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Non-blood sources of cell-free DNA for cancer molecular profiling in clinical pathology and oncology

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ABSTRACT

Liquid biopsy can quantify and qualify cell-free (cfDNA) and tumour-derived (ctDNA) DNA fragments in the bloodstream. CfDNA quantification and mutation analysis can be applied to diagnosis, follow-up and therapeutic management as novel oncologic biomarkers. However, some tumor-types release a low amount of DNA into the bloodstream, hampering diagnosis through standard liquid biopsy procedures. Several tumors, as such as brain, kidney, prostate, and thyroid cancer, are in direct contact with other body fluids and may be alternative sources for cfDNA and ctDNA. Non-blood sources of cfDNA/ctDNA useful as novel oncologic biomarkers include cerebrospinal fluids, urine, sputum, saliva, pleural effusion, stool and seminal fluid. Seminal plasma cfDNA, which can be analyzed with cost-effective procedures, may provide powerful information capable to revolutionize prostate cancer (PCa) patient diagnosis and management. In the near future, cfDNA analysis from non-blood biological liquids will become routine clinical practice for cancer patient diagnosis and management.

1. Introduction

Over recent decades there has been a rapid expansion of knowledge in the field of molecular biology and biochemistry, leading to the development of precision medicine and tailored therapies.(Lu and Liang, 2016) The understanding of oncogenic pathways and neoplastic genetic signatures shed the conceptual basis for the development of liquid biopsy procedures and oncological-targeted therapies. Today, immunohistochemical and biochemical tumor characterization are part of the routine process to define patient diagnosis and prognosis.(Ponti et al., 2016; Hofman et al., 2018; Ponti et al., 2018d)

The identification of new diagnostic and prognostic markers from non-tumoral biological samples is an interesting field in continuous evolution. Liquid biopsy procedures have been developed to identify oncologic biomarkers in several body fluids such as blood, plasma, cerebrospinal liquid, seminal fluid, saliva and urine.(Ulrich and Paweletz, 2018; Stewart et al., 2018; Burgener et al., 2017) The concept that body fluids may reveal the presence of several systemic diseases dates back to ancient Greek and Indian history. The development of humoral theory was attributed to Hippocrates (ca. 460–370 BCE), and its concepts were the base of Western medicine from antiquity through to the 19th century. “Humoral” derives from the word “humor,” which in ancient greek means “fluid”, and health was defined as the proper humoral balance for that individual. The most extensively studied body fluids are blood and urine, but many other liquids, such as cerebrospinal fluid, saliva and seminal fluids have been analyzed in order to determine their diagnostic and prognostic potential.

An example of a routinely applied liquid biopsy for patient management is the dosage of prostate specific antigen (PSA) in the bloodstream. However, the PSA protein is present both in healthy and in neoplastic prostate cells and therefore has a low sensitivity and specificity for prostate cancer (PCa) diagnosis. In contrast, tumor-derived cell-free DNA (ctDNA) based techniques are more specific and can be applied to many body fluids in order to identify novel oncologic biomarkers for diagnosis, prognosis and monitoring of tumor therapeutic response.

The presence of DNA within the non-cellular fraction of peripheral blood, termed cell-free DNA (cfDNA), was initially identified more than 50 years ago.(Ulrich and Paweletz, 2018; Stewart et al., 2018; Burgener et al., 2017) Decades passed before cfDNA levels were found to be elevated within the serum of cancer patients, with at least a portion of the cfDNA being tumor-derived (ctDNA). The advantage of ctDNA analysis from liquids other than blood is the collection of targeted information from a body fluid directly in contact with the tumor source (eg. cerebrospinal fluid for central nervous system neoplasm). Furthermore, contrary to what occurs with ctDNA in the bloodstream,

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ctDNA in other bodily fluids are not diluted and higher concentrations of ctDNA are derived from active secretion and/or necrotic phenomena.

ctDNA harbors cancer-specific genetic and epigenetic alterations that allow its detection and quantification using a variety of emerging techniques. The promise of convenient non-invasive access to the complex and dynamic molecular features of cancer through peripheral blood has galvanized translational researchers around this topic with compelling routes to clinical implementation, particularly in the post -treatment surveillance setting. (Ulrich and Paweletz, 2018; Stewart et al., 2018; Burgener et al., 2017) Although analysis methods must contend with small quantities of ctDNA present in most patients and the relative over-abundance of background cfDNA derived from normal tissues, recent technical innovations have led to dramatic improvements in the sensitivity of ctDNA detection. The collection of biologic samples to isolate cfDNA is often repeatable, allowing for real-time and dynamic monitoring of molecular changes.(Zeng et al., 2018) As a result, ever more studies are investigating the clinical utility of ctDNA for applications in treatment response assessment,(Dawson et al., 2013) identification of emerging resistance mechanisms,(Mok et al., 2015; Tabernero et al., 2015) and minimal residual disease detection.(Guo et al., 2016) In addition, ctDNA is released from multiple tumor regions, and may thereby represent the whole molecular picture of a patient's malignancy, potentially solving the problem of intra-tumor heterogeneity,(L De Mattos-Arruda et al., 2014; Leticia De Mattos-Arruda et al., 2015; Jamal-Hanjani et al., 2016) which may lead to false-negative results and suboptimal therapy selection, and characterization of clonal heterogeneity and selection. (Ulrich and Paweletz, 2018; Stewart et al., 2018; Burgener et al., 2017).

In this review we explore the application of non-blood derived ctDNA and cfDNA in the discovery of novel oncological biomarkers, describing the existing evidence and focusing on the recent development of seminal plasma analysis.

2. Biology of cell free DNA and cell tumoral DNA

Cell-free DNA (cfDNA) was first identified in human plasma in 1948 and is believed to be released from cells throughout the body into the blood stream.(MANDEL and METAIS, 1948) Most cfDNA in the blood originates from hematopoietic cells(Allam et al., 2014; Stroun et al., 1989) however many other organs and tissues contribute to the total amount of blood cfDNA.(Stroun et al., 2001)

The origin of cfDNA has not fully been explored, but there are many mechanisms by which DNA gets into the circulation. Blood cfDNA fragments of healthy individuals are primarily of lymphoid and myeloid origin.(Anker et al., 1999) Once outside of the cell, cfDNA is steadily degraded by nucleases, possibly with the help of macrophages(Chused et al., 1972) and excreted into urine via the renal system.(Thierry et al., 2016; Botezatu et al., 2000) Multiple studies have demonstrated that the majority of cfDNAs are short molecules around 166-167bp, although longer fragments also exist.(Jiang and Lo, 2016; Snyder et al., 2016) While necrosis results in longer fragments (> 10.000 bp), apoptosis is associated with DNA fragments of about 180bp, or multiples of this length, which appear as a ladder electrophoresis pattern. cfDNA is likely associated with nucleosomes as the length of 167 bp corresponds approximately to the length of DNA wrapped around a histone.(Snyder et al., 2016) In addition to cell death, neutrophils can mediate the immune response by releasing neutrophil extracellular traps (NETs) that can trap and kill various pathogens.(Kaplan and Radic, 2012) These are extracellular network structures composed of both nuclear and mitochondrial DNA fibers, which are covered by various proteins such as histones and proteases.(Kaplan and Radic, 2012) Another way of releasing DNA into the circulation is active release of newly synthesized DNA via vesicles and lipoproteonucleotide complexes.(Fuchs et al., 2012) Based on the appearance in the circulation, cfDNA molecules can be divided into three basic categories: free DNA fragments, vesicle-bound DNA and DNA-macromolecular complexes.

In cancer, a portion of cfDNA originates from tumor cells, referred to as circulating-tumor DNA (ctDNA), and can harbor specific mutations corresponding to the patient's tumor. ctDNA profiling has recently become an area of increasing clinical relevance in oncology, in particular due to advances in the sensitivity of sequencing technologies, allowing a reliable identification of cancer mutations. Several studies showed high concordance between individual mutations found in ctDNA samples and tumor tissue.(Bettegowda et al., 2014) Cancer patients have much higher cfDNA concentrations (0 to > 1000 mg/mL) than healthy individuals (0–100 ng/mL) and the total amount varies among patients with comparable cancer type and stage.(Bettegowda et al., 2014) Direct correlations between ctDNA level and tumor burden, stage, vascularity and therapy response have been described for several cancers.

cfDNA can be found in plasma as well as other body fluids such as urine, cerebral spinal fluid (CSF), pleural fluid, and saliva, among others.(Stewart and Tsui, 2018) Depending on tumor type, different rates of cfDNA/ctDNA have been reported in blood samples. CtDNA was detected in over 75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers. However, levels below 50% were found in primary brain tumors, in kidney, prostate, and thyroid cancers, (Bettegowda et al., 2014) and may be due to the influence other factors, such as blood-brain barrier restriction mechanisms, inflammatory or autoimmune processes that increase non-tumor cfDNA fraction, effectively diluting the ctDNA amount. The identification of ctDNA from biologic fluids other than blood, enables prompt diagnosis and a reliable genetic identification of cancer types, characterized by shedding low concentrations of ctDNA into the blood stream. These tumors are often in direct contact with other body fluids, such as urine, saliva, cerebrospinal fluid, pleural effusion or seminal liquid, releasing a considerable amount of ctDNA and creating a privileged source for non-invasive ctDNA quantification and characterization.

3. Sources of cfDNA in oncology

3.1. cfDNA in saliva and sputum

Saliva provides good-quality genomic DNA, which is comparable to blood as a template for genotyping.(Bahlo et al., 2010; Hu et al., 2012; Siravegna et al., 2017) Salivary DNA has been used for the detection of germline mutations in various screening studies, such as for breast and brain cancers.(Dean et al., 2015; Adel Fahmideh et al., 2015) Numerous studies have shown that sputum tumor DNA could be a promising tool for early detection of lung cancer. Recent studies have shown proof-of-principle for ctDNA as a biomarker in head and neck squamous cell carcinoma (HNSCC), with both blood and saliva serving as a diagnostic medium. In a cohort of 93 HNSCC patients, plasma or saliva samples were collected to identify somatic mutations (TP53, PIK3CA, CDKN2A, HRAS, NRAS) and HPV (HPV-16, -18).(Wang et al., 2015a) Tumor DNA was detected in 76% of saliva samples (n = 93) and 87% of plasma samples (n = 47) respectively, and TP53 was the most common mutant detected (86%). Patients with oral cavity cancers were all shown to have tumor-specific DNA in their saliva samples (100%, n = 46). Saliva was shown to be a more sensitive predictor than plasma for early stage disease, with 100% detection in saliva versus 70% in plasma.(Wang et al., 2015a)

3.2. cfDNA in urine

Several reports have analyzed the value of urinary cfDNA (UCF-DNA) for the diagnosis of bladder cancer (BC).(Todenhöfer et al., 2018) At the present time, the standard diagnostic methods for BC are cystoscopy and urine cytology. However, urine cytology has poor sensitivity (except for high grade tumors)(Bier et al., 2018) and, although

flexible cystoscopy has been recently introduced, the procedure is both invasive and uncomfortable. Therefore, there is growing interest in new non-invasive diagnostic tools that have better sensitivity and specificity for BC. To overcome these difficulties, several urine-based biomarkers, such as bladder tumor antigen, nuclear matrix protein 22 (NMP22), and fluorescence in situ hybridization (FISH), have been studied but none of them have been able to demonstrate superiority to cystoscopy or cytology.(Breen et al., 2015)

The main source of UCF-DNA is thought to be apoptotic and necrotic cancer cells.(Bryzgunova and Laktionov, 2019) UCF-DNA might be gathered as a result of renal cfDNA transport from the blood or direct contact from urinary tracts. Most of the UCF-DNA of urinary tract cancers patients is not derived from blood, but is rather derived directly from tumor cells (ctDNA). Kim TW et al. demonstrated that the non-invasive quantification of IQ motif containing GTPase activating protein 3 (IQGAP3) urinary nucleic acids (NA) could be a suitable tool for distinguishing between BC patients and patients with non-cancer-associated hematuria.(Kim et al., 2018) In other reports, UCF-DNA profiling using PCR-based detection or high throughput sequencing technology of tumor-associated genes, has been indicated as a technique to yield promising results in bladder and PCa. Recently Christensen et al. applied a digital droplet PCR (ddPCR) approach for the detection of common mutations in UCF-DNA, suggesting that the levels of mutant ctDNA in the urine of non-muscle-invasive bladder cancer (NMIBC) patients were positively correlated with tumor stage, grade and size, and that a high initial level of mutant urinary ctDNA was predictive of future disease progression.(Christensen et al., 2018) In a cystectomy patient group, high mutant UCF-DNA was able to predict future disease recurrence, the association being more pronounced with ctDNA.

PCa represents one of the most common tumors in European men and one of the leading causes of male cancer associated deaths.(Alberts et al., 2015) At present, the only noninvasive approach currently used for the diagnosis of PCa is the determination of PSA (prostate-specific antigen) in blood, which has been shown to reduce PCa mortality. However, the use of PSA has recently been questioned because of its low accuracy, especially in terms of specificity.(Alberts et al., 2015; Ilic et al., 2018) Previous studies identified UCF-DNA integrity as a potentially good marker for the early diagnosis of noninvasive PCa, with an overall diagnostic accuracy of about 80% in small patient series. (Casadio et al., 2013; Salvi et al., 2015) UCF-DNA is easily quantifiable in PCa patients and could prove to be an important source of biomarkers, such as gene mutations or epigenetic modifications, that may accurately assist in distinguishing PCa from other benign diseases of the urogenital tract.

3.3. cfDNA in cerebrospinal fluid

Tumor-derived DNA typically constitutes a small fraction of all cfDNA in plasma, while the proportion of such DNA in cerebrospinal fluid (CSF) is much higher due to the lower background of normal DNA. (Leticia De Mattos-Arruda et al., 2015; Wang et al., 2015b) Owing to the blood–brain barrier, CSF cfDNA is unable to circulate fully within the blood system, resulting in a limited amount of cfDNA from central nervous system (CNS) being released into blood plasma. Thus, blood plasma is not the best biologic liquid for the detection and characterization of intercranial lesions.(Pentsova et al., 2016; Pan et al., 2015; Li et al., 2018; Yang et al., 2014; Alix-Panabières and Pantel, 2016) CSF circulates throughout the CNS and may provide potential information on intracranial lesions. Few studies indicated that CSF could be an important method of liquid biopsy in patients with CNS cancers, even though lumbar puncture is an invasive procedure that should be performed only if necessary. Moreover, selected gene profiles in CSF could be consistent with their primary tumors.(Pentsova et al., 2016; Pan et al., 2015; Li et al., 2018; Yang et al., 2014; Alix-Panabières and Pantel, 2016) Li YS et al. demonstrated that CSF liquid biopsy has potential clinical applications for diagnosis and characterization of leptomeningeal metastasis (LM) of non-small cell lung cancer (NSCLC) with mutated Epidermal Growth Factor Receptor (EGFR).(Li et al., 2018) Moreover, CSF cfDNA has a potential role in revealing dynamic changes of the tumor burden of LM throughout treatment, and further studies should be conducted to explore its roles in prediction and prognosis.(Li et al., 2018)

3.4. cfDNA in stool

Colorectal cancer (CRC), a common cause of cancer-related mortality worldwide, is preventable with effective screening and removal of precursor lesions.(Berger and Ahlquist, 2012) Yet, screening efforts have been hampered by low participation rates and by performance limitations of the screening tools themselves. Stool DNA testing has emerged as a biologically rational and user-friendly strategy for the non-invasive detection of both CRC and critical precursor lesions. (Berger and Ahlquist, 2012; Ahlquist et al., 2008; Redwood et al., 2016) Unlike most conventional screening tools, stool DNA testing detects proximal and distal colorectal neoplasms with higher sensitivity compared to fecal immunochemical testing for hemoglobin (FIT), for the detection of screening-relevant colorectal neoplasia (SRN). Several key technical advances have led to increasingly accurate approaches for stool DNA testing, including the use of a DNA preservative buffer with stool collection, efficient target capture and amplification methods, broadly informative multi-marker panels, and automated assay components.(Imperiale et al., 2004, 2014; Ahlquist et al., 2008) Based on previous studies, advanced multi-marker stool DNA tests including methylated markers, mutation markers and an assessment of faecal haemoglobin, have been shown to detect CRC at sensitivities of 85% and higher and adenomas > 1 cm at 60% and higher in a case-control environment.(Berger and Ahlquist, 2012) In a large population cross-sectional study, Imperiale et al. demonstrated that the sensitivity of the DNA test for the detection of both CRC (92.3%) and advanced precancerous lesions (42.4%) exceeded that of FIT by an absolute difference of nearly 20 percentage points.(Imperiale et al., 2014)

3.5. CtDNA in pleural fluids

Pleural effusion is a common complication of lung cancer. The collection of pleural effusion fluid or of bronchial washing samples with physiological saline solutions is currently used in diagnosing cancers of the respiratory system.(Kimura et al., 2006; Soh et al., 2006) The detection of *EGFR* mutations in cytological samples of pleural effusion fluid is feasible, although often difficult owing to the limited number of cancer cells that are usually available for analysis. Kimura et al. demonstrated that pleural effusion cfDNA can be used to detect *EGFR* mutations and that the *EGFR* mutation status may be a useful predictor of the gefitinib response in patients with non-small cell lung cancer (NSCLC).(Kimura et al., 2006) In another study, the feasibility of identifying *EGFR* mutations in tumor derived DNA collected through bronchial washings, termed cytology cell free DNA (ccfDNA), was examined.(Kawahara et al., 2015) The results demonstrated the high sensitivity and specificity (88% and 100%, respectively) of this approach compared with the analysis of DNA from tumor tissue, suggesting that activating *EGFR* mutations can be accurately detected in ccfDNA. Thus, ccfDNA might be a valuable alternative to cytological samples, although larger investigations are needed to validate this diagnostic approach. A limited number of studies have investigated the diagnostic, prognostic, or predictive value of miRNAs in pleural effusion fluid from patients with NSCLC.(Han et al., 2013; Wang et al., 2012) In one study, the authors found that a signature comprising five miRNAs in the effusion samples was predictive of the overall survival of patients with NSCLC and malignant pleural effusion.

3.6. CtDNA in ascites

Detection and characterization of cfDNA in ascites has only been analyzed in one preliminary study.(Husain et al., 2017) Husain H. et al. isolated cfDNA from ascites and demonstrated the presence of tumorigenic copy number variations (CNVs) in cancer-associated genes in a small series of 6 metastatic cancer patients, providing a rationale for the study of ascites as a source of ctDNA for the comprehensive analysis of relevant targets.(Husain et al., 2017) In addition, in their small patient series, the ascitic cfDNA of one patient was characterized by a 15-fold amplification of the *EGFR* gene in ascites cfDNA analysis, which was not present in two separate lung biopsies of the tumor. Even though the significance of detecting discordant alterations in primary tissue versus blood versus ascites is unclear, the authors hypothesized that ascites may provide important genomic information regarding an individual's cancer that may complement and expand data obtained from tissue biopsies.(Husain et al., 2017)

3.7. cfDNA in seminal plasma

The quantification of serum and plasma cfDNA in PCa patients has been found to be significantly higher with respect to age-matched healthy controls.(Ponti et al., 2018c) It has been proven that increased levels of cfDNA are released and can be isolated from serum and plasma in PCa patients. Further, the level of cfDNA in PCa patients was found to be significantly higher than in benign prostatic hyperplasia (BPH) patients.(Wyatt et al., 2017; Feng et al., 2013)

It has recently been demonstrated for the first time that human seminal fluid can be a valuable source of cfDNA for the identification of novel oncological biomarkers. Seminal plasma cfDNA from PCa patients is significantly more concentrated than age-matched healthy individuals, but tumor stage has been found to be independent of other parameters, such as age at diagnosis. (Ponti et al., 2018g, e; Ponti et al., 2018f) Fluorometric and electrophoretic assessments allow a reliable quantification and qualification of seminal plasma cfDNA, that could be routinely adopted for PCa screening programs. In the same study, seminal cfDNA of PCa patients yielded higher values than those of age-matched healthy volunteers, 1721.27 ng/µl ng/µl and 75.4 ng/µl, respectively.(Ponti et al., 2018a) This allows a reliable characterization and differentiation between a cohort of patients affected by PCa and the age-matched control group. The aforementioned average seminal cfDNA is notably higher than average blood cfDNA concentrations of PCa patients, being approximately 100 times higher compared to mean blood cfDNA values reported in literature (1.8–35 ng/µl).(Costa et al., 2017) In another study, seminal cfDNA was compared to BPH patients, revealing a significant difference in the concentration levels of cfDNA in PCa and BPH patient cohorts. A possible cut-off level of 450 ng/ul seminal cfDNA was proposed as able to discriminate between the two distinct groups. In addition, the electrophoresis of seminal plasma cfDNA enabled the discrimination between PCa patients and BPH or age-matched healthy individuals, because of a distinct electrophoretic pattern in PCa patients.

Seminal cfDNA levels are a potential clinical biomarker in early PCa diagnosis, referring important information regarding tumor characterization, patient prognosis and management, for the determination of therapeutic strategies and subsequent follow-up.

4. cfDNA and cancer patient management

There are several important applications in which liquid biopsy might confer an advantage to patients with cancer: the potential use of cfDNA as a biomarker include applicatyions in the diagnostic, prognostic and predictive settings. Therefore, CfDNA quantification and analysis should be included in a management strategy that considers the patient's clinical status, the clinical relevance of test results, and local feasibility of the different testing methods. Liquid biopsy can be considered at the time of initial diagnosis in all patients who need tumor molecular profiling, but it is particularly recommended when tumor tissue is scarce, unavailable, or a significant delay is expected in obtaining tumor tissue.(Stewart and Tsui, 2018) CfDNA originating from biologic samples such as blood or other biological body fluids can be analyzed through quantitative and qualitative analysis, guiding cancer patient management, as demonstrated in many cancer types. For instance, in NSCLC treatment-naive patients, *EGFR* and *ALK* rearrangement assessment using ctDNA is currently a recommended procedures for cancer management. (Rolfo et al., 2018; Marmarelis et al., 2017)

Several biologic fluids are under investigation for the diagnostic, prognostic and therapeutic value of cfDNA quantification and analysis. UCF-DNA and scfDNA are the most striking examples of non-blood biologic fluids, containing large amounts of cfDNA, whose quantification has a great prognostic and therapeutic value.

Cancer is a dynamic disease and ctDNA released by tumor cells strictly reflects the heterogeneity of both primary cancer and metastases.(Stewart et al., 2018; Stewart and Tsui, 2018) In this scenario, to improve the standard of patient care, the institution of a Molecular Tumor Board (MTB) has been proposed in several hospitals to discuss the best treatment option for the patient, considering molecular testing results, including those from liquid biopsies. Some initial experiences have been reported.(Rolfo et al., 2018; Harada et al., 2017) Rolfo et al. created an MTB that retrospectively evaluated 141 patients, recommending a treatment in 78 (55%) of patients.(Rolfo et al., 2018) The group of Harada et al. also used MTB for the selection of cancer patients who should be advised to perform genetic testing; resulting in the approval of 132/191 cases for NGS analysis.(Harada et al., 2017)

4.1. Measuring disease burden

The clinical utility of liquid biopsy has been explored in different clinical phases for several tumors, from early disease detection to the identification of prognostic factors in early stages and to the molecular characterization of metastatic disease and eventual relapse. The presence of ctDNA itself is indicative of disease and the amount of ctDNA can also be an indicator of the amount of disease. As previously discussed, the amount of ctDNA is correlated with tumor stage, and many groups have observed that higher levels of ctDNA have been associated with worse survival outcomes in patients(Lecomte et al., 2002; Gray et al., 2015; Gautschi et al., 2007; Stewart and Tsui, 2018), and can be used as a measure of disease burden, along with imaging studies. Overall, ctDNA has been shown to be a better predictor of prognosis than other tumour markers and that ctDNA concentration increase correlates with poorer clinical and radiological outcomes.

In CRC patients and in PCa patients, those with higher ctDNA levels for selected genes had worse outcomes with respect to those with lower ctDNA levels.(Romanel et al., 2015) While this observation is probably due to the correlation between ctDNA levels and tumor burden, the ability of cancer cells to shed DNA in circulation may also reflect disease aggressiveness.

4.2. Patient stratification and prediction of therapeutic response

Identifying molecular biomarkers in early tumor patients is needed in order to develop more personalized follow-up and treatment schedules. Detection of gene mutations in liquid biopsy for early detection of recurrence requires highly sensitive techniques. Molecular profiling tests prior to treatment provides the possibility of stratifying patients based on prognosis for the administration of adjuvant therapy(Romanel et al., 2015; Scherer et al., 2016) or for the selection of specific targeted therapies. By measuring several mutations, either in blood or in other body fluids, the changes in their ratios can provide some insight into the tumor's evolution and continued heterogeneity during treatment. (Misale et al., 2012; Chabon et al., 2016) This can also be extended to

identify the appearance of resistance mechanisms. Serial studies of CRC found positive selection of blood *KRAS* mutations during anti-EGFR therapy and a decline in their representation after withdrawal.(Misale et al., 2012) Similar results have been recorded in NSCLC patients treated with EGFR inhibitors, where resistance mutations were identified in ctDNA prior to clinical progression.(Sorensen et al., 2014) A 54-gene panel detected ctDNA in 58% of patients with multiple types of cancer, with 68% of those having an actionable mutation by an FDA-approved drug.(Schwaederle et al., 2016) Interestingly, DNA derived from NSCLC tumors can be detected with high sensitivity in urine and plasma, enabling diagnostic detection and monitoring of therapeutic responses from non-blood body fluids.(Reckamp et al., 2016)

ctDNA has also been shown to be relevant to predict recurrence after resection of locally advanced rectal cancer or liver metastases from CRC.(Parkinson et al., 2016) Overman et al. showed that post-operative detection of ctDNA was significantly correlated with relapse-free survival (RFS). Tie et al. showed that ctDNA analysis appears to be strongly predictive of recurrence among patients with both lower (pathological complete response) and higher risk (node positive) disease. (Parkinson et al., 2016) In this context, ctDNA could be exploited to closely monitor patients before and after surgery to identify high-risk patients for disease recurrence.

5. Future directions: potential non-invasive sources of DNA in clinical pathology and oncology: Oral mucosa and skin

Guthrie/FTA card-based blood spots, buccal scrapes, and finger nail clippings are DNA-containing specimens that are uniquely accessible and thus attractive as alternative tissue sources.(Klassen et al., 2012) As an alternative source to blood or buccal swabs, or both, there have been many trials to discover a non-invasive method to collect DNA reference samples from various specimens of the human body, such as nails, hair and skin scales.(Hogervorst et al., 2014; Blumenberg, 2012; Bond, 2007; Ghatak et al., 2013; Albujja et al., 2018) The value of DNA profiling from skin or adnexal samples has been previously analyzed and reviewed for forensic purposes only and crime scene investigations, however the application of these DNA analysis methods to skin oncology may allow direct genomic tumor profiling from tumor derived skin scales, nail clippings of nail tumors or hair sampled from the tumoral skin surface.

Previous trials have attempted to overcome the disadvantages of traditional samples (eg. blood samples) with the advantages of new, less invasive sampling materials to ensure the largest possible inclusion of subjects or volunteers in the studies. In particular, some attempts have been made to collect reference samples from skin surface cells (Zamir et al., 2004; Kopka et al., 2011) supporting this methodology as easy and simple in an accessible area. Such a method could be extremely useful among certain cultures (e.g., in the Middle East), where the use of traditional samples or buccal swabs can be considered invasive or is otherwise socially unacceptable. Skin lifts are a promising source of non-blood tumoral DNA. In previously mentioned studies, all the genetic profiles generated from skin lifts and swabs were consistent with their corresponding buccal swab and blood samples, indicating that both body regions and recovery methods yield accurate profiles (Zamir et al., 2004; Kopka et al., 2011). The accuracy of these newer techniques is necessary for future evaluations of the diagnostic value of the tumoral DNA recovered from alternative non-blood sources.

6. Conclusion

Applications of liquid biopsies in oncology have emerged and developed at an incredible rate over the past 5 years. Many studies have shown that cfDNA quantification is able to improve cancer diagnosis and patient management, however, several tumor-types, as such as kidney, prostate, and thyroid cancer, shed a low amount of cfDNA into the bloodstream hampering the diagnosis through standard liquid biopsy procedures. Research on cfDNA quantification from other body fluids is a promising field for the identification of new biomarkers that will allow prompt diagnosis and a better management of cancer patients. The current authors recently demonstrated, for the first time, that human seminal fluid can be a valuable source of cfDNA for the identification of novel oncological biomarkers and seminal plasma cfDNA from PCa patients is significantly more concentrated than age-matched healthy individuals and patients affected by BPH.(Ponti et al., 2018e)

In the near future, the quantification and analysis of cfDNA will probably become part of routine diagnostic and clinical management of cancer patients. In order to accomplish this goal, it will be important to standardize ctDNA quantification methods and allow ctDNA detection for rare molecular alterations in order to anticipate drug resistance.

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