemistry</td>
<td>[80, 103, 121, 128]</td>
</tr>
<tr>
<td>Heparin</td>
<td>Plasma</td>
<td>Clinical chemistry, immunochemistry</td>
<td>[80, 103, 104]</td>
</tr>
<tr>
<td>EDTA</td>
<td>Plasma/ whole blood</td>
<td>Hematology, immunochemistry</td>
<td>[20, 65, 69, 110]</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>Plasma/ whole blood</td>
<td>Hemostasis, platelets</td>
<td>[24, 129–131]</td>
</tr>
</tbody>
</table>

EDTA, ethylenediaminetetraacetic acid.

Protease inhibitors

Some cardiovascular analytes are avidly bound and degraded by circulating proteases that are not well-inactivated in vitro by EDTA. Therefore, understanding sensitivity to degradation is important when evaluating new biomarkers. A prime example of an important cardiac marker subject to rapid in vitro decay is atrial natriuretic peptide (ANP) whose concentrations rapidly fall following phlebotomy in part due to degradation by neprilysin; brain natriuretic peptide (BNP) is also subject to in vitro degradation but this is slower. For biomarkers vulnerable in this manner, collection in tubes with specialized protease inhibitor cocktails, cold handling and processing, and storage at −80°C may provide optimal results [35].

Serum vs. plasma

Plasma and serum are obtained by centrifugation of whole blood; however, to obtain serum, blood clotting is necessary before centrifuging. Importantly, this makes the protein profile different between plasma and serum [36].

Respect to serum, during clot formation, several proteins are non-specifically adsorbed or randomly captured in the clot, and cellular elements can secrete components [36, 37], which could lead to obtaining false positives for differentially expressed proteins in serum [38]. In the other hand, human plasma contains the most complex and complete representation of the human proteome [39, 40], 3,500 detectable proteins according to the last HUPO PPP [41]. Accordingly, the use of serum samples is not recommended for peptidomic biomarker discovery, although it can be useful for validation purposes in order to improve specificity [27, 39, 42].

Traditionally, plasma has been recommended in emergency clinical laboratories over serum because anticoagulated blood samples can be centrifuged immediately after collection, enabling rapid analysis and reducing turnaround time. For example, the 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation recommend a turnaround time of 1 h or less for cTn [43]. This cannot be achieved with serum samples, where it is necessary to wait 30–60 min for the correct clot formation [44] or 20 min if BD RST tubes are used [45]. Moreover, the time lapse to obtain serum is not always easy to standardize in real world conditions. The use of plasma is also advantageous because is more stable than serum in several processes [46]. However, there are disadvantages associated with plasma use: (a) the collection tube must be well mixed for the anticoagulant to be effective, (b) plasma does not always withstand freeze-thaw cycles well [47, 48], and (c) most routine laboratories use serum. In addition, specifically heparin- and EDTA-plasma interfere with many LC-MS/MS and potentiometric methods, respectively.

Blood handling

The pre-analytical phase known as handling refers to the time from extraction to processing. It is important to ensure the optimal time and temperature handling is to avoid impacts on precision and quality.

Ease of obtaining samples

Not only can traumatic blood draws cause hemolysis but traumatic draws will stimulate tissue thromboplastin and by doing so, activate clotting. Thus, samples obtained for the measurement of coagulation related tests may be influenced despite an appropriate anticoagulant in the tube [49].

Blood handling time

Classic standard guidelines for blood sample handling recommend that plasma and serum should be separated from cells within the first 2 h after drawing the blood [50], though some current guidelines specify that the time from collection to centrifugation should not exceed 1 h, or as quickly as possible for plasma biomarker studies [28]. If the centrifugation process is delayed, keeping the sample at 4°C is better to maintain the stability of certain analytes, while in other cases it produces a negative effect [51]. For this reason, it is important to consider which biomarkers should be evaluated to assess if the sample can be kept refrigerated in case of delay or not.
For example, in the specific case of cytokines, which play an important role in the progression of cardiovascular disease, it has been proven that keeping blood samples for a long time at room temperature or lower can significantly alter plasma cytokine levels, so is crucial to minimize the blood handling time in these cases [52, 53].

**Blood handling temperature**

Storage temperature before centrifugation is also an important factor to consider. Several studies have demonstrated that the concentration of some biomarkers can vary depending on the storage temperature prior to the centrifugation process [28, 54, 55]. Current standard guidelines for blood sample handling recommend that, if the sample can be centrifuged within a short time, the samples should be kept at room temperature to minimize the platelet activation that occurs at low temperatures. In some situations, in response to temperature, the analyte of interest may be cleaved from its normal molecular position affecting measured values [56].

**Blood processing**

Various guidelines based on expert opinion or manufacturer recommendations are available for sample processing [50, 57, 58]. To avoid disturbances in the blood sample during processing, the speed, temperature, and centrifugation time must be considered. To obtain serum, once the clot formation time has passed, the classic recommendation is to centrifuge the sample at 1,500–2,000 g at room temperature for 10 min [46]. To obtain plasma, blood samples are typically centrifuged at 1,000–1,200 g for 10 min; however, often a second centrifugation at a higher speed of 2,000–3,000 g is required to obtain platelet-poor plasma. Residual platelet contamination is a significant confounding source of circulating miRNAs [59–61]; therefore, in this case obtaining platelet-poor plasma is crucial for obtaining reliable results. Similarly, sample processing plays a crucial role in circulating cell free DNA (ccfDNA) studies to avoid genomic DNA contamination, and samples must be completely cell free [62–64].

**Storage**

Proper specimen storage is critical to maintaining specimen integrity, as inappropriate specimen storage can change the sample properties and affect the final results. Appropriate storage conditions include controlling the number of freeze-thaw cycles, duration of thaw events, time from last thaw to processing, and temperature between last thaw and processing. This is usually accomplished by processing the sample and aliquoting it into multiple different tubes with somewhere between 250 and 500 μL so that individual samples can be thawed for measurement without impairing the ability to measure other analytes that might be impacted by the thawing process per se. It is important to differentiate samples that will be processed following centrifugation in the routine clinical laboratory and those that will be stored in biobanks for future studies.

**Routine clinical laboratory**

If the samples are not going to be stored, samples must be transferred directly to the analyzer in the routine clinical laboratory to avoid alterations in the stability of the analytes, considering that sample stability depends on temperature and it is different depending on the analyte. For example, N-terminal proBNP (NT-proBNP), cardiac troponins (cTn), cancer antigen 125 (CA125), soluble ST2 (sST2), and galectin-3 are stable analytes under a wide-ranging of storage temperatures [51, 65–68], whereas BNP is only stable in whole blood for at about 4 h at room temperature [69] and ANP is even more unstable [70–73].

Sometimes results from clinical laboratories are collected in databases directly from routine analysis. For instance, parameters as cTn, NT-proBNP, or C-reactive protein are included in many clinical profiles and analyzed in fresh samples for patient management. This has the advantage of not been subjected to freeze-thaw cycles, but several considerations have to been made: (a) results may be obtained from different laboratories or analyzers; (b) in long lasting studies reactive and calibrators lots may change, and (c) operators and quality specifications may vary.

Taking this into consideration, for stable analytes it should be recommended to process all samples in a same batch and analyzer, regardless previous results. This is particularly useful in novel immunoassays, that may not have an established standard for calibration.

**Biospecimen biobanking**

The banking of samples should not be neglected. The use of retrospective serum and plasma samples may introduce significant variability in the molecular composition of the samples solely because of heterogeneity in storage procedures. Storage temperature, storage time, and freeze-thaw cycles may interfere with the sample composition.
Storage temperature and time. To lessen the effects of storage temperature, it must remain stable over time. Liquid nitrogen maintains stable ultra-low temperatures; however, most samples are stored in non-cycling freezers with temperatures ranging between −70 and −80 °C. Temperature variations in −20 °C or −40 °C freezers are more likely to affect sample stability to a greater degree, particularly as many of these freezers are cycling, meaning their temperature rises and falls to reduce frost formation [74]. In any case, it is important to have a centralized record of freezer temperatures, with measures taken from independent probes. Furthermore, the short-term thermal exposure that may occur while removing individual samples from a biobank needs to be considered. In this case, cooling systems, such as dry ice, or working in low temperature rooms to avoid heating the samples while searching for specific samples is recommended. Zander et al. demonstrated that short-term thermal exposures of 5 min while working in low temperature rooms do not alter the levels some biomarkers while others are affected [75].

Freeze-thaw cycles. Typically, it is recommended to aliquot the samples and store at −80 °C. It is important to find a balance between the number of aliquots and the volume of each and the available storage space. The availability of several aliquots allows the effect of the number of freeze-thaw cycles to be mitigated, allowing analysis of different biomarkers and analytes in different time frames. Most of the commercial manuals specify avoiding several defrost cycles without specifying an exact number, and studies are not always available in the literature. Repeated freeze-thaw cycles affectation will also depend on the analysis technique used [76–78].

Pre-analytical considerations in biomarker research

As described in the previous section, there are multiple pre-analytical variables that can interfere with or alter the results of clinical investigations carried out by our staff in hospitals, primary care and research centers. Table 2, summarizes the pre-analytical recommendations for the most relevant cardiac biomarkers to date; however, we will try to go deeper into it depending on its clinical value.

Myocyte stress

Natriuretic peptides

Nowadays, NPs are the gold standard clinical indicators in the diagnosis and prognosis of heart failure.

As we have discussed previously, ANP concentrations decrease rapidly following phlebotomy, it is very unstable [70–73], like BNP, although to a lesser degree. In these cases it is essential to carry out the sample collection in tubes with specialized protease inhibitor cocktails and cold handling to provide optimal results [35]. In addition, BNP must be measured in EDTA-plasma or whole blood drawn into plastic tubes due to proteolytic degradation in vivo [79] and it is only stable in whole blood for at about 4 h at room temperature [69].

NT-proBNP is less vulnerable to degradation. NT-proBNP can be measured in serum or heparin-plasma with similar results [80] and it can be also evaluated in EDTA-plasma, but in this case, it may yield lower values compared to serum and heparin-plasma, depending on the assay method [80, 81]. In addition, NT-proBNP is stable under a wide-range of storage temperatures [51, 65–68], analyte levels are not influenced by moderate hemolysis (0.6 g/L) [16] and it is not significantly affected by freeze-thaw cycles [66].

NPs are a clear example of the importance of understanding sensitivity to degradation and the correct selection of the blood collection tube to evaluate new biomarkers.

Inflammation

Cytokines

Cytokines have been reported to participate in different pathophysiological mechanisms of cardiovascular disease. Cytokines play diverse roles with both, detrimental and positive effects on the cardiovascular system. Pro-inflammatory cytokines, such interleukin-1 (IL-1) and IL-18, are involved in the development of cardiac pathologies and are suggested to be potential therapeutic targets. There are also cytokines with pleiotropic functions, including IL-6, that play duals role in CVD. All of them could be predictors of adverse outputs. Determine the implication of each cytokine in the progression of cardiovascular disease is key to developing new therapeutic
agents that aim to treat the inflammatory processes associated with this pathology. Therefore, it is important to know how pre-analytical variables can affect cytokine concentrations in clinical research.

Cytokines are highly labile to temperature, so it is advisable to measure them as soon as possible after collection [82]. Binnington et al. described absence of alterations in cytokine concentrations in whole blood or plasma stored refrigerated up to 10 days [83]; however, also it has been proven that keeping the samples for a long time at room temperature or lower can significantly alter plasma cytokine levels [52, 53]. Then, minimizing the blood handling time seems crucial in cytokine research.

Regarding freeze-thaw cycles, it has been shown that IL-6, IL-10, Interferon-γ, and IL-2 levels are stable in plasma over three freeze-thaw cycles [84]; but, there are other cytokines whose concentrations increase or decrease due to thawing cycles. IL-1β, IL-4, and IL-10 circulating levels increase at least 3–5-fold following one freeze/thaw cycle, stabilizing at these levels during a subsequent nine freeze/thaw cycles [85].

Therefore, minimizing the pre-analytical variables as much as possible in the study of interleukins is very important to obtain valid and reliable results.

### Table 2: Recommended pre-analytical variables of relevant cardiac biomarkers.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Clinical value</th>
<th>Sample matrix</th>
<th>Storage temperature stability</th>
<th>Freeze-thaw cycles stability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP</td>
<td>Myocyte stress</td>
<td>EDTA-plasma</td>
<td>4 h at RT</td>
<td>Affected very significantly</td>
<td>[69, 79]</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>Myocyte stress</td>
<td>EDTA-plasma</td>
<td>3 days at RT</td>
<td>Not significantly affected</td>
<td>[66, 80, 81]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparin-plasma serum</td>
<td>6 days at 2–8 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 months at –20 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANP</td>
<td>Myocyte stress</td>
<td>EDTA-plasma</td>
<td>Few days at –80 °C</td>
<td>Affected very significantly</td>
<td>[70–73]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 month at –196 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sST2</td>
<td>Stress, inflammation, fibrosis</td>
<td>EDTA-plasma</td>
<td>48 h at RT</td>
<td>Up to three cycles</td>
<td>[65, 90–92]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparin-plasma serum</td>
<td>7 days at 2–8 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18 months at –20 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsTn</td>
<td>Injury</td>
<td>EDTA-plasma</td>
<td>24 h at 2–8 °C</td>
<td>Up to four cycles</td>
<td>[103–106]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparin-plasma serum</td>
<td>12 months at –20 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA125</td>
<td>Congestion</td>
<td>EDTA-plasma</td>
<td>7 days at 2–8 °C</td>
<td>Up to three cycles</td>
<td>[51, 132]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparin-plasma Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galectin-3</td>
<td>Fibrosis</td>
<td>EDTA-plasma serum</td>
<td>15 days at RT</td>
<td>Up to six cycles</td>
<td>[19, 20, 65, 127]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 days at 2–8 °C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*EDTA- and heparin-plasma samples should not be exchanged with serum samples. EDTA, ethylenediaminetetraacetic acid; BNP, brain natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; ANP, atrial natriuretic peptide; sST2, soluble ST2; CA125, cancer antigen 125.

sST2

sST2, also known as interleukin 1 receptor-like 1 (IL1RL-1), is produced in response to stress and overload by cardiac fibroblasts and cardiomyocytes [86]. sST2 plays an important role in many inflammatory diseases and prevents fibrosis and inflammatory response when interacting with IL-33.

Especially, sST2 is gaining attention in the management of chronic heart failure. Lupón et al. developed The Bio-Heart Failure Risk Calculator (BCN BIO-HF Calculator) that incorporated available biomarkers, including ST2, reflecting different pathophysiological pathways in heart failure [87]. Really, sST2 is a novel biomarker that could be used for diagnosis and management of patients with several cardiovascular diseases [88, 89].

Regarding the pre-analytical variables that we have been commenting on, sST2 is a very stable biomarker in different aspects. The effects produced by hemolysis on sST2 levels are minimal and not significant [90]. In addition, serum, lithium heparin-, and EDTA-plasma are validated samples for the Presage ST2 assay, the only approved test by the FDA for clinical use [65, 91, 92]; however, the new SEQUENT-IA ST2 assay has yet been evaluated only in serum and EDTA-plasma samples.
Respect to storage, sST2 is stable under a wide-ranging of storage temperatures, 48 h at RT, 7 days at 2–8 °C and 18 months at −20 °C [65, 67, 68] and it is stable up to three freeze-thaw cycles [65].

The pre-analytical stability of ST2 makes it easier to investigate this promising biomarker in cardiovascular physiopathology.

**GDF-15**

Growth differentiation factor 15 (GDF-15) is a member of the transforming growth factor-cytokine superfamily that has emerged as a stress potential biomarker in cardiovascular disease with potential implications for risk and patient management. It has been recognized as a biomarker of mortality in patients with acute coronary syndrome [93–95].

GDF-15 has favorable pre-analytic characteristics. The anticoagulant matrix does not affect the analyte measurement since no differences are observed in the concentration of GDF-15 between serum and EDTA-, heparin-, or citrate-plasma [96, 97]. In addition, GDF-15 immunoreactivity is stable in serum and whole blood at room temperature for at least 48 h, which facilitates its use in routine laboratories. Regarding the analysis of GDF-15 in frozen samples, it must be considered that it is stable until the fourth freeze-thaw cycle [96, 97].

Both the pre-analytical characteristics of GDF-15 and its analytical stability make research with it advantageous.

**Injury**

**Cardiac troponins**

Cardiac troponins I and T (cTnI, cTnT) are the primary cardiac biomarkers used for the diagnosis of myocardial injury, as they are sensitive and specific markers of cardiomyocyte injury and more specific than creatine kinase. Serial measurements of cTn are useful to understand kinetics, peak values and percentage changes. Clinical implications of high-sensitivity cTn (hs-cTn) assays are widely known and detailed in the 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation.

Daves et al. reported in cTnI and cTnT were not influenced by moderate hemolysis (0.6 g/L) [16]; however, the development of very high sensitive methods could lead to notable differences [98]. In the recent years, it has been described that hemolysis can produce both negative and positive interferences in cTn [99]. Respect cTnT, higher degrees of hemolysis decrease its concentrations in a hemoglobin concentration-dependent manner [18], causing false negatives that make it difficult to interpret results [98, 100], surely because hemolysis could release proteases that cleave the cTnT antigenic regions recognized by the immunoassays [17]. In contrast, Harley et al. reported that hemolysis interference can be positive or negative depending on the high-sensitivity assay and the concentration range for cTnI [101]. This positive interference has been corrected in most immunoassays; however, it has recently been published that hs-cTnI concentrations in heparin-plasma are increased by hemolysis (>4 g/L) with the Ortho hs-cTn assay only at the low concentration range [101, 102]. The release of multiple proteins from erythrocytes increases matrix complexity and this may influence immunoassays in different ways, depending on the monoclonal antibodies, solid phase characteristics, and incubation times [100].

cTn are also a good example to demonstrate how the use of serum or plasma can influence concentration levels. Initial reports indicated that for some assays cTn levels were significantly lower in heparin-plasma samples than in serum, probably because heparin may bind to troponin, decreasing immunoreactivity [103, 104]. Roche Diagnostics, manufacturer of cTnT assay, recommended customers to avoid using heparin-plasma samples in their assay until new test formulations corrected this variability [103, 105]. At present, Elecsys Troponin T hs package insert specify that plasma (EDTA and heparin) and serum samples cannot be interchanged because there are differences in the values that are obtained [106]. With the increasing number of cTn assays reaching the market, this issue needs to be revisited whenever a new assay is put in use.

**Cardiac circulating cell free DNA**

Dying cells release nucleosome-size fragments of genomic DNA to the blood system. Identification of specific circulating cell free DNA (ccfDNA) from cardiomyocytes would allow a liquid biopsy to be performed, reflecting the physical state of myocardial tissue.

Recently, Zemmour et al. identified specific cardiomyocyte methylation markers that allow to identify ccfDNA release by death cardiomyocytes after ischemia ensues [64].

Cardiac ccfDNA could be a promising non-invasive clinical marker and diagnostic tool in acute myocardial infarction but more clinical studies are required and it is important to consider different pre-analytical variables.

Free hemoglobin has been reported to correlate with ccfDNA concentrations, then ccfDNA levels are increased.
in hemolyzed samples [107]. However, Streleckiene et al. demonstrated that the effect of hemolysis on ccfDNA levels depended on the isolation kit used [108]. In this regard, it would be interesting to see if hemolysis has any effect on the concentrations of ccfDNA released specifically from cardiomyocytes.

Several studies have shown higher ccfDNA concentrations in serum than in plasma samples due to the clotting process [109–112]. For that, to study ccfDNA it is more advisable to use plasma samples, and more specifically, EDTA-plasma [110].

Regarding sample storage, DNA is stable at 4 °C for several weeks, at −20 °C for months, and at −80 °C for years [113, 114]. Specifically for ccfDNA, storage time has been reported to have no influence on the detection of specific sequences or mutations after several years [115].

**Other**

**microRNAs**

microRNAs (miRNAs) are endogenous and conserved non-coding RNAs that are involved in several pathways in the cardiovascular system. miRNAs are characterized by great stability, resisting degradation in blood-derived specimens kept at room temperature for up to 24 h after collection [116, 117]. The stability of miRNAs in serum and plasma and their resistance to degradation make them promising biomarkers for diagnosis and prognosis of cardiovascular diseases [118]; however, several pre-analytical variables could influence in circulating miRNA identification and quantification [119].

Regarding sample matrix, it has been demonstrated that EDTA is the best anticoagulant for studying circulating miRNA profiling [120], as heparin and citrate may interfere with the enzyme activity in PCR-based assays. However, in the event that EDTA-sample is not available, Basso et al. demonstrated that miRNA quality is comparable in serum and EDTA- and citrate-plasma [121].

Processing in this case is also a crucial step due to differential processing could alter miRNAs quantitation [122]. In addition, platelets represent a source of contamination for circulating miRNAs, then obtaining platelet-poor plasma is crucial to avoid miRNA contamination. Platelets also can interfere with stability during storage, despite several miRNAs resist degradation in plasma that is frozen and thawed multiple times [116, 123], Muth et al. reported that freeze-thaw cycles can affect miRNA stability if platelet depletion is inadequate [61]. This is another reason why proper platelet removal is necessary.

Actually, research with this type of biomarker requires a thorough bibliographic review. Faraldi et al. demonstrated that different miRNAs are differently affected even if the same collection method or storage condition are performed [119]. Establishing standardization protocols for the study of each miRNA independently is crucial for the clinical implementation of miRNAs as biomarkers.

**CA125**

CA125 is emerging as a novel congestion biomarker in heart failure. It has traditionally been a biomarker of ovarian and endometrial cancer, however, recent publications demonstrate the usefulness of CA125 to monitor or guide the treatment of heart failure [124, 125].

As its use is established for the diagnosis and management of different cancers, the implication of pre-analytical variables in its stability is well studied. Sandhu et al. found that CA125 levels were lower if samples handled at 4 °C, so it is advisable to keep the samples at room temperature before centrifugation [51]. They also showed that plasma or serum samples can be used interchangeably and that CA125 remains stable under a wide-range of storage temperatures after centrifugation [51].

Respect to freeze-thaw cycles, it has been described that CA125 is stable up to three cycles of freezing and thawing [51].

**Galectin-3**

Galectin-3 is a β-galactoside-binding member of the lectin family implicated in cardiac fibrosis. It is considered a prognostic biomarker that identifies increased risk of death and heart failure [126].

To evaluate circulating levels of galectin-3 it is crucial to avoid hemolyzed samples. Hemolysis produces a positive interference due to intracellular release of galectin-3 from white blood cells [19]. Assessment of galectin-3 levels in hemolyzed specimens may confuse the identification of the risk of death and heart failure.

Both serum and EDTA-plasma have been validated for galectin-3 measurement [65]. La’ulu et al. demonstrated similar galectin-3 levels between serum and EDTA-plasma [20], while Gaze et al. showed higher galectin-3 values in plasma than serum samples [127] using the same type of assay and analyzer, so we would not recommend exchanging both types of sample until further evidence is published.

Respect analyte stability, it has been reported that galectin-3 is stable under a wide-range of storage temperatures, and it is stable up to six freeze-thaw cycles [65].
Discussion

There is a great need to discover and validate new biomarkers that help clinicians to manage the patient with cardiovascular disease; however, it is necessary to consider that there are multiple pre-analytical variables that can interfere in the clinical investigations carried out by our staff in hospitals, primary care, and research centers (Table 2). The ability to manage and track pre-analytical variations impacting biospecimen integrity is crucial to high quality and reliable results. As we have seen in this review, there are multiple differences between the different types of plasma and the concentration of certain analytes can vary between serum and plasma. The different levels of circulating cTn between plasma and serum found with the first automated immunoassays are a clear example of the importance of correctly selecting the sample matrix, in the same way that the BNP instability exemplifies the importance of standardizing the handling time.

The stability and matrix effect of promising new biomarkers needs to be studied to facilitate clinical trials. The analysis methodology and the objective must also be considered, because if the study biomarker will be used in emergency laboratories, it should be validated with plasma for the reasons we have previously explained.

Nowadays, large multicenter global studies where samples are collected in different recruitment centers and sent to the promoting center for analysis, are increasingly abundant. For a correct reproducibility between derivation and validation cohorts it is necessary that the process of obtaining and handling the sample is completely standardized between centers. The formation of working groups that standardize and control the quality of pre-analytical handling of blood samples would be advisable.

It is necessary to introduce academic clinicians and investigators to basic considerations for biospecimen management and essential pre-analytical recommendations to use this biological material in the most appropriate and efficient way to obtain valid conclusions from new promising cardiovascular biomarker research.

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