Opinion Paper

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Extra-analytical sources of uncertainty: which ones really matter?

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Abstract: Since the endorsement by ISO15189:2012 of measurement uncertainty (MU) for the estimation of error in measurement procedures, the debate has been ongoing with questions concerning which method should be used for estimating MU and the benefits of using MU over other error methods. However, only limited attention has been given to extra-analytical sources of uncertainty and, currently, a clear standpoint is still missing. This opinion paper aims to evaluate whether extra-analytical variables could be included in MU. Considering coagulation tests as an example, the possible sources of preanalytical variations are evaluated by using a fishbone diagram. After excluding preanalytical errors, additional sources of uncertainty are divided into amenable to standardization/harmonization and/or possible random sources, which are not standardizable nor harmonizable. Finally, sources of uncertainty are evaluated for a possible inclusion into MU. In addition, postanalytical uncertainty is discussed, particularly considering the laboratory results calculated through a mathematical equation, derived from one or more quantities affected by their specific uncertainty.

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Introduction

The endorsement of measurement uncertainty (MU) by ISO15189:2012 for the estimation of measurement error has triggered a debate concerning the implementation of MU in clinical laboratories [1]. Two recently published opinion papers of Westgard [2] and Farrance et al. [3] underlined that the agreement around a scientifically sound model for estimating MU, suitable to be applied to a large number of measurement procedures, is still undefined [1–8]. However, one of the aspects that remained only marginally covered in both papers, was the inclusion of extra-analytical variations on MU estimation. The clause 5.5.1.4 of ISO15189:2012 clearly stated that "*The relevant uncertainty components are those associated with the actual measurement process, commencing with the presentation of the sample to the measurement procedure and ending with the output of the measured value*" [8], emphasizing the notion that MU estimation should be focused on the analytical aspects of a measurement (and not on the extra-analytical). Based on these premises, our viewpoint is not to promote criticisms to the ISO15189:2012 statement, but to discuss if and how MU estimation should be accommodated by including potential sources of uncertainty, originating in the extra-analytical phases. Indeed, the combination of several standard uncertainty components, including the extra-analytical phase variations, is advocated by the bottom-up approach proposed by the JCGM 100:2008 document [9].

The aim of this paper is to evaluate whether extra-analytical variables could be included in the MU. To this purpose, some preanalytical and postanalytical variables are firstly evaluated to distinguish sources of uncertainty from errors. Secondly, sources of uncertainty are discussed for standardization/harmonization feasibility and eventually for their inclusion to MU estimation. Coagulation

tests are used as example, even if discussion is extended to other laboratory tests extra-analytical variables.

Notably, the "within the laboratory wall" uncertainty represents only a part of the whole diagnostic uncertainty, which might cause physicians to give different diagnoses to the same patients, over-testing, unnecessary surgeries, more hospitalizations and referrals and increased health care expenditures [10]. Indeed, diagnostic uncertainty is a small part in the whole clinical reasoning that leads to decision-making and includes past experience of physicians, the pre-test probability of a disease or the disease prevalence, the uncertainty originating from the measuring procedure and from the interpretation of the results in view of the patient's clinical parameters or comorbidities and in differential diagnosis. This aspect, however, has not been considered in the present study.

The starting point for the evaluation of the MU of a test result includes the analysis of all the possible sources of uncertainty, which should be preferentially performed by a cause and effect diagram (also known as a fishbone diagram or Ishikawa diagram) [9]. Conceptually, extra-analytical uncertainty regards all the measurable sources of variations attributable to the preanalytical and postanalytical phases that can influence tests results.

Preanalytical uncertainty

A non-exhaustive list of possible sources of preanalytical uncertainty to consider are: patient preparation, blood collection, sample transportation handling and storage, possible interferences cause by hemolyzed, lipemic and clotted samples, wrong anticoagulant-sample volume ratio, within- and between-subject biological variation. Figure 1 shows the Ishikawa diagram of the major contributing factors to preanalytical variations for coagulation tests, such as prothrombin time (PT) and activated partial thromboplastin time (aPTT). These tests were chosen as illustrative examples because in hemostasis, similarly to other laboratory areas, the quality of results is closely related to the preanalytical aspects. The first point to highlight in Figure 1 is that many of the reported contributing factors to preanalytical variations, represent preanalytical errors. For example, blood collection from central venous lines might lead to heparin contamination and/or partially clotted samples. Both conditions represent preanalytical errors, even if the effects on results of coagulation tests might be different. Indeed, considering heparin contamination as an example, it might be critical for aPTT testing if monitoring therapy, while this will be less relevant for PT results, as most reagents nowadays incorporate heparin neutralizers up to 1 IU/mL [11]. Another example of preanalytical error is incorrect tube filling, that may cause serious consequences to coagulation test results; especially under-filled tubes might cause significant sample dilution and provide falsely prolonged clotting times due to the excess calcium-binding citrate [11]. Other common preanalytical errors, not only specific for coagulation tests, might be incorrect request, incorrect blood collection device, and wrong sample handling, etc.

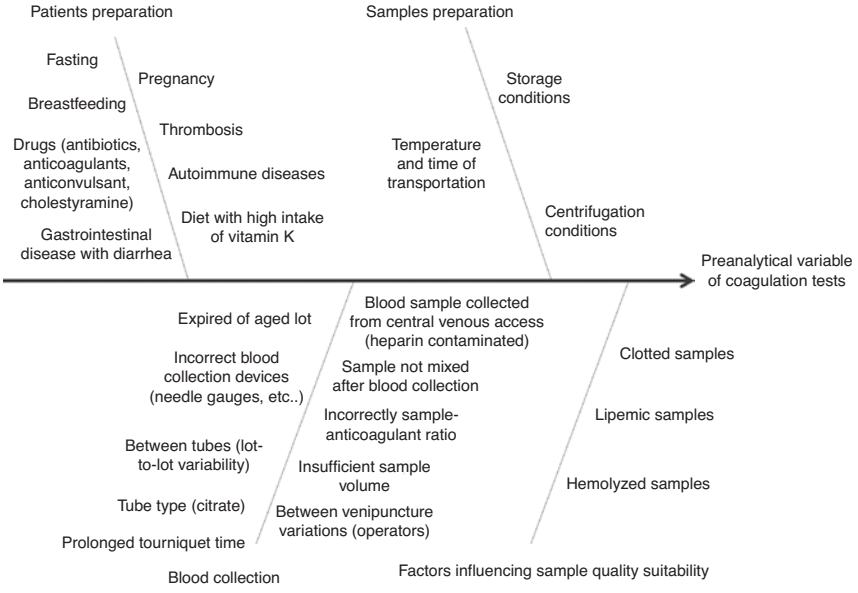

Figure 1: Ishikawa (fishbone) diagram reporting the major contributing factors to the preanalytical phase variations of coagulation tests.

All these preanalytical factors should ever be considered errors rather than sources of variations of test results; in fact, they derive from the complex interplay of system-related and human factors, including the lack of compliance with standard operating procedures (SOP) and may occur despite laboratory efforts in the field of risk management for the prevention, detection and mitigation of adverse events through the analysis of errors. Therefore, MU should not be confused with errors or mistakes [12]. According to this viewpoint, laboratories should comply with proper guidelines and SOPs for handling all specimens' issues originating errors, including rejection of samples.

Besides preanalytical errors, remaining sources or variation of laboratory tests in the preanalytical phase can be further divided in (a) amenable to standardization/harmonization or (b) random occurring sources of variation, which are not standardizable/not harmonizable (Table 1). Standardizable/harmonizable sources are represented by those factors for which the side effects on test results can be removed/minimized by well-written procedures [13, 14]. On the contrary, not standardizable/not harmonizable sources of variation can be only partially minimized, even if controlled. For factors occurring randomly, effects cannot be estimated *a priori* as neither the time nor the strength of the effect they may exert are fully quantifiable. Examples of standardizable/harmonizable sources of variations regarding patients are: patients' preparation (posture, fasting and physical exercise), venipuncture (e.g. tourniquets time, withdraw tube order, tube filling volume, sample mixing, clotting time), time and temperature of sample during transportation, centrifugation force and time (Figure 1, Table 1). Further, other cases of standardizable/harmonizable conditions for laboratory tests can be taken into consideration. For example, physiological changes during pregnancy result in alterations in many laboratory parameters. Pregnancy is described as causing variations not only of hormones levels, but also to several measurands levels, such as fibrinogen, coagulation factors (factors VII, VIII, X, von Willebrand factor [VWF]), metabolites (amino acids, urea), electrolytes (calcium, magnesium, iron, zinc, copper), etc… [15, 16]. Therefore, a laboratory tests request during pregnancy may become an example of inappropriate test requesting

Table 1: Preanalytical sources of variation for laboratory tests.

Preanalytical sources of variations			
Patients	**Blood collection**	**Sample handling**	**Analytical interferences**
Standardization/harmonization possible			
– Posture during venipuncture – Fasting – Daily and age-related circadian rhythms[a] – Drugs (e.g. antibiotics, anticoagulants, hormonal contraceptives, etc.)[b] – Pathophysiological alterations (thrombosis, autoimmune disease, etc.)[b] – Elective cases such as pregnancy, breastfeeding and menstrual cycle – Dietary factors or food supplements known to cause an effect on test result (e.g. biotin) – Physical exercise	– Between-operators venipuncture variations (e.g. tourniquets usage, tube order, etc.)	– Centrifugation conditions (time, force and temperature) – Temperature of sample transportation – Time of sample transportation	– Hemolyzed samples – Lipemic samples – Clotted samples – Icteric samples
Occurring random or standardization/harmonization not possible			
– Circadian seasonal rhythms – Dietary factor or food supplements not known to cause an effect on test result	– Between-part and between-lot variations in blood collection tubes (such as, e.g. aging of blood collection device variations) – Between operator (e.g. individual choice of the type of the blood collection device based on the specific patients)	– Pneumatic tube transportation	– Possible heterophile antibodies interferences[c]

Sources are divided in (a) amenable to standardization/harmonization or (b) random occurring, which are not standardizable/not harmonizable. [a]Standardizable/harmonizable by using different reference intervals. [b]Determinable by questionnaire. [c]They may affect some specialized hemostasis assays.

depending on the situation, although in other situations there may be a need for laboratory testing during this period.

Drugs intake, such as antibiotics, hormonal contraceptives, etc. may also cause effects on several measurand levels and hemostasis. Therefore, to standardize this preanalytical variable, the consumption of drugs that may affect laboratory tests should be ideally stopped for a period before laboratory testing. However, suspending drug consumption is not always possible and testing is often performed whilst on medications; in these cases, drug intake should be recorded on a questionnaire directly supplied to the patients before blood collection. A different situation is represented by tests requested to measure or monitor therapy, for example, international normalized ratio (INR) (warfarin) and aPTT (heparin anticoagulant therapy), when testing is performed without stopping medications. In therapeutic treatments or in the presence of pathophysiological alterations, phlebotomists should collect these patients' information, especially when they are of value for interpretation of results.

Besides drug intake, the questionnaire should also record other information like dietary factors or food supplements that may represent standardizable/harmonizable preanalytical variables, in order to allow laboratory professionals to carefully consider their possible effects on specific measurand levels.

Other avoidable events by standardization are the presence of some endogenous analytical interferents, which should be determined/measured automatically by the preanalytical workstations or by analytical instrumentations, for example, by "serum indices" [17]. For analytical methods known to be influenced by hemolysis, such as laboratory tests employing spectrophotometric measurements, hemolysis increases the background absorbance reading of plasma, thus affecting the accuracy of test results; in these cases, when *in-vitro* hemolysis is suspected and spectrophotometric methods are used, samples should be rejected as unsuitable samples. For coagulation tests, if testing must be pursued, for example, when *in-vivo* hemolysis is present or suspected, analysis can be performed by a mechanical end point detection system, although it should be noted that results may still be compromised as cell lysis products include tissues factors that may activate coagulation. Further, the management of icteric or lipemic samples is also challenging, as high concentrations of triglycerides and bilirubin can cause various degrees of interference in some laboratory tests, although they are often indicative of physiologic and clinically important disorders [17, 18]. In addition, some sources of biological variation could be minimized by carefully standardized protocols, namely the daily-related and age-related components of the biological variation; the fist, for example, like for cortisol and melatonin, could be minimized/eliminated by standardizing the collection time; the second, could be dealt with estimating age-dependent reference intervals either by direct or – when not possible – by indirect methods.

Conversely, other preanalytical sources of variations, such as those significant variations demonstrated to exist between different lots of blood collection tubes and between venipunctures can be only minimally reduced by standardized procedures [19, 20]. The study by Sylte et al. evaluated the between venipunctures differences for 15 measurands in 20 healthy volunteers, by using a standardized study protocol and found that the between venipuncture variations were significantly high with respect to other preanalytical sources, especially for some measurands such as glucose. The authors finally concluded that several causes of variations can be included into the between venipuncture sources of variabilities, such as the strength of the tourniquet, the position of the arm, the duration of the venipunctures, while the differences obtained in different arms were not found to be statistically significant [20]. In addition, pneumatic tube systems can cause preanalytical variations, mainly due to the increases of foam and bubble formation, potentially causing specimens hemolysis.

Dietary factor effects on test results are not completely understood yet, as recently pointed out for food supplements containing biotin (vitamin B7), which was demonstrated to exert an effect which is variable in magnitude and can skew results to be either falsely high or falsely low depending on the assay design and conditions [21]. For example, diets with highly variable vitamin K food intake may have a significant effect on PT/INR results for patients on warfarin therapy [22]. Another uncontrollable source of variation can be caused by the presence of heterophilic antibodies interferences, which although not known to affect routine coagulation tests, may affect some specialized hemostasis assays, and the effects of which on assay results are not estimable *a priori* [23]. A peculiar case of uncertainty might be represented by the international sensitivity index (ISI) and the mean normal PT (MNPT), which are both used to calculate INR from PT and to harmonize laboratory test results by taking into account reagent and instrumentation variability [24]. The raised issues on the accuracy of these indices might be at least partially accommodated by including uncertainty of ISI and MNPT in the MU of INR, although a detailed discussion of this topic is beyond the scope of this study.

Importantly, apart from the diurnal and age-related variation, the remaining variations due to the biological factors causing random fluctuation around a homeostatic setting point should not (theoretically) be included in the MU estimation. In fact, as measurements are defined by the international vocabulary of metrology as the process for obtaining a quantity value, biological variation is not a true part of the measurement, but rather has an effect on the patients' results and their interpretation [25]. Therefore, the biological variation affects more specifically the interpretation of test results rather than the test result itself. Tools such as reference change values or reference intervals, both containing biological variations in themselves, are usually used by clinicians for test result interpretation as they may be included in laboratory reports, while a proposal for interfacing the MU value with these two tools has recently been developed and explained [5, 7].

In should be emphasized that laboratories have the overall responsibility for the measurement procedure and the associated uncertainty, as defined by the ISO15189:2012 [8]. Therefore, in some circumstances, some preanalytical events can effectively be considered sources of variations of test results (such as slight hemolysis, prolonged storage time or above the normal range of specimen temperature) and therefore included in the MU estimation. For example, an attempt for handling the uncertainty of test results for the effects of interferences on specimens, such as hemolysis, either by applying a correction factor or by increasing measurement uncertainty, have been proposed by Jones and Hawkins [26]. These authors evaluated the possibility of reporting results corrected for hemolysis interferences, under certain defined and limited circumstances, by including in MU the uncertainty of the correction factor. They concluded that, if results should be provided, issuing results corrected for the effect of interference may be better than reporting uncorrected results when the uncertainties of each final result are included in the decision. However, this strategy should be suggested only when there is a linear response between the effect of sample interferent on test results, for example, cell-free hemoglobin concentrations interferences in potassium measurement. Similarly, Kouri et al. evaluated the effect of prolonged sample transportation (up to 3 days at 4 °C) and found that the uncertainty component varies from 0.4% of serum cholesterol to 17% of serum CRP [25]. Interestingly, with the uncertainty being dependent on the measurand type, on the storage time and temperature, it is virtually impossible to derive a unique function able to accommodate MU with storage time and temperature uncertainties. Extrapolated data on uncertainty for other measurands appear to be difficulty to obtain. Therefore, each test would have its own function, which should be experimentally estimated by adequate data and included in the MU estimation only for clinically relevant situations (e.g. specific test performed by hub laboratories in vast regional areas with several spoke laboratories) and only when laboratories have evaluated all the relevant effects.

Postanalytical uncertainty

Postanalytical uncertainty includes all the sources of variation that originate from the postanalytical phase, from the generation of results to their communication to physicians and subsequent interpretation. One example is represented by the estimation of glomerular filtration rate (eGFR), which is calculated using a formula including age, gender, creatinine levels and mathematical parameters [27]. When mathematical equations are applied to any quantities measured with uncertainty, such as measurands levels, volumes or weight, the law of propagation of uncertainties should be used to estimate the total uncertainty, as suggested by the JCGM 100:2008 document [9]. However, one alternative for estimating the eGFR MU can be represented by Monte Carlo simulations, which are both easy to be understood and implementable by spreadsheets [9, 28]. Interesting, postanalytical uncertainty will represent a challenge for tailoring of medical treatments to individual patients based on demographic information, genetic testing and other molecular sciences, namely proteomics or metabolomics, or single cell analyses. According to this view, it should be noted that precision medicine may give the impression of providing certainty, with "better diagnosis" and "best patients care". However, as Hunter recently pointed out, this is a false perception. Precision medicine results usually include a greater tolerance of uncertainty in diagnostic testing, especially when different results are combined together for calculating disease probabilities, using more specialized facilities due to complexity [29]. Importantly, the interpretation of that probability, which would assist physicians in making diagnosis will require approaches to data presentation, risk quantification and communication of uncertainty [29].

Conclusions

In conclusion, extra-analytical phases include many steps, most of which are potentially represented by preanalytical

errors. Considering the remaining preanalytical variables, it might be possible to minimize or limit their uncertainty by introducing well-defined procedures developed by laboratories for standardizing the total process. A few aspects of the preanalytical phase still may deserve consideration as possible sources of variability, though the accurate mathematical estimation of these sources of uncertainty is problematic. Postanalytical sources of uncertainty should also be considered, especially when multiple quantities measured uncertainty are used and combined for generating results utilized in the medical decision-making processes.

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