Chapter 2

Preanalytical Issues in Hemostasis and Thrombosis Testing

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Abstract

Hemostasis testing is critical to many hemorrhagic and thrombotic disorders, wherein laboratory diagnostics can provide critical information for diagnosis, prognostication, and therapeutic monitoring. Due to this crucial role in modern medicine, hemostasis tests should be carried out at their highest degree of quality, thus encompassing standardization and monitoring of all phases of the testing process. It is now clearly established that the preanalytical phase is the most critical and vulnerable part of the total testing process, since up to 70% of diagnostic errors are due to highly manual activities encompassing patient preparation and collection of biological samples, as well as handling, transportation, preparation and storage of blood specimens. Due to the peculiar sample matrix required for hemostasis testing (i.e., plasma anticoagulated with buffered sodium citrate), additional critical issues may impair the reliability of these tests. Therefore, this article aims to provide an updated overview of the most important preanalytical variables that may ultimately impair the quality of hemostasis and thrombosis testing.

Key words Preanalytical issues, Preanalytical variables, Hemostasis testing, Thrombosis testing

1 Introduction

The results of hemostasis testing are crucial for clinical decision making, for therapeutic monitoring, and for prognostication of most hemorrhagic and thrombotic disorders [1]. Although several lines of evidence indicate that laboratory diagnostics is not foolproof, the vast majority of perceived laboratory errors are actually attributable to the extra-analytical phases of testing, and especially emerge from the manually intensive activities characterizing the preanalytical phase. This phase is conventionally defined as that entailing distinct but inter-related processes involving collection, handling, transport, and preparation of diagnostic specimens destined for testing; some of these processes are inherently peculiar to the field of hemostasis [2].

Since many preanalytical variables impact heavily the quality of the testing process, a deepened understanding of the essential aspects of the preanalytical phase will provide considerable benefits to ensure the reliability of test results [3, 4]. It is now clearly

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established that many preanalytical errors are due to system flaws and/or insufficient training of operators with specimen collection and handling responsibilities. Subsequent sections of this chapter are therefore devoted to critically analyze the most important sources of preanalytical variability in hemostasis testing, and to also provide potential preventive measures and solutions.

2 Sample Collection

Blood collection is an unavoidable preliminary activity, finalized with the attainment of the biological matrix (i.e., plasma anticoagulated with sodium citrate) that is needed for most tests of hemostasis. The technique for collecting blood was revolutionized at the beginning of the twentieth century by the introduction of safer and more effective needle and syringe systems, thus allowing abandonment of the former approach, essentially based on so-called venesection [5]. However, it was not until the early 1980s that blood collection by means of ordinary straight needles and syringes was also subsequently abandoned in favor of disposable needles and so-called closed systems (i.e., evacuated blood collection tubes directly connected to the needle), thus representing another breakthrough in terms of safety (of both patients and operators) and importantly also the quality of the specimens. These innovative devices consisted then, and still consist now, of a double-pointed needle, a disposable plastic holder, an adapter, and a series of evacuated blood collection tubes with perforable stoppers. The obvious safety advantage is represented by the fact that blood can flow directly from the vein into the blood tube, with no external contact. The further recent adoption of safety needles equipped with retractable sheathes has also allowed sequential drawing of many tubes with little chance of blood leakage [6].

Before sample collection, some basic requirements should be fulfilled to prevent generating undue variability in sample quality. These typically entail a fasting status (e.g., overnight fast, lasting 8 h before blood collection) [7, 8], avoidance of high-intensity physical activity [9], and emotional stress [10].

3 The Procedure

Unlike many other areas of medicine, no definitive indications have so far been provided regarding best practice for performing a venipuncture. Despite a set of general indications having been published, the technique remains largely operator-dependent, in that each phlebotomist tends to establish an individual practice for obtaining their sample and thus what they perceive to be the best possible result [11]. Although the larger and fuller median cubital

and cephalic veins of the arm are used most frequently, the basilic vein on the dorsum of the arm or dorsal hand veins are also acceptable. Wrist and hand veins are sometimes used, but less frequently. Foot veins, other than otherwise recommended (*see* below), are a last resort because of the higher probability of complications. Blood collection from veins of the metacarpal plexus should be particularly discouraged due to the higher risk of blood cell injury [5]. Venipuncture sites that must be avoided comprise those containing extensive scars from burns and surgery, upper extremity on the side of a previous mastectomy, hematomas, sites of intravenous (IV) therapy or blood transfusions, along with edematous extremities.

The most accurate procedure for venipuncture entails palpating and tracing the path of the veins with a finger (arteries pulsate, are most elastic, and have a thick wall). When superficial veins are not readily apparent, blood can be forced into the vein by massaging the arm from wrist to elbow, taping the site with index and second finger, applying a warm, damp washcloth to the site for 5 min, or lowering the extremity over the bedside to allow the veins to fill. Fist clenching does not seem to have a clinically meaningful influence on the quality of testing, but should be precautionarily avoided since it has been associated with abnormalities of some blood parameters [12, 13]. Recently, the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) working group for the preanalytical phase (WG-PRE) has planned and disseminated a specific questionnaire aimed to establish the state-of-the-art of phlebotomy practices across Europe, showing that there is a compelling necessity to (a) assess the quality of current practices, compliance to the available standards and to identify some critical steps occurring during phlebotomy in different healthcare settings; (b) adopt and locally use existing phlebotomy guidelines (e.g., CLSI H3-A6) in all countries that do not have their own guidelines; (c) engage national societies in basic training program development and continuous education of healthcare phlebotomy staff with blood collection responsibilities [14].

As for any other area of in vitro diagnostic testing, the quality of the venipuncture has a dramatic impact on reliability of hemostasis tests. Despite the new generation of clotting tests reagents and analyzers being less vulnerable to the leading analytical interferences (i.e., hemolysis, icterus, and lipaemia) [15], test results may still be biased when cell-free hemoglobin, bilirubin, and triglycerides exceed a certain plasma concentration [16]. Visual inspection has long been used for establishing the quality of the specimen, but this practice is inherently biased by a variable degree of intra-observer and inter-observed variability [17]. The gradual introduction of automated and objective means for evaluating these types of interference (i.e., so-called serum or plasma indices) is now commonplace in chemistry and also in many coagulation analyzers, and its use should hence be encouraged for

standardizing phlebotomy practices and also for obtaining useful information about the individual phlebotomy performance [18].

3.1 Venipuncture Site Cleaning

All existing guidelines regarding blood collection [19–21] recommend that the skin in proximity of the venipuncture site be cleaned with a sterile disinfectant (preferably 70% isopropyl or ethyl alcohol) applied to gauze, swab, or a cotton ball, using a firm but gentle pressure, starting from the center of the venipuncture site and moving downward and outward to cover an area of approximately one inch. After cleansing has been done, the alcohol should be allowed to dry completely for up to 30 s, or gently removed with clean gauzes or cotton balls; this is because excess alcohol at the venipuncture site may be a source of discomfort (e.g., producing a "burning" feeling during skin perforation) and, especially, that aspiration of alcohol into evacuated blood tubes may be assumed to cause spurious hemolysis. However, recent evidence attests that avoidance of wiping alcohol from the site of venipuncture does not really impact the quality of the diagnostic specimen, nor does it cause in vitro hemolysis during blood drawing [22].

3.2 Tourniquet Placing

Once a suitable vein has been located, but especially when this is less feasible, the application of the tourniquet accompanies the vast majority of venipunctures. Although this practice cannot be universally discouraged, since it is seen as an unquestionable aid for identifying a suitable vein in patients with challenging venous accesses, there are some basic precautions that should be utilized in order to prevent excessive venous stasis, hemoconcentration, and generation of bias in test results from tourniquet use [23]. Basically, the tourniquet should be applied approximately 10 cm above the intended site of venipuncture, should be tight enough to stop the venous flow but not so tight so as to impede arterial circulation (i.e., should not cause pain or discomfort to the patient), should not be left in situ for more than 2 min (when more time is required for the venipuncture, the tourniquet should be released for a few seconds and then reapplied), and should be immediately released once the last tube has been collected. Transilluminator devices are an alternative approach that more reliably identifies a suitable vein without the use of the tourniquet, thus completely avoiding the risk of generating prolonged venous stasis. A recent study has concluded that the use of these devices is practical and analytically suitable for routine venipuncture performance [24].

3.3 Type of Needles

Rather understandably, the needle is one of the most essential parts of a blood collection device/system, since it is absolutely required to perforate the skin, the vein wall and establish a continuum for flowing the blood from the inside of the vein to the blood collection tube. Needles are currently available for evacuated systems and for use with syringes, single draw, or butterfly systems. Some

general calibration characteristics have been conventionally established for identifying size and scope of phlebotomy needles. Basically, the needles are classified by gauge (G), referring to the overall diameter of the needle expressed in mm. Accordingly, the larger the G, the smaller the diameter of the needle. Venipunctures are generally carried out using needles with a diameter ranging between 19 G (the largest acceptable) and 25 G (the smallest acceptable). Needles with a diameter comprised between 19 G and 21 G are mainly used for collecting blood from antecubital veins, 23 G needles for smaller antecubitals, medium size forearm, hand veins, whereas the use of 25 G needles is typically limited to collecting blood from small veins or else for drawing blood in newborns and children. Small needles are sometimes used when drawing blood from anxious patients or in those suffering from the so-called needle phobia [25]. As regards hemostasis testing, it has now been acknowledged that the use of small bore needles does not introduce a clinically meaningful bias in clotting tests and platelet count, provided that an appropriate technique is used [26]. Interestingly, even the use of the so-called butterfly devices does not seem to impair the quality of the specimens for hemostasis testing, provided that the air contained in the tubing is not drawn into the evacuated blood tube (*see* below) [27].

3.4 The Blood Tubes

The blood tube is another essential part of the phlebotomy toolbox, since it is the container in which the blood will be drawn and sent to the laboratory for testing. Unlike widespread perception, not all tubes are alike. The first and foremost difference is the concentration of the anticoagulant used for the clotting test. For a long time, the standardization of hemostasis testing has been plagued by the use of two different concentrations of buffered sodium citrate, i.e., 0.105 mol/L (i.e., 3.2%) and 0.129 mol/L (i.e., 3.8%). Many previous experiences demonstrated that clotting tests performed using these two different concentrations yielded clinically meaningful bias from each other [28], so that the CLSI has finally endorsed the use of the lower concentration for most applications [29]. Beside the anticoagulant concentration, an additional and potentially important source of variability is represented by the brand of the blood tube. A recent study, using as many as five evacuated blood collection tubes for hemostasis testing manufactured by five different in vitro diagnostic companies, has demonstrated the existence of clinically significant bias in clotting times for both APTT and PT among the different containers [30]. Another important and virtually unresolved issue in hemostasis testing is the use of the discard tube prior to collecting the citrated blood tube. Despite this practice having long been endorsed by many national and international organizations, there is now reliable evidence that the use of a discard tube may be unnecessary, at least for routine and specialized clotting tests. The only two known

exceptions to this rule are represented by collection of blood tubes for platelet function testing or due to the need to discard the fluids or the air contained in the tubing of intravenous lines and butterfly devices [31].

3.5 Local or Remote Transportation of Blood Tubes

Once the blood tubes have been collected, they must obviously be transported to the site of testing which, unlike facilities equipped with point of care testing (POCT) devices, may be housed some distance from the site of collection. Importantly, the organization of many laboratories around the world has undergone a considerable revolution over the past decades, shifting toward the so-called hub and spoke paradigm [32]. This networking approach finds its origin in 1955, when Delta Air Lines established the main hub in Atlanta (Georgia), which encompasses a central facility (i.e., the "hub") where both routine and specialized tests are performed, and secondary facilities (i.e., the "spokes") which usually perform routine testing and are required to ship the specimens for specialized analyses to the hub, generally because of specialization or economies in testing [33]. The use of transport boxes has thus become commonplace for shipping biological samples, despite the requirement that the quality of these devices must be preliminarily checked [34] and the transport condition must be accurately standardized and monitored to prevent deterioration [35]. Indeed, some basic criteria must be fulfilled to maintain the quality of plasma during shipment, including separation of plasma from blood cells immediately after collection and not later than 4 h for most tests [36], maintaining tubes and aliquots safely capped in a vertical position during transport, use of validated biohazard containers, use of first/second/third level containers, assurance of stable conditions of temperature (i.e., 18–25 °C) and humidity during transportation, avoidance of excessive light exposure, prevention of physical trauma to the specimens, deliverance to the reference laboratory as soon as possible, recording of transportation time (from start to arrival to the laboratory), and monitoring of sample conditions throughout transportation. Obviously, all samples received in the laboratory without assurance of quality transportation must be rejected to prevent the generation of unsuitable results which may ultimately jeopardize the clinical decision making [37].

Indeed, the various steps of shipment of diagnostic blood specimens should be carefully standardized for ensuring that their quality is preserved upon reaching the final testing sites. The more popular means of sample shipment encompass hand transportation (for near distances), wheel transportation (i.e., by means of bicycles, motorcycles, cars, vans, or tracks), but also aircraft or helicopters for longer routes. Recently, another opportunity has emerged, that is using the so-called drones, also known as unmanned aerial vehicle (UAV) or unmanned aerial systems (UAS). Basically, these

devices are aircraft without a human pilot aboard, initially developed for military purposes and then extended to be used also in healthcare, (e.g., for delivering medicals, equipment, goods, and foods during natural—or human—disasters such as earthquakes, tsunamis, and wars). Recently, the use of these devices has been proposed also for flying specimens, by demonstrating that hemostasis tests are not biased by conveying plasma specimens with drones [38]. Although this should be seen as an intriguing opportunity, it seems by far too premature to suggest its routine use for shipping plasma samples due to the many catastrophic crashes that have been recently recorded and the still insufficient evidence that the quality of all hemostasis tests is not really impaired [39].

As regards local transportation, an already consolidated approach is based on pneumatic transport systems (PTSs), originally invented in the nineteenth century for purposes of delivering paper documents, but rapidly extending to many healthcare settings for shipping medical and laboratory specimens. The initial drawbacks of these systems, principally attributable to the injury of blood cells for high acceleration or deceleration forces, have been virtually overcome with the development of a new generation of PTS. Therefore, other than samples for platelet function testing, blood specimens for routine and specialized hemostasis testing can now be readily transported by this approach [40, 41].

3.6 Centrifugation

One foremost aspect in performing hemostasis testing is that the clotting tests must be carried out in plasma, once this has been separated from corpuscular elements of blood (i.e., platelets, erythrocytes, and leukocytes). The CLSI currently mandates that blood tubes for hemostasis assays should be centrifuged at 1500 × *g* for no less than 15 min, in order to obtain a platelet poor plasma (PPP) containing $<10 \times 10^9$ platelets per liter [29]. It is also recommended that a swing-out bucket rotor should be used to minimize remixing of plasma and platelets. These indications are largely used by most laboratories around the globe, and no compelling evidence has been brought forward that they should be revised. However, stat (i.e., urgent) hemostasis testing represents a possible exception to this rule, wherein some clotting tests need to be notified to the requesting physician in the shortest possible time to manage an urgent health condition. Under this circumstance, it has been reliably demonstrated that a 5–10 min centrifugation time at 1500 × *g* may be suitable for obtained quality plasma specimens for urgent coagulation testing [42]. Other similar "short" protocols have been validated locally (McVicker and Favaloro, unpublished data), with the caveat that samples cannot be frozen for subsequent follow-up tests unless plasma is separated and undergoes a second centrifuge to ensure adequate removal of cellular elements. This is now underscored by the fact that frozen plasma aliquots, rather than fresh specimens, are often used for

specialized coagulation testing, so generating an additional challenge to the quality of results of hemostasis tests.

Interestingly, a recent study showed that the platelet count in fresh plasma samples was virtually identical to that of plasma specimens centrifuged at 1000 × *g*, but was significantly decreased in those centrifuged at 3000 × *g* and significantly higher in those centrifuged at 500 × *g*. Although APTT values were not biased by residual platelets in plasma, results of both PT and fibrinogen were consistently impaired in plasma centrifuged at 1000 and 500 × *g*. Even more importantly, test results of factor VIII and factor FIX showed a positive bias in frozen plasma, with major differences observed for samples centrifuged at 3000 × *g* [43].

As regards the use of the centrifuge brake, another recent study showed that PT clotting times may be slightly prolonged (mean bias 0.2 s) and fibrinogen values may also be higher (mean bias 0.29 g/L) using the centrifuge brake set to on, compared to centrifugation with the brake disabled [44]. This clearly indicates that centrifugation of blood specimens for routine coagulation testing should be preferably carried out with the centrifuge brake set to off or, otherwise that all the centrifugation processes in the laboratory should be uniformed to enabling or disabling the centrifuge brake in order to produce comparable data.

4 Consequences of Poor Processes in Blood Collection and Processing

The previous sections have provided guidance on optimal blood collection and sample processing. In addition to what has already been described, it may be worthwhile briefly highlighting the consequences of poor adherence to such guidance [45].

4.1 Wrong Sample Collected

As noted above, the correct sample for most tests of hemostasis is 3.2% sodium citrate. However, alternate samples may be recommended for specific tests (e.g., 3.8% sodium citrate or hirudin anticoagulant for some platelet function tests). Test samples that generally are unsuitable for hemostasis include EDTA (ethylenediaminetetraacetic acid; used for complete blood counts), heparin (used for some biochemistry tests), and no-additive/clot enhancing agent (to produce serum). This is since 3.2% sodium citrate produces a plasma sample that is weakly anticoagulated and which can be easily reversed by the addition of a small and standard amount of pro-coagulant material to initiate coagulation in the test (usually calcium chloride or thrombin). EDTA and heparin represent stronger anticoagulants that are more difficult to reverse, and which will not be reversed using standard assay concentrations of calcium chloride or thrombin. Therefore, such a

sample will generally yield abnormal test results and yield false suggestion of hemostasis disease. In contrast, serum represents the end stage of most hemostasis assays, and thus serum samples cannot be used to examine hemostasis. Examples of the effects of such samples on hemostasis and on the testing of such samples are given in Table 1. Sample type can be identified by either performing additional tests to assess presence or absence of fibrinogen (?serum) and heparin, or presence or absence of potassium, sodium or calcium (?EDTA) [46].

4.2 Under-Filling or Overfilling of Blood Tubes

Under-filling of sodium citrate blood tubes should not in general occur, as vacutainer systems generally deliver adequate fills; however, a traumatic or difficult collection may lead to under-filling due to obstruction or collapse of the venous system or early withdrawal of the needle. An under-filled tube is usually considered to be one that is <90% filled. Under-filling will lead to two linked events—dilution of the sample (given sodium citrate is in liquid form and meant to represent 10% by volume of the total blood collection) and over-anticoagulation of the sample. Both adversely affect hemostasis tests and lead to inaccurate test results that may lead to false conclusion of hemostatic disease or its false exclusion (Table 1).

Similarly, over-filling should not occur with vacutainer-type systems, but may occur with needle collection, and subsequent transfer to blood collection tubes. Over-filling may lead to insufficient anticoagulation of the sample, or inability to adequately mix the sample, and thus to pretest clotting events, which may increase or shorten clot-times in different tests depending on the balance of sample activation or clotting that has occurred (Table 1). Over-filling may also indicate that several partial draw citrate tube samples have been pooled, which may mean a doubling of citrate anticoagulant and over-anticoagulation of the sample, as in the under-fill blood collection tube.

4.3 Hemolysis

Hemolysis may result from in-vivo red cell lysis, but is usually a consequence of poor blood collection or processing. Hemolysis is the leading cause of hemostasis test interference [47] and causes both biological and spectral interference in hemostasis testing, with consequent inaccurate test results potentially leading to false conclusion of hemostatic disease [16].

5 Conclusions

Hemostasis testing has a foremost importance for the managed care of patients with many blood coagulation or platelet disorders, either hemorrhagic or thrombotic. However, the potential impact

Table 1
Consequences of inappropriate sample, poor collection, and/or poor sample processing

Event	How generated?	Effect on routine assays	Effect on specialized assays	Effect on patient?
Clotted sodium citrate blood sample or serum	Inadequate sample mixing, overfilled tube, sample transferred late from syringe collection or after being collected into clot-tube	False prolonged coagulation test times, low levels, or zero fibrinogen	False low factor assays (especially FII, FV, FVIII), false suggestion of VWD, flow obstructions in PFA-100.	Potential false diagnosis of fibrinogenemia, factor deficiency/ hemophilia, VWD, platelet dysfunction (depending on extent of effect and test(s) performed)
Partial sample clot		False shortening or prolongation in coagulation test times	False low or high factor assays, false suggestion of VWD, flow obstructions in PFA-100.	
Heparin contaminated sample	Sample collected into "heparin tube" and then transferred to citrate tube; sample collected post blood gas collection using heparinized needle; sample collected from heparin flush line	Raised coagulation test times, reduced fibrinogen	Reduced clotting factors (especially APTT based: FVIII, FIX, FXI, FXII); suggestion of factor inhibitors	False identification of dysfibrinogenemia/ hypofibrinogenemia; potential for false identification of LA, factor deficiency/ factor inhibitors
EDTA contaminated sample	Sample collected into "EDTA tube" and then transferred to citrate tube; EDTA plasma sent as frozen aliquot	False prolongation in coagulation test times	Reduced clotting factors (especially FV and FVIII); suggestion of factor inhibitors	False identification of factor deficiency/ factor inhibitors
Transport issues	Delayed transport or transport under poor conditions	False shortening or prolongation in coagulation test times	False prolonged clotting times and false low factor levels	
	Refrigerated transport	False shortened clotting times	Platelet activation (false shortening or obstructions in PFA-100), loss of FVIII (false low level), activation of FVII (false high level)	Potential false diagnosis of factor deficiency/ hemophilia, VWD, platelet dysfunction (depending on extent of effect and test(s) performed)

(continued)

Table 1
(continued)

Event	How generated?	Effect on routine assays	Effect on specialized assays	Effect on patient?
Centrifugation issues	Poor application including braking with high level platelet and RBC contamination of plasma and post freeze/thaw sample issues including hemolysis	False shortening or prolongation in coagulation test times	False negative LA, false high/low factor levels	Potential false diagnosis/exclusion of factor deficiency/hemophilia, LA, VWD (depending on extent of effect and test(s) performed)
Storage issues	Storage of whole blood for excess time or inappropriately, storage of plasma in frost free freezers with thaw/freeze cycling, storage of plasma in −20 °C freezers for excessive time prior to testing	False prolonged coagulation test times	False low factor assays, false suggestion of VWD	Potential false diagnosis of factor deficiency/hemophilia, VWD (depending on extent of effect and test(s) performed)
hemolysis	Poor sample collection (traumatic) or processing, freezing of plasma with cells remaining (e.g., excessive centrifuge brake)	False shortening or prolongation in coagulation test times	False negative LA, false high/low factor levels	Potential false diagnosis/exclusion of factor deficiency/hemophilia, LA, VWD (depending on extent of effect and test(s) performed)

This is not meant to be an exhaustive assessment, but aims to provide some examples that may be more commonly encountered.
Abbreviations: *APTT* activated partial thromboplastin time, *EDTA* ethylenediaminetetraacetic acid, *LA* lupus anticoagulant, *PFA* platelet function analyzer, *RBC* red blood cells, *VWD* von Willebrand disease

of many preanalytical variables (Fig. 1) must not be discounted, wherein such bias may ultimately lead to the generation of unreliable tests results, leading to incorrect conclusions about patient hemostasis status, and ultimately jeopardizing patient safety [1]. Consideration could also be given to providing mechanisms to improve practice in the preanalytical phase by initiation of quality indicators and corrective action [48].

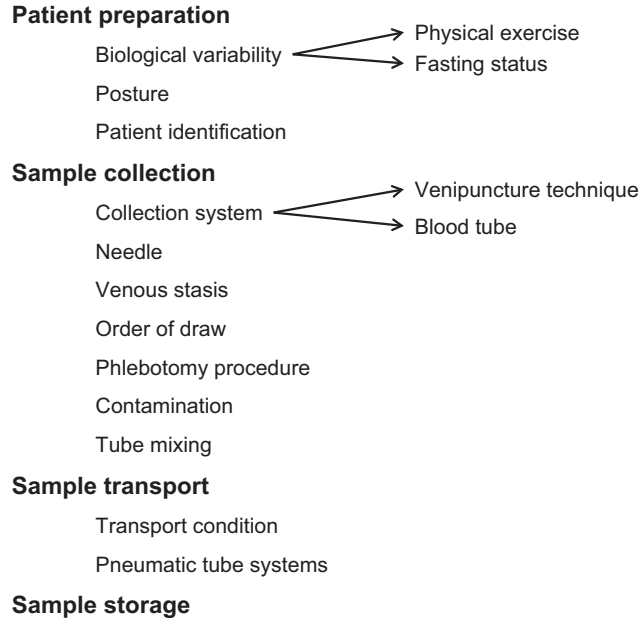

Fig. 1 Sources of preanalytical variability in hemostasis testing

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