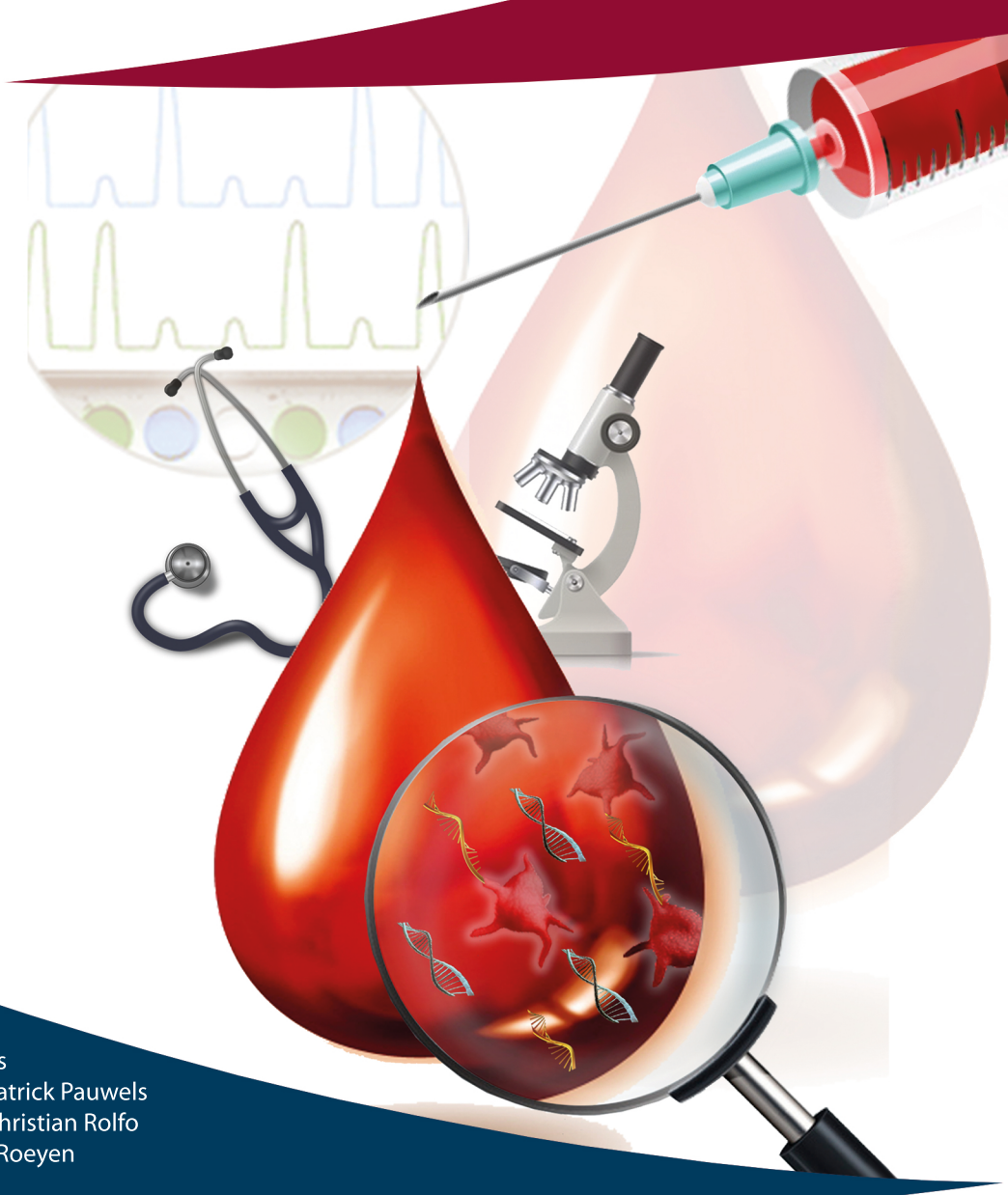
Optimization of the Liquid Biopsy Workflow: from Research to Clinical Practice

Dissertation submitted to obtain the academic degree of
Doctor in Medical Sciences at the University of Antwerp
to be defended by

Laure Sorber

Mentors
Dr. Karen Zwaenepoel
Dr. Julie Jacobs



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Optimalisatie van de vloeibare biopsie procedure: van onderzoek naar klinische routine

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List of abbreviations

AF	allele frequency
ALK	anaplastic lymphoma kinase
ARMS	amplification-refractory mutation system
Assay sensitivity	maximal attainable sensitivity
ASCO	American society of clinical oncology
BBB	blood-brain barrier
BEAMing	beads, emulsion, amplification, and magnetics
BELAC	Belgian accreditation institution
Bp	base pairs
CAPP-Seq	cancer personalized profiling by deep sequencing
CEN	European Committee for Standardization
cDNA	complement DNA
cfNA	circulating cell-free nucleic acids
cfDNA	circulating cell-free DNA
cfRNA	circulating cell-free RNA
CRC	colorectal cancer
CTC	circulating tumor cell
ctDNA	circulating cell-free tumor DNA
ctRNA	circulating cell-free tumor RNA
Cq	quantitation cycle
ddPCR	digital droplet PCR
dPCR	digital PCR
EDTA tubes	BD Vacutainer K_2EDTA tubes
EGFR	epidermal growth factor receptor
EML4	echinoderm microtubule-associated protein-like 4
EQ	epiquick™ circulating cell-free DNA isolation kit
FDA	US food and drug administration
FFPE	formalin-fixed paraffin embedded
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

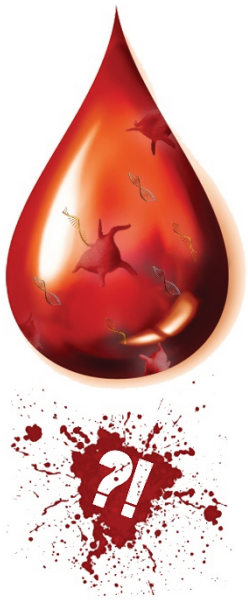
gDNA	genomic DNA
HR	hazard ratio
LoD	limit of detection
LINE-1	long interspersed nuclear elements
MABS	monoclonal antibodies
MAF	minor allele frequency
MET	mesenchymal-epithelial transition factor
MetctDNA	methylated ctDNA
mPCR-NGS	multiplexed PCR and NGS
MRI	magnetic resonance imaging
NGS	next-generation sequencing
NIP testing	non-invasive prenatal testing
$NpM_{V1/V2}$	NEXTprep-Mag™ cfDNA isolation kit, two consecutive versions
NSCLC	non-small cell lung cancer
ORR	overall response rates
OS	overall survival
PAXgene tubes	PAXgene Blood ccfDNA tubes
PDAC	pancreatic ductal adenocarcinoma
PET	positron emission tomography
PFS	progression-free survival
PME	PME free-circulating DNA extraction kit
PPP	platelet poor plasma
PRP	platelet rich plasma
QIA	QIAamp® circulating nucleic acid kit
qPCR	real-time quantitative PCR
R	correlation coefficient
Roche tubes	Cell-Free DNA Collection tubes
RSC	Maxwell® RSC ccfDNA plasma kit
RT	room temperature
Safe-SeqS	safe-sequencing system
SCLC	small cell lung cancer
SQI	semi-quantitative index

Streck tubes	Cell-Free DNA BCT® tubes
Tam-Seq	tagged-amplicon deep sequencing
TAT	turnaround time
TEP	tumor educated platelets
tDNA	tumor DNA
TKI	tyrosine kinase inhibitor
Transit time	number of days from blood collection until arrival at UZA
tRNA	tumor RNA
TEPs	tumor educated platelets
UID	unique identifier
UZA	Antwerp University Hospital
WBC	white blood cell
WT	wild type

INTRODUCTION

CHAPTER 1

Liquid Biopsy Introduced



Rationale

According to estimates from the World Health Organisation (WHO), cancer incidence and mortality are increasing across the globe [1]. In 2018, the International Agency for Research on Cancer (IARC) provided a status report on the cancer burden worldwide, based on GLOBOCAN 2018. This is an online database which provides estimates of incidence and mortality in 185 countries for 36 types of cancer. Lung cancer was found to be the most commonly diagnosed cancer (11.6% of all cases), as well as the leading cause of cancer-related mortality (18.4% of the total cases). Together with female breast cancer and colorectal cancers, it is responsible for one-third of the cancer incidence and mortality burden worldwide [2].

The discovery of targetable molecular alterations and effective drugs have led to the implementation of personalized therapy in patients with advanced cancer [3]. One of the most well-known examples of targeted therapy are tyrosine kinase inhibitors (TKIs) which selectively target specific mutant forms of Epidermal Growth Factor Receptor (*EGFR*) in non-small cell lung cancer (NSCLC). Clinical trials revealed the efficacy of several EGFR TKI generations, which has led to the approval of these drugs in *EGFR*-mutated NSCLC patients [3, 4]. Similarly, the TKIs crizotinib, ceritinib, and alectinib have been established as first-line therapy for Anaplastic Lymphoma Kinase (*ALK*)-translocated, metastatic NSCLC. Crizotinib has also been approved for use in case of *ROS1*-translocations [5]. The expanding array of effective targeted therapies also include monoclonal antibodies (mAB), of which several have been implemented in clinical practice – for example, trastuzumab in *HER2*-amplified breast cancer, and cetuximab in *RAS* wild type (WT) colorectal cancer (CRC)[3, 6]. Sadly, resistance to targeted therapy is nearly universal. In some cases, the underlying resistance mechanisms have already been unraveled, resulting in the development of next-generation drugs [3]. Molecular profiling has become indispensable in providing patients with personalized therapy throughout their disease course.

The current clinical gold standard to diagnose and manage cancer are tissue biopsies as they enable histological as well as genetic characterization of the tumor [7]. However, mutational analysis in tissue is encumbered by several limitations. First of all, the procedure to obtain tumor tissue can put the patient at risk and is, in some cases, rendered impossible

due to the performance status of the patient. Secondly, tissue biopsies provide a snap-shot of the tumor in location and time due to inter- and intratumoral heterogeneity and evolution, respectively. Taking multiple biopsies simultaneously to overcome tumor heterogeneity is not possible due to ethical and practical considerations. In contrast, in the setting of acquired resistance to therapy, rebiopsy at different locations is vital in order to detect therapeutic targets. Lastly, tissue biopsies don't always provide sufficient material for molecular analysis, especially in lung cancer [7, 8]. Liquid biopsy, consisting of circulating cell-free (tumor) DNA (cfDNA/ctDNA) and RNA (cfRNA/ctRNA) analysis in plasma, has emerged as a complementary technique. Its minimally invasive character coupled with novel and highly sensitive analysis platforms make it the perfect tool for real-time follow-up of the genetic landscape of the tumor [8, 9]. However, due to a lack of standard operating procedures, especially in the case of circulating RNA, reported results of liquid biopsies remain ambiguous. Not only analytical, but also pre-analytical variables such as the use of different blood collection tubes, transportation, and processing, can affect mutation detection rates [10]. Several of these aspects will be further explored throughout this thesis. This study will focus on optimizing the workflow of circulating cell-free nucleic acid (cfNA) based liquid biopsy for implementation in routine clinical practice.

Aims and outline of the thesis

The general aim of this thesis was to determine the optimal workflow of circulating cell-free nucleic acid (cfNA) based liquid biopsy for implementation in routine clinical practice.

In **chapter 2**, we start by providing an **overview** of the **potential use of cfNA-based liquid biopsy in personalized treatment in non-small cell lung cancer (NSCLC) patients**. We discuss several molecular testing techniques which are sufficiently sensitive and accurate to enable cfNA analysis. Furthermore, we highlight various genetic alterations with targeted therapies already available and which cfNAs are most appropriate for the detection of these alterations.

We have divided this dissertation in two major sections. In the first part of this dissertation, we started by investigating several pre-analytical variables of the liquid biopsy workflow. It was opted to focus on *KRAS*-mutated cancer patients as they are associated with a high incidence rate in several cancer types, such as pancreatic ductal adenocarcinoma (PDAC, 90 %)[11], NSCLC (40%)[12], and colorectal cancer (CRC, 40%)[13]. Furthermore, *KRAS* mutations likely occur early in tumorigenesis, which makes them a suitable marker for overall tumor burden [7]. In **chapter 3**, we compared **the isolation efficiency** of **five cfDNA isolation kits**. Total cfDNA concentration and integrity were determined by digital droplet PCR (ddPCR) and real-time quantitative PCR (qPCR), respectively. As there is a slight difference in fragmentation pattern between cfDNA and ctDNA, we also determined the *KRAS*-mutated ctDNA fraction isolated by each kit.

The focus of **chapter 4** was set on determining the **optimal centrifugation protocol** for both **cfDNA and cfRNA analysis**. Five centrifugation protocols were selected, which differed in centrifugation speed, temperature, time, and number of centrifugation steps. These protocols were evaluated in the standard BD Vacutainer K_2EDTA (EDTA) tubes and the Cell-Free DNA BCT® (Streck) cfDNA collection tubes.

In **chapter 5** we performed a systematic review of literature concerning the **cfDNA stabilizing efficiency of (specialized) blood collection tubes (BCTs)** prior to blood processing. Next, we evaluated the performance of PAXgene, Roche, and Streck tubes in a situation most resembling a clinical setting. Blood samples collected in EDTA tubes and processed immediately were taken as gold standard.

In the second part of this dissertation, we focused on the clinical applicability of liquid biopsy in NSCLC and PDAC. We set out to assess the influence of several (pre-) analytical variables on ctDNA analysis in a real-life setting. In **chapter 6** we present an **optimized workflow for liquid biopsy in a clinical setting**. In a **multicenter study**, we collected 549 blood samples of 234 NSCLC patients over the course of their treatment. We proposed specifications for pre-analytical variables, including centrifugation protocol, transit time, and temperature. We further specified recommendations regarding analytical and biological variables, such as reached sensitivity and metastatic site location. In addition, we present guidelines on how to interpret ctDNA analysis results in concordance with the patients' therapy status.

In **chapter 7**, we further highlight **the potential of liquid biopsy in a clinical setting**. We present a case study in which we demonstrate the added value of liquid biopsy as a complementary tool in detecting (atypical) disease progression under targeted therapy.

Finally, **chapter 8** discusses the presented results in relation to relevant literature and gives clear insights on future perspectives.

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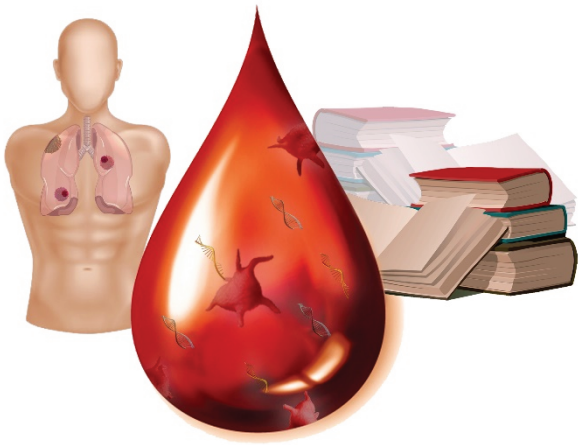
CHAPTER 2

Circulating Cell-Free Nucleic Acids and Platelets as a Liquid Biopsy in the Provision of Personalized Therapy for Lung Cancer Patients.



Sorber L, Zwaenepoel K, Deschoolmeester V, Van Schil P, Van Meerbeeck J, Lardon F, Rolfo C, Pauwels P. Circulating cell-free nucleic acids and platelets as a liquid biopsy in the provision of personalized therapy for lung cancer patients. Lung Cancer, Volume 107, Pages 100-107, 2017.

Abstract

Lung cancer is the predominant cause of cancer-related mortality in the world. The majority of patients present with locally advanced or metastatic non-small-cell lung cancer (NSCLC). Treatment for NSCLC is evolving from the use of cytotoxic chemotherapy to personalized treatment based on molecular alterations. Unfortunately, the quality of the available tumor biopsy and/or cytology material is not always adequate to perform the necessary molecular testing, which has prompted the search for alternatives. This review examines the use of circulating cell-free nucleic acids (cfNA), consisting of both circulating cell-free (tumoral) DNA (cfDNA – ctDNA) and RNA (cfRNA), as a liquid biopsy in lung cancer. The development of sensitive and accurate techniques such as Next-Generation Sequencing (NGS); Beads, Emulsion, Amplification, and Magnetics (BEAMing); and Digital PCR (dPCR), have made it possible to detect the specific genetic alterations (e.g. *EGFR* mutations, *MET* amplifications, and *ALK* and *ROS1* translocations) for which targeted therapies are already available. Moreover, the ability to detect and quantify these tumor mutations has enabled the follow-up of tumor dynamics in real time. Liquid biopsy offers opportunities to detect resistance mechanisms, such as the *EGFR* T790M mutation in the case of EGFR TKI use, at an early stage. Several studies have already established the predictive and prognostic value of measuring ctNA concentration in the blood. To conclude, using ctNA analysis as a liquid biopsy has many advantages and allows for a variety of clinical and investigational applications.

Introduction

Lung cancer is the world's leading cause of cancer-related mortality. Approximately 80% of all lung cancer cases are non-small-cell lung cancer (NSCLC) patients, the majority of whom present with locally advanced or metastatic disease [1]. Over the last decade, several oncogenic driver mutations have been discovered in the histological subtypes of NSCLC, particularly adenocarcinoma, with mutations found in *KRAS* (32.2%), *EGFR* (11.3%), *ALK* (3.9%), *MET* exon 14 (4.2%), *BRAF* (7%), *PIK3CA* (2%), *ROS1* (2%), *HER2* (2%) and *RET* (1%), and amplification of *MET* (2.2%) and *HER2* (0.9%) [2, 3]. Squamous cell carcinoma has also been associated with mutations in *PIK3CA* (12%), *PTEN* (10%) and amplification of *FGFR1* (20%) [3]. This molecular understanding has resulted in a treatment paradigm shift from "one treatment fits all" regimens, such as chemotherapy, to personalized treatment strategies developed on the basis of molecular alterations in tumor DNA [4]. The latter approach consists in screening for genetic aberrations in specific oncogenic signaling pathways, such as the RAS-RAF-MEK-ERK pathway. These pathways can be targeted to reverse the resulting uncontrolled growth, proliferation and survival when up-regulated [5]. These targeted therapies have been found to have a much higher clinical efficacy compared to standard therapy [6]. Genetic profiling of tumors has therefore become indispensable in the development of personalized therapies. Tissue biopsy is the gold standard for tumor genotyping, yet there are several limitations associated with mutational analysis in tissue. For example, lung cancer biopsies often provide limited, low-quality material, which is less suitable for molecular analysis [7]. Furthermore, single tumor biopsies do not always reveal the entire genomic landscape of tumor, heightening the need for personalized diagnosis and treatment [8]. As mentioned above, tissue biopsy remains the gold standard for assessing the mutational status of tumors. However, liquid biopsy, which allows for the analysis of several blood-based biomarkers, has emerged as a useful complementary technique.

This review examines the use of circulating cell-free nucleic acids (cfNA), consisting of both DNA (cfDNA) and RNA (cfRNA), as a liquid biopsy aiding in the provision of personalized therapies for lung cancer patients.

Liquid Biopsy: cfDNA

The first identification of cfDNA in blood was reported by Mandel and Metais in 1948 [9]. Since then, cfDNA has found applications in many disciplines of medicine, but particularly in the evaluation of fetal DNA in the circulation of expectant mothers as a form of non-invasive prenatal (NIP) testing [10]. Liquid biopsy has also produced promising results in the field of targeted cancer therapy, given that the analysis of cfDNA and especially the fraction derived from tumor cells, namely circulating cell-free tumor DNA (ctDNA) enables the detection of specific genetic alterations as potential targets for targeted therapy [11]. CfDNA and ctDNA is released into the bloodstream through various mechanisms, including apoptosis, necrosis and even active shedding [12]. CtDNA is thought to be able to provide a broader picture of the genomic landscape of tumors throughout the body [13, 14]. Murtaza *et al.* has demonstrated the potential of ctDNA in real-time sampling of multifocal clonal evolution [15]. The minimally invasive sampling required for liquid biopsy also offers the opportunity to detect the molecular changes that cancer cells undergo during treatment [13, 16], and to monitor tumor burden and the occurrence of relapse [17]. Several investigational studies have reported that ctDNA levels increase rapidly as the disease progresses, and decline in the case of resective surgery and/or successful medical therapy [14, 16, 18]. The low concentrations of ctDNA in blood, especially in the early stages of disease, and its potential as a liquid biopsy have prompted the development of specialized collection vessel, highly efficient isolation techniques and sensitive detection method. Consequently, patients can be monitored closely and provided with personalized treatment regimens. Liquid biopsy not only allows for frequent sampling [19], but also provides an alternative to tissue biopsy among critically ill patients or when tissue specimens are limited or unavailable at the time of diagnosis. In the phase 3 IPASS and INTEREST studies, it was seen that only 42% and 31% of patients had tissue available or tissue could be obtained for molecular testing, respectively [20, 21].

Practical approach to performing Liquid Biopsy in the clinic

cfDNA preparation

At present, EDTA tubes are most commonly used for blood collection. The anticoagulant in the tubes prevents clotting and acts as an indirect DNase I inhibitor [22]. CfDNA in blood is associated with a high turn-over due to the presence of nucleases, which means that plasma preparation must be performed within 1 to 2 hours of blood sampling [23, 24]. Plasma remains the matrix of choice, however, since higher cfDNA concentrations are observed in serum due to contamination by genomic DNA released by white blood cells during the clotting process [23]. Alternative blood collection tubes containing preservative reagents, such as Cell-Free DNA BCT® (Streck tubes) and PAXgene Blood DNA tubes (Qiagen), have also been developed. The formaldehyde-free preservative reagents in these tubes not only stabilize nucleated blood cells, preventing the release of cellular genomic DNA, but also inhibit the nuclease-mediated degradation of cfDNA [25, 26]. Toro *et al.* demonstrated that Cell-Free DNA BCT® tubes, in particular, make it possible to keep blood samples at room temperature for several days before plasma preparation, which has led to their application in NIP testing of fetal DNA and the analysis of ctDNA in cancer patients [26, 27].

In order to ensure clinically meaningful sensitivity, it is essential to isolate cfDNA from plasma efficiently. Only then can the cfDNA yield be compared and normalized reliably when screening for the range of biomarkers associated with targeted therapies. CtDNA load has been reported to correlate with disease stage [28]. CtDNA can make up as little as 0.01% of the total cfDNA (minor allele frequency (MAF)) [29]. The fragment size profile of the cfDNA and ctDNA can differ. CfDNA primarily consists of uniform fragments, whereas the ctDNA fragment size depends on the cellular process causing its release into the circulation [30, 31]. Jiang *et al.* reported that short DNA fragments preferentially carry tumor-associated aberrations [31]. Several kits have been developed to specifically isolate smaller fragments. Comparison in terms of their isolation efficiency, reproducibility and representation of smaller DNA fragments, has revealed that the QIAamp® circulating nucleic acid kit (Qiagen) performs best. Isolation kits designed to extract high-integrity genomic DNA from blood cells or virions, were less effective [32-34]. Our own research group compared the Qiagen kit with the Maxwell® RSC ccfDNA Plasma (Promega),

which displayed similar results of cfDNA yield and ctDNA detection, where the Promega kit has the advantage of a fully automated protocol over the labor intensive Qiagen kit [35]. Standardization of ctDNA analysis requires a uniform centrifugation protocol as well as normalization of the amount of plasma input. Several protocols describe two sequential spins of blood samples (varying from 800 – 2000 g for 10 – 15 min) to separate plasma from buffy coat [36, 37]. Page *et al.* described the need for a third bench top spin (1000 g for 5 min) to remove any remaining cells, platelets, and cellular debris [38]. High plasma input is necessary to generate sufficient ctDNA concentrations to be able to detect low MAF [32]. Besides high isolation efficiency, ctDNA analysis also requires methods with a low limit of detection (LoD).

ctDNA analysis

Several platforms have been developed to analyze ctDNA for the presence of particular biomarkers, including real-time quantitative PCR (qPCR); the Scorpion Amplification-Refractory Mutation System (ARMS); Beads, Emulsion, Amplification and Magnetics (BEAMing); digital PCR (dPCR); and Next-Generation Sequencing (NGS) (Table 1).

Table 1

Overview of the various techniques used for cfDNA analysis of cancer patients.

Method	Approach	Mechanism	Sensitivity	References
qPCR	Candidate Gene	Specific TaqMan probes	10%	[39]
Scorpion ARMS	Candidate Gene	Specific Scorpion primer-probe complex	1%	[40, 41]
dPCR	Candidate Gene	Specific TaqMan probes; compartmentalization in droplets/wells	0.01%	[42, 43]
BEAMing	Candidate Gene	Magnetic beads – partitioning in water-in-oil emulsion	0.01%	[44, 45]
NGS	Screening of the genome	Parallel sequencing of millions of DNA templates	≤0.04%	[46, 47]

qPCR = quantitative PCR; ARMS = Amplification-Refractory Mutation System; BEAMing = Beads, Emulsion, Amplification and Magnetics; dPCR = digital PCR; NGS = Next-Generation Sequencing

➢ *Real-time quantitative PCR*

Real-time qPCR, consisting of both TaqMan based and Scorpion ARMS assays, are the most widely used platforms for the quantification of nucleic acids [48-51]. ARMS is a standard PCR method for detecting of single-base mutations and small deletions. It involves amplifying the target DNA using sequence-specific primers that only trigger this amplification when the target allele is contained within the sample. The presence or absence of a PCR product therefore indicates the presence or absence of the target [52]. Because this method is relatively insensitive in the detection of low levels of mutation, most likely due to primer competition, it is often combined with Scorpion technology, making it a qPCR-based technique. Scorpions consist of a PCR primer covalently linked to a probe, which in turn interacts with a quencher. The presence of a mutation can be detected by the Scorpion ARMS in a real-time PCR setting [40, 53]. While qPCR is a standardized, relatively inexpensive, and technically straightforward test, it is unable to match the high sensitivity of other techniques such as dPCR [42, 54].

➢ *Digital PCR (dPCR)*

The dPCR approach is similar to real-time qPCR. Instead of amplifying the sample as a whole, however, the DNA templates are partitioned into thousands of individual, parallel PCR reactions. The detection of the signal indicates the presence (positive) or absence (negative) of the target sequence. In this manner, dPCR allows for the detection of mutated ctDNA in a high background of wild type cfDNA, rendering it highly sensitive. Various array-loading platforms have been designed, in which partitioning is based on capillary action, either microfluidic-chamber or microfluidic-well chip based (Thermo Fisher OpenArray and Fluidigm). Other platforms include droplet-based dPCR (Bio-Rad® and Raindance®), in which samples are dispersed into 20,000 and 10,000,000 droplets, respectively, and which are screened individually by flow cytometry for the presence of signal after amplification [55-57]. Besides high sensitivity, dPCR also has a relatively easy workflow, which can be implemented in the clinic. DPCR only screens for known mutations, however, several mutations can be assessed simultaneously [58, 59]

➢ *Beads, emulsion, amplification and magnetics (BEAMing)*

BEAMing is based on the same principle as emulsion dPCR, during which streptavidin-biotin interactions are used to bind DNA templates to magnetic microbeads before emulsion into droplets. After amplification, magnetics are employed to release and purify the PCR products [60]. In this manner a number of known mutations can be detected in a high background of wild type cfDNA [24]. Besides the fact that BEAMing only screens for known mutations, it also has a complex workflow and high cost per sample, making implementation in routine clinical settings less feasible.

➢ *Next-generation sequencing (NGS)*

NGS is based on the production of short sequences from single DNA molecules and their comparison to a reference sequence. This parallel sequencing of millions of DNA templates results in the sequencing of a large portion of the genome. Whole genome sequencing allows us to screen not only for mutations, but also for rearrangements and copy number aberrations, providing a genetic survey of an individual's (cancer) genome [61]. Newman *et al.* developed an ultrasensitive method for the quantification of ctDNA, namely the Cancer Personalized Profiling by deep Sequencing (CAPP-Seq), which consists of a 'selector' that covers multiple classes of somatic alterations and can identify mutations in over 95% of tumors [47]. Tagged-amplicon deep sequencing (Tam-Seq) described by Forshew *et al.* is based on the amplification and deep sequencing of genomic regions covering thousands of bases, even from individual copies of fragmented DNA. They were able to identify cancer mutations at allele frequencies as low as 2%, with sensitivity and specificity of more than 97% [62]. The Safe-Sequencing System (Safe-SeqS) was developed to increase the sensitivity of massively parallel sequencing system instruments for identifying rare variants. Each template molecule is assigned a unique identifier (UID) and amplification of each uniquely tagged template molecule is used to create UID families. All amplification products are sequenced. The variant is considered to be a true mutation only if ≥95% of the PCR fragments with the same UID contain an identical mutation [63]. These specialized and modified NGS techniques ensure that NGS has adequate sensitivity to be useful in ctDNA analysis. Despite its high sensitivity, NGS remains an expensive

technique, and is time intensive. Furthermore, extensive data analysis requires a highly experienced bioinformaticians for evaluating and interpreting the results.

The best approach seems likely to involve a combination of these techniques. In addition to determining the mutational profile, it is essential to follow up both the mutational load and the occurrence of resistance mechanisms in order to provide a personalized treatment. Since knowledge of the entire molecular make-up allows us to select the optimal therapy, NGS appears to be the most appropriate technique for use at the time of diagnosis. For follow up, on the other hand, a candidate-gene approach involving mutation-specific detection techniques, such as dPCR, might be more suitable because of the lower cost, simpler workflow, and easier data management. To give an example; *MET* amplification is one of the resistance mechanisms associated with *EGFR*-mutated NSCLC. Tumors containing preexisting clones of *MET* amplification would undergo positive selection during EGFR tyrosine kinase inhibitor (TKI) therapy [64]. Paweletz *et al.* have reported the detection of *MET* amplification in ctDNA of NSCLC patients using a bias-corrected targeted NGS technique. *MET* amplification was detected in a patient harboring an *EGFR* exon 19 deletion, with acquired resistance to erlotinib and afatinib. Amplification was also detected in ctDNA of an advanced NSCLC patient with a high clinical suspicion of harboring a targetable genomic alteration. In both cases *MET* amplification was also detected in the tumor tissue [65]. Detection of these *MET* subclones at the time of diagnosis would enable specific screening for the occurrence of this resistance mechanism during targeted therapy (Figure 1B) [66].

Clinical applications of Liquid Biopsy

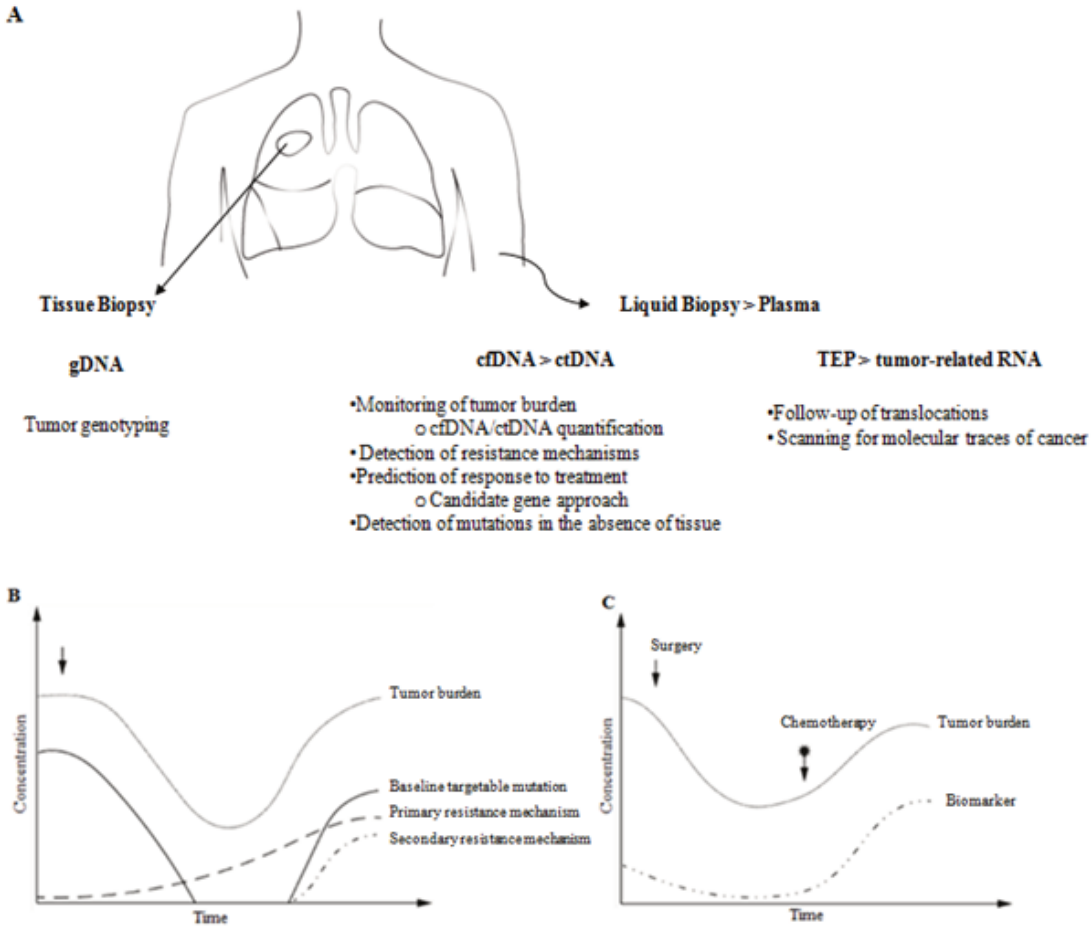


Figure 1: Applications of Liquid Biopsy in providing personalized treatment and the follow up of NSCLC patients. A Tissue biopsy is the gold standard in providing genomic DNA for tumor genotyping. Plasma based liquid biopsy is a source for both cfDNA, which contains the ctDNA, and platelets carrying the tumor-related RNA. Combining tissue and liquid biopsy might be the best strategy in providing patients with personalized treatment; **B** at baseline a targetable mutation (e.g. *EGFR* exon 19 deletion) together with a primary resistance mechanism in a small subclone (e.g. *MET* amplification) might be detected by tissue and/or liquid biopsy. Liquid Biopsy enables follow up of these markers during targeted therapy as well as screening for (known) secondary resistance mutations (e.g. *EGFR* T790M). **C.** Other applications include monitoring of the tumor burden both in targeted therapy setting as with surgery and chemotherapy, where detection of certain biomarkers (e.g. *KRAS* mutations) might be indicative of minimal residual disease and in some cases a predictor of prognosis to treatment. *cfDNA: circulating cell-free DNA; ctDNA: circulating cell-free tumor DNA; gDNA: genomic DNA; TEP: tumor educated platelets.*

Monitoring tumor burden

One of the first aspects of disease management in which liquid biopsy offers great potential is the monitoring of tumor burden (Figure 1B-C). Radiological imaging is currently the standard technique used for post-treatment follow-up that aims to detect either signs of residual disease or local or distant recurrences. However, detection of micrometastases is hampered by the limited sensitivity of the imaging techniques used. Furthermore, frequent monitoring is difficult since imaging techniques are costly and often expose patients to radiation [67, 68]. Several investigational studies have demonstrated the potential of cfDNA, and especially ctDNA, as a surrogate marker for tumor burden [12, 62, 69]. Sozzi *et al.* was able to discriminate patients with radically resected primary NSCLC (stages I-III) from healthy individuals. Even stage IA patients were found to display higher mean values of ctDNA than those of the controls. They were also able to detect early recurrence based on ctDNA quantification and analysis of microsatellite alterations [70]. The development of highly sensitive techniques such as the CAPP-Seq has expanded the range of sensitivities at which ctDNA can be detected. In the study of Newman *et al.*, ctDNA levels in NSCLC patients were found to be closely correlated with tumor volume. CtDNA was detected in 100% of patients with stage II-IV NSCLC and in 50% of patients with stage I NSCLC, with 96% specificity for mutant allele fractions down to approximately 0.02%. Furthermore, ctDNA levels also enabled earlier response assessment than radiographic approaches [47]. However, some controversy remains with regard to whether ctDNA is a simple measure of tumor burden, given the fact that cfDNA levels also rise in a number of benign disorders such as infections, diabetes, cardiovascular diseases and non-neoplastic lung diseases [71, 72]. Variations in cfDNA concentrations between individuals with advanced disease remains poorly understood [12, 69]. Nygaard *et al.* found no significant correlation between total cfDNA and total tumor burden using Positron Emission Tomography (PET) in advanced NSCLC [73]. However, there are many indications that ctDNA does have potential as a liquid biopsy in the monitoring of tumor burden [74, 75]. Further improvements both in technology and in our understanding of the kinetics associated with cfDNA and ctDNA release might help to provide clarification.

Detecting resistance mechanisms

At present, the most widely known TKI targets in NSCLC are *EGFR* activating mutations, 90% of which consist of in-frame deletions in exon 19 and L858R point mutations in exon 21 [76]. First-line treatment of patients harboring *EGFR* activating mutations with EGFR TKIs gefitinib, erlotinib or afatinib has resulted in superior overall response rates (ORR), progression-free survival (PFS) and quality of life compared to chemotherapy [6, 77, 78]. Unfortunately, however, the majority of patients develop secondary (acquired) resistance within a year. The acquisition of the T790M mutation is the most frequent resistance mechanism, responsible for nearly 60% of cases [45, 79, 80]. In this setting the advantage of using liquid biopsy is that it makes frequent sampling possible. Several studies have reported the reduction or even disappearance during TKI treatment of *EGFR* activating mutations in plasma, which had reappeared together with the T790M resistance mutation. In these studies, the T790M mutation in plasma was detected 15 to 344 days before disease progression was established based on RECIST criteria [16, 81, 82]. Consequently, frequent sampling for the detection of resistance to targeted therapy could potentially be the most important application of liquid biopsy in personalized medicine (Figure 1_B). Liquid biopsy can thus allow clinicians to adopt more effective, alternative treatment strategies [11]. One of the first clinically useful examples emerged with the development of drugs such as CO-1686 and AZD9291, which are highly selective, irreversible EGFR TKIs that target the T790M resistance mutation [83-85]. Unfortunately, even the use of third generation TKIs has led to new resistance mechanisms. Thress *et al.* recently reported the occurrence of AZD9291 resistance through the mechanism of the *EGFR* C797S mutation. These authors performed NGS on ctDNA from seven AZD9291 resistant NSCLC patients, one of whom exhibited an acquired *EGFR* C797S mutation. Subsequently they performed digital dPCR on serial ctDNA samples collected from fifteen AZD9291-treated NSCLC patients harboring the T790M mutation. Three molecular subtypes of AZD9291 resistance were detected. Six cases exhibited the C797S mutation, five cases had the T790M mutation without the C797S mutation and in four cases only the underlying *EGFR* activating mutation was found [86]. In addition, tracking the dynamics of the various mechanisms of resistance of subpopulations might allow patients to be re-challenged with the same or similar drugs when the resistance-associated allele has been lost in the absence of targeted

therapy. The release of treatment pressure was observed to result in the loss of the molecular resistance mechanism in three NSCLC patients, which facilitated the re-treatment of these patients with *EGFR* TKIs [87, 88]. Additional EGFR TKI resistance mechanisms in NSCLC can also be detected with a candidate-gene approach, in which liquid biopsy can be employed to screen for *MET* amplification, as well as mutations in *PIK3CA*, *HER2*, and *BRAF* [4, 65]. Transformation to small-cell lung cancer (SCLC) is known as a rare resistance mechanism [89], which at present cannot be detected using liquid biopsy. Furthermore, the development of highly sensitive techniques has made it possible to detect extremely low amounts of biomarkers whose clinical significance is not always clear. Watanabe *et al.* detected the pretreatment T790M mutation in 373 surgically resected tumor samples from *EGFR*-mutated NSCLC patients using droplet dPCR. Approximately 80% of the patients exhibited the mutation at an ultra-low allele frequency of between 0.001% and 0.1%, where the mutation was more frequently detected in patients with larger tumors and those who harbored the common *EGFR* mutations, exon 19 deletion and L858R mutation. As this was the first large-scale study to use and ultra-sensitive method, however, the clinical significance of this pretreatment T790M mutation remains unclear [85].

Predicting response to treatment

Marchetti *et al.* were the first to describe a correlation between clinical response and a semi-quantitative index (SQI) of mutated *EGFR* levels in the first days of treatment. The SQI reflected a trend in the proportion of mutated versus wild-type copies of the *EGFR* gene. Serial ctDNA specimens were prospectively collected from 20 NSCLC patients harboring activating *EGFR* mutations during *EGFR* TKI treatment. In 95% of the cases, a progressive decrease in *EGFR* SQI was observed, from the fourth day of treatment onwards, with an average decrease of 63.5% by day 14 after treatment initiation. A distinction was made between rapid and slow responders, where rapid responders displayed a decrease in mutant plasma *EGFR* levels of at least 50% by day 14. A rapid decrease was associated with radiologically significant tumor shrinkage at two months, whereas a lower mean percentage of tumor shrinkage was observed in the slow responders. In two slow responders, mutation clearance was not reached and an early increase in the levels of

T790M-mutated ctDNA was observed. Over the course of 60 days after treatment initiation, no T790M mutations were detected in the serial cfDNA specimens of the rapid responders. Although the long-term impact of TKI treatment on rapid and slow responders in relation to PFS and overall survival (OS) requires further clinical validation, Marchetti *et al.* have demonstrated the potential of liquid biopsy to determine the effectiveness of targeted therapy in the first two weeks of treatment [19]. Thus, ctDNA analysis can aid in selecting the best treatment regimen for each patient (Figure 1B).

The potential of liquid biopsy to predict response to treatment has also been described in a multivariate analysis, in which 246 advanced stage NSCLC patients were screened for *KRAS* mutations in plasma before initiation of first-line carboplatin combined with vinorelbine; patients who harbored *KRAS*-mutated ctDNA were found to have significantly shorter OS and PFS than the wild type patients [90]. However, no significant differences in OS or PFS were observed with regard to the presence or absence of *KRAS*-mutated ctDNA in a study of 308 advanced stage NSCLC patients treated with a combination of cisplatin and docetaxel [91]. This difference might be related to the fact that subtypes of *KRAS*-mutated NSCLC respond differently to chemotherapy regimens [92]. A meta-analysis of 41 relative publications determined that mutated *KRAS* is a weak, yet valid predictor of poor prognosis and treatment outcomes in NSCLC [93] (Figure 1C).

Detecting mutations in the absence of tissue

At the time of diagnosis most NSCLC patients present with unresectable disease. Tissue for genotyping can then be obtained using procedures such as bronchoscopic biopsy, aspiration cytology or CT-guided percutaneous needle biopsy [94]. These procedures do not always generate enough material for diagnostic tests, or may be rendered impossible by the poor performance status of the patient. Furthermore, certain clinical trials of targeted therapy for NSCLC often mandate repeat biopsies at progression. In these cases liquid biopsy could represent an alternative when screening for biomarkers associated with targeted therapy [95]. Where possible, however, the complementary use of both liquid and tissue biopsy may be the best choice. Thress *et* al. compared multiple platforms for the analysis of ctDNA of NSCLC patients during treatment with AZD9291. The plasma samples were compared to matched tumor samples before the start of treatment. The

tissue-plasma discordance and 'reduced' T790M specificity of the plasma assays they described might have been related not only to technological limitations, but also to tumor heterogeneity. Mutations missed in the localized tumor samples could have been picked up by the plasma given that it mirrors the entire tumor burden and vice versa. This is corroborated by the fact that the rate of clinical response to AZD9291 was found to be almost identical in patients harboring T790M mutations according to plasma and tumor genotyping [96].

Liquid Biopsy: Platelet tumor-related RNA

Rearrangements in the *ALK* gene are associated with high sensitivity to ALK TKIs crizotinib [97]. Platelets might be a good matrix for detecting of these rearrangements. Tumor-related RNA is released into the blood by several microvesicle-dependent or –independent mechanisms, and research has shown that platelets can sequester this RNA as well as other tumor-associated biomolecules, resulting in tumor-educated platelets (TEPs) [98, 99]. Platelets have emerged as key players in tumor cell growth, invasion, spread, migration, intravasation, extravasation, and establishment of distant metastasis [100, 101]. Nilsson *et al.* were the first to detect the Echinoderm Microtubule-associated protein-Like 4 (*EML4*)-Anaplastic Lymphoma Kinase (*ALK*) rearrangements in the platelets of patients with NSCLC [102]. They screened for *EML4-ALK* rearrangements in the platelets and plasma consisting of both cfRNA and RNA contained in exosomes of 77 NSCLC patients, 38 of whom had *EML4-ALK*-rearranged tumors. Intriguingly, in a subpopulation of 29 patients with *EML4-ALK*-rearranged tumors treated with the ALK TKI crizotinib, the detection of these rearrangements in platelets was found to be correlated with lower PFS and OS. In total, sensitivities and specificities of 65% and 100% in platelets and 21% and 100% in plasma RNA were achieved for the detection of *EML4-ALK* rearrangements. This difference in sensitivity is most likely due to the rapid degradation of the cfRNA or to the low abundance of exosomes containing rearranged EML4-ALK. Besides platelet tumor-related RNA's potential for detecting ALK rearrangements, it may also be a useful tool in the prediction and monitoring of response to crizotinib treatment. One patient was

monitored for *EML4-ALK* rearrangements over a period of 30 months; the rearrangement was again detected in platelet RNA two months before radiological progression could be demonstrated.

TEPs are characterized by altered RNA profiles, a fact which enabled the research group of Best *et al.* to distinguish 228 patients with localized and metastasized tumors from 55 healthy individuals with 96% accuracy [99]. Sequencing of mRNA allowed the researchers to scan for general molecular traces of cancer and also provided strong indications on tumor type and molecular subclass. This was demonstrated by the 71% rate of accuracy achieved when pinpointing the location of the primary tumor across six different tumor types as well as by the accurate identification of *MET* or *HER2*-positive tumors, and mutant *KRAS*, *EGFR,* and *PIK3CA* tumors. While further research and clinical validation is needed, these studies highlight the potential of platelets as liquid biopsies in the detection of targets associated with personalized therapy (Figure $1_{A\text{-}B}$).

Discussion & future directions

Liquid biopsy has been shown to be a high-quality blood-based biomarker for use in many aspects of cancer treatment, as well as a complementary or even alternative method for providing material for mutation analysis in clinical settings. Due to its predictive properties as a biomarker, it could also contribute to the assessment of new targeted therapies [19, 86]. The remaining issues related to liquid biopsy are now being resolved by the development of more specific and even more sensitive techniques [47, 62, 63]. The combination of multiplexed PCR and NGS (mPCR-NGS system), as seen with the TAM-Seq system, was used by Jamal-Hanjani *et al.* to identify ubiquitous and heterogeneous mutations in the ctDNA of early-stage NSCLC patients [103]. Only a small cohort was used, hence clinical validation of mPCR-NGS is needed to define the use of ctDNA analysis in the detection of early-stage cancers. The highly sensitive techniques might offer opportunities for developing cancer-screening strategies in the future. Other research lines related to the diagnostic biomarkers of lung cancer include the detection of

hypermethylation and or hypomethylation in certain genes, such as *p16*, *DAPK, APC, MGMT* and *BCAT1*, *CDO1*, *TRIM58* and *ZNF177* [104, 105].

In this review we have emphasized the role of liquid biopsies in providing personalized treatment and improving the follow-up of patients in clinical setting. It is evident that liquid biopsy has a role to play both in clinical trials and in the clinic. However, more research is still needed to enable the development of specific and sensitive detection techniques and specific biomarkers for use in this fascinating and rapidly evolving field.

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PART I: OPTIMIZATION OF THE LIQUID BIOPSY WORKFLOW

CHAPTER 3

A Comparison of CfDNA Isolation Kits: Isolation and Quantification of Cell-Free DNA in Plasma.

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Abstract

The analysis of cell-free DNA (cfDNA) as a sensitive biomarker for cancer diagnosis and monitoring has resulted in a need for efficient and standardized cfDNA isolation. In this study we compared the isolation efficiency of the QIAamp® circulating nucleic acid kit (QIA) with four other cfDNA isolation kits; the PME free-circulating DNA Extraction Kit (PME), the Maxwell® RSC ccfDNA Plasma Kit (RSC), the EpiQuick™ Circulating Cell-Free DNA Isolation Kit (EQ) and two consecutive versions of the NEXTprep-Mag™ cfDNA Isolation Kit ($NpM_{V1/2}$). CfDNA was isolated from ten plasma samples, of which five contained *KRAS*-mutated cell-free tumor DNA (ctDNA). Digital droplet PCR (ddPCR) was used to quantify the total cfDNA concentration as well as the *KRAS*-mutated ctDNA fraction. CfDNA integrity was assessed with real-time quantitative PCR (qPCR). The QIA and the RSC kits displayed similar isolation efficiencies of both *KRAS*-mutated ctDNA and non-mutated cfDNA, whereas the yield generated by the PME and NpM_{V2} kits was significantly lower. qPCR indicated the presence of ddPCR inhibiting agents in the eluates of the NpM_{V1} and EQ kits. To conclude, this study presents two highly efficient isolation kits for cfDNA isolation, of which the RSC kit has the advantage of a fully automated protocol over the labor intensive QIA kit.

Introduction

In oncology, circulating cell-free DNA (cfDNA) in plasma has emerged as a new and sensitive biomarker. A fraction of this cfDNA is derived from tumor cells, also known as circulating cell-free tumor DNA (ctDNA). This ctDNA fraction is believed to reflect the genetic aberrations found in tumor tissue, e.g. *KRAS* mutations. As a consequence, cfDNA, which contains ctDNA, can be used a 'liquid biopsy'. High isolation efficiency is vital for successful ctDNA analysis, particularly in early disease. CtDNA levels have been reported to correlate with tumor burden [1-3] and ctDNA as minor allele frequency (MAF) can make up as little as 0.01% of the total cfDNA [4]. Fragment size profile is another factor to consider when dealing with ctDNA/cfDNA isolation. CfDNA consists primarily of uniform fragments of 185 to 200 bp, whereas, ctDNA can differ in fragment size profile depending on the cellular process causing its release into the circulation [5, 6]. This could explain the inefficiency of some isolation methods which were developed to extract high-integrity genomic DNA from blood cells or virions, instead of smaller DNA fragments [7, 8].

In this study we compared cfDNA isolation efficiency of one of the most commonly used cfDNA isolation kits, namely the QIAamp® circulating nucleic acid kit (QIA – Qiagen)[9-12], with four newly launched cfDNA isolation kits; the PME free-circulating DNA Extraction Kit (PME – Westburg, Leusden, Netherlands), the Maxwell® RSC ccfDNA Plasma Kit (RSC – Promega, Leiden, Netherlands), the EpiQuick™ Circulating Cell-Free DNA Isolation Kit (EQ – Epigentek, New York, United States), and two consecutive versions of the NEXTprep-Mag™ cfDNA Isolation Kit ($NpM_{V1/2}$ – SanBio B.V., Uden, Netherlands). Ten plasma samples (Biobank@UZA), including five with a *KRAS* mutation, of patients treated for a pancreatic pathology, were examined using these isolation kits. To determine the isolation efficiency, the QX200™ Droplet Digital™ PCR system (ddPCR – Bio-Rad) was used for the quantification of the total cfDNA and the *KRAS*-mutated ctDNA fraction. Fragment size profile was analyzed using a qPCR assay consisting of amplicons of different lengths.

Material and Methods

Plasma samples

Ten plasma samples were collected from nine patients treated at the Antwerp University Hospital (UZA): two patients with benign pancreatic cysts, three pancreatic ductal adenocarcinoma (PDAC) patients who underwent surgery, and four PDAC patients with metastasized disease, including one patient of whom a second blood sample was taken during follow-up (Table 1). All patients provided written informed consent. The human biological material used in this publication was provided by Biobank@UZA, Antwerp, Belgium; ID: BE71030031000 [13].

Table 1

Plasma samples: overview of *KRAS* mutational status

ID no.	**Group**	***KRAS* mutational status**	
		Blood	**Tissue**
1	Stage IV PDAC	Mutated	NA
2		Mutated	NA
3	Stage IV PDAC	Mutated	NA
4	Stage IV PDAC	Mutated	NA
5	Stage IV PDAC	Mutated	NA
6	Surgery (Stage III PDAC)	WT	WT
7	Surgery (Stage IIb PDAC)	WT	WT
8	Surgery (Stage IIb PDAC)	WT	Mutated
9	Surgery (Benign pancreatic cyst)	WT	WT
10	Surgery (Benign pancreatic cyst)	WT	WT

NA: not available; PDAC = pancreatic ductal adenocarcinoma; Plasma samples 1 and 2: samples taken at different time points from the same patient; WT: wild type

Determination of KRAS mutational status

The *KRAS* mutational status of these patients was determined prior to this study on both the ctDNA and resection specimen of the patients who underwent surgery (Table 1). The QIA kit was used to extract cfDNA from 1 mL of plasma with an elution volume of 100

μL. DNA was isolated from the formalin-fixed paraffin embedded (FFPE) tissue with the Cobas® DNA Sample Preparation Kit (Roche, Anderlecht, Belgium), according to the manufacturer's instructions. *KRAS* mutation analysis was performed with the ddPCR™ *KRAS* Screening Multiplex Kit (Bio-Rad) on the QX200™ Droplet Digital™ PCR system (ddPCR – Bio-Rad) (see Material and methods – digital droplet PCR). All patients with metastasized disease were *KRAS* mutated, whereas only one patient from the group of surgically resected tumors showed a *KRAS* mutation (Table 1).

cfDNA isolation

In this study we compared the isolation efficiency of the most commonly used isolation kit, namely the QIA kit with four specific cfDNA isolation kits: the PME, RSC, the EQ and two consecutive versions of the NpM kit ($NpM_{V1/2}$) (Table 2). The QIA kit is based on cfDNA binding to a silica membrane on a vacuum manifold, whereas cfDNA capture by the PME kit is based on polymer mediated enrichment. The RSC, the EQ and the $NpM_{V1/2}$ kits are all magnetic beads-based methods, with the protocol of the RSC kit being fully automated. The QIA kit as well as the RSC and $NpM_{V1/2}$ kits include a lysis step before DNA binding to either a membrane or magnetic beads; whereas the lysis step of the other kits occurs after DNA binding. The QIA kit can be partially automated, however, the lysis and vacuum steps still have to be performed manually.

Each kit processed a volume of 0.5 mL of plasma with an elution volume of 50 μl; with the exceptions of the EQ kit and the second version of the NpM kit (NpM_{V2}), where an elution volume of 20 μL and 12 μL were used, respectively. All experiments were performed according to the manufacturer's protocol, including the optional addition of carrier RNA for both the QIA and the PME kit. The PME kit contains two different Lysis/Binding Solution systems, namely the GS/VL and the SE/SBS system. In our hands the Lysis /Binding Solution SBS system was shown to have a better isolation efficiency (data not shown).

All samples were quantified by Qubit® dsDNA HS Assay kit (Thermo Fisher Scientific, Aalst, Belgium) according to the manufacturer's manuscript (supplementary Table 1). The lower elution volume of the EQ and the NpM_{V2} kits were corrected by diluting the samples 1:2.5 and 1:4.17, respectively. In total, 5 μL of each sample was loaded.

Table 2

Overview of cfDNA isolation kits

Kit	Method	Lysis step before cfDNA capture	Protocol
QIA	Silica based column	Yes	Manual
PME	Polymer mediated enrichment	No	Manual
RSC	Magnetic beads	Yes	Automatic
EQ	Magnetic beads	No	Manual
NpM_{V1}/NpM_{V2}	Magnetic beads	Yes	Manual

EQ: EpiQuick™ circulating cell-free DNA isolation kit; NpM_{V1}/NpM_{V2} : two consecutive versions of the NEXTprep-Mag™ cfDNA isolation kit; PME: PME free-circulating DNA extraction kit; QIA: QIAamp® circulating nucleic acid kit; RSC: Maxwell® RSC ccfDNA plasma kit.

Digital droplet PCR

The QX200™ Droplet Digital™ PCR system (ddPCR – Bio-Rad) was used for quantification of the total cfDNA and the *KRAS*-mutated ctDNA fraction. All samples were screened with the ddPCR™ *KRAS* Screening Multiplex Kit (Bio-Rad) for the presence of seven *KRAS* mutations (G12A/C/D/R/S/V and G13D) and one specific wild type sequence within exon 2. TaqMan PCR reaction mixtures were assembled by 10 µL 2x ddPCR™ Supermix for Probes (Bio-Rad), 1 µL ddPCR™ *KRAS* Screening Multiplex Kit and 10 µL of isolated sample. To correct for the use of the lower elution volume with the EQ and the NpM_{V2} kit, 7.5 and 9.5 µL of elution buffer was added to 5 and 3 µL of sample, respectively. CfDNA from human plasma is considered to be too diluted for accurate spectrophotometric analysis. Hence, the maximum amount of each sample is loaded, regardless their concentration determined by Qubit analysis. Final-volume reactions of 20 µL were loaded into sample wells of a DG8™ cartridge (Bio-Rad) with 70 µL of Droplet Generation Oil for Probes (Bio-Rad). Droplets were generated by the QX200™ Droplet Generator (Bio-Rad); and 40 µL of the generated droplets was manually transferred with a multichannel pipette into a 96-well PCR plate and amplified in a C1000 Touch™ thermal cycler (Bio-Rad). The thermal cycling conditions were as follows: 95°C for 10 min, 40 cycles of 94°C for 30 s, then 55°C for 1 min, followed by 98°C for 10 min and cooling to 4°C. Droplets were analyzed using the QX200 Droplet Reader (Bio-Rad). Data analysis was performed with the QuantaSoft™ version 1.7.4.0917,

which uses the number of positive and negative droplets to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% confidence intervals.

Real-time quantitative PCR

CfDNA integrity was assessed by real-time quantitative PCR (qPCR). Sample quality was estimated based on two primer sets that produce amplicons of 105 and 236 base pairs (bp) fragments from non-overlapping target sites in the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. Primer sequences of the 105 bp and the 236 bp fragments are as follows: (5'-GGCTGAGAACGGGAAGCTTG-3' and 5'-ATCCTAGTTGCCTCCCCAAA-3') (5'-CGGGTCTTTGCAGTCGTATG-3' and 5'-GCGAAAGGAAAGAAAGCGTC-3') (Agilent Technologies). qPCR reaction mixtures were assembled by 4 μL LightScanner® Mastermix (Idaho Technologies), 1 μL of 3.76 μM primer sets, 3 μL nuclease-free water and 2 μL of isolated cfDNA. The cfDNA isolated by both the EQ and NpM$_{V1/2}$ kits was corrected for the lower elution volume in the same manner as described above. The concentrations of the cfDNA generated by Qubit analysis were not taken into account when loading the samples. Final-volume reactions of 10 μL were placed in a Cobas® 4800 System (Roche). The thermal cycling conditions for the 105 bp fragment were as follows: 95°C for 2.5 min, 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 3 min. The conditions for the 236 bp fragment were similar, with the exception of 50 cycles of amplification instead of 45. The Lightcycler® 480 SW was used to calculate the quantitation cycle (Cq).

Statistical analyses

The difference in isolation efficiency and *KRAS* mutation detection of the QIA kit versus the newly launched cfDNA isolation kits was determined by the comparison of the amount of total cfDNA and the percentage of *KRAS*-mutated ctDNA, respectively. CfDNA integrity of the cfDNA isolation kits was established by the comparison of the Cq values of both the short and long fragments of *GAPDH* isolated by the newly launched cfDNA isolation kits versus the QIA kit. Additionally, the Cq values of the short fragments were compared to those of the long fragments within each cfDNA isolation kit. This difference

in Cq values was also compared between the QIA and the other kits. The Mann Whitney U-test was performed using the IBM SPSS Statistics version 23.0 to assess whether there are statistical significant differences ($p \leq 0.05$) in isolation efficiency, *KRAS* mutation detection and cfDNA integrity.

Results

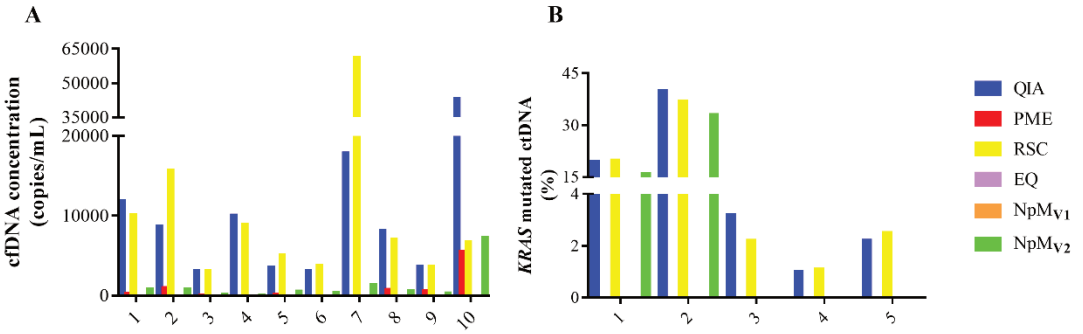
Isolation efficiency

The isolation efficiency of four newly launched cfDNA isolation kits (PME, RSC, EQ, $NpM_{V1/2}$) were compared to the QIA kit. DdPCR was used to quantify both the total cfDNA as well as the *KRAS*-mutated ctDNA fraction, with the results reported as copies/µL. The RSC kit displayed similar cfDNA yields as the QIA kit, both significantly exceeding the signals generated by cfDNA isolated by the PME and NpM_{V2} kit (Figure $1_{A\&C}$). An average yield of 56.49 (QIA), 62.57 (RSC, p=0.853), 4.06 (PME, p<0.0005) and 6.08 (NpM_{V2}, p=0.001) copies/µL was detected. In three cases the cfDNA isolation yield generated by the PME kit was too low to be detected by ddPCR. Two of these samples were taken from patients with resectable PDAC and one sample from a patient with metastasized disease. No amplification was detected in the samples isolated by the EQ and NpM_{V1} kits.

KRAS mutation detection

DdPCR was used to detect the presence of seven *KRAS* mutations (G12A/C/D/R/S/V and G13D) and one specific wild type sequence within exon 2 in the samples. Mutational analysis of the blood and tissue samples (if available), was performed previously. *KRAS* mutations were found in all patients with metastasized disease. Only one patient with resectable disease carried a *KRAS* mutation, which was not present in the blood. *KRAS* mutations were detected in all samples of stage IV PDAC patients by the QIA and the RSC kits, with the mutated fraction ranging from approximately 1 to 40% (p=1.000). The NpM_{V2} kit was able to detect *KRAS* mutations in two samples with the highest mutational load (p=0.245), whereas the PME kit did not detect any *KRAS*-mutated ctDNA (Figure

1.B). On the other hand, none of the samples isolated by the EQ and the NpM$_{V1}$ kit generated any positive signals (Table 3).



C

	CfDNA (copies/mL)										*KRAS* allele frequency (%)				
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5
QIA	11820	8700	3080	10020	3540	3080	17800	8100	3640	43200	19.5	40	3.2	1	2.2
PME	240	940	46	0	160	0	0	700	580	5460	0	0	0	0	0
RSC	10120	15680	3100	8880	5020	3740	61200	7020	3680	6700	19.9	37	2.2	1.1	2.5
EQ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NpM$_{v1}$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NpM$_{v2}$	760	840	132	46	520	380	1360	560	280	7280	16	33	0	0	0

Figure 1: Comparison cfDNA isolation kits. A Comparison of four cell-free DNA (cfDNA) isolation methods with the amount of total cfDNA in copies/mL. The high yields of samples 7 and 10, generated by the RSC and QIA kits, respectively, are significantly different from the yields generated by the other kits. This is most likely because of contamination of these samples by genomic DNA of the buffy coat. No signals were generated by the samples isolated by the EQ and NpM$_{V1}$ kits; **B** The percentage of *KRAS*-mutated cell-free tumor DNA. None of the samples isolated by the PME, EQ, and NpM$_{V1}$ kit generated a signal: **C** Overview of values displayed in **A** and **B**. *EQ: EpiQuick™ circulating cell-free DNA isolation kit; NpM$_{V1}$/NpM$_{V2}$: two consecutive versions of the NEXTprep-Mag™ cfDNA isolation kit; PME: PME free-circulating DNA extraction kit; QIA: QIAamp® circulating nucleic acid kit; RSC: Maxwell® RSC ccfDNA plasma kit.*

CfDNA integrity

CfDNA integrity was determined based on qPCR reactions of 105 and 236 bp fragments (Figure 2). All samples isolated by the QIA, PME, RSC, NpM$_{V1}$ and NpM$_{V2}$ kit displayed amplification of both fragments. Only two of the samples isolated by the EQ kit displayed Cq values, with the only signal detected corresponding to either the 105 bp or the 236 bp fragment. The Cq values of the 105 bp and 236 bp fragments of the QIA and the RSC kit were similar (p=0.762 and p=0.119, respectively). The fluorescent signals generated by the fragments isolated by the PME, NpM$_{V1}$ and NpM$_{V2}$ were all significantly higher for the 105 bp and lower for the 236 bp fragment in comparison to those isolated by the QIA kit

(Table 3). Furthermore, the difference in Cq values between the 105 bp and 236 bp fragments is similar between the QIA and the RSC kit (p=0.112) and smaller in the PME, EQ, NpM_{V1}, and NpM_{V2} kits in comparison to the QIA kit (Table 3). This demonstrates a lower isolation efficiency of short fragments compared to the QIA kit. Intriguingly, all the samples isolated by the NpM_{V1} kit displayed significant fluorescent signals, with the Cq values of the 105 bp and 236 bp fragments similar to those of the NpM_{V2} kit (p=0.821 and p=0.677, respectively). DdPCR analysis did not generate any signals for the samples isolated by the NpM_{V1} kit, which suggests the presence of ddPCR inhibiting agents.

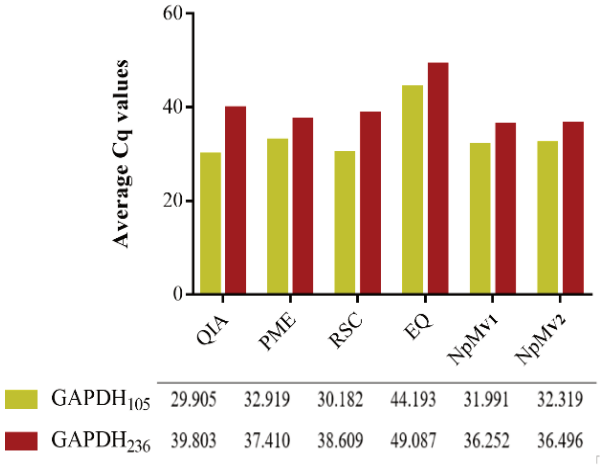


Figure 2: Comparison of the average quantitation cycle (Cq) values of the 105 bp and 236 bp fragments generated by the cfDNA isolation kits.

The highly deviating average Cq values of the EQ kit are because of the fact that for each fragment only one sample generated a significant fluorescent signal. The samples are expected to differ from each other, because they can originate from patients with metastatic of early-stage disease, or from patients with benign pancreatic cysts. Statistical analysis was performed using all samples. *EQ: EpiQuick™ circulating cell-free DNA isolation kit; GAPDH: glyceraldehyde-3 phosphate dehydrogenase; NpM_{V1}/NpM_{V2}: two consecutive versions of the NEXTprep-Mag™ cfDNA isolation kit; PME: PME free-circulating DNA extraction kit; QIA: QIAamp® circulating nucleic acid kit; RSC: Maxwell® RSC ccfDNA plasma kit.*

Table 3

Overview of cfDNA analysis

Kit	Digital Droplet PCR		Real-time qPCR			
	Average yield of total cfDNA [copies/mL]	Average percentage of *KRAS*-mutated ctDNA [%]	Average yield of $GAPDH_{105}$ [Cq]	Average yield of $GAPDH_{236}$ [Cq]	Average yield of $GAPDH_{105vs236}$ [Cq]	Difference of average yield of $GAPDH_{105-236}$ [Cq]
QIA (reference	11,298	13.18	29.905	39.803	$p = 0.001$	-9.898
PME	812.6 ($p<0.0005$)	0 ($p=0.013$)	32.919 ($p = 0.007$)	37.41 ($p = 0.028$)	$p = 0.001$	-4.491 ($p<0.001$)
RSC	12,514	12.54	30.182	38.609	$p < 0.001$	-8.427
EQ	/	/	44.193 ($p < 0.001$)	49.087 ($p < 0.001$)	$p < 0.001$	-4.894 ($p=0.001$)
NpM_{V1}	/	/	31.991 ($p = 0.002$)	36.252 ($p = 0.01$)	$p < 0.001$	-4.261 ($p<0.001$)
NpM_{V2}	1,215.8 ($p=0.001$)	9.8	32.319 ($p = 0.008$)	36.496 ($p = 0.002$)	$p = 0.001$	-4.177 ($p<0.001$)

Results were statistically significant when $p \leq 0.05$.

Cq: quantitation cycle; EQ: EpiQuick™ circulating cell-free DNA isolation kit; NpM_{V1}/NpM_{V2}: two consecutive versions of the NEXTprep-Mag™ cfDNA isolation kit; PME: PME free-circulating DNA extraction kit; QIA: QIAamp® circulating nucleic acid kit; RSC: Maxwell® RSC cfDNA plasma kit; /: no results obtained

Discussion & Conclusion

To be able to use cfDNA as a biomarker for cancer diagnosis and monitoring, reproducible and standardized methods for isolation are crucial. Since the increasing interest in cfDNA, various non-cfDNA specific isolation kits have been compared to commercially available or lab-made cfDNA isolation kits. At present the QIA kit has been reported to produce high cfDNA yields [7, 14, 15]. In this study we compared the QIA kit with four newly launched, specific cfDNA isolation kits, which are based on various methods of cfDNA recovery, as mentioned earlier. The protocol of the QIA, RSC, NpM_{V1} and NpM_{V2} kit consists of lysis of the sample before binding to either the column of magnetic beads. In this way they might also capture the nucleic acids which are adhered to proteins or trapped in vesicles and/or exosomes. According to literature, tumor-derived exosomes not only contain mRNAs and microRNAs, but also double stranded DNA [16]. Hence, we believe that this lysis step and consequent binding of more (cf)DNA, is responsible for the higher isolation efficiency of the QIA and the RSC kit, and to a lesser extent the NpM_{V2} kit, versus the PME kit. Furthermore, the presence of seven *KRAS* mutations in the isolated ctDNA was investigated. The QIA and RSC kit were able to detect *KRAS* mutations in all the stage IV PDAC patients. However, none of the isolation kits were able to detect a *KRAS* mutation in the ctDNA of the stage IIB PDAC patient whose tumor tissue was mutated. CtDNA analysis in early disease stages is associated with lower sensitivity due to the fact that ctDNA levels are associated with disease stage [17]. No statistical significant difference was found in total cfDNA yield, *KRAS*-mutated ctDNA fraction and Cq values between the QIA and RSC kits. Despite the fact that the NpM_{V2} kit failed to detect *KRAS* mutations in three of the five samples, no statistical significant difference was found either. The MAF of all three samples classified as wild type was below 5%, whereas the other two samples had a high mutational load (sample 1 and 2). Due to the lower isolation efficiency of the NpM_{V2} kit compared to the QIA kit, there is less cfDNA, and consequently less mutated ctDNA, isolated. In this manner, the concentration of the mutated fraction is too low to be detected. We expect that if the number of samples is increased, the yield of the *KRAS* mutational fraction isolated by the NpM_{V2} kit would most likely be significantly different from that of the QIA kit. The lower isolation efficiency might also explain the false

negative results generated by the PME kit. Interestingly, a higher prevalence of cancer-associated DNA mutations has been reported among shorter fragments [6, 18]. Both the PME and NpM_{V1} kit isolated less small fragments than the QIA kit, which might also explain the lower *KRAS* mutation detection. Hence, the NpM_{V2} and PME kit are less suited for detection of low mutated fractions such as in the case of early disease detection[17] or early detection of resistance [19]. The samples isolated by the NpM_{V1} kit did not generate any result in the ddPCR analysis, however, the Cq values for both the 105 and 236 bp amplicon were similar to those of the NpM_{V2} kit. Therefore, we believe that the eluate of the NpM_{V1} kit contains ddPCR inhibiting agents, which do not interfere with qPCR analysis. Given that ddPCR analysis of samples isolated by the EQ kit generated no signal and qPCR analysis resulted in only two positive signals, the eluate might hinder multiple PCR-based methods. However, the absence of signal might also be due to the fact that there was only a low amount of cfDNA isolated. Magnetic beads-based manual protocols are associated with an extensive learning process, making it less interesting for fast implementation in a clinical setting. This might also play a role in the lower cfDNA isolation efficiency of the NpM_{V2} kit in comparison to the QIA kit. CfDNA has become an interesting biomarker for cancer since mutational analysis of ctDNA allows for the detection of (actionable) mutations present in the tumors [20]. To be able to detect these cancer-associated mutations, highly sensitive PCR-based methods have been developed, such as ddPCR [21], beads, emulsion, amplification and magnetics (BEAMing) [22] and multiplexed PCR coupled with next-generation sequencing (mPCR-NGS) [23]. The presence of PCR inhibiting agents in the cfDNA isolate poses a difficulty for mutational analysis. We demonstrated that ddPCR as a downstream procedure is not feasible with the first version of the NEXTprep-Mag™ cfDNA Isolation kit (NpM_{V1} – SanBio B.V.) and the Epiquick™ Circulating Cell-Free DNA Isolation Kit (EQ – Epigentek). The use of plasma samples from patients instead of plasma from healthy subjects, spiked with mutated DNA generated from cell lines provides us with an ideal matrix which enables us to make valid conclusions despite our limited sample size.

This study presents two highly efficient isolation kits, namely the QIAamp® circulating nucleic acid kit (QIA – Qiagen) and the Maxwell® RSC ccfDNA Plasma Kit (RSC – Promega), of which the RSC kit has the advantage of a fully automated, magnetic beads

based protocol over the labor intensive QIA kit. We can also conclude that the choice of downstream ctDNA analysis is important. Some isolation kits might not be compatible with certain analysis techniques. Besides the need for high isolation efficiency, a minimal and easy workflow is indispensable, especially with regards to the implementation of cfDNA analysis in a clinical setting.

Acknowledgements

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Supplementary data

Supplementary Table 1

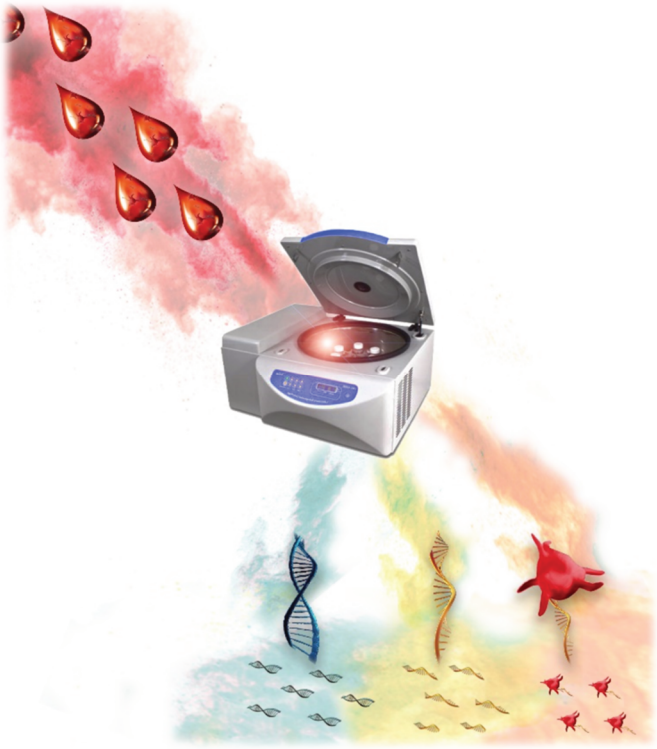
Qubit analysis

Sample	QIA	PME	RSC	EQ	NpM_{V1}	NpM_{V2}
1	0.976	0.988	0.456	/	0.280	0.227
2	1.370	1.190	0.716	0.170	0.556	0.269
3	0.277	1.030	0.113	0.092	0.086	0.048
4	0.556	1.280	0.300	0.101	0.084	/
5	0.218	1.110	0.157	0.121	0.488	0.123
6	0.318	0.996	0.152	0.100	0.408	0.098
7	0.668	1.200	2.030	0.060	2.00	0.456
8	1.820	1.590	8.520	1.580	1.220	1.000
9	1.110	0.712	1.880	1.660	0.708	0.712
10	2.460	1.780	28.800	2.290	3.630	9.160

Concentration of the isolated cfDNA in ng/μL generated by EQ: EpiQuick™ circulating cell-free DNA isolation kit; GAPDH: glyceraldehyde-3 phosphate dehydrogenase; NpM_{V1}/NpM_{V2}: two consecutive versions of the NEXTprep-Mag™ cfDNA isolation kit; PME: PME free-circulating DNA extraction kit; QIA: QIAamp® circulating nucleic acid kit; RSC: Maxwell® RSC ccfDNA plasma kit.

CHAPTER 4

Circulating cell-free DNA and RNA Analysis as Liquid Biopsy: Optimal Centrifugation Protocol.



Sorber L, Zwaenepoel K, Jacobs J, De Winne K, Goethals S, Reclusa P, Van Casteren K, Augustus E, Lardon F, Roeyen G, Peeters M, Van Meerbeeck J, Rolfo C, Pauwels P. Cancers, Volume 11, Issue 4, 2019.

Abstract

The combined analysis of circulating cell-free (tumor) DNA (cfDNA/ctDNA) and circulating cell-free (tumor) RNA (cfRNA/ctRNA) shows great promise in determining the molecular profile of cancer patients. Optimization of the workflow is necessary to achieve consistent and reproducible results. In this study, we compared five centrifugation protocols for the optimal yield of both cfDNA/ctDNA and cfRNA/ctRNA. These protocols varied in centrifugation speed, ambient temperature, time, and number of centrifugation steps. Samples from 33 participants were collected in either BD Vacutainer K_2EDTA (EDTA) tubes or cell-free DNA BCT® (Streck) tubes. cfDNA concentration and fragment size, and cfRNA concentration were quantitated in all samples by digital droplet PCR (ddPCR) and quantitative PCR (qPCR). The *KRAS*-mutated ctDNA and ctRNA fraction was determined via ddPCR. In EDTA tubes, the protocol generating both plasma and platelets was found to produce high quality cfDNA and cfRNA concentrations. Two-step, high-speed centrifugation protocols were associated with high cfDNA but low cfRNA concentrations. High cfRNA concentrations were generated by a one-step, low-speed protocol. However, this coincided with a high amount of genomic DNA (gDNA) contamination. In Streck tubes, two-step, high-speed centrifugation protocols also generated good quality, high cfDNA concentration. However, these tubes are not compatible with cfRNA analysis.

Introduction

The analysis of circulating cell-free (tumor) DNA (cfDNA/ctDNA) as a liquid biopsy has shown tremendous potential in many aspects of oncology, ranging from minimally invasive molecular profiling and treatment monitoring to the detection of resistance mutations [1]. Several limitations of ctDNA-based liquid biopsies have been addressed over the last few years. Complications include the short half-life of cfDNA [2] as well as wild type (WT) DNA contamination from leukocyte lysis [3]. In order to detect the often-low concentration and highly variable ctDNA fraction, several techniques with high analytical sensitivity and specificity have been developed [4]. Furthermore, blood preservation tubes have been designed, which stabilize white blood cells and inhibit nuclease activity [5]. ctDNA has been established as an excellent source of mutation and methylation analysis [4], however, it might be less appropriate for the detection of gene rearrangements. Even though the group of Russo *et al.* were successful in detecting gene-fusions in ctDNA [6], extensive deep sequencing or break-point encompassing targeted assays are necessary to detect rearrangements. Circulating cell-free (tumor) RNA (cfRNA/ctRNA) analysis could complement ctDNA in determining the molecular profile. Analysis of cfRNA in circulation or embedded in vesicles or tumor-educated platelets (TEPs) has shown great potential in the detection of several cancer-associated aberrations [7-9]. Standardization of the liquid biopsy workflow is necessary for clinical implementation [10]. Several studies have been performed to determine optimal pre-analytical conditions for cfDNA [11-13] and, to a lesser extent, for cfRNA analysis [14]. However, studies which address the generation of both cfDNA and cfRNA are lacking. In this study, we compared five centrifugation protocols with varying centrifugation speed, temperature, time, and number of centrifugation steps for the optimal cfDNA and cfRNA yield. These protocols were evaluated in two of the most commonly used blood collection tubes, namely BD Vacutainer K_2EDTA tubes (EDTA tubes; BD, Erembodegem, Belgium) and cell-free DNA BCT® tubes (Streck tubes; Streck, Biomedical Diagnostics, Antwerp, Belgium). To this purpose, both cfDNA and cfRNA were isolated from healthy volunteers and patients with *KRAS*-mutated metastatic cancer. CfDNA concentration and fragment size, and

cfRNA concentration were quantitated in all samples. The fraction of *KRAS*-mutated ctDNA and ctRNA was also determined.

Material and Methods

Study Design

In total, 15 patients with *KRAS*-mutated advanced cancer and 18 healthy volunteers were included. Each participant contributed five 10 mL blood samples, which were collected in either EDTA (n = 95) (BD, Erembodegem, Belgium) or Streck (n = 70) (Streck, Biomedical Diagnostics, Antwerp, Belgium) tubes. In the former, samples were processed within 2 h after blood collection, whereas Streck tubes were left at room temperature for 24 h before plasma generation. The study was conducted according to the Declaration of Helsinki, and ethical committee approval (B300201422715) was obtained from the ethical committee of Antwerp University Hospital (UZA). All participants provided written informed consent.

This study consisted of three phases (Table 1). Healthy volunteers were included in phase one. In phase two, the blood samples from healthy volunteers were spiked with *KRAS*-mutated tumor DNA in order to simulate ctDNA. DNA was isolated from *KRAS*-mutated NSCLC formalin-fixed paraffin embedded (FFPE) tissue samples, because in contrast to high-quality cell line-derived DNA, the fragmented and partly degraded properties of FFPE-derived DNA are a more suitable ctDNA alternative. The resulting spike mix consisted of 900 mutated DNA copies per μL, of which 1 μL was injected into the blood samples immediately after blood collection. The third phase consisted of known *KRAS*-mutated NSCLC, colorectal cancer (CRC), and pancreatic ductal adenocarcinoma (PDAC) patients to validate our findings. Total cfDNA and cfRNA concentration and cfDNA fragment analysis were investigated in all phases.

Table 1

Study cohort

Phase	Participants	EDTA (N = 19)		Streck (N = 14)	
		cfDNA samples	**cfRNA samples**	**cfDNA samples**	**cfRNA samples**
1	Healthy volunteers • cfDNA: total cfDNA concentration quantification & fragment analysis; • cfRNA: total cfRNA concentration quantification.	n° = 48	n° = 55	n° = 12	n° = 7
2	Healthy volunteers – spiked with KRAS-mutated DNA • cfDNA: total cfDNA concentration quantification & fragment analysis; • spiked tumor DNA: tDNA concentration & allele frequency quantification; • cfRNA: total cfRNA concentration quantification.	n° = 24	n° = 28	n° = 24	n° = 28
3	*KRAS*-mutated metastatic cancer patients • cfDNA: total cfDNA concentration quantification & fragment analysis • ctDNA: ctDNA concentration & allele frequency quantification • cfRNA: total cfRNA concentration quantification • ctRNA: ctRNA concentration & allele frequency quantification	n° = 42	n° = 47	n° = 48	n° = 53

CfDNA: circulating cell-free DNA; cfRNA: circulating cell-free RNA; ctDNA: circulating cell-free tumor DNA; ctRNA: circulating cell-free tumor RNA; N: number of subjects; n°: number of samples; tDNA: tumor DNA.

Plasma was generated from matched blood samples with different centrifugation protocols varying in centrifugation speed, temperature, time, and number of centrifugation steps (Table 2). This study was performed in collaboration with the Biobank UZA/UAntwerpen. Therefore, the standard (CP_{Basic}) protocol from the Biobank UZA/UAntwerpen was included. As most studies recommend two centrifugation steps [12, 13, 15], an adapted version of this protocol was also added, of which both the plasma ($CP_{AdBasic}$) and resulting pellet ($CP_{AdBasic\_P}$) were analyzed. We also selected the "Isolated circulating cell-free DNA from plasma protocol, CEN/TS 16835-3" as advised by the European committee for standardization (CEN; CP_{CEN}). This protocol requires for blood samples to be put on ice immediately after blood collection and centrifugation at 4 °C. The adapted protocol (CP_{AdCEN}) can be completely performed at room temperature. As Streck tubes are not

compatible with low temperatures, we included the manufacturer's instructed protocol for Streck tubes (CP_{Streck}) [16]. Lastly, we selected a centrifugation protocol which produces both plasma (CP_{Plat}) and platelets (CP_{Plat_P}) [7]. Plasma samples were stored at -80 °C by the Biobank UZA/UAntwerpen (Antwerp, Belgium; ID: BE71030031000; Belgian Virtual Tumourbank funded by the National Cancer Plan, BBMRI-ERIC; No. Access: 1; Last: 13 June 2018) [17].

Table 2

Centrifugation protocols for cfDNA / cfRNA analysis

ID	Protocol	Specifications	Details	Temperature	Matrix
CP_{Basic}	Basic (Biobank UZA/UAntwerpen)	1. 10' – 400 g		RT	Plasma
$CP_{AdBasic\_P}$ **$CP_{AdBasic}$**	Basic (adapted)	1. 10' – 400 g 2. 1' – max speed	2nd centrifugation step after storage at -80°C	RT	Pellet Plasma
CP_{Plat} **CP_{Plat_P}**	Platelet	1. 20' – 120 g 2. 20' – 360 g 3. 5' – 360 g	Platelets are washed with PBS in 3rd centrifugation step	RT	Plasma Platelets
CP_{Streck}	Streck	1. 10' – 1.600 g 2. 10' – 6.000 g		RT	Plasma
CP_{CEN}	CEN	1. 10' – 1.900 g 2. 10' – 16.000	Blood samples on ice immediately after blood collection	4°C	Plasma
CP_{AdCEN}	CEN (adapted)	1. 10' – 1.900 g 2. 10' – 16.000		RT	Plasma

A smooth braking profile was used in all centrifugation protocols to prevent disruption of the buffy coat layer. Plasma (and platelets) from each centrifugation protocol was snap frozen and stored at -80°C. *CEN: European Committee for Standardization; cfDNA: circulating cell-free DNA; cfRNA: circulating cell-free RNA; CP: centrifugation protocol; Platelets: only RNA isolation; RT: room temperature; 1: first; 2: second, and; 3: third centrifugation step, respectively.*

cfDNA Isolation and Analysis

cfDNA isolation was performed using the Maxwell RSC ccfDNA large volume plasma kit (Promega, Leiden, The Netherlands) according to the manufacturer's protocol. Detailed procedures are described elsewhere [18]. Plasma samples were processed immediately after thawing, with the exception of the adapted basic protocol. Prior to processing, the plasma was centrifuged at maximum speed for one minute. The plasma was separated from the resulting pellet, which was hereafter dissolved in 1 mL nuclease-free water. For cfRNA, 0.5 mL of all plasma samples and the dissolved pellet were reserved.

Samples were screened with the ddPCR *KRAS* Screening Multiplex Kit (Bio-Rad, Temse, Belgium) to determine total cfDNA concentration and *KRAS* mutational status. In total, 10 µL of template was used per sample. Detailed procedures can be found elsewhere [19]. The concentration of these samples was not determined prior to ddPCR analysis. Total cfDNA was also quantified by a qPCR assay consisting of an 82-base pair (bp) *Long Interspersed Nuclear Elements* (*LINE-1*) sequence [20]. The samples were brought to the same concentration for cfDNA sample quality assessment using a 425:82 bp ratio. The 425 bp amplicon was designed to encompass the 82 bp amplicon; representing mainly genomic DNA released from blood cells and cfDNA, respectively. Hence, a high ratio is indicative of gDNA contamination [21]. A long fragment contamination curve consisting of cell line and digested FFPE tissue material was used to determine the percentage of long fragment contamination. All samples were run in duplicate. qPCR mixtures were assembled by 4 µL LightScanner Mastermix (Bioké, Leiden, The Netherlands), 1 µL of 1 µM primer sets, and 5 µL (diluted) cfDNA. Cycle conditions were 95 °C for 2.5 min, then 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min cycled 45 times, and 72 °C for 3 min. The LightCycler 480 SW (Roche, Vilvoorde, Belgium) was used to calculate the quantitation cycle (Cq).

cfRNA Isolation and Analysis

cfRNA was isolated from 200 µL of plasma or directly from platelets and converted to complement DNA (cDNA) using the miRNeasy Serum/Plasma Kit and miScript II RT Kit, respectively, as per manufacturer's instructions (Qiagen, Antwerp, Belgium). Samples were analyzed using the ddPCR *KRAS* Screening Multiplex kit in the same manner as the

cfDNA samples, with the exception of the platelet cDNA. Only 1 μL of template was used instead of 10 μL.

Statistical Analysis

The Kruskal–Wallis test was performed to ascertain whether there was a significant difference between the centrifugation protocols and blood collection tubes for total cfDNA and cfRNA yield, percentage of long DNA fragments, and *KRAS*-mutated ctDNA fraction. The Mann–Whitney U test was used to determine the effect of the individual centrifugation protocols. Statistical analysis was performed using IBM SPSS Statistics version 24.0 (IBM, Brussels, Belgium). p-values of <0.05 were considered statistically significant. Figures were made with Graphpad Prism7 (Graphpad Software Inc., La Jolla, CA, USA).

Results

In this study, five centrifugation protocols were compared for the optimal yield of both cfDNA/ctDNA and cfRNA/ctRNA in either EDTA or Streck tubes (Table 2).

Total cfDNA Concentration and Characteristics

The cfDNA yield, as well as genomic DNA (gDNA) contamination, were examined per centrifugation protocol. Significant differences in total cfDNA concentration were observed by digital droplet PCR (ddPCR) ($p < 0.001$ and $p = 0.004$) and quantitative PCR (qPCR) analysis ($p = 0.001$ and $p = 0.017$) in both EDTA and Streck tubes, respectively. Centrifugation protocols were also found to influence DNA integrity in EDTA ($p < 0.001$) and Streck tubes ($p < 0.001$), as determined by fragment size analysis. The individual differences between these protocols was further investigated (Figure 1; individual *p*-values are provided in Table S1).

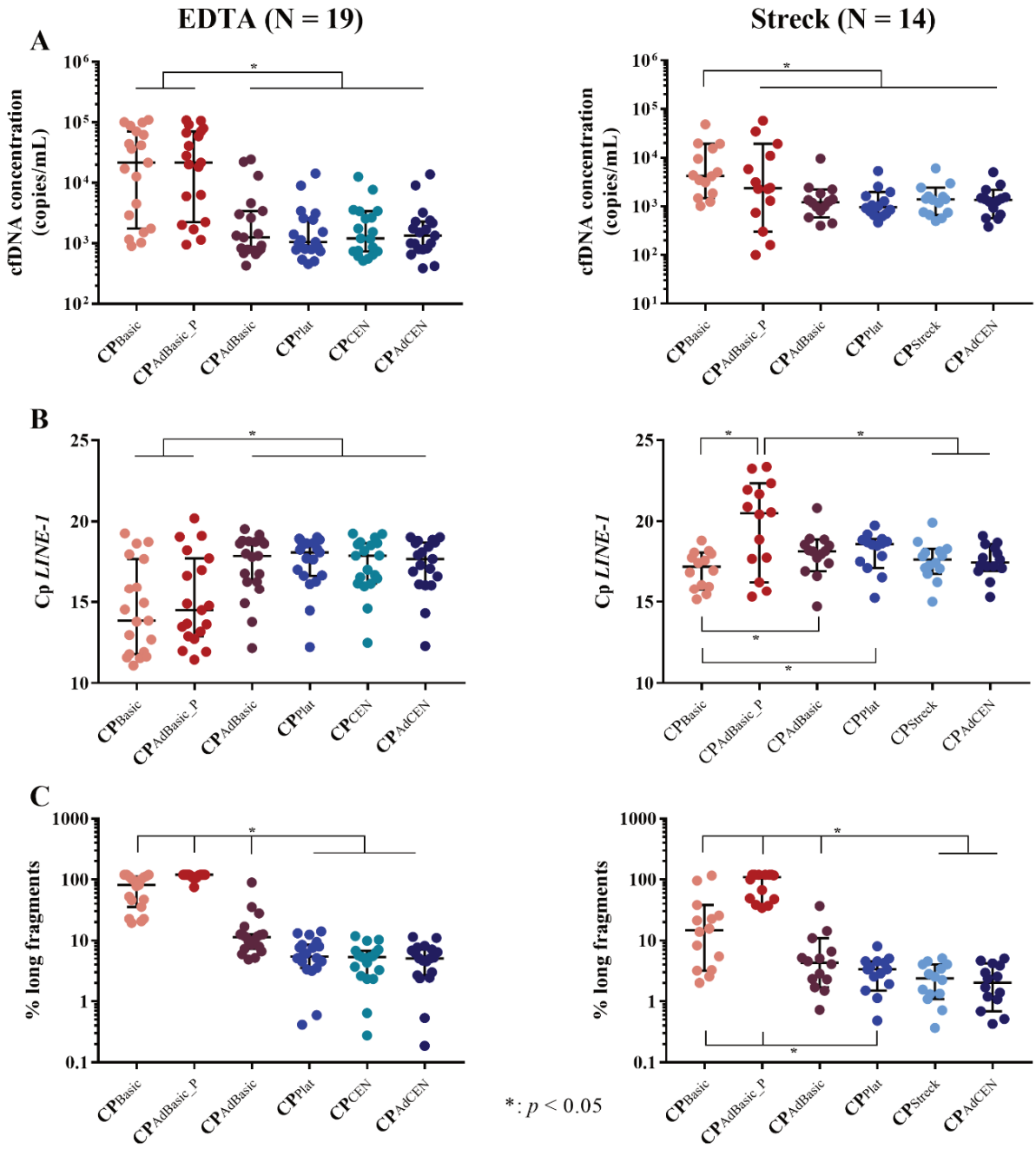


Figure 1. Comparison of total cfDNA yield per centrifugation protocol in EDTA and Streck tubes. In all samples, **A** digital droplet PCR (ddPCR) (copies/mL) and **B** *LINE-1* 82 bp quantitative PCR (qPCR) were performed to determine total cfDNA concentration, as well as **C** cfDNA integrity analysis by qPCR to determine the percentage of long (> 400 bp) fragments. *N: number of participants.*

cfDNA Yield

DdPCR analysis revealed that the highest cfDNA concentrations in EDTA tubes were generated by the basic (CP_{Basic}; one-step, low-speed) centrifugation protocol and the pellet of the adapted basic protocol ($CP_{AdBasic\_P}$). No significant differences were observed between the other centrifugation protocols (Figure 1_A). Similar results were observed by qPCR analysis (Figure 1_B). In Streck tubes, ddPCR analysis revealed that the basic centrifugation protocol (CP_{Basic}) alone was responsible for the highest cfDNA concentration. The pellet of the adapted basic protocol ($CP_{AdBasic\_P}$) generated a very variable cfDNA yield and did not differ from the other centrifugation protocols (Figure 1_A). More differences were observed with qPCR (Figure 1_B). cfDNA concentration seemed to be similar between the basic protocol (CP_{Basic}), the Streck (CP_{Streck}), and adapted CEN (CP_{AdCEN}) protocols. However, while the basic protocol had a significantly higher cfDNA yield than the platelet-generating protocol (CP_{Plat}), the plasma ($CP_{AdBasic}$), and pellet ($CP_{AdBasic\_P}$) of the adapted basic protocol, only the latter ($CP_{AdBasic\_P}$) was found to be lower than that of the Streck (CP_{Streck}) and adapted CEN (CP_{AdCEN}) protocols.

cfDNA Integrity

Next, we investigated DNA integrity (Figure 1_C). In EDTA tubes, the pellet of the adapted basic protocol ($CP_{AdBasic\_P}$) generated the highest concentration of long fragments (gDNA contamination), followed by the basic protocol (CP_{Basic}) itself. The plasma of the adapted basic protocol generated significantly fewer long fragments, but still retained higher concentrations than the other centrifugation protocols. Interestingly, no differences in DNA integrity were detected between the platelet generating (CP_{Plat}) and (adapted) CEN ($CP_{(Ad)CEN}$) protocols, despite their differences in centrifugation speed and time.

Similar results were detected in Streck tubes. The highest concentration of long fragments was detected in the pellet of the adapted basic protocol ($CP_{AdBasic\_P}$), followed by the basic protocol (CP_{Basic}). However, there was no significant difference in DNA integrity between the plasma of the adapted basic ($CP_{AdBasic}$) and the platelet-generating (CP_{Plat}) protocol. As seen in EDTA tubes, the two-step, high-speed (adapted CEN (CP_{AdCEN}), Streck (CP_{Streck})) protocols, as well as the two-step, low-speed, platelet-generating protocol (CP_{Plat}), were found to generate good-quality cfDNA (low amounts of long fragments). Even though

comparing the performance of EDTA and Streck tubes was not part of the aim of this study, a significant difference in cfDNA concentration and DNA integrity per centrifugation protocol was detected between these tubes, with the exception of the tube specific protocols (CP_{CEN} and CP_{Streck}). The pellet of the adapted basic protocol ($CP_{AdBasic\_P}$) generated significantly higher cfDNA concentrations in EDTA tubes according to ddPCR ($p = 0.015$) and qPCR ($p < 0.001$) analysis. Similar distributions were observed in the basic protocol (CP_{Basic}), although, only by qPCR analysis ($p = 0.013$). In contrast, DNA integrity was found to be significantly different in all centrifugation protocols, except for the pellet of the adapted basic protocol ($CP_{AdBasic\_P}$) (Figure 2; individual *p*-values are provided in Table S2). Finally, a higher percentage of long fragments was detected in EDTA tubes compared to Streck tubes.

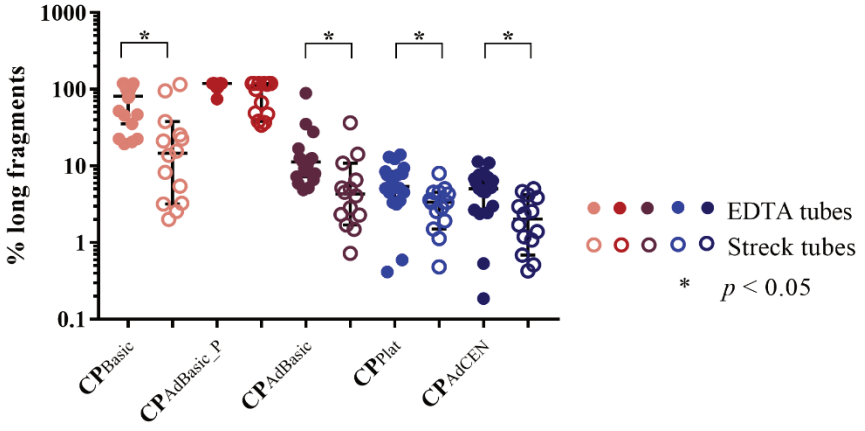


Figure 2. cfDNA integrity in EDTA (N = 19) vs. Streck (N = 14) tubes.

N: number of participants.

KRAS-Mutated ctDNA

To investigate the effect of the centrifugation protocol on the ctDNA fraction, we quantified the *KRAS*-mutated tDNA and ctDNA in spiked samples from healthy volunteers and *KRAS*-mutated metastatic cancer patients, respectively. In a first phase, spiked *KRAS*-mutated tDNA was evaluated by ddPCR analysis in samples from 8 participants collected in EDTA (4) and Streck (4) tubes. Next, the same number of samples were collected from *KRAS*-mutated metastatic cancer patients. As the results from spiked and cancer patient samples differed significantly, it was opted to proceed with the data from cancer patient samples only (Figure 3). All samples were investigated for the presence of *KRAS* mutations. In 7 out of 8 patients, *KRAS*-mutated ctDNA could not be detected

in the pellet of the adapted basic protocol (CP*AdBasic_P*). The exception was patient 4, who had a very high mutated *KRAS* allele frequency (AF; ranging from 20.6 to 51.9% in the other protocols). No significant differences in *KRAS* ctDNA concentration or AF could be observed between the other centrifugation protocols. However, the sample generated by the basic protocol (CP_{Basic}) of patient 2 was defined as *KRAS* WT, while *KRAS*-mutated ctDNA was detected in the plasma generated by the other centrifugation protocols. Despite the number of patients, it seems obvious that both the basic protocol (CP_{Basic}) and the pellet of the adapted basic protocol ($CP_{AdBasic\_P}$) are not suitable for ctDNA analysis.

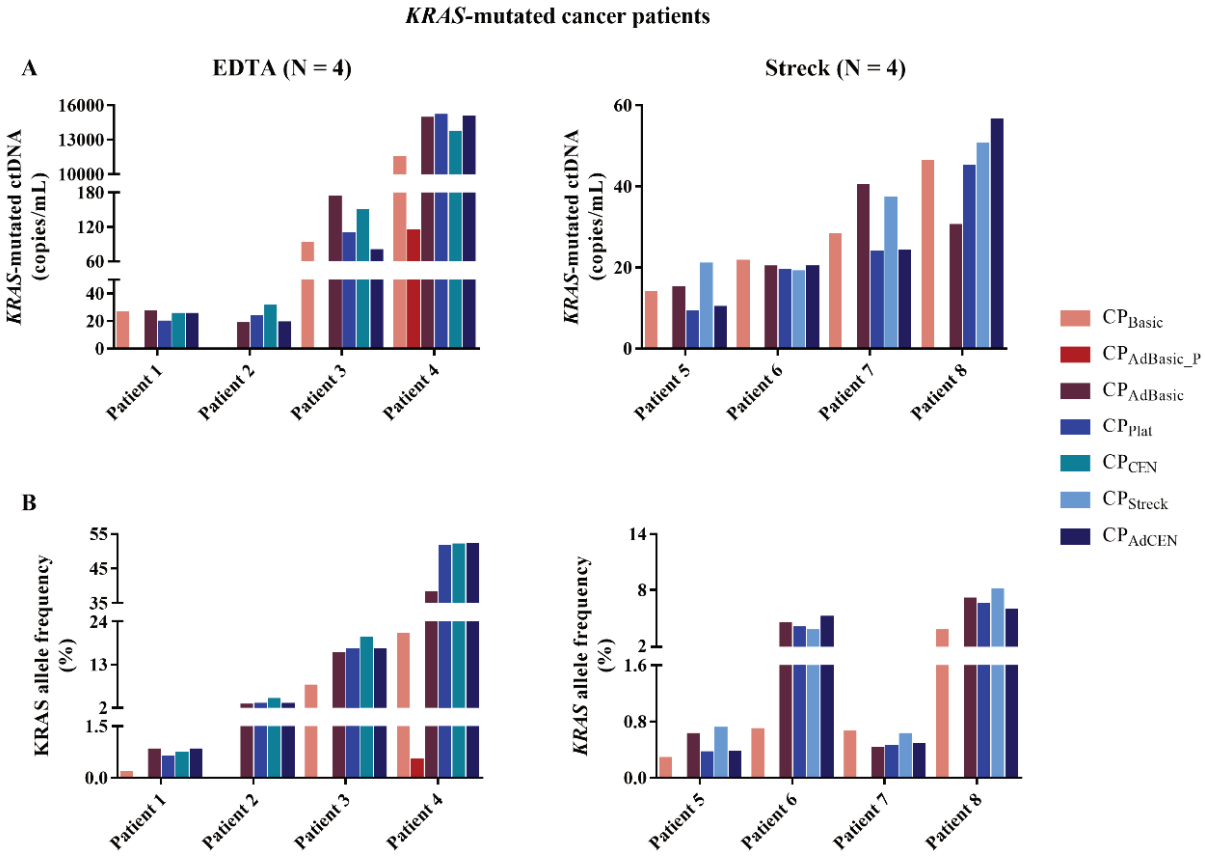


Figure 3. ctDNA analysis of *KRAS*-mutated metastatic cancer patients (8) per centrifugation protocol (Table 2) in EDTA (left) and Streck (right) tubes. A Concentration of *KRAS*-mutated ctDNA (copies/mL); **B** KRAS-mutated ctDNA allele frequency (%). *N: number of participants.*

Total cfRNA Concentration

Next, the total cfRNA yield was determined using ddPCR (Figure 4). In EDTA tubes, significant differences in cfRNA concentration were observed between the different centrifugation protocols ($p < 0.0001$; individual p-values are provided in Table S3). As expected, platelets (CP_{Plat_P}) provided the highest cfRNA yield, while the platelet-rich plasma (PRP) basic protocol (CP_{Basic}) still generated more cfRNA than the other centrifugation protocols. No differences could be observed between the pellet ($CP_{AdBasic\_P}$) and the platelet-poor plasma (PPP) of the adapted protocol ($CP_{AdBasic}$) and the platelet-generating protocol (CP_{Plat}). These protocols still generated higher cfRNA concentrations compared to the adapted and original CEN protocols ($CP_{(Ad)CEN}$), which were also found to be similar in cfRNA yield. In Streck tubes, no cfRNA or only very low concentrations could be detected across all centrifugation protocols.

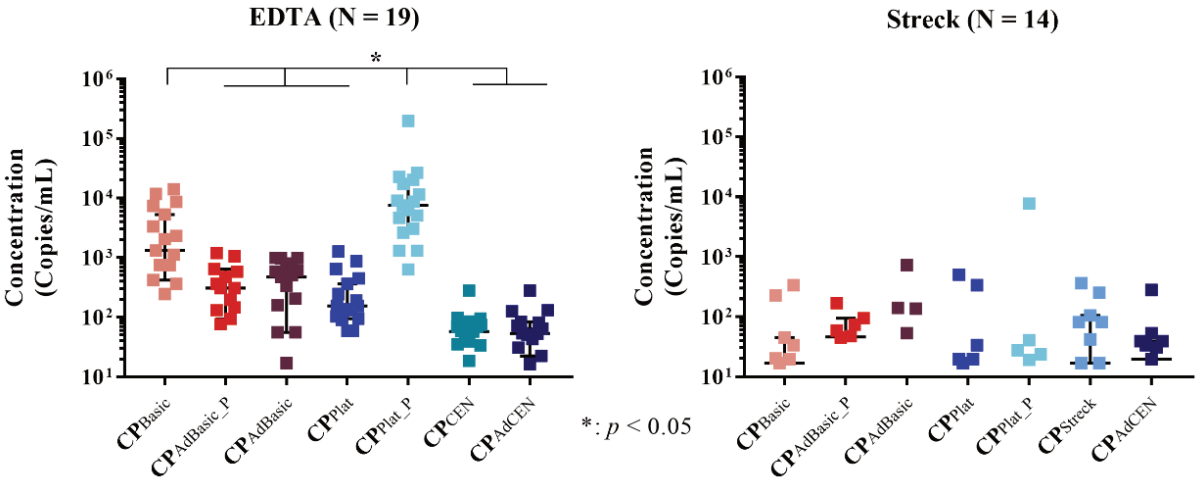


Figure 4. Comparison of cfRNA yield per centrifugation protocol in EDTA and Streck tubes. *N: number of participants.*

KRAS-Mutated ctRNA

To further investigate the effect of the centrifugation protocol on the ctRNA fraction, the samples were also screened for the presence of *KRAS* mutations. In EDTA tubes, *KRAS*-mutated ctRNA could only be detected in the platelets (CP_{Plat_P}) (Figure 5) in 3 out of 4 *KRAS*-mutated metastatic cancer patients (as depicted in Figure 3). In Streck tubes, no

KRAS-mutated ctRNA could be detected in any centrifugation protocol, as only extremely low cfRNA concentrations, or no cfRNA at all, were generated.

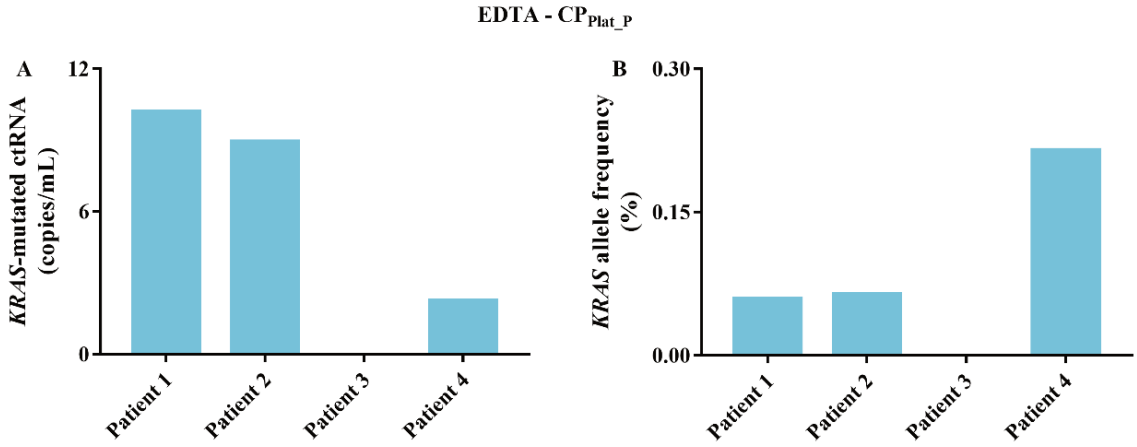


Figure 5. ctRNA analysis of *KRAS*-mutated metastatic cancer patients (4). A Concentration of *KRAS*-mutated ctRNA (copies/mL); **B** *KRAS*-mutated ctRNA allele frequency (%).

Discussion & Conclusion

The purpose of this study was to determine the optimal centrifugation protocol for the generation of high-quality cfDNA and cfRNA in two commonly used blood collection tubes; EDTA and Streck tubes. Despite the relatively small sample size of this study, significant differences in cfDNA and cfRNA yield and quality could be observed. In EDTA tubes, the plasma and platelets from the platelet-generating protocol were found to produce both good quality cfDNA and high cfRNA concentration. cfDNA analysis in Streck tubes generated similar results in all centrifugation protocols, however, at best, very low levels of cfRNA could be generated.

In EDTA tubes, the cfDNA characteristics, namely cfDNA concentration and contamination with long DNA fragments, from the platelet-generating protocol were found to be similar to those of the adapted and original CEN protocols. *KRAS*-mutated ctDNA concentration and AF also seemed similar. While the platelet-generating protocol consists of two slow-speed centrifugation steps, the (adapted) CEN protocols have a two-step, high-speed procedure. Most studies recommend a double centrifugation protocol,

consisting of an initial slow centrifugation to separate plasma from buffy coat, followed by a fast centrifugation to clear any remaining cellular material and generate high quality cfDNA [12, 13, 15]. Although there is no consensus with regards to actual centrifugation speed [22, 23], both centrifugation steps described in these studies, together with the adapted and original CEN protocols, are much faster in comparison to the platelet-generating protocol. We speculate that the longer centrifugation time (20 min versus 10 min) might be responsible for the low contamination with long DNA fragments, despite the slow centrifugation speed. In contrast, the plasma of the adapted basic protocol, generated by a first slow-speed centrifugation step, followed by a 1-min high-speed centrifugation post-thaw, was found to contain significantly more long DNA fragments. Hence, the interplay between centrifugation speed and time probably has a stronger influence on cfDNA generation than these other factors alone. We did not detect an influence of temperature, as both cfDNA characteristics and ctDNA detection were similar between the adapted and original CEN protocols. Furthermore, no differences between these four centrifugation protocols could be observed in mutated ctDNA concentration and AF. It is important to note that, despite our limited number of patients with detectable *KRAS*-mutated ctDNA, the basic protocol and the pellet of the adapted basic protocol were found to be less suitable for cfDNA analysis. The pellet of the adapted basic protocol seems to consist almost exclusively of long DNA fragments. This is to be expected, as previous research highlights that the second centrifugation step removes any remaining cellular material [12]. Hence, the resulting plasma will mostly contain the cfDNA fraction. Our data support this, as *KRAS*-mutated ctDNA was only detected in the pellet of a patient with a very high ctDNA concentration. The mutated *KRAS* ctDNA AF in the other centrifugation protocols ranged between 20.6% and 50.9%. This is highly unusual, as ctDNA is most often present in very low (<1%) percentages [2]. The plasma of the basic protocol contains both the remaining cellular material as well the cfDNA fraction, as indicated by the high percentage of long fragments. In this manner, the ctDNA is diluted by WT DNA, resulting in the classification of one patient sample as WT despite the use of the highly sensitive ddPCR technique [24]. Furthermore, there are several disorders and physiological processes, such as autoimmune disorders, exhaustive exercise, or trauma, that are known to contribute to the total cfDNA concentration [25]. This might explain the low

AF detected in all centrifugation protocols from patient 7 (Figure 3). In these cases, further dilution of the mutated ctDNA with WT gDNA would be detrimental to analytical success. In Streck tubes, cfDNA analysis revealed similar results as in EDTA tubes. Interestingly, no difference in the generated plasma volume was observed in EDTA or Streck tubes. This was unexpected, especially in the case of the Streck tubes. In a multicenter study, we observed a negative influence on plasma volume upon longer storage of blood samples in Streck tubes. Increased centrifugation speed was found to negate this effect [16, 18], which is recommended by Streck. As plasma volume is directly associated with cfDNA concentration [26], it is doubtful whether protocols based on low centrifugation speed, such as the platelet-generating and (adapted) basic protocol, will generate sufficient material for the detection of low abundant aberrations. Even though there were no significant differences in cfDNA characteristics detected between the platelet-generating protocol and the Streck and adapted CEN protocol, it seems that there was still a slight shift towards more long fragments in the former. This might explain the fact that no significant differences were observed between this protocol and the plasma of the adapted basic protocol. The Streck and adapted CEN protocols seem to be the most appropriate centrifugation protocols for cfDNA analysis in Streck tubes. Both are two-step, high-speed protocols, with a first centrifugation step of <2000 g for 10 min. However, the second step differs significantly (6000 vs. 16,000 g). This corresponds to previously reported data in which no difference in cfDNA concentration was observed between a second centrifugation step of 3000 g or >10,000 g [23]. Hence, there is no need to have access to a full spectrum of centrifuges with higher centrifugation speeds. Interestingly, significant differences in percentage of long fragments were observed between centrifugation protocols of EDTA vs. Streck tubes, with the exception of the pellet of the adapted basic protocol. Van Dessel *et al.* reported a background level of leukocyte lysis, which is responsible for low levels of gDNA [11]. Hence, leukocyte lysis might be diminished by the cell stabilizing effect of Streck tubes [16].

In EDTA tubes, the highest cfRNA concentration, as well as *KRAS*-mutated ctRNA, were detected in the platelet fraction of the platelet-generating protocol. Our data corresponds to previous research in which platelets were highlighted to be an excellent source for tumor RNA (tRNA) [7, 8]. Nilsson *et al.* also reported a higher sensitivity for the detection of tumor-associated aberrations in platelet RNA than in the plasma of the platelet-generating

protocol. Even though no *KRAS*-mutated ctRNA could be retrieved from the other centrifugation protocols, the basic protocol might be the best alternative in the absence of platelets. The proposed workflow for PPP consists of two centrifugation steps prior to freezing; namely to remove the bulk of circulating cells and the residual platelets [27]. Hence, the basic protocol most likely still contains some platelets due to its single slow-speed centrifugation procedure. Furthermore, a single freeze/thaw cycle of plasma samples containing (residual) platelets has been demonstrated to release significant quantities of miRNA and platelet microparticles [28]. In contrast, the adapted and original CEN protocols both generate PPP, resulting in the lowest cfRNA concentrations of all protocols. Similar cfRNA yields were detected between the plasma of the platelet-generating protocol, and the pellet and plasma of the adapted basic protocol, despite differences in centrifugation speed and time. These findings also highlight the importance of both factors on cfDNA and cfRNA generation. It is important to note that in this study, the entire platelet fraction was used for cfRNA isolation. This fraction was generated from the entire blood sample (10 mL). In the case of plasma samples, only 200 µL of plasma could be used, which corresponds to approximately 500 µL of blood. The pellet of the adapted basic protocol was re-dissolved in 1 mL of nuclease-free water, of which 200 µL was also is used. Hence, this represents approximately 2 mL of blood. By increasing the input volume, higher cfRNA concentrations might be obtained [29]. Recently, RNA isolation kits with increased input volumes have been developed and are currently being tested, among other pre-analytical variables, in a Belgian extracellular RNA quality control (exRNAQC) study [30]. These findings will aid in the standardization of the cfDNA- and cfRNA-based liquid biopsy workflow. Another issue that needs to be addressed is the low *KRAS*-mutated ctRNA concentration and AF compared to ctDNA. This might be the result of the reactivity of isolated platelets, or contamination with residual cells and microparticles [31]. Red blood cells have been shown to contain a plethora of RNAs [32], which might interfere with these results. Furthermore, centrifugation alone might not be sufficient to generate a pure platelet fraction. The group of Rikkert *et al.* demonstrated that the pellet of the platelet generating protocol not only contains platelets (71%), but also large (tumor-derived) extracellular vesicles (EVs; 22%), circulating tumor cells (CTCs), smaller (tumor-derived) EVs, and cfDNA (<3%) [33]. Even though we were able to detect *KRAS*-mutated ctRNA in platelets in 3 out of 4 patients with detectable *KRAS*-mutated

ctDNA, further research is necessary to increase the quality of cfRNA from platelets and plasma.

However, the advantage of the platelet-generating protocol is the generation of sufficient cfRNA concentration and high quality cfDNA from 10 mL of blood. In this manner, the amount of molecular information would be maximized with limited material from the patient. The true potential of cfRNA analysis lies in the detection of gene fusions and amplifications, for which cfDNA is a less appropriate matrix [7]. An example of the potential of this approach is the real-time follow up of *Anaplastic Lymphoma Kinase* (*ALK*) rearranged non-small cell lung cancer (NSCLC). *ALK*-rearranged NSCLC is a validated molecular target of ALK tyrosine kinase inhibitors (TKIs) [34]. Sadly, ALK TKI resistance is an issue, with acquired *ALK* mutations responsible for a large subset of resistant cases [35]. In these cases, it is an advantage to have the cfRNA as well as the cfDNA. The high quality cfDNA can be screened for any resistance mutations, while translocation can be detected in the platelet cfRNA by targeted assays [7] or via sequencing [36].

In contrast, little to no cfRNA was generated in Streck tubes, irrespective of centrifugation protocol. It is likely that the cell-free DNA BCT® (Streck) tubes do not sufficiently stabilize cfRNA. This could be expected, as cell-free RNA BCT® tubes have already been developed [37]. The low yield might also indicate that the preservative of the Streck tubes is not compatible with the cfRNA isolation kit we used. Presently, there are studies ongoing in which the efficiency of several cfRNA tubes and isolation techniques are being evaluated [30, 38]. A blood collection tube which preserves both cfDNA and cfRNA would maximize the detection of several cancer-associated aberrations based on one blood sample.

To conclude, the platelet-generating protocol provides high quality cfDNA and cfRNA concentrations in EDTA tubes. However, the selected centrifugation protocol will be dependent on the downstream analysis. Therefore, we recommend this protocol when looking at cfRNA or multiple aberration types (e.g., translocations and acquired resistance mutations). In the case of cfDNA screening alone, the adapted CEN protocol (two-step, high-speed) would be more appropriate, as it is a shorter and less laborious procedure. In terms of biobanking, where blood samples can be collected without a definite downstream analysis, the basic protocol (one-step, low-speed) might offer the most possibilities. In this

case, the generated platelet-rich plasma will permit cfRNA isolation, while a second high-centrifugation step will clear away most of the remaining cellular material. Cell-free DNA BCT® (Streck) tubes should only be used for cfDNA analysis. Both the Streck and adapted CEN protocols generate high quality cfDNA. Further research might reveal blood preservation tubes which generate both good quality cfDNA and cfRNA from one blood sample.

Acknowledgements

The authors would like to thank everybody involved in this study, especially the patients who were willing to provide these blood samples. Special thanks to Franscica Kohl, Peggy De Clercq, Sven De Keersmaeker, Tim Mertens, and all the staff from Oncology for collecting these samples. Furthermore, the authors would also like to thank the Biobank UZA/UAntwerpen for the close collaboration. Lastly, special thanks to Dr. Abraham Lin for proofreading our manuscript.

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Table S1

Statistical analysis of total cfDNA

CfDNA	EDTA						Streck				
	CP	AdBasic_P	AdBasic	Plat	CEN	AdCEN	**CP**	AdBasic_P	AdBasic	Plat	Streck	AdCEN
Copies/mL	Basic	0.956	**0.001**	**0.000123**	**0.000311**	**0.000222**	Basic	0.168	**0.001**	**0.001**	**0.002**	**0.002**
	AdBasic_P		**0.000278**	**0.00006**	**0.000139**	**0.000097**	AdBasic_P		0.383	0.291	0.491	0.358
	AdBasic			0.715	0.651	0.930	AdBasic			0.312	0.679	0.818
	Plat				0.599	0.559	Plat				0.550	0.635
	CEN					0.965	Streck					0.836
Cp *LINE-1*	Basic	0.474	**0.004**	**0.002**	**0.003**	**0.003**	Basic	**0.012**	**0.043**	**0.013**	0.28	0.27
	AdBasic_P		**0.016**	**0.016**	**0.019**	**0.026**	AdBasic_P		0.108	0.098	**0.043**	**0.031**
	AdBasic			0.849	0.827	0.965	AdBasic			0.435	0.251	0.312
	Plat				0.77	0.715	Plat				0.085	0.081
	CEN					0.977	Streck					0.963
% long fragments	Basic	**0.000164**	**0.000002**	**1.36E-07**	**1.36E-07**	**1.36E-07**	Basic	**0.000127**	**0.024**	**0.004**	**0.001**	**0.000338**
	AdBasic_P		**1.13E-07**	**9.59E-08**	**9.59E-08**	**9.59E-08**	AdBasic_P		**0.000008**	**0.000006**	**0.000006**	**0.000006**
	AdBasic			**0.003**	**0.00011**	**0.00011**	AdBasic			0.358	**0.035**	**0.031**
	Plat				0.358	0.35	Plat				0.215	0.154
	CEN					0.827	Streck					0.748

CP: centrifugation protocol; numbers in bold: statistically significant ($p < 0.05$).

Table S2

CfDNA integrity of EDTA vs Streck tubes

CP	**Basic**	**AdBasic_P**	**AdBasic**	**Plat**	**AdCEN**
EDTA vs Streck	**0.001**	0.085	**0.002**	**0.003**	**0.011**

CP: centrifugation protocol; numbers in bold: statistically significant (p <0.05).

Table S3

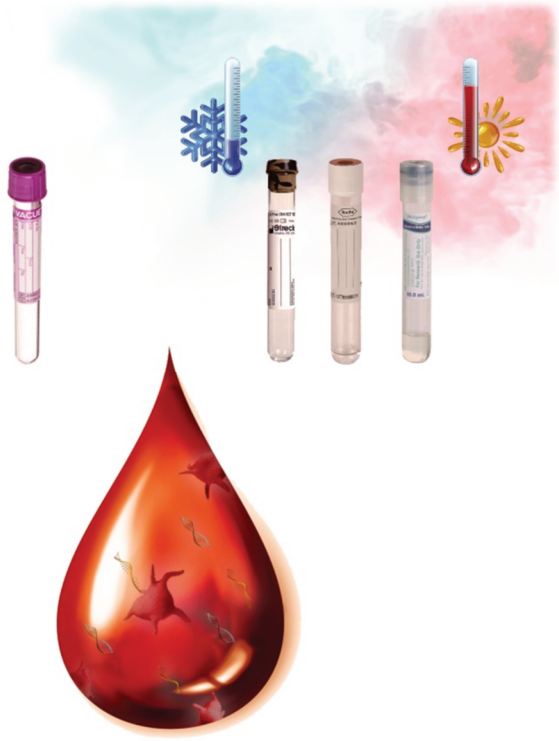
Statistical analysis of total cfRNA in EDTA tubes

	CP	AdBasic_P	AdBasic	Plat	Plat_P	CEN	AdCEN
cfRNA (copies/mL)	Basic	**0.001**	**0.005**	**0.000387**	**0.006**	**0.000012**	**0.000012**
	AdBasic_P		0.333	0.972	**0.000001**	**0.012**	**0.014**
	AdBasic			0.361	**0.000002**	**0.006**	**0.004**
	Plat				**0.000001**	**0.000437**	**0.001**
	Plat_P					**6.38E-07**	**6.39E-07**
	CEN						0.843

CP: centrifugation protocol; numbers in bold: statistically significant (p <0.05).

CHAPTER 5

Specialized Blood Collection Tubes for Liquid Biopsy: Improving the Pre-Analytical Conditions.



Sorber L, Zwaenepoel K, Jacobs J, De Winne K, Van Casteren K, Augustus E, Lardon F, Prenen H, Peeters M, Van Meerbeeck J, Roeyen G, Rolfo C, Pauwels P. Molecular Diagnosis & Therapy, accepted, DOI: 10.1007/s40291-019-00442-w

Abstract

The potential of circulating cell-free DNA (cfDNA) analysis as a liquid biopsy has led to the development of several specialized tools. We started by performing a systematic review of the cfDNA stabilizing efficacy in standard EDTA and specialized blood collection tubes (BCTs), namely: CellSave, Norgen, PAXgene, Roche, and Streck tubes. Next, we evaluated the efficacy of the latter three BCTs in a situation resembling the clinical setting. Blood samples were collected from 10 KRAS-mutated metastatic cancer patients and stored for 72 hours. During this time, samples were shaken and kept at either 6 °C or at room temperature for 24 hours to mimic transport. Conclusions could be drawn despite differences in methodological approaches between the studies. While cfDNA levels in EDTA tubes are only stable for a couple of (≤ 6) hours, they could be sustained for at least 48 to 72 hours in all three specialized BCTs, irrespective of temperature. This timespan enables a fast turn-around-time, which is one of the advantages of liquid biopsy. To conclude, the choice between these specialized BCTs is less vital when they are processed correctly within a few days.

Introduction

The analysis of circulating cell-free (tumor) DNA (cfDNA / ctDNA) present in the blood offers various opportunities for cancer management. By its nature, liquid biopsy can provide molecular stratification, identify resistance mutations, and monitor tumor response in a fast and minimally invasive manner [1, 2]. There are already two liquid biopsy-based assays Food and Drug Administration (FDA) approved; the cobas® EGFR Mutation Test v2 [3] and the Epi proColon® [4].

Interest in cfDNA-based liquid biopsy continues to grow, which is reflected in the scientific output [5]. Over the past years, specialized blood collection tubes (BCTs) have been developed which contain a preservative that stabilizes blood cells and cfDNA concentration. Therefore, in the first part of this study we performed a systematic review to assess the efficacy of various BCTs in stabilizing cfDNA. The second phase of this study consisted of an evaluation of three specialized BCTs; the cell-free DNA BCT® tubes (Streck tubes; Streck, Biomedical Diagnostics, Antwerp, Belgium), Cell-Free DNA Collection tubes (Roche tubes; Roche Diagnostics, Vilvoorde, Belgium), and PAXgene Blood ccfDNA tubes (PAXgene tubes; PreAnalytix, BD Biosciences, Erembodegem, Belgium). We compared their cfDNA stabilizing efficacy to that of BD Vacutainer K2EDTA (EDTA tubes; BD, Erembodegem, Belgium), which were immediately processed at baseline and taken as gold standard. Based on observations from a multicenter study [6] , we selected certain transport and handling conditions in order to mimic a situation most resembling the clinical setting. Blood samples were collected from metastatic cancer patients of whom the KRAS-mutational status was verified on tissue biopsies. cfDNA concentration and integrity as well as the fraction of KRAS-mutated ctDNA were quantitated in all samples.

Material and Methods

Literature review

In the first part of this study, we performed a literature review in the PubMed® database regarding blood collection tube efficacy for cfDNA-based liquid biopsy in an oncology setting. Research articles were selected based on the following search string: (ccfDNA OR cfDNA OR "cell-free DNA" OR "cell free DNA" OR "circulating free DNA" OR "circulating DNA" OR "circulating tumor DNA" OR ctDNA OR "plasma free DNA") AND (blood collection tubes OR blood collection tube OR blood collection protocols OR blood collection protocol OR preservative tubes OR preservative tube OR stabilizing reagent) NOT ("fetal DNA" OR "maternal plasma" OR "fetal"). The resulting selection was filtered further to fully match the aim of this study. This strategy resulted in 25 research articles (Table 1).

Blood Sample Collection and Processing

In the second part of this study, 10 patients with KRAS-mutated advanced cancer were included. Blood was collected in the standard EDTA tubes and long-term storage BCTs from different manufactures, namely: Streck (Streck), Roche (Roche Diagnostics), and PAXgene (PreAnalytix) tubes. Blood samples collected in EDTA tubes were processed immediately. The long-term storage BCTs were inverted 10 times immediately after blood collection as per manufacturers' instructions. First, samples were stored upright at RT for 24 hours. Next, samples were placed on a Rocking Platform (VWR, Oud-Heverlee, Belgium) and shaken (50 RPM) for 24 hours either at 6°C or RT to mimic transport conditions. Finally, samples were again stored upright at RT for 24 hours. Plasma preparation was performed according to manufacturer's instructions and the experience of the research group [7] (Table 2). After centrifugation, plasma samples were immediately stored at -80°C until further use. This study was conducted according to the Declaration of Helsinki, and ethical committee approval (B300201422715) was obtained from the ethical committee of Antwerp University Hospital (UZA). All participants provided written informed consent.

Table 2

Sample processing

Tube type	Plasma preparation	1st centrifugation step	2nd centrifugation step
EDTA	< 2 hrs	1.900 g - 10'	16.000 g - 10'
Streck	72 hrs	1.600 g - 10'	6.000 g - 10'
Roche*	72 hrs	1.600 g - 10'	16.000 g - 10'
PAXgene	72 hrs	1.900 g - 15'	1.900 g - 10'

*A smooth braking profile was used to prevent disruption of the buffy coat layer. *: adapted centrifugation protocol. The second centrifugation step was added as the manufacturer's instructions only specified the first step.*

cfDNA Isolation and Analysis

Prior to cfDNA isolation, plasma samples were photographed to compare the level of hemolysis (Figure 1). cfDNA was isolated from up to 5 mL of plasma using the QIAamp circulating nucleic acid kit (Qiagen, Antwerp, Belgium) according to manufacturer's instructions.

cfDNA and KRAS-mutated ctDNA concentration were quantified using the ddPCR KRAS Screening Multiplex Kit (Bio-Rad, Temse, Belgium). cfDNA integrity was assessed by determining the percentage of long fragments (425 base pairs) via qPCR. Detailed procedures can be found elsewhere [7].

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics, version 25.0 (IBM, Brussels, Belgium). The Kruskal-Wallis test was used to ascertain whether there were significant differences between tube types in plasma volume, cfDNA yield and characteristics. The Chi-Square test was performed to determine whether the occurrence of hemolysis is associated with tube type. p-values of <0.05 were considered statistically significant. Figures were made with Graphpad Prism7 (Graphpad Software Inc., La Jolla, CA, USA).

Results

Literature review

In total, 25 research articles matched our search criteria regarding BCT efficacy for cfDNA-based liquid biopsy in an oncology setting (Table 1). These studies compared specialized tubes (Cell-Free DNA BCT® tubes (Streck), Cell-Free DNA Collection Tubes (Roche), CellSave Preservative Tubes (CellSearch), PAXgene Blood ccfDNA Tubes (PreAnalytix), cf-DNA/cf-RNA Preservative Tubes (Norgen)) to EDTA tubes as well as amongst each other. CfDNA yield and mutated (artificial) ctDNA detection was investigated in 24 [8-31] and 13 [8, 9, 11, 13, 14, 21-23, 25-27, 29, 32] out of 25 studies, respectively. Two studies evaluated methylation levels in either Streck or PAXgene tubes compared to EDTA tubes [10, 24]. Furthermore, cfDNA integrity was also examined in 9 studies [11, 15-17, 19, 20, 27, 29, 30].

Plasma preparation

In 22 out of 25 studies, two-step centrifugation protocols were used to generate plasma [8-11, 14-31]. One study even had two consecutive centrifugations prior to plasma separation [20]. The first centrifugation step was lower than 1,000 g in six studies [15, 18, 21, 22, 24, 28], ranging between 300 and 820 g. In one of these studies, the original centrifugation speed of 550 g was increased to 1,000 g for samples processed after 7 days [24]. Parpart-Li et al. even reported a time-dependent decrease of plasma volumes in both EDTA and Streck tubes [21]. The group of Norton et al. published two papers in which they investigated the efficacy of EDTA and Streck tubes under different conditions [18, 19]. In the second study, the centrifugation speed of both steps were increased; from 300 g followed by 5,000 g to 1,600 g and 16,000g, respectively. Different centrifugation protocols were also used in the study of Ahlborn et al [8]. The centrifugation speed of the first step was increased, while the second was decreased in Streck tubes (processed after 72 hours) versus EDTA tubes (at baseline); 2,250 g vs 1,600 g and 18,000 g vs 20,000 g, respectively. Although all studies performed the centrifugation steps in a consecutive manner, it is important to note that plasma samples can be centrifuged a second time post-thaw [7, 28,

33]. In 5 out of 25 studies, centrifugation was performed at 4 °C instead of at RT [8, 9, 17, 30, 31].

BCT efficacy

The cfDNA stabilizing efficacy of EDTA tubes at different time points and conditions was evaluated in 15 studies [14, 16-19, 21-25, 27, 28, 30-32]. In 4 out of 8 studies, decreased cfDNA stability was detected after 24 hours at RT [14, 16, 19, 21]. One study even reported a decrease in cfDNA integrity after 8 hours [17]. The remaining 7 studies processed blood samples after a couple of hours (≤ 6 hrs), while the following time point in which samples were processed was several days after blood collection (≥ 2 d) [18, 23-25, 30-32]. None of them reported a decrease in cfDNA stability in the first hours. Five studies evaluated the influence of low temperatures (4 – 6 °C) on cfDNA stability [14, 17, 18, 21, 22]. In 3 of these studies, storage of EDTA tubes at low temperatures resulted in stable cfDNA levels for a longer period of time [17, 21, 22]. However, there was no consensus between the different studies with regards to maximum time-to-processing, ranging between 6 and 72 hours.

Twenty-two studies evaluated the efficacy of Streck tubes [8-11, 13-22, 25-32]. cfDNA levels were stable up to the last collection point (5 – 14 days) in 8 out of 13 studies ([11, 17, 20-22, 26, 28, 32] vs [9, 18, 19, 29, 31]). However, 4 studies reported increased cfDNA concentrations and/or occurrence of hemolysis after as little as 3 to 5 days [13, 25, 29, 30]. The influence of temperature was also evaluated in 4 other studies [11, 14, 17, 18]. Low temperatures (4 – 8 °C) only influenced cfDNA stability after prolonged storage (≥ 3 days). Increased genomic DNA (gDNA) contamination and hemolysis were also only observed after 5 days at 40 °C [11]. It is important to note that a heat shock (5 hours at 39 °C, followed by 19 hours at RT) or fluctuating, higher temperatures (12 hours at 30 °C, 12 hours at RT for up to 7 days) did not influence cfDNA stability [17]. One study also investigated methylated cfDNA levels [10]. Lower methylated cfDNA levels were observed in Streck tubes after 48 hours compared to EDTA tubes processed at baseline.

The efficacy of Roche tubes was evaluated in 6 studies [9, 12, 17, 20, 29, 31]. Thereof, 5 studies compared Roche tubes to EDTA and/or other specialized BCTs [9, 17, 20, 29, 31]. In 3 out of 6 studies, cfDNA levels were stable up to the last collection point (2 – 14 days)

[9, 12, 31]. Results varied on whether Roche or Streck tubes had superior cfDNA stabilizing efficacy. However, in contrast to Streck tubes, Roche tubes were affected by fluctuating temperatures (12 hours at 30 °C, 12 hours at RT), with gDNA contamination detected after 6 days. Heat shock or storage at 4 °C for 48 hours didn't seem to influence cfDNA stability [12, 17].

PAXgene tubes were evaluated in 6 studies [9, 17, 24, 26, 29, 30]. Only 1 study compared PAXgene to EDTA tubes only [24], while the other studies compared them to Streck (2) [26, 30] or to Streck and Roche tubes (3) [9, 17, 29]. In 4 out of 6 studies, cfDNA stability was maintained until the last time point (4 – 7 days) [9, 17, 29, 30]. No consensus could be reached on whether PAXgene tubes have superior cfDNA stabilizing compared to Streck and/or Roche tubes. Fluctuating temperatures for 7 days or heat shock did not have an effect on tube efficacy [17]. Lastly, 1 study reported no influence on methylation levels after storage for 7 days [24].

CellSave tubes, which were primarily designed to stabilize circulating tumor cells (CTCs), were evaluated in 4 studies [14, 23, 27, 28]. Even though 1 study reported a marginally significant decrease in cfDNA stability after 48 hours [14], the other studies demonstrated stable cfDNA concentrations up to the last time point (4 – 5 days) [23, 27, 28]. Temperature or methylation analysis was not investigated in any study.

Norgen tubes were compared to PAXgene, Roche, and Streck tubes in 1 study [29]. Significantly shorter cfDNA fragments were detected in these tubes, which were stabilized up to 7 days (the last time point).

Table 1

Literature review – BCT efficacy

Author	Subjects (n)	BCT	Processing	Centrifugation		cfDNA analysis	BCT efficacy
				1st	2nd		
Parackal (2019) [20]	Healthy (20)	Roche Streck	<2 hrs, 4, 7, 10, 14 days [RT]	200 g – 10, 1,600 g – 10	1,600 g – 10	• yield • integrity	• R: 7 d • S: 14 d
Enko (2019) [12]	Healthy (30)	Roche	0, 24, 48 hrs [4 – 8 °C / RT]	1,600 g – 15'	/	• yield	• 48 hrs: 4°C – RT
Ward (2019) [29]	Healthy (8)	Norgen PAXgene Roche Streck	4 hrs, 1, 5, 7 days [RT]	1,300 g – 20'	12,000 g – 10'	• yield • mutated ctDNA • integrity	• N: 7 d (significantly smaller cfDNA fragments) • P: 7 d • R: ≤ 5 d (hemolysis at 7 d, even slight traces at 5 d) • S: 24 hrs (hemolysis at 5 d)
Zhao (2019) [31]	Healthy (6)	EDTA Roche Streck	0 hrs, 3, 7, 14 days [± RT (20 - 30°C)]	2,000 g – 10' (4°C)	16,000 g – 10' (4°C)	• yield	• E: 0 hrs • R: 14 d • S: 7 d Hemolysis & WBC rupture don't occur concurrently; no significant influence on quantity and complexity of NGS libraries
Nikolaev (2018) [17] (1/2)	Healthy (ns)	EDTA	0 hrs, 8, 16, 24, 32, 40, 48 hrs, 7 days [RT] 5 hrs [39 °C] / 19 hrs [RT] 0 hrs, 1, 2, 3, 4, 5, 6, 7 days [4 °C]	1,600 g – 10' (4°C)	16,000 g – 10' (4°C)	• yield • integrity	• E o RT: < 8 hrs o 4°C: 48 hrs o Heat shock: significant gDNA contamination

Author	Subjects (n)	BCT	Processing	Centrifugation		cfDNA analysis	BCT efficacy
				1st	2nd		
Nikolaev (2018) [17] (2/2)	Healthy (ns)	PAXgene	0 hrs, 7 days [RT]	1,600 g – 10' (4°C)	16,000 g – 10' (4°C)	• yield • integrity	• P: 7 d, irrespective of higher temperature (30 °C) / heat shock • R: < 7 d RT, < 6d at higher temperature, though not influenced by heat shock • S: 7 d, irrespective of higher temperature (30 °C) / heat shock
		Roche	0 hrs, 1, 2, 3, 4, 5, 6, 7 days [30 °C 12 hrs / RT 12 hrs]				
		Streck	5 hrs [39 °C] / 19 hrs [RT]				
Risberg (2018) [22]	Cancer pts (± 30, ± 13)	EDTA	0, 6, 24, 48, 96 hrs, 1 week [RT]	820 g – 10'	14,000 g – 10'	• yield • mutated ctDNA	• E o RT: 24 hrs o 4°C: > 24 hrs • S: 7d No influence of shipping & no induction of base substitutions
			0, 24, 48, 96 hrs [RT / 4 °C]				
			0, 96 hrs, 1 week [RT]				
		Streck	48 hrs (exceptions 2 = 96 hrs, 1 = 5 days) [RT / shipped]				
Markus (2018) [15]	Healthy (23)	EDTA	< 1 hr [RT]	820 g – 10'	16,000 g – 10'	• yield • integrity	• E: < 1 hr • S: 48 hrs No induction of background noise
		Streck	24, 48 hrs [RT]				
Bartak (2019) [10]	Cancer pts / adenoma (5 / 5)	EDTA	< 4 hrs [RT]	1,350 g – 12'	1,350 g – 12'	• yield • methylation	• E: < 4 hrs • S: 48 hrs Lower methylated levels than in EDTA tubes
		Streck	48 hrs [RT]				

Author	Subjects (n)	BCT	Processing	Centrifugation		cfDNA analysis	BCT efficacy
				1st	2nd		
Alidousty (2017) [9]	Healthy (ns)	PAXgene	0, 1, 2, 3, 4, 7 days [RT]	1,600 g – 10' (4°C)	16,000 g – 10' (4°C)	• yield • mutated artificial ctDNA	• P: 7 d • R: 7 d • S: ≤ 7 d Lower stabilization efficacy with low (0.5 ng) artificial ctDNA concentrations
		Roche					
		Streck					
Van Ginkel (2017) [28]	Cancer pts (2; 2; 2)	EDTA	<1 hr (E), 1, 2, 5 days [RT]	380 g – 20'	20,000 g – 10'	• yield	• E: 24 hrs • C: 5 d • S: 5 d
		CellSave					
		Streck					
Mehrotra (2017) [16]	Cancer pts (31)	EDTA	2, 4, 16, ≥ 24 hrs [RT]	2,000g – 10'	2,000 g – 10'	• yield • integrity	• E: 16 hrs • S: ≥ 24 hrs
		Streck					
Warton (2017) [30]	Healthy (8, 9 collections)	EDTA	1 hr [4 °C], 4 days [RT]	2,500 g – 10' (4°C)	3,500 g – 10' (4°C)	• yield • integrity	• E: 1 hr at 4°C • P: 4 d • S: ≤ 4 d (slight increase in high-molecular weight)
		PAXgene					
		Streck					
Ahlborn (2017) [8]	Cancer pts (5; 25 collections)	EDTA	< 15 min [RT]	1,600 g – 20' (4°C)	20,000 g – 20' (4°C)	• yield • mutated ctDNA	• E: < 15 min • S: 72 hrs
		Streck	72 hrs [RT]	2,250 g – 20' (4°C)	18,000 g – 20' (4°C)		

Author	Subjects (n)	BCT	Processing	Centrifugation		cfDNA analysis	BCT efficacy
				1st	2nd		
Schmidt (2017) [24]	Cancer pts (29)	EDTA	< 2-3 hrs, 7 days [RT]	550 g – 15'	3,000 g – 15'	• yield • methylation	• E: < 2-3 hrs • P: ≤ 7 d No influence on methylation quantification
		PAXgene		1,000 g – 15'			
Van Dessel (2017) [27]	Cancer pts (16)	EDTA	< 1 hr, 1, 4 days [RT]	1,711 g – 10'	12,000 g – 10'	• yield • mutated ctDNA • integrity	• E: 24 hrs • C: 4 d • S: 4 d
		CellSave					
		Streck					
Rothwell (2016) [23]	Healthy / Cancer pts (20 / 35)	EDTA	(healthy) < 4 hrs [RT], 4 days [shipped]	2,000 g – 10'	2,000 g – 10'	• yield • aberrant ctDNA	• E: 4 hrs • C: 4 d No induction of SNPs
		CellSave	(cancer pts) < 4 hrs (E), 4 days [RT]				
Diaz (2016) [11]	Healthy / cancer pts (82 / 21)	EDTA	2 hrs [RT]	1,600 g – 10'	16,000 g – 10'	• yield • mutated (artificial) ctDNA • integrity	• E: 2 hrs • S: 5 d Increased gDNA contamination at ≤ 10 °C (3d) & 40 °C (5d). No induction of base changes
		Streck	2 hrs, 3 & 5 days [4 °C / RT / 40°C]				

Parpart-Li (2016) [21]	Cancer pts (9; 45 collections)	EDTA	< 4 hrs, 1, 3, 5, 7 days [RT]	375 g – 10'	18,400 g – 10'	• yield
						• mutated ctDNA
							• E
							  ○ RT: < 4 hrs
							  ○ 4°C: 3 d
							• S: 7d
							Decrease of plasma volume after long storage in both tube types

		Streck	< 4 hrs, 3, 7 days [4 °C EDTA / RT]				

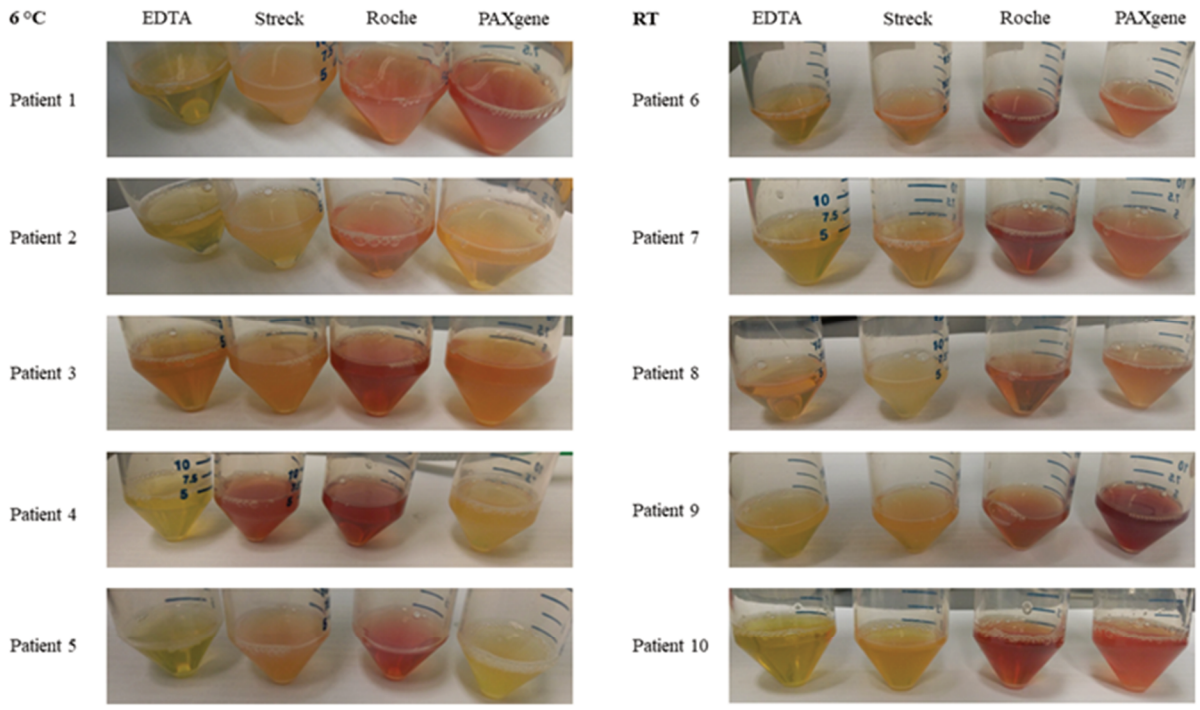
Author	Subjects (n)	BCT	Processing	Centrifugation		cfDNA analysis	BCT efficacy
				1st	2nd		
Hrebien (2016) [13]	Cancer pts (71)	EDTA	< 2 hrs [RT]	1,600 g – 20'	/	• yield • mutated ctDNA	• E: 2 hrs • S: ≤ 48 to 72 hrs (slight increase in total cfDNA in 10.3% which influenced mutation calling) Discordance observed in case of low allele frequency
		Streck	48 to 72 hrs [RT]				
Kang (2016) [14]	Cancer pts (10)	EDTA	2, 6, 48 hrs [4 °C (E&S) / RT]	2,500 g – 10'	16,000 g – 10'	• yield • mutated ctDNA	• E: 6 hrs • C: ≤ 48 hrs (decreased stability marginally significant) • S: 48 hrs
		CellSave					
		Streck					
Sherwood (2016) [25]	Cancer pts (20)	EDTA	2 hrs, 3 days [RT]	2,000 g – 10'	2,000 g – 10'	• yield • mutated ctDNA	• E: 2 hrs • S: 2 hrs (3 d: low, but significant increase in cfDNA but superior detection of mutated ctDNA to EDTA tubes)
		Streck					
Toro (2015) [26]	Cancer pts (10)	EDTA	2 hrs [RT]	Two-step (ns)		• yield • mutated ctDNA	• E: 2 hrs • P: 2 hrs • S: 7 d
		PAXgene	2 hrs, 7 days (S&P) [RT]				
		Streck					
Denis (2015) [32]	Cancer pts (10)	EDTA	2 hrs, 4, 7, 10 days [RT]	2,000 g – 10'	/	• mutated ctDNA	• E: 2 hrs • S: 10 d
		Streck					

Author	Subjects (n)	BCT	Processing	Centrifugation		cfDNA analysis	BCT efficacy
				1st	2nd		
Norton (2013) [19]	Healthy (12, 6)	EDTA	0 hrs, 1, 2, 3, 7, 14 days [RT]	1,600 g – 20'	16,000 g – 10'	• yield • integrity	• E: < 24 hrs • S: 7 d Shipping has no influence (3d)
		Streck	48 hrs (RT / shipped (13 – 23 °C))				
Norton (2013) [18]	Healthy (5, 6, 8 & 10)	EDTA	0 hrs, 7, 14 days [6 °C / RT / 37 °C]	300 g – 20'	5,000 g – 10'	• yield • rare events	• E: 24 hrs, unshaken • S: 7 d Shaking/shipping has no influence, decreased stability when stored at 6°C
		Streck	0, 3, 6, 24 hrs [RT / on shaker / still vs shipped]			• yield	

BCT: blood collection tube; C: CellSave tubes; E: EDTA tubes; n: number of subjects; ns: not specified; P: PAXgene tubes; R: Roche tubes; S: Streck tubes; WBC: white blood cell

BCT efficacy in a real-life setting

In the second part of this study, we evaluated the cfDNA stabilizing efficacy of three specialized BCTs (PAXgene, Roche, Streck) for up to 72 hours, during which time they were kept at 6 °C or RT and shaken for 24 hours. Their efficacy was also compared to EDTA tubes, which were processed immediately after blood collection and were regarded as gold standard. First, all plasma samples were inspected for the presence of hemolysis (visible by the naked eye) based on images taken prior to cfDNA isolation. Each sample was attributed a hemolysis score (Figure 1).



T	6 °C			
Patient	EDTA	Streck	Roche	PAXgene
1	0	1	3	3
2	0	0	3	1
3	2	2	3	2
4	0	3	3	0
5	0	1	3	0
Total	2	7	15	6

T	RT			
Patient	EDTA	Streck	Roche	PAXgene
6	0	1	3	2
7	0	1	3	2
8	1	0	2	1
9	0	1	3	3
10	0	0	3	3
Total	1	3	14	11

Figure 1: Hemolysis evaluation plasma samples. Samples were visually inspected for the presence of hemolysis and were subsequently attributed a score.; 0: clear, yellow plasma, 1: cloudy, yellow to slightly orange plasma, 2: (dark) orange plasma, 3: red plasma. *PAXgene: PAXgene Blood ccfDNA Tubes; Roche: Cell-Free DNA Collection Tubes; RT: room temperature; Streck: Cell-Free DNA BCT® tubes; T: temperature.*

Hemolysis was found to be associated with tube type ($p < 0.001$). This association was observed both at 6 °C ($p = 0.034$) and at room temperature ($p = 0.008$). Hemolysis was observed in all samples collected in Roche tubes. The majority of samples in PAXgene tubes were also affected, although, not to the same extent. However, no statistically significant differences in cfDNA yield (Figure 2A) or integrity (Figure 2B) were observed between BCTs, irrespective of whether storage temperature was taken into account. In total, 8 out of 40 samples (20%) had more than 5% of long (> 425 bp) fragments; half of which were collected in PAXgene tubes. The remaining four were two Streck samples, one Roche and one EDTA sample. Two of the samples collected in PAXgene tubes had 54 and 74% long fragments, respectively, while all the other samples had less than 15%.

Next, we investigated the stabilizing effect of the BCTs on the ctDNA fraction by quantifying the KRAS-mutated ctDNA. Seven out of 10 patients were found to have detectable KRAS-mutated ctDNA levels. Similar concentrations (Figure 2C) and allele frequencies (AFs; Figure 2D) were observed in all BCTs, both at 6 °C and at RT. Unsurprisingly, the PAXgene sample which contained over 70% of long fragments, had much lower KRAS-mutated ctDNA concentrations and AF compared to the samples collected in the other BCTs, 1144 vs 4278.52 copies/mL and 1.76 vs 21.2%, respectively.

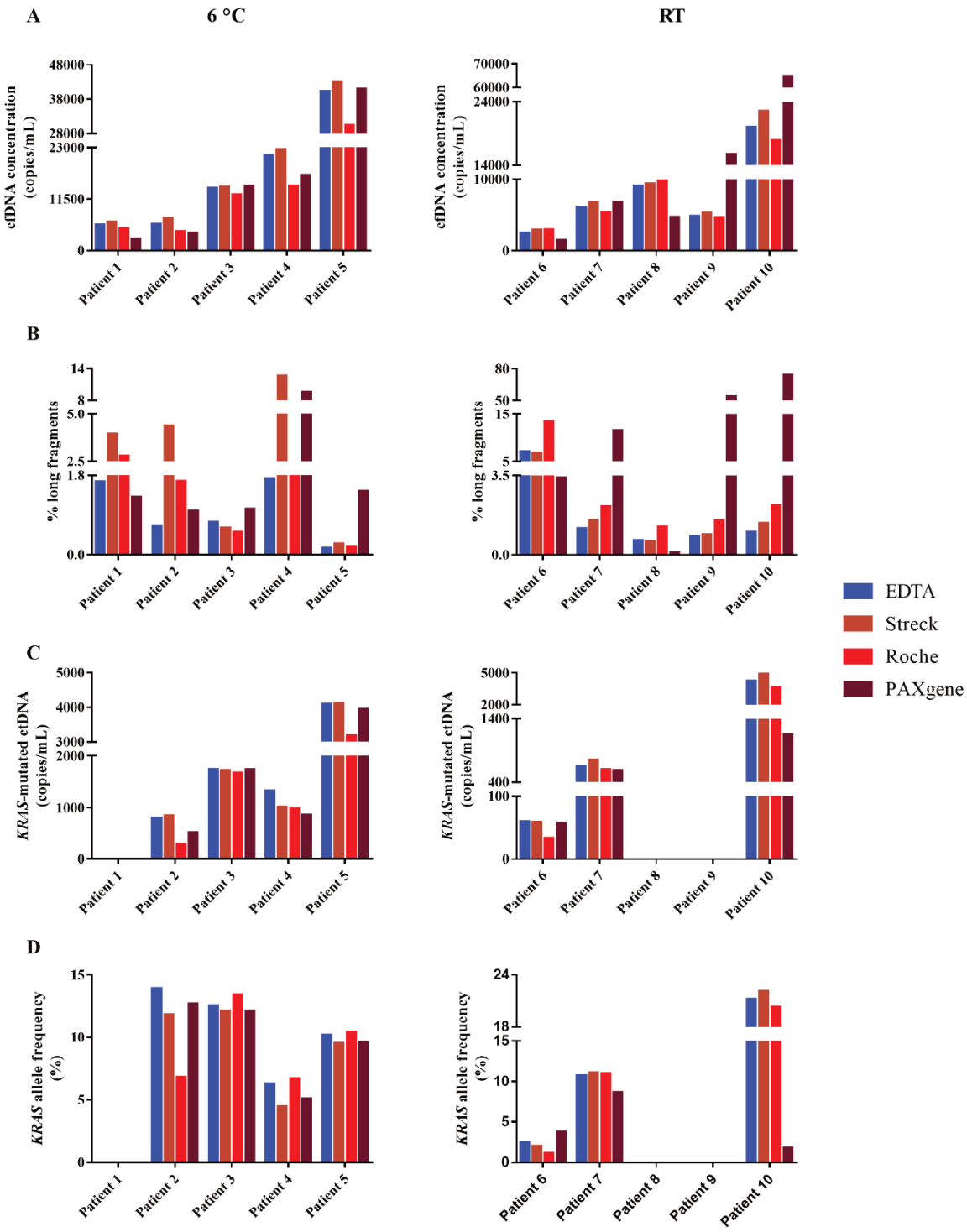


Figure 2: Comparison of BCT efficacy. EDTA tubes were processed at baseline, while specialized BCTs (Streck, Roche, PAXgene) were stored for 72 hours prior to processing. During this time, they were agitated for 24 hours at 6 °C or at RT. **A** cfDNA yield (copies/mL); **B** cfDNA integrity analysis based on the ratio of long (> 425 bp) versus short (82 bp) DNA fragments (%); **C** *KRAS*-mutated ctDNA concentration (copies/mL); and **D** allele frequency (%); *BCT: blood collection tubes, PAXgene: PAXgene Blood ccfDNA Tubes; Roche: Cell-Free DNA Collection Tubes; Streck: Cell-Free DNA BCT® tubes.*

Discussion & Conclusion

In the first part of this study, we performed a systematic review to determine the influence
In the first part of this study, we performed a systematic review to determine the influence
of the delay in processing of blood samples collected in (specialized) BCTs on cfDNA. Twenty-five studies matched our search criteria [8-32]. EDTA tubes were compared to one or more specialized BCTs (CellSave, PAXgene, Norgen, Roche, and Streck tubes) in 21 studies [8, 10, 11, 13-19, 21-28, 30-32], three studies evaluated specialized BCTs only [9, 20, 29], and one study investigated Roche tubes only [12]. In 6 out of 21 studies, EDTA tubes were processed at baseline, within a time span of 15 minutes up to 4 hours [8, 10, 11, 13, 15, 26]. The remaining 15 studies included several time points on which samples were processed, ranging from a couple of hours up to two weeks. There were no reports of decreased cfDNA stability within a 6-hour delay in processing. However, no consensus with regards to maximum time-to-processing could be reached [14, 16-19, 21-25, 27, 28, 30-32]. This holds true with regards to influence of low temperatures. While 3 out of 4 studies reported cfDNA stability up to 24 hours when stored at 4 – 6 °C [17, 21, 22], no significant improvement in cfDNA stability was observed in the fourth study [14].

No major differences in efficacy could be observed between CellSave, Norgen, PAXgene, Roche, and Streck tubes. Nearly all studies reported cfDNA stability for at least 48 to 72 hours [8-12, 15, 17-24, 27, 28, 30-32]. Only three studies demonstrated a (low, but significant) decrease in cfDNA stability in this time span, namely two in Streck [13, 25], and another one in CellSave tubes [14]. In the study of Hrebien et al., the slight increase of cfDNA could also be (partially) attributed to the lack of a second centrifugation step [13]. A double centrifugation would remove most of the cellular debris [7, 25]. No consensus could be reached on maximum time-to-processing. One of the major advantages of liquid biopsy is the ability to acquire material in a fast and minimally invasive way, thereby decreasing turnaround time. Hence, it would be advantageous to decrease transport time as much as possible to provide physicians and patients with results as soon as possible. The specialized BCTs were found to be affected by temperature only in extreme circumstances [11, 12, 14, 17, 18]. This was corroborated by our findings. The cfDNA stabilizing efficacy of PAXgene, Roche, and Streck tubes after 72 hours were similar compared to each other

as well as to EDTA tubes processed at baseline. Furthermore, cfDNA concentration and plasma volume were similar between the specialized BCTs despite differences in volume and/or additive volume of preservative (data not shown). While both Streck and PAXgene tubes have a blood draw volume of 10 mL, the volume of preservative differed strongly (0.2 mL vs 1.5 mL, respectively). In contrast, Roche tubes have a blood draw volume of 8.5 mL (no information regarding additive volume of preservative was available). Temperature was also shown to have no significant influence on cfDNA stability as the specialized BCTs were only subjected to shaking and temperature differences for 24 hours. Despite the persistent presence of hemolysis in Roche tubes, no significant increase in gDNA was observed. Zhao et al. reported that rupture of white blood cells (WBCs), the main cause of gDNA contamination, is not necessarily synchronized with hemolysis [31]. This would indicate that red blood cells (RBC) aren't stabilized as efficiently as WBCs and cfDNA in Roche tubes. It remains unclear whether this is due to the stabilizing properties of the preservative or due to mechanical lysis during collection as a result of the larger vacuum of these tubes. PAXgene tubes were also observed to be prone to the occurrence of hemolysis. In two samples, more than 50% of the total cfDNA was made up by gDNA. Even though this could have resulted from WBC rupture, this seems less likely. While strong hemolysis occurred in the sample of patient 1, no strong increase in gDNA was detected. In contrast, the plasma from patient 4 was similar to that of the EDTA tube, yet a clear increase in gDNA could be observed (9.54 vs 1.73%). The strong gDNA contamination (> 50%) could also be attributed to the centrifugation protocol used to generate plasma from samples collected in PAXgene tubes. The speed of the second centrifugation step is much lower compared to the other protocols (1.900 g vs ≥ 6.000 g). It is possible that the remaining cellular debris isn't cleared as efficiently or that the resulting layer of debris is rather loose. Hence, gDNA could be accidentally transferred along with the plasma after centrifugation.

Two studies investigated methylation levels in Streck and PAXgene tubes, respectively. While lower methylation levels were detected in Streck compared to EDTA tubes [10], no effect on methylation quantification was observed in PAXgene tubes [24]. Furthermore, another study evaluated both the cfDNA and microRNA stabilizing efficacy of specialized BCTs [29], with the Norgen and PAXgene tubes performing the best. However, further

research remains vital to evaluate whether the specialized BCTs are fully compatible with methylation and RNA analysis.

One of the major limitations of this study is the sample size. The small number of (KRAS-mutated) samples limited the power to observe slight effects of for example total blood draw volume, additive volume or preservative, or conditions during transport. Furthermore, the presence of hemolysis was only evaluated in a visual manner. Measuring the hemoglobin concentration might be a better alternative as it provides more quantitative data. Finally, the influence of specialized BCTs on methylation and RNA analysis was not evaluated in this study. However, despite these limitations, our study corroborated the findings previously reported in literature.

To conclude, PAXgene, Roche, and Streck tubes have similar cfDNA stabilizing efficacy, irrespective of temperature, when blood samples are processed within 48 to 72 hours. Processing within this time frame will enable liquid biopsy to provide molecular information in a fast and minimally invasive way. When assessing the efficacy of BCTs, it is important to note that hemolysis might not be the most optimal criteria. Lastly, a second centrifugation step of sufficient speed will reduce gDNA contamination to a minimum.

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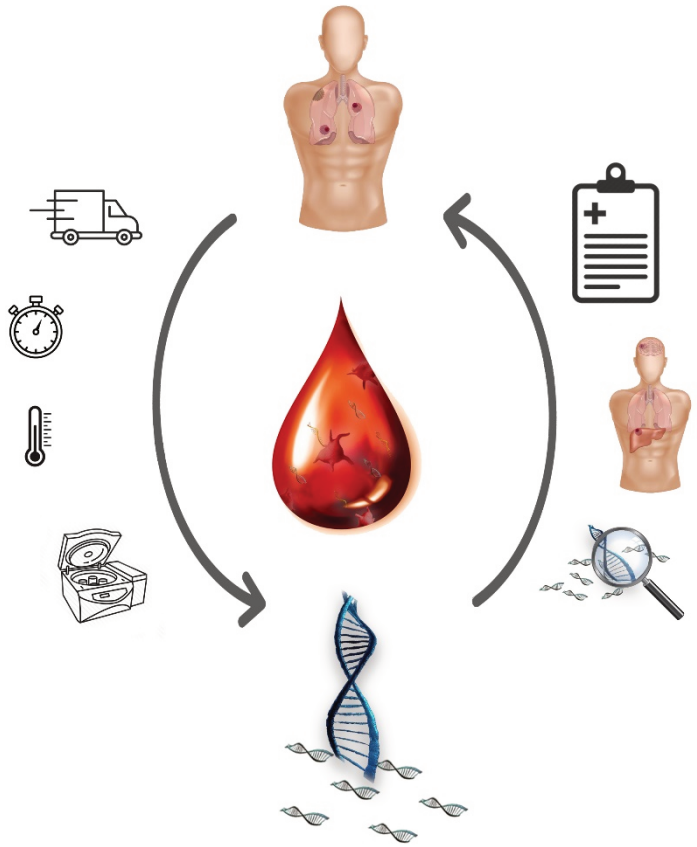
PART II: CLINICAL APPLICABILITY OF LIQUID BIOPSY

CHAPTER 6

A Multicenter Study to Assess *EGFR* Mutational Status in Plasma: Focus on an Optimized Workflow for Liquid Biopsy in a Clinical Setting.



Sorber L, Zwaenepoel K, De Winne K, Van Casteren K, Augustus E, Jacobs J, Zhang XH, Galdermans D, De Droogh E, Lefebure A, Morel AM, Saenen E, Bustin F, Demedts I, Himpe U, Pieters T, Germonpré P, Derijcke S, Deschepper K, Van Meerbeek J, Rolfo C, Pauwels P. Cancers (Basel), Volume 10, Issue 9, 2018.

Abstract

A multicenter study was performed to determine an optimal workflow for liquid biopsy in a clinical setting. In total, 549 plasma samples from 234 non-small cell lung cancer (NSCLC) patients were collected. Epidermal Growth Factor Receptor (*EGFR)* circulating cell-free tumor DNA (ctDNA) mutational analysis was performed using digital droplet PCR (ddPCR). The influence of (pre-) analytical variables on ctDNA analysis was investigated. Sensitivity of ctDNA analysis was influenced by an interplay between increased plasma volume ($p < 0.001$) and short transit time ($p = 0.018$). Multistep, high-speed centrifugation both increased plasma generation ($p < 0.001$) and reduced genomic DNA (gDNA) contamination. Longer transit time increased the risk of hemolysis ($p < 0.001$) and low temperatures were shown to have a negative effect. Metastatic sites were found to be strongly associated with ctDNA detection ($p < 0.001$), as well as allele frequency ($p = 0.034$). Activating mutations were detected in a higher concentration and allele frequency compared to the T790M mutation ($p = 0.003$, and $p = 0.002$, respectively). Optimization of (pre-) analytical variables is key to successful ctDNA analysis. Sufficient plasma volumes without hemolysis or gDNA contamination can be achieved by using multistep, high-speed centrifugation, coupled with short transit time and temperature regulation. Metastatic site location influenced ctDNA detection. Finally, ctDNA levels might have further value in detecting resistance mechanisms.

Introduction

Non-small cell lung cancer (NSCLC), which makes up approximately 80% of all lung cancer cases, remains one of the world's leading causes of cancer-related mortality [1]. The latest American Society of Clinical Oncology (ASCO) guidelines concerning systemic therapy for stage IV NSCLC highlight that tumors should be examined for the presence of targetable molecular alterations [2]. Detection of activating Epidermal Growth Factor Receptor (*EGFR)* mutations warrants treatment with EGFR tyrosine kinase inhibitors (TKIs), such as afatinib, erlotinib, or gefitinib. Despite high response rates and durable responses, most patients develop resistance after a median period of 12 months [3]. The *EGFR* T790M mutation accounts for approximately half of the instances in acquired resistance to first line EGFR TKI therapy [4], in which case osimertinib (third generation EGFR TKI) is recommended [2]. Hence, genotyping is important in order to provide patients with the most optimal treatment. Although tissue biopsies remain the gold standard, it is associated with several limitations. Taking a biopsy is an invasive procedure, which can be potentially harmful for the patient. In addition, the tumor cell content can be very low. Liquid biopsy, consisting of circulating cell-free (tumor) DNA (cfDNA/ctDNA) analysis, has emerged as viable approach to guide therapeutic decisions and provide real-time follow-up [5, 6]. The development of both targeted, digital-PCR based and (non-) targeted, genome-wide analyses have increased the potential of ctDNA analysis. Several studies have demonstrated high concordance of mutational profiles between tissue, plasma, and most recently urine samples [7, 8]. Importantly, detection of *EGFR* mutations in a liquid biopsy is sufficient proof to warrant EGFR TKI therapy [7, 9-11]. Patients have been found to display similar clinical response to EGFR TKI therapy, irrespective of whether *EGFR* mutations were identified in plasma, urine, or tissue samples. The group of Sacher *et al.* demonstrated the feasibility of plasma genotyping using digital droplet PCR (ddPCR), which is a high-throughput technology with a high level of sensitivity and specificity [12]. This study also reported a shorter turnaround time (TAT) in comparison to tissue biopsies, making it a promising approach for guiding precision medicine. In 2016, the cobas® EGFR Mutation Test v2 (Roche Molecular Systems, Inc., Branchburg, New Jersey, USA) was the first liquid biopsy test to acquire US Food and Drug Administration (FDA) approval,

which was a major advance in the ctDNA liquid biopsy field [10]. At present, it remains the only FDA approved ctDNA analysis test, though more companion diagnostic liquid biopsies are being developed and validated [13].

Liquid biopsy has great potential for molecular pathology in cancer, but standard operating procedures have to be established in order to facilitate broad clinical implementation [6]. In this multicenter study, we have investigated various (pre-) analytical variables to provide an optimized workflow for implementation of liquid biopsy in a clinical setting.

Material and Methods

Study Design

This study was designed as a real-life observational trial of NSCLC patients in several hospitals in Belgium. The objective was to determine the optimal (pre-) analytical settings to assess *EGFR* mutational status using liquid biopsy in a clinical setting. The study was conducted according to the Declaration of Helsinki. Ethical committee approval for this study was obtained from each site with the Ethical committee of the UZA as primary committee (B300201526045, 26/10/2015). All patients provided written informed consent. At inclusion, patients were separated in three groups based on *EGFR* mutational status determined by tissue analysis, namely; (i) tissue analysis was unsuccessful or impossible, (ii) *EGFR* wild type (WT), and (iii) *EGFR* mutated. Patients of the first two groups provided only one blood sample, but, if *EGFR*-mutated ctDNA was detected in this sample, they were reassigned to the third group. Multiple blood samples were collected before and during (targeted) therapy and at radiologic disease progression. Feedback with regards to *EGFR* mutation status was provided to the treating thoracic oncologist. Additional information with regards to stage, therapy and the presence or occurrence of brain and extrathoracic metastases was requested.

Sample Collection and Processing

The majority of blood samples were collected using Cell-Free DNA BCT® tubes (Streck tubes; Streck, Biomedical Diagnostics, Antwerp, Belgium). These tubes were shipped to

the UZA by post. The number of days from blood collection until arrival at UZA (transit time) and average monthly temperature during transport was recorded. Upon arrival, the blood samples were centrifuged and the generated amount of plasma and presence of hemolysis were documented. In the first part of this study, a one-step, low-speed centrifugation protocol (400× *g* for 10 min) was used. As part of the constant optimization process of the liquid biopsy workflow, this protocol was altered based on in-house comparison [14]. This protocol consists of a two-step, high-speed centrifugation (1600× *g* for 10 minutes followed by a second step, either 16,000× *g* for 10 min or maximum speed for 1 min). At the start of this study, patients were also included using samples in EDTA tubes. Plasma was generated within 2 h of blood collection, pooled and transported on dry ice. Samples from a small subset of in-house patients were also included using EDTA tubes. Sample collection, processing and storage was performed by Biobank@UZA (Antwerp, Belgium; ID: BE71030031000; Belgian Virtual Tumourbank funded by the National Cancer Plan, BBMRI-ERIC; No. Access: 1, Last: 25 September 2017) [15]. All plasma samples were stored at −80°C prior to cfDNA isolation.

CfDNA Isolation and Analysis

CfDNA isolation was performed using the Maxwell RSC ccfDNA Large Volume Plasma Kit (Promega, Leiden, the Netherlands) according to the manufacturer's protocol. The cfDNA was eluted in 65 μL of buffer, irrespective of input plasma volume, ranging from 0.5 to 4 mL. Samples were stored at −20 °C until genotyping. *EGFR* mutational analysis was performed by ddPCR (BioRad, Temse, Belgium). Two multiplex ddPCR assays were designed to detect (i) exon 19 hotspot deletions and the T790M resistance mutation and (ii) the L858R, L861Q and G719A/C/S activating mutations, respectively. The assays were composed of primers and probes described by Oxnard *et al.* [16] and in-house design (sequences, concentrations, ddPCR mix and thermal cycling conditions are available in Supplementary data). Detailed procedures can be found elsewhere [17]. In case of (suspected) progressive disease, samples were also analyzed using the T790M primer/probe assay from BioRad, according to the manufacturer's instructions. These samples were analyzed in triplicate to decrease sampling error and increase detection rate. In 2016, these assays and the related workflow received accredited status by BELAC (the

Belgian Accreditation Institution). Samples were grouped based on maximal attainable sensitivity (reached assay sensitivity), which is dependent on the previously determined threshold of a positive target event (e.g., T790M) and total cfDNA input. The classification was based on detectable *EGFR* mutation frequencies as seen in in-house data and literature [18]. Hence, samples were categorized as non-informative (>3%), restricted (0.5–3%), or adequate (<0.5%) for the multiplex assays. Thresholds for the T790M assay were lower, namely >1%, 0.1–1%, and <0.1%, respectively. cfDNA isolation, analysis and subsequent feedback to the treating thoracic oncologist were performed once a week. Hence, a TAT of approximately five working days, from sample arrival at UZA until feedback, was generally achieved.

Statistical Analysis

Linear regression was performed to determine the influence of plasma volume on reached assay sensitivity. The effect of pre-analytical variables, including transit time, centrifugation protocol, and average temperature, on plasma generation and the occurrence of hemolysis was investigated using linear and logistic regression, respectively. Next, the impact of all these variables, including plasma volume and hemolysis, on reached assay sensitivity was examined using multivariate analysis. In a subset of samples, one-way ANOVA was performed to determine the effect of temperature on reached assay sensitivity. Whether the presence of brain and/or extrathoracic metastases influenced ctDNA detection was analyzed using chi square analysis, with the Fisher's exact test to determine the effect of these metastases in relation to each other. Furthermore, the Mann–Whitney U test was performed to investigate their effect on ctDNA concentration and allele frequency, as well as the dynamics between the activating and T790M mutation, ctDNA prior to therapy and at radiologic progression, and lines of treatment. Survival estimates and hazard ratio were calculated according to the Kaplan–Meier method and Cox regression, respectively. ROC curve analysis was used to assess whether ctDNA concentration and allele frequency prior to therapy has an influence on the occurrence of radiologic progression. Statistical analysis was performed using IBM SPSS Statistics version 24.0 (IBM, Brussels, Belgium). *p*-values of <0.05 were considered statistically significant. Figures were made with Graphpad Prism7 (version 7, Graphpad Software Inc, La Jolla, CA, USA).

Results

Patient Cohort

In total, 234 NSCLC patients were included (Table 1), of which 549 plasma samples were collected. These include 77 (32.91%) patients classified as *EGFR* wild type (WT) according to tissue analysis and 51 patients (21.79%) without any information regarding *EGFR* mutational status. *EGFR* mutations were detected in plasma samples of 1 (1.30%) and 3 (5.88%) patients, respectively. The 110 *EGFR*-mutated patients (47.01%) were identified by tissue and/or plasma analysis. Of these patients, 38 provided one plasma sample (34.55%) and 68 (61.82%) provided on average six follow-up plasma samples. 101 patients received targeted therapy before or during the study. The *EGFR* T790M resistance mutation was detected in tissue and/or plasma of 34 (30.91%) *EGFR*-mutated patients, of which 19 arose during follow-up. In the other patients, the presence of the T790M mutation was already established or suspected at inclusion.

Sample Characteristics

The plasma samples (n = 549) were collected primarily in Streck tubes (472, 85.98%). In some cases, BD Vacutainer K_2EDTA (EDTA) tubes (77, 14.03%) were used (at the start of the multicenter study and in-house inclusion). All samples were grouped based upon the reached assay sensitivity, which is the maximal detectable *EGFR* mutation frequency; non-informative ([>3%] 45, 8.20%), restricted sensitivity ([0.5–3%] 217, 39.52%), and adequate sensitivity ([<0.5%] 287, 52.28%). In total, 106 (19, 3%) samples were hemolytic upon plasma generation. During the study, the centrifugation protocol for Streck tubes (n = 472) was altered, with 369 (78.18%) samples processed using the original protocol and 103 (21.82%) with the two-step protocol (Table S1).

Pre-Analytical Variables

Reached assay sensitivity, which is calculated based on total cfDNA input, was significantly influenced by the amount of generated plasma ($p < 0.001$), irrespective of the type of collection tube. In addition, a negative correlation with the presence of hemolysis was

observed (correlation coefficient (R) = −0.136, p = 0.001). Most samples were collected using Streck tubes. Based on the data obtained from

samples collected in these tubes, the effect of several pre-analytical variables (centrifugation protocol, transit time, and ambient temperature (average temperature per month) (Table S1)) on reached assay sensitivity was investigated.

- ***Effect of Pre-Analytical Variables on Plasma Volume and Hemolysis***

The two-step centrifugation protocol resulted in a significant increase in the amount of generated plasma ($p < 0.001$). A longer transit time was associated with a lower amount of plasma in the original centrifugation protocol (p = 0.005). This effect was not observed in the two-step protocol. Average temperature did not seem to influence plasma generation. Hemolysis was found to be significantly correlated with a lower amount of plasma ($p < 0.001$). Hence, the effect of the pre-analytical variables (centrifugation protocol, transit time and temperature) on the occurrence of hemolysis was also determined. Transit time was the only factor to have a significant impact (p <

Table 1

Overview of patient cohort

Parameters	**N (%)**
***EGFR* WT (n = 124)**	
Median age (years)	67
Stage at inclusion	
I	13 (10.48)
II	5 (4.03)
III	26 (20.97)
IV	75 (60.78)
Unspecified	5 (4.03)
***EGFR* mutated (n = 110)**	
Median age (years)	67.5
Stage at inclusion	
I	4 (3.64)
II	4 (3.64)
III	10 (9.09)
IV	89 (80.91)
Unspecified	3 (2.73)
EGFR	
DEL19	63 (57.27)
L858R	32 (29.09)
L861Q	3 (2.73)
G719X	4 (3.64)
Other	8 (7.27)
T790M	34 (30.91)
Therapy (N = 101*)	
erlotinib	42
PFS (months)	12.3
gefitinib	20
PFS (months)	12.7
afatinib	21
PFS (months)	11.4
osimertinib	16
PFS (months)	8.5
Metastases (at inclusion)	
Brain	
yes	26 (23.64)
no	75 (68.18)
na	9 (8.18)
Extrathoracic	
yes	40 (36.36)
no	61 (55.46)
na	9 (8.18.45)

Na: not available; PFS: progression-free survival; WT: wild type; *: two patients were included in a study 'erlotinib vs osimertinib in first line', hence no information with regards to their therapy was available.

0.001). 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.

➢ *Effect of Pre-Analytical Variables on Reached Assay Sensitivity*

Multivariate analysis was performed to determine the effect of the aforementioned pre-analytical variables on reached assay sensitivity (maximal detectable *EGFR* mutation frequency). Centrifugation protocol was significantly associated with reached assay sensitivity ($p < 0.001$). The original protocol resulted in a seemingly higher sensitivity than the two-step protocol, as additional long fragment DNA is removed in case of the latter. An increase in transit time in samples processed using the original protocol was found to negatively influence reached assay sensitivity ($p = 0.001$), whereas the opposite effect was observed in samples from the two-step protocol ($p = 0.016$). A trend was observed towards decreased sensitivity when hemolysis occurred ($p = 0.047$). In conclusion, sensitivity is influenced by the interplay between centrifugation protocol, amount of plasma, and transit time ($p < 0.001$).

The average temperature was found to have no significant effect on either plasma generation or reached assay sensitivity. Interestingly, when looking at the samples processed using the original centrifugation protocol, a significant difference in average temperature per reached assay sensitivity was observed ($p = 0.005$). An average temperature of 8 °C was recorded for samples which generated a non-informative result, while 11.5 ($p = 0.004$) and 10.8 °C ($p = 0.009$) was recorded for samples with restricted and adequate sensitivity, respectively.

cfDNA Analysis

CtDNA Detection

In this study, the *EGFR*-mutated patient cohort ($n = 110$) provided 416 plasma samples, of which 50 samples were taken prior to therapy, 69 at radiologic progression and 297 during follow-up. Mutated ctDNA was detected in 34 (68%) baseline samples and 51 (73.91%) samples at radiologic progression. Detectable levels of mutated ctDNA seemed to be more often found in samples with adequate sensitivity (*EGFR* mutation can be

detected at frequencies below 0.5% of the total cfDNA), higher amount of generated plasma and shorter transit time (Table 2). However, no statistical differences between mutated and WT samples were observed with regards to these pre-analytical variables. In 63.16% of the samples with detectable mutated ctDNA, the sample was classified as adequate sensitivity.

Table 2

Detection of mutations in plasma samples taken prior to therapy and at radiologic progression in relation to pre-analytical variables.

		ctDNA sensitivity ***adequate***	**Plasma volume** ***# mL***	**Transit time** ***days***	**Average temperature** ***°C***	**Centrifugation protocol** ***two-step***	**Hemolysis status** ***present***
PtT (50)	WT (16)	9 (56.3%)	3.00	1.77	13.0	5 (38.5%)	3 (18.8%)
	MUT (34)	21 (61.8%)	3.00	1.59	11.0	10 (34.5%)	7 (20.6%)
PD (69)	WT (21)	12 (60%)	2.85	1.67	11.6	8 (40%)	6 (28.6%)
	MUT (48)	29 (61.7%)	3.00	1.34	12.5	14 (35.9%)	5 (10.4%)

Centrifugation protocol – original (one-step, low-speed) vs two-step (high-speed); ctDNA: circulating, cell-free tumor DNA; adequate sensitivity (< 0.5%); MUT: mutated; PD: progressive disease; PtT: prior to therapy; WT: wild type.

The detection of ctDNA was found to be significantly associated with the presence of extrathoracic metastases both prior to treatment ($p < 0.001$) and at radiologic progression ($p < 0.001$). This association was also seen in the absence ($p = 0.001$, and $p = 0.004$) and presence ($p = 0.038$, and $p= 0.006$) of brain metastases in these two settings, respectively. The presence of brain metastases did not influence the detection of mutated ctDNA prior to therapy, irrespective of extrathoracic metastases. Unexpectedly, the presence of brain metastases at radiologic progression was found to positively influence the ctDNA detection ($p = 0.009$). However, this association was no longer detected when taking the presence or absence of extrathoracic metastases into account.

ctDNA Characteristics

The concentration (copies/mL) and allele frequency (%) of *EGFR*-mutated ctDNA (both activating mutations and T790M resistance mutation) in samples taken at radiologic disease progression or prior to therapy were found to be similar in these two groups. Previous

treatment schedules also did not seem to influence ctDNA levels. Interestingly, extrathoracic metastases were found to have a significant influence on allele frequency. Furthermore, higher concentrations were detected compared to brain metastases alone (Figure 1).

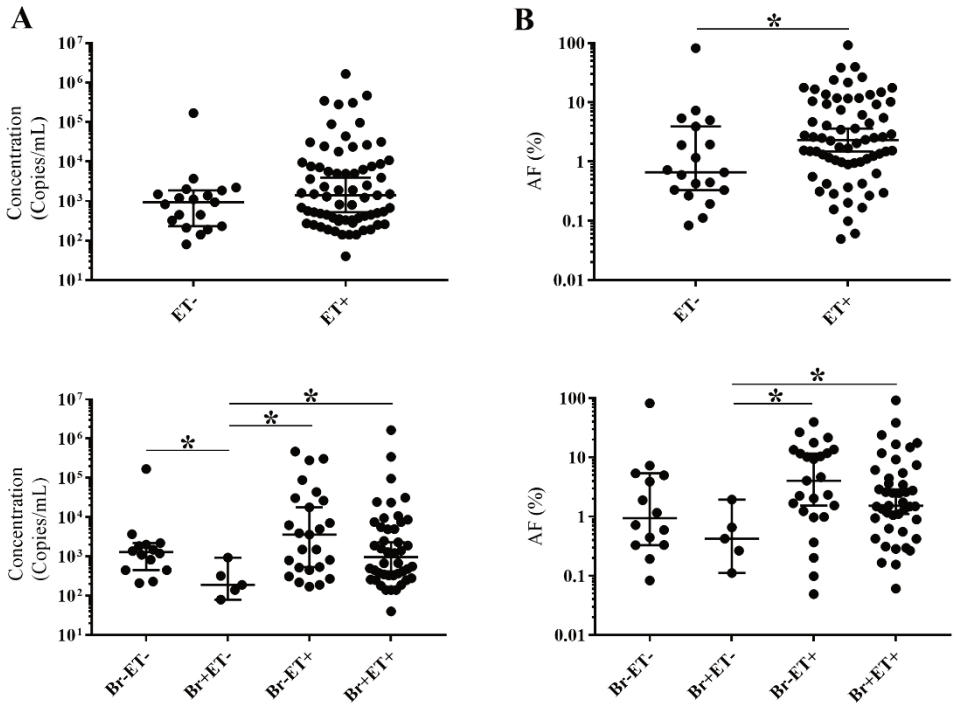


Figure 1: Influence of metastases on detectable *EGFR*-mutated ctDNA levels. A Distribution of concentration (copies/mL); **B** Distribution of allele frequency (%)**.** *Br: brain metastases; ET: extrathoracic metastases; AF (%): percentage of allele frequency; +: present; -: absent; median with 95% confidence interval (CI);* *******: $p < 0.05$.

The dynamics between activating mutations and the T790M mutation were also investigated. The T790M mutation was detected in 29 samples taken at radiologic progression. Both the concentration and allele frequency were lower than those of the activating mutation ($p < 0.001$, and $p < 0.001$, respectively) (Figure $2_{A\&C}$). Furthermore, the T790M mutation was also detected in 14 follow-up samples without radiologic progression to EGFR TKI therapy, in which a similar distribution was detected ($p = 0.037$, and $p = 0.002$, respectively) (Figure $2_{B\&D}$). A clear difference in both the concentration and allele frequency was observed between the activating mutation and T790M mutation ($p < 0.001$, and $p < 0.001$, respectively) in 40 out of 43 plasma samples. In three cases, the activating

mutation was not detected, mostly due to technical failure of the assay which screens for the activating mutations.

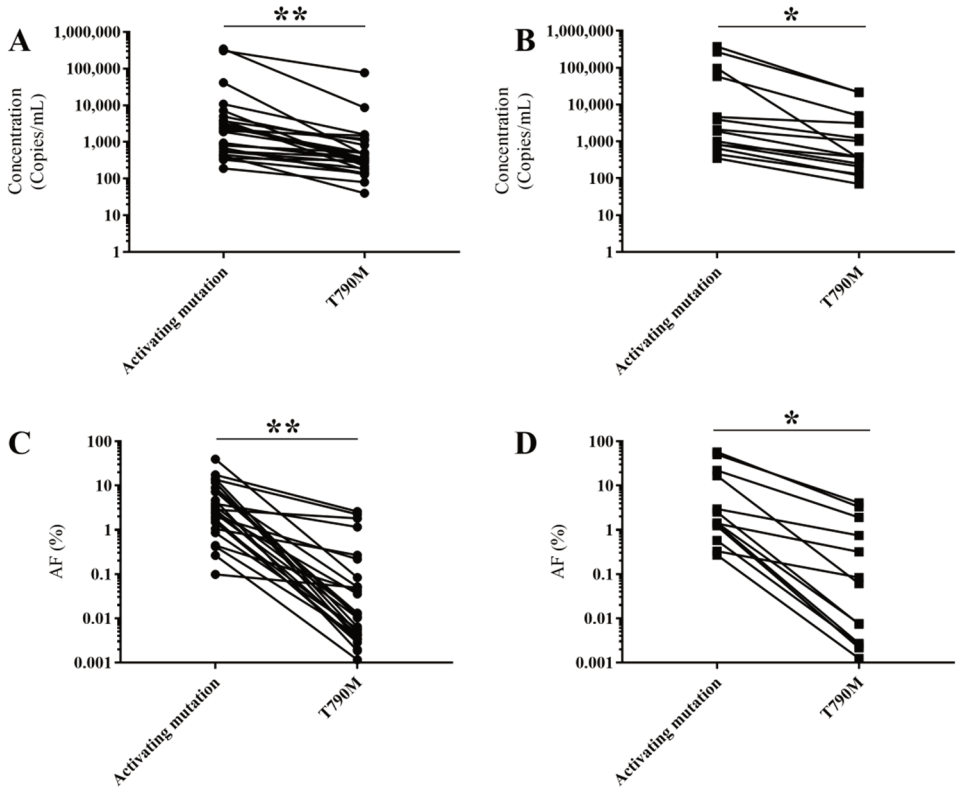


Figure 2: Comparison of ctDNA (activating mutations vs T790M mutation) (n = 43). A-B Concentration (copies/mL) of *EGFR* activating mutations (ex19del, L858R, L861Q, and G719X) versus T790M mutation detected in samples taken at radiological progression (n = 29) (**A**) and during follow up (n = 14) (**B**), respectively. The lowest detectable concentration was 190 and 40 copies/mL, with a median of 1950 and 360 copies/mL, respectively; **C-D** Distribution of the allele frequencies detected in samples taken at radiological progression (n = 29) (**C**) and during follow up (n = 14), respectively. The lowest allele frequency detected was 0.0985 and 0.0012 %, with a median of 2.530 and 0.0107 %, respectively. * $p < 0.05$; ** $p < 0.001$.

A significant difference in progression-free survival (PFS) was detected between patients with or without detectable ctDNA levels (5.8 months vs 13.3 months; hazard ratio (HR) = 3.646, 95% CI 1.201 – 11.071; $p = 0.029$) and with or without extrathoracic metastases prior to therapy (8.2 months vs 14.4 months; HR = 2.082, 95% CI 1.303 – 3.326; $p = 0.002$). High concentration and allele frequency of the mutated ctDNA at baseline was also found to have an influence on radiologic progression ($p = 0.004$, and $p = 0.017$,

respectively). The presence of brain metastases at the start of therapy was not found to affect PFS.

Discussion & Conclusion

In this study, several (pre-) analytic variables were investigated in order to provide an optimized and standardized workflow to implement liquid biopsy in a clinical setting (Table 3).

Reached assay sensitivity was calculated based on total cfDNA input; however, it is important to note that no distinction could be made between WT ctDNA, normal cfDNA, and genomic DNA (gDNA). The categorization of reached assay sensitivity was based on the required thresholds to detect clinically relevant ctDNA levels, as seen in in-house data and literature [18]. Sensitivity of cfDNA analysis was found to be increased by an interplay between increased plasma volume and short transit time. We hypothesize that the efficacy of Streck tubes is impaired with increased transit time. The main function of these tubes is to stabilize (i) cfDNA and (ii) blood cells and subsequently prevent hemolysis and gDNA contamination. According to Streck, longer storage of blood samples requires an increase of centrifugation speed [19, 20], which was also observed in our study. Furthermore, the multistep, high-speed centrifugation not only increased plasma volume, but also decreased the gDNA contamination [21]. This might explain the lower sensitivity in these samples, as well as the seemingly contradictory effect of transit time on reached assay sensitivity per centrifugation protocol. The fact that hemolysis was found to be strongly associated with an increased transit time and coincided with lower plasma volumes supports our hypothesis of impaired function of Streck tubes. Only a slight negative effect on sensitivity was observed, as we did not differentiate between hemolytic samples due to biological or technical causes.

Table 3

Optimized workflow for liquid biopsy in a clinical setting.

Variables	Protocol	Specifications
Pre-analytical variables	**Streck tubes**	
	Centrifugation protocol	Two-step, high speed: ↑ plasma volume & ↓ gDNA contamination
	Transit time	Short: to ensure proper cell and cfDNA stabilization in Streck tubes
	Temperature	>10 °C: to ensure proper cell and cfDNA stabilization in Streck tubes
	EDTA tubes	
	Processing	Within 2 hours: no liquid biopsy-specific preservatives present
	Centrifugation protocol	Two step: ↓ gDNA contamination
Analytical & biological variables	**ddPCR**	
	Reached assay sensitivitiy	Indication of cfDNA concentration
	T790M mutation	Test the majority of the isolated cfDNA: lower concentration & AF than activating mutation↑ ctDNA detection
	Metastases	
	Extrathoracic	
	Intrathoracic	Very high sensitivity is necessary due to low ctDNA concentrations
	Brain	Disruption of BBB ↑ ctDNA detection
Interpretation	**ctDNA detection**	
	No *EGFR* mutation	Tissue biopsy
	EGFR activating mutation	
	Prior to therapy	EGFR TKI therapy is recommended
	Without T790M mutation at PD to EGFR TKI therapy	
	Low AF	New blood sample in a few weeks time
	High AF	Tissue biopsy
	EGFR (activating &) T790M mutation	Osimertinib therapy is recommended

AF: allele frequency; BBB: blood brain barrier; cfDNA: circulating cell-free DNA; ctDNA: circulating cell-free tumor DNA; gDNA: genomic DNA; PD: progressive disease; ↑: increase; ↓ decrease.

According to the manufacturer's instructions, Streck tubes remain effective for up to 14 days at 6 °C to 37 °C [20]. However, it has been demonstrated that storage of these tubes

at 10 °C or lower was associated with an increase of gDNA as well as hemolysis and lower plasma volumes [22]. Even though we only recorded the average monthly temperature, temperatures below 10 °C were noted to have a detrimental effect on sensitivity in samples processed using the original centrifugation protocol. As the temperature in north western Europe often drops below 10 °C, optimization of transport conditions, such as other types of cfDNA collection tubes or more insulated transport, might aid in the standardization of liquid biopsy in a clinical setting. Further research might reveal specific, favorable temperature intervals by shipping a data logger with each blood sample. In this manner, differences between the outside temperature and the temperature inside the transport box, duration of exposure to aberrant temperatures, etc. can be closely monitored.

The majority of samples in our cohort in which mutated ctDNA was detected were classified as "adequate sensitivity". This indicates that the characterization is a good indication. Optimization of these pre-analytical variables will ensure the accuracy of our characterization of actual maximum mutated ctDNA detection.

Pre-analytical variables as well as biological factors have an impact on cfDNA analysis and interpretation. The detection and allele frequency of mutated ctDNA were strongly associated with the presence of extrathoracic metastases. Furthermore, the concentration and allele frequency of mutated ctDNA in these patients were significantly higher than those in patients who only had brain metastases. This is in line with previously reported data in which the relationship between mutated ctDNA detection and both number of metastatic sites and the presence of liver and bone metastases were highlighted [12]. This indicates that tumor load has a significant impact on ctDNA in the circulation and that extrathoracic metastases are responsible for a significant part of the total cfDNA concentration. No effect of brain metastases themselves on the detection, concentration or allele frequency of mutated ctDNA could be discerned. The presence of the blood–brain barrier (BBB) as a physical obstacle could prevent ctDNA from entering the circulation [23]. Only a limited number of patients were included who had brain metastases without an extrathoracic component. Furthermore, it is difficult to detect a negative influence of these brain metastases on the ctDNA concentration in the circulation, especially in the presence of the positive effect of extrathoracic metastases. It is also important to note that information with regards to disruption of the blood brain barrier, or progressive disease due to brain and/or extrathoracic metastases was not available in all

cases. However, we speculate that we have been able to detect the moment of BBB penetration in one patient (Figure 3$_{A}$).

Generally, a higher concentration and allele frequency of the activating mutation was detected compared to the T790M mutation. These findings confirm the data of Oxnard *et al.* [16], highlighting the importance of testing the majority of the cfDNA isolated from 10 mL of blood in case of T790M ddPCR analysis. In this study, we did not explore whether this difference was due to technical aspects (e.g. assay sensitivities). Furthermore, a recent study reported the correlation of the T790M ratio to the activating mutation and response to osimertinib [24]. Follow-up samples in our study revealed that the T790M mutation closely follows the activating mutation, albeit at a lower concentration and allele frequency (Figure 3$_{B}$). The activating mutation was also found to be very informative when tracking progressive disease. In a subset of patients, this mutation was detected at very low allele frequency, without the T790M mutation. A blood sample in a few weeks' time resulted in the detection of both the T790M and activating mutation, the latter in a higher allele frequency (Figure 3$_{C\text{-}D}$). We hypothesize that the T790M mutation was already present in the first sample, but the concentration was too low to detect. Interestingly, increasing and/or high allele frequency of the activating mutation was detected in some patients, without evidence of the T790M mutation (Figure 3$_{E\text{-}F}$). Tissue analysis confirmed absence of the T790M mutation despite the presence of the activating mutation. Further research might determine a clinically relevant threshold in which it is advisable to screen for other resistance mechanisms. In three samples, the T790M mutation was detected without the activating mutation. Presence of the T790M mutation was verified by tissue analysis. In two cases, the activating mutation was missed due to technical issues. To increase the detection of low levels of T790M mutation, it is key to analyze the majority of the cfDNA obtained from 10 mL of blood. Even though there have been reports of T790M detection without the activating mutation [25], most studies highlight coexisting mutations [26, 27]. Absence of both mutations was taken as an indication of very low levels of ctDNA in the circulation.

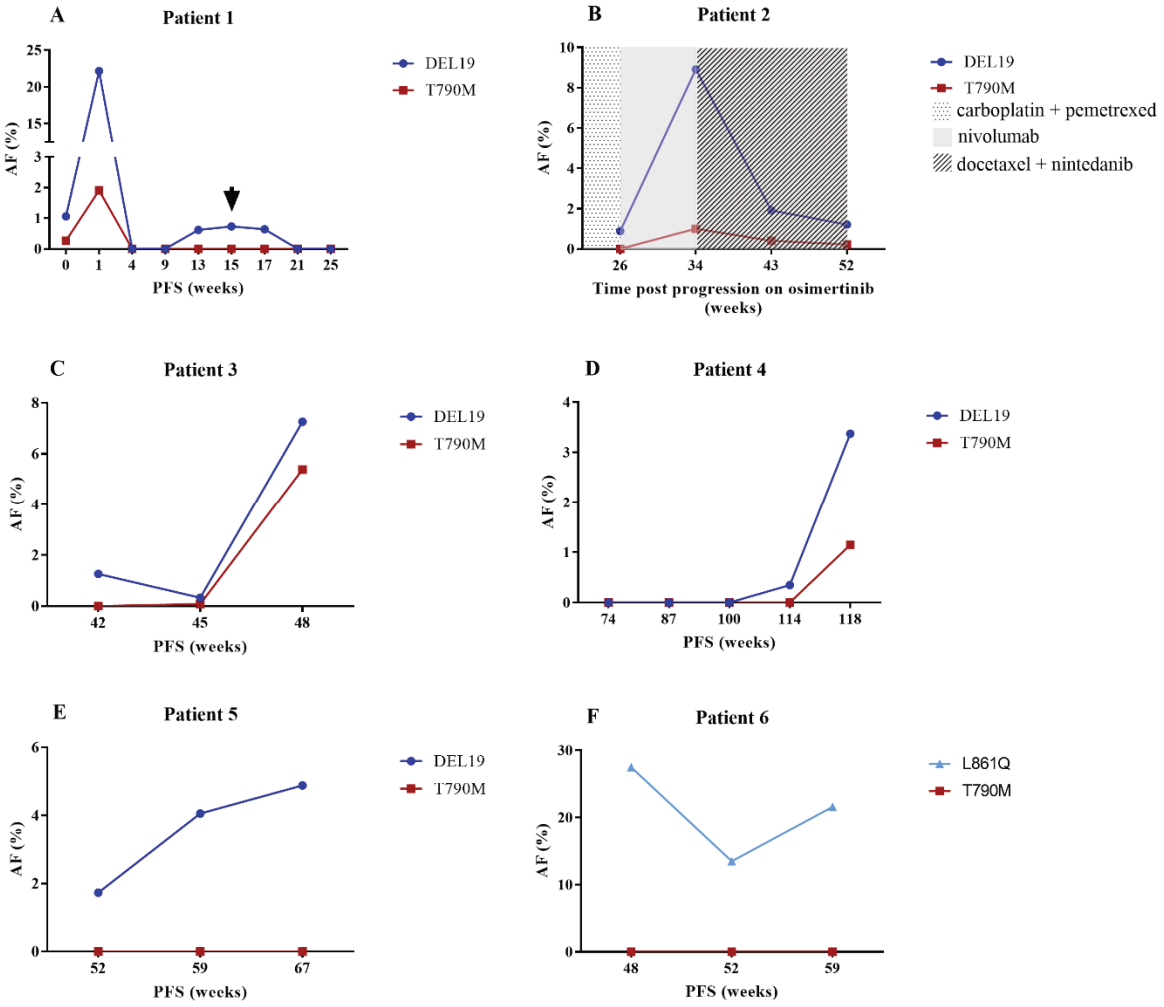


Figure 3: Kinetics of mutated ctDNA. A Initial increase and decrease until detectable levels of mutated ctDNA after osimertinib therapy. A spike of the activating mutation was detected from week 13 until week 17. At week 15, brain metastases with penetration of the blood brain barrier were detected via brain magnetic resonance imaging (MRI); **B** The T790M mutations follows the pattern of the activating mutation throughout several treatment schemes, however, at lower allele frequencies; **C-D** The activating mutation was detected at very low allele frequencies. A blood samples in a few weeks' time resulted in the detection of increased allele frequency of this mutation together with the T790M resistance mutation. This corresponded with progressive disease to first- and second-generation EGFR TKI therapy, respectively; **E-F** Increasing and extremely high AFs of the activating mutations were detected, respectively. Due to progressive disease under first generation EGFR TKI therapy, tissue biopsies were performed at the last time point, which confirmed the absence of the T790M mutation. *AF (%): percentage of allele frequency; PFS: progression-free survival in weeks;* ↓*: brain MRI.*

In this study, reached assay sensitivity and detection of the *EGFR* activating mutations were used to determine good quality samples with sufficient ctDNA concentrations. As

EGFR mutations only represent a fraction of the total ctDNA and are susceptible to EGFR TKI therapy, other biomarkers might be more suitable. Methylated ctDNA (metctDNA) has been shown to be a promising surrogate biomarker of tumor burden in liquid biopsy samples of colorectal cancer patients [28]. Furthermore, metctDNA could function as a prognostic marker, together with mutated ctDNA. The potential prognostic value of mutated ctDNA (concentration) has already been proposed [29, 30]. However, discordant results [31] highlight the need for further research to fully optimize liquid biopsy analysis.

Acknowledgements

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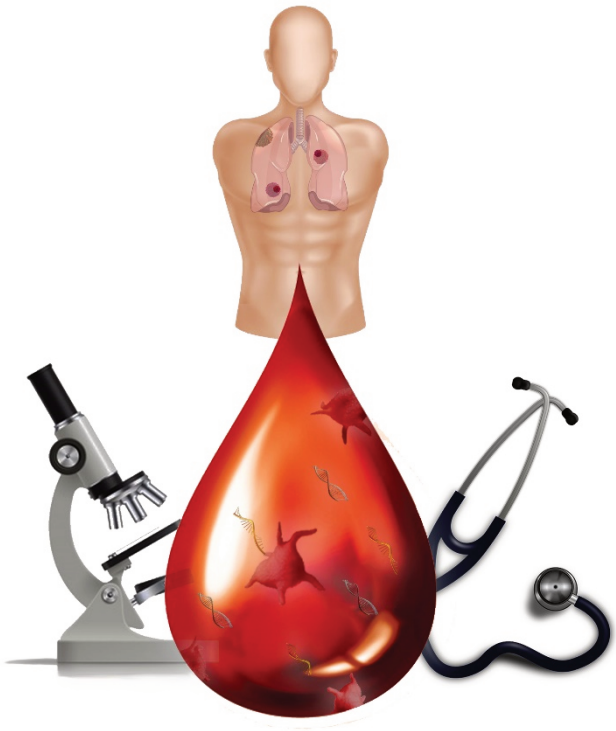
Supplementary Data

Supplementary data are available online.

CHAPTER 7

Monitoring EGFR TKI Resistance in Real-Time using ddPCR-based Liquid Biopsy: A Case Report.



Sorber L, Zwaenepoel K, Wouters A, Janssens A, Hiddinga B, Van Meerbeeck J, Lardon F, Pauwels P, Rolfo C. Monitoring EGFR TKI resistance in real-time using ddPCR-based liquid biopsy: a case report. Journal of Clinical Pathology, Volume 71, Issue 8, Pages 754-756, 2018.

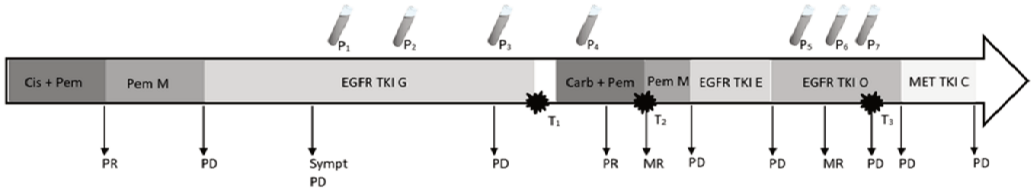
Introduction

Liquid biopsies, consisting of the analysis of circulating cell-free (tumor) DNA (cfDNA/ctDNA), circulating tumor cells (CTCs), or tumor-derived exosomes, have shown great potential in providing personalized therapy. Presently, the analysis of ctDNA has enabled the detection and real-time follow-up of specific genetic alterations in non-small cell lung cancer (NSCLC) patients, for which targeted therapies are available. Epidermal Growth Factor Receptor (*EGFR*) activating mutations are one of the most widely known examples of targets of tyrosine kinase inhibitors (TKIs). Several studies have described the detection of the *EGFR* T790M resistance mutation in blood samples 15-344 days prior to disease progression based on RECIST criteria [1]. Importantly, similar response rates to TKI therapy have been described whether the *EGFR* mutations were detected in blood or tissue samples [2]. These findings highlight the potential of liquid biopsy as a less invasive alternative to provide personalized therapy. However, relatively few studies report the detection of other resistance mechanisms in follow-up blood samples. In this case report, we highlight a case in which digital droplet PCR (ddPCR) based liquid biopsy aids in the detection of an atypical disease progression.

Case Report

We present the case of a 46-year-old woman diagnosed with stage IV (extra-cranial metastases) *EGFR*-mutated lung adenocarcinoma in June 2013. Tissue samples were taken throughout the disease course as part of clinical care. The patient provided written informed consent that blood samples could be taken at several time points (Figure 1). The human biological materials used in this publication were provided by Biobank@UZA (Antwerp, Belgium; ID: BE71030031000; Belgian Virtual Tumourbank funded by the National Cancer Plan; BBMRI-ERIC; No. Access: (1), Last: January 18, 2017)[3]. We performed ddPCR to detect *EGFR* mutations and Mesenchymal-Epithelial Transition factor (*MET*) amplification in tissue and plasma samples (Figure 2A-C).

A



B

Timeline of RECIST criteria, tissue and liquid biopsies

Date (dd/mm)	2013			2014						2015								
RECIST	NA	NA/12	02/07			05/11		25/02	02/04	22/06		10/08		28/08	16/09	09/11		
P				16/07	08/10	05/11	18/02				08/07	10/08	27/08					
T							20/11		03/04					28/08				

C

Date	Treatment (Initiated)	Best response RECIST	Best response Clinical response	DOR	Imaging
22/06/'15	Osimertinib (22/06/'15)	Baseline	ECOG1	NA	
10/08/'15	Osimertinib	MR	ECOG1 CB	1.5 months	
16/09/'15	Osimertinib	PD	ECOG2 CP	2.5 months	
09/11/'15	Crizotinib (16/09/'15)	PD	ECOG3 CP	Death after 2 months	

Figure 1. Case report. A An overview of the treatment schedule of the patient and reported RECIST during the disease course; **B** Tissue and liquid biopsies, consisting of circulating cell-free tumor DNA analysis, have been taken at several time points; **C** CT scans before and during osimertinib and subsequent crizotinib therapy. *C: crizotinib; Carb: carboplatin; CB: clinical benefit; Cis: cisplatin; CP: clinical progression; DOR: duration of response; E: erlotinib; ECOG: performance scale developed by the Eastern Cooperative Oncology Group; G: gefitinib; MR: mixed response; NA: not available; RECIST: Response Evaluation Criteria In Solid Tumors; Sympt PD: symptomatic progressive disease; T: tissue biopsy; TKI;*

The patient was treated with chemotherapy from June 2013 until the following December, when imaging displayed progressive disease. A tissue biopsy was sent to the Antwerp University Hospital (UZA) for molecular analysis, however, this sample was not retained for ddPCR analysis. The *EGFR* activating L858R mutation was identified for which gefitinib, a first-generation EGFR TKI, was administered from July 2014 onwards, at which time blood samples were collected at the treating thoracic oncologist's discretion. After 11 months of gefitinib therapy, the patient displayed disease progression. Both the *EGFR* activating L858R and T790M resistance mutation were detected in tissue (T_1) and blood samples (P_3). However, osimertinib, a third-generation EGFR TKI, was not available at that time. Treatment with chemotherapy was initiated January 2015. Initially, the patient displayed a partial response, which was also detected by liquid biopsy (P_4). In April 2015, radiological progression was detected and tissue biopsy (T_2) revealed the *EGFR* L858R mutation with T790M loss, prompting treatment with erlotinib. Remarkably, this was followed by an atypical fast radiological and clinical progression, whereas *EGFR* T790M mutated tumors are generally associated with slower growth [4].

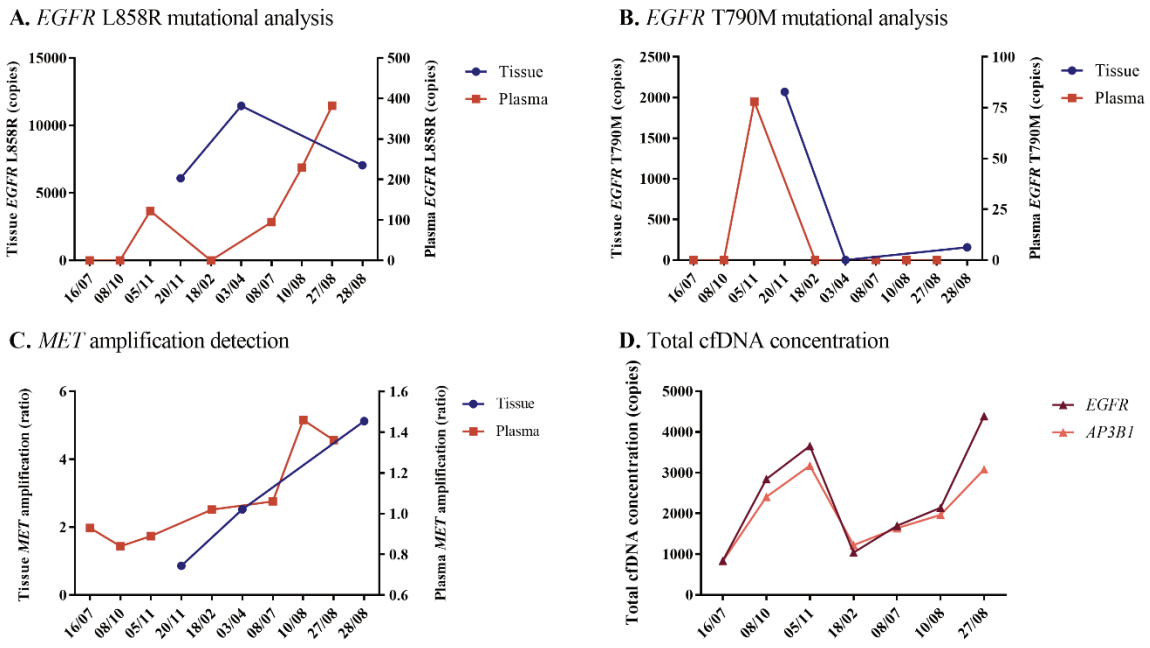


Figure 2: Results of tissue and liquid biopsy analysis using ddPCR. A *EGFR* L858R mutation concentration detected in tissue and plasma samples; **B** *EGFR* T790M concentration detected in tissue and plasma samples; **C** Ratio of the concentration of the *MET* gene versus the household *AP3B1* gene detected in tissue and plasma samples; **D** The total amount of cfDNA concentration determined by the *EGFR* and *AP3B1* assays detected in plasma samples. *cfDNA: cell-free DNA; ddPCR: digital droplet PCR.*

Subsequent treatment with osimertinib resulted in a mixed response and progressive disease was detected within two months (Figure 1_C). No *EGFR* T790M mutation could be detected in the blood samples (P_{5-7}) taken during treatment, however, the presence of *MET* amplification was revealed. As the presence of *MET* amplification was confirmed in tissue biopsy (T_3, Figure 2_C), crizotinib therapy was initiated in medical need. Unfortunately, the patient still displayed progressive disease. She chose to halt targeted therapy and passed away shortly after.

Discussion & Conclusion

In approximately 60% of *EGFR*-mutated NSCLC patients, the additional *EGFR* T790M mutation is the mechanism of acquired resistance to EGFR TKI therapy [1]. The average reported progression-free survival (PFS) of these patients treated with osimertinib ranges between 9.6 and 12.3 months [5, 6]. In this case report, we highlight a patient who displayed an atypical fast progression to first generation EGFR TKI. Furthermore, despite the presence of the *EGFR* T790M mutation, osimertinib treatment resulted in a short and mixed response. The *EGFR* T790M mutation could no longer be detected in the blood samples before and during osimertinib therapy. In this case report, it was not further investigated whether this was due to technical or biological issues/parameters, such as assay sensitivity and response to therapy or small subset of T790M-mutated cells, respectively. DdPCR analysis did reveal the presence of *MET* amplification, which has already been reported as a resistance mechanism to both first- and third-line EGFR TKI therapy [7]. Interestingly, previous reports have suggested a reciprocal relationship between the *EGFR* T790M mutation and *MET* amplification. However, in case of increasing *MET* amplification, this overlap becomes less frequent [8].

Liquid biopsy might be a promising source to fully understand mechanisms of acquired resistance, without being hampered by metastatic heterogeneity. Both *EGFR* and *MET* ddPCR assays provided us with an absolute quantification of total cfDNA. The decrease and increase of the total detected cfDNA corresponded with the RECIST reported throughout the patients' disease course (Figure 2_D). Furthermore, the detection of *MET*

amplification was (partly) correlated with the therapy resistance and fast disease progression.

CtDNA analysis using ddPCR assays is a relatively easy, cheap, and high-throughput method for real-time follow-up of patients in a clinical setting. Previous reports of *MET* amplified ctDNA detection using ddPCR displayed relatively low concordance rates with tissue [9]. Sensitivity might be increased by further optimizing *MET* ddPCR assays, such as the use of multiple reference genes. The presence of wild type (WT) cfDNA might hamper the detection of low amounts of *MET* amplification. By analyzing the circulating cell-free RNA (cfRNA) in comparison to (several) reference genes, small amounts of gene amplification might be detected using liquid biopsy.

To conclude, we hypothesize that the fast, atypical progression observed in this case study was most likely due to *MET*-amplified clones. We have highlighted the use of ctDNA analysis as liquid biopsy and the importance of combining both molecular and clinical data in order to provide personalized therapy. In this manner, tissue biopsies can be complemented by liquid biopsies in the screening for EGFR TKI resistance mechanisms.

Acknowledgements

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DISCUSSION

CHAPTER 8

General Discussion and Future Perspectives.

General Discussion

The expanding application of targeted therapies based on tumor-specific aberrations has made molecular profiling essential for cancer management [1]. As tissue biopsies are encumbered by several limitations, new ways to observe the genetic landscape of the tumor and its evolution were introduced. The term liquid biopsy implies the use of blood or other bodily fluids such as urine, saliva, pleural effusions, and cerebrospinal fluid, to provide diagnostic, predictive, and prognostic information. The analysis of circulating tumor cells (CTCs), circulating cell-free nucleic acids (cfNAs), exosomes, and tumor-educated platelets (TEPs) has shown great potential in several aspects of cancer patient management [1-3].
The literature review in **chapter 2** highlighted the potential of cfNA based liquid biopsy in providing personalized therapy to non-small cell lung cancer (NSCLC) patients [4]. Furthermore, we described several highly sensitive techniques which have been specifically designed to detect the often low and highly variable circulating cell-free tumor DNA (ctDNA) fraction. However, despite these advances, these techniques remain limited by the number of genome equivalents per sample. The detection of a true mutation by either a targeted assay or (targeted) next-generation sequencing (NGS) technique, often requires more than one signal or read, respectively. Higher sensitivity is not necessarily obtained by using more material as analytical success is often compromised by degradation of cfDNA or contamination with wild type (WT) genomic DNA (gDNA) from normal cells [5]. Hence, optimization of pre-analytical conditions is crucial to ensure high cfDNA quality and yield for downstream molecular applications. As a result, in this doctoral thesis we have focused on the optimization and standardization of the liquid biopsy workflow.

We started this project by determining the optimal ctDNA isolation kit as the ctDNA fraction is highly variable and can make up as little as 0.01% of the total cfDNA (**chapter 3**). Five cfDNA isolation kits were selected based on i) column- or magnetic beads-based DNA capture, ii) lysis step prior to or after DNA capture, and iii) manual or automated protocol [6]. The QIAamp Circulating Nucleic Acid Kit (Qiagen) and the Maxwell RSC ccfDNA Plasma Kit (Promega) were found to have the highest cfDNA isolation efficiency. Despite the fact that the former is column-based and the latter consists of a magnetic

beads-based cfDNA capture, both kits had a lysis step prior to DNA capture and were found to favor shorter cfDNA fragments. One of the main findings of this study was the importance of the lysis step prior to DNA capture. CfDNA has been demonstrated to be present in the circulation in different forms, namely as solitary molecular or macromolecular complexes (linked to serum proteins or to the cell-free membrane parts or internalized in vesicles, exosomes, apoptotic bodies or microparticles) [7]. Hence, lysis of the sample will result in a larger fraction of cfDNA that can be captured. This also highlights the advantage of using plasma samples from patients with detectable mutant ctDNA over plasma samples with spiked tumor DNA. The spiked DNA might not represent the entire cfDNA content and is vulnerable to DNAse activity in plasma. In line with our results, the Qiagen kit has been consistently reported to be one of the better performing kits [8-11]. We detected similar cfDNA concentrations between the Qiagen and Promega Kit. This was also reported by Pérez *et al.* [12], while Diefenbach *et al.* described a higher cfDNA isolation efficiency of the Qiagen kit [11]. The latter study consisted of spiked plasma samples, which might explain the discrepancy. Furthermore, both kits were found to favor shorter cfDNA fragments. This fraction has been reported to contain the majority of ctDNA [13]. Hence, both kits were able to detect *KRAS* mutated ctDNA in all plasma samples from stage IV PDAC patients. Even though both kits were found to have a high cfDNA isolation efficiency and a larger sample volume, the Promega kit has the advantage of a semi-automated protocol. We opted to use the Promega kit throughout this dissertation.

The next obvious step in the optimization of the liquid biopsy workflow was the appropriate generation of plasma. Whereas ctDNA has been established as an ideal source for mutation and methylation analysis, ctRNA in the circulation or embedded in vesicles or tumor-educated platelets (TEPs) seems to be a more appropriate matrix for the detection of gene fusions and amplifications. Hence, we selected five centrifugation protocols differing in centrifugation speed, time, temperature, and number of centrifugation steps and compared their cfDNA/ctDNA and cfRNA/ctRNA yield (**chapter 4**).

In EDTA tubes, the platelet-generating protocol, which produces plasma and platelets, was found to generate high quality cfDNA and high cfRNA concentrations, respectively [14].

Currently, the recommended centrifugation protocol for cfDNA consists of two centrifugation steps; a first centrifugation step to separate plasma and a second to remove any remaining cells or cellular debris. The reported conditions range between 800 – 2000 g for 10 – 20 minutes, followed by 2000 – 16,000 g [5]. Hence, it was unexpected to find high quality cfDNA in the plasma of the platelet-generating protocol, as it consisted of two very low (< 400 g) centrifugation steps. We believe that cfDNA quality is influenced by the interplay between centrifugation speed and duration. This is also highlighted by the cfDNA generated by the low-speed (400 g) protocol coupled with a 1-min high-speed centrifugation post-thaw. This second step significantly reduced the genomic DNA (gDNA) contamination compared to the one-step, low-speed centrifugation. However, the resulting cfDNA quality was still lower that the quality of the cfDNA generated by the platelet-generating and two-step, high-speed centrifugation protocols. This might be further improved by increasing the durations of the post-thaw centrifugation step. On the other hand, the one-step centrifugation protocol, aside from platelets, was found to generate the highest cfRNA concentration. As demonstrated by previous research, platelets are an excellent source of cfRNA and ctRNA [15, 16]. Due to the low centrifugation speed (400 g), the one-step protocol generates plasma that is relatively rich in platelets. Platelet rich plasma (PRP) can be obtained by a centrifugation speed < 1000 g, with the highest platelet recovery at 100 g for 10 minutes [17, 18]. In contrast, the other centrifugation protocols consist of high centrifugation speed and/or two centrifugation steps, resulting in platelet poor plasma (PPP). PPP generally contains a platelet count of $< 10*10^3$ platelets / μL [19]. A limitation of this study was that only 200 μL of plasma, representing 500 μL of blood, was used for cfRNA isolation compared to the entire platelet fraction, which corresponds to 10 mL of blood. Despite the fact that cfRNA isolation kits with increased input volume are being developed, we believe that the platelet-generating protocol will remain the optimal protocol. The entire plasma fraction of one blood sample can be used to isolate cfDNA, while cfRNA can be generated from the platelets. In this manner, high concentrations and quality of both cfDNA and cfRNA can be obtained, thereby maximizing the molecular information with limited material from the patient.

We also compared the efficiency of these centrifugation protocols in one of the most commonly used stabilizing blood collection tubes (Streck tubes). Similar to EDTA tubes, two-step, high-speed protocols and the platelet-generating protocol were found to have

the highest cfDNA isolation efficiency. In this study, samples collected in Streck tubes were kept at room temperature for 24 hours prior to centrifugation. It is important to note that longer storage of blood samples in these tubes requires higher centrifugation speed to ensure sufficient plasma generation [20, 21]. Hence, it is unlikely that any of the low-speed centrifugation protocols can be used in a real-life setting. We also found that little or no cfRNA could be isolated in Streck tubes. As specific cell-free RNA BCT® (Streck) tubes have been developed [22], it seems likely that the DNA Streck tubes don't sufficiently stabilize cfRNA. In this manner, two different collection tubes would have to be used to perform a complete molecular analysis. Similar to EDTA tubes, a blood collection tube stabilizing both cfDNA and cfRNA would provide maximal molecular information from one blood sample.

Lastly, we evaluated the effect of delayed processing of blood samples and the choice of (specialized) blood collection tubes (BCTs) on cfDNA (**chapter 5**). We started by performing a systematic review. In total, 25 studies met our criteria and were examined in detail. Although there were strong differences in methodology between the studies, we were able to draw certain conclusions. Standard EDTA tubes are the recommended tube type for in-house collection, providing samples are processed as soon as possible, preferably within 4 to 6 hours. Storage at low temperatures (4 – 6 °C) might enable longer stability, however, maximum time-to-processing remains controversial. In case of delayed processing, specialized BCTs are indicated as they have been designed to stabilize blood cells as well as cfDNA. In an interval of 48 to 72 hours, no major differences in efficacy were observed between CellSave, PAXgene, Norgen, Roche, and Streck tubes. Similar to EDTA tubes, the reported maximum time-to-processing varied strongly between studies. However, since a short turnaround time (TAT) is one of the major advantages of liquid biopsy, it would be undesirable to delay processing beyond 72 hours. Temperature was only reported to have an influence on the BCT efficacy in extreme circumstances (e.g. heat shock). To validate these findings, we investigated the efficacy of PAXgene, Roche, and Streck tubes in a situation most resembling the clinical setting. No differences in cfDNA stability were observed between these tubes after 72 hours, irrespective of temperature. Furthermore, shaking, performed to mimic shipping, did not affect cfDNA and *KRAS*-mutated ctDNA concentrations or cfDNA characteristics. The outcome was similar to

those detected in EDTA tubes, which were processed immediately and considered as gold standard. Although Roche and PAXgene tubes were prone to hemolysis, this was not reflected in cfDNA concentration [23]. This could be due to the fact that the rupture of white blood cells (WBCs), which are the main cause of gDNA contamination, doesn't necessarily coincide with hemolysis [24]. Furthermore, samples were processed using their recommended centrifugation protocol, which might improve the cfDNA quality [21]. Based on the literature review and our own data, we could conclude that the efficacy of these specialized BCTs was similar providing they were processed within 48 to 72 hours and according to the appropriate protocol. Hence, the choice between these specialized BCTs is of less importance than the selection of the optimal processing protocol.

In the second part of this dissertation, we evaluated the potential of liquid biopsy in a clinical setting. Therefore, we set up a multicenter study in Belgium in which we performed liquid biopsy analysis in 234 NSCLC patients to assess their *EGFR* mutational status (**chapter 6**). In total, 549 blood samples were collected and processed at the Antwerp University Hospital (UZA). The majority of blood samples was collected in Streck tubes. Plasma was generated immediately upon arrival and stored at -80 °C prior to cfDNA isolation. The *EGFR* mutation status was assessed by two in-house developed and one T790M-specific digital droplet PCR (ddPCR) assays, all of which are BELAC (the Belgian Accreditation Institute) accredited. A TAT of one work-week upon arrival of the blood samples was achieved [21]. Based on our results, we present the optimal conditions for successful cfDNA analysis:

- *Pre-analytical variables*

Elevated cfDNA concentration can be achieved by increased plasma volume coupled with shorter transportation time. Thereby, blood samples collected in Streck tubes should be processed within 48 hours, to ensure their cell and cfDNA stabilization efficiency. Two-step, high-speed centrifugation in EDTA and Streck tubes are necessary to prevent gDNA contamination, as demonstrated in chapter 4. In case of the latter, high centrifugation speed is crucial to generate sufficient plasma volume.

- *Analytical and biological variables*

The detection of genetic aberrations is not only dependent on assay sensitivity, but also on the amount of available genome equivalents in a plasma sample [5]. Our categorization of

reached assay sensitivity (i.e. the previously established threshold of a positive target (e.g. T790M) versus total cfDNA input) is a good indication of maximum mutated ctDNA detection. It is important to note that this approach does not differentiate between cfDNA and gDNA. A sample with gDNA contamination due to improper pre-analytical conditions can be falsely categorized as high sensitivity. A possible solution would be to screen for a so-called 'passive' aberration that occurred at the initiation from tumorigenesis. As it provides little to no selective advantage, it represents the overall tumor burden [25]. Aberrant DNA methylation has been described as an early event in carcinogenesis [26, 27] and was shown to accurately represent tumor burden [28]. Hence, the detection of methylated ctDNA (metctDNA) could serve as a safeguard in determining the actual ctDNA content. Furthermore, we recommend testing the majority of the isolated cfDNA for the *EGFR* T790M mutation, to ensure detection in case of lower allele frequencies (AF) than the original *EGFR* activating mutations [21]. Liquid biopsy is subject to sampling errors; only 10 – 20 mL is collected from approximately 5 L of normal blood volume and the cfDNA concentration and quality is affected by several pre-analytical conditions (as highlighted in chapters 3, 4, 5, and 6). Hence, when screening of other biomarkers (expected to be present at low AFs), testing the majority of the cfDNA might be the most appropriate approach as well.

Besides technical considerations, biological variables should also be considered. In line with our findings, tumor burden and metastatic site location have been reported to influence cfDNA concentration [29, 30]. Both number of metastatic sites and the presence of bone or liver metastases were found to be correlated with increased sensitivity [29]. Hence, exceedingly sensitive techniques are necessary for cfDNA detection in case of localized disease. In case of central nervous system (CNS) metastases, the cfDNA detection in plasma is hampered by the blood-brain barrier (BBB). Pons et al. reported that in the absence of extra-CNS tumors, mutations could not be detected in the plasma cfDNA [30]. Hence, cfDNA analysis in patients with extensive disease, and BBB disruption in case of CNS metastases, will be less likely to generate false-negative results [21].

- *Interpretation*

It is generally accepted to perform tissue genotyping in case of a negative result. Despite the development of highly sensitive techniques and the recent interest in pre-analytical conditions, the exact mechanism of cfDNA release in the circulation remains unknown [5].

As such, it is impossible to discriminate between a WT sample and a sample with little to no cfDNA. On the other hand, the detection of *EGFR* (activating) mutations in treatment-naïve NSCLC patients is sufficient to warrant EGFR tyrosine kinase inhibitor (TKI) therapy. Furthermore, we found that the AF of *EGFR* activating mutations in patients displaying progressive disease under EGFR TKI might be indicative of the resistance mechanism. High AF without T790M detection were associated with other resistance mechanisms. In case of low AF, the T790M concentration was often too low to be detected and was revealed in a blood sample at a later time [21]. According to the 2018 ESMO clinical practice guidelines, detection of the T790M mutation is mandatory to warrant Osimertinib (a third-generation EGFR TKI) treatment [31]. Recently, the results of the FLAURA trial were published. Osimertinib was shown to have a higher efficacy than the standard EGFR TKIs, erlotinib and gefitinib, in the first-line treatment of EGFR mutated NSCLC patients [32]. Furthermore, the frequency of CNS metastases was found to be lower in the osimertinib group. Even though the second-generation EGFR TKI afatinib was not included in this study, Osimertinib has become the standard first-line treatment in this patient cohort. Even though the detection of the T790M mutation will become of less importance, the results we presented in chapter 6 will still have merit in the detection of resistance mechanisms. First of all, there are currently still a large number of NSCLC patients who are being treated with first- or second-generation EGFR TKIs. Upon progression, the AF of the *EGFR* activating mutation might be indicative of the resistance mechanism. Second, the *EGFR* C797S has already been described as a resistance mechanism to Osimertinib in a second-line setting. Furthermore, resistance mechanisms to Osimertinib therapy in a first-line setting still need to be characterized. The interplay between the AF of the *EGFR* activating mutations and resistance mechanism might not only provide information in this setting, but also with other targetable aberrations and/or tumor types.

In **chapter 7**, we highlighted the potential of liquid biopsy in unraveling resistance mechanisms [33]. The results of the cfDNA analysis throughout the patients' disease course, closely follows the patterns observed in the previous chapter. The first time the patient progressed during first-generation EGFR TKI therapy, the EGFR L858R and T790M mutations were detected in the liquid biopsy sample as well as in the tissue biopsy.

In both sample types, the AF of the T790M mutation was lower than the activating mutation, confirming our results from chapter 6. However, the second time the patient displayed progressive disease under first-generation EGFR TKI therapy, the activating mutation was detected in multiple liquid biopsy samples with increasing AF without the T790M mutation. It was revealed that a *MET* amplification was responsible for the atypical fast progression. A tissue biopsy confirmed the presence of a strong *MET* amplification coupled with an extremely low T790M AF. Both the T790M mutation and *MET* amplification have been described as resistance mechanisms to first- and, in case of the latter, third-generation EGFR TKI therapy [34-36]. This patient developed a heterogeneous response to the treatment, which was revealed by combining the clinical data with the molecular information from both the tissue and liquid biopsies.

It is important to note that the patient had a high tumor burden as well as a high level of *MET* amplification. If the amount of *MET* amplified ctDNA in the circulation was lower, it might not have been detected with cfDNA-based ddPCR. As indicated previously, cfRNA is a more appropriate matrix for the detection of gene amplifications and fusions. Hence, the combination of cfDNA and cfRNA will further increase the potential of liquid biopsy in unraveling resistance mechanisms of targeted therapies and providing patients with personalized treatment.

Future perspectives

There is increasing evidence of the clinical utility of liquid biopsy analysis in a routine setting. Prospective studies have reported that the response rate (RR) based on cfDNA analysis are similar to those described in tissue-based studies [37, 38]. Furthermore, the detection of targets for targeted therapies was at least as high as in tissue-based biopsies and with a shorter TAT [39]. Our work on devising the optimal workflow will aid in the standardization and further implementation of liquid biopsy in both research settings and clinical routine. Presently, there are already a number of cfDNA-based interventional studies ongoing. In the UK plasma MATCH trial [NCT03182634] ctDNA screening is used to identify subgroups of advanced breast cancer patients based on their sensitivity to targeted therapies. Furthermore, the safety and activity of these targeted treatments are also

verified [40]. There are also two studies ongoing in which therapy is switched based on cfDNA results. In the CAcTUS trial [NCT03808441], *BRAF*-mutated metastatic melanoma patients are initially treated with targeted therapy. Changes in *BRAF*-mutated ctDNA levels are used to inform when to switch to immune therapy [41]. The objective of the CHRONOS trial [NCT03227926] is to assess the efficacy and safety of a re-challenge with anti-EGFR therapy based on the clonal evolution of *RAS*-mutated ctDNA. Blood samples of *RAS*-extend WT metastatic CRC patients are monitored for the presence of *RAS*-mutated ctDNA upon secondary resistance to anti-EGFR therapy and during progression to second line chemotherapy [42]. It is important to note that liquid biopsy can also have an added value in earlier cancer stages. In the DYNAMIC-III trial [ACTRN12617001566325], the detection of methylated ctDNA is instructive in (de-) escalating adjuvant chemotherapy in stage III CRC patients who underwent surgery with curative intent [43].

It is important to note that liquid biopsy in these approaches is hampered by the variability of cancer mutation profiles in patients. Aberrant methylation profiles present an advantage, as they tend to be tissue- and region-specific, and occur early in carcinogenesis [28, 44]. The *SEPT9* gene methylation assay was the first, and currently only, FDA-approved blood test for CRC screening [45]. Although the results of prospective trials are somewhat less encouraging [46, 47], the combination of several markers could further increase the sensitivity and specificity of liquid biopsy. In a study by Bachet *et al.* mutational analysis was coupled with methylation detection to assess the presence of ctDNA in metastatic CRC patients [48]. Combination of cfDNA- and cfRNA-based genomic screening with cfDNA-based methylation analysis could substantially increase the number of patients who might benefit from real-time follow-up by liquid biopsy. One example in which liquid biopsy could provide much needed information is the early detection of response to therapy. The clonal evolution of the ctDNA, based on genetic and epigenetic screening, might inform whether the patient will show a durable response to therapy. The increased number of patients who can be screened by liquid biopsy would facilitate large observational and interventional studies, and accelerate the implementation of liquid biopsy in clinical routine.

The added value of combining several biomarkers has also been highlighted in the CancerSEEK study [49]. The CancerSEEK blood test is able to detect eight common

cancer types by screening for genetic alterations and protein biomarkers. This was assessed in 1005 patients with non-metastatic, clinically detected cancers of the liver, pancreas, lung, ovary, stomach, breast, esophagus, or lung and 812 healthy controls. The specificity of the CancerSEEK test was greater than 99%, while the sensitivities ranged from 69 to 98% for five cancer types (esophagus, pancreas, stomach, liver, and ovary). In these cancer types, there are currently no screening tests available for average-risk individuals. Inclusion of other biomarkers, such as methylation analysis, might further increase sensitivity. The group of Shen *et al.* have developed a novel method to analyze the cfDNA methylome for the detection and classification of early-stage cancers [50].

While blood is currently the standard concept of liquid biopsy, there are other bodily fluids which can be used to investigate tumor-derived material. These include urine, sputum, saliva, cerebrospinal fluid, and pleural effusions [51]. The completely non-invasive character of the former three together with the possibility of collection at the patient's own home would greatly improve the patients' comfort, especially in a follow-up setting. Several studies have already demonstrated good concordance between these fluids, plasma, and tissue [51-53]. Interestingly, the group of Wu et al. observed a difference in genomic profiles detected per type of liquid biopsy samples [53]. However, further research is still necessary to validate this finding as well as establish standardized work flows.

To conclude, further studies into the combination of several (liquid biopsy-based) biomarkers will pave the way towards personalized therapy for the majority of patients.

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42. *Rechallenge With Panitumumab Driven by RAS Dynamic of Resistance.*
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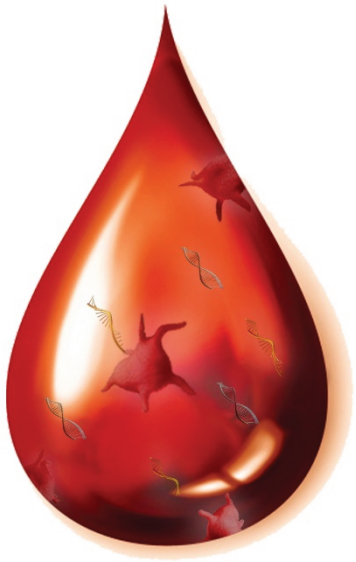
CHAPTER 9

Summary

Samenvatting



Summary

Personalized treatment of advanced cancers has been revolutionized by the discovery of targetable molecular alterations and associated, effective drugs. Due to the clonal evolution of the tumor, characterization of the tumor has become necessary at several time points during the patients' disease course. Although tissue biopsies, the gold standard, provide both histological and genetic information, they are encumbered by several limitations. The inter- and intratumor heterogeneity and clonal evolution coupled with the risk of the procedure itself can make tissue biopsies less suitable for real-time follow-up. Liquid biopsy, consisting of the analysis of several biomarkers in the blood, is a minimally invasive technique to assess the molecular landscape of the tumor. The development of highly sensitive analysis platforms has enabled the analysis of several of these biomarkers, such as circulating cell-free (tumor) DNA (cfDNA / ctDNA) and RNA (cfRNA / ctRNA). In **chapter 2**, we described the potential applications of circulating cell-free nucleic acids-based (cfNAs) liquid biopsy in non-small cell lung cancer (NSCLC), a tumor type with several targetable alterations. However, the lack of standard operating procedures has limited its implementation in a clinical setting. In this PhD dissertation we have focused on identifying key steps to compose an optimized liquid biopsy workflow.

As even highly sensitive techniques are dependent on the number of genome equivalents, we started by evaluating several cfDNA isolation kits (**chapter 3**). We found that a lysis step prior to DNA binding is crucial to capture both the circulating cfDNA fraction as well as the cfDNA adhered to or captured in vesicles of protein aggregates. Moreover, we also demonstrated the importance of using clinically relevant samples when evaluating cfDNA isolation efficiency. The QIAamp Circulating Nucleic Acid Kit (Qiagen) and the Maxwell RSC ccfDNA Plasma Kit (Promega) were found to be the best performing kits. As the latter consisted of a semi-automated protocol, we opted to use this kit throughout this dissertation, with the exception of chapter 5.

In **chapter 4**, we assessed the generation of cfDNA and cfRNA by several centrifugation protocols in both standard EDTA tubes and stabilizing blood collection (Streck) tubes. In

EDTA tubes, the protocol which generates both plasma and platelets was found to be highly suitable for cfDNA and cfRNA analysis, respectively. We could demonstrate that cfDNA characteristics were identical between the recommended two-step, high-speed centrifugation protocols and this platelet-generating protocol, which consists of two slow centrifugation steps. CfDNA generated by a one-step, low speed protocol is hampered by a high genomic DNA (gDNA) contamination. A second high-speed centrifugation post-thaw will improve the cfDNA quality. On the other hand, platelets are an excellent source of cfRNA, followed by this one-step, low-speed protocol. In contrast, the high-speed centrifugation protocols generated very low cfRNA concentrations. We also revealed that cfRNA is not sufficiently stabilized in Streck tubes. Therefore, we can conclude that the selected centrifugation protocol is dependent on the downstream analysis. When analyzing both cfDNA and cfRNA, the platelet-generating protocol is the most appropriate protocol to use with EDTA tubes, followed by the one-step, low-speed protocol. On the other hand, a two-step, high-speed centrifugation is the most efficient protocol to generate cfDNA of high quality and quantity in both tube types.

Another important aspect of the liquid biopsy workflow is the time-to-processing of blood samples and the choice of blood collection tube (BCT). In **chapter 5**, we performed a systematic review to evaluate the effect of delayed processing of blood samples collected in different (specialized) BCTs on cfDNA. Standard EDTA tubes should be processed as soon as possible, as cfDNA stability after 4 to 6 hours can't be guaranteed. Stability might be increased by storing samples at low temperatures (4 – 6 °C). Similar cfDNA stabilizing efficacies were reported for CellSave, PAXgene, Norgen, Roche, and Streck tubes when processed within 48 to 72 hours. Temperature only affected the specialized BCTs' efficacy in extreme circumstances (e.g. heat shock). This was confirmed by our study, in which we evaluated the efficacy of PAXgene, Roche, and Streck tubes in a situation resembling the clinical setting. No significant differences were observed between these tubes when processed correctly within 48 to 72 hours. This time frame also facilitates a short turn-around time (TAT), which is one of the advantages of liquid biopsy.

The ability of liquid biopsy to detect genetic aberrations as targets for targeted therapy has enormous potential in routine clinical setting. Therefore, we evaluated the liquid biopsy

workflow for the assessment of *EGFR* mutations in non-small cell lung cancer (NSCLC) patients in a multicenter study (**chapter 6**). In total, we investigated the influence of several (pre-) analytical variables on the cfDNA in 549 plasma samples of 234 NSCLC patients. The use of a multistep, high-speed centrifugation protocol coupled with short transit time ensured high plasma volumes. Furthermore, this was also associated with decreased gDNA contamination, prevented hemolysis, and negated the negative effect of low temperatures during transport. These findings confirmed the conclusions of the previous chapters on how to generate high quality cfDNA. We were also able to demonstrate that the *EGFR* T790M resistance mutation is almost always present in lower concentrations and allele frequency (AF) than the original *EGFR* activating mutations. Hence, we recommend to analyze the majority of the cfDNA when using liquid biopsy to detect resistance mechanisms to EGFR tyrosine kinase inhibitor (TKI) therapy. Furthermore, biological variables were also found to influence liquid biopsy sensitivity. CfDNA analysis is more likely to be successful if extrathoracic metastases are present, instead of a single brain metastasis. Tissue genotyping is always indicated in the case of a negative result, while the detection of *EGFR* mutations in treatment-naïve NSCLC patients is sufficient to warrant EGFR TKI therapy. We also demonstrated that the AF of the original *EGFR* activating mutation might be indicative of the resistance mechanism responsible for progressive disease under EGFR TKI therapy. To summarize, we have evaluated the optimal liquid biopsy workflow to ensure successful cfDNA analysis.

We further highlighted the potential of liquid biopsy in a case report (**chapter 7**). As described in chapter 6, we could show that the evolution of the original *EGFR* activating mutation was indicative of the resistance mechanism responsible for the patients' atypical fast progression. We clearly demonstrated the advantage of combining clinical data with molecular information from tissue and liquid biopsy to provide patients with personalized treatment.

To conclude, in this PhD dissertation we constructed an optimized workflow for plasma-based liquid biopsy. The combination of several biomarkers is becoming more important. Hence, our work on both cfDNA and cfRNA will aid in the standardization and further implementation of liquid biopsy in routine clinical setting. Furthermore, future research

will reveal whether this workflow is stringent enough to also enable urine- or sputum-based liquid biopsy. Thereby providing patients with a completely non-invasive tool for real-time follow-up.

Samenvatting

De ontdekking van moleculaire veranderingen als doelwitten van gerichte en efficiënte antikankertherapie hebben de behandeling van kankerpatiënten in een laat stadium grondig veranderd. Tumoren ondergaan ook klonale evolutie. Dit maakt het belangrijk om de tumor te karakteriseren op meerdere momenten tijdens het ziekteverloop van de patiënt. Weefselbiopten, waarbij er een stukje van de tumor wordt weggenomen, zijn de gouden standaard om tumoren te karakteriseren. Ondanks dat ze histologische en genetische informatie voorzien, hebben deze biopten enkele belangrijke beperkingen. De aanwezigheid van inter- en intratumor heterogeneiteit, klonale evolutie en het feit dat het nemen van een biopsie niet zonder risico is voor de patiënt, kan weefselbiopten minder geschikt maken voor real-time opvolging. Vloeibare biopsie, waarbij verschillende biomerkers in het bloed onderzocht worden, is een minimaal invasieve techniek om het moleculaire landschap van de tumor te bekijken. De ontwikkeling van zeer gevoelige platformen maakt het mogelijk verschillende biomerkers te onderzoeken, waaronder circulerend cel-vrij (tumor-) DNA (cfDNA / ctDNA) en RNA (cfRNA / ctRNA). In **hoofdstuk 2** hebben we de verschillende toepassingen beschreven van vloeibare biopsie op basis van circulerend cel-vrij nucleïnezuren in niet-kleincellige longkanker (NSCLC). NSCLC is een tumortype met verschillende moleculaire doelwitten. Spijtig genoeg is de implementatie van vloeibare biopsie in klinische routine beperkt door het ontbreken van standaard procedures die gepaard gaan met verlies in kwalitatief cfDNA/cfRNA. In dit proefschrift zijn we op zoek gegaan naar de belangrijke stappen om een geoptimaliseerde vloeibare biopsie-procedure op te stellen.

Gezien zelfs de zeer gevoelige technieken afhankelijk zijn van het aantal genoomequivalenten, zijn we gestart met de evaluatie van verschillende cfDNA isolatiekits (**hoofstuk 3**). We konden vaststellen dat een lysisstap voor het binden van DNA noodzakelijk is om zowel de circulerende cfDNA-fractie als het cfDNA dat gebonden is in of aan vesikels en eiwitaggregaten te isoleren. Meer nog, we hebben aangetoond dat het belangrijk is om klinisch relevante stalen te gebruiken bij het evalueren van cfDNA isolatie-efficiëntie. De QIAamp Circulating Nucleic Acid Kit (Qiagen) en de Maxwell RSC

ccfDNA Plasma Kit (Promega) bleken de best functionerende kits. Gezien deze laatste bestaat uit een semi-geautomatiseerd protocol, hebben we besloten om deze kit doorheen dit proefschrift te gebruiken, met uitzondering van hoofdstuk 5.

In **hoofdstuk 4** hebben we de cfDNA and cfRNA opbrengst van verschillende centrifugatieprotocollen onderzocht in de standaard EDTA-tubes and de stabilizerende bloedcollectietubes (Streck). We hebben aangetoond dat in EDTA-tubes het protocol dat zowel plasma als bloedplaatjes genereert, uitermate geschikt is voor respectievelijk cfDNA- en cfRNA-analyse. We konden aantonen dat de cfDNA eigenschappen identiek waren tussen de aanbevolen twee-staps, hoge snelheid centrifugatieprotocollen en dit plaatjes-genererende protocol, dat bestaat uit twee trage centrifugatiestappen. Het cfDNA gegenereerd door het éénstaps, lage snelheid centrifugatieprotocol wordt echter gehinderd door een hoge genomisch DNA- (gDNA) contaminatie. Een extra hoge snelheid centrifugatie na het ontdooien verbetert de cfDNA-kwaliteit. Anderzijds zijn bloedplaatjes een uitstekende bron van cfRNA, gevolgd door dit eenstaps, lage snelheid protocol. De hoge snelheidsprotocols genereerden echter zeer lage cfRNA concentraties. We konden ook vaststellen dat cfRNA niet voldoende gestabiliseerd wordt in Streck tubes. Op basis van deze resultaten konden we concluderen dat de keuze van centrifugatieprotcol afhankelijk is van het type analyse. In het geval van zowel cfDNA- als cfRNA-analyse, is het plaatjes-genererende protocol het meest aangewezen in EDTA-tubes, gevolgd door het éénstaps, lage snelheidsprotocol. Anderzijds is een twee-staps, hoge snelheid centrifugatie het meest efficiënte protocol om cfDNA van hoge kwaliteit en kwantiteit te genereren in beide tubetypes.

Een ander belangrijk aspect van de vloeibare biopsie-procedure is de tijd-tot-verwerken van de bloedstalen en de keuze van bloed collectie tubes (BCTs). In **hoofdstuk 5** hebben we een gestructureerd literatuuronderzoek uitgevoerd om het effect van de tijd-tot-verwerken na te gaan op verschillende (gespecialiseerde) BCTs. De standaard EDTA-tubes dienen zo snel mogelijk verwerkt te worden, gezien de cfDNA-stabiliteit niet gegarandeerd is na 4 tot 6 uur. De stabiliteit zou verhoogd kunnen worden door de stalen op een lage temperatuur (4 – 6 °C) te bewaren. Gelijkaardige cfDNA-stabiliserende effectiviteit werd gerapporteerd voor CellSave-, PAXgene-, Norgen-, Roche-, en Streck-tubes wanneer deze

binnen de 48 tot 72 uur verwerkt werden. Temperatuur beïnvloedde de werking van de gespecialiseerde BCTs alleen in extreme omstandigheden (bvb bij een hitteschok). Dit werd bevestigd in onze eigen studie, waarbij we de effectiviteit van PAXgene-, Roche-, en Streck-tubes evalueerden in een situatie waarbij de klinische situatie nagebootst werd. Er konden geen significante verschillen aangetoond worden tussen deze tubes wanneer deze op een correcte manier binnen de 48 tot 72 uur verwerkt werden. Deze termijn maakt een korte uitvoeringstijd mogelijk, wat een van de voordelen is van vloeibare biopsie.

Vloeibare biopsie is in staat om genetische afwijkingen te detecteren die als doelwit dienen van gerichte antikankertherapie. Dit biedt enorme mogelijkheden voor de klinische routine. Daarom hebben we de vloeibare biopsieprocedure om *EGFR* mutaties op te sporen in NSCLC-patiënten onderzocht in een multicentrische studie (**hoofdstuk 6**). In totaal hebben we de invloed van verschillende (pre-) analytische factoren op het cfDNA onderzocht in 549 plasmastalen van 234 NSCLC-patiënten. Het gebruik van een meerstaps, hoge snelheid centrifugatieprotocol tesamen met een korte transporttijd verzekerde grote plasmavolumes. Meer nog, dit was geassocieerd met een vermindering van gDNA contaminatie, voorkwam hemolyse, en deed het negatieve effect van lage temperaturen teniet. Deze bevindingen bevestigden de conclusies van de vorige hoofdstukken omtrent de beste manier om cfDNA van hoge kwaliteit te bekomen. We hebben ook aangetoond dat de *EGFR* T790M resistentie mutatie bijna altijd aanwezig is in lagere concentraties en allelfrequenties (AF) dan de originele *EGFR*-activerende mutaties. Hierdoor raden we aan om de meerderheid van het cfDNA te analyseren wanneer vloeibare biopsie gebruikt wordt om resistentiemechanismen tegen EGFR tyrosine kinase inhibitoren (TKI) op te sporen. Voorts hebben we aangetoond dat biologische factoren ook een invloed hebben op de gevoeligheid van vloeibare biopsie. CfDNA-analyse is vaker succesvol in de aanwezigheid van extrathoracale metastasen vergeleken met één enkele hersenmetastase. In het geval van een negatief resultaat is een weefselbiopt altijd aangewezen. Anderszijds is de detectie van *EGFR* mutaties in behandelingsnaïeve NSCLC-patiënten voldoende om EGFR TKI-therapie op te starten. We hebben ook aangetoond dat de AF van de originele *EGFR*-activerende mutatie een indicatie kan geven over het resistentiemechanisme verantwoordelijk voor progressieve ziekte tijdens EGFR TKI-

therapie. Samenvattend, hebben we in dit hoofstuk de optimale condities voor succesvolle cfDNA analyse achterhaald.

We hebben het potentieel van vloeibare biopsie nogmaals aangehaald in een casestudie (**hoofdstuk 7**). Zoals reeds beschreven in hoofdstuk 6 was de evolutie van de originele *EGFR*-activerende mutatie indicatief voor het verantwoordelijke resistentiemechanisme en de atypisch snelle ziekteprogressie van de patiënt. We hebben duidelijk het voordeel aangetoond van het combineren van klinische data met de moleculaire informatie van weefsel- en vloeibare biopten in het voorzien van een op maat gemaakte behandeling voor de patiënt.

In conclusie hebben we in dit proefschrift een geopmaliseerde procedure opgesteld voor vloeibare biopsie op basis van plasma. De combinatie van verschillende biomerkers blijft belangrijker worden. Vandaar dat ons werk op zowel cfDNA als cfRNA zal helpen in de standaardisatie en verdere implementatie van vloeibare biopsie in de klinische routine. Bovendien zal toekomstig onderzoek uitwijzen of deze procedure goed genoeg is om ook vloeibare biopsie op basis van urine of speeksel toe te laten. Op deze manier kunnen we patiënten van een volledig niet-invasieve manier voorzien om hen in real-time op te volgen.

CURRICULUM VITAE

Personalia

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Education and scientific career

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2013 – 2014 **Trainee**

"Genetic Research into the Role of the *NPY2R* Gene and the *NPY4R* Gene in the Development of Obesity"

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2014 Certificate of Laboratory Animal Science, FELASA Category C

2012 – 2014	Master of Science in Biomedical Sciences Major: Molecular and Cellular Biomedical Sciences University of Antwerp *Magna cum laude*
2008 – 2012	Bachelor of Science in Biomedical Sciences University of Antwerp

Scientific output

Articles in peer reviewed journals

2019 **Sorber, L.**, Zwaenepoel, K., Jacobs, J., De Winne, K., Van Casteren, K., Augustus, E., Lardon, F., Prenen, H., Peeters, M., Van Meerbeeck, J., Roeyen, G., Rolfo, C., Pauwels, P. (2019) Specialized Blood Collection Tubes for Liquid Biopsy: Improving the Pre-Analytical Conditions. Molecular Diagnosis & Therapy, accepted, DOI: 10.1007/s40291-019-00442-w

Sorber, L., Zwaenepoel, K., Jacobs, J., De Winne, K., Goethals, S., Reclusa, P., Van Casteren, K., Augustus, E., Lardon, F., Peeters, M., Van Meerbeeck, J., Rolfo, C., Pauwels, P. (2019). CfDNA and cfRNA Analysis as Liquid Biopsy: Optimal Centrifugation Protocol. Cancers (Basel), 11. doi:10.3390/cancers11040458

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2017 **Sorber, L.**, Zwaenepoel, K., Deschoolmeester, V., Roeyen, G., Lardon, F., Rolfo, C., & Pauwels, P. (2017). A Comparison of Cell-Free DNA Isolation Kits: Isolation and Quantification of Cell-Free DNA in Plasma. J Mol Diagn, 19(1), 162-168. doi:10.1016/j.jmoldx.2016.09.009

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2016 Reclusa, P., Giallombardo, M., Castiglia, M., **Sorber, L.**, Van Der Steen, N., Pauwels, P., & Rolfo, C. (2016). P2.06: "Exosomal miRNA Analysis in Non-Small Cell Lung Cancer: New Liquid Biomarker?", Track: Biology and Pathogenesis. J Thorac Oncol, 11(10S), S219-S220. doi:10.1016/j.jtho.2016.08.080

Reclusa, P., Sirera, R., Araujo, A., Giallombardo, M., Valentino, A., **Sorber, L.**, Bazo, IG., Pauwels, P., Rolfo, C. (2016). Exosomes genetic cargo in lung cancer: a truly Pandora's box. Transl Lung Cancer Res, 5(5), 483-491. doi:10.21037/tlcr.2016.10.06

Poster presentations

2019 De Winne, K., **Sorber, L.,** Lambin, S., Keulen, L., Broeckx, G., Pauwels, P., Zwaenepoel, K. Results of a first panTRK IHC ringtrial – Molecular Analysis for Personalised Therapy Congress, London

Sorber, L., Zwaenepoel, K., Jacobs, J., De Winne, K., Goethals, S., Van Casteren, K., Augustus, E., Lardon, F., Peeters, M., Van Meerbeeck, J., Rolfo, C., Pauwels, P. The best of Both Worlds: Optimal Centrifugation Protocol for cfDNA and cfRNA.

EACR-ESMO Joint Conference on Liquid Biopsies, Bergamo

BACR conference, Antwerp

Augustus, E., Van Casteren, K., Zwaenepoel, K., **Sorber, L.**, Van Dam, P., Roeyen, G., Peeters, M., Pauwels, P. The Effect of Centrifugation Protocols and the Use of First Void on the Concentration of Cell-Free DNA and Genomic DNA Contamination in Urine Samples – EACR-ESMO Joint Conference on Liquid Biopsies, Bergamo

2018 **Sorber, L.**, Zwaenepoel, K., Jacobs, J., De Winne, K., Goethals, S., Van Casteren, K., Augustus, E., Lardon, F., Peeters, M., Van Meerbeeck, J., Rolfo, C., Pauwels, P. The best of Both Worlds: Optimal Centrifugation Protocol for cfDNA and cfRNA – Exosomes & Liquid Biospies Europe, Rotterdam

Sorber, L., Zwaenepoel, K., Demedts, I., Pieters, T., Germonpré, P., Derijcke, S., Deschepper, K., Van Meerbeeck, J., Rolfo, C., Pauwels, P. A Multicenter Study to Assess *EGFR* Mutational Status in Plasma: Focus on an Optimized Workflow – EACR25: From Fundamental Insight to Rational Cancer Treatment, Amsterdam

2017 **Sorber, L.**, Zwaenepoel, K., Wouters, A., Janssens, A., Hiddinga, B., Van Meerbeeck, J., Lardon, F., Rolfo, C., Pauwels, P. Detecting Resistance Mechanisms in Patients on EGFR TKI Treatment by Liquid Biopsy – AACR conference, Washington

Sorber, L., Zwaenepoel, K., Wouters, A., Janssens, A., Hiddinga, B., Van Meerbeeck, J., Lardon, F., Rolfo, C., Pauwels, P. The Use of Liquid Biopsy in the Detection of Resistance Mechanisms in Patients on EGFR TKI Treatment – BACR conference, Leuven

2016 **Sorber, L.**, Zwaenepoel, K., Deschoolmeester, V., Roeyen, G., Goethals, S., Lardon, F., Rolfo, C., Pauwels, P. A Comparison of cfDNA Isolation Kits: Isolation and Quantification of Cell-Free DNA in Plasma – AACR conference, New Orleans

Sorber, L., Zwaenepoel, K., Deschoolmeester, V., Goethals, S., Lardon, F., Van Meerbeeck, J., Rolfo, C., Pauwels, P. Prospective of Liquid Biopsy in the Follow up of Cancer Patients: An Illustrative Case – 37th EORTC-PAMM Winter Meeting, Antwerp

Sorber, L., Zwaenepoel, K., Deschoolmeester, V., Roeyen, G., Goethals, S., Lardon, F., Rolfo, C., Pauwels, P. Isolation and Quantification of Cell-Free DNA in Plasma: A Comparison of cfDNA Isolation Kits – BACR conference, Brussels

Oral presentations

2019 Molecular Pathology Course for Residents in Pathology, Antwerp, 29 March – Liquid Biopsy

2018 AstraZeneca ddPCR Workshop, Antwerp, 11 December – Liquid Biopsy: Optimal Pre-Analytical Conditions

Molecular Oncology, 1st Master in Biomedical Sciences, Antwerp, 5 November – Liquid Biopsy: ddPCR

Molecular Pathology Course for Residents in Pathology, Antwerp, 15 June – Liquid Biopsy

2017 Liquid Biopsy Meeting, Brussels, 8 December – Liquid Biopsy in NSCLC & PDAC: Pre-analytics & Analysis

Molecular Oncology, 1st Master in Biomedical Sciences, Antwerp, 21 November – Liquid Biopsy: ddPCR

Round Table AstraZeneca, Liège, 20 June – Realizing the Full Potential of Liquid Biopsy for EGFR Testing

AstraZeneca Masterclass, Antwerp, 17 May – *EGFR* T790M mutation testing

Molecular Diagnostics Spring Meeting, Brussels, 9 April – A Comparison of Cell-Free DNA Isolation Kits: Isolation and Quantification of Cell-Free DNA in Plasma

2016 AstraZeneca Round Table, Bruges, 12 December – Liquid Biopsy: *EGFR* T790M testing

Symposium voor Pathologie, Aalst, 2 December – De Detectie van Predictieve Merkers in Circulerend Tumor DNA uit Celvrij Bloedplasma: Mogelijkheden, Toepassingen, Pitfalls en Standaardisatie

Molecular Oncology, 1st Master in Biomedical Sciences, Antwerp, 23 November – Liquid Biopsy: ddPCR

AstraZeneca Molecular Board Meeting, Brussels, 11 February – Liquid Biopsy: *EGFR* Testing

TOGA Meeting, Antwerp, 16 October – Liquid Biopsy: IRIS

Amgen Preceptorship, 15 & 17 March – RAS Test Future Directions: Blood Sample and Circulating Tumor Cells / Cell-Free Tumor DNA

2015 Molecular Oncology, 1st Master in Biomedical Sciences, Antwerp, 16 October – Liquid Biopsy: ddPCR

Molecular Pathology Day, Antwerp, 3 October – Liquid Biopsy: An Introduction

2014 Molecular Oncology, 1st Master in Biomedical Sciences, Antwerp, 20 October – Liquid Biopsy: ddPCR

Science communication

2019 Tutorial video liquid biopsy workflow – in collaboration with Boehringer-Ingelheim

Video "Bloed als Boodschapper in de Strijd tegen Kanker" – Science Figured Out, project by Scriptie vzw

Press>>Speak Wedstrijd Wetenschapscommunicatie, Antwerp, 29 March – Bloed als Boodschapper in de Strijd tegen Kanker.

Scientific grants

2019 Research Grants from Kom op tegen Kanker to finish PhD thesis (Emmanuel van der Schueren) – The Optimal Liquid Biopsy Workflow for Implementation in Research and Clinical Setting

2017 Research Grant from Kom op tegen Kanker – Analysis of Plasma, Platelets, and Urine Nucleic Acids: the Value of Liquid Biopsy to Improve Diagnosis, Follow-up, and Monitoring of Treatment Response of Lung Cancer and Pancreatic Cancer Patients

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about Bavet as I am, or as you call it: Laure's restaurant ☺. You really know how to throw a housewarming / game night. I just realized that I still have footage of the dancing part of the evening. In more work-related matters, you really are an amazing post-doc! Dit brengt me bij de vierde bewoner van deze bureau, de immer fashionable **Elly**. Bedankt om met me te gaan winkelen voor een kleed voor een trouwfeest, zonder jou had ik het zelfs nooit uit de rekken gehaald! Je enthousiasme voor lopen werkt echt aanstekelijk ☺, hoeveel mensen je hebt weten overtuigen om tijdens de middag te gaan lopen. Ik wens je heel veel succes met je project in Australië (ik wou dat ik ook naar daar kon)! Ik wil me niet inbeelden hoe bruin jij gaat zijn als je terugkomt.

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ik kan je niet genoeg bedanken om mijn figuren te maken! Zonder jou had het maar iets triestigs geworden ^^. Sorry om je zoveel werk te bezorgen, maar ik denk dat deze figuren een van de dingen uit mijn doctoraat zijn waar ik het meest trots op ben! Dat we nog vaak samen onze verjaardag kunnen vieren ☺. **Putte** (aka Michaël, Jos), ook jou moet ik bedanken om mij al al die jaren te steunen! Hoe vaak dat je niet hebt geluisterd als ik ergens over moest ventileren of me ergens zorgen over maakte. Bedankt om mee te gaan in mijn pasta-cravings (al moet je toegeven dat je de smaak ook te pakken hebt gekregen), mij altijd blij te maken met het aanduiden van schattige diertjes, elk jaar een nieuwe "katlender" en voor alle mooie reizen die we al samen hebben gemaakt ☺. Ook een dikke merci aan jouw familie voor de warme ontvangsten, steun en leuke momenten. Dit brengt me tot mijn familie, waarbij iedereen een welgemeende dikke dankuwel verdient! Iedereen bedankt om altijd te vragen hoe het met mijn doctoraat stond, te luisteren (en op z'n minst interesse te veinzen ^^) als ik erover vertelde en me altijd succes te wensen! **Tante Marijke**, langsgaan op de gang van het ethisch commité was altijd een beetje leuker als ik dan verder ging en u tegenkwam ☺. Mijn studentenjaren zijn tot hun einde aan het komen, al kom ik graag nog eens op ons oude schoolbankje zitten bij u en **nonkel Walter**! **Matthias**, ik ben blij dat jij ondertussen ook mee deel uitmaakt van de Bavet-fanclub (ik ben de tel kwijt hoeveel volk ik al mee naar daar heb genomen). Ook **Ome Serge**, **Laurent** en **Eric** moet ik bedanken om altijd naar mijn verhalen te luisteren. Neefjes, hopelijk heb ik jullie iets kunnen bijbrengen over de "multifunctionele brij" ☺. Lollie, je bent niet meer de enige dr. Sorber L (allé, hopelijk toch, nog even duimen ^^), al staat er bij jou wel nog een ir. bij (die tensoren toch). **Mama** en **Opa**, ook al zijn jullie er niet meer bij, ik denk aan jullie! Dan hebben we nog de SorberSquad. **Cedric** en **Gina**, ook al ben ik ondertussen de kleinste van ons drie (Gina, echt maar met 1 cm é), ik blijf de oudste. Waar is de tijd dat Gina ons dwong om mee naar die verschrikkelijke barbiefilms te kijken? Ik heb me enorm geamuseerd met jullie op Gina haar vrienden- en familiecantus en nadien nog in de Klimax, dat doen we nog eens! Allebei bedankt om er te zijn en ik wens jullie ook nog heel veel succes! Cedric, jij met je mooie appartement en Gina, jij met je studies (hint: de nervus vagus is geen stofje ^^) en je studentenleven. De Sorberkids geraken er wel ☺. **Ania**, enorm bedankt om er echt altijd te zijn voor ons! Hoeveel dat je ons altijd geholpen hebt ☺, daarom nemen we je plagen er graag bij (ik heb nog alle prinsessensokjes die je voor me gekocht hebt ^^). **Papa**, ik wil je heel hard bedanken om me altijd te steunen en te zorgen dat ik alles kon

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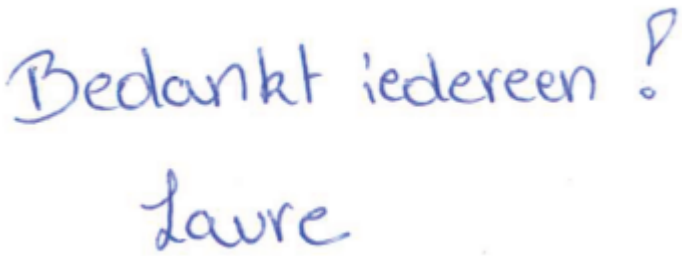
Bij deze nogmaals, iedereen enorm enorm bedankt! Zoals elk klein stapje in de liquid biopsy workflow belangrijk en onmisbaar is, zijn jullie dat voor mij ook!



P.S. Papa, ik weet niet of je het je nog herinnert, maar dit is nog steeds de roze pen gegraveerd met mijn naam die ik jaren (en jaren) geleden van u en mama heb gekregen ☺.