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Pre-analytical issues in liquid biopsy – where do we stand?

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Abstract: It is well documented that in the chain from sample to the result in a clinical laboratory, the pre-analytical phase is the weakest and most vulnerable link. This also holds for the use and analysis of extracellular nucleic acids. In this short review, we will summarize and critically evaluate the most important steps of the pre-analytical phase, i.e. the choice of the best control population for the patients to be analyzed, the actual blood draw, the choice of tubes for blood drawing, the impact of delayed processing of blood samples, the best method for getting rid of cells and debris, the choice of matrix, i.e. plasma vs. serum vs. other body fluids, and the impact of long-term storage of cell-free liquids on the outcome. Even if the analysis of cell-free nucleic acids has already become a routine application in the area of non-invasive prenatal screening (NIPS) and in the care of cancer patients (search for resistance mutations in the *EGFR* gene), there are still many unresolved issues of the pre-analytical phase which need to be urgently tackled.

Keywords: cell-free nucleic acids; extracellular nucleic acids; liquid profiling; pre-analytical phase.

Introduction

Recently, new European Union regulations on medical devices and in vitro diagnostic medical devices entered into force and will apply fully for in vitro diagnostics in May 2022 [1]. The requirements for in vitro diagnostics will change significantly and will also include the definition of pre-analytical parameters.

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The analysis of cell-free nucleic acids (which has misleadingly been coined liquid biopsy, more precisely would be “liquid profiling”) may be useful in different areas, for the diagnosis of patients with different diseases and especially for cancer patients, treatment monitoring, tracking of minimal residual disease (early appearance of treatment resistance), and for the establishment of the prognosis for these patients. While the latter points might be within tangible reach, its usefulness as a diagnostic tool is still heavily debated. The reasons for this include, but are not limited to:

- the very low concentration of DNA with tumor-associated alterations in the blood/body fluids of early-stage cancer patients;
- the insufficient sensitivity and specificity of tests for the detection/quantification of tumor-associated alterations (especially in a diagnostic setting);
- a gap in our understanding of which factors contribute to the formation of changes which might lead to a tumor development (age-dependent methylation changes which can be detected in tumor patients and in older but otherwise healthy people alike).

In a recently published review, the prospects and challenges for liquid biopsy for the African continent with poor resources and limited access to medical infrastructures were looked at [2]. Although the statement that liquid biopsy “eliminates the need for surgical biopsies” is highly understandable, it remains unrealistic. A literature survey on the analysis of circulating tumor DNA (ctDNA) issued by the American Society of Clinical Oncology and the College of American Pathologists stated that “some ctDNA assays are clinically useful for certain types of advanced tumors” [3]. In contrast, this type of analysis does not yet hold any clinical utility for ctDNA analysis in early-stage cancer, therapy monitoring or residual disease detection. Additionally, the authors stressed the need for closing the gaps in our understanding of pre-analytical factors. We are still far away from recognizing the importance of pre-analytical influences on the outcome of laboratory tests. This was demonstrated in a paper (just to give an example) on the challenges for the implementation of

methods for the screening for fetal aneuploidy in pregnant women with the aid of cell-free DNA (cfDNA) in which the issue of pre-analytics is not even mentioned [4].

The first meeting on Circulating Nucleic Acids in Plasma and Serum (CNAPS) that was organized 20 years ago by the founders of the liquid biopsy field Drs. Stroun and Anker, already called for a standardization of the numerous pre-analytical issues. By now, the approach of liquid biopsy is not only commercialized (as a screening method for a non-invasive prenatal detection of fetuses with numeric chromosomal aberrations like trisomy 21) but is also approved by several regulatory agencies for taking care of cancer patients (such as the detection of emerging resistance mutations against first-line *EGFR* therapy). A recently published review lists 14 clinical trials focusing on the use of cfDNA in tumor patients, but none of them deals with pre-analytical issues [5].

A striking example for the urgent need for a standardization of sample handling and processing is the paper by Trigg et al. [6] in which 50 randomly sampled papers (all published in 2015) were reviewed. A third of them did not provide any information on the method for the plasma/serum isolation, 19/50 did not detail the specification for the centrifugation steps to free the matrix from blood cells, and 18 of them gave no information on the cfDNA quantification method. Also, when browsing the literature for this survey, we stumbled upon some papers in which plasma was used for the analysis but was called serum and *vice versa*. Additionally, our knowledge about the biology of cell-free nucleic acids, their mechanisms of liberation, the composition and interaction with other molecules (like proteins, lipids and fatty acids), which may play an important role in transportation and their removal from the body, is still very rudimentary.

The aim of the paper is to review and critically summarize the data on previous attempts to set up standard operating procedures (SOPs) in the field of liquid biopsy. We will focus on papers dealing with cell-free nucleic acids and exclude work done on circulating tumor cells.

Biology of cfDNA

Cell-free nucleic acids are not only found in the circulation (i.e. plasma/serum and lymphatic fluid) but also in all examined body fluids [7]. Among them is urine [8–10], cerebrospinal fluid (CSF) [11, 12], pleural fluid including ascites [13, 14], saliva [15, 16], bronchial lavage fluid [17] and the cell-free supernatant from fine needle aspiration of thyroid nodules [18]. The ubiquitous occurrence

of cell-free nucleic acids leads one to assume that these molecules might have a biological meaning. Nevertheless, there are still more questions than answers and our knowledge on this subject is very limited.

Gahan and Stroun demonstrated that newly synthesized DNA/RNA-lipoprotein complexes (termed *virtosome*) are released from eukaryotic cells into the environment [19]. These complexes are easily taken up and are able to modify the recipient cells. This transfer of genetic information from T to B lymphocytes was demonstrated in mice which were injected with cfDNA from human T cells which had previously been exposed to different viruses [20]. A few days later, these mice that were never exposed to the actual pathogen produced anti-virus-specific human antibodies. In a different set of experiments, it was shown that tumor DNA injected into mice is able to induce the development of a tumor [21]. In a report published some years ago, Gartler had demonstrated that *in vitro* cultivated cells are able to incorporate labeled DNA. He postulated the possibility for a “non-cellular transfer of genetic information in higher organisms” [22]. Cell-free fetal DNA was also shown to have an immune-modulating effect *in vivo* in pregnant mice [23], and the priming of dendritic cells by DNA from exosomes was demonstrated in a mouse model [24]. Later, Garcia-Olmo and coworkers reported that cell-free nucleic acids isolated from the plasma of colorectal cancer (CRC) patients could induce an oncogenic transformation of susceptible *in vitro* cultivated cells [25]. Additionally, this group also performed several *in vitro* and *in vivo* experiments which led them to conclude that cell-free nucleic acids might play a role in metastasis [26, 27]. Mittra et al. demonstrated that cell-free nucleic acids and cell-free chromatin particles can enter healthy cells, integrate into their genomes and elicit DNA double-strand breaks and apoptotic responses [28]. Because of genomic integration, the NF- κ B pathway is activated leading to the induction of a systemic inflammation, which in turn is seen as the cause of different acute and chronic diseases. Among them is chemotherapy-induced toxicity, which is assumed to be caused by the release of a large amount of cell-free chromatin from dying cells inducing DNA damage, apoptosis and inflammation in healthy bystander cells. The administering of substances able to neutralize and/or degrade cell-free chromatin led to a decrease in inflammatory cytokines, a reduction in apoptosis and inflammation, and the prevention of prolonged neutropenia after chemotherapy treatment [29]. A stress signaling function for oxidized cfDNA was recently described [30]. A good review on the functional aspects of cfDNA was recently published by Aucamp and coworkers [31]. In addition, the size of cfDNA originating from different sources (such as

cell-free fetal DNA or ctDNA released from tumor cells) matters and needs to be considered.

Basic principles of cell-free nucleic acids: release, half-life and removal

In the first paper describing the presence of extracellular nucleic acids in humans, 10 healthy people and 15 persons with a variety of different health issues (but no cancer patients) were analyzed [32]. Interestingly, only a pregnant woman (third trimester) demonstrated an increased amount of nucleic acids differing from all other subjects. This is in contrast to many later published papers, which show an increased quantity of cell-free plasma DNA in almost all cancer patients [33].

Even if plasma and serum are frequently the first choice to look for cell-free nucleic acids, they were also detected in other body fluids like urine [10, 34, 35]. Interestingly, DNA from urine supernatants has not only been used in patients with genitourinary cancers (such as prostate, bladder and kidney) but seems to be useful in patients with non-urolological cancers as well (reviewed in Jain et al. [36]).

From the very beginning of the analysis of cell-free nucleic acids, it was an obvious assumption that DNA and RNA are released into the cellular environment by apoptosis and necrosis. These mechanisms play an important role, but an active release of nucleic acids was shown as well [37]. There are several reports demonstrating that nucleic acids are attached on the outside of cells and can be released by a mild ethylenediaminetetraacetic acid (EDTA) or trypsin treatment [38, 39]. A recent report states that the majority of plasma cfDNA is located inside of exosomes [40]. The examination of supernatants from *in vitro* cultivated cells for the presence of cell-free nucleic acids showed that

- the amount of released DNA follows a tight regulation,
- the quantity of cfDNA is independent of the number of apoptotic and necrotic cells, and
- there is a correlation between the number of cells in the G1 phase and the quantity of cfDNA [31, 41, 42].

Additional evidence for an active and regulated release of cfDNA was reported by Toth et al. who described a circadian rhythm for the release of cell-free total DNA and methylated *SEPTIN 9* DNA in a small group of CRC patients [43]. When they collected blood at four different

times (12 pm, 6 am, 12 am and 6 pm), there was a modest variation for DNA in one healthy subject and one patient with adenoma, whereas the variation was larger in CRC patients with stage II–IV. The peak for *SEPTIN 9* DNA was at midnight or 6 am for stage I/II patients, whereas for stage III/IV patients it was 12 am or 6 pm. Summarizing their data, they found that samples taken at midnight were always positive for cell-free *SEPTIN 9* DNA but only in 78% of the samples taken at the other time points. These are interesting observations, but unfortunately the study population is too small (11 subjects in total) to draw any significant conclusions. Rasmussen and co-workers analyzed plasma and serum samples taken from healthy volunteers 3 times a day (8 am, 12 am and 3 pm) for the quantity of nucleosomes containing 5-methylcytosine (5mC) DNA or the histone modification H3K9Me3, but did not find any fluctuation [44]. When they looked for differences in these markers in serum samples obtained on different days (day 1, 8, 15, 22 and 29), the intra-individual variation was 12.7% and 11.5% for 5mC and H3K9Me3, respectively. A similar observation was reported for the amount of cfDNA in urine [45]. The quantity of cfDNA from male and female healthy subjects, which was isolated over several days, demonstrated a large day-to-day variation in both sexes (the urine was collected always at the same time of the day, i.e. in the morning between 9 am and 10 am).

Garcia-Olmo and coworkers [46] used an animal model (tumor-bearing rats injected with colon tumor cells) to demonstrate a time-dependent fluctuation of cell-free tumor DNA. They also observed a fluctuation in the amount of cell-free tumor DNA when compared to the total DNA quantity but were unable to demonstrate any relationship to tumor size. A similar fluctuation of shedding tumor cells into urine was recently reported in prostate cancer patients [8]. Gasparello et al. used several *in vitro* cultivated cell lines and tumor xenografts from these cell lines for the analysis of mutated cfDNA [47]. They observed that LS174T cells secrete much less DNA into cell-culture supernatants than LoVo cells, but the amount of mutated DNA from LS174T xenografts was much higher than from LoVo xenografts. Additional studies of tumor tissue from xenograft mice showed that ctDNA release operates independently from housekeeping functions, which are connected to cell proliferation, tumor necrosis and vascularization. Their conclusion was that the amount of cfDNA seems to reflect a cell line-dependent property.

The published data on half-life and clearance mechanisms are summarized in Table 1.

The overarching message from this compilation is that cell-free nucleic acids demonstrate a short half-life

Table 1: Half-life of cfDNA.

Reference	System	Nucleic acid	Detection method	Remarks
[48]	Mice model	IV injection of tritium-labeled DNA	TCA precipitation and measurement of specific radioactivity	More than 99% of the total radioactivity was removed from the circulation in the first 30 min (rapid phase) and was excreted through the kidney
[49]	Mice model	IV injection of ¹²⁵ I-labelled mononucleosomes	TCA precipitation and measurement of specific radioactivity	Rapid removal of mononucleosomes from the circulation when up to 11 µg DNA was injected; with increasing doses of injected mononucleosomes, slopes of removal curves decreased; the liver was the major organ for removal (71%–84% in 10 min)
[50]	Mice model	IV injection of tritium-labeled genomic DNA and poly I-poly C preparations	Precipitation with specific antibodies or TCA	Intact and denatured DNA is very rapidly cleared (first 3 min after injection), then elimination rate gradually decreases; at 60 min only 10% DNA detectable, serum nucleases not important for DNA removal, liver and spleen show fast and high uptake of administered DNA
[51]	Mice model	IV injection of dsDNA and ssDNA of purified calf thymus DNA, ¹²⁵ I-labeled, large fragments (1–2 kb) + small fragments (75–100 bp), oligonucleotides (5–15 bases)	TCA precipitation	Removal of all DNA preparation followed an exponential curve (two components), 1 st component = organ uptake, 2 nd component = excretion of breakdown products (same slope for all DNA preparations), initial clearance of large ssDNA was significantly faster than dsDNA; max persistence in the circulation was 20 min (ssDNA) and 40 min (dsDNA), ssDNA is mainly removed by the liver, dsDNA + oligonucleotides are mainly removed by soft tissue
[52]	Mice model	Injection of a soluble form of chromatin into retro-orbital sinus, ¹²⁵ I-labeled	TCA precipitation	Liver uptake is the most important factor for histone clearance, Kupffer cells + macrophages specifically needed, pretreatment of mice with DNA strongly inhibits chromatin clearance (same receptors responsible for chromatin + DNA clearance)
[53] ^a	Pregnant women	Male fetal DNA, <i>SRY</i> sequence	q-PCR	Mean half-life for cell-free fetal DNA is 16 min (4–30 min), DNA removal consists of two phases (one initial rapid phase + a slower 2 nd phase)
[54]	Mice model and pregnant women, cancer patients	Injection of irradiated Raji cells or 32P-labeled λ-phage DNA into mice	PCR for detection of different targets (λ DNA, Raji cells), <i>DYZ1</i> gene (pregnant women), <i>K-ras</i> mutant	Majority of injected DNA is recycled, only 3% is excreted into urine over 3 days, Raji-specific DNA is detectable in urine as well, 77 bp amplicons of Y-chromosome-specific sequences found in urine of pregnant women as well as mutated <i>K-ras</i> -amplified DNA from pancreas of carcinoma patients
[55]	Patients with NPC	Quantification of EBV DNA in patients receiving radiotherapy	q-PCR	Median half-life of plasma EBV DNA was found to be 3.8 days, this value seems to reflect the cell death rather than the actual clearance of EBV DNA from plasma
[56]	Patients with NPC	Quantification of EBV DNA in patients who are surgically treated	q-PCR	Median half-life of cell-free EBV DNA in NPC patients was 139 min
[57]	Patients with kidney transplants	Male-specific DNA	q-PCR	Cell-free transplant-derived kidney DNA was found in urine and plasma, no male-specific DNA was found in urine by day 7 after transplantation, male-specific kidney-derived cell-free DNA disappeared much faster from plasma than from urine (no detailed data provided)
[58]	Patients with renal disease	Quantification of total cell-free DNA before and after hemodialysis	q-PCR	In the majority of patients (16/18), the pre-hemodialysis DNA level was reached 30 min after end of the procedure

Table 1 (continued)

Reference	System	Nucleic acid	Detection method	Remarks
[59] ^b	<i>In vitro</i> stability experiment	Quantification of <i>Candida albicans</i> DNA	q-PCR	Incubation of purified (QIAamp DNA Blood Mini Kit) cell-free <i>Candida</i> DNA in human or rabbit plasma for up to 3 days at RT demonstrated no change in DNA concentration
[60] ^c	Healthy subjects and cancer patients	Quantification of total cell-free DNA	Quantification by Hoechst 33258 dye	In healthy people, different levels of DNase activity was found in combination with low levels of cfDNA (range 0–66 ng/mL), in 18 cancer patients no DNase activity was demonstrated but an increased amount of cell-free DNA (range 0–> 1000 ng/mL)
[61]	Patients with colorectal cancer	Quantification of total and mutant cell-free DNA before and after treatment	q-PCR (total DNA), BEAMing (tumor-associated DNA)	Kinetic analysis of the quantity of ctDNA in one (!) patient showed a half-life of 114 min after surgery
[62]	Pregnant women	Quantification of cell-free fetal and maternal DNA	Paired-end sequencing	Two phases of fetal DNA clearance (fast = 0–2 h and slow = 6–18 h), fetal DNA half-life of the phases was 1 h and 13 h, respectively, clearance via kidney up to 19% of fetal DNA only
[63] ^d	<i>In vitro</i> system	Naked DNA and DNA-protein complexes were spiked into serum, saliva or urine	Amplification of a panel of short tandem repeat loci	Half-lives of DNA with/without protein in serum 158 min/31 min at 37 °C, half-lives of DNA with protein in saliva = 176 min at 37 °C and 252 min at RT, half-lives of DNA in urine (with and without protein) were too short to be detectable
[64]	Rabbit model	Injection of tumor cells (VX2) into the animal	Quantification of total and tumor-associated (VX2) cell-free DNA by q-PCR	Surgical resection of the tumor ctDNA levels decreased with a half-life of 23–52 min
[65]	Dog model	Quantification of total cell-free DNA before and after surgical procedures (6 h to 3 days)	Qubit dsDNA HS Assay Kit	Half-life of cell-free DNA was determined to be 5.6 h

^aThe authors performed an additional experiment in which they collected blood in “plain tubes” before cesarean section and at 2 h after delivery, processed these samples immediately after blood draw (centrifugation) and incubated the supernatant for 2 h at 37 °C (only the pre-cesarean samples). In 3/10 samples, there was almost no change in fetal DNA concentration while the other seven samples showed values of 31%–74% of pre-cesarean values. In contrast, in 9/10 samples taken 2 h after cesarean section, no cell-free fetal DNA was detectable. As the authors used the word “plasma”, it is not clear whether the blood was sampled in EDTA tubes. In this case, their conclusion “that plasma nucleases play only a partial role in the removal of circulating fetal DNA in most subjects” might not be correct as the EDTA inhibits nuclease action. ^bIt is not clear why purified *Candida albicans* DNA behaves differently when compared with DNA from other organisms demonstrating a much higher stability in plasma. ^cIn other reports, it was shown that the majority of cell-free nucleic acids is taken up by the liver and the importance of DNase activity in the removal is not clear, especially as DNA released in complexes with proteins, lipids or other molecules might be protected from degradation. In addition, Koizumi et al. detected no DNase II activity in serum but in urine (Expt Anim 1995). ^dThis report is in contrast to other papers in which the presence of cell-free nucleic acids in urine has successfully been described. A possible explanation for this discrepancy is that the rather large PCR products chosen for amplification (only 2/19 amplified STR loci generate an amplicon shorter than 100 bp) are suboptimal for total cfDNA quantification. cfDNA, cell-free DNA; dsDNA double-stranded DNA; EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; PCR, polymerase chain reaction; q-PCR, quantitative PCR; RT, room temperature; ssDNA, single-stranded DNA; TCA, trichloroacetic acid.

only. There seem to be several mechanisms involved in their clearance (liver uptake, removal through the kidney, degradation by nucleic acid degrading enzymes), but there are still open questions (just to name a few of them) as follows:

- Are the clearance mechanisms the same for all kinds of different forms of cfDNA?

- Does the half-life of cell-free nucleic acids in diseased patients differ from the ones in healthy people?
- Are there any differences in the half-life of cell-free nucleic acids in different body fluids and what are the clearance mechanisms in these fluids?
- A deeper understanding of the mechanisms involved in the liberation of extracellular nucleic acids into the

- environment (including but not limited to circadian rhythms),
- Composition and physical, chemical and biological properties of particles associated with cell-free nucleic acids,
- Half-life of these different particles, which contain cell-free nucleic acids and mechanisms of their clearance.

How to choose an optimal control population

In many studies aiming to establish genetic marker/s useful in a diagnostic setting, healthy people (not too seldom young blood donors) are used as controls. This might not be a good idea, as it is important to discriminate cancer patients not from healthy people but specifically from patients with benign diseases affecting the same organ. Apart from age and different environmental influences (factors that are taken into consideration in some studies), chronic non-malignant diseases have an effect on the genetic makeup of cells which should not be underestimated. This applies in particular to inflammatory processes, which are associated with genetic and epigenetic alterations playing a role in the development of a tumor. The presence of chromosomal translocations frequently found in lymphomas had been described in healthy people already some years ago [66–68]. In addition, an allelic imbalance and an overexpression of *TP53* and *CCND1* (*CYCLIN D1* protein) was found in normal lung tissue from a patient with lung cancer [69]. Mutations of the *TP53* and *KRAS* genes were also detected in plasma samples prospectively collected in a large group of healthy subjects [70]. Upon follow-up examinations, 16/33 people harboring a mutation in one of the two genes developed a tumor approximately 1.5 years after blood collection. This demonstrates that about 50% of this group was not healthy when they were included in the study. The analysis of cell-free plasma DNA also detected *TP53* mutations in 11.4% of the non-cancer controls [71], and in 7.4% of healthy subjects analyzed for the presence of *KRAS* mutations [72]. In peritoneal fluids of women without cancer, low frequency of *TP53* gene mutations and an age-dependent increase were found [73]. Whole-exome sequence analysis of 12,380 people without a malignant disease showed a clonal hematopoiesis with somatic mutations in 3111 of them and a strong correlation with age [74]. It was demonstrated that people with clonal hematopoiesis were more likely to receive a diagnosis of hematologic cancer, but so far there is no method to differentiate between this group and people who live

with this alteration and not being diagnosed with a tumor (although this might change in long-term follow-up trials). Many mutations found in primary tumor samples were also detected in genomic DNA from white blood cells (WBCs) as background mutations (germline polymorphisms and mutations with low allele frequency [AF], i.e. <10%), which will make it harder to differentiate between people with an early stage of cancer and healthy people [8, 75]. In addition, there are several papers in which genetic alterations frequently found in tumor cells were detected in normal tissues (apart from WBC) [76–78].

It is expected that an increase in the analytical sensitivity of methods for the detection of low-level genetic alterations not only in tissue but in body liquids as well will lead to an increasing number of reports demonstrating the presence of tumor-associated alterations (genetic and epigenetic) in healthy subjects. Therefore, the choice of controls (such as the appropriate use of spike-in controls or other reference material) and the determination of cut-off values are becoming even more important.

Preparation of cell-free body fluids

According to the data collected in Table 2, our own experience, and a survey conducted at the CNAPS IX meeting, this step is probably the first one on which to agree on a standard operational procedure. In order to remove not only intact cells but also debris and other large particles, it is absolutely necessary to perform two centrifugation steps. The second centrifugation can be performed after thawing plasma samples without any loss of information [81], and even a third centrifugation step does not introduce any bias. Alternatively, a filtration step can be included which has been shown to work equally well but takes more time and needs a filtration device [79].

Recommendation: In order to make sure that plasma samples are cell-free, they should be re-centrifuged at high speed before DNA extraction. This holds especially for samples which are collected and processed in several locations and are shipped (frozen) for analysis.

Comparison of blood-drawing tubes with different anticoagulants

In the early days of cfDNA analysis, a few papers were published comparing different anticoagulants and it was shown that EDTA and citrate performed equally well (Table 3).

Table 2: Methods for the generation of cell-free plasma.

Reference	Samples	Centrifugation method	Detection method	Remarks
[79]	EDTA blood, pregnant women	Medium speed (1600g) alone or in combination with high speed (16,000g) or filtration, low speed (800g) alone or in combination with high speed (16,000g) or filtration, Percoll gradient alone or in combination with high speed or filtration	Quantification of total and fetal DNA conc. (q-PCR)	Incomplete removal of WBC leads to high total DNA conc. but does not affect fetal DNA, double spin (low/medium speed + high speed) or spinning + filtration gives best results
[80]	EDTA blood, sex-mismatched bone marrow transplantation patients	400g up to 16,000g for 10 min	Quantification of total DNA conc. (q-PCR)	No change of total cell-free DNA conc. in all samples
[81]	EDTA blood (7 mL and 10 mL tubes), pregnant women	800g or 800g + 16,000g (7 mL tubes) 800g or 800g + 16,000g (10 mL tube)	Quantification of total DNA conc. (q-PCR)	Low-speed spinning removes WBC in 7 mL tubes but not in 10 mL tubes (double spin necessary), 2 nd spin can be performed after thawing of plasma sample (!)
[82]	EDTA blood, banked and prospectively collected samples from healthy people and cancer patients	1600g 5 min (banked plasma) 2 × 1600g 10 min (prospectively collected plasma)	Quantification of total DNA conc. (q-PCR)	Prospectively collected samples had lower DNA concentrations than banked samples, no difference in cfDNA conc. between cancer patients and controls
[83]	EDTA, healthy female donors	2 × 2000 rpm for 10 min, 2000 rpm for 10 min + 13,500 rpm for 5 min	PCR	Amplification products of >300 bp are not seen after high-speed spinning, this step (high-speed centrifugation) can be performed after thawing, i.e. before DNA isolation
[84]	EDTA, healthy female donors	2 × 1000g for 10 min, 1000g for 10 min + 1000g, 2000g or 10,000g for 10 min each	Quantification of total DNA conc. (q-PCR)	CfDNA yield was the same for all spinning protocols, two-step spinning with high speed (10,000g) reduced the number of quantifiable miRNAs from 195 to 138
[85]	EDTA, NSCLC patients	1 × vs. 2 × spin at 2000g for 10 min	Quantification of total DNA conc. (q-PCR)	Spinning protocol (1 × vs. 2 × centrifugation) had no influence on cfDNA yield when samples were processed 2 h after blood draw, at 3 day processing double spinning reduced cfDNA
[86]	EDTA, healthy people and cancer patients	1 × 800g for 10 min 800g for 10 min + 11,000g 1 min 800g for 10 min + 11,000g 1 min (after thawing) 380g for 20 min + 20,000g 10 min 380g for 20 min + 20,000g 10 min (after thawing)	NanoDrop spectrophotometry, ddPCR	First protocol gave the highest yield of cfDNA (72 copies/μL), all other methods were comparable (range 28–40 copies/μL)
[87]	EDTA, cancer patients	820g for 10 min + 14,000g for 10 min 1600g for 10 min + 14,000g for 10 min 1600g for 10 min + 3000g for 10 min	Total DNA quantification (dPCR) and mutant detection (targeted amplicon sequencing)	No difference for all spinning protocols regarding total amount of cell-free DNA and mutant allele fraction

Table 2 (continued)

Reference	Samples	Centrifugation method	Detection method	Remarks
[88]	EDTA, healthy people and cancer patients with mutated <i>KRAS</i>	1 × 400g for 10 min 400g for 10 min + max speed for 1 min 120g for 20 min + 360g for 20 min + 360g for 5 min 1600g for 10 min + 6000g for 10 min 1900g for 10 min + 16,000g for 10 min (4 °C or RT)	ddPCR <i>KRAS</i> screening multiplex kit for total and mutant DNA conc.	For biobanking without predefined downstream application, the first protocol is recommended; for the analysis of cfDNA, the last protocol (high speed at 4 °C or RT) are optimal

Anker et al. [89]: even high-speed centrifugation (45,000 rpm) does not seem to harm cells (liberation of their nucleic acids) but brake should be reduced! cfDNA, cell-free DNA; ddPCR, droplet digital PCR; dPCR, digital PCR; EDTA, ethylenediaminetetraacetic acid; miRNAs, micro RNAs; NSCLC, non-small cell lung carcinoma; q-PCR, quantitative PCR; RT, room temperature; WBC, white blood cell.

Table 3: Comparison of different anticoagulants.

Reference	Tube/anticoagulant	Plasma samples	Detection method	Remarks
[90]	EDTA, citrate	Healthy volunteers	q-PCR	cfDNA quantity was compared to serum, no data on comparison between EDTA and citrate
[91]	EDTA, heparin, citrate	Healthy volunteers	q-PCR	No difference in plasma cfDNA conc. in all tubes when stored for up to 6 h, at 24 h cfDNA quantity higher in all tubes, but smallest increase in EDTA tubes
[92]	EDTA, plain tubes (serum)	Pregnant women	q-PCR for quantification of fetal DNA (<i>DYS-14</i>)	DNase activity was much higher in serum than in EDTA tubes, adding EDTA to serum samples lead to inhibition of DNase activity, serum storage at 37 °C lead to strong decrease in cell-free fetal DNA, much less in EDTA tubes
[93]	EDTA, citrate	Healthy volunteers and cancer patients	Fluorometric quantification (total cfDNA), mutation detection with mutation-biased PCR and quench probe system	Stabilization of cfDNA after RT storage for 1 or 3 days was somewhat better in citrate than EDTA

cfDNA, cell-free DNA; EDTA, ethylenediaminetetraacetic acid; q-PCR, quantitative PCR; RT, room temperature.

Most research groups advice against the use of heparin as it is known to inhibit DNA polymerase and might lead to negative polymerase chain reaction (PCR) results. It did not take a long time to demonstrate that a delayed processing of blood-drawing tubes (EDTA, citrate, heparin, plain tubes for serum extraction) has a negative effect on cfDNA (such as a reduced detection rate for mutant sequences). Very soon, several companies made an effort to develop tubes that stabilize blood samples (see Table 4). While Streck Inc. (Omaha, NE, USA) was the first company to market a blood collection tube specifically designed to prevent lysis of nucleated blood cells after prolonged storage, some other companies like Qiagen and Roche followed. The basic principle of all these tubes is to stabilize WBCs and to prevent the “contamination” of cfDNA with

genomic DNA. When DNA and blood samples were treated with the reagent used for cell stabilization (contained in blood collection tubes [BCTs]) for up to 2 weeks, there was no effect seen on amplification by PCR whereas formaldehyde or glutaraldehyde led to a significant decrease in DNA amplification [120].

From the data compiled in Tables 3 (comparing EDTA- and citrate-containing tubes) and 4, it can be concluded that

- EDTA or citrate perform equally well when samples are processed within a reasonable time after blood draw (4 to max. 6 h) and storage at 4 °C to ambient temperature (<30 °C), and
- The use of one of the blood-stabilizing tubes is highly advisable when samples need to be stored/shipped before processing.

Table 4: Tubes with blood-stabilizing properties for cfDNA analysis.

Reference	Tubes		# Samples/patients	Storage time/ temperature	Method	Remarks
	EDTA ^a	Streck BCT Tube type, Brand/company				
[94]	x	x	26 Pregnant women, 6 non-pregnant women	Up to 2 weeks, RT	Total DNA quantification and detection of SRY sequence (hypermethylated in fetal DNA) with q-PCR	Strong increase in total DNA in EDTA, no change in BCT, reduction in percentage of cell-free fetal DNA in EDTA, not in BCT
[95]			10 Pregnant women	Shipped (3 days), RT or with frozen gel packs	Total DNA and fetal DNA (SRY) quantification (PCR)	Shipping at RT had no effect on total DNA in both tubes, shipping of EDTA and BCT (4 °C) led to decreased cell-free fetal DNA
[96]	x	x	10 Cancer patients	24 h, RT	q-PCR, total DNA, <i>p53</i> mutation detection, methylation analysis	EDTA plasma worked better than BCT plasma in combination with Qiagen DNA blood mini kit
[97]	x	x	Healthy people	Up to 2 weeks, 6 °C, 22 °C or 37 °C	Total DNA quantification (q-PCR)	BCT plasma performed well under all different conditions
[98]	x	x	240 Pregnant women	Up to 2 weeks, RT (BCT only)	Total and fetal DNA quantification with PCR and MALDI-TOF MS	Storage at RT had only modest effect on total and fetal DNA in BCT, storage for 1 day at 30 °C or 37 °C decreased fetal DNA fraction
[99]	x	x	10 Cancer patients PAXgene ^b	7 days, RT (PAXgene+BCT)	<i>PIK3CA</i> mutation detection	Strong increase in <i>PIK3CA</i> wild type DNA in PAXgene tubes
[100]	x	x	Pregnant women	5 days, RT, shipping for 2–5 days at 0 °C–30 °C or –20 °C to 0 °C	Total DNA and fetal DNA (SRY) quantification (q-PCR)	Shipping at all different temperature conditions in BCT had no effect on fetal DNA
[101]	x	x	10 Cancer patients	Up to 10 days, RT	<i>BRAF</i> mutation detection (PCR)	Storage in BCT had no effect on mutation detection while EDTA samples failed
[102]	x	x	10 Cancer patients CellSave tube ^c	Up to 2 days, RT	Total DNA quantification (Qubit) and ddPCR (<i>TP53</i> and <i>PIK3CA</i> mutation detection)	An increase in WT DNA and a decrease in mutant DNA was seen in EDTA tubes when stored for 2 days, no change in BCT and CellSave tubes
[85]	x	x	20 Cancer patients	Up to 3 days, RT	Total DNA quantification (q-PCR) and <i>K-ras</i> mutation detection (ARMS technology)	Low but significant increase in total DNA was seen in BCT after a 3-day storage, decreased sensitivity of <i>K-ras</i> mutation detection was seen in BCT at day 3
[103]	x	x	21 Cancer patients, 8+6 healthy donors	72 h (BCT, cancer patients), up to 5 days/ 4 °C, RT, 40 °C (BCT, healthy people)	Total DNA quantification (q-PCR) and mutation detection (BEAMing)	3-day RT storage showed reliable detection of mutant DNA in spiking expts + in cancer patient plasma, storage of BCT at 4 °C or 40 °C for up to 5 days not recommended
[104]	x	x	9 Cancer patients	Up to 2 days, RT	Detection of copy-number alterations and mutational profiling	ctDNA level did not change (not even in EDTA), same with CNA analysis and mutational profiling
[105]	x	x	5 Cancer patients	72 h (BCT), RT (?)	Mutation detection with ddPCR	Plasma DNA from both tubes work equally well

Table 4 (continued)

Reference	Tubes		# Samples/patients	Storage time/ temperature	Method	Remarks
	EDTA ^a	Streck BCT	Tube type, Brand/company			
[106]	x	x	ccfDNA	8 Healthy people	4 days, RT	Total DNA quantification (q-PCR) + DNA integrity test (q-PCR)
[107]	x	x		Cancer patients	Up to 7 days, RT and up to 3 days, 4 °C	Prolonged storage had no effect on yield and integrity in ccfDNA plasma samples, slight increase in high MW DNA was seen in BCT at day 4
[108]	x	x		31 Diverse advanced cancer patients	>24 h, RT	No change in total and mutant cfdNA in BCT over storage time, strong increase in total DNA + decrease in mutant DNA in EDTA
[109]	x	x	ccfDNA	Healthy donors	Up to 7 days, RT	Storage up to 16 h gave comparable DNA yield, integrity and NGS performance for detection of somatic variants
[105]	x	x		5 Cancer patients	3 days (BCT only), (probably) RT	ARMS PCR for detection of spiked mutant <i>EGFR</i> DNA
[110]	x	x	CellSave tube ^c	16 Cancer patients	Up to 4 days, RT	Total DNA quantification (ddPCR for <i>BRAF</i> + <i>PIK3CA</i> WT loci), mutation detection (same genes, ddPCR)
[111]	x	x	CellSave tube ^c , Heparin, Citrate	Three pooled plasma samples, 46 healthy people, 18 cancer patients	Up to 1 day, RT (EDTA, heparin, citrate), up to 5 days, RT (BCT, CellSave)	No change in cfdNA concentration in BCT + CellSave, significant decrease in variant allele frequency in EDTA but not the other tubes
[112]	x	x	ccfDNA	29 Cancer patients	7 days, RT	Total cfdNA conc. remained stable in EDTA for 1 day at RT, citrate plasma yielded less cfdNA than EDTA
[113]	x	x		3 Pediatric cancer patients	Same day processing, no storage	Increase in total DNA in EDTA tubes (not in ccfDNA), methylation analysis possible
[114]	x	x	PAXgene ^b	Healthy people	7 days, RT, 7 days (12 h 30 °C, 12 h 22 °C), 1 day (5 h 39 °C, 19 h 22 °C)	All samples work equally well, also for SNP detection
[115]	x	x		Healthy people, adenoma + colorectal cancer patients	2 days, RT (Streck)	BCT and PAXgene worked well with all different temp. conditions, Roche tube showed some contamination with high MW DNA
						No difference in total cfdNA quantity, methylation analysis worked better with EDTA

Table 4 (continued)

Reference	Tubes		# Samples/patients	Storage time/ temperature	Method	Remarks
	EDTA ^a	Streck BCT	Tube type, Brand/company			
[87]	x	x		Up to 1 week, RT	Total DNA quantification (q-PCR), detection of mutant + WT <i>TP53</i> and <i>PIK3CA</i> (q-PCR), targeted amplicon sequencing	BCT plasma worked well with all assays, shipment of BCT had no effect on mutant allele fraction and global copy number changes
[93]	x	x	Sodium citrate	Up to 3 days (RT), up to 1 week (4 °C)	Capillary electrophoresis system, <i>EGFR</i> mutation detection (PCR)	Sodium citrate works better than EDTA (4 °C for 3 days) and comparable to BCT and PAXgene
[116]	x	x	Roche	Up to 2 weeks, RT	Total DNA quantification and NGS analysis	Strong increase in total DNA in EDTA tubes as compared to Roche + Streck (day 14), no difference for NGS when stored for 3 days
[117]			Roche	Up to 2 days, RT and 4°–8 °C	Total DNA quantification with ddPCR	All samples worked equally well, no difference was seen
[118]	x	x	Roche+Norgen	7 days, RT	Total DNA quantification (Qubit) and determination of <i>TP53</i> + <i>PIK3CA</i> (ddPCR)	No change in total DNA + integrity in all tubes, tumor-associated genes were detectable in all samples
[88]	x	x	Roche + Norgen	1 day RT + rocking platform (1 day) 6 °C or RT) + 1 day RT (Σ = 72 h)	CFDNA integrity test (qPCR) + K-ras mutation quantification (ddPCR)	No differences in DNA yield + integrity observed (both temp), comparable number of mutated K-ras observed in all tubes independent of temperature
[119]	x	x	ccfDNA	Immediate processing + storage at RT for 6 h and 24 h	Pathogenic DNA was quantified by target-specific qPCR	Processing delay led to increased Ct values of all pathogens in Streck and ccfDNA tubes but only significant for <i>M. tuberculosis</i> + <i>S. enterica</i> , processing of EDTA blood after 24 h (single spin plasma preparation) did not change Ct values of any pathogen

^aInclude K₂EDTA, K₃EDTA and tubes from different vendors. ^bPAXgene tubes are not designed to be used for cell-free DNA but for the isolation of genomic DNA from stabilized cells. ^cCellSave preservative tubes were originally developed for blood samples to be used for the CellSearch circulating tumor cell test and are commercialized by Menarini Silicon Biosystems. ARMS, amplification refractory mutation system; BCT, blood collection tube; ccfDNA, circulating cell-free DNA; cfdNA, cell-free DNA; ddPCR, droplet digital PCR; EDTA, ethylenediaminetetraacetic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; MW, molecular weight; NGS, next-generation sequencing; PCR, polymerase chain reaction; q-PCR, quantitative PCR; RT, room temperature; SNP, single-nucleotide polymorphism; WT, wild type.

What happens when processing of blood or biological fluids is delayed?

The data compiled in Table 5 demonstrate convincingly that blood samples drawn into EDTA tubes can be stored at room temperature (RT) for a few hours without losing any information when analyzed for tumor-associated alterations. As mentioned before, cfDNA can be isolated from different body fluids like urine, CSF, saliva, pleural effusion fluids and bronchial washings [8, 132, 133]. Unfortunately, there are almost no data on how to handle these fluids in a way to optimize the isolation and characterization of extracellular nucleic acids.

In a “seven at one blow” approach, Sorber and colleagues tried to analyze the effect of a variety of different pre-analytical factors on the sensitivity of a test for the detection of *EGFR* mutations in the plasma of lung cancer patients [88]. One of their conclusions reads that “A longer transit time was associated with a lower amount of plasma” in combination with a one-step low-speed centrifugation protocol for plasma processing. As it was not stated in the paper how and where (outside the lab?) the tubes were stored before processing, it is not possible to comprehend this result. Additionally, it is stated that “In conclusion, transit time had a significant impact on plasma generation and the occurrence of hemolysis. However, this effect on plasma generation could be negated by using a two-step, high-speed centrifugation protocol.” For the reader, it is not clear why or how a two-step high-speed centrifugation for the plasma preparation might prevent hemolysis of the sample.

Recently, a multidisciplinary working group of specialists from different fields from Canada published a paper with recommendations to improve *EGFR T790M* testing for tissue and cfDNA (liquid biopsy). One statement is considered critical from our point of view, i.e. the proposition to isolate the plasma from EDTA tubes within 6 to 48 h after blood draw [134]. It has been demonstrated in several papers that storage of EDTA blood for more than 24 h (independent of the storage temperature) led to increase in DNA caused by lysis of blood cells and should be avoided by all means. If the number of mutant DNA molecules is high, this might not have an influence on the test result. In samples with a low mutant allele fraction, the increase in DNA from healthy cells might mask the detection of mutant molecules due to an increased noise. We therefore recommend to use EDTA tubes only if the plasma can be prepared within 6 h after blood draw. Otherwise, the application of cfDNA stabilization tubes is indicated.

Factors which can impact the quantity/quality of cfDNA

Companies like Sarstedt and Sartorius sell blood collection tubes without a vacuum, which needs to be generated manually by the phlebotomist. In contrast, many other producers developed blood collection tubes with a vacuum. So far, there are no data or a head-to-head comparison on the influence these different tubes might have on cfDNA. Other questions for which we do not have an answer yet include the following:

- Does it make a difference whether patients are fasting vs. eating?
- Does the position during blood draw (sitting vs. lying) make a difference? (It is known that the change of position from supine to sitting or *vice versa* has an effect on some laboratory parameters [135], but so far we do not know whether this is true for cell-free nucleic acids as well.)
- Does tourniquet stasis have an influence on the quantity/quality of cell-free nucleic acids (there is only one report which analyzed the amount of nucleosomes harboring either methylated DNA or a histone modification after blood draw with/without stasis and no difference was detected [44])?
- Does the needle size used for blood drawing (apart from being big enough to prevent hemolysis) have an effect? The recently introduced 25-gauge BD Vacutainer® UltraTouch™ needles have a diameter somewhat larger than the 23-gauge needle and perform equally well in terms of (not) causing hemolysis [136]. As these needles just entered the market, they have not yet been tested for cfDNA applications.

Other factors which might have an impact on liquid biopsy results are age, gender, ethnicity, body mass index, organ health, smoking, physical activity, diet, glucose levels, oxidative stress, medication status, infections, menstruation and pregnancy.

Long-term storage of cell-free matrices and freeze-thaw cycles

The results compiled in Table 6 give an unclear image and do not allow a firm data-based conclusion for an optimal storage method. The decay rates, which were determined in these papers, are highly variable and there is no trend (not to mention congruence) at all. We assume that the main

Table 5: Influence of delayed blood processing on cfDNA.

Ref	Tube	Storage time	Storage temperature	Samples	Method	Remarks
[80]	EDTA	Up to 6 h	RT	Healthy people	q-PCR for total DNA conc.	No difference in total DNA quantity when stored for 6 h
[121]	EDTA	Up to 24 h	4 °C (8 + 24 h) and RT (up to 8 h)	Healthy people	q-PCR	Almost no change in cfDNA conc. in all samples, strong increase in serum tubes past 2 h at RT
[122]	EDTA	15 min–24 h	RT	Pregnant women	q-PCR for total and fetal DNA conc.	Strong increase in total DNA conc. after 6 h, no change in fetal DNA conc. up to 1 day
[123]	EDTA	6 h, 24 h	4 °C or RT	Healthy volunteers	q-PCR for total DNA conc., DNA integrity assay (q-PCR)	Increase in total DNA after 24 h but not 6 h (4 °C and RT), altered DNA integrity (201/105 bp PCR) at 24 h RT but not 4 °C
[124]	unknown	More than 14 days	Not stated	Pregnant women	<i>RHD</i> genotyping (PCR)	67/1896 samples were inconclusive or false negative, most of them had been stored for more than 2 weeks + showed high maternal background DNA
[125]	EDTA	Up to 1 day	On ice or at RT	Healthy volunteers	PicoGreen assay and q-PCR	Increase in cfDNA was seen starting at 4 h storage independent from storage temperature
[126]	EDTA	Up to 8 days shipping (median 2 days)	RT (?)	Pregnant women	<i>RHD</i> genotyping (PCR)	Steep increase in total cfDNA conc. starting with day 2 of storage but almost no change in amount of fetal DNA in samples shipped for 6 days
[95]	EDTA	Shipping for max of 3 days	RT or in boxes with frozen gel packs	Pregnant women	q-PCR for total and fetal DNA (<i>SRY</i>) conc.	Quantity of total amount of cfDNA was much higher at 3 days independent from shipping temperature, almost no change in amount of fetal DNA in shipped samples independent of temperature
[127]	EDTA	Up to 2 days	RT	Pregnant women	Multiplexed sequencing (MPS)	Plasma samples stored for 1 day can reliably be used for MPS analysis (this method enriches for short DNA fragments thus removing longer ones from lysed maternal cells)
[128]	EDTA	Shipping from 2 to 9 days		Pregnant women	<i>RHD</i> genotyping (PCR)	Outdoor temperature (–5 °C to 25 °C) did not affect fetal DNA quantity, total DNA level at mean average temperature 20°–24 °C was significantly less than at mean average temperature of 24°–30 °C
[129]	EDTA	At least 1 day	4 °C	Pregnant women	<i>RHD</i> genotyping (PCR)	All samples worked fine for quantification of <i>SRY</i> sequence
[85]	EDTA	3 days	RT	NSCLC patients	Total cfDNA quantification (q-PCR) + <i>K-ras</i> mutation detection (ARMS PCR)	Strong increase in total DNA quantity at 3 days, detection rate for mutant <i>K-ras</i> dropped from 80% to 40% after a 3-day storage
[104]	EDTA	Up to 2 days	5 °C or RT	Prostate cancer patients	Targeted sequencing (panel of 305 genes of interest)	CtDNA level did not change at 2-day storage, copy number alterations and mutational profiling identical in all samples independent of storage time and temperature
[102]	EDTA	Up to 2 days	4 °C or RT	Breast cancer patients	Total cfDNA + ctDNA, DNA quantification by ddPCR	3/6 Patients showed increased amount of total cfDNA at 2 days' storage (both temperatures), 2/4 patients had decreased tumor allele frequencies at 2 days' storage (both temperatures)
[44]	Serum	Up to 3 days	Storage on ice or RT	Healthy people and cancer patients	Quantification of cell-free nucleosomes containing 5-methylcytosine DNA or histone modification H3K9Me3 (ELISA)	Samples kept at RT had significantly higher values than samples stored on ice independent of storage time

Table 5 (continued)

Ref	Tube	Storage time	Storage temperature	Samples	Method	Remarks
[130]	EDTA	1 day	4 °C or RT	NSCLC patients	Total DNA quantification (Qubit) + mutation detection (ddPCR)	No difference in total cfDNA amount, same for mutation detection
[110]	EDTA	Up to 4 days	RT (?)	Cancer patients	Variant allele frequency was determined by Sanger sequencing or NGS	There was a significant increase in total cfDNA conc. in samples processed at 4 days, these were predominantly larger DNA fragments, nevertheless somatic variants detected in the 1-h sample were found in 96-h samples as well
[111]	EDTA	Up to 1 day	RT	Healthy people and NSCLC patients	Total DNA quantification (Qubit)	No change in total cfDNA amount over time
[87]	EDTA	Up to 1 week	4 °C or RT	Cancer patients	Total DNA quantification + mutant detection (<i>TP53</i> + <i>PIK3CA</i>) with digital PCR	Levels of total cfDNA increased over time while fraction of ctDNA decreased (absolute number of mutant DNA copies were stable over time), storage at 4 °C for up to 2 days increased the level of total cfDNA quantity but less than storage at RT, 4-day storage at 4 °C showed significant increase in total cfDNA
[131]	EDTA	Up to 12 h	RT	NSCLC patients	Total DNA quantification (Qubit) + mutant detection (NGS-targeted sequencing)	Increased level of total cfDNA conc. was found as early after 8–12 h storage, storage for >4 h lead to decreased detection of mutant DNA which contrasts the majority of other papers

ARMS, amplification refractory mutation system; cfDNA, cell-free DNA; CtDNA, circulating tumor DNA; ddPCR, droplet digital PCR; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; NGS, next-generation sequencing; NSCLC, non-small cell lung carcinoma; PCR, polymerase chain reaction; q-PCR, quantitative PCR; RT, room temperature.

reason for these contradicting results is caused by the use of individual plasma samples from healthy and diseased subjects. It is commonplace that blood is an extremely complex liquid and its composition is highly variable. For this reason, the use of plasma/serum pools instead of single blood samples for long-term storage experiments might be a good alternative. Recently, it was demonstrated that plasma samples with a low mutant AF degrade faster during storage than samples with a high mutant AF, supporting the assumption that individual samples behave very differently [93]. The storage of extracted cfDNA in a dry form (such as paper-based matrices) as compared to the usual storage in a liquid might be a viable alternative as shown by Heider and colleagues [149].

So far, only a few papers were published in which the effect of freeze-thaw cycles on cfDNA was analyzed. The effect of freezing and thawing of plasma samples up to 3 times did not have an effect on plasma cfDNA concentration [123]. Additionally, freezing and thawing of

plasma samples once did not change the DNA integrity index (DNA-II) (measured as the ratio of the amount of 201 bp and 105 bp amplicons of the *LEPTIN B* gene). In contrast, freezing and thawing of plasma samples up to 3 times had a significant effect on DNA integrity probably due to fragmentation, but treatment of extracted DNA the same way (3×freeze-thaw) had no effect. In one of the first reports on the detection of cell-free serum RNA, it was demonstrated that tyrosinase mRNA survived one freezing/thawing cycle but was undetectable after three cycles [138]. The decision to aliquot samples in larger (>3 mL and more) or smaller (<100 µL or less) volume tubes is strongly dependent on the downstream application and should be performed accordingly. If it were known that an analysis method requires the usage of a large plasma volume and frozen samples have to be thawed only once, it would be a waste of time (not to mention increased costs) to aliquot samples in small-volume containers as usually recommended.

Table 6: Effects of long-term storage of cell-free matrix on cfDNA.

Reference	Samples	Storage condition	Detection method	Remarks
[137]	Plasma and serum from healthy volunteers and cancer patients	−70 °C	Nested PCR and gel electrophoresis	In 4/16 plasma/serum samples stored for more than 6 years <i>K-ras</i> mutations were found (no data presented on decay rate/loss over time)
[138]	Serum from cancer patients	−70 °C	Reverse transcription, nested PCR and gel electrophoresis	Tumor-associated gene expression (tyrosinase mRNA) was detected in sera stored for >2 years
[139]	Sera and amniotic fluid from pregnant women	−20 °C	q-PCR (<i>DYS1</i> locus)	There was a loss of 0.66 GE/mL per month of storage in serum, this was not seen in amniotic fluid samples
[140]	Plasma from healthy volunteers, lung cancer patients and heavy smokers	−80 °C for plasma and −20 °C for isolated DNA	q-PCR (<i>hTERT</i>)	Yearly decay rate for isolated DNA was 30.5% and for DNA from stored plasma 30.7%
[123] ^a	Plasma from healthy volunteers	−80 °C	q-PCR (<i>leptin</i> gene)	No change in DNA conc. and in DNA integrity (201 bp/105 bp, 365 bp/105 bp) was seen (after 2 weeks storage)
[141]	Plasma from pregnant women	−20 °C for at least 4 years	Total and fetal DNA conc. (q-PCR)	Amplification of fetal DNA (amplicon sizes from 63 bp to 524 bp) showed no change after 4 years of storage (compared to samples analyzed 1 month after blood draw)
[142]	Serum samples from cancer patients	−70 °C	Quantitation of nucleosomes by ELISA	Nucleosome loss was 7%/year at 70 °C (5-year period)
[143]	Serum from cancer patients	−20 °C and −80 °C	Panel of different miRNAs	10-day storage did not change miRNA conc. at either temperature, 20-month storage did not change total RNA conc. but several miRNAs were affected by storage conditions (temperature), storage of up to 10 years at −20 °C showed no decrease at 4 years but significant decrease after 6 years and longer
[84]	Plasma from cancer patients	−80 °C for more than 12 years	Panel of miRNA (TLDA)	In a fresh sample pool, 202 miRNA were detected and in the stored pool 177 miRNA, the C_T values were comparable in both pools
[144] ^b	Plasma from healthy individuals	Short range: Up to 3 h at 80 °C, −20 °C, +4 °C, and RT long range: plasma storage at −80 °C for 9 months, storage of extracted cfDNA for up to 5 months at −20 °C	q-PCR targeting BRAF (short amplicon 105 bp, long 288 bp)	3 h experiment: Storage at 4 °C gave the highest yield for long amplicon, no change for short-sized amplicon, storage at 4 °C and RT led to slight decrease in DNA integrity, Long-term experiment: no change in cfDNA conc. after 9 months storage, slight decrease in conc. after storage of extracted cfDNA for 5 months
[145]	Plasma from cancer patients	−80 °C for up to 21 months	Quantification of total amount of cfDNA by q-PCR	Mean decrease of 38% in total cfDNA was seen
[146]	Plasma from pregnant women	RT, 4 °C, −80 °C	Quantification of total + fetal DNA conc. (q-PCR)	RT storage of plasma for 2 weeks did not change fetal DNA quantity, holds true for storage at 4 °C for 3 days and −80 °C for 2 weeks
[147]	Plasma from healthy people spiked with DNA from MCF-7 or SKBR3 cells, sodium bisulfite-treated DNA from spiked plasma (MCF-7 cells) was stored at −80 °C	−80 °C for up to 8 months (spiked plasma)	Real-time MSP or WGA	Plasma storage for 3 months had no influence on ctDNA analysis, 8-month storage led to a decrease in methylated ctDNA, bisulfite-converted DNA can be stored for 1 year at −20 °C or −80 °C without any effect on real-time MSP analysis

Table 6 (continued)

Reference	Samples	Storage condition	Detection method	Remarks
[93]	Plasma from cancer patients	–20 °C (isolated cfDNA) or –80 °C (plasma)	Detection of <i>EGFR</i> gene mutations (L858R and T790M) by mutation-biased PCR and quench probe system (in-house)	Decrease in <i>EGFR</i> mutation in isolated cfDNA was 20–25% and in plasma 35–40% reduction, respectively, after 7 years of storage
[119]	Whole blood from healthy donors drawn into K ₂ EDTA, Streck cell-free DNA tube or PAXgene Blood ccfDNA tube was spiked with DNA from different pathogens	24 weeks at –80 °C	Real-time PCR with pathogen-specific primers	No change in Ct values when compared to fresh samples
[148]	Plasma from breast cancer patients	14 days at –80 °C	Copy number alteration analysis (NGS) and SNV analysis (QIASeq Target DNA Breast Cancer Panel)	Freezing showed no significant statistical difference on the cfDNA yield, total number of aligned reads, percentage of aligned reads, and CNV quality score and did not affect the results of SNV analysis

^aThe authors also conclude that DNA appears to be more resistant to fragmentation when stored in DNA extraction solution than in plasma.

^bThe decrease in the yield of long-sized amplicons after storage for 3 h at –20 °C or –80 °C is probably not due to storage itself but caused by freezing and thawing the samples once. cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; ELISA, enzyme-linked immunosorbent assay; GE, genome equivalents; miRNAs, micro RNAs; MSP, methylation-specific PCR; NGS, next-generation sequencing; PCR, polymerase chain reaction; q-PCR, quantitative PCR; SNV, single-nucleotide variation; TLDA, TaqMan low-density array; WGA, whole genome amplification.

As the impact of long-term storage has not been examined in detail so far, we need more studies and data to answer these questions:

- What are the optimal storage conditions (time, temperature) for cell-free matrices and extracted cfDNA samples?
- Does the thawing procedure (duration, i.e. fast vs. slow, temperature) have an effect on cfDNA quantity/quality?

Proposals: In order to make results from longitudinal analyses comparable, the time of sampling should be about the same for the whole group.

It would be very helpful to develop decay markers as a surrogate for deterioration of cfDNA upon handling and storage.

Is DNA quantification and determination of cfDNA fragmentation a useful tool?

As stated before, an increase in the quantity of cfDNA is not only seen in tumor patients but in other conditions as well. These are (just to mention a few examples) patients with myocardial infarction [150], an ischemic stroke [151],

trauma patients [152] and healthy subjects who exercise extensively [153]. Additionally, it was demonstrated that the amount of cfDNA depends on many intrinsic as well as extrinsic factors (such as DNA extraction method, see following text) and that there is large overlap in the quantity of cfDNA in patients with a malignant or benign disease and frequently also with healthy people [154]. There are several different quantification methods in use differing in their DNA concentration (see below) which needs to be considered [155].

In one of the earliest reports analyzing the fragment size of cfDNA, these molecules were examined by electron microscopy and a median strand length of 273 nm in healthy subjects and 185 nm in pancreatic cancer patients was described [156]. This observation was later confirmed and found that cfDNA from tumor patients is more fragmented than from healthy people and that tumor-associated cfDNA (ctDNA) consists of smaller fragments [157–159]. On the other hand, there are reports in which mutant ctDNA was also detected in long fragments (1–9 kb) and that the yield of mutant ctDNA is dependent on the isolation method [160]. Plasma samples from hepatocellular carcinoma (HCC) patients showed a correlation between the amount of short DNA fragments (less than 150 bp) and a larger amount of tumor DNA and *vice versa*, i.e. a lower quantity of tumor DNA is associated with increased fragment length [161]. In addition, Vagner et al. [162] isolated large extracellular vesicles (EV) from

prostate cancer patients and showed that they contain chromosomal DNA of up to 2×10^6 bp long, thereby reflecting the complete genetic make-up of the cells of origin. These large vesicles seem to be tumor-specific, as they were not found in plasma from healthy people.

The measurement of the DNA-II (ratio of ALU 115 bp vs. 247 bp amplicon) obtained from cell-free supernatants from subjects with undiagnosed pleural effusions demonstrated a higher DNA-II in patients with a malignant pleural effusion when compared to patients with a benign effusion. It was concluded that this method could be useful especially in cases where the pleural aspirate cytology is negative [163]. This approach could be a useful tool in clinical practice, but unfortunately there exist so far no more data to confirm this result.

These observations lead us to the question whether the measurement of DNA fragmentation in plasma samples from patients is a meaningful tool. Wang and colleagues were the first to describe an increased DNA integrity in plasma from cancer patients as compared to a control group consisting of patients with a variety of different non-neoplastic diseases as well as healthy people [164]. The authors conclude that “the increased DNA integrity in plasma DNA is associated with cancer, and measurement of DNA integrity may provide a simple and inexpensive measure for cancer detection, including their ability to discriminate this group from a healthy control population”. Another group confirmed this data but found a very large overlap in the DNA-II between healthy people and women with a benign breast disease [165]. After the first description on the usefulness of the measurement of DNA integrity for the discrimination of cancer patients from controls, a surge of more than 50 papers were published, some of them corroborating the original results while others were not able to find a difference between patients with and without cancer.

From our own experience, the quantification of isolated cfDNA makes sense particularly when time-consuming and or expensive downstream applications are planned. In these cases, we would recommend a quantitative PCR (q-PCR) (or even digital PCR [dPCR] for absolute quantification) instead of spectrophotometry or fluorometric methods as it was shown that there is a good correlation between serum cfDNA concentration measured by PicoGreen and qPCR but only a weak correlation between spectrophotometry and PicoGreen [166]. Others confirmed this observation in that the accordance between PicoGreen staining and different real-time PCR methods (SybrGreen and TaqMan probe) was rather weak [167].

In conclusion, we need a much deeper understanding about the release mechanisms of cfDNA, what these

molecules look like and in which forms they are present in the blood stream. As long as we have only a rudimentary understanding of the factors determining these mechanisms, we will not be able to answer the question whether the determination of the DNA integrity might be a clinically meaningful tool.

Choice of matrix – plasma vs. serum vs. other body fluids

Cell-free nucleic acids (of which the majority is usually from healthy cells) containing DNA with tumor-associated alterations (ctDNA) can be isolated not only from plasma and serum, but from other body fluids like ascites, breast milk, lymphatic and peritoneal fluids, bone marrow aspirates, urine, prostatic fluid, peritoneal lavage, sputum, cerebrospinal fluid, gastric juice, and biliary and stool samples as well [154]. In patients with hematological tumors, it seems obvious to use plasma or serum as starting material for a genetic analysis of cfDNA. The same holds for urine in patients with a disease of the urogenital tract [8, 168]. Sputum is an interesting target liquid to be used specifically for patients with head and neck diseases, with respiratory tract or bronchopulmonary cancers [169–171]. An interesting approach was recently published by Wu and colleagues [170] who looked for mutant driver gene alterations by next-generation sequencing (NGS) in tissue, plasma, sputum and urine of non-small cell lung carcinoma (NSCLC) patients. They found a concordance rate between tissue and cfDNA from the different liquids of 86% (plasma) to 70% (urine). When they combined the data for all liquids, the concordance rose to 90%. Unfortunately, this approach might not be useful for a daily routine as it takes more time and effort and is associated with higher costs. The analysis of cfDNA in a malignant pleural effusion from cancer patients was shown to be as successful as the examination of plasma from these patients. This holds not only for lung cancer patients [172] but has been demonstrated in a gastric cancer patient also [173].

The observation that the amount of cfDNA in serum vs. paired plasma is several fold higher had been described by Lo et al. in pregnant women [53, 174]. This was later confirmed in healthy people [90, 121, 111], in bone marrow transplantation patients [80] and in patients with a CRC metastasized to the liver [175]. For the question whether plasma or serum is better suited for a molecular analysis of cfDNA, there can be no universal “correct” answer, as this depends on the target/s and the method of analysis.

Vallee and coworkers answered this question in favor of plasma when they looked for mutations in the *EGFR* gene in NSCLC patients [176]. They found an activating *EGFR* mutation in 95% of the plasma samples and 72% in serum. A similar observation (i.e. higher quantity of ctDNA in plasma than serum) was made in a mouse xenograft model in which the amount of mutant DNA (*KRAS* and *PSAT1*) in serum and plasma was compared [177]. When Kobayashi and coworkers looked for *EGFR T790M* mutations in plasma and serum of NSCLC patients, they found a higher specificity in serum (67%) than in plasma (50%), but the test sensitivity was the same for both liquids [178]. Mayo de Las Casas also looked for *EGFR* mutations in cfDNA and detected them in higher frequencies in plasma than in serum, but in 8% of the patients these alterations were found exclusively in serum [179]. These patients would not have received a targeted therapy if only plasma had been analyzed. Similar observations were reported for *BRAF* mutations in melanoma patients in whom the amount of tumor-derived DNA in plasma was significantly higher than in serum [180]. The comparison of plasma and serum from patients with metastatic CRC demonstrated that the fraction of ctDNA is higher in plasma and this matrix is better suited for the methylation and mutation analysis [181]. For the examination of EV, plasma and serum samples have been used [182], whereas many groups seem to prefer serum for the analysis of micro RNAs (miRNAs). Therefore, it looks as if plasma is the preferred choice for the detection of mutations in cfDNA.

When compared to the data on how to handle plasma/serum in a way to enable the optimal analysis of cfDNA, other body liquids do very poorly. Recently, it has been demonstrated that CSF is a valuable source for the molecular genetic analysis in patients who suffer from primary brain tumors or from metastases to the brain. Pan et al. [183] looked for mutations in CSF and plasma and observed a higher mutant AF in the former. In addition, they detected some alterations in plasma not found in CSF. So far, it is unknown whether the mutant AF in these patients was higher in plasma and/or whether the blood-brain barrier played a role. When both fluids are available, it might therefore be a good idea to analyze both [13]. Pentsova et al. [184] sequenced cfDNA from cell-free CSF supernatant and the corresponding genomic DNA from the cell pellet and found a higher percentage of sequence reads in the supernatant than in the CSF pellet (a similar observation was described for the analysis of microsatellites in cell-free bronchial lavage fluid and the corresponding cell pellet by Schmidt et al.) [17]. For patients afflicted with a brain tumor, the examination of CSF seems to be more useful than plasma as more tumor-associated

alterations are found in the former liquid than in plasma [157, 185–187]. A similar observation was made not only for patients with a primary brain tumor but also in lung cancer patients with leptomeningeal metastases in whom more mutations were found in CSF than in plasma [188]. Unfortunately, there is no optimized standard protocol for CSF collection, preparation, biomarker isolation and data analysis [12].

Urine is a body fluid which can be collected non-invasively and seems to be a valuable source for cfDNA in patients with a benign and a malignant disease as well as for the isolation and analysis of cell-free fetal DNA [189]. Nevertheless, our knowledge on the best strategy for the acquisition, handling and processing of urine for the analysis of extracellular nucleic acids from this fluid is still very limited. This starts with the question when to collect urine (time of the day), the usage of first-void morning samples rather than second void, midstream vs. total void and whether to use preservatives or not. So far, it is not clear whether urine needs to be processed as soon as possible after collection or if storage is possible, and when the answer is yes, under which conditions. Bosschietter et al. aimed to establish a protocol for urine collection and storage before analysis. When the samples were stored at 4 °C for 2 days, no preservation substances were necessary but storage at RT for 7 days without any additive (either 40 mM EDTA or urine conditioning buffer from Zymo Research) led to a strong decrease in cfDNA (Qubit dsDNA kit) [190]. Freezing the samples at –20 °C or –80 °C with or without any preservative did not lead to a decrease in the total DNA quantity. Additionally, when urine from a group of patients with NSCLC or bladder cancer was stored for 7 days at RT in the presence/absence of EDTA, a strong decrease in methylated DNA was found in the samples which were stored without any additive. Similar results, i.e. no change in the total amount of DNA in urine supernatant from bladder cancer patients, which had been stored for 7 days at RT in containers containing a proprietary preservative, were reported by Li et al. [191].

DNA isolation

As we have to assume that in the majority of cases the quantity of ctDNA in body liquids is very low, that cfDNA is *a priori* in a “poor shape” (i.e. highly fragmented) and that there is no method to predict the percentage of ctDNA, it is absolutely necessary to aim for the highest DNA isolation efficiency. In recent years, several companies developed a range of DNA isolation kits of which only the “dedicated”

kits were recommended for the extraction of cfDNA [192]. In several papers, a variety of different kits were compared [84, 85, 93, 193–204]. From these results, one can conclude that bead-based isolation methods worked somewhat better than membrane-based kits and that the former ones are especially useful for the isolation of low-molecular-weight DNA. A similar conclusion was recently reported for the isolation of cfDNA from urine [45]. It is important to keep in mind that the DNA yield and purity of these “dedicated” kits show a high variability depending on the method of the isolation procedure. In addition, these kits show a variety depending on the body liquid used for DNA isolation (plasma, urine, CSF).

In order to establish a reliable standard for q-PCR or dPCR quantification, we would recommend the use of DNA resembling cfDNA as much as possible (such as highly fragmented genomic DNA). As this field is very much in movement and there are constantly new DNA isolation kits introduced, it seems too early to recommend a specific kit for a specific task/method.

Trials for the establishment of quality assessments

Already in 2015, the European Committee for Standardization published the Technical Specification DIN CEN/TS 16835 (<https://www.cen.eu/work/products/TS/Pages/default.aspx>). In part 3 (Isolated circulating cell-free DNA from plasma), it is stated that the storage time before plasma processing should not exceed 6 h. The authors of this guideline also recommend the use of blood collection tubes stabilizing cfDNA, especially if there is a longer delay between blood draw and plasma preparation. Additionally, a two-step centrifugation protocol (low- and high-speed centrifugation) for cell removal is recommended.

Recently, two external quality assessment schemes for the isolation and analysis of ctDNA harboring an *EGFR* mutation were published [205, 206]. These initiatives are highly welcome but unfortunately take the second step before the first as the focus is on the analytical quality, thus leaving out all pre-analytical steps associated with probe sampling and handling. A study initiated by Malentacchi and colleagues a few years ago had sent out plasma samples to participating laboratories across Europe and analyzed the quantity and quality of different isolation methods used by q-PCR [192]. A similar approach was recently published by Keppens et al. who had distributed plasma samples with spiked-in mutant DNA (*EGFR* and/or *K-ras/N-ras* mutation) to 32 laboratories [207]. The labs

then used their preferred method for DNA extraction and analysis and reported the results back. As important and interesting as these trials are, they still leave us in a state of limbo on all preceding pre-analytical steps. In addition to these initiatives, a working group of the European Federation for Clinical Chemistry and Laboratory Medicine is trying to improve the harmonization in the pre-analytical phase across European member societies [208–210].

Final remarks

The generated data over the recent years on pre-analytical issues related to the use of cell-free nucleic acids are indeed impressive. Nevertheless, there are large gaps in our understanding of the basic mechanisms of cfDNA such as release, composition and distribution in different body liquids, stability and their biological function. In addition, we need to identify the most critical pre-analytical parameters for the preparation of body liquids to be used for the analysis of cell-free nucleic acids [211]. Only if we close these gaps we will be able to establish SOPs for the optimal handling of liquid biopsy samples to obtain a maximum of information in a molecular analysis of cell-free nucleic acids. We expect that an agreement on the optimal methods for the use of blood-drawing tubes (EDTA vs. blood-stabilizing tubes) to enable a delayed processing, and the efficient and complete removal of cells and cell debris from body liquids will be the easiest to achieve. This will be the first step for a broad application of liquid biopsy as part of a daily clinical routine method.

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