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Understanding Preanalytical Variables and their Effects on Clinical Biomarkers of Oncology and Immunotherapy

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Abstract

Identifying a suitable course of immunotherapy treatment for a given patient as well as monitoring treatment response is heavily reliant on biomarkers detected and quantified in blood and tissue biospecimens. Suboptimal or variable biospecimen collection, processing, and storage practices have the potential to alter clinically relevant biomarkers, including those used in cancer immunotherapy. In the present review, we summarize effects reported for immunologically relevant biomarkers and highlight preanalytical factors associated with specific analytical platforms and assays used to predict and gauge immunotherapy response. Given that many of the effects introduced by preanalytical variability are gene-, transcript-, and protein-specific, biospecimen practices should be standardized and validated for each biomarker and assay to ensure accurate results and facilitate clinical implementation of newly identified immunotherapy approaches.

Keywords

Preanalytical variability; Biospecimen Science; Cancer immunotherapy; Biomarker

Introduction

Recent advances in immuno-oncology have brought immunotherapy to the forefront of clinical medicine as a viable approach for cancer treatment [1]. Cancer immunotherapy encompasses several different strategies that range from immunomodulating drugs to protein-specific inhibitors. Translation of research gains to new therapies has been rapid, as Food and Drug Administration (FDA)-approved cancer immunotherapies now include monoclonal antibodies against a specific protein involved in cancer progression, such as trastuzumab (Herceptin®) that targets the HER2 protein [2]; antibody-drug conjugates, such

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6.0 Conflict of interest

The authors declare that there are no conflicts of interest.

as ado-trastuzumab emtansine (Kadcyla®) that contains both an antibody specific to HER-2's extracellular domain and the cytotoxic drug DM-1 [3]; and antibodies that target checkpoint inhibitors, such as the anti-PD-1 antibody pembrolizumab [4], (Keytruda®), nivolumab (Opdivo®) and atezolizumab (Tenectriq®). Chimeric antigen receptor (CAR) T-cell immunotherapy, CTL019-KYMRIAH™ (tisagenlecleucel), a new therapy for pediatric and young adult B-cell acute lymphocytic leukemia (ALL) patients, also recently gained FDA approval [5].

While these new immunotherapy options represent novel breakthroughs in cancer treatment, they remain reliant on the same biospecimen collection practices utilized by more traditional treatment options. The methods used to collect, process and store biospecimens can vary widely within and across different medical institutions and individual laboratories. These variations, or preanalytical factors, in biospecimen handling have been shown in the literature to markedly affect the detection of clinically relevant diagnostic biomarkers [6]. For example, how a blood or tissue biospecimen is collected, processed and stored can affect its cellular, molecular, and proteomic profiles. Preanalytical factors include the type and size of the biospecimen, vessel used, duration and temperature of a delay to processing, preservation method, temperature and duration of storage, and number of freeze-thaw cycles. Identifying and minimizing effects introduced by preanalytical variability is difficult as such effects are often not global in nature but instead can be specific to the type of biospecimen used, the analytical platform employed, and the gene, transcript, or protein affected. Effects are often also dependent upon the specific preanalytical factor; for example, different proteins may be affected by a delay that induces tissue ischemia vs. the storage conditions of a preserved specimen. The variety of preanalytical factors known to affect the accurate assessment of biomarkers within a biospecimen illustrates the complexity of challenges faced by basic and translational research as well as clinical laboratories testing patient samples.

Accurately and reliably assessing DNA, RNA and proteins in a biospecimen allows clinicians to appropriately stratify patients with the same disease, make optimal treatment decisions, and avoid potentially serious complications for patients. This review will focus on preanalytical variables that affect detection of basic and clinical biomarkers relevant to immunotherapy in both tissue and blood biospecimens.

1.0 Preanalytical variables and Tissues

Predictive and prognostic biomarkers stand at the forefront of precision medicine, as they guide patient stratification and impact clinical decisions. Suboptimal or variable practices in the collection, handling and processing of tissues can lead to significant variability in molecular and proteomic profiling, which includes immunological biomarkers. Regardless of whether treatment is immune-, hormonal-, or chemotherapy-based, molecular and proteomic biomarkers are crucial to cancer diagnosis and in predicting treatment response of a tumor. Tissue biospecimens that are used in basic and clinical research to identify, validate, and apply such biomarkers include both snap-frozen and formalin-fixed, paraffin-embedded (FFPE) biospecimens. Damage to proteins and nucleic acids may be introduced by both types of preservation, as well as during tissue handling, processing, and storage procedures.

It is crucial that the potential impact of preanalytical variability on biomarkers used to direct and gauge immunotherapy is not overlooked. Programmed cell death protein 1 (PD-1) is an immunoinhibitory receptor expressed on the surface of CD4+ and CD8+ T-cells, B cells, and some tumor infiltrating lymphocytes (TILs). As PD-1 functions as a late-phase response immune checkpoint, PD-1 and its physiological ligands (PD-L1 and PD-L2) are current targets of cancer immunotherapy [7, 8]. Immunotherapy employing monoclonal antibodies against PD-1 and cytotoxic associated antigen 4 (CTLA-4) have garnered attention as immune checkpoint inhibitors in several cancer types including melanoma, NSCLC, RCC, bladder cancer and Hodgkin's disease [9–14]. A recent review estimates that inhibition of PD-1 has been investigated using nine different antibodies in more than 500 clinical studies [8]. Tumor expression of PD-L1 protein may be predictive of anti-PD-1 treatment response for some tumor types [15, 16]. Thus, immunohistochemical staining of PD-L1 in tissue and CD3, CD4, and CD8 in TILs are routinely performed on biopsies obtained pre and/or post-treatment to understand the biology of the tumor and to gauge the tumor's response to treatment. Evidence supporting the prognostic and predictive value of TILs identified via immunohistochemistry is strong for a number of solid tumors, including melanoma, renal cell carcinoma, prostate carcinoma, urothelial carcinoma, head and neck squamous cell carcinoma, ovarian carcinoma, non-small cell lung carcinoma, gastric carcinoma, and colorectal carcinoma [17]. There are currently three PD-L1 expression assays that have been approved by the US-FDA for use in FFPE tumor tissue as companion diagnostic assays (Dako, IHC 22C3; Dako, IHC 28-8; Ventana Sp142 assay) and clinically validated as a predictor of anti-PD-1 therapy response. However, it has been reported that different PD-L1 assays may generate different results even when case-matched samples are analyzed per the manufacturer's instructions [18]. This may be partially attributable to absence of non-standardized conditions for tissue collection, fixation, processing, scoring and interpretation. Such studies suggest that significant challenges remain regarding optimizing and validating PD-L1 assays. Despite the emergence of PD-1 as a promising new immunotherapy for cancer treatment, we were unable to locate a study investigating potential effects of preanalytical factors on PD-L1 expression although two recent reviews outlined the importance of considering and minimizing preanalytical variability when quantifying PD-L1 expression [18, 19]. Notably, PD-L1 expression is sensitive to analytical variability, with significant differences in immunohistochemical scoring reported for three FDA-approved companion and complementary assays for PD-L1 expression in NSCLC [20].

Given the rapid implementation of immunotherapy approaches in cancer treatment, it is not surprising that evidence is sparse regarding potential susceptibility of related biomarkers to preanalytical effects. However, effects observed among non-immunologic biomarkers may be indicative of how immunotherapy-relevant biomarkers are affected by preanalytical variability given a shared reliance on the same clinical biospecimen types and established analytical platforms for tumor characterization across cancer treatment options. For example, estrogen receptor (ER) and progesterone receptor (PR) status of breast tumors, as determined by immunohistochemistry, is a valuable clinical tool used to determine appropriate treatment options. The American Society of Clinical Oncology and the College of American Pathologists estimated that up to 20% of immunohistochemical analyses worldwide may be inaccurate, a problem that has partially been attributed to preanalytical

variability [21]. A false-negative or false-positive ER status may not only result in inappropriate hormone therapy, but may (in the future) also affect the efficacy of immunotherapy. It was recently reported that cytokine-based immunotherapy in conjunction with anti-estrogen therapy elicited a synergistic response in patients diagnosed with ERα-positive metastatic breast cancer [22]. Specific steps and factors in the collection, preservation, processing and storage of FFPE specimens that have been shown to adversely affect immunohistochemical staining of clinically relevant and immunological biomarkers are reviewed below, but have also been described in detail previously [23]. Non-immunological companion biomarkers also often represent the first line of tools employed for patient stratification regarding treatment eligibility. Immunotherapy biomarkers could then be explored secondarily in the same FFPE tissue specimens to gauge eligibility or to assess a patient's response to immunotherapeutic agents.

Importantly, effects of preanalytical variability associated with FFPE tissue biospecimens are not limited to immunohistochemistry. With the advent of next-generation sequencing (NGS), it has been possible to identify somatic mutations in large fragments of DNA isolated from FFPE biospecimens using whole exome sequencing (WES) and whole genome sequencing (WGS). WES has also been used to identify neo-epitopes which present as major histocompatibility complex (MHC) class I and class II targets for T-cell receptors [24]. A high frequency of tumor somatic mutations in melanoma and NSCLC correlates with a clinical benefit from treatment with checkpoint inhibitors [25, 26]. However, preanalytical factors can affect NGS findings. For example, the number of nucleotide variants identified by NGS has been shown to differ as a result of a delay to fixation, time in formalin and the pH of the formalin solution used [27]. Apart from WES, the utility of microsatellite instability (MSI) as a predictive biomarker for response to a PD-1 blockade has been demonstrated in CRC patients [28] and holds great potential in mismatch repair (MMR) deficient tumors to predict clinical benefit in patients. The strength of microsatellite instability signals, however, can be affected by the method of tissue preservation [29]. We have reviewed the literature for preanalytical factors associated with FFPE processing, and have summarized below reported effects on downstream molecular and proteomic analyses. Detailed summaries of preanalytical effects on proteomic and molecular assays [30] have been reviewed previously.

1.1 Delay to fixation/prefixation

1.1.1 Effect on proteins—Cold ischemic time is a critical factor that determines the suitability of a tissue for immunoassays. While several reports agree that a cold ischemic time (delay to formalin fixation) of ≤ 12 h is optimal for immunohistochemistry [31–34], ultimately an acceptable duration of cold ischemia will depend upon the protein or phosphoprotein of interest and characteristics of the biospecimen used for analysis, including the type of tumor or tissue, the size of the tumor collected, and method of collection [23]. A delay to fixation can affect companion-diagnostic testing of biomarkers like ER, HER-2, p-Akt, and pERK 1/2 by immunohistochemistry. For ER, the false negative rate increased when a specimen was tested more than 24 h after collection [35], or when the specimen was subjected to longer cold ischemia as occurs when collected by surgical resection versus biopsy [36]. A similar effect has been shown for HER-2, as FISH signals

[31] and immunohistochemical staining [37] were reduced after delays to fixation of 2 and 3 h, respectively. Conversely, significant alterations in levels of phosphorylation sites and phosphoproteins have been reported after much shorter cold ischemia times of 5 min [38] and 10–20 min [39], respectively. The scope of effects included approximately 6% of the more than 23,000 phosphorylation sites examined by LC-MS/MS in ovarian carcinoma specimens subjected to cold ischemia times of 5–60 min [38]. Significant changes induced by cold ischemia included both increases and declines in phosphorylation site levels, although timing of changes varied between phosphosites and tumors [38]. Expression levels of p-Akt and p-ERK 1/2 serve as crucial pharmacodynamic endpoints and serve as treatment efficiency biomarkers in clinical trials that involve Akt/ERK1/2 inhibitors. Cold ischemia induced changes in phosphoprotein levels were modest but significant, and included declines in ERK 1/2, MEK, and c-MET phosphorlyation after colon tumors were subjected to delays of 10–20 min [39], and instability in levels of p-mTOR, pAKT, and p-MAPK in approximately 50% of breast, lung and colon tumors subjected to delays of 10–60 min [40]. Immunohistochemical staining of p-Akt and p-ERK 1/2 is markedly reduced with progressive delays to fixation of up to 80 min, which could lead to both false-negative and false-positive results in a clinical setting [41]. Other biomarkers that show increased expression with increased delay to fixation include Caspase-3 (apoptosis) [42] and the STAT- 1 transcription factor [43] as well as phosphorylated cleaved caspase-3 Asp-175 [44] and IRS-1 Ser-612 [44]. Some markers, e.g. phosphorylated Ser-1177 nitric oxide synthase (NOS), do not change with a delay to tissue preservation [44]. A study of protein analysis of FFPE tissues by Western blot concluded that larger specimens (1.2–3.5 mm^3) are preferred over small punch biopsies (0.7 mm^3) [45].

Development of a quality assessment tool and an intrinsic control for FFPE tissue would allow specimen suitability to be determined prior to costly analysis and could improve quality assurance in clinical settings. General guidelines are available that describe analyte stability and laboratory quality control for analyzing tissue-based molecular biomarkers [46].

1.1.2 Effect on DNA—Next-generation sequencing (NGS), which is commonly used for somatic tumor mutational analysis, has been found to be a sensitive method of identifying and quantifying DNA variants in FFPE tissue biospecimens [47–49]. Detection of a KRAS mutation (G12D), a protoconcogene that has been targeted by adoptive T-cell therapy in cases of metastatic colorectal cancer [50], by NGS has been shown to be influenced by method of tissue acquisition. KRAS translocation status was successfully determined in fewer specimens procured by fine-needle aspiration (FNA) than by core-needle biopsy (46 versus 67%), largely due to insufficient tumor content in FNA specimens [51]. DNA obtained from FFPE samples is prone to degradation, and C>T transitions introduced during fixation and embedding have been documented [52]; both of these issues may affect downstream NGS-based testing. Other factors that determine NGS success include optimal DNA input, percentage of tumor content, and macro-dissection [53]. PCR success rates were higher when DNA was extracted from specimens 3–10 mm in diameter as opposed to smaller specimens [54]. Decalcification using EDTA has also been found to be beneficial

when compared to acid-based methods, as it allows amplification of longer PCR products, reduced background staining and resulted in stronger FISH signals [55–57].

1.1.3 Effect on RNA—RNA gene expression signatures can serve as prognostic and predictive biomarkers in immunotherapy and can be used to characterize immune cells and tissue compartments. Several clinically validated RNA expression tests are either in development or are FDA-approved (Prosigna and Mammaprint) (http://prosigna.com/docs/Prosigna_Product_Data_Sheet_US.pdf; http://www.agendia.com/healthcare-professionals/breast-cancer/mammaprint/)[58, 59]. Since RNA is prone to rapid degradation by nucleases, special precautions must be taken when collecting and mounting FFPE tissue sections for RNA analysis. Accurate indicators and tools are lacking for evaluating FFPE biospecimens for fit-for-purpose analysis. In frozen specimens, RNA quality is commonly assessed by RNA Integrity Number (RIN), the product of an algorithm largely based on the ratio of rRNA 28S and 18S subunits [60]; however, these rRNA subunits are highly degraded in FFPE specimens [61]. Alternative quality metrics, including DV200 [62–64] and a Bioanalyzer RNA fragmentation assay [48], have been used to assess the suitability of RNA from FFPE specimens for RNAseq by measuring fragment length. The paraffin-embedded RNA metric (PERM) also shows promise when assessing the suitability of RNA isolated from FFPE specimens for real-time PCR analysis, although it was developed using a mouse animal model and its applicability for clinical specimens has (to the best of our knowledge) not been demonstrated [65]. Studies investigating delay to fixation in tissues report similar RINs from FFPE specimens subjected to a 0 and 2 h delay [66] and similar relative expression of transcripts in FFPE specimens subjected to a 0 and 12 h delay [67].

1.2 Fixation

1.2.1 Effect on Proteins—In general, immunohistochemical staining is reported to be optimal when tissue specimens are fixed in 10% to 15% neutral buffered formalin (NBF) [68–70]. Also, several studies report optimal immunostaining when tissue is fixed for 6–24 h at ambient temperature, while fixation for 3 days or more was detrimental [71–75]. For PD-L1 expression, fixation for 12–72 h in 10% NBF is recommended for consistent immunohistochemical staining; fixation for ≤ 3 h results in variable PD-L1 signals [58]. Mutations in the BRCA1 gene, which codes for a protein responsible for repairing double-stranded DNA breaks, are associated with increased risk of breast and ovarian cancer [76–78]. Similarly, PARP and ATM are enzymes that also aide in the repair of damaged DNA. Studies have also shown that the level of tissue fixation can have a profound effect on expression levels of cancer diagnostic biomarkers including BRCA1, PARP and ATM, as enhanced detection by immunohistochemistry is observed with longer fixation times [78]. Difficulties with immunostaining of cyclin dependent kinase inhibitor ($p27^{kip-1}$), a potential prognostic biomarker for prostrate, breast and gastrointestinal tumors, has been attributed to poor tissue quality as a result of inadequate formalin fixation [79]. While several studies report superior immunostaining with formalin fixation at 4°C in comparison to ambient temperatures [80, 81], optimal fixation temperatures may be protein-specific and the time required for optimal fixation may have to be adjusted accordingly. These studies together suggest that development of fixation-specific quality control standards for FFPE tissue

biospecimens could improve the quality of specimens analyzed and thus increase the accuracy of predictions for prognosis and therapy responsiveness.

1.2.2 Effect on DNA—The composition of formalin used for fixation can affect DNA analysis. DNA extracted from tissue fixed in NBF generated higher yields than those fixed in unbuffered formalin, as well as greater genotype determination and in situ hybridization (ISH) success rates [82, 83]. The duration of formalin fixation should be < 72 h to avoid adverse effects on DNA integrity and yield, and success of downstream assays such as PCR, SNP and ISH [83–86]. Also, fixation at temperatures higher than ambient (37°C or 60°C) have resulted in reductions in DNA yield, integrity and PCR success [85, 87].

1.2.3 Effect on RNA—Several studies agree that the optimal fixation duration for FFPE biospecimens is between 8 and 48 h when RNA is to be analyzed by RT-PCR [88, 89] or ISH [86]. Similar to results found for DNA, formalin fixation at a temperature higher than ambient (37°C) results in poor RNA quality [85].

1.3 Processing and Storage

1.3.1 Effect on Proteins—The reagents, temperatures, and durations of processing are specific to the tissue processor used and to customized protocols utilized in individual laboratories, creating a great deal of heterogeneity in tissue processing. Sub-optimal dehydration is reported to be a particularly important concern for successful immunohistochemical staining [90]. Optimal dehydration reagents and procedures are dependent upon the type of antigen targeted for immunohistochemistry. While isopropanol is reported to be optimal for the detection of glycoproteins [70], several different dehydration reagents gave equivalent ER immunostaining among specimens [91]. The temperature of dehydration can affect immunohistochemical staining; although temperatures both above (45°C) and below (4°C, −20°C) ambient have been reported to be preferred, the optimal temperature will depend upon the reagent used [70, 72, 92]. With respect to embedding, immunostaining has been found to be superior with low melting point (45°C) as opposed to high melting point polymer paraffin (65°C) [70]. Tumor positioning during embedding as well as conditions of FFPE slide drying may also affect and impact the detection of immunological markers, such as CD3, CD4, and CD8 immunostaining of TILs within the tumor margin versus its center. In cases of both melanoma and colorectal cancer, it has been demonstrated that the location of TIL staining is directly correlated with response to PD-1 inhibitors [93–95].

The literature evidence suggests that immunostaining profiles of paraffin blocks stored for several years (4–68 yrs) can remain stable [96–98]; however, given that individual proteins may exhibit different stabilities, it is recommended that each antigen be evaluated for changes over time. In general, long-term storage should be avoided for analyses that require protein extraction, as some studies demonstrate as much as a 50% decrease in protein yield obtained from archival FFPE specimens compared to those stored for less than 1 y [45]. Storage of slide-mounted sections should be avoided, as protein detection by immunohistochemistry is impaired after 1–3 weeks of storage [99, 100]. It is therefore recommended that fresh sections from FFPE blocks be used for analysis when possible, or

conversely, immunostaining be experimentally validated in stored sections for each antigen of interest.

1.3.2 Effect on DNA—While little evidence is available on how DNA analysis is impacted by dehydration and clearing reagents and conditions, long-term storage has been studied more extensively. While the majority of the studies report that FFPE block storage has a limited effect on downstream DNA analysis [101–104], it can affect lengths of amplifiable DNA [105] and whole genome amplifiable fragments [106]. While one study reported stable ISH analysis of FFPE slide-mounted sections stored for up to 1 y [107], information is limited on how other methods of DNA analysis may be affected by slide storage.

1.3.3 Effect on RNA—The impact of FFPE block storage on RNA endpoints has been extensively studied. Reports indicate that FFPE blocks stored for 2–20 years demonstrated decreased mRNA amplification efficiency (determined by reduced amplicon size) [101, 108–111], and reduced RNA integrity as determined by RIN [88, 109], when compared with FFPE blocks stored for 1 year or less. Regarding storage of FFPE slide-mounted sections, qRT-PCR success rates were superior when FFPE sections were stored at ambient temperature for < 90 days as opposed to storage at 4°C or −80°C [112], although another study found no observable difference between slide storage at ambient and 4°C [113].

2.0 Snap-frozen versus FFPE specimens

While snap-freezing may be impractical in clinical settings due to processing and storage constraints, snap-frozen tissue remains the gold standard for biomarker discovery studies, DNA and RNA analyses, and protein analysis by mass spectromtry. However, formalin fixation and paraffin embedding is often preferred for structural staining and immunohistochemistry as it results in superior preservation of morphological details and is a convenient and cost-effective method of specimen preservation and storage, one that also enables the detection of immunologically relevant proteins by immunohistochemistry. Genomic, proteomic and transcriptomic biomarker profiles have been compared between tissue biospecimens preserved by snap-freezing and FFPE. Regarding genomic amplification, the choice of priming method during whole genome amplification (WGA) (degenerate oligonucleotide priming, random primer amplification, omniplex amplification, multiple displacement amplification, restriction and rolling circle aided rolling circle amplification) more strongly influences the quantity, accuracy and prevalence of amplification artifacts in FFPE tissues than in snap-frozen tissue [106, 114–116]. Priming method can thus substantially increase the template required for genotyping/copy number analysis. It has been demonstrated that successful amplification of DNA from FFPE tissues is dependent on the anticipated amplicon size, which in turn is influenced by the FFPE processing regime and extraction method employed [105]. Studies have also demonstrated discordance in mutational analysis, SNV, mismatch in SNP arrays, as well as the presence of sequencing artifacts in FFPE specimens [117–119] when compared to snap-frozen biospecimens. Differences in copy number have also been reported when data obtained from FFPE specimens is compared to frozen specimens, although results may also be influenced by analytical platform [119, 120]. While high quality histopathology can facilitate molecular diagnostics in glioblastoma [121]; when FFPE neurosurgical biopsy specimens were

analyzed by PCR for O6-methylguanine-DNA methyltransferase gene (MGMT) promoter methylation status, poor reliability and reproducibility were observed [122], highlighting the need for alternative methods of tissue fixation and more predictive quality assessment tools.

Activating mutations in codons of the *KRAS* gene are present in approximately 40% of CRCs, 30% in NSCLC adenocarcinoma and up to 95% in pancreatic carcinoma. Patients harboring mutations in *KRAS* have significantly less overall survival as compared to patients with wild type KRAS [123, 124]. Importantly, KRAS mutational status can be predictive of whether the tumor will respond to EGFR targeted therapy [125], and could in the future predict response to KRAS targeted adoptive T-cell immunotherapy [50]. However, discordance between frozen and FFPE specimens for KRAS mutation status has been reported and could compromise accurate clinical testing. While 6% of KRAS variants were misclassified as wild type in FFPE specimens with high-resolution melting analysis [126], 18–20% were misclassified as wild type with traditional sequencing [126, 127]. Further, 9% of FFPE specimens exhibited a KRAS mutation that was not identified in the case-matched frozen specimen [126]. Similar sequencing artifacts consistent with FFPE-induced deamination have been reported for BRAF [128], another proto-oncogene targeted by specific drug inhibitors and a promising target of monoclonal antibody immunotherapy [129]. The occurrence of sequencing artifacts in FFPE specimens was reduced by uracil-DNA glycosylase pretreatment [27, 128]. The quantity of DNA isolated from FFPE specimens may also be a confounding factor; insufficient DNA levels was identified as the cause of missed BRAF mutations by Sanger sequencing in 3 out of the 4 FFPE specimens engineered to express them [130].

With regard to RNA, some studies have reported differential mRNA expression between tumor and normal adjacent biospecimens in FFPE but not frozen tissue [131]. Also, it has been reported that mRNA levels determined by microarray may show weak [132] or strong correlations [66] between frozen and FFPE biospecimens. An increase in the relative abundance of GC-rich transcripts in FFPE tissues relative to matched frozen controls further suggests that transcript-specific biomarkers should not be evaluated in FFPE specimens without proper standardization and validation [133]. While microRNAs (miRNA) have been proposed as promising diagnostic markers for some cancers [134, 135], miRNA stability in tissue is affected by preanalytical variability. A delay to fixation, method of preservation (snap-frozen vs. FFPE), and type of stain used before microdissection have all been shown to impact analysis of miRNA levels [136]. Conversely, while miRNA profiles appear to remain stable with FFPE block storage [136], individual miRNAs may be unstable; levels of miR-494 and miR-513 are reported to be vulnerable to prolonged storage [137].

While considerable efforts have been made to ensure the accuracy of data generated with FFPE specimens, data generated with FFPE tissue should be validated against a snap-frozen cohort when investigating new analytes or employing a novel assay or technique.

3.0 Preanalytical variables and Blood Biomarkers

Blood biospecimens are a valuable source of diagnostic and prognostic biomarkers for both blood cancers and solid tumors. The concept of a liquid biopsy, i.e. sampling blood as an

alternative or complement to diagnostic testing of a solid tumor, is an appealing approach currently being embraced in clinical settings given its minimally invasive nature, fast turnaround time, and reduced cost in comparison to tissue biopsy. Determining the mutational status of a tumor, whether directly through analysis of a tissue biopsy or indirectly through analysis of a blood specimen, can identify suitable treatments. One concern of relying solely on blood specimens is whether mutations detected in circulating tumor DNA (ctDNA) are reflective of the same mutations expressed within the solid tumor. However, a high level of agreement between the mutational status of the solid tumor and ctDNA has been reported for RAS mutations in cases of metastatic colorectal cancer [138]; and PIK3CA, TP53, ERBB2, BRCA1 and BRCA2 mutations in cases of metastatic breast cancer [139]. Further, levels of a BRAF mutation in ctDNA served as an early indicator of response to T-cell transfer immunotherapy among patients diagnoses with metastatic melanoma [140]. Recently, the FDA has also approved the cobas® EGFR Mutation Test v2, for the detection of EGFR mutations, which can be used with tumor specimens as well as well as with plasma collected from patients diagnosed with metastatic NSCLC. It is the first FDA-approved test that relies on results obtained from a liquid biopsy to predict treatment response of a solid metastatic tumor to a EGFR tyrosine kinase inhibitor (https://molecular.roche.com/assays/cobas-egfr-mutation-test-v2-ce-ivd/). The presence and number of circulating tumor cells (CTCs)[141–143] and levels of circulating microRNAs (miRNA) [144] also hold promise as biomarkers of disease, disease progression and patient survival for several types of cancer. FDA-approved CTC assays include the CELLSEARCH® Circulating Tumor Cell assay, which quantifies epithelial-based CTCs in whole blood collected from patients diagnosed with metastatic breast, prostate or colorectal cancer (https://www.cellsearchctc.com). Several circulating miRNA species also have diagnostic and prognostic value as biomarkers of solid tumors [144]. Liquid biopsy is also a promising option for monitoring immunotherapy response. Levels of a BRAF mutation detected in ctDNA by digital PCR correlated with tumor response to anti-PD-1 immunotherapy in melanoma patients, and also corresponded to results of radiological and clinical testing [145]. In light of current challenges associated with the occurrence of tumor pseudoprogression in response to immunotherapy, ctDNA levels may be a useful supplemental tool to diagnostic imaging to assess tumor burden. KRAS-mutated ctDNA levels were lower in plasma from patients with pseudoprogression and elevated in patients experiencing tumor progression [146]. Immunologically-relevant cell populations and their surface markers can also provide beneficial information regarding a cancer's response to a specific treatment. A higher frequency of myeloid-derived suppressor cells (MDSCs) and CD4+, CD25+, FoxP3+ regulatory T-cells (or Tregs) were associated with an improvement in overall patient survival among melanoma patients receiving Ipilimumab [147]. Accumulation of regulatory T-cells also holds prognostic significance in cases of colorectal cancer [148], and CD8+ T cell number is directly correlated with improved outcome for patients diagnosed with lung cancer and colorectal cancer [149, 150].

Importantly, preanalytical variables associated with blood collection, processing, handling and storage have been associated with irreproducible biomarker results largely due to a lack of standardization and validation of preanalytical procedures [136, 151–154], and preanalytical variability has been recognized as a major challenge in biomarker development

[155]. Biospecimen preanalytical variability also affects clinical medicine; Lippi et.al. estimates that up to 93% of diagnostic errors are attributable to a lack of standardized biospecimen collection and handling procedures [156]. The potential for variable or suboptimal processing begins with collection tube type and processing, as the choice between whole blood, plasma and serum is governed by both the analyte of interest and the assay used. Another key preanalytical factor, specimen preservation, is often driven by need and circumstance. Immune cell populations are often investigated by flow cytometry, which has been shown to be susceptible to sample collection, handling and processing practices [157, 158]. While fresh peripheral blood mononuclear cells (PBMCs) are preferred for analysis by flow cytometry, analysis of fresh biospecimens is often unpractical in large clinical trials. Cryopreserved biospecimens are thus utilized, requiring standardization and validation of cryopreservation methods. Guidelines are available that describe optimal PBMC collection as well as details of preanalytical factors that should be considered [159, 160]. Monitoring cytokines, chemokines, growth factors, soluble molecules like antibodies, matrix metalloproteinases (MMP) and adhesion molecules in serum and plasma are an effective method of monitoring tumor progression and therapy response [161], as well as clinical outcome [162–165]. ELISPOT, for example, is a quantitative immunological assay used to analyze and monitor B, T and NK cells and monocytes, as well as secretion of cytokines, chemokines, cytotoxic mediators, and antigen-specific T-cell stimulation [166]. However, biospecimen collection and preparation are critical steps in this pipeline, and standardized practices are needed to improve the accuracy of data generated. It's also important to consider that effects attributable to preanalytical variability are not necessarily global, but rather can affect individual proteins and peptides while leaving the proteomic profile intact [167–171]. Below we review preanalytical factors that have been shown to affect biomarker detection and quantification.

3.1 Tube type/anticoagulant

Cytokines, immune cell signaling molecules that can also enhance immune recognition of tumor cells, are being investigated as both immunotherapy targets and sentinels of response [172]. However, choice of the type of blood specimen analyzed (serum versus plasma) and which anticoagulant is used to obtain plasma have clear effects on cytokine detection [173, 174]. Agreement between detection levels in serum and plasma varied based on the cytokine measured, with some studies reporting a strong correlation [161] while another reported significant differences in 19 of the 60 cytokines detected in healthy individuals [173]. The optimal choice of anticoagulant for analysis of plasma specimens appears to be cytokine-specific, as levels of IL-8 and IL-10 were higher in heparin compared to EDTA plasma [175], while IFN-γ was higher in EDTA than heparin plasma [175]. While EDTA and citrate plasma displayed similar results, heparin plasma exhibited significantly higher levels for 10 cytokines and lower levels for two cytokines than EDTA and citrate samples [173].

For proteomics, EDTA plasma with protease inhibitors was preferable to serum, other anticoagulants, or EDTA without protease inhibitors [176, 177], whereas higher quality DNA and RNA were obtained from citrate plasma [176]. For metabolomic analyses by NMR, heparin was preferred over other anticoagulants [178], but substantial effects were not observed with different anticoagulants [179]. Investigations of individual protein

biomarkers, such as proposed immunotherapy targets CD40 [180] and VEGF [181], support concerns over blood specimen type (serum versus plasma) and anticoagulant choice, as levels of both CD40 [182] and VEGF [183] were significantly higher in serum than plasma, and VEGF levels were significantly higher in EDTA plasma than Hirudin or ACD plasma [183]. Levels of miRNA-21, a biomarker of immunotherapy response, were also affected by anticoagulant choice, as it was undetectable in lithium heparin plasma but was comparable among serum, EDTA and citrate plasma [184]. miRNA-21 levels were also higher in buffy coat and red blood cells in comparison to EDTA plasma [184]. Anticoagulant choice may also play a factor in immune cell profiling, specifically T-cells, B-cells and NK-cells, as sodium heparin has been found to preserve the integrity of cell surface antigens [185]. New and potentially better collection preservatives and media additives are also being developed for the optimal preservation of cellular subsets of blood as part of the liquid biopsy approach that targets circulating tumor cells (CTC's) [186], cell-free DNA [187], circulating tumor DNA (ctDNA)[188], and exosomes [189]. Early analysis indicates that Cell-free DNA BCT Streck, CellSave, and EDTA tubes do not differ in DNA yield if specimens are processed within 2 h, but the delay-induced effects observed in EDTA plasma after longer delays are avoided with Streck Cell-free DNA BCT, and CellSave tubes tubes [188, 190, 191]. Differences in the profile and abundance of cell-free DNA were also observed among plasma and serum specimens [192]. Single cell network profiling (SCNP) is a flow cytometry-based approach used to measure the functional signaling capacity of a targeted subset of cells, which allows for analysis of multiple types of immune cells without the need for physical cell isolation or novel sample processing procedures. For SCNP analysis, sodium heparin tubes are preferred and PBMCs are prepared by Focal gradient and stored using a specific cryopreserved procedure [193]. Thus, blood collection methods must be optimized and validated for the biomarker of interest.

3.2 Delayed Processing

Effects associated with a delay in blood processing on the total proteome have been thoroughly examined and several delay-sensitive biomarkers have been identified [116]. Metabolites, peptides and proteins have also been reported in the literature to be sensitive to processing delays, with levels of ascorbic acid [194], amylase, AST, HDLC, alkaline phosphatase, calcium, creatinine, ferritin, folate, glucose, phosphate, potassium, sodium and LDH [195, 196] either decreasing or increasing in comparison to immediately processed controls. Serum levels of GGT and LDH are sensitive to both a delay from collection to processing and a delay from processing to freezing of plasma and serum samples [197]. VEGF, an important endothelial oncology biomarker and proposed immunotherapy target [181] was reduced in EDTA plasma after a delay of 4 h or longer at room temperature [183], and in serum specimens after a 1 h delay at room temperature or 4°C [198]. Levels of the surface receptor CD40 decreased with both the duration and temperature of delay in plasma [199], and was also affected by centrifugation speed during plasma processing [182], as well as a post-centrifugation delay in serum processing [200]. Prostate stimulating antigen (PSA) (free, total, and ACT) was stable in serum, heparin and EDTA whole blood for up to 4 h at room temperature [201], although significant declines were observed in free PSA after a 5.5 h delay [201], and free and total PSA after a 1 h delay at 37°C [202]. Plasma levels of IFNγ

declined when the delay to processing occurred at room temperature, while specimens stored at 4°C remained stable [203].

DNA integrity, determined by qPCR, has been reported to be stable in whole blood subjected to a delay of up to 24 h at 4°C [204]. Similarly, levels of cell-free DNA are stable for up to 6 h regardless of whether plasma was collected in Streck, BCT, or EDTA tubes [188]; an increase was observed in EDTA specimens after a 24 h [192] or 48 h delay to processing at room temperature [205].

Studies investigating RNA expression report changes in gene expression patterns in serum and plasma samples subjected to a processing delay of 3 h [206, 207]. Levels of miRNA-21 were reduced by approximately 20% after a 3 h delay at room temperature [208]. Further, miRNA-21 levels were also adversely affected by a high degree of hemolysis [209].

3.3 F/T and Cryostorage

Freeze-thaw cycling, defined as the number of times a specimen or sample is frozen and thawed, has affected several hormones with documented cancer involvement, including estradiol, estrone, and testosterone [210]. Sex hormone-binding globulin (SHBG) is also affected by both freeze-thaw cycling and frozen storage [210, 211], and brain natriuretic peptide is sensitive to frozen storage [212]. VEGF, a proposed target of immunotherapy, is also sensitive to freeze-thaw cycling, although reports conflict as to whether levels increase [183] or decrease [198] in EDTA plasma specimens frozen and thawed two or more times. Multiple freeze-thaw cycles are detrimental to some but not all cytokines. Levels of IL-4 and TNF-α increased while levels of IL-13, Il-15, Il-17, IFN-γ and IL-8 decreased after one or more freeze-thaw cycles, although IL-6 and IL-10 remained stable for up to four freeze-thaw cycles [213]. The magnitude of change for IL-8 was a 3–5 -fold increase in serum and plasma following one freeze-thaw cycle relative to fresh specimens [174]. Clinical chemistry analytes, such as AST, BUN, GGT and LDH, measured in both serum and plasma are also sensitive to repeated freeze-thaw cycling [197].

Acceptable durations of frozen storage are an important consideration for cancer immunotherapy. Because blood specimens collected during a immunotherapy clinical trial may not be analyzed immediately, the stability of cytokines and other immunologically relevant proteins during prolonged storage is an important consideration that may affect downstream results. Many cytokines, including IL-13, IL-15, IL-17 and IL-8, exhibited evidence of degradation within one year of storage of sodium heparin plasma at −80°C, although IL-2, IL-4, IL-12 and IL-18 remain stable for up to 3 years [213]. Notably, the type of specimen being stored may have an impact on stability, as miRNA-21 levels declined significantly in whole blood stored at −80°C for 9 months although no effect of storage was observed for EDTA plasma [184]. Plasma stored at −80°C and analyzed by nuclear magnetic resonance (NMR) spectrometry remained stable for up to 19 months at −80°C, but storage for 20 to 30 months resulted in a 2% increase in cholesterol and modest changes in the levels of N-acetyl glycoproteins and creatinine [178].

3.4 Chimeric antigen receptor T-cell therapy

Chimeric antigen receptor (CAR) T-cell therapy, a form of adoptive cell therapy in which a patient's T-cells are modified and cultured *ex vivo*, has emerged as a promising and novel immunotherapy for cancer treatment [214]. While the early results of clinical trials are promising, several hurdles exist that could hamper widespread clinical adoption. Considering that expertise and resources for such a treatment would be limited, cryopreservation and shipment of biospecimens would likely be required. Shipment of whole blood resulted in temperature dependent declines in cell recovery, viability and immune function in one study [215], while others reported adverse effects on NK and B cells although T-cells and antitumor activity were uncompromised [216, 217]. Other preanalytical factors, including time to processing, thawing, resting time, and stimulation time affected PBMC recovery and viability, while only stimulation time affected T-cell response [218]. While cryopreservation may be one method to circumvent delays required for specimen transport, it does not exclude the potential for preanalytical effects. T-cells are actually more sensitive to cryopreservation than other PBMC cell types [219]. More specifically, the type and temperature of cryopreservation media used has been shown to affect PBMC viability and T-cell response [220], as did temperature fluctuations during frozen storage [221].

4.0 National Cancer Institute Biospecimen Science studies

The National Cancer Institute's (NCI) Biorepositories and Biospecimen Research Branch (BBRB) has actively addressed preanalytical challenges associated with collection, processing, and storage of human biospecimens and their influence on subsequent molecular analyses. Past BBRB research initiatives conducted under the NCI Biospecimen Research Network (BRN) focused on effects of cold ischemia, prolonged fixation, the temperature and duration of frozen storage, as well as the identification of biomarkers of such effects and the development of new quality assessment methods [167, 222–228]. A major study of tissue preanalytical factors, the Biospecimen Preanalytical Variables project (BPV), is underway.

Recent BRN-sponsored research indicates that while serum and plasma samples remain relatively stable at room temperature (~ 20°C) for up to 24 h prior to centrifugation, longer delays lead to significant changes in a subset of the proteome. Both the temperature and duration of frozen storage should also be considered by basic and clinical research investigators, as several proteins exhibit changes after plasma and serum were stored for 6 or 18 months and changes were more prevalent with storage at −20°C than −80°C. Subjecting plasma and serum to multiple freeze-thaw cycles also altered levels of specific proteins, although specimens collected in P100 and serum tubes with a mechanical separator were found to be the most protected from preanalytical effects. Results such as these from the BRN highlight preanalytical factors of concern for the analysis of blood samples and provide data that contributes to the development of evidence-based guidelines on blood processing and storage [229].

Discussion

Clinical options for cancer treatment have recently expanded to include several different immunotherapy approaches. Despite promising results, the percentage of treated patients that respond favorably to immunotherapy remains lower than expected and varies across cancer types [230](http://www.npr.org/sections/health-shots/2016/09/17/494230963/cancer-immunotherapy-at-a-crossroads; http://blog.aacr.org/why-does-immunotherapy-not-benefit-everyone-long-term/). While predictive biomarkers of treatment response are currently being explored as a potential solution, their effectiveness hinges in part on the biospecimens used for patient testing. Experience indicates that the biomarkers that guide cancer treatment can be compromised by suboptimal or variable biospecimen collection, processing, and storage practices [21, 35]. Although cancer immunotherapy represents a new and exciting treatment avenue, it remains reliant on the same biospecimen collection, processing, and storage procedures used for decades, and in many cases, the same analytical platforms for biomarker quantification. In the present review we have highlighted several preanalytical variables that have reported effects on the detection of immunologically-relevant biomarkers in both tissue and blood biospecimens. Susceptible analytes include both predictors of response as well as characteristics of phenotypical and functional assays that are used to monitor immunotherapy response *in situ* and *ex vivo*. Potential effects of preanalytical variability apply to both tissue and blood biospecimens, and ramifications of suboptimal or variable specimen handling extend to both basic research and clinical settings.

While formalin-fixation and paraffin-embedding (FFPE) has been widely used for more than 50 years by both researchers and pathologists, it remains a complex multi-step process and reagents, temperatures, and durations often differ between laboratories. Assessing the analytical impact of suboptimal or variable FFPE processing is further complicated by the fact that the majority of effects reported to date are gene-, transcript-, and protein-specific as opposed to global changes in quality, yield, or profile. For this reason it is important that any biomarker be experimentally validated for a given FFPE processing regime and analytical assay. Concerns over the potential impact of preanalytical variability on biomarkers of immune function and response are supported by reported effects of preanalytical factors such as time in fixative, formalin composition, and embedding reagents and conditions on leukocyte cell surface antigens, such as CD3, 4, and 8 [70]; a delay to fixation and time in fixative on the protein VEGF [183, 231]; and discrepancies in the detection of KRAS and BRAF variants in FFPE specimens compared to those that are snap-frozen [126–128]. While a systematic evaluation of preanalytical effects has not been reported to date for predictive biomarkers of immunotherapy response, such as PD-L1, the successful implementation of immunohistochemistry-based assays for their detection warrants careful consideration of preanalytical factors such as a delay to fixation; duration and condition of formalin fixation; reagents, durations and conditions of tissue processing; safeguards against nucleic acid degradation during sectioning when applicable; and storage durations and conditions [18].

Sources of analytical variability also warrant consideration and standardization, which include the biomarker assay/kit used, manual versus automated staining, internal and external quality assurance methods, and the interpretation of results [18, 19]. A few initiatives are underway in an effort to minimize effects attributed to analytical variability,

such as The Blueprint Programmed Death Ligand 1 (PD-L1) Immunohistochemistry (IHC) Assay Comparison Project that represents a collaboration between academic and commercial organizations that aims to compare clinical and analytical results obtained with each of the four PD-L1 IHC assays used in clinical trials [232]. A blueprint proposal for companion diagnostic comparability for PD-L1 also been published by the FDA (https://www.fda.gov/downloads/medicaldevices/newsevents/workshopsconferences/ucm439440.pdf). In addition, control tissues with known IHC performance characteristics (representing true positive and true negatives) can be included in the specimen cohort, as any variation in assay performance would be indicative of an unforeseen issue that arose during the preanalytical or analytical phase. As the clinical emphasis of immunohistochemistry shifts from single marker to multiplex analysis, a thorough understanding of the roles that preanalytical and analytical variables play in the accuracy of results will be crucial for the accurate delineation of cellular interactions between tumor and immune cells.

While several preanalytical factors have documented effects on potential immunotherapy biomarkers in FFPE tissue biospecimens, the preservation process itself introduces inherent artifacts. In most cases, such artifacts can be minimized by antigen retrieval, including a demodification step, and optimization of extraction conditions or the bioinformatic pipeline, but analysis of archival specimens with an unknown processing history should be avoided. Formalin-free fixatives are viable alternatives to formalin that enable accurate molecular analysis while minimizing fixation-related artifacts; however, given their relatively new emergence compared to formalin, we still have an incomplete understanding of how molecular and proteomic end points may be affected by preanalytical variability.

Blood biospecimens, as well as their derivatives, are also susceptible to effects introduced during suboptimal or variable collection, processing, and storage practices. Immunotherapy-relevant biomarkers analyzed in blood, plasma or serum include the TCR repertoire; cytokines; genotype; and circulating proteins, miRNA and ctDNA. Immune cell subtypes may express different sensitivities to different preanalytical factors, and as such, collection, processing, and storage practices should be optimized and standardized. Available evidence indicates that the quantity of some immunologically relevant biomarkers differs greatly between plasma and serum [174, 182, 192, 203]; therefore, it’s advisable to evaluate and standardize the type of blood specimen used for a given biomarker throughout the biomarker validation process [233]. When plasma is used for analysis, it is important to standardize the anticoagulant, collection tube, and centrifugation protocol used, as these have documented effects on immunologically relevant cytokine [175, 203], miRNA [184], and protein [174, 175, 182, 183, 198] biomarkers, as well as on levels of detectable ctDNA [188, 191, 192]. For any blood derived biospecimen, delays prior to processing, after centrifugation, as well as freeze-thaw cycling should be minimized, as excessive delays and freeze-thaw events have been reported to adversely affect immunologically relevant cytokine [174, 203, 213, 234], miRNA [184, 198], and protein [174, 182, 198, 200] biomarkers, as well as ctDNA levels [188, 191, 192]. Proper cryopreservation is also crucial if analysis or culture of immune cell subsets or PBMCs is anticipated given that analysis of fresh whole blood and PBMCs is often not practical during clinical trials. While chimeric antigen receptor (CAR) T-cell therapy has not received much attention in the literature in terms of preanalytical effects, steps in processing required for cell isolation, engineering, culture, cryopreservation,

storage, and transport prior to administration to the patient warrant detailed investigation given that when many of these steps were examined in isolation, adverse effects were reported for cell viability and response [215, 217, 219–221]. Rapid isolation is required to avoid contamination and loss of function when enzyme-linked immunospot (ELISPOT) assays are used to monitor B, T and NK cell responses [185]. In order to distinguish true immunological changes from technical and preanalytical/analytical artifacts, global standardization is needed when assessing cellular response from a subset of immune cells.

Tumor mutational analysis of DNA using NGS is also currently widely used for mutational analysis of both tissues and blood in the field of immuno-oncology. Optimal analytical and clinical performance of these assays is dependent on several preanalytical factors including sample type and the method of DNA extraction, quantification and quality assessment. As such, these preanalytical factors should be carefully monitored to preserve the sensitivity and reproducibility of these assays [235]. Efforts have been made to standardize DNA extraction for both formalin-fixation and snap-freezing [48, 236–240]. However, DNA degradation is of particular concern when FFPE specimens are analyzed, given the low levels of DNA obtained and the increase in C>T artifacts. RNA-based analysis of tissues and blood pose further challenges given RNA is prone to degradation and sensitive to nuclease activity. RNA quality methods such as DV200 and qRT-PCR amplification of differently sized transcripts are proving to be more informative than RIN, particularly in the case of FFPE specimens. While definitive markers of specimen quality have yet to be validated, several potential QC assays for qualification of banked plasma, serum, and other biospecimens have been proposed by the ISBER Biospecimen Working Group; such assays may serve to stratify clinical biospecimens based on a fit-for-purpose approach regarding a specific analyte or analytical platform [241]. Ultimately, each specimen will need to be determined if it is fit-for-purpose, as the accuracy and reproducibility of data is also influenced on the platform used for analysis, as is the case with RNA sequencing [242].

Many instances of preanalytical effects can be minimized by thorough standardization of specimen handling practices both within an individual laboratory or biobank and between collection and processing sites. In this regard, biobanks stand at the forefront of personalized medicine as they represent a primary source of specimens used in both basic and clinical research. While the vast majority of biobanks utilize some form of SOP for specimen handling, protocols are usually institution-specific. Such differences in specimen handling and resultant specimen quality has yielded a new initative, harmonization of biobanking practices. Rather than strict adherence to a single collection of SOPs for specimen handling, strategies for international biobank harmonization focus on increasing transparency and cooperative sharing of data, specimens, and resources [243]. The National Cancer Institute has released several resources to facilitate both standardization and harmonization to ensure that specimens are fit-for-purpose. NCI’s BBRB recently released an updated version of the “NCI Best Practices for Biospecimen Resources”, which outlines operational, technical, ethical, legal, and policy best practices (https://biospecimens.cancer.gov/bestpractices/). Developing and implementing infrastructure to monitor and document the history of a biospecimen, such as SOP deviations, are also critical. Documenting such deviations would also allow erroneous analytical results to be traced back to key events during specimen collection, processing or storage. Resources and strategies for implementing infrastructure

related to specimen annotation has been developed by the ISBER Biospecimen Science Working Group. The Standard PREanalytical Code (SPREC) is a seven item code that reflects the type, collection, handling and storage of the biospecimen in question, which can be integrated into existing quality management systems currently used by biobanks [244, 245]. Guidance on immunophenotyping assays [246] and single cell immunological assays is also available [160]. NCI's Early Detection Research Network (EDRN) has also developed and released consensus SOPs for the collection, processing, handling, and storage of serum and plasma specimens for biomarker discovery and validation [247]. NCI has also recently invested in funding programs that investigate preanalytical factors involved in clinical biomarker qualification and validation [248], as well as the development of a new initiative on the establishment of Cancer Immune Monitoring and Analysis Centers (CIMACs) (https://grants.nih.gov/grants/guide/rfa-files/RFA-CA-17-005.html). NCI's Biorepositories and Biospecimen Research Branch (BBRB) publishes a series of evidence-based procedural guidelines termed "Biospecimen Evidence-Based Practices (BEBP)" [249]. The International Society for Biological and Environmental Repositories (ISBER) Biospecimen Science Working Group also released a report identifying promising biomarkers of biospecimen quality to enable fit-for purpose use of archived FFPE blocks and biobanked serum and plasma [250]. The Society for Immunotherapy of Cancer (SITC) biomarkers task force also recently organized a joint FDA/NCI workshop in an effort to harmonize standard operating procedures on specimen collection, processing and storage for samples that will ultimately be used to investigate immunotherapy biomarkers. The taskforce recommends using standardized functional assays, and banking sufficient amounts of blood, serum, DNA, and RNA to determine patient genotype and tumor gene expression profiles. A standardized approach for TIL scoring in any solid tumor has also been proposed based upon recommendations made by an International Immuno-Oncology Biomarker working group on TIL assessment in breast carcinoma [251]. In an effort to standardize documentation across published studies, NCI's BBRB has published Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines that define key details relating to patient consent and biospecimen handling that should be described accordingly in manuscripts that use human biospecimens [252]. Transitioning a newly developed and promising molecular assay to clinical use involves proper biomarker qualification and validation, and requires knowledge of and experience with regulatory issues and policies. Thus, it is crucial that each newly developed test be carefully standardized in terms of preanalytical variability, between patient variability, and assay reproducibility, sensitivity, and specificity. Some assays may also require validation in a CLIA-certified laboratory, which itself may necessitate a team composed of multidisciplinary participants.

In conclusion, it is clear that clinical implementation of newly identified biomarkers to predict and monitor response to cancer immunotherapy requires accurate and reproducible quantification in tissue and blood biospecimens. However, given what evidence is available, immunologically relevant biomarkers are susceptible to effects introduced during suboptimal or variable specimen collection and handling. To facilitate the success of such biomarkers, specimen practices must be standardized and validated for a given gene, transcript, protein, or cell type of interest and the analytical assay used for detection and/or characterization. While significant efforts are underway to better understand and mitigate the effects of

preanalytical variability on clinically relevant biomarkers, researchers and clinicians should remain vigilant as to potential effects of preanalytical variability and base decisions on specimen collection, processing and storage on evidence that is currently available.

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Table 1

Preanalytical factors affecting markers of immune therapy in tissue specimens

Marker	Preanalytical Factor	Method(s)	Reference(s)
BRAF	Post-extraction treatments		[121]
CD3	Dehydration and clearing, Fixation duration, Fixative buffer and type, paraffin-embedding	IHC	[63]
CD4	Dehydration and clearing, Fixation duration, Fixative buffer and type, paraffin-embedding	IHC	[63]
CD8	Dehydration and clearing, Fixation duration, Fixative buffer and type, paraffin-embedding	IHC	[63]
KRAS G12D	Specimens collection method Preservation (FFPE vs Frozen)		[45] [119, 120]
miR-21	Preservation		[240]
PD-1	Time in fixative	IHC	[52]
VEGF	Cold ischemia Fixation duration		[175] [223]

Table 2

Preanalytical factors affecting markers of immune therapy in blood specimens

Marker	Preanalytical Factor	Method(s)	Reference(s)
BRAF mutation detection ctDNA	Processing Delay		[241]
	Processing Delay	Digital PCR, Real-time PCR	[180, 183, 184]
	Tube type (Preservative tube)	Digital PCR, Real-time PCR	[180, 183, 184]
	Plasma versus serum	Digital PCR	[184]
	Freeze-thaw cycling	Immunoassay	[205]
	Anticoagulant	Immunoassay	[167]
IFN-gamma	Processing delay	RIA	
	Anticoagulant	RIA, Clin chem	
	Plasma versus serum	RIA	
	Freeze-thaw cycling	Immunoassay	[205]
IL-1a	Processing delay	Immunoassay	[205]
	Freeze-thaw cycling	Immunoassay	[205]
	Freeze-thaw cycling	Clin chem	[166]
IL-4	Anticoagulant		[167]
	Freeze-thaw cycling		[166, 205]
IL-5	Anticoagulant	Immunoassay	[167]
	Freeze-thaw cycling		[205]
	Anticoagulant	Immunoassay	[167]
	Processing delay		
IL-8	Anticoagulant	Immunoassay	[167]
	Tube type	Clin chem	[166]
	Processing delay		[166]
	Freeze-thaw cycling		[226]
	Storage	Immunoassay	[205]
	Freeze-thaw cycling	Clin chem	[166]
	Anticoagulant	Clin chem	[167]
	Storage	Immunoassay	[205]
	Freeze-thaw cycling	Immunoassay	[205]
IL-15	Storage	Immunoassay	[205]
	Freeze-thaw cycling	Immunoassay	[205]
IL-17	Anticoagulant	Clin chem	[167]
	Storage	Immunoassay	[205]
	Freeze-thaw cycling	Immunoassay	[205]

Marker	Preanalytical Factor	Method(s)	Reference(s)
miR-21	Anticoagulant	Real-time PCR	[176]
	Plasma versus serum	Real-time PCR	[176]
	Hemolysis		[201]
	Processing delay	Real-time PCR	[176, 190]
	Storage (serum or plasma)		[176, 200]
	Freeze-thaw cycles	Real-time PCR	[176, 190]
sCD-40L	Anticoagulants		[174]
	Processing delay	Immunoassay	[174, 192]
	Centrifugation		[174]
	Freeze-thaw cycles		[174]
	Storage (Serum)		[174, 191]
TNF-alpha	Anticoagulant	Clin chem	[167]
	Tube type	Clin chem	[166]
	Processing delay		
	Storage (serum or plasma)	Clin chem	[166]
	Freeze-thaw cycling	Immunoassay	[205]
VEGF	Anticoagulant		[175]
	Tube type	Clin chem	[166]
	Processing delay		[166, 175, 190]
	Centrifugation speed		[190]
	Storage		[166, 242]
	Freeze-thaw cycling		[175, 190, 226]
VEGF-R2	Freeze-thaw cycling		[242]
	Storage (Serum)		[242]
T-Cell recovery	Processing Delay		[207]
	Freezing/Freezing parameters		[211, 212]
	Post-thaw storage		[211]
	Frozen Storage		