Preanalytical Considerations in the Design of Clinical Trials and Epidemiological Studies

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Clinical trials and epidemiologic and most types of clinical research include the acquisition of biological samples to be analyzed either immediately after collection or in the future for biomarkers related to the study hypotheses. Tested biomarkers include those used to monitor subjects’ health and to detect harmful side effects (e.g., liver and kidney function tests) or to follow the impact of therapeutic interventions. These samples may also be used for biomarker discovery. Whatever the study goals may be, the quality of study outcomes will depend heavily on the quality of the samples obtained and subsequent analysis. Since the largest component of total error in the clinical laboratory has been found to be associated with the preanalytical phase, it is probably safe to assume that the same conclusion applies to clinical trials and epidemiologic studies. Although standardization of preanalytical variables is not a trivial manner, it is essential to ensure successful outcomes of these studies. The competency of personnel performing such testing, and their training and understanding of sample collection and processing procedures, must be assured. Samples must be handled in an identical fashion at all times and in all locations, and procedures must be in place to avoid sample mix-ups. These are only few of the challenges encountered during the preanalytical phase. In this Q&A, 5 experts with a vast experience in national and international biomarker studies provide their insights into the issue of preanalytical variables and how these can impact the design of clinical and epidemiologic studies.

Do you think that preanalytical variables are given enough consideration when designing clinical trials or epidemiologic studies?

David Morrow: This is an area of vulnerability for biomarker testing in clinical studies. Although some investigators are very experienced and map out the preanalytical considerations thoroughly, in many cases, such planning does not occur either because of insufficient appreciation of preanalytical variables or the retrospective nature of many biomarker studies.

Christina Ellervik: Preanalytical standard operating procedures (SOPs) are as important in research as they are for the clinical laboratory. SOPs must be clearly defined in every trial or epidemiologic study, and if specimens do not meet SOP standards they should either be destroyed or flagged. It is important that all preanalytical variations be described for every specimen. Preanalytical errors in a study may cause delays in reporting results because samples have to be recollected.

Evan Stein: With the development over the last 2 decades of national and global central laboratories focused on supporting clinical and epidemiological trials, there has been a tremendous, and needed, improvement of all the laboratory related aspects. In clinical laboratories offering analysis for clinical trials the preanalytical variables related to transit time from patient to laboratory are a huge factor. A large
amount of our time and effort in the central laboratory was spent on assessing and documenting the effect of sample handling and sample stability under various conditions before analysis. This is something we never even thought of doing in our hospital laboratory.

Ann Hsing: In most epidemiologic studies, we give a lot of thought to our design, including covariates such as age, sex, race, socioeconomic status, body size, medication use, and other factors, before we start a biomarker study. We also pay meticulous attention to factors that can potentially affect sample integrity, including time of collection, blood separation and processing, and storage. So, yes, we do give careful consideration to variables, procedures, and QC issues before we start the study, collect samples, or assay samples in the laboratory. For epidemiologic studies, this is a standard practice.

Amar Sethi: Unfortunately, I would have to say, no. Laboratory data obtained during clinical trials are affected by the same shortcomings encountered for data generated in the clinical laboratory. The clinician is most likely to question a result if it fails to agree with the patient history and the overall clinical picture. This often leads to the questioning of the analytical rather than the preanalytical integrity of the system. Similar questions may be raised when the mean, median, or trend does not correlate with other related biomarkers.

David Morrow: There is substantial variability to the depth of such consideration. In the clinical literature, I believe that the potential impact of preanalytical issues is often given limited consideration and sometimes not recognized at all.

Christina Ellervik: Preanalytical variables should be taken into consideration when interpreting results, especially when results differ between similarly conducted studies. Discrepant results may arise from applying different preanalytical SOPs or breaches of SOPs. It’s important to remember that medical research is for the patient. Since conclusions may be drawn on diagnosis, staging, prognosis, stratification, etc., the results of a study could actually result in clinical guideline changes; thus, we must assure the highest standards in sample integrity. Otherwise there could be potential harm to patients from incorrect diagnoses or choice of treatment.

Evan Stein: As discussed above, there has been significant improvement in consideration and standardization of preanalytical variables into protocols. However, there has been less attention and focus when it comes to interpreting and publishing the results of trials, which are highly dependent on biomarker results. This is often a result of the lack of involvement of reviewers with expertise in laboratory science during the peer review process. Most high-impact journals have for the last few decades included a statistician as a reviewer for all clinical trial publications, but none that I am familiar with include a laboratory professional with knowledge and experience with the analytical aspects of the biomarkers being reported.

Amar Sethi: A set approach is often employed that addresses some, but not all preanalytical issues, and the considerations may be exactly the same for all analytes. Specimen type, processing instructions, storage conditions, and stability under predefined conditions are usually considered. However, implementing alternative conditions for analytes that require more stringent preanalytical handling may greatly impact the integrity of the study. For example, if platelet-poor plasma is required for a particular test, the trial sponsor may request an alternative procedure to process the sample, “because the trial site does not have the infrastructure to carry out complex processing protocols.” Although the alternative procedure might not have been studied thoroughly before implementation, the results would be expected to be equally robust as those obtained with the more established approach to sample preparation. These kinds of compromises cause the data obtained to lose statistical integrity and power and jeopardize their potential to adequately address the scientific question under study.

Do you think that preanalytical variables are given enough consideration in the clinical decision-making process when discussing the clinical impact of results from clinical trials or epidemiologic studies?
What strategies can be used to minimize preanalytical variables in clinical trials or epidemiologic studies?

David Morrow: Most important is prospective determination and planning for the potential impact of preanalytical variables on biomarker or other assessments. After this initial planning phase, implementation of a formalized set of operational instructions for sample collection and handling, verification of adequate facilities, and training of study staff, whether single-center or multinational, are all important.

Evan Stein: The single best strategy to reduce and anticipate such issues is to incorporate a knowledgeable laboratory professional from an experienced central laboratory in the drafting of the protocol and continue to keep this person involved throughout the trial. A specific laboratory manual and training of clinical sites is also an important aspect of all trials.

Christina Ellervik: Conducting a real-time pilot study that includes all the steps from sample collection to storage, retrieval, and analysis often identifies the weak spots, which then can be corrected before the real study begins.

Ann Hsing: In epidemiologic studies, we try to learn as much as possible about the determinants of a specific marker or a panel of markers before embarking on an analytic study (i.e., measuring a marker or a panel of markers in cases and controls). This is a standard procedure. For example, we conduct pilot or methodological studies to assess intra- and interperson variation of a specific marker, such as melatonin, sex steroids, or metabolomic profiles/patterns, within and between assay variations, and the impact of covariates on marker concentration or variation. These studies are usually carried out in healthy individuals (controls) first. We then evaluate factors, such as age, race, sex, body size, medication use, chronic conditions, and other factors that might impact the levels of this marker or a panel of markers.

Amar Sethi: It is important to realize that statistical analysis of laboratory data must not be based solely on analytical accuracy and precision. If possible, studies should be done to evaluate the effect of preanalytical variations on the resulting accuracy (difference from analyte measurement at collection and analysis) and precision. If a minimal accuracy criterion must be met to avoid exclusion of individuals from further trial participation, more stringent handling and processing procedures should be instituted, especially when the safety of participants is of concern. When higher imprecision is seen as a result of increased variation in preanalytical procedures, such information needs to be considered in statistical power calculations.

The effect of variation in specimen processing (e.g., centrifuge speed, platelet contamination, time from collection until processing) or from processing to storage (short-term stability at room temperature, 4 °C, etc.) should be studied. The potential for enzymic (e.g., proteases or nucleases) degradation or for nonspecific loss of the analyte of interest (e.g., binding to the surface of the collection tube) should be investigated and, if possible, remedied by adding stabilizers. Finally, a situation that is usually overlooked in clinical trials is the potential of interference by a novel drug, which can affect the accuracy of determining some analytes.

What resources are available for investigators when they begin to design their trials or sample selection processes?

David Morrow: For the most part, as I see this as dependent on involvement or consultation of individuals with sufficient experience and expertise. Commercial research laboratories often have well-vetted procedures for standard analytes but may have limited or no experience with investigational assays.

Christina Ellervik: The Biospecimen Reporting for Improved Study Quality (BRISQ) report is a useful tool. In this report, critical issues in biospecimen handling are described, and I think you will easily understand, if you’re a clinician and not a specialist in clinical chemistry, that consulting a clinical chemistry laboratory is essential in planning a trial or epidemiologic study.

Evan Stein: These resources are now fairly standardized for large NIH and major pharmaceutical trials but are not that easily available for individual researchers or for the many small biotech companies whose main focus and experience have been in preclinical drug development. In my experience, the very large central laboratories actually have limited numbers of professionals with experience in conduct of clinical trials, preanalytical variables, and protocol development and conduct. These aspects are often handled by the central lab’s business development, resulting in minimal direct contact or discussion between the laboratory professionals and the pharmaceutical team developing and running the trial. There are fortunately some midsize and smaller central and academic laboratories where such expertise is readily available.

Ann Hsing: I rely on my laboratory collaborators. They are the experts in the specific marker we are planning to measure, so I learn from them and tap into their expertise. We work together to design a sound and better study. Collaboration and knowledge sharing are the keys.
to success. It is critical to share credit fairly with our laboratory collaborators to build a long-term collaborative partnership.

I also recommend the National Cancer Institute’s Biorepository and Biospecimen Research Branch best practices and SOPs (http://biospecimens.cancer.gov/default.asp); and the International Society for Biological and Environmental Repositories information on best practices (http://www.isber.org/?page=BPR).

Amar Sethi: An expert laboratorian with knowledge of and experience in the measurement of the biomarkers to be studied should be consulted and potential preanalytical sources of error should be identified. The data gathered from these efforts should be provided to the investigators and statisticians for incorporation into trial design.

Sample and analyte integrity can be a difficult issue in longitudinal studies since samples often are collected long before it is known which markers might be tested. Is there a technology or a process that you recommend for samples being collected now that will improve stability of most analytes so that future investigations will not suffer from poor sample quality?

David Morrow: For our studies, in which we are assessing predominantly protein biomarkers, we place an emphasis on short processing times, and we transition to storage at −70 °C as soon as possible. We store longer term in the vapor phase of liquid nitrogen. Use of sampling tubes with protease inhibitors is also an option but can be costly and also may have unanticipated impact for certain analytes. We also aim to dictate appropriate centrifuge times to obtain platelet poor plasma.

Christina Ellervik: Technology and processes will depend on the type of samples you collect and what immediate or future downstream analyses you anticipate to carry out. Generally, the less human handling and the more standardization and automation (i.e., in sample processing, transport, storage, and laboratory information systems) the better.

Evan Stein: This is an important question. One can never predict what new biomarker will be discovered in the future and what will be the most optimal patient preparation, sample type, sample processing, and storage conditions. One can only suggest that as many types of samples (serum, plasma, etc.) be collected at baseline and selected other visits, be processed as quickly as possible, and stored at −70 °C in numerous aliquots. This is, and will remain, a challenge for everyone involved in clinical trials.

Ann Hsing: We do not know what the future holds or what markers we will be able to test in 2030 or thereafter. For example, 30 years ago, we did not have metabolomic assays or the knowledge that we would be able to measure circulating tumor DNA in plasma. There will always be new technology and new methodology for testing new markers, so it is not possible to have a process that will assure integrity of all markers to be tested in the future. The key is to be as rigorous as possible in our process, and exercise caution by processing specimens as soon as possible, usually within 2–3 h, to minimize potential degradation. Data from methodologic studies are helpful. The more we know about the stability and characteristics of a specific marker, the better we can design our study. For example, certain sex steroids are robust and do not degrade much at −20 °C, whereas others require −80 °C storage.

Amar Sethi: Stabilizers or preservatives may improve stability of some analytes, but unfortunately cannot protect all analytes from the effects of long-term storage. One retrospective approach for understanding the stability of a novel molecule is to begin long-term stability analysis using the preanalytical processes implemented in the original study. Back calculations can determine the original value of the analyte once the rate of change is identified. This retrospective approach may be slow; however, it provides a high degree of confidence due to the specificity of the study with regard to the preanalytical processing, the analyte, and the method employed.

More work should be done during qualification of biomarkers for use in epidemiologic studies and clinical trials, as well as for use in the in vitro diagnostic sphere. This work would include studies that document the best specimen type, collection, processing, and preservative addition(s). Furthermore, an effective and rational storage protocol and study of short- and long-term stability of biomarker in the matrix of choice should be developed. Much of this work could be undertaken by the Foundation for the NIH Biomarker Consortium through a systematic analysis of specimen collection, processing, and storage protocols for a limited number of novel biomarkers.

Hospital laboratories are a potential rich source of samples for biomarker explorations. What are the limitations to use these types of samples?

David Morrow: These samples are often stored refrigerated, rather than frozen, and may sit at room temperature for prolonged periods. These aspects tend to limit the scope of analytes that we can reliably assess.

Christina Ellervik: Hospital samples are usually not collected for research purposes. Nevertheless, the preanalyti-
cal considerations to these samples should not differ from those in a research setting. To use these samples, we must know as much about the patients as in a specific research study. In some countries these data can be difficult to track, but in others, extensive linkages to electronic records or registries are available making them easier to use. However, in some countries ethical issues may prevent these samples from being used for research purposes.

**Evan Stein:** Hospital laboratories do provide a very rich potential source of samples but such samples present major limitations for any type of clinical research. These problems start with the lack of approval to use these samples for anything other than what has been requested by the patient’s physician and the difficulty and time needed to obtain ethics review and approval for any other use. Second, hospital laboratories have limited ability and storage space, and virtually none to retain samples at −70 °C for any period of time. In general, hospital laboratories keep samples for 7 days at 4 °C and then discard them. A third issue is that the information technology (database and identification) systems are not designed for clinical trials. Consequently, the effort, cost, and expertise needed to search and create patient disease databases to find and select out specific samples are not a priority and are perceived as adding to laboratory costs; this is compounded by the additional, and specialized personnel and storage facilities needed. Having started our clinical trial work in a large hospital academic center as a way to increase resources for funding training programs, it soon became apparent that trying to do both routine patient service and clinical research were incompatible and led to our group leaving to focus on clinical trials.

**Ann Hsing:** Samples from hospital laboratories, mostly clinical samples, are great resources for biomarker exploration. They often come in handy as pilot samples and are useful for certain clinical assessments. The usefulness of these samples depends on the research question. If we are interested in prevalence of certain infections, average or range of a biomarker (such as sex steroids or cytokine concentrations) in the population (healthy individuals), or cholesterol concentration in a population or certain groups, these clinical samples would not be the best source. Since these samples generally come from individuals who visit the hospitals for certain conditions (chronic or acute), the concentrations would be skewed and not representative of those seen (means, ranges, or prevalence) in the general population.

**Amar Sethi:** The kinds of novel biomarkers that are often employed in clinical trials are generally not stable under the handling and processing protocols commonly used in hospital laboratories. If specimens from clinically interesting subjects are collected for routine testing, they are unlikely to be useful for evaluation of many low-abundance proteins, complex lipids, metabolites, and many nucleic acids.

**Besides the previously discussed issues with sample integrity and storage, are there preanalytical issues related to the information that accompanies samples?**

**David Morrow:** There certainly can be. In general, variability in recovery of analyte resulting from preanalytical issues tends to introduce “noise” and thus bias observations toward the null. We are much more likely to miss an important observation than to detect a false association. However, the absolute concentrations of an analyte across a population can certainly be shifted by preanalytical issues. For example, decreased recovery of brain natriuretic peptide from use of glass tubes or handling at higher temperatures might lead to the systematic recording of lower concentrations in an entire population of samples.

**Christina Ellervik:** Some papers have attempted to address, list, and prioritize all the preanalytical variations that need to be taken into consideration and documented for samples in studies. I think the take-home message in this Q&A is that any possible variation in the preanalytical process that you can think of will impact the study result. And the sum of all preanalytical errors, analytical errors, and biological variation will definitely impact your study results; thus, it’s equally important to address the potential preanalytical issues pre- and poststudy instead of overlooking their existence.

**Evan Stein:** This is an important question that can impact the interpretation and validity of the results. For example, it is important in diabetic and lipid trials to know the time of the last meal before sample collection as this significantly influences blood sugar and triglycerides (usually used in calculating low-density lipoprotein cholesterol). These parameters, which these days are often programed and determined by the central laboratory, may also impact patient inclusion or exclusion in a clinical trial.

**Ann Hsing:** For most samples used in epidemiologic studies, we usually require information on 1) time of collection, 2) whether the blood was collected during the fasting state, 3) time elapsed between blood collection and processing, 4) anticoagulant used, 5) stabilizing agents used (EDTA or ascorbate), 6) blood processing method, 7) storage temperature and condition, 8) freeze-and-thaw cycles, and 9) special procedure used during collection or processing. We also record whether the samples are hemolyzed or are hyperlipemic. These factors may interfere with the assay of specific markers. We also
ask about shipping methods and temperature during shipping to ensure that specimens are kept frozen before the assay.

For all of our studies, we always include blind replicates to evaluate within- and between-batch assay variation. This is a standard QC procedure in epidemiologic studies. If within-batch variation (usually assessed by CV) is too high (usually >15%, depending on the biological nature of the marker), we would request re assay. If an assay does not require too much serum/plasma volume, we usually ask for replicate measurements for each subject and then use the average of the 2 measurements for analysis. This is not always possible as serum/plasma samples from prospective cohort studies are precious and every drop needs to be used with great caution and justification.

Amar Sethi: In addition to the previously discussed issues, information about the status of the subject supplying the specimen can be very helpful when deciding whether data from a particular sample should be collected or included in a study. For example, drug and nutritional supplement intake, diet, fasting status, and time of collection are essential to establish if interferences or diurnal variation could impact results. Beyond that, a host of general demographic data could be especially helpful in the epidemiologic domain. Another practical issue related to potential errors is the kind of storage tube in which a specimen might be stored. The type (even brand and lot) of tube and its construction material could be important in some circumstances.

Can you give an example of an “I wish I had thought of that” experience from one of your trials or studies?

David Morrow: We have had many “hard knocks” with our platelet studies. Because of the ex vivo release of platelet markers during activation of residual platelets, it is critically important to obtain platelet-poor plasma if one wishes to assay true circulating concentration of a biomarker. Soluble CD40 ligand is a good example of a biomarker where very disparate results are obtained from serum (where there is substantial ex vivo release during coagulation), plasma, and platelet poor plasma. In my view, it was the challenge with preanalytical variables that contributed strongly to the failure of CD40 ligand to be reproducibly useful across studies and led to substantial confusion for researchers in the field.

Evan Stein: Unfortunately, wishing one had collected and stored a sample, an additional sample, or a different one (perhaps plasma plus serum; or stored at −70°C instead of −20°C) is a far too common occurrence over the years. We never know what biomarkers or genetic markers will be discovered in the next year, let alone 5 or 10 years, and often wish we had appropriate samples, properly collected and stored, that we could quickly “pull out of the freezer” and assess. While we would like to collect every type of sample possible, and store them under optimal conditions, this is just not possible owing to both financial and human ethics committee restrictions. Such concerns should be carefully considered both by the funders as well as investigators and laboratory professionals very early on in the planning of all trials.

Ann Hsing: The process and quality of the samples are usually satisfactory, but I always wish I could have collected one more tube of blood for additional testing or had the resources to include more samples for testing, so we would have sufficient power to make a definitive statement about certain subgroups. For example, in many of our studies, we often do not have a large enough sample size for minorities, including African Americans, Hispanics, or Asians, making it difficult to generalize the findings to these important subgroups. In epidemiologic studies, for example, for metabolomic studies, we need large samples (approximately 1000 samples) to have sufficient power to tease out modest effects. Since resources are limited, it is important to be highly selective, careful, and meticulous in what we do to maximize the values of these precious samples.

Amar Sethi: It is the duty of those directing the work in the laboratories providing testing for clinical trials and epidemiologic studies to inform investigators of the preanalytical issues that could affect the quality of their studies. I cannot count the number of times investigators have come to our laboratory wondering why the results they had obtained from a study did not meet expectations. After analysis of their study protocols we had to tell them about the potential for diurnal variation, fasting status, or a stability issue that was not controlled, and which likely adversely affected their study findings. To prevent study directors from telling us, “I wish I had thought of that” we need to educate our biomedical community colleagues about the issues we have discussed here.

Christina Ellervik: Don’t let the preanalytical issues be an afterthought; the preanalytical considerations are the cornerstones of the biochemical analyses. If you can’t describe the preanalytical process in your study, don’t analyze your samples.

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