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Editorial

Paramount importance of sample quality in pre-clinical and clinical research – need for standard operating procedures (SOPs)

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1. Pre-analytical errors contribute to irreproducible data

Irreproducibility of biomedical research is an overarching problem, where several key reasons have been identified, including: i) problems with study design, ii) variability in biological tools (such as antibodies, cell lines), iii) data quality, iv) biased data analysis and interpretation of results, and iv) inadequate documentation and reporting of protocols (Freedman et al. 2015) and further often a lack of publicly available raw data. In post-genomic era biomedical research often depends on collection of biological materials including body fluids and tissue samples from animal models or humans followed by state-of-the-art transcriptomic, proteomic or metabolomics profiling. Omics studies of body fluids (i.e. blood, urine, saliva, cerebrospinal fluid, follicular fluid, peritoneal fluid) are crucial for biomarker discovery, as well as for understanding systemic disease mechanisms, while molecular profiling of tissue samples have essential roles for deciphering disease pathophysiology and for identification of tissue biomarkers and novel drug targets.

The scientists might not be aware that differences in collection, processing and storage of biological materials can alter molecular composition and can influence experimental outcomes, and thus can lead to irreproducible, misleading and erroneous results. It has been reported that in routine clinical diagnostics as much as 60-80% of errors occur in the pre-analytical phase (Lehmann 2015), (Carraro *et al.* 2012). Sensitive and very expensive downstream targeted or non-targeted omics analyses can thus be jeopardized by the pre-analytical errors, due to low quality samples (Yin *et al.* 2015). However, it has to be emphasized that omics approaches differ considerably in their susceptibility to pre-analytical errors, with metabolomics being the most sensitive to pre-analytical confounders (Lehmann 2015), (Anton *et al.* 2015).

2. Standard operating procedures ensure sample quality

After collection, biological materials may undergo pronounced changes of transcriptome, proteome and metabolome, therefore detailed protocols, so called "standard operating procedures" (SOP) are needed to control and harmonize all pre-analytical steps. By following SOPs good quality of samples can be assured and the pre-analytical variability can be significantly reduced (Moore *et al.* 2011a). SOPs have crucial roles for collection of all biospecimens either for a biobank or individually for a particular pre-clinical or clinical study. SOP for collection of different biospecimens can be obtained from different sources including Biospecimen Research Database (BRD) at National Cancer Institute (https://brd.nci.nih.gov/brd/), where evidence-based biospecimen SOPs are available as also information about effects of individual pre-analytical factors on specific molecular analyses (Moore *et al.* 2011a). A SOP for collection of a particular biospecimen has to be adapted and optimized to the individual study and to downstream analyses and also to the limitations of the individual institution or institutions, "balancing ideal with practical/workable" (Jackson and Banks 2010). This should be done in close collaboration between clinical-biochemists, clinicians and analytical experts to ensure feasibility and consequently strict compliance with SOP, which is prerequisite for sample quality (Yin *et al.* 2015). Evidence-based SOPs should thus define the best practices and have to be updated on a regular basis according to new findings in biospecimens research (Moore *et al.* 2011a). It is also highly recommended that

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SOP is pretested and that staff involved in sample acquisition, preparation, storage and transportation is appropriately trained before starting with sample collection (Kühn *et al.* 2014).

3. Pre-analytical errors jeopardize sample quality

As biomedical research most often relies on molecular profiling of blood samples and tissue samples, we below provide a list of pre-analytical errors associated with collection of these bio-specimens.

3.1. Pre-analytical errors in collection of blood samples

When performing blood molecular profiling scientists should be aware of multiple factors before and during sample collection that can affect omics data. These factors include: i) nutritional state of the individual, ii) circadian rhythm, iii) exercise before specimen collection, iv) diet, v) life style factors (e.g. smoking), vi) dietary supplements, vii) medication, etc. (Lehmann 2015), (Yin *et al.* 2015). Therefore the relevant data considering an individual patient, healthy individual or study participant has to be gathered using appropriate questionnaires to allow inclusion of these potential confounders or effect modifiers in further analysis.

During sample collection pre-analytical bias may originate from: i) sample processing times and temperatures; ii) type of blood collection tubes and additives, time of coagulation; iii) haemolysis of samples; iv) sample storage conditions; and v) freeze-thaw cycles.

3.1.1. Sample processing times and temperatures

The exposure of whole blood samples to room temperatures affects metabolites (Kamlage *et al.* 2018) and protein profiles (Kaisar *et al.* 2016) (Lehmann 2015), due to metabolic activity of blood cells (e.g. anaerobic glycolysis in erythrocytes increasing levels of lactate and decreasing levels of glucose; pentose phosphate pathway increasing concentrations of glucose-6-phosphate; proteinase activity leading to protein degradation and increased concentrations of amino acids, phospholipase A2 activity increasing concentrations of lysophospholipids, etc.). The difference in time of exposure to room temperature thus changes concentrations of metabolites and proteins and introduces pre-analytical bias. For instance, MS-based metabolomics profiling revealed that 6 h incubation of a whole blood at room temperature affected 24% of 255 tested metabolites (Kamlage *et al.* 2018). Storage of serum and plasma samples at 4° or on cool packs for up to 24 hours significantly stabilised metabolites (Breier *et al.* 2014). On the other hand prolonged incubation at room temperature had less pronounced effects on plasma proteome as determined by quantitative LC-MS/MS (Kaisar *et al.* 2016).

3.1.2. Blood collection tubes and additives, time of coagulation

Also blood collection tubes (glass *versus* polypropylene or polycarbonate), time of coagulation (serum) and different anticoagulants (plasma) can affect omics data as shown for their proteomics and metabolomics profiles (Jackson and Banks 2010). Due to inherent instability of RNA blood transcriptomics require special collection tubes with proprietary stabilizing reagent, which allow immediate lyses of whole blood cells and stabilization of RNA molecules (Duale *et al.* 2012). As microRNA molecules are packed in microparticles or lipoproteins and can be associated with RNA-binding proteins (e.g. Argonaute 2) they are remarkably stable and can also be isolated directly from plasma samples (Mitchell *et al.* 2008). The differences in the anticoagulants used might change the sample composition; e.g., heparin binds certain proteins (Jackson and Banks 2010), while Li-heparin may cause matrix effects when studying metabolites (Yin *et al.* 2015). As compared to plasma samples, the serum samples are more prone to pre-analytical variability. Blood clotting is namely associated with release of various metabolites and proteins from activated platelets – thus the time of coagulation has to be strictly controlled (Lehmann 2015).

3.1.3. Haemolysis of samples

The most common pre-analytical error is haemolysis, which can also affect metabolomics and proteomic profiles as it causes the release of intracellular constituents including metabolites and enzymes (Jackson and Banks 2010), (Lehmann 2015), (Yin *et al.* 2015). Haemolysis can result from strong aspiration, vigorous shaking, centrifugation at high velocity, and also by transportation by pneumatic tube system and these factors have to be taken into consideration (Lehmann 2015).

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3.1.4. Sample storage conditions

Also exposure of serum and plasma samples to room temperature affect molecular profiles, most probably due to the enzymes released or secreted from blood cells before centrifugation. Targeted metabolomics study reported increased levels of amino acids and lysophospholipids when serum samples were kept for prolonged time at room temperature before freezing (Anton *et al.* 2015). The storage temperature is crucial, as the freezing of samples alone might not prevent alterations to the proteome, metabolome and transcriptome (Jackson and Banks 2010).

Storage at -20ºC leads to substantial proteolysis and other modifications of a subset of proteins in serum samples (Lee *et al.* 2010). It is thus essential to store serum or plasma samples at -80ºC or below. Also prolonged storage at -80°C may affect metabolic profiles. An analysis of long term storage of plasma samples for over 5 years at -80°C revealed overall good metabolite stability (55 metabolites out of 111 remained unchanged). The effects were different in distinct classes: concentrations of amino acids increased by 15% and acyl-alkyl-phosphatidylcholines decreased by 13% for example (Haid *et al.* 2017).

3.1.5. Freeze/ thaw cycles

It is also important to note that freeze-thaw cycles can alter sample composition and thus molecular profiles, as these cause aggregation, adsorption to tubes, and precipitation (Jackson and Banks 2010). It is thus highly recommended to mix the sample and subsequently prepare multiple aliquots before freezing. If thawing cannot be avoided, samples should be thawed stepwise and kept on ice for as short time as possible (Yin *et al.* 2015). For serum samples one freeze-thaw cycle did not affected the metabolites while two freeze-thaw cycles revealed significantly decreased concentrations of 11 out of 159 metabolites as demonstrated by targeted metabolomics (Breier *et al.* 2014). Further studies showed that some classes of molecules like oxilipins might be heavily affected (Dorow *et al.* 2016).

3.1.6 Transportation

Finally, sample transportation is also important and may introduce pre-analytical bias due to fluctuations in temperatures or sample thawing. Samples have to be transported to a biobank or the analytical laboratory on a sufficient quantity of dry ice by a reliable courier or logistic company (Kühn *et al.* 2014).

3.2. Pre-analytical errors in collection of tissue samples

Also molecular profiling of tissue samples can be affected by different pre-analytical factors. The factors to be considered overlap with those summarized for collection of blood samples (Jackson and Banks 2010). The pre-analytical bias can be associated with factors considering the patient or study participant, among others, medications applied before or during surgery. During sample collection the quality of tissue samples may depend on the surgical procedure, especially the time between ligation of the main artery, which causes ischemia, and removal of the biospecimen (Spruessel *et al.* 2004). In colon tissue from cancer patients it has been shown that 30 minutes of ischemia affected expression of 20% of 18,400 transcripts and 30% of proteins, where most changes occurred within 15 minutes of ischemia (Spruessel *et al.* 2004). Also processing and storage may affect the quality of samples, e.g. size of tissue sample, delay to processing and freezing, method of freezing or type of fixative, temperature and duration of storage, freeze and thaw cycles (Jackson and Banks 2010).

4. Quality control measures

To improve the reproducibility of biomedical research it is essential that biospecimens are collected according to evidence-based SOP, and that handling of biospecimens is reported in a thorough and standardized manner. At present harmonization of biobanking standards have already been achieved in certain areas of medical research (Rahmioglu *et al.* 2014; Adishesh *et al.* 2017) and also Biospecimen Reporting for Improved Study Quality (BRISQ) Guidelines have been prepared for body fluids and solid tissues (Moore *et al.* 2011b). To identify and record the main pre-analytical factors that may affect integrity of clinical fluids and tissue biospecimens during collection, processing and storage biobanks and individual laboratories can benefit from Standard Preanalytical Code (SPREC), which has been developed by International Society for Biological and Environmental Repositories. The ability to track and control pre-analytical variations is fundamental for the provision of high quality biospecimens for biomedical research. As biobanks have no proof of actual sample quality and pre-analytical variables have not been recognized in the early years of biobanking (Nussbeck *et al.* 2013) (Lehmann 2015), it is of utmost importance to have appropriate markers to determine the quality

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of samples before expensive downstream analyses. Potential markers for sample quality have been studied using proteomics and metabolomics approaches (Anton *et al.* 2015) (Kong *et al.* 2017). Recently, Liu et al identified (4E,14Z)-sphingadienine-C18-1-phosphate as a biomarker that reflects deviations from SOPs during sample processing and can be used for assessing the quality of serum and plasma samples (Liu *et al.* 2018). To the best of our knowledge no biomarkers for assessing the quality of tissue samples have so far been reported.

5. Closing remarks

Good quality samples is of uttermost importance for reliable and reproducible biomedical research. Scientists should be aware that small differences in the processing and handling of specimens can introduce pre-analytical bias and can have dramatic effects on analytical reliability and reproducibility (Jackson and Banks 2010). Thus the levels of proteins or metabolites in biospecimens may not always originate from the pathophysiological processes but may result from different pre-analytical factors (Lehmann 2015). When collecting biospecimens, evidenced based SOP, optimized for a particular omics approach, should be strictly followed as regards to collection, processing and storage of samples. If possible, a sample quality test should also be performed before performing downstream "omics" analyses. By following these recommendations pre-analytical errors should be minimized, which will contribute to better quality and reproducibility of individual studies and biomedical science as a whole.

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