Revealing traces of tumour DNA in the circulation

Beagan, Jamie John

2022

document version
Publisher's PDF, also known as Version of record

Revealing traces of tumour DNA in the circulation

Towards clinical implementation

Jamie J. Beagan

Revealing traces of tumour DNA in the circulation

Towards clinical implementation

Jamie J. Beagan

Financial support for printing of this thesis was kindly provided and supported by Cancer Center Amsterdam.

ISBN: 978-94-6458-093-8
Cover design and layout: Publiss | www.publiss.nl
Printed by: Ridderprint | www.ridderprint.nl

VRIJE UNIVERSITEIT

Revealing traces of tumour DNA in the circulation

Towards clinical implementation

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor of Philosophy aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. J.J.G. Geurts,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op donderdag 7 april 2022 om 13.45 uur
in een bijeenkomst van de universiteit,
De Boelelaan 1105

door

Jamie John Beagan

geboren te Southampton, Verenigd Koninkrijk

promotor:	prof.dr. B. Ylstra
copromotor:	dr. D.M. Pegtel
promotiecommissie:	prof.dr. J. Cloos dr. J.B. Tuynman dr. R.J.A. Fijneman prof.dr. E. Schuuring prof.dr. R.D.M. Steenbergen prof.dr. N. Gilbert

Table of contents

Chapter 1

General Introduction

Thesis rationale

Cancer is an insidious disease that can arise at an unpredictable time and location in the body. Too often, it is detected at a late stage when treatment options are limited and the chance of survival is poor. After the diagnosis of cancer has been made, monitoring the growth of tumours and determining prognosis remains a considerable challenge. Certain types of solid tissue cancer, such as melanoma and prostate, can be detected by visual and physical examination. For breast, colon and cervical cancer, population screening is widely used to detect early tumour development. For less accessible tissues such as the liver, pancreas and lungs, imaging techniques such as PET-CT, X-ray and ultra-sound are relied upon for initial tumour detection. Subsequently, a tissue biopsy of a suspected tumour is taken for pathological evaluation by morphology, often supported by immunohistochemistry and genetic analysis [1]. Unfortunately, small tumours can be missed, while a biopsy only provides a partial view of the tumour's pathological features. In addition, the invasive nature of any biopsy carries the risk of complications as well as causing pain and discomfort to the patient.

Prior to this PhD project, breakthrough research had opened the way for a new approach to diagnose and monitor cancer. Recent technical advances had facilitated the detection in plasma of genetic material derived from tumours [2–4], allowing the indirect analysis of tumour genetics from a standard blood draw; the so-called 'liquid biopsy' [5].

This PhD thesis focuses on tumour DNA free in the blood, referred to as circulating tumour (ct)DNA. All malignant solid tumours in a patient's body can theoretically shed ctDNA into the circulation, allowing cancer detection and a more complete picture of the tumour genetic burden. Importantly, the complete genetic heterogeneity in a single tumour could be represented in ctDNA, rather than only the part of the tumour analysed in a solid tissue biopsy. The minimally invasive nature of a blood draw reduces the burden on the patient and allows for more frequent sampling and ultimately a more flexible treatment regimen. It is conceivable to use ctDNA to inform the prognosis of a patient's cancer, identify the most relevant treatment options and monitor the response of tumours to treatment over time. Liquid biopsy may also enable earlier detection of cancer than is currently possible. A patient's chance of survival could be drastically increased by early intervention and their quality of life improved by a reduced treatment burden.

At the start of this project, the promise of ctDNA analysis had been demonstrated in research laboratories but it was not clear how it could be applied in a diagnostic setting. To be clinically informative, molecular diagnostic testing must be highly

reproducible, sensitive, specific and adhere to strict quality standards. However, important questions surrounded the suitability of ctDNA analysis to meet these requirements. Most challenging was the observation that ctDNA exists in a background of cell-free (cf)DNA shed from dead, previously healthy cells and tissues, complicating the detection of rare tumour-derived molecules. Numerous other clinical and technical variables throughout the implementation process had to be assessed, quantified and controlled to improve the reliability of ctDNA analysis for diagnostics.

Liquid biopsy is urgently needed to inform timely clinical decisions, allow selection of the most effective treatments and improve the efficient use of healthcare resources. The work described in this translational thesis is primarily intended to evaluate the initial implementation of ctDNA analysis in a clinical setting and assess the extent to which it may benefit patients. Through doing this, we also aimed to learn more about the nature of ctDNA to advance the field of liquid biopsy more broadly.

Introduction to liquid biopsy

Solid tumours can shed whole cells or parts of cells, such as DNA, RNA and proteins, into the blood stream. If these cellular components have characteristics associated with cancer, they could serve as circulating cancer biomarkers that could theoretically be detected and analysed. However, due to the high degree of similarity between tumour and healthy cells, cancer biomarkers can be challenging to identify. Reliable detection is further complicated by a typically low concentration in the circulation, which is often below the sensitivity limit of commonly used diagnostic tests.

Depending on the context, non-genetic cancer biomarkers such as proteins are the easiest to detect in the blood and have thus been used as liquid biopsies in the clinic for some time. Notable examples are the prostate-specific antigen (PSA) for prostate cancer and the carcinoembryonic antigen (CEA) for colorectal cancer, both of which are routinely analysed during treatment [6,7]. Although tests for these antigens are technically highly sensitive, their utility is limited by a weak correlation between PSA or CEA detection and the presence of progressive cancer [8,9]. Protein-based tests are therefore used as an indication of progressive disease but always require confirmation by other techniques such as imaging.

As cancer is the result of errors in DNA, arguably the most informative cancer biomarkers are genetic alterations such as point mutations, copy number aberrations (CNAs), translocations and aberrant methylation. Molecular techniques have typically lacked the sensitivity to reliably detect DNA or RNA mutations in

the circulation, requiring a solid tissue biopsy as the material for genetic analysis. However, recent technical advances have enabled the identification of genetic circulating cancer biomarkers with huge potential to improve cancer management. Notable among these are DNA and RNA in circulating tumour cells (CTCs) [5], DNA and micro RNAs (miRNAs) in extracellular vesicles (EVs) [10] and RNA in tumour-educated platelets (TEPs) [11].

Cell-free (cf)DNA

Cell-free (cf)DNA is a by-product of dead cells, mostly healthy leukocytes, that have reached the end of their productive life and been eliminated by apoptosis [12,13]. After cellular decomposition, cfDNA remains in the blood plasma until degradation by the DNase I enzyme, excretion by the kidneys into urine or removal through uptake by the liver and spleen and subsequent degradation by macropohages [14].

The existence of cfDNA was first described in 1948 but techniques sufficiently sensitive for reproducible analysis have only become available in recent years [14,15]. One of the earliest diagnostic techniques was shallow whole genome sequencing (sWGS), a form of Next Generation Sequencing (NGS), to analyse relative copy number aberrations across the genome. The discovery in 1997 that cfDNA from a developing foetus could be detected in the blood of pregnant women led to Non-Invasive Prenatal Testing (NIPT), the first major clinical use of cfDNA analysis [16]. In NIPT, sWGS is used to screen cfDNA from maternal blood for foetal trisomies of chromosomes 13, 18 and 21, which cause Patau's, Edwards' and Down's syndrome respectively [17]. This form of prenatal screening is now performed in several developed countries, including in more than 42% of pregnancies a year in the Netherlands [18].

By screening thousands of cfDNA samples from healthy pregnant women, genomic aberrations typically associated with cancer were incidentally discovered [19]. These chromosomal imbalances have led to the detection of pre-symptomatic cancer, suggesting a portion of their cfDNA came from a hidden tumour [19]. In one exemplar report, several chromosomal imbalances were identified in a blood sample. Following physical examination and biopsy of an enlarged lymph node, a diagnosis of Hodgkin's Lymphoma was made in this individual [20]. These cases demonstrate that cfDNA from cancer can be present in the blood and is detectable using a clinically validated technique.

Circulating tumour (ctDNA)

Circulating tumour (ctDNA) is generally defined as the portion of cfDNA that contains cancer-associated mutations alterations. CtDNA is released mainly by apoptosis of cancer cells and to a lesser extent by necrosis and active secretion [12].

Unlike in NIPT, where the fetal fraction is typically around 10% at the time of sampling, the proportion of ctDNA in the circulation of cancer patients varies greatly and can be below 1% [21,22]. This low fraction and the frequent lack of CNVs makes ctDNA technically challenging to distinguish from the healthy background.

Mutations

CtDNA is typically detected by analysis of cancer-associated alterations. However, mutation detection does not guarantee that ctDNA is present, as mutations associated with cancer frequently occur in non-cancerous cells [23]. Conversely, the absence of a mutation does not guarantee the absence of ctDNA. An alternative way to characterise ctDNA is to analyse the fragment length, an approach known as fragmentomics. In living cells, nuclear DNA is wrapped tightly around histone proteins linked together like beads on a string. When a cell dies and breaks down, enzymes cut the DNA linking the histones. The DNA wrapped around histones is protected from degradation, resulting in fragments typically 166bp in length from healthy cells [24]. In cancer cells, the DNA is usually wrapped differently around histones, giving a higher proportion of shorter fragments around 144bp in length [24]. Fragment length analysis combined with mutation or chromosomal aberration detection can therefore increase the confidence that a ctDNA fragment originates from a cancer cell.

Methylation

Another means to detect ctDNA independently of the nucleotide sequence is to determine the ctDNA methylation status. The addition of a methyl group to cytosine, mostly at CpG dinucleotides, functions as a regulator of gene expression. Cancer genomes have a characteristically different pattern of methylation across the genome compared to healthy cells, enabling its exploitation as a cancer biomarker. For example, hypermethylation in CpG-rich promotor regions can silence the transcription of tumour suppressors such as *CDKN2A*, *RB* and *MLH1* [25]. Analysis of methylation status using methylation microarrays, quantitative methylation-specific PCR or bi-sulphite sequencing can detect ctDNA. Due to well characterised tissue-specific methylation patterns, it is even possible to infer the cell type from which the ctDNA originated and thus infer the patient's cancer type [26].

Liquids containing ctDNA

Blood has traditionally been used to study cancer biomarkers due to its accessibility, affordability of sampling and well-established use in clinical settings. However, other liquids outside of the circulation also contain detectable levels of ctDNA. Cerebrospinal fluid can be a rich source of ctDNA from neurological tumours, although it is less accessible and more invasive to collect compared to

blood [27]. Some tissues can even be self-sampled away from the clinic, such as through cervical swabs for cervical cancer screening [28]. Studies of urine and saliva have demonstrated the capacity of these fluids to carry ctDNA, with the advantage of entirely non-invasive collection outside the healthcare setting [29].

Tissue-guided ctDNA detection

A reliable way to detect ctDNA is to use previously identified point mutations, copy number aberrations and translocations from a patient's solid tumour tissue to guide analysis. Pathology departments routinely analyse tumour tissue of certain cancer types to identify mutations and chromosomal aberrations. These biomarkers can then be targeted in blood using highly sensitive and specific techniques. Comparative analyses have shown a high concordance between mutations in solid tissue and blood. In advanced non-small cell lung cancer (NSCLC) for example, concordance can be over 90% [30]. The extent of concordance probably depends upon various technical factors including assay sensitivity and specificity, pre-analytical processing, as well as biological factors such as tumour: location, burden, tissue of origin, stage, aggressiveness, angiogenesis and metastatic behaviour.

Technical developments for ctDNA detection

Quantitative PCR

Sensitive and specific techniques are essential for the reliable detection of low concentrations of ctDNA in plasma. Traditional PCR followed by Sanger sequencing is a widely used molecular technique but can currently only detect the presence of minor alleles above 20% frequency and is not quantitative [31]. A high proportion of DNA in a sample must therefore share a mutation to enable detection by this approach. This may be sufficient to detect clonal mutations in solid tumour tissue but not at a low frequency in cfDNA. Real-time quantitative PCR is highly sensitive and specific and is proven to be capable of identifying mutations in cfDNA down to a sensitivity of around 10% [32]. The first mutation-specific liquid biopsy technique to receive FDA approval was the qPCR-based Cobas *EGFR* Mutation Test (Roche Diagnostics) for *EGFR* mutations in plasma from NSCLC patients [33].

Digital PCR

Digital PCR has further increased analytical sensitivity by dividing a sample into thousands of smaller reactions in the same tube. Leading techniques are droplet digital (dd) and BEAMing (Beads, Emulsion, Amplification and magnetics) PCR, both of which use droplets of oil to partition the reaction. Different approaches are then used to analyse the reaction in the individual droplets to identify ctDNA down to a sensitivity of around 0.01-0.03% for BEAMing and 0.04-1% for ddPCR [34–36].

The highly targeted nature of these techniques makes them most effective when mutations are already known, or a specific clinically actionable mutation is under investigation. The useability and relatively low cost of ddPCR compared to BEAMing has made it one of the most adopted techniques for ctDNA point mutation analysis in the research setting.

Next Generation Sequencing - mutation detection

Next Generation Sequencing (NGS) allows a greater number and type of mutations to be targeted in a single assay. Several NGS assays that analyse mutations in one or more genes have received FDA approval for use in the clinic [37]. Although generally less sensitive for individual mutations than ddPCR or BEAMing, the ability to interrogate many nucleotide positions increases the likelihood of detecting a point-mutation from a limited cfDNA input. Innovative methods of labelling individual cfDNA fragments combined with sophisticated computational techniques have further increased the sensitivity over recent years. However, NGS for mutation detection is a more expensive option that impedes large scale routine clinical implementation.

Next Generation Sequencing - copy number analysis

Copy number analysis, on the other hand, offers a relatively cheap and quick option to screen for the presence of ctDNA in a sample. Studies have shown that copy number aberrations can be detected at a sensitivity of <1-5% in cancer patients [38]. A disadvantage is that high throughput copy number screening requires many samples per NGS run and a substantial investment in laboratory equipment and bioinformatic infrastructure, especially compared to ddPCR. Fortunately, this infrastructure already exists in hospitals that perform NIPT, lowering the barrier to implementation in this setting.

Pre-analytical conditions

The often-low quantity of ctDNA in a blood sample requires the most sensitive assays to operate at their limit of detection in a background of non-cancer cfDNA. Dilution of the ctDNA fraction can be exacerbated by blood collection in EDTA-containing tubes, which are commonly used to collect blood for molecular analysis. The lack of a cellular preservative in EDTA-containing tubes can cause lysis of healthy leukocytes and the subsequent release of a high and unwanted background DNA from healthy cells into the sample. The result is a further dilution of the ctDNA fraction. Sensitivity can be improved by careful consideration of blood collection methods and other pre-analytics, the conditions under which samples are processed before analysis. The type of blood collection tube, the time and temperature of blood storage before plasma separation and the methods of cfDNA isolation are all important pre-analytical conditions that must be controlled. These factors were not fully known at the start of this project. The work presented in this thesis includes testing and optimisation of these variables, an essential process to ensure reliable results.

Aim of this thesis

The aim of this thesis is to improve the pre-analytical and analytical conditions for ctDNA detection and develop assays to assess the feasibility of implementation in a clinical setting.

This aim is achieved through the following three-point strategy:

1. Set up and validate workflows for point mutation and copy number aberration (CNA) analysis to detect ctDNA (Figure 1)
    a. Point mutations: Biobanked plasma from non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) patients is used to set-up and evaluate a ddPCR workflow for mutation detection. (Pre)analytical variables such as blood sample collection and processing are optimised.
    b. CNAs: Development of a PCR-free method for ctDNA copy-number analysis to assess its suitability for high-throughput clinical use in a sub-set of NSCLC and Lymphoma patients.

2. Detect point mutations in ctDNA that were previously identified in solid tissue
    a. Clinical and pathological information from the records of NSCLC patients is used to identify patients with a tumour that harbours *EGFR* mutations. ddPCR is used to target these *EGFR* mutations in cfDNA.
    b. Solid tissue from colorectal cancer patients selected for Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (CRS-HIPEC) is sequenced by NGS to identify mutations. These mutations are targeted in cfDNA using ddPCR.
3. Assess the utility of ctDNA detection to address real-world clinical needs. The trend between ctDNA detection and the following clinical characteristics is investigated:
    a. Tumour response to the *EGFR*-inhibitor osimertinib in NSCLC patients.
    b. Disease recurrence in colorectal cancer patients eligible for CRS-HIPEC.

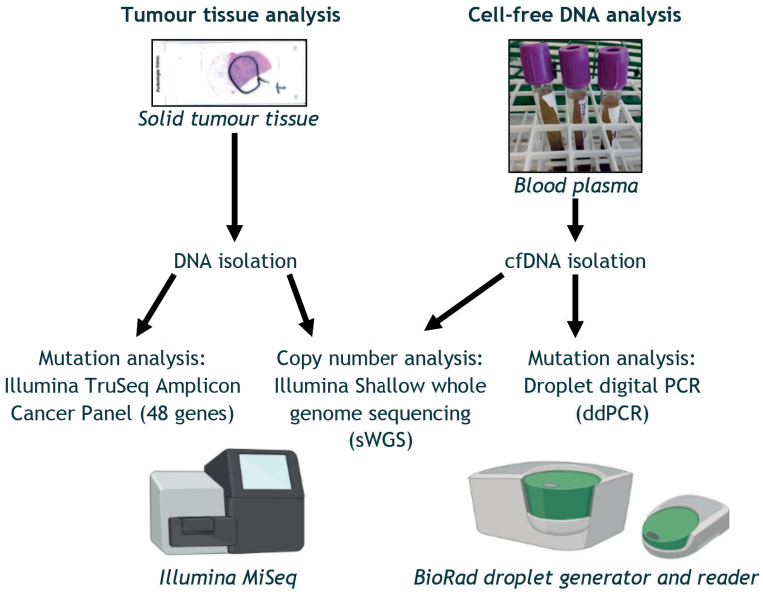


Figure 1. A graphical abstract of the analysis workflow. *Graphics created with BioRender.com.*

References

1. Matias-Guiu X, Stanta G, Carneiro F, et al. The leading role of pathology in assessing the somatic molecular alterations of cancer: Position Paper of the European Society of Pathology. *Virchows Arch*. 2020;476(4):491-497. doi:10.1007/s00428-020-02757-0
2. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224):224ra24-224ra24. doi:10.1126/scitranslmed.3007094
3. Chan KCA, Jiang P, Zheng YWL, et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. *Clin Chem*. 2013;59(1):211-224. doi:10.1373/clinchem.2012.196014
4. Thierry AR, Mouliere F, El Messaoudi S, et al. Clinical validation of the detection of *KRAS* and BRAF mutations from circulating tumor DNA. *Nat Med*. 2014;20(4):430-435. doi:10.1038/nm.3511
5. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: challenges and perspectives. *Trends Mol Med*. 2010;16(9):398-406. doi:10.1016/j.molmed.2010.07.001
6. Ruiz-Bañobre J, Kandimalla R, Goel A. Predictive Biomarkers in Metastatic Colorectal Cancer: A Systematic Review. *JCO Precis Oncol*. 2019;(3):1-17. doi:10.1200/PO.18.00260
7. French WW, Wallen EM. Advances in the diagnostic options for prostate cancer. *Postgrad Med*. 2020;132(sup4):52-62. doi:10.1080/00325481.2020.1822067
8. Bryce AH, Chen Y-H, Liu G, et al. Patterns of PSA versus clinically progressive disease in the E3805 CHAARTED trial. *J Clin Oncol*. 2018;36(15_suppl):5046. doi:10.1200/JCO.2018.36.15_suppl.5046
9. Fakih MG, Padmanabhan A. CEA monitoring in colorectal cancer. What you should know. *Oncology (Williston Park)*. 2006;20(6):579-587; discussion 588, 594, 596 passim.
10. Bebelman MP, Smit MJ, Pegtel DM, Baglio SR. Biogenesis and function of extracellular vesicles in cancer. *Pharmacol Ther*. 2018;188:1-11. doi:10.1016/j.pharmthera.2018.02.013
11. Best MG, Wesseling P, Wurdinger T. Tumor-Educated Platelets as a Noninvasive Biomarker Source for Cancer Detection and Progression Monitoring. *Cancer Res*. 2018;78(13):3407-3412. doi:10.1158/0008-5472.CAN-18-0887
12. Grabuschnig S, Bronkhorst AJ, Holdenrieder S, et al. Putative Origins of Cell-Free DNA in Humans: A Review of Active and Passive Nucleic Acid Release Mechanisms. *Int J Mol Sci*. 2020;21(21). doi:10.3390/ijms21218062
13. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell*. 2016;164(1):57-68. doi:https://doi.org/10.1016/j.cell.2015.11.050
14. Bronkhorst AJ, Ungerer V, Holdenrieder S. The emerging role of cell-free DNA as a molecular marker for cancer management. *Biomol Detect Quantif*. 2019;17:100087. doi:https://doi.org/10.1016/j.bdq.2019.100087
15. MANDEL P, *MET*AIS P. [Nuclear Acids In Human Blood Plasma]. *C R Seances Soc Biol Fil*. 1948;142(3-4):241-243.
16. Lo YM, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet (London, England)*. 1997;350(9076):485-487. doi:10.1016/S0140-6736(97)02174-0

17. Kater-Kuipers A, Bakkeren IM, Riedijk SR, et al. Non-invasive prenatal testing (NIPT): societal pressure or freedom of choice? A vignette study of Dutch citizens' attitudes. *Eur J Hum Genet*. 2021;29(1):2-10. doi:10.1038/s41431-020-0686-9

18. van der Meij KRM, Sistermans EA, Macville MVE, et al. TRIDENT-2: National Implementation of Genome-wide Non-invasive Prenatal Testing as a First-Tier Screening Test in the Netherlands. *Am J Hum Genet*. 2019;105(6):1091-1101. doi:https://doi.org/10.1016/j.ajhg.2019.10.005

19. Amant F, Verheecke M, Wlodarska I, et al. Presymptomatic identification of cancers in pregnant women during noninvasive prenatal testing. *JAMA Oncol*. 2015;1(6):814-819. doi:10.1001/jamaoncol.2015.1883

20. Ballova V V, Conrad B, Schäffer L, Menk M, Caspar C. Noninvasive prenatal testing and incidental detection of maternal Hodgkin's lymphoma. *Hematol Oncol*. 2017;35(S2):305-306. doi:https://doi.org/10.1002/hon.2439_40

21. van der Pol Y, Mouliere F. Toward the Early Detection of Cancer by Decoding the Epigenetic and Environmental Fingerprints of Cell-Free DNA. *Cancer Cell*. 2019;36(4):350-368. doi:10.1016/j.ccell.2019.09.003

22. Ashoor G, Syngelaki A, Poon LCY, Rezende JC, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: relation to maternal and fetal characteristics. *Ultrasound Obstet Gynecol*. 2013;41(1):26-32. doi:https://doi.org/10.1002/uog.12331

23. Martincorena I, Fowler JC, Wabik A, et al. Somatic mutant clones colonize the human esophagus with age. *Science*. 2018;362(6417):911-917. doi:10.1126/science.aau3879

24. Wan JCM, Massie C, Garcia-Corbacho J, et al. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. *Nat Rev Cancer*. 2017;17(4):223-238. doi:10.1038/nrc.2017.7

25. Cerchietti L, Melnick A. DNA Methylation-Based Biomarkers. *J Clin Oncol Off J Am Soc Clin Oncol*. 2017;35(7):793-795. doi:10.1200/JCO.2016.71.0616

26. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet*. 2019;20(2):71-88. doi:10.1038/s41576-018-0071-5

27. Mouliere F, Mair R, Chandrananda D, et al. Detection of cell-free DNA fragmentation and copy number alterations in cerebrospinal fluid from glioma patients . *EMBO Mol Med*. 2018;10(12):1-6. doi:10.15252/emmm.201809323

28. Maver PJ, Poljak M. Primary HPV-based cervical cancer screening in Europe: implementation status, challenges, and future plans. *Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis*. 2020;26(5):579-583. doi:10.1016/j.cmi.2019.09.006

29. Peng M, Chen C, Hulbert A, Brock M V, Yu F. Non-blood circulating tumor DNA detection in cancer. *Oncotarget*. 2017;8(40):69162-69173. doi:10.18632/oncotarget.19942

30. Schwartzberg LS, Horinouchi H, Chan D, et al. Liquid biopsy mutation panel for non-small cell lung cancer: analytical validation and clinical concordance. *npj Precis Oncol*. 2020;4(1):15. doi:10.1038/s41698-020-0118-x

31. Monzon FA, Ogino S, Hammond MEH, Halling KC, Bloom KJ, Nikiforova MN. The Role of *KRAS* Mutation Testing in the Management of Patients With Metastatic Colorectal Cancer. *Arch Pathol Lab Med*. 2009;133(10):1600-1606. doi:10.5858/133.10.1600

32. Chen M, Zhao H. Next-generation sequencing in liquid biopsy: cancer screening and early detection. *Hum Genomics*. 2019;13(1):34. doi:10.1186/s40246-019-0220-8

33. O'Donnell P, Shyu J, Ferguson J, et al. A Real-time PCR Assay for the Detection of EGFR Mutations in FFPE Tissue and Plasma Samples of NSCLC Patients. *J Clin Oncol*. 2016;34(15_suppl):e23251-e23251. doi:10.1200/JCO.2016.34.15_suppl.e23251

34. Yang X, Zhuo M, Ye X, et al. Quantification of mutant alleles in circulating tumor DNA can predict survival in lung cancer. *Oncotarget*. 2016;7(15):20810-20824. doi:10.18632/oncotarget.8021

35. Garcia J, Forestier J, Dusserre E, et al. Cross-platform comparison for the detection of RAS mutations in cfDNA (ddPCR Biorad detection assay, BEAMing assay, and NGS strategy). *Oncotarget*. 2018;9(30):21122-21131. doi:10.18632/oncotarget.24950

36. Diehl F, Schmidt K, Durkee KH, et al. Analysis of Mutations in DNA Isolated From Plasma and Stool of Colorectal Cancer Patients. *Gastroenterology*. 2008;135(2):489-498.e7. doi:10.1053/j.gastro.2008.05.039

37. Adashek JJ, Janku F, Kurzrock R. Signed in Blood: Circulating Tumor DNA in Cancer Diagnosis, Treatment and Screening. *Cancers* . 2021;13(14). doi:10.3390/cancers13143600

38. Keller L, Belloum Y, Wikman H, Pantel K. Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. *Br J Cancer*. 2021;124(2):345-358. doi:10.1038/s41416-020-01047-5

1

Chapter 2

Circulating Tumor DNA Analysis: Clinical Implications for Colorectal Cancer Patients. A Systematic Review

Sander Bach*
Nina R Sluiter*
Jamie J Beagan*
Joost M Mekke
Johannes C F Ket
Nicole C T van Grieken
Renske D M Steenbergen
Bauke Ylstra
Geert Kazemier*
Jurriaan B Tuynman*

*Contributed equally as first and senior authors, respectively

JNCI Cancer Spectr. 2019 Jun 19;3(3):pkz042

Abstract

Background

Liquid biopsies could improve diagnosis, prognostication, and monitoring of colorectal cancer (CRC). Mutation, chromosomal copy number alteration, and methylation analysis in circulating tumor DNA (ctDNA) from plasma or serum has gained great interest. However, the literature is inconsistent on preferred candidate markers, hampering a clear direction for further studies and clinical translation. This review assessed the potential of ctDNA analysis for clinical utility.

Methods

A systematic review according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines was conducted up to December 3, 2018, followed by methodological quality assessment. Primary endpoints were accuracy for detection, prognostication, and monitoring.

Results

Eighty-four studies were included. For CRC detection, sensitivity was 75% using ctDNA mutation analysis and up to 96% using copy number analysis. Septin 9 (*SEPT9*) hypermethylation analysis showed sensitivities of 100% and specificities of 97%. Regarding prognostication, ctDNA *KRAS* mutations were associated with oncological outcome and could predict response to anti–epidermal growth factor receptor therapy. For monitoring, sequential ctDNA *KRAS* mutation analysis showed promise for detection of relapses or therapy resistance.

Conclusions

This comprehensive overview of ctDNA candidate markers demonstrates *SEPT9* methylation analysis to be promising for CRC detection, and *KRAS* mutation analysis could assist in prognostication and monitoring. Prospective evaluation of marker panels in clinical decision making should bring ctDNA analysis into practice.

Colorectal cancer (CRC) is the third most common cancer in the Western world (1,2) and the incidence is still rising (3). In recent decades, oncological outcomes have improved because of the implementation of screening programs, improvement of surgical procedures, and introduction of novel systemic regimens. However, CRC is still the second leading cause of cancer-related death (1,2). Further innovation is needed to improve diagnosis, patient-specific treatment selection, and disease monitoring.

The stage of disease at diagnosis is the most important prognostic factor for survival in CRC (4). It is therefore of utmost importance to detect CRC at an early stage, which requires improved screening approaches. The value of current screening methods is hampered by the low sensitivity of the fecal occult blood test (FOBT) and the invasive nature and costs of colonoscopy (5).

A second challenge concerns selection of the most suitable treatment, warranting better prognostic markers. The current decision process for systemic therapy is largely based on clinicopathological characteristics, leaving a substantial number of patients under- or overtreated. Genetic subtyping (6) and expression profiling (7) enhance patient selection. However, improved approaches are needed to further subclassify patients by their risk of recurrence and suitability for adjuvant therapies.

A third major area of interest is disease monitoring after initial curative treatment or during systemic therapy. Up to 40% of CRC patients will experience disease recurrence despite curatively intended treatment (8). Unfortunately, recurrences are often detected at advanced stages, excluding these patients from potentially curative rescue treatments. Current follow-up consists of serial carcinoembryonic antigen (CEA) measurements in serum, imaging, and colonoscopy (9). Unfortunately, the value of CEA for follow-up is limited by its low accuracy (10,11), with only marginal benefit observed when combined with computed tomography (CT) scans (12). The value of CT imaging is limited to the detection of large lesions, illustrated by a sensitivity of 11% for nodules smaller than 5 mm (13). Colonoscopy provides a high level of sensitivity (>95%) but can evaluate only endo luminal disease (5). These issues stress the urgent clinical need for a robust and noninvasive diagnostic marker facilitating CRC detection and prediction of treatment response.

Liquid biopsies are a rapidly developing field of research focused toward the analysis of cancer biomarkers isolated from nonsolid tissues. Various tumor-derived products can be detected in blood, including circulating tumor cells, circulating tumor DNA (ctDNA), circulating RNAs, exosomes, and tumor educated platelets (14–16). Of these tumor-derived products, ctDNA has been investigated most extensively and has shown promising accuracies for cancer detection (17–20). These DNA fragments originate from tumor cells and are released into the circulation

through apoptosis, necrosis, and secretion (17). Accordingly, tumor-specific (epi-) genetic alterations such as driver mutations, chromosomal copy number alterations (CNAs), and methylation can be detected in ctDNA and could be of high value for cancer detection, prognostication, and treatment monitoring (17–20).

The primary challenge of ctDNA analysis is to detect tumor-derived molecules in a high background of cell-free DNA (cfDNA) from healthy cells. Currently, ctDNA detection techniques mainly revolve around real-time polymerase chain reaction (PCR) and sequencing approaches (14,15). Allele-specific quantitative PCR has a high sensitivity for ctDNA detection, with a detection limit of 0.014–0.004% (21). Emulsion PCR methods such as droplet digital PCR (ddPCR) and beads, emulsion, amplification, and magnetics are most sensitive, with a detection limit of 0.01–0.001% (22,23). The disadvantage of PCR-based methods is the limited number of foci that can be assessed, relying on the initial identification of patient-specific solid-tumor tissue alterations. Sequencing platforms including next-generation sequencing (NGS) allow for broader genomic coverage. However, this method is time consuming and expensive, hampering clinical implementation. An overview of the main methods to detect ctDNA is depicted in Figure 1.

Several Food and Drug Administration (FDA)–approved assays are commercially available for ctDNA-based cancer diagnostics, including a PCR kit for detection of epidermal growth factor receptor (*EGFR*) mutations in non-small cell lung cancer patients (Cobas v2) (24) and a PCR assay measuring methylated *SEPT9* in blood to detect CRC (Epi ProColon) (25). Copy number analysis of circulating DNA is currently routine diagnostic practice in several countries, including the Netherlands, for noninvasive prenatal testing (26). Numerous studies claim a potential clinical role for ctDNA, but the diverse and sometimes contradictory results and recommendations hamper widespread translation into daily practice of CRC patients. Therefore, the aim of this study is to systematically review the current literature on the potential role of ctDNA mutation, copy number, and methylation analysis for CRC diagnosis, prognostication, and monitoring.

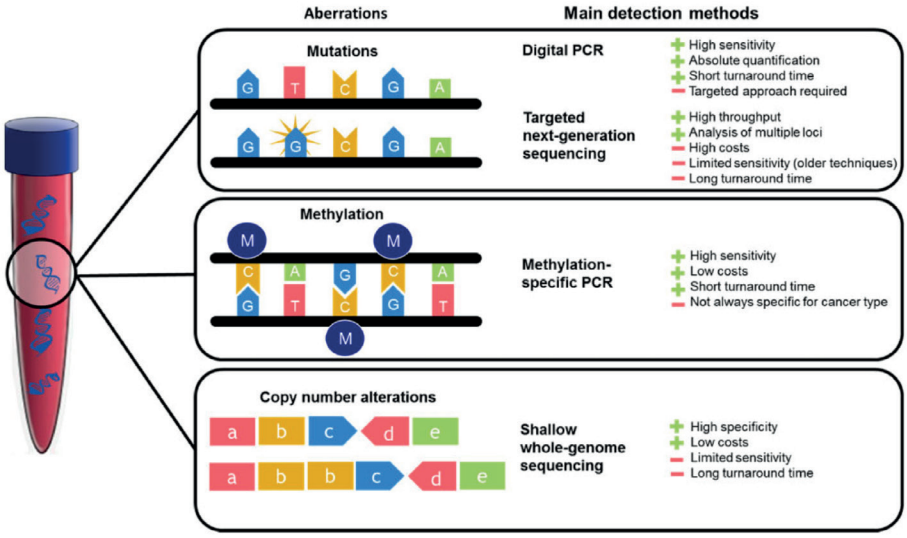


Figure 1. The three types of circulating tumor DNA aberrations covered in this review. For every DNA aberration, commonly used techniques to determine its presence in plasma or serum are depicted. PCR = polymerase chain reaction.

Materials and Methods

Search Strategy

A systematic literature review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses statement (27). Systematic searches were performed in the bibliographic databases PubMed, Embase.com, and Clarivate Analytics/Web of Science up to December 3, 2018, by SB, NRS, and JCFK (Supplementary Table 1). The search query included indexed terms and free-text words for "DNA" and "variation" or "methylation" and "blood" or "serum" and "colorectal cancer."

Study Selection

Screening and study selection was independently performed by three reviewers (JMM, NRS, SB). If necessary, articles were discussed to achieve consensus. All full-text articles in English, Dutch, French, German, or Russian on ctDNA mutation, copy number, or methylation analysis in the serum or plasma of CRC patients were considered eligible. Human studies assessing therapy-naive patients with a minimum age of 18 years that allowed determination of sensitivity were included. Literature reviews, case reports, and studies in which ctDNA analysis was performed in fewer than 10 CRC patients or in patients with hereditary CRC or inflammatory bowel disease were excluded. If overlapping data were reported, either the most recent study or that with the most complete data on our outcomes of interest was included.

Data Extraction

Primary outcomes were sensitivity and specificity of ctDNA analysis for CRC detection, subdivided according to several clinical settings: diagnosis, prognostication, and monitoring. Sensitivity was defined as the percentage of CRC patients in whom a specific ctDNA aberration was detected. Specificity was defined as the percentage of healthy control individuals without detected ctDNA. Additionally, the technical concordance was extracted, defined as the percentage of agreement between ctDNA and solid-tumor tissue analysis. Data on single mutations in sequencing panels were extracted if two or more studies reported this mutation.

Quality Assessment

Risk of bias assessment of all included studies was independently performed by three reviewers (JMM, NRS, SB). Risk of bias was scored as low, high, or unclear using the validated Quality Assessment of Diagnostic Accuracy Studies 2 tool (QUADAS-2) (28). Custom criteria were created, and agreement among reviewers was initially determined in a pilot of 10 studies. Disagreement was resolved by discussion with all reviewers present (JMM, NRS, SB). To ensure high-quality assessment of the described literature, articles were excluded from further analysis in case one domain was scored as "high" in combination with "unclear" or "high" risk at a second domain of the QUADAS-2. Review Manager 5 software (The Nordic Cochrane Centre, Copenhagen, Denmark) was used for managing the QUADAS-2 results.

Results

The search identified 8478 eligible abstracts. After removal of duplicates, 5567 studies were excluded by title and abstract screening. Subsequently, 382 articles were excluded by full-text evaluation, leaving 134 studies, all in English, for risk of bias assessment. Figure 2 depicts the study selection procedure.

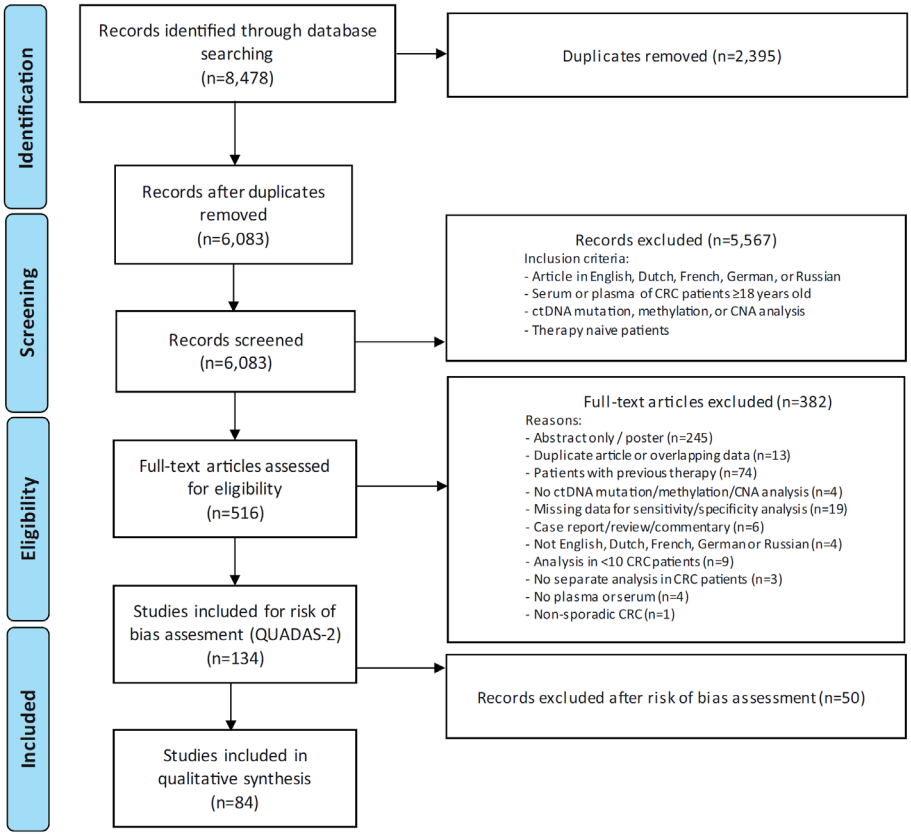


Figure 2. The Preferred Reporting Items for Systematic Reviews and Meta-analyses flowchart for inclusion of the studies. The risk of bias assessment using the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) was incorporated in the flowchart. CNA = copy number alteration; CRC = colorectal cancer; ctDNA = circulating tumor DNA.

Fifty studies were excluded based on quality assessment using QUADAS-2, leading to the inclusion of 84 studies. The majority of studies (123 of 134) scored unclear or high risk of bias on at least one domain, mainly study design or index test. Only 11 studies scored low risk on all domains (29–39). Most papers scored low risk on applicability concerns, reference standard (histological assessment), and flow and timing. The main findings of the risk of bias assessment are depicted in Supplementary Figures 1, A and B (detailed overview, available online).

An overview of the clinical implications with the main markers of interest is provided in Figure 3.

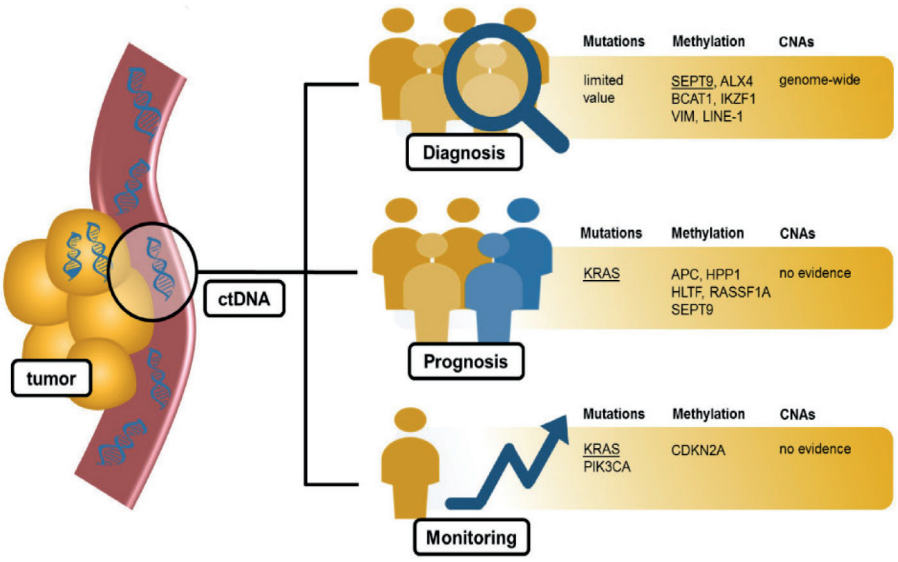


Figure 3. A graphical overview of the evidence for circulating tumor DNA (ctDNA) use in clinical practice. The most promising markers are presented for each clinical implication. Markers considered to be of special interest are underlined. Other markers depicted in the figure are promising but require further research. CNA = copy number alteration.

Accuracy of ctDNA Analysis for CRC Diagnosis

Current screening methods consist of FOBT and colonoscopy and have an overall sensitivity of 51% for individuals experiencing clinical symptoms and 19% at earlier stages (40). The present section describes ctDNA aberrations that could aid in CRC detection. Tables 1 and and 2 present an overview of the identified candidate mutation and methylation markers (Table 1) and CNAs (Table 2) in ctDNA with sensitivities per stage, specificities, and concordance rates.

Table 1. An overview of the sensitivity and specificity for CRC detection of all ctDNA mutation, hypermethylation, and hypomethylation markers included in this review*

Marker	Sensitivity					Specificity	Concordance with primary tumor
	Stage I	Stage II	Stage III	Stage IV	Stage not reported		
Mutation							
APC (41–48)	0–50%	6–57%	3–46%	15–75%	14–18%	NA	16–100%
BRAF (34,35,42, 45,48–53)	50%	0–9%	33%	3–29%	2–12%	NA	33–100%
ERBB2 (44,54)	NA				5–9%	NA	NA
KRAS (32,34,35,41–46,48–53,55–64)	0–67%	3–46%	5–50%	5–73%	8–71%	70–100%	25–100%
NRAS (35,52)	NA				12%	NA	100%
PIK3CA (44,45,48,49,51)	NA			19%	0–21%	NA	0%–100%
tp53 (41–46,48)	0–25%	22–30%	17–49%	38–67%	6–50%	100%	14–100%
Hypermethylation							
AKAP12 (65)	NA				48%	92.0%	NA
ALX4 (66–70)	75%	83%	82%	100%	29–83%	66–99%	NA
	30%		60%				
APC (67,71–74)	24%		60%	54%	20–57%	68–100%	50%
BCAT1 (31,75)	21%	62%	68%	81%	57–65%	95–97%	NA
BMP3 (67)	NA				29%	89%	NA
BNC1 (67)	NA				12%	87%	NA
BRCA1 (67)	NA				25%	78%	NA
CDH1 (72)	NA				60%	84%	NA
CDH4 (76)	NA				70%	100%	83%
CDKN2A (55,67,71,77–79)	15%	50–67%	50–67%	10–75%	9–61%	70%–96%	70–82%
CRABP1 (75,80)	NA				50%	NA	NA
DAPK1 (72)	50.0%			NA		74%	80%
DLC1 (81)	36%		48%		42%	91%	NA
ERCC1 (82)	60%			NA	NA	93%	90%
EYA4 (80)	NA				50%	NA	NA
FBN2 (83)	9%	7%	8%	NA	9%	NA	8%
FGF5 (75)	NA				85%	83%	NA
FHIT (72,74)	NA				20–50%	84%	40%
GATA5 (84)	46%		83%		61%	NA	NA
GRASP (75)	NA				54%	93%	NA

Marker	Sensitivity					Specificity	Concordance with primary tumor
	Stage I	Stage II	Stage III	Stage IV	Stage not reported		
HIC1 (67)					6%	99%	NA
HLTF (67,85–89)	8–20%	15–16%	9–16%	24–47%	11–30%	96–100%	41–42%
hMLH1 (67,77,89)	27%	0–24%	25–27%	12–40%	16–29%	100%	33%
HPP1 (85,87–90)	3–7%	0–6%	5–9%	52–53%	13–72%	NA	56%
IKZF1 (31,75)	28%	41%	55%	94%	48–68%	95–99%	NA
IRF4 (75)			NA		59%	96%	NA
ITGA4 (84)		24%		54%	37%	81%	NA
LRR3CB (74)			NA		15%	NA	23%
MAL (80)					50%	NA	NA
MGMT (67,82)		58%		NA	6%	95–99%	94%
MLH1 (67,77,89,91)			NA		45%	57%	33%
NELL1 (80)				NA	33%	NA	NA
NDRG4 (67,92)		54%		56%	9–55%	NA	NA
NEUROG1 (67,87)	31%	28%	26%	20%	21–26%	NA	NA
NGFR (93)	20%	25%	36%	36%	38%	91.4%	NA
NPTX2 (67)			NA		70%	41%	NA
OSMR (67,94)		74%		77%	11–75%	86–93%	79%
p73 (77)			NA		25%	NA	NA
PCDH10 (36)		71%		54%	63%	NA	67%
PDX1 (75)			NA		45%	70%	NA
PHACTR3 (67)			NA		15%	94%	NA
PPENK (67)			NA		10%	96%	NA
RAR-β (67)					25%	30%	
RASSF1A (67,73)		14%	47%	45%	11–34%	84–100%	NA
RUNX3 (95)		33%		50%	42%	100%	NA
SDC2 (67,75)			NA		24–59%	84–94%	NA
SEPT9 (25,29,30,32, 33,66,67,75, 80,93,94,96–107)	14–84%	50–100%	38–100%	68–100%	24–96%	73–97%	80–88%
		20–57%		52–70%			
		64%		NA			
SFRP1 (67)					22%	93%	
SFRP2 (67,84)		42%		71%	20–54%	72–82%	NA
SHOX2 (103)		NA		44%	21%	NA	NA
SMAD4 (72)			NA		52%	64%	NA
SOX21 (75)			NA		80%	50%	NA

Marker	Sensitivity					Specificity	Concordance with primary tumor
	Stage I	Stage II	Stage III	Stage IV	Stage not reported		
SPG20 (67)			NA		16%	82%	NA
SST (67,80)			NA		30–50%	69%	NA
TAC1 (67,80)			NA		50–53%	53%	NA
TFPI1 (108)			NA		7%	98%	NA
FPI2 (67)	0%	10%	13%	58%	18%	100%	NA
THBD (67)			NA		10%	99%	NA
TMEFF2 (66,93)	5%	22%	47%	45%	30–71%	90–95%	NA
VIM (67,109,110)	50–52%	55–67%	40%	86%	18–71%	60–93%	78%
WIF1 (67)			NA		10%	96%	NA
WNT5A (67)			NA		6%	95%	NA
Hypomethylation							
CBS (111)			NA		56%	NA	NA
LINE-1 (112)	63%		68%		66%	90%	NA
Panels							
Hypermethylation: *ALX4* + *BMP3* + *NPTX2* + *RARB* + *SDC2* + *SEPT9* + *VIM* + female sex + age>66 (67)	89%		NA		91%	73%	NA
Mutations: sequencing panel including *TP53* + *APC* + *KRAS* (45)			NA		100%	NA	NA
Mutations: *APC* + *KRAS* + *TP53* (46)	0%	22%	49%	67%	35%	100%	46%
Hypermethylation: *APC* + *MGMT* + *RASSF2A* + Wif-1 (86)	87%			NA		92%	NA
Hypermethylation: *BCAT1* + *IKZF1* (38)	41%	76%	59%	71%	62%	92%	NA
Hypermethylation: *ALX4* + *SEPT 9* + *TMEFF 2* (66)			NA		84%	88%	NA

*The number of studies reporting a specific marker is represented next to the target gene. If possible, the sensitivity was presented separately for each disease stage. Concordance was defined as the percentage of agreement between ctDNA analysis and mutation or methylation analysis in the primary tumor. CRC = colorectal cancer; ctDNA = circulating tumor DNA; NA = not available, for when no data were available in a specific category.

Table 2. An overview of the sensitivity and specificity for CRC detection of all analyzed potential ctDNA markers

				Sensitivity					
				Stage I	**Stage II**	**Stage III**	**Stage IV**	**Overall**	**Specificity**
Detection of any CNA (37,39,113)				41%		73%		56%	66–87%
Chr	**Arm**	**Locus**	**Gene**	50–100%	45–100%	45–91%	58–100%	49–96%	
Copy number gains									
1	q			20%	33%	9%	0%	17%	100%
1	p			20%	17%	0%		9%	100%
2	q			20%	17%	9%	0%	13–19%	100%
2	p			20%	33%	9%	0%	16–17%	100%
3	q				0%		0%	9%	100%
4	q			40%	17%	0%		4%	100%
5	q			0%	17%	0%		4–19%	100%
5	p			20%	17%	18%	0%	17–18%	100%
6	p	21.1	*CCND3*		0%		15%	4%	NA
6	q				0%	9%	0%	4%	100%
6	p			20%	50%	18%	0%	26%	100%
7	q	21.2	*CDK6*		0%	5%	10%	4%	NA
7	q	34	*BRAF*		0%	5%	15%	4%	NA
7	q				0%	9%	0%	4%	100%
7	p			0%	33%	9%	0%	9%	100%
8	p	11.21	*KAT6A*			NA		20%	NA
8	q	23.1	*RSPO2*	0%	0%	5%	40%	11%	NA
8	q	24.21	*MYC*		0%		35%	9%	NA
8	p	11.21	*IKBKB*		0%		20%	4%	NA
8	q				0%	18%	0%	9%	100%
9	q				0%	9%	0%	4%	100%
9	p					NA		28%	NA
10	q			0%	33%	0%		13%	100%
10	p			0%	33%	36%	0%	13–30%	100%
11	q	13.3	*CCND1*		0%		20%	4%	NA
12	p	13.33	*KDM5A*		0%		15%	4%	NA
12	p	12.1	*KRAS*		0%		15%	4%	NA
12	p			0%	33%	9%	100%	22%	100%
13	q	12.13	*CDK8*		0%		30%	8%	NA
13	q	13.1	*BRCA2*		0%		30%	8%	NA

				Sensitivity					
				Stage I	Stage II	Stage III	Stage IV	Overall	Specificity
Detection of any CNA (37,39,113)				41%		73%		56%	66–87%
Chr	**Arm**	**Locus**	**Gene**	50–100%	45–100%	45–91%	58–100%	49–96%	
13	q	34	*IRS2*	0%		5%	25%	8%	NA
13				0%		27%	100%	22%	100%
15				20%	17%	9%	0%	13%	100%
17				0%	33%	45%	0%	30%	100%
17	p			NA				13%	NA
18				20%		0%		4%	100%
19				0%	33%	55%	100%	39%	100%
19	q			NA				28%	NA
19	p			NA				16%	NA
20	q	13.2	*AURKA*	0%		5%	20%	13%	NA
20	q	11.23	*SRC*	0%		5%	45%	13%	NA
20				20%	0%	18%	0%	13%	100%
20	p			NA				16%	NA
21				0%	17%	0%		4%	100%
22				20%	17%	18%	0%	17%	100%
Copy number losses									
1	p			0%		9%	0%	4–16%	100%
2	p			0%		9%	0%	4%	100%
3	q			0%		9%	0%	4%	100%
3	p			0%		18%	0%	9–13%	100%
4	q			0%		9%	0%	4%	100%
4	p			20%	33%	18%	0%	22%	100%
5	q			0%		9%	0%	4%	100%
5	p			20%	33%	18%	0%	22%	100%
6	p			NA	17%	0%		4–16%	100%
6	q			NA				28%	NA
7	q			20%		0%		4–13%	100%
7	p			0%		9%	0%	4%	100%
8	q			0%	17%	0%	0%	4%	100%
8	p			20%	50%	45%	100%	25–43%	100%
9	q			0%	33%	18%	100%	22%	100%
9	p			20%	50%	27%	0%	30%	100%

				Sensitivity					
				Stage I	Stage II	Stage III	Stage IV	Overall	Specificity
Detection of any CNA (37,39,113)				41%		73%		56%	66–87%
Chr	Arm	Locus	Gene	50–100%	45–100%	45–91%	58–100%	49–96%	
10	q			0%	33%	0%		9%	100%
10	p			0%	17%	0%		4%	100%
11	q			0%	17%	9%	0%	9%	100%
11	p			0%	33%	18%	0%	17%	100%
12	p				NA			13%	NA
12	q			20%	0%	0%	0%	4–13%	100%
12	p			20%	33%	0%	0%	13%	100%
14				0%	17%	0%	0%	4%	100%
14	q				NA			25%	NA
14	p				NA			13%	NA
15				20%	17%	0%		9%	100%
16				20%	83%	9%	0%	13–26%	100%
17	p	13.1	AURKB		0%		20%	4%	NA
17	p	13.1	*TP53*	0%		5%	25%	8%	NA
17				20%	17%	9%	0%	17%	100%
18	q	22.2	SOCS6		0%		30%	8%	NA
18				0%	33%	55%	0%	39%	100%
19				80%	66%	9%	100%	39%	100%
20				0%	33%	9%	0%	13%	100%
21				0%		18%	0%	9%	100%
22				40%	17%	36%	0%	30%	100%

The number of studies reporting on a specific marker is represented next to the target gene. If possible, the sensitivity was presented separately for each disease stage. CNA = copy number alteration; CRC = colorectal cancer; NA = not available, no data were available in a specific category.

In general, the analysis of ctDNA mutations showed a limited sensitivity of up to 57% in stage I–III disease, although a higher sensitivity of 75% was found in stage IV CRC using analysis of *APC* mutations. Detection of CRC by use of ctDNA copy number analysis showed promising sensitivities up to 96% but was described by only three studies. Analysis of *SEPT9* hypermethylation resulted in high sensitivities (up to 100%) and specificities up to 97%. The methylation markers adenomatous polyposis coli (*APC*), vimentin (*VIM*), branched chain amino acid transaminase 1 (*BCAT1*), Aristaless-like homeobox 4 (*ALX4*), IKAROS family zinc finger 1 (*IKZF1*), and *LINE-1* showed potential but were described by a limited number of studies ($n < 5$).

Mutation Marker Candidates

The mutational landscape of CRC is very heterogeneous, but several well-studied hot-spot mutations in genes with a crucial role in the progression of adenoma to carcinoma are known (6). Inactivating mutations in the tumor-suppressor gene *APC* are present in 30–70% of sporadic CRC (114). *KRAS* and *BRAF* mutations are found in 30% and 10% of CRC, respectively (114). The presence of these mutations is both a reflection of tumor biology (qualitative information) and tumor burden (quantitative information). Detection of these mutations is therefore an attractive approach for cancer diagnosis.

KRAS

For diagnostic purposes, point mutations of the *KRAS* gene were most frequently evaluated (n = 25 articles), resulting in sensitivities between 0 and 73% for stage I–IV CRC (32,34,35,41–46,49–53,55–64,115). Fourteen studies reported a sensitivity of more than 30% using various detection methods (32,34,35,41,42,44,53,55,56,59 ,61,63,64,115). The largest and most recently published studies found sensitivities between 32% and 41% in patients with stage I–IV CRC using ddPCR or Intplex allele-specific PCR in plasma (34,62,64). Two recently published studies using ddPCR to analyze ctDNA from plasma (n = 150 patients) (64) and allele-specific PCR on ctDNA from serum (n = 50 patients) (62) found sensitivities of 41% and 32%, respectively, that increased to 48% and 53% in stage IV CRC. *KRAS* mutations were rarely detected in ctDNA from healthy control individuals, illustrated by specificities ranging between 70% and 100% (32,46,49,58–60,62). Technical concordance between ctDNA and solid-tumor tissue analysis was heavily influenced by the analytical platform and ranged between 25% and 100% (32,34,35,42–44,46,49,50,52,56,57,59,61–64,115). Higher concordance rates (>60%) were reported by recent studies using ddPCR in plasma (34,35,62,64). In summary, the use of *KRAS* mutation analysis in ctDNA is hampered by low sensitivities of less than 50% for detection of CRC despite relatively good specificities and concordance rates.

BRAF

Detection of CRC by *BRAF* mutation analysis in ctDNA was evaluated in 10 studies, all reporting relatively low sensitivities of 0–50% independently of the technique used (34,35,42,45,49–53,115). The largest cohort study on *BRAF* ctDNA analysis found a *BRAF* mutation in only one of the 115 CRC patients using nested-PCR in serum (50), and a recent study in 97 locally advanced rectal cancer patients reported *BRAF* ctDNA mutations in the plasma of only two patients using ddPCR (34). Another recent study in 21 stage IV CRC patients reported a higher sensitivity of 29% for detection of *BRAF* mutations using an NGS panel of 90 oncogenes in plasma (45). None of these studies provided data to determine specificity. Concordance rates varied heavily among studies, but the only two studies evaluating *BRAF* mutations

with ddPCR found a concordance of 100% (35,53). Nevertheless, because of the low frequency of *BRAF* mutations in ctDNA of CRC patients, analysis of this aberration is not suitable for large-scale CRC screening.

APC

Four of eight studies investigating *APC* mutations in ctDNA reported sensitivities greater than 35% for CRC diagnosis using various detection methods (41,42,44–47,115,116). In the largest cohort (n = 133 patients), a sensitivity of 8% was found for detection of stage I–IV CRC and 15% for stage IV disease using a MassArray assay in plasma (43). A recent study showed a comparable sensitivity of 18% using an NGS panel in plasma of stage I–IV patients (44). A specificity of 100% was reported by only one study using single-strand conformation polymorphism-PCR for ctDNA detection in serum (46). The concordance for detection of *APC* mutations ranged from 16% to 100% (42–44,46,47,115). Four of the six studies describing concordance reported rates lower than 50% (43,44,46,47), none of them describing ddPCR. The low sensitivity makes *APC* an unattractive marker for CRC detection.

Copy Number Alterations

Aneuploidy, an abnormal number of chromosomes, is a common causal event in CRC. Several CNA patterns have been identified, including deletions of both arms of chromosome 17 and 18 in 56% and 66% of CRC patients, respectively (6). Analysis of copy numbers uses a genomewide approach so does not rely on detecting nucleotide-specific changes that may occur below the detection threshold in a cfDNA sample. Furthermore, large (>3 Mb) or high-level (≥4 copies) CNAs are absent in healthy individuals, allowing a high level of specificity (117).

So far, a limited number of studies have investigated the use of ctDNA for CRC detection. The three included studies on CNAs in blood of CRC patients are the most recent and reported inconsistent results using shallow whole-genome sequencing methods (Table 2) (39,113,118). Depending on the study, detection of CNAs was described on the level of a whole chromosome, chromosome arm, and/or a specific gene. One study reported copy number gains or losses across the whole genome in the plasma of 96% of stage I–IV CRC patients and 100% of stage IV CRC patients (113). Other studies reported lower sensitivities of 49% (39) and 56% (118) for detection in plasma of stage I–IV CRC patients. When focusing on CNAs of specific chromosomes, copy number losses on chromosome 18q and both gains and losses on chromosome 19 were found in the plasma of 39% of CRC patients (113). Furthermore, a specificity of 66–87% was reported (113,118). Because studies did not provide data to determine CNA concordance, this is not reported in Table 2. In summary, the analysis of genomewide CNAs is a promising method for noninvasive CRC detection but requires more research.

Methylation Marker Candidates

Hypermethylation in promotor regions of genes associated with tumorigenesis is a common phenomenon in CRC that mainly occurs in CpG islands, concentrated regions of DNA sequences susceptible to methylation. Fifteen percent of sporadic colorectal tumors are characterized by high methylation levels, referred to as CpG island methylation phenotype (119). However, CpG island methylation phenotype–negative tumors also have recurrent patterns of DNA methylation, which could allow methylation to be exploited for CRC detection (120).

SEPT9

Hypermethylation of the *SEPT9* promotor region was frequently investigated in large cohorts. Most of the 23 studies (25,29,30,32,33,66,67,75,80,93,94,96–107) that analyzed *SEPT9* hypermethylation by various methods demonstrated it to be among the most accurate candidate markers, reporting sensitivities greater than 50% for stage I–IV CRC (25,30,32,33,66,75,93,94,96–105,107). The analysis of *SEPT9* hypermethylation in ctDNA in plasma using quantitative methylation-specific PCR (qMSP) showed sensitivities of 61–62% in three recent large cohorts (n = 98, n = 123, and n = 187 patients) (94,104,105). Several other large-cohort studies showed potential for a commercially available test using qMSP for analysis of *SEPT9* hypermethylation in plasma, reporting sensitivities between 73% and 87% for stage I–IV CRC (30,33,97,99,101,102,107). The sensitivity gradually increased with higher stages and was reported to be 100% in stage IV CRC patients in several studies (30,99,101). In most recent studies, specificities of 82–95% were found (29,67,94,99,104). The few studies describing concordance reported rates of approximately 80% (32,94,97). Overall, detection of hypermethylated *SEPT9* seems promising for CRC detection considering its high accuracy.

CDKN2A (p16)

All six studies evaluating cyclin-dependent kinase inhibitor 2A (*CDKN2A*) hypermethylation in ctDNA of CRC patients used MSP. The most recent study used qMSP and reported a sensitivity of 9% for stage I–IV CRC detection (67). Other studies published in the past decade did not find specificities exceeding 35% (71,77,78). One study reported a specificity of 96% (67). Concordance rates of 70% and 82% were described in two studies (78,79). Taken together, only a limited number of studies provided an overall picture of the potential value of *CDKN2A* hypermethylation analysis in ctDNA for CRC detection. Detection of hypermethylated *CDKN2A* by MSP does not show potential for CRC detection considering its low sensitivity.

HLTF

All six studies on helicase-like transcription factor (*HLTF*) hypermethylation analysis for the purpose of CRC detection used qMSP and described large cohorts of more than 100 patients (67,85–89). The most recent study found a sensitivity of 11% for analysis in plasma (67), which was supported by the majority of other studies describing sensitivities of less than 20% (67,85–88). Two studies reported specificities (96% and 100%) (67,89), and two studies reported concordance rates (41% and 42%) (86,88). Taken together, this candidate marker is not considered to be of value for CRC detection because of the low observed sensitivities.

Other Candidate Methylation Markers

Several less frequently described candidate markers presented in Table 1 showed high sensitivities, supporting their further investigation. Of particular interest for further validation are (studies with highest reported sensitivity) across stages I–IV: *ALX4* [sensitivity 83%, specificity 70% (68)], *APC* [sensitivity 57%, specificity 86% (72)], *BCAT1* [sensitivity 65%, specificity 97% (75)], *IKZF1* [sensitivity 68%, specificity 95 (76)], and *VIM* [sensitivity 71%, specificity not reported (109)]. Furthermore, hypomethylation of *LINE-1* [sensitivity 66%, specificity 90% (112)] and cystathionine-beta-synthase (CBS) [sensitivity 56%, specificity not reported (111)] are of interest and require further study.

Marker Panels

The simultaneous analysis of multiple ctDNA mutation, copy number, and/or hypermethylation markers potentially results in higher accuracy for CRC detection. Most evidence arises from studies evaluating panels of hypermethylation markers. Combined analysis of *APC*, O-6-methylguanine-DNA methyltransferase (*MGMT*), Ras association domain family member 2 (*RASSF2A*), and WNT inhibitory factor 1 (*Wif-1*) hypermethylation was evaluated in 243 stage I–II CRC patients and demonstrated a sensitivity of 87% and a specificity of 92% (86). In a more recent study (n = 193 patients), a panel of the plasma hypermethylation markers *ALX4*, bone morphogenetic protein 3 (*BMP3*), neuronal pentraxin 2 (*NPTX2*), retinoic acid receptor beta (*RARB*), syndecan 2 (*SDC2*), *SEPT9*, and *VIM* analyzed with MSP showed a sensitivity of 91% for stage I–IV and 89% for stage I–II CRC using a multifactorial model accounting for sex and age (67). This study reported a specificity of 73%. The largest described panel was an NGS panel of 90 oncogenes including the most common CRC mutations. With this panel, one to six mutations were found in all 21 studied CRC patients (sensitivity 100%) without providing information on specificity (45). None of the studies reported technical concordance rates for these panels. Overall, the use of marker panels for CRC detection resulted in high accuracy.

ctDNA for Prognostication and Treatment Selection in CRC

Pre therapeutic Analysis

Pre- as well as post-therapeutic ctDNA analysis have the potential to improve clinical decision making. Quantification of ctDNA before treatment could serve as a prognosticator because of a strong correlation with tumor burden. In the included studies, ctDNA analysis in therapy-naive patients allowed profiling of mutation patterns and detection of *KRAS* mutations before anti-*EGFR* therapy. Additionally, the presence of ctDNA was correlated with clinicopathological parameters (Figure 3), supporting its use in treatment planning. None of the included studies reported on detection of posttherapeutic ctDNA CNAs.

Figure 4. Associations between the presence of preoperative circulating tumor DNA and clinicopathological variables and oncologic outcomes. The percentage of patients with a positive marker is represented for the categories of the variables. Green: all studies reporting on the specific marker found statistically significant associations; orange: part of the studies found statistically significant and part found statistically nonsignificant (NS) associations; pink: all studies found statistically NS associations. The overall (OS) and disease-free survival (DFS) is presented for patients with a positive (+) and a negative (−) marker. # = no percentage of patients or median or mean OS or DFS provided, * = median, ** = mean. TNM = tumor (T), nodes (N), and metastases (M).

Quantitative analysis showed that ctDNA mutations in *KRAS*, *APC*, and *TP53* genes (41,46) and hypermethylation of multiple genes (*APC*, GATA binding protein 5 (*GATA5*), *HLTF*, hyperpigmentation, progressive, 1 (*HPP1*), integrin subunit alpha 4 (*ITGA4*), protocadherin 10 (*PCDH10*), Ras association domain family member 1 (*RASSF1A*), *SEPT9*, short stature homeobox 2 (*SHOX2*), and secreted frizzled-related protein 2 (*SFPR2*) are frequently present in patients with late-stage CRC (29,36,46,73,84,89,103,106). The detection of *KRAS* mutations (60) and hypermethylation of the *HLTF*, *HPP1*, tissue factor pathway inhibitor 2 (*TFPI2*), *SEPT9*, *SHOX2*, and *VIM* genes (89,103,108,109) in ctDNA was associated with the presence of distant metastases. Accordingly, the presence of ctDNA as detected by mutation [90-gene NGS panel (45), *KRAS*, *APC*, tumor protein P53 (*TP53*) (46,60)], copy number (113), or hypermethylation analysis [*APC*, *HLTF*, *HPP1*, *RASSF1A* (73,89,90)] was associated with worse progression-free and overall survival. Qualitative ctDNA analysis showed that presence of *KRAS* mutations in ctDNA could predict the effectiveness of targeted therapies, illustrated by an absence of clinical response to anti-*EGFR* therapy in stage IV CRC patients with *KRAS* mutations detected in pretherapeutic blood samples (61).

Post therapeutic Analysis

The detection of ctDNA after therapy could qualify patients for additional therapies by indicating residual disease or recurrence. The studies included in this review showed that the posttherapeutic detection of ctDNA mutations was correlated with poor oncologic outcome and, accordingly, may reflect (residual) tumor load after tumor resection. The detection of ctDNA using an NGS panel of 90 oncogenes after start of systemic treatment was found to be an independent risk factor for poor survival in 21 stage IV CRC patients (45). In seven CRC patients, the postoperative presence of driver gene mutations in plasma ctDNA, as detected by an 85-gene NGS panel, was associated with a poor prognosis (44). Another study (n = 60 patients) demonstrated that the persistence of serum *KRAS* mutations after surgery was associated with an increased risk of recurrence (59).

Postoperative ctDNA hypermethylation was found to be associated with poor oncologic outcome. In 79 CRC patients, *SEPT9* methylation levels dropped to barely detectable amounts after surgery in all patients except those with distant metastases or positive resection margins (103). In another study (n = 16 patients), the two patients with methylated *SEPT9* in postoperative ctDNA both presented with a recurrence during follow-up (104). Furthermore, in a study describing 82 CRC patients, postoperative detection of *SEPT9* hypermethylation in plasma was associated with increased mortality (107). Several other methylation markers were proposed as indicators of residual disease. Postoperative detection of *HPP1* hypermethylation was associated with poor survival in 337 CRC patients (90).

Elevated *VIM* methylation plasma levels were associated with residual disease after surgery in patients with colorectal liver metastases, whereas CEA levels had returned to normal levels after surgery (110). Another proposed method to detect residual disease is combined analysis of plasma *BCAT1* and *IKZF1* hypermethylation. Tumor resection resulted in reduced methylation levels of these genes with complete elimination of the signal in 10 of 26 patients (31). Taken together, postoperative presence of ctDNA suggests residual disease. However, included studies consist of small cohorts and clinical validation is warranted.

ctDNA for CRC Monitoring

Monitoring of disease by serial liquid biopsies to assess treatment response and detect recurrences during follow-up is a promising and valuable companion to current detection methods. Quantitative detection of ctDNA levels potentially allows early detection of recurrences (121). Qualitative analysis of ctDNA mutations and CNAs could find therapeutic targets and help detect therapy resistance (121).

Six studies evaluated the potential of ctDNA analysis during follow-up after surgery or during systemic treatment of CRC patients (45,55,59–61,115), all of which had small sample sizes. Five studies reported data on ctDNA mutation analysis (45,59–61,115) and one study investigated a combination of hypermethylation and mutation markers (55). No articles reported on CNAs for the use of CRC patient monitoring.

An increase in ctDNA levels, as detected by an NGS panel of 90 oncogenes, could detect resistance to chemotherapy (45). Additionally, quantitative analysis of *KRAS* mutations allowed detection of recurrences with 100% sensitivity in patients with *KRAS*-positive solid tumors (60,61,115) and improved monitoring compared with current diagnostic modalities (61). In three of seven metastatic CRC patients with a recurrence, reappearance of plasma *KRAS* mutations was detected before a diagnosis could be made using conventional methods. Moreover, in eight patients with acquired resistance during anti-*EGFR* therapy, *KRAS* mutations were detectable in plasma 3 months before disease progression was seen on CT scans (61). Furthermore, newly diagnosed *KRAS* and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations were found up to 4 months before radiological progression in two stage IV CRC patients receiving systemic therapy (115). The combined analysis of *KRAS* mutations and *CDKN2A* methylation analysis in plasma of CRC patients increased diagnostic accuracy (55). At this moment, however, conclusions of all studies are hampered by small sample sizes.

Discussion

Analysis of ctDNA in peripheral blood samples, so-called liquid biopsies, has the potential to realize early-stage detection of CRC and serve as a prognostic, predictive, and monitoring tool. The present systematic review is the first to evaluate the use of the most promising types of ctDNA analysis in a clinical setting. To date, the highest accuracy for CRC detection has been obtained by *SEPT9* hypermethylation analysis, especially in combined panels. For diagnostic purposes, analysis of single ctDNA mutations does not yet allow for clinical decision making. For the purposes of prognostication and disease monitoring, the most robust results were obtained by consecutive sampling and subsequent *KRAS* mutation ctDNA analysis. The analysis of CNAs could be promising for clinical use as well but is still in its infancy.

The present findings provide a starting point for implementation of ctDNA analysis into the clinic by setting out promising candidate markers. The high sensitivities of up to 100% and specificities of up to 97% of *SEPT9* methylation ctDNA analysis suggest a diagnostic role for this candidate marker. Even higher sensitivities could theoretically be obtained in combination with other promising methylation markers such as *APC*, *VIM*, *BCAT1*, *ALX4*, *IKZF1*, and *LINE-1*. Cancer detection through copy number analysis in ctDNA has great potential for CRC detection, with sensitivities up to 96% and specificities up to 100%. However, only a small number of included studies reported on CNAs in ctDNA, hampering solid conclusions. In contrast, analysis of single-gene ctDNA mutations showed disappointing sensitivities of less than 50% with highly variable specificities so is unlikely to increase the accuracy of current screening methods. The low sensitivities are probably due to the relatively low proportion of cfDNA fragments carrying the tumor-specific mutation, described as the variant allele frequency, or due to the absolute number of mutant DNA molecules in the sample (17,122,123).

For prognostication and disease monitoring, mutation ctDNA analysis is considered the most valuable. For prognostication, pre- and posttherapeutic analyses alike of *KRAS* and *APC* mutations provided information on tumor load (quantitative analysis) and allowed molecular profiling (qualitative analysis) to guide treatment decisions by determining the indication for (neo-)adjuvant therapies (19,20,124). Owing to correlation with oncologic outcomes, ctDNA detection after tumor resection suggests the presence of residual disease undetectable with conventional methods (17). This potentially enables accurate identification of patients for adjuvant systemic therapies. Additionally, the presence of *KRAS* mutations in ctDNA could predict treatment response to anti-*EGFR* therapy (61). The detection of ctDNA at higher stages could result from increased shedding of ctDNA or occult micrometastases (17). For monitoring purposes, consecutive

analysis during follow-up showed high accuracy for detection of recurrences in patients with known pre-therapeutic detectable *KRAS* mutations (55,59–61,115). Additionally, *KRAS* mutation analysis in ctDNA allows repeated analysis of tumor mutations to identify acquired resistance (61) and emerging potential therapeutic targets (121). In this way, ctDNA analysis could guide tailored treatment. None of the included studies investigated CNAs for monitoring of CRC. Theoretically, serial copy number analysis could be useful as well because it does not target a specific genomic site but measures across the entire genome.

Clinical implementation of liquid biopsies for population-based screening also has high potential. Limitations of current studies are the small cohorts and poorly defined or absent healthy control individuals. Moreover, there is a lack of studies focusing on detection of precursor lesions. Before widespread implementation for screening, adequately powered validation studies comparing ctDNA with the FOBT and colonoscopy are essential. The current literature on liquid biopsies for CRC mainly consists of nonrandomized retrospective studies, with only a few markers tested in validation cohorts. A technical issue is the mutational heterogeneity observed in CRC. Accurate mutation monitoring requires expensive panel-based NGS approaches to test many genes before start of therapy and subsequent consecutive analyses of specific mutations. For this process, multiple-gene testing and highly robust assays for individual mutations are warranted, impeding widespread use. However, large-scale whole-genome mutation analysis in blood as a liquid biopsy will be feasible in the near future, enabling not only monitoring of recurrences but also evaluation of clonal evolution to adjust therapeutic approaches. Cost-effectiveness analysis and clinical validation in prospective trials are currently ongoing.

To our knowledge, this is the first systematic review assessing candidate mutation, CNA, and methylation markers in blood samples for clinical use in CRC patients. These approaches could not only complement each other but also be combined to achieve higher accuracy (125). In line with the present review, the value of methylation analysis for CRC detection is supported by a systematic review reporting hypermethylation of the *APC*, neurogenin 1 (*NEUROG1*), *RASSF1A*, *RASSF2A*, *SDC2*, *SEPT9*, tachykinin precursor 1 (*TAC1*), and thrombomodulin (*THBD*) genes in ctDNA to be detectable in early-stage CRC patients (20). However, in contrast to the low sensitivities reported for ctDNA mutation analysis, in a recent review the use of *KRAS* and *APC* mutation analysis in ctDNA was advocated for early CRC detection, with particular interest in *APC* mutations because of their presence in precursor lesions (126). Notably, this review was not performed systematically and no quality assessment was performed, impeding the authors' conclusions.

Unfortunately, it was not possible to conduct a meta-analysis because of the variability of methods. Furthermore, the use of other liquid biopsy substrates such

as circulating RNAs or circulating tumor cells was beyond the scope of this study. The focus on ctDNA was chosen because it has been investigated most extensively and is proposed as the most promising reproducible method for CRC detection with high accuracy (17–19). We included analysis of copy numbers because this is a promising, novel, and relatively simple method to detect ctDNA (15,127). Moreover, we did not include studies on other ctDNA sources currently being explored, such as urine, stool, and saliva (128,129). Similarly, other noninvasive approaches to genetic diagnosis of CRC were also omitted despite widespread clinical use. For example, the FDA-approved Cologuard (Exact Sciences) test analyzes mutations and methylation changes in DNA from stool (130). However, because stool is not a source of ctDNA, it was not covered by the scope of this study.

Translation of ctDNA into clinical daily practice is still awaited. The use of ctDNA for therapy guidance has already been suggested for locally advanced rectal cancer patients (131) and could help clinicians decide whether additional intervention is required after local excision of early-stage rectal cancer (132). A prospective comparison of current guidelines for adjuvant treatment with a novel approach based on residual ctDNA should be carried out and is currently being planned for advanced rectal cancer patients (Dynamic-Rectal study— ACTRN12617001560381). Prospective combined analysis of (epi-) genomic markers integrated with other biomarker substrates such as proteomics or metabolomics could facilitate cancer detection with higher accuracy (133). Such innovative blood tests should be designed using an "-omics" approach (134), opening up potential combinations of other candidate biomarkers. Furthermore, implementation of ctDNA analysis is promoted by novel detection methods that are being developed at a rapid pace. Techniques that are currently too expensive for routine use, such as personalized ctDNA sequencing, might become feasible within years (135). However, the use of highly sensitive and specific single-locus assays such as ddPCR, which is currently a more straightforward and cost-effective method, are still expected to be relevant (136,137), particularly for repeated measurements in patients with known tumor mutations in a tissue-guided manner (18). Finally, collaboration between academia and industrial partners is becoming increasingly important for the transition of biomarkers into the clinic, but a solid cost-effectiveness analysis is key for this purpose (138).

In conclusion, the present overview of literature proposes ctDNA analysis of methylation panels including *SEPT9* as the most valuable option for CRC detection. The use of liquid biopsies for disease monitoring seems even more promising. *KRAS* mutation analysis appears of particular interest for prognostication and monitoring of CRC patients to provide treatment guidance and tailored therapies. CNAs can be detected in the blood of CRC patients at various stages. Owing to its genomewide rather than gene-specific approach, copy number analysis could potentially be useful as a companion for early detection or monitoring. However,

more research is needed. Creation of approaches combining various types of ctDNA analyses could further enhance accuracy. Prospective studies, preferably in a randomized setting in which clinical decisions depend on ctDNA results of the currently proposed candidate markers, should provide the definitive evidence to bring ctDNA analysis to clinical practice.

Funding

This work was supported by intramural funding from the VUmc-CCA Foundation, Edli Foundation, and the Weijerhorst Foundation. This research did not receive any other specific grants from funding agencies in the public or commercial sectors.

Notes

Affiliations of authors: Department of Surgery, Cancer Center Amsterdam (SB, NRS, JMM, GK, JBT), Department of Pathology, Cancer Center Amsterdam (JJB, NCvG, RDMS, BY) and Medical Information Specialist/Literature Researcher Medical Library (JCK), Amsterdam UMC, Vrije Universiteit Amsterdam, Vrije Universiteit, Amsterdam, the Netherlands.

References

1. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer. 2013;49(6):1374–1403.
2. Siegel RL, Miller KD, Jemal A.. Cancer statistics, 2016. CA Cancer J Clin. 2016;66(1):7–30.
3. Brouwer NPM, Bos A, Lemmens V, et al. An overview of 25 years of incidence, treatment and outcome of colorectal cancer patients. Int J Cancer. 2018;143(11):2758–2766.
4. Brenner H, Kloor M, Pox CP.. Colorectal cancer. Lancet. 2014;383(9927):1490–1502.
5. McLoughlin RM, O'Morain CA.. Colorectal cancer screening. World J Gastroenterol. 2006;12(42):6747–6750.
6. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330–337.
7. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. Nat Med. 2015;21(11):1350–1356.
8. Böhm B, Schwenk W, Hucke HP, et al. Does methodic long-term follow-up affect survival after curative resection of colorectal carcinoma? Dis Colon Rectum. 1993;36(3):280-286.
9. Werkgroep Colorectaal Carcinoom. Richtlijn Colorectaal Carcinoom http://www.oncoline.nl/colorectaalcarcinoom. Accessed January 11, 2019.
10. Duffy MJ. Carcinoembryonic antigen as a marker for colorectal cancer: is it clinically useful? Clin Chem. 2001;47(4):624–630.
11. Rockall TA, McDonald PJ.. Carcinoembryonic antigen: its value in the follow-up of patients with colorectal cancer. Int J Colorectal Dis. 1999;14(1):73–77.
12. Primrose JN, Perera R, Gray A, et al. Effect of 3 to 5 years of scheduled CEA and CT follow-up to detect recurrence of colorectal cancer: the FACS randomized clinical trial. JAMA. 2014;311(3):263–270.
13. Koh JL, Yan TD, Glenn D, et al. Evaluation of preoperative computed tomography in estimating peritoneal cancer index in colorectal peritoneal carcinomatosis. Ann Surg Oncol. 2009;16(2):327–333.
14. Normanno N, Cervantes A, Ciardiello F, et al. The liquid biopsy in the management of colorectal cancer patients: current applications and future scenarios. Cancer Treat Rev. 2018;70:1–8.
15. Heitzer E, Haque IS, Roberts CES, et al. Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet. 2018;20(2):71–88.
16. Best M, Sol N, Kooi I, et al. mRNA-sequencing of tumour-educated platelets allows for multiclass liquid biopsy-based diagnosis of cancer. J Extracell Vesicles. 2015;4:169–170.
17. Wan JC, Massie C, Garcia-Corbacho J, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer. 2017;17(4):223–238.
18. Ignatiadis M, Lee M, Jeffrey SS.. Circulating tumor cells and circulating tumor DNA: challenges and opportunities on the path to clinical utility. Clin Cancer Res. 2015;21(21):4786–4800.
19. Nordgård O, Tjensvoll K, Gilje B, et al. Circulating tumour cells and DNA as liquid biopsies in gastrointestinal cancer. Br J Surg. 2018;105(2):e110–e120.
20. Rasmussen SL, Krarup HB, Sunesen KG, et al. Hypermethylated DNA as a biomarker for colorectal cancer: a systematic review. Colorectal Dis. 2016;18(6):549–561.

21. Thierry AR, Mouliere F, El Messaoudi S, et al. Clinical validation of the detection of *KRAS* and BRAF mutations from circulating tumor DNA. Nat Med. 2014;20(4):430–435.

22. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;14(9):985–990.

23. Sausen M, Phallen J, Adleff V, et al. Clinical implications of genomic alterations in the tumour and circulation of pancreatic cancer patients. Nat Commun. 2015;6:7686.

24. Jenkins S, Yang JC, Ramalingam SS, et al. Plasma ctDNA analysis for detection of the EGFR T790M mutation in patients with advanced non-small cell lung cancer. J Thorac Oncol. 2017;12(7):1061–1070.

25. Warren JD, Xiong W, Bunker AM, et al. Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. BMC Med. 2011;9:133..

26. van Schendel RV, van El CG, Pajkrt E, et al. Implementing non-invasive prenatal testing for aneuploidy in a national healthcare system: global challenges and national solutions. BMC Health Serv Res. 2017;17(1):670..

27. Moher D, Shamseer L, Clarke M, et al. Preferred Reporting Items for Systematic Review and Meta-analysis protocols (PRISMA-P) 2015 statement. Syst Rev. 2015;4:1..

28. Whiting PF, Rutjes AW, Westwood ME, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med. 2011;155(8):529–536.

29. Chen CH, Yan SL, Yang TH, et al. The relationship between the methylated septin-9 DNA blood test and stool occult blood test for diagnosing colorectal cancer in Taiwanese people. J Clin Lab Anal. 2017;31(1). doi: 10.1002/jcla.22013.

30. Jin P, Kang Q, Wang X, et al. Performance of a second-generation methylated *SEPT9* test in detecting colorectal neoplasm. J Gastroenterol Hepatol. 2015;30(5):830–833.

31. Pedersen SK, Symonds EL, Baker RT, et al. Evaluation of an assay for methylated *BCAT1* and *IKZF1* in plasma for detection of colorectal neoplasia. BMC Cancer. 2015;15:654..

32. Danese E, Minicozzi AM, Benati M, et al. Comparison of genetic and epigenetic alterations of primary tumors and matched plasma samples in patients with colorectal cancer. PLoS One. 2015;10(5):e0126417..

33. Song L, Jia J, Yu H, et al. The performance of the m*SEPT9* assay is influenced by algorithm, cancer stage and age, but not sex and cancer location. J Cancer Res Clin Oncol. 2017;143(6):1093–1101.

34. Sclafani F, Chau I, Cunningham D, et al. *KRAS* and BRAF mutations in circulating tumour DNA from locally advanced rectal cancer. Sci Rep. 2018;8(1):1445..

35. Sefrioui D, Beaussire L, Perdrix A, et al. Direct circulating tumor DNA detection from unpurified plasma using a digital PCR platform. Clin Biochem. 2017;50(16–17):963–966.

36. Danese E, Minicozzi AM, Benati M, et al. Epigenetic alteration: new insights moving from tissue to plasma - the example of *PCDH10* promoter methylation in colorectal cancer. Br J Cancer. 2013;109(3):807–813.

37. Xu JF, Kang Q, Pan YM, et al. A novel method to detect early colorectal cancer based on chromosome copy number variation in plasma. Cell Physiol Biochem. 2018;45(4):1444–1454.

38. Symonds EL, Pedersen SK, Baker RT, et al. A blood test for methylated *BCAT1* and *IKZF1* vs. a fecal immunochemical test for detection of colorectal neoplasia. Clin Transl Gastroenterol. 2016;7(1):e137..

39. Li J, Dittmar RL, Xia S, et al. Cell-free DNA copy number variations in plasma from colorectal cancer patients. Mol Oncol. 2017;11(8):1099–1111.

40. Lansdorp-Vogelaar I, van Ballegooijen M, Boer R, et al. A novel hypothesis on the sensitivity of the fecal occult blood test: results of a joint analysis of 3 randomized controlled trials. Cancer. 2009;115(11):2410–2419.

41. Hsieh JS, Lin SR, Chang MY, et al. *APC*, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance. Am Surg. 2005;71(4):336–343.

42. Lilleberg SL, Durocher J, Sanders C, et al. High sensitivity scanning of colorectal tumors and matched plasma DNA for mutations in *APC*, *TP53*, K-RAS, and BRAF genes with a novel DHPLC fluorescence detection platform. Ann N Y Acad Sci. 2004;1022:250–256.

43. Lin JK, Lin PC, Lin CH, et al. Clinical relevance of alterations in quantity and quality of plasma DNA in colorectal cancer patients: based on the mutation spectra detected in primary tumors. Ann Surg Oncol. 2014;21(suppl 4):S680–S686.

44. Sun X, Huang T, Cheng F, et al. Monitoring colorectal cancer following surgery using plasma circulating tumor DNA. Oncol Lett. 2018;15(4):4365–4375.

45. Yamauchi M, Urabe Y, Ono A, et al. Serial profiling of circulating tumor DNA for optimization of anti-VEGF chemotherapy in metastatic colorectal cancer patients. Int J Cancer. 2018;142(7):1418–1426.

46. Wang JY, Hsieh JS, Chang MY, et al. Molecular detection of *APC*, K- ras, and p53 mutations in the serum of colorectal cancer patients as circulating biomarkers. World J Surg. 2004;28(7):721–726.

47. Diehl F, Schmidt K, Durkee KH, et al. Analysis of mutations in DNA isolated from plasma and stool of colorectal cancer patients. Gastroenterology. 2008;135(2):489–498.

48. Kim ST, Lira M, Deng SB, et al. *PIK3CA* mutation detection in metastatic biliary cancer using cell-free DNA. Oncotarget. 2015;6(37):40026–40035.

49. Kidess E, Heirich K, Wiggin M, et al. Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget. 2015;6(4):2549–2561.

50. Pu X, Pan Z, Huang Y, et al. Comparison of *KRAS*/BRAF mutations between primary tumors and serum in colorectal cancer: biological and clinical implications. Oncol Lett. 2013;5(1):249–254.

51. Li X, Yang T, Li CS, et al. Surface enhanced raman spectroscopy (SERS) for the multiplex detection of Braf, *KRAS*, and Pik3ca mutations in plasma of colorectal cancer patients. Theranostics. 2018;8(6):1678–1689.

52. Beranek M, Sirak I, Vosmik M, et al. Carrier molecules and extraction of circulating tumor DNA for next generation sequencing in colorectal cancer. Acta Medica (Hradec Kralove). 2016;59(2):54–58.

53. Sakai K, Tsurutani J, Yamanaka T, et al. Extended RAS and BRAF mutation analysis using next-generation sequencing. PLoS One. 2015;10(5):e0121891..

54. Loree JM, Bailey AM, Johnson AM, et al. Molecular landscape of *ERBB2*/ERBB3 mutated colorectal cancer. J Natl Cancer Inst. 2018;110(12):1409–1417.

55. Frattini M, Gallino G, Signoroni S, et al. Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. Cancer Lett. 2008;263(2):170–181.

56. Kuo YB, Chen JS, Fan CW, et al. Comparison of *KRAS* mutation analysis of primary tumors and matched circulating cell-free DNA in plasmas of patients with colorectal cancer. Clin Chim Acta. 2014;433:284–289.

57. Liu P, Liang H, Xue L, et al. Potential clinical significance of plasma-based *KRAS* mutation analysis using the COLD-PCR/TaqMan((R)) -MGB probe genotyping method. Exp Ther Med. 2012;4(1):109–112.

58. Perrone F, Lampis A, Bertan C, et al. Circulating free DNA in a screening program for early colorectal cancer detection. Tumori. 2014;100(2):115–121.

59. Ryan BM, Lefort F, McManus R, et al. A prospective study of circulating mutant *KRAS*2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in postoperative follow up. Gut. 2003;52(1):101–108.

60. Shin SJ, Chun SM, Kim TI, et al. Feasibility of multiplexed gene mutation detection in plasma samples of colorectal cancer patients by mass spectrometric genotyping. PLoS One. 2017;12(5):e0176340..

61. Yamada T, Iwai T, Takahashi G, et al. Utility of *KRAS* mutation detection using circulating cell-free DNA from patients with colorectal cancer. Cancer Sci. 2016;107(7):936–943.

62. Kloten V, Ruchel N, Bruchle NO, et al. Liquid biopsy in colon cancer: comparison of different circulating DNA extraction systems following absolute quantification of *KRAS* mutations using Intplex allele-specific PCR. Oncotarget. 2017;8(49):86253–86263.

63. Ono Y, Sugitani A, Karasaki H, et al. An improved digital polymerase chain reaction protocol to capture low-copy *KRAS* mutations in plasma cell-free DNA by resolving 'subsampling' issues. Mol Oncol. 2017;11(10):1448–1458.

64. Tian F, Liao Y, Zhang Y.. Variations in transrenal DNA and comparison with plasma DNA as a diagnostic marker for colorectal cancer. Int J Biol Markers. 2017;32(4):e434–e440.

65. Liu W, Guan M, Su B, et al. Rapid determination of AKAP12 promoter methylation levels in peripheral blood using methylation-sensitive high-resolution melting (MS-HRM) analysis: application in colorectal cancer. Clin Chim Acta. 2010;411(13–14):940–946.

66. He Q, Chen HY, Bai EQ, et al. Development of a multiplex MethyLight assay for the detection of multigene methylation in human colorectal cancer. Cancer Genet Cytogenet. 2010;202(1):1–10.

67. Rasmussen SL, Krarup HB, Sunesen KG, et al. Hypermethylated DNA, a circulating biomarker for colorectal cancer detection. PLoS One. 2017;12(7):e0180809..

68. Ebert MP, Model F, Mooney S, et al. Aristaless-like homeobox-4 gene methylation is a potential marker for colorectal adenocarcinomas. Gastroenterology. 2006;131(5):1418–1430.

69. Herbst A, Rahmig K, Stieber P, et al. Methylation of *NEUROG1* in serum is a sensitive marker for the detection of early colorectal cancer. Am J Gastroenterol. 2011;106(6):1110–1118.

70. Salehi R, Atapour N, Vatandoust N, et al. Methylation pattern of *ALX4* gene promoter as a potential biomarker for blood-based early detection of colorectal cancer. Adv Biomed Res. 2015;4(1):252..

71. Erdem B, Kucukyildirim S, Saglar E, et al. Promoter hypermethylation of p16 and *APC* in gastrointestinal cancer patients. Turk J Gastroenterol. 2014;25(5):512–517.

72. Pack SC, Kim HR, Lim SW, et al. Usefulness of plasma epigenetic changes of five major genes involved in the pathogenesis of colorectal cancer. Int J Colorectal Dis. 2013;28(1):139–147.

73. Matthaios D, Balgkouranidou I, Karayiannakis A, et al. Methylation status of the *APC* and *RASSF1A* promoter in cell-free circulating DNA and its prognostic role in patients with colorectal cancer. Oncol Lett. 2016;12(1):748–756.

74. Kondratov AG, Ne*KRAS*ov KA, V LL, et al. Comparative analysis of epigenetic markers in plasma and tissue of patients with colorectal cancer. Biopolym Cell. 2014;30(2):129–134.

75. Mitchell SM, Ho T, Brown GS, et al. Evaluation of methylation biomarkers for detection of circulating tumor DNA and application to colorectal cancer. Genes (Basel) 2016;7(12):125.

76. Miotto E, Sabbioni S, Veronese A, et al. Frequent aberrant methylation of the CDH4 gene promoter in human colorectal and gastric cancer. Cancer Res. 2004;64(22):8156–8159.

77. Kim JW, Park HM, Choi YK, et al. Polymorphisms in genes involved in folate metabolism and plasma DNA methylation in colorectal cancer patients. Oncol Rep. 2011;25(1):167–172.

78. Sakamoto J, Fujiya M, Okamoto K, et al. Immunoprecipitation of nucleosomal DNA is a novel procedure to improve the sensitivity of serum screening for the p16 hypermethylation associated with colon cancer. Cancer Epidemiol. 2010;34(2):194–199.

79. Zou HZ, Yu BM, Wang ZW, et al. Detection of aberrant p16 methylation in the serum of colorectal cancer patients. Clin Cancer Res. 2002;8(1):188–191.

80. Liu Y, Chew MH, Tham CK, et al. Methylation of serum SST gene is an independent prognostic marker in colorectal cancer. Am J Cancer Res. 2016;6(9):2098–2108.

81. Wu PP, Zou JH, Tang RN, et al. Detection and clinical significance of DLC1 gene methylation in serum DNA from colorectal cancer patients. Chin J Cancer Res. 2011;23(4):283–287.

82. Shalaby SM, El-Shal AS, Abdelaziz LA, et al. Promoter methylation and expression of DNA repair genes *MGMT* and ERCC1 in tissue and blood of rectal cancer patients. Gene. 2018;644:66–73.

83. Hibi K, Mizukami H, Saito M, et al. FBN2 methylation is detected in the serum of colorectal cancer patients with hepatic metastasis. Anticancer Res. 2012;32(10):4371–4374.

84. Zhang X, Song YF, Lu HN, et al. Combined detection of plasma *GATA5* and SFRP2 methylation is a valid noninvasive biomarker for colorectal cancer and adenomas. World J Gastroenterol. 2015;21(9):2629–2637.

85. Herbst A, Wallner M, Rahmig K, et al. Methylation of helicase-like transcription factor in serum of patients with colorectal cancer is an independent predictor of disease recurrence. Eur J Gastroenterol Hepatol. 2009;21(5):565–569.

86. Lee BB, Lee EJ, Jung EH, et al. Aberrant methylation of *APC*, *MGMT*, *RASSF2A*, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer. Clin Cancer Res. 2009;15(19):6185–6191.

87. Philipp AB, Nagel D, Stieber P, et al. Circulating cell-free methylated DNA and lactate dehydrogenase release in colorectal cancer. BMC Cancer. 2014;14:245..

88. Philipp AB, Stieber P, Nagel D, et al. Prognostic role of methylated free circulating DNA in colorectal cancer. Int J Cancer. 2012;131(10):2308–2319.

89. Wallner M, Herbst A, Behrens A, et al. Methylation of serum DNA is an independent prognostic marker in colorectal cancer. Clin Cancer Res. 2006;12(24):7347–7352.

90. Herbst A, Vdovin N, Gacesa S, et al. Methylated free-circulating *HPP1* DNA is an early response marker in patients with metastatic colorectal cancer. Int J Cancer. 2017;140(9):2134–2144.

91. Grady WM, Rajput A, Lutterbaugh JD, et al. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. Cancer Res. 2001;61(3):900–902.

92. Xiao W, Zhao H, Dong W, et al. Quantitative detection of methylated NDRG4 gene as a candidate biomarker for diagnosis of colorectal cancer. Oncol Lett. 2015;9(3):1383–1387.

93. Lofton-Day C, Model F, Devos T, et al. DNA methylation biomarkers for blood-based colorectal cancer screening. Clin Chem. 2008;54(2):414–423.

94. Yuan P, Cheng XJ, Wu XJ, et al. OSMR and *SEPT9*: promising biomarkers for detection of colorectal cancer based on blood-based tests. Transl Cancer Res. 2016;5(2):131–139.

95. Zheng Y, Zhang Y, Huang X, et al. Analysis of the RUNX3 gene methylation in serum DNA from esophagus squamous cell carcinoma, gastric and colorectal adenocarcinoma patients. Hepatogastroenterology. 2011;58(112):2007–2011.

96. Ahlquist DA, Taylor WR, Mahoney DW, et al. The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. Clin Gastroenterol Hepatol. 2012;10(3):272–277 e1.

97. Church TR, Wandell M, Lofton-Day C, et al. Prospective evaluation of methylated *SEPT9* in plasma for detection of asymptomatic colorectal cancer. Gut. 2014;63(2):317–325.

98. Potter NT, Hurban P, White MN, et al. Validation of a real-time PCR-based qualitative assay for the detection of methylated *SEPT9* DNA in human plasma. Clin Chem. 2014;60(9):1183–1191.

99. Song L, Peng X, Li Y, et al. The *SEPT9* gene methylation assay is capable of detecting colorectal adenoma in opportunistic screening. Epigenomics. 2017;9(5):599–610.

100. Tänzer M, Balluff B, Distler J, et al. Performance of epigenetic markers *SEPT9* and *ALX4* in plasma for detection of colorectal precancerous lesions. PLoS One. 2010;5(2):e9061..

101. Tóth K, Sipos F, Kalmár A, et al. Detection of methylated *SEPT9* in plasma is a reliable screening method for both left- and right-sided colon cancers. PLoS One. 2012;7(9):e46000..

102. Johnson DA, Barclay RL, Mergener K, et al. Plasma Septin9 versus fecal immunochemical testing for colorectal cancer screening: a prospective multicenter study. PLoS One. 2014;9(6):e98238..

103. Bergheim J, Semaan A, Gevensleben H, et al. Potential of quantitative *SEPT9* and *SHOX2* methylation in plasmatic circulating cell-free DNA as auxiliary staging parameter in colorectal cancer: a prospective observational cohort study. Br J Cancer. 2018;118(9):1217–1228.

104. Fu B, Yan P, Zhang S, et al. Cell-free circulating methylated *SEPT9* for noninvasive diagnosis and monitoring of colorectal cancer. Dis Markers. 2018;2018:6437104.

105. Xie L, Jiang XY, Li Q, et al. Diagnostic value of methylated septin9 for colorectal cancer detection. Front Oncol. 2018;8:247.

106. Kim H, Lee JK, Hong YJ, et al. Detection of methylated *SEPT9* in Korean colorectal cancer patients: comparison with previous studies. Clin Lab. 2018;64(9):1573–1579.

107. Song L, Guo S, Wang J, et al. The blood m*SEPT9* is capable of assessing the surgical therapeutic effect and the prognosis of colorectal cancer. Biomarkers Med. 2018;12(9):961–973.

108. Hibi K, Goto T, Shirahata A, et al. Detection of *TFPI2* methylation in the serum of colorectal cancer patients. Cancer Lett. 2011;311(1):96–100.

109. Shirahata A, Sakuraba K, Goto T, et al. Detection of vimentin (*VIM*) methylation in the serum of colorectal cancer patients. Anticancer Res. 2010;30(12):5015–5018.

110. Li M, Chen WD, Papadopoulos N, et al. Sensitive digital quantification of DNA methylation in clinical samples. Nat Biotechnol. 2009;27(9):858–863.

111. Xue G, Lu CJ, Pan SJ, et al. DNA hypomethylation of CBS promoter induced by folate deficiency is a potential noninvasive circulating biomarker for colorectal adenocarcinomas. Oncotarget. 2017;8(31):51387–51401.

112. Nagai Y, Sunami E, Yamamoto Y, et al. *LINE-1* hypomethylation status of circulating cell-free DNA in plasma as a biomarker for colorectal cancer. Oncotarget. 2017;8(7):11906–11916.

113. Molparia B, Oliveira G, Wagner JL, et al. A feasibility study of colorectal cancer diagnosis via circulating tumor DNA derived CNV detection. PLoS One. 2018;13(5):e0196826..

114. Armaghany T, Wilson JD, Chu Q, et al. Genetic alterations in colorectal cancer. Gastrointest Cancer Res. 2012;5(1):19–27.

115. Kim ST, Lee WS, Lanman RB, et al. Prospective blinded study of somatic mutation detection in cell-free DNA utilizing a targeted 54-gene next generation sequencing panel in metastatic solid tumor patients. Oncotarget. 2015;6(37):40360–40369.

116. Lin PC, Lin JK, Lin CH, et al. Clinical relevance of plasma DNA methylation in colorectal cancer patients identified by using a genome-wide high-resolution array. Ann Surg Oncol. 2015;22(suppl 3):S1419–S1427.

117. Feuk L, Carson AR, Scherer SW.. Structural variation in the human genome. Nat Rev Genet. 2006;7(2):85–97.

118. Xu JF, Yang L, Ma XY, et al. Chromosome copy number variation analysis to detect early colorectal cancer with liquid biopsy. Clin Gastroenterol Hepatol. 2017;15(1):e46–e47.

119. Juo YY, Johnston FM, Zhang DY, et al. Prognostic value of CpG island methylator phenotype among colorectal cancer patients: a systematic review and meta-analysis. Ann Oncol. 2014;25(12):2314–2327.

120. Freitas M, Ferreira F, Carvalho S, et al. A novel DNA methylation panel accurately detects colorectal cancer independently of molecular pathway. J Transl Med. 2018;16(1):45..

121. Greaves M, Maley CC.. Clonal evolution in cancer. Nature. 2012;481(7381):306–313.

122. Vaughn CP, Zobell SD, Furtado LV, et al. Frequency of *KRAS*, BRAF, and NRAS mutations in colorectal cancer. Genes Chromosom Cancer. 2011;50(5):307–312.

123. de Cuba EM, Snaebjornsson P, Heideman DA, et al. Prognostic value of BRAF and *KRAS* mutation status in stage II and III microsatellite instable colon cancers. Int J Cancer. 2016;138(5):1139–1145.

124. Ocaña A, Díez-González L, García-Olmo DC, et al. Circulating DNA and survival in solid tumors. Cancer Epidemiol Biomarkers Prev. 2016;25(2):399-406.

125. Bachet JB, Bouche O, Taieb J, et al. RAS mutation analysis in circulating tumor DNA from patients with metastatic colorectal cancer: the AGEO RASANC prospective multicenter study. Ann Oncol. 2018;29(5):1211–1219.

126. Howell JA, Khan SA, Knapp S, et al. The clinical role of circulating free tumor DNA in gastrointestinal malignancy. Transl Res. 2017;183:137–154.

127. Mouliere F, Chandrananda D, Piskorz AM, et al. Enhanced detection of circulating tumor DNA by fragment size analysis. Sci Transl Med. 2018;10(466).

128. Janku F, Vibat CR, Kosco K, et al. BRAF V600E mutations in urine and plasma cell-free DNA from patients with Erdheim-Chester disease. Oncotarget. 2014;5(11):3607–3610.

129. Peng M, Chen C, Hulbert A, et al. Non-blood circulating tumor DNA detection in cancer. Oncotarget. 2017;8(40):69162–69173.

130. Imperiale TF, Ransohoff DF, Itzkowitz SH, et al. Multitarget stool DNA testing for colorectal-cancer screening. N Engl J Med. 2014;370(14):1287–1297.

131. Tie J, Cohen JD, Wang Y, et al. Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. Gut. 2019;68(4):663–671.

132. Borstlap WA, Tanis PJ, Koedam TW, et al. A multi-centred randomised trial of radical surgery versus adjuvant chemoradiotherapy after local excision for early rectal cancer. BMC Cancer. 2016;16:513.

133. Cohen JD, Li L, Wang Y, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science. 2018;359(6378):926–930.

134. Hasin Y, Seldin M, Lusis A.. Multi-omics approaches to disease. Genome Biol. 2017;18(1):83..

135. Strickler JH, Loree JM, Ahronian LG, et al. Genomic landscape of cell-free DNA in patients with colorectal cancer. Cancer Discov. 2018;8(2):164–173.

136. Denis JA, Guillerm E, Coulet F, et al. The role of BEAMing and digital PCR for multiplexed analysis in molecular oncology in the era of next-generation sequencing. Mol Diagn Ther. 2017;21(6):587–600.

137. Baker M. . Digital PCR hits its stride. Nat Methods. 2012;9(6):541–544.

138. Asadullah K, Busch A, Gottwald M, et al. Industry-academia collaborations for biomarkers. Nat Rev Drug Discov. 2015;14(12):805–806.

Supplementary material

Supplementary Table 1

Search strategy for PubMed (3 December 2018)
[Mesh] = Medical subject headings
[tiab] = words in title OR abstract

Search	Query	Items found
#5	#4 NOT (animals[mh] NOT humans[mh])	**2,194**
#4	#1 AND #2 AND #3	**2,393**
#3	"Blood"[Mesh] OR "blood" [Subheading] OR plasma*[tiab] OR "Hematologic Tests"[Mesh] OR hematolog*[tiab] OR haematolog*[tiab] OR blood[tiab] OR serum*[tiab] OR seral*[tiab]	**4,431,122**
#2	"DNA Methylation"[Mesh] OR "DNA Copy Number Variations"[Mesh] OR (("DNA"[Mesh] OR dna[tiab] OR dnas[tiab] OR kras[tiab] OR braf[tiab] OR sept9[tiab] OR "sept 9"[tiab] OR cdkn2a[tiab] OR p16[tiab] OR hltf[tiab] OR alx4[tiab] OR alex4[tiab] OR bcat1[tiab] OR ikzf1[tiab] OR vim[tiab] OR pik3ca[tiab] OR tp53[tiab] OR "tp 53"[tiab] OR apc[tiab] OR cfdna*[tiab] OR ccdna*[tiab] OR ctdna*[tiab] OR ftdna*[tiab] OR fcdna*[tiab] OR cpdna*[tiab]) AND ("Methylation"[Mesh] OR "Mutation"[Mesh] OR methylati*[tiab] OR mutation*[tiab] OR (copy number*[tiab] AND (variation*[tiab] OR variant*[tiab] OR change*[tiab])) OR abberati*[tiab] OR alterati*[tiab] OR amplificati*[tiab] OR gain*[tiab] OR loss*[tiab] OR deleti*[tiab]))	**451,201**
#1	"Colorectal Neoplasms"[Mesh] OR "Appendiceal Neoplasms"[Mesh] OR ((carcinoma*[tiab] OR neoplas*[tiab] OR tumour*[tiab] OR adenocar*[tiab] OR adenoid*[tiab] OR tumor[tiab] OR tumora*[tiab] OR tumorb*[tiab] OR tumorc*[tiab] OR tumord*[tiab] OR tumore*[tiab] OR tumorf*[tiab] OR tumorg*[tiab] OR tumorh*[tiab] OR tumori*[tiab] OR tumorj*[tiab] OR tumork*[tiab] OR tumorl*[tiab] OR tumorm*[tiab] OR tumorn*[tiab] OR tumoro*[tiab] OR tumorp*[tiab] OR tumorq*[tiab] OR tumorr*[tiab] OR tumors*[tiab] OR tumort*[tiab] OR tumoru*[tiab] OR tumorv*[tiab] OR tumorw*[tiab] OR tumorx*[tiab] OR tumory*[tiab] OR tumorz*[tiab] OR cancer*[tiab] OR malignan*[tiab]) AND ("Intestine, Large"[Mesh:NoExp] OR "Cecum"[Mesh] OR "Colon"[Mesh] OR "Rectum"[Mesh] OR colorectal*[tiab] OR colon*[tiab] OR rectal*[tiab] OR appendi*[tiab] OR cecum*[tiab] OR coecum*[tiab] OR caecum*[tiab] OR cecal*[tiab] OR coecal*[tiab] OR caecal*[tiab] OR sigmoid*[tiab]))	**323,289**

Search strategy for Embase.com (3 December 2018)

/exp = EMtree keyword with explosion

/de = EMtree keyword without explosion

/mj = EMtree keyword as major subject

:ti,ab = words in title or abstract

Search	Query	Items found
#5	#4 NOT ('animal cell'/de OR 'animal experiment'/de OR 'animal model'/de OR 'animal tissue'/de OR 'cancer cell culture'/de OR 'case report'/de OR 'human cell'/de OR 'in vitro study'/de OR 'nonhuman'/de)	3,221
#4	#1 AND #2 AND #3	5,462
#3	'blood'/exp OR 'blood examination'/exp OR plasma*:ab,ti,kw OR hematolog*:ab,ti,kw OR haematolog*:ab,ti,kw OR blood:ab,ti,kw OR serum*:ab,ti,kw OR seral*:ab,ti,kw	5,379,508
#2	'dna methylation'/exp OR 'dna methylation assay'/exp OR 'copy number variation'/exp OR (('dna'/exp OR dna:ti,ab,kw OR dnas:ti,ab,kw OR kras:ti,ab,kw OR braf:ti,ab,kw OR sept9:ti,ab,kw OR 'sept 9':ti,ab,kw OR cdkn2a:ti,ab,kw OR p16:ti,ab,kw OR hltf:ti,ab,kw OR alx4:ti,ab,kw OR alex4:ti,ab,kw OR bcat1:ti,ab,kw OR ikzf1:ti,ab,kw OR vim:ti,ab,kw OR pik3ca:ti,ab,kw OR tp53:ti,ab,kw OR 'tp 53':ti,ab,kw OR apc:ti,ab,kw OR cfdna*:ti,ab,kw OR ccdna*:ti,ab,kw OR ctdna*:ti,ab,kw OR ftdna*:ti,ab,kw OR fcdna*:ti,ab,kw OR cpdna*:ti,ab,kw) AND ('mutation'/exp OR 'methylation'/exp OR methylati*:ti,ab,kw OR mutation*:ti,ab,kw OR (('copy number*' NEAR/3 (variation* OR variant* OR change*)):ti,ab,kw) OR abberati*:ti,ab,kw OR alterati*:ti,ab,kw OR amplificati*:ti,ab,kw OR gain*:ti,ab,kw OR loss*:ti,ab,kw OR deleti*:ti,ab,kw))	568,751
#1	'colon tumor'/de OR 'colon cancer'/exp OR 'colorectal tumor'/exp OR 'large intestine tumor'/de OR 'appendix tumor'/exp OR 'cecum tumor'/exp OR 'large intestine cancer'/exp OR 'rectum tumor'/exp OR ((carcinoma*:ab,ti OR neoplas*:ab,ti OR tumour*:ab,ti OR adenocar*:ab,ti OR adenoid*:ab,ti OR tumor*:ab,ti OR cancer*:ab,ti OR malignan*:ab,ti) AND ('large intestine'/de OR 'cecum'/exp OR 'colon'/exp OR 'rectum'/exp OR colorectal*:ab,ti OR colon*:ab,ti OR rectal*:ab,ti OR appendi*:ab,ti OR cecum*:ab,ti OR coecum*:ab,ti OR caecum*:ab,ti OR cecal*:ab,ti OR coecal*:ab,ti OR caecal*:ab,ti OR sigmoid*:ab,ti))	486,547

Search strategy for Clarivate Analytics/Web of Science Core Collection (3 December 2018)

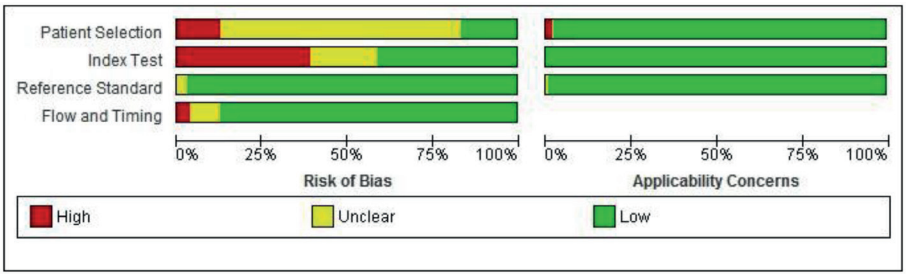
Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years

TOPIC = words in title, abstract or author keywords

NEAR/x = words near to each other, x places apart

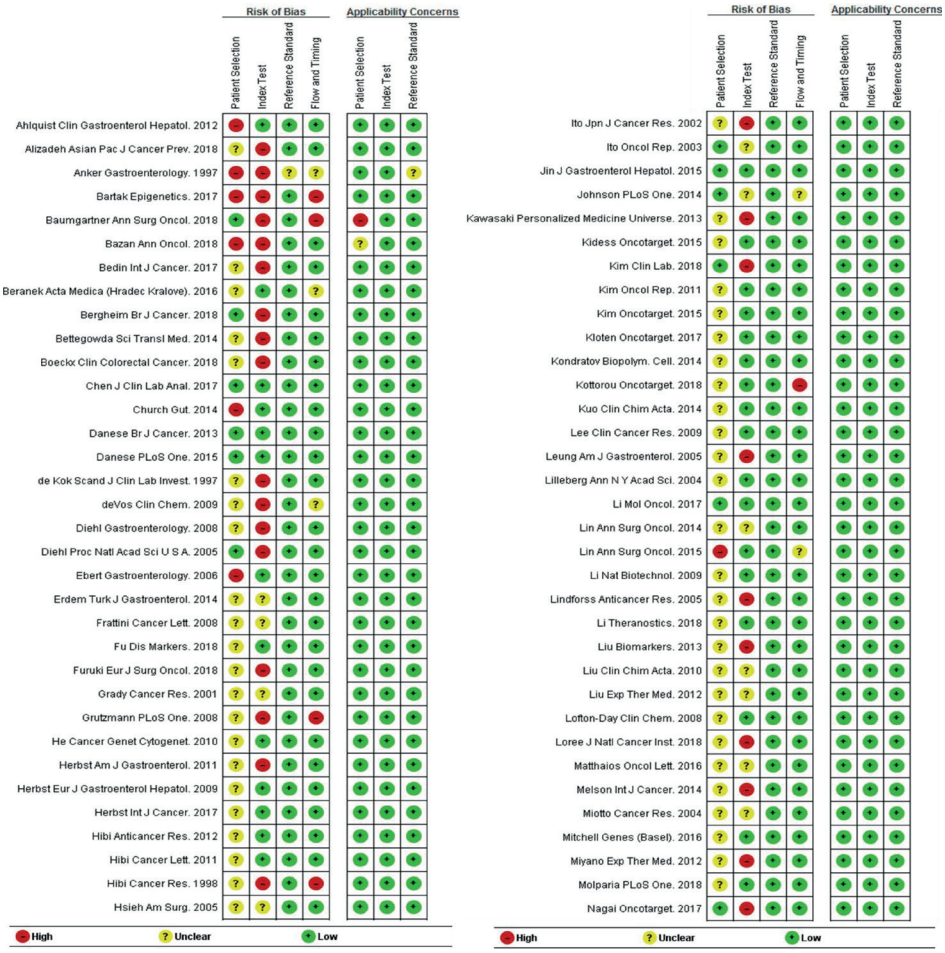
Search	Query	Items found
#5	#4 AND #3 AND #2 AND #1	3,063
#4	TOPIC: (plasma* OR hematolog* OR haematolog* OR blood OR serum* OR seral*)	3,582,437
#3	TOPIC: (methylati* OR mutation* OR ("copy number*" AND (variation* OR variant* OR change*)) OR abberati* OR alterati* OR amplificati* OR gain* OR loss* OR deleti*)	3,332,539
#2	TOPIC: (dna OR dnas OR kras OR braf OR sept9 OR "sept 9" OR cdkn2a OR p16 OR hltf OR alx4 OR alex4 OR bcat1 OR ikzf1 OR vim OR pik3ca OR tp53 OR "tp 53" OR apc OR cfdna* OR ccdna* OR ctdna* OR ftdna* OR fcdna* OR cpdna*)	1,381,156
#1	TOPIC: (((carcinoma* OR neoplas* OR tumour* OR adenocar* OR adenoid* OR tumor* OR cancer* OR malignan*) NEAR/3 (colorectal* OR colon* OR rectal* OR appendi* OR cecum* OR coecum* OR caecum* OR cecal* OR coecal* OR caecal* OR sigmoid*)))	289,433

Supplementary Figure 1a. Risk of bias assessment according to the QUADAS-2 for all 134 initially included studies.

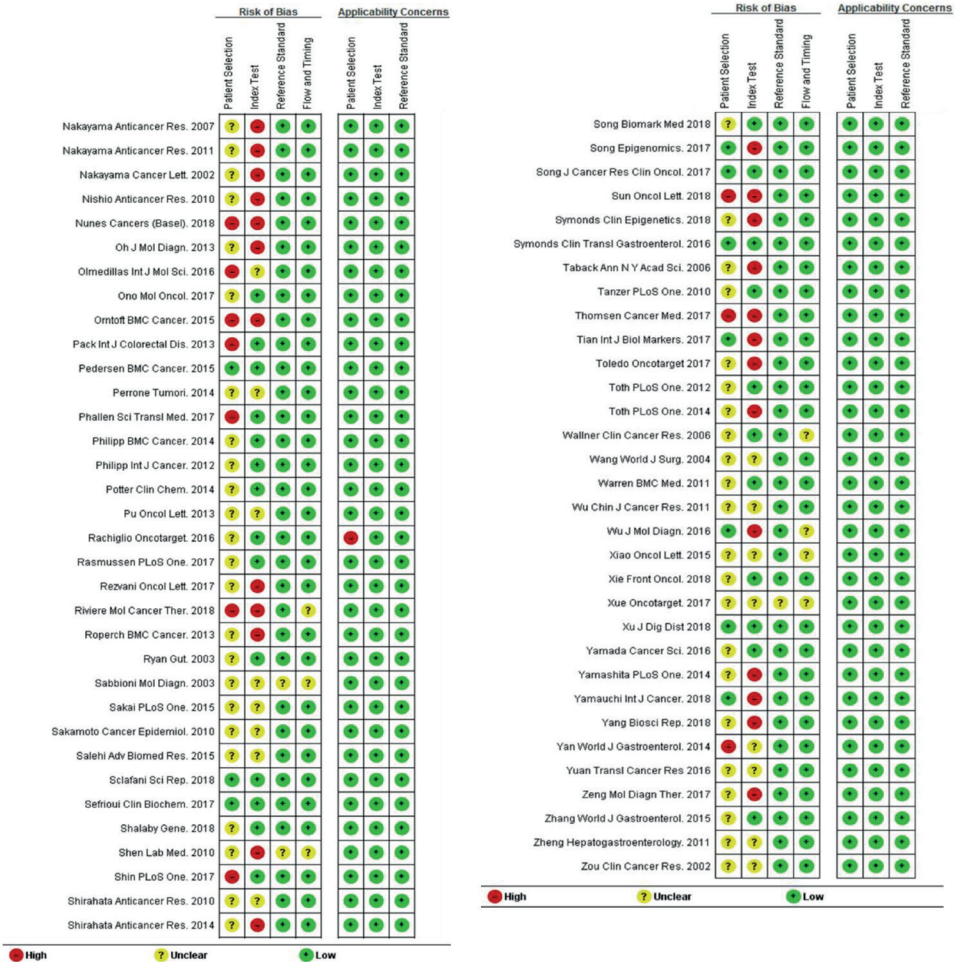


Supplementary Figure 1b

A detailed overview of the risk of bias assessment according to the QUADAS-2 for each study.



Supplementary Figure 1b (continued)



Chapter 3

Circulating Tumor DNA as a Preoperative Marker of Recurrence in Patients with Peritoneal Metastases of Colorectal Cancer: A Clinical Feasibility Study

Jamie J Beagan*
Nina R Sluiter *
Sander Bach
Paul P Eijk
Stijn L Vlek
Daniëlle A M Heideman
Miranda Kusters
D Michiel Pegtel
Geert Kazemier
Nicole C T van Grieken
Bauke Ylstra*
Jurriaan B Tuynman*

*Contributed equally as first and senior authors, respectively

J Clin Med. 2020 Jun 4;9(6):1738

Abstract

Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy (CRS-HIPEC) may be curative for colorectal cancer patients with peritoneal metastases (PMs) but it has a high rate of morbidity. Accurate preoperative patient selection is therefore imperative, but is constrained by the limitations of current imaging techniques. In this pilot study, we explored the feasibility of circulating tumor (ct) DNA analysis to select patients for CRS-HIPEC. Thirty patients eligible for CRS-HIPEC provided blood samples preoperatively and during follow-up if the procedure was completed. Targeted Next-Generation Sequencing (NGS) of DNA from PMs was used to identify bespoke mutations that were subsequently tested in corresponding plasma cell-free (cf) DNA samples using droplet digital (dd) PCR. CtDNA was detected preoperatively in cfDNA samples from 33% of patients and was associated with a reduced disease-free survival (DFS) after CRS-HIPEC (median 6.0 months vs median not reached, $p = 0.016$). This association could indicate the presence of undiagnosed systemic metastases or an increased metastatic potential of the tumors. We demonstrate the feasibility of ctDNA to serve as a preoperative marker of recurrence in patients with PMs of colorectal cancer using a highly sensitive technique. A more appropriate treatment for patients with preoperative ctDNA detection may be systemic chemotherapy in addition to, or instead of, CRS-HIPEC.

Keywords: CRS-HIPEC; circulating tumor DNA; colorectal cancer; droplet digital PCR; liquid biopsy; peritoneal metastases.

1. Introduction

Metastatic colorectal cancer (CRC) remains the second most common cause of cancer-related death, despite improvements in treatment over recent decades [1]. Peritoneal metastases (PMs) are diagnosed in 10–25% of CRC patients [2,3,4], either at the time of primary tumor diagnosis or during subsequent investigations. If restricted to the peritoneum, referred to as isolated PMs, treatment with systemic chemotherapy confers a median overall survival (OS) of 12–18 months [5,6,7,8]. Patients with isolated PMs that are limited in their spread throughout the peritoneum may qualify for Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy (CRS-HIPEC) treatment, which is associated with a disease-free survival (DFS) of 13.1 months and an increased median OS of 35–45 months [9,10,11,12]. Patients with the least extensive peritoneal spread have the potential to experience the greatest benefit from CRS-HIPEC, reflected in a median OS of 56 months [13]. The addition of HIPEC following CRS did not show significant survival benefit in the recent large randomized-controlled PRODIGE-7 trial [14]. However, unlike the patients included in our study, patients in the PRODIGE-7 trial had received neo-adjuvant systemic therapy prior to CRS-HIPEC and had received oxaliplatin rather than mitomycin C during HIPEC [14]. Nevertheless, CRS performed in high-volume expert centers resulted in an OS of 41 months [14]. This demonstrates that a dedicated centralized multimodality approach to treatment offers improved survival for patients with PMs.

Unfortunately, shortcomings in modalities of detection and quantification of PMs often result in diagnosis at an advanced stage, when the peritoneal spread is extensive. These patients typically see reduced benefit from CRS-HIPEC because of a higher incidence of recurrence after the procedure [15,16] and because they experience greater treatment-related morbidity from extensive CRS [17]. Timely preoperative detection of PMs could help decide if any potential survival benefit outweighs the treatment-related morbidity. Similarly, recurrence of PMs after CRS-HIPEC is a common phenomenon [13] that also tends to be diagnosed at an advanced stage. Improved detection in this situation could indicate an early start to chemotherapy to reduce disease symptoms, or a repeat CRS-HIPEC with potentially curative intent if the recurrence is isolated and limited [18].

If PMs are not detected during resection of the primary tumor, initial detection is by subsequent (PET)-CT imaging, which is limited to a sensitivity of 72% and further decreases to 11% for nodules smaller than 5 mm [19]. Carcinoembryonic antigen (CEA) levels can be elevated in the blood of CRC patients, particularly in those with metastases. However, the sensitivity of the CEA test is limited in patients with isolated PMs [20,21]. If PMs are suspected, diagnostic laparoscopy (DLS) is often performed preoperatively to determine the Peritoneal Cancer Index (PCI),

a numeric score ranging from 1–39 that combines lesion size with the number of affected abdomino-pelvic regions [10,22]. Although DLS offers a higher sensitivity and specificity for detection, it is invasive and often still underestimates the extent of PMs [23]. The most reliable measure of PMs is an intraoperative assessment performed immediately before the intended CRS-HIPEC. However, up to 25% of patients are disqualified at this point due to irresectable PMs, reflected by a PCI higher than 20, so undergo an open–close procedure whereby the abdomen is closed without CRS-HIPEC [24]. There is an urgent clinical need for less invasive and more accurate tools to detect and quantify the extent of PMs. Novel approaches include improved imaging techniques such as diffusion-weighted (DW) MRI [25] and liquid biopsy analysis.

Recent technical advances have enabled circulating tumor (ct) DNA, the fraction of cell-free (cf) DNA that originates from tumor cells, to be detected in plasma with high sensitivity and specificity. Analysis can serve as a dynamic marker in CRC patients by the quantification of cfDNA levels and the identification of tumor-specific genetic and epigenetic markers including; mutations, structural variations and methylation [26,27,28]. Studies that used digital PCR methods have shown ctDNA to be detectable in up to 100% of CRC patients with systemic metastases and in 73% of those without evidence of systemic metastasis [29]. However, in a study that used a next-generation sequencing (NGS) panel to test patients with resectable PMs of diverse cancer types, ctDNA was only detectable preoperatively in 39% of cases [30]. The lower plasma ctDNA representation in CRC patients with isolated PMs compared to systemic metastases is probably due to inherent biological differences in ctDNA shedding.

The detection and quantification of relatively low quantities of ctDNA in plasma from CRC patients with PMs requires a highly sensitive technique. Droplet digital (dd) PCR has emerged as one of the most sensitive and specific methods of ctDNA analysis in the oncology setting [31]. By specifically targeting genomic loci in a tissue-guided manner, ddPCR can precisely quantify DNA fragments that contain either the mutant or wild-type nucleotide in a relatively simple, fast and cost-effective work-flow. Although this approach has previously been applied to colorectal malignancies [32,33], to our knowledge, it has not been used as a biomarker in a well-defined cohort of CRC patients who are candidates for CRS-HIPEC.

In this pilot study, we aimed to explore the feasibility of ctDNA analysis in a clinical situation to select patients for CRS-HIPEC. We assessed the capability of ctDNA to quantify the extent of PMs and its suitability as a preoperative prognostic marker of recurrence.

2. Experimental Section

2.1. Study Design and Patients

Patients diagnosed with synchronous or metachronous PMs of colorectal adenocarcinoma and considered eligible for CRS-HIPEC following standard work-up including imaging and DLS, were initially included in this study at the Cancer Center Amsterdam of Amsterdam University Medical Center (location VUmc) between August 2016 and March 2018 [11,34]. Patients were preoperatively excluded from the study if: the estimated extent of PMs was deemed to be irresectable by subsequent (PET-) CT or DLS; systemic metastases were detected (excluding resectable liver metastases with minimal tumor burden) [35]; or PMs removed during a previous procedure were found by histological assessment to be non-colorectal in origin or were not adenocarcinoma. Patients were excluded from the study on a technical basis if no mutations were identified in PMs. Clinical and pathological data were retrospectively obtained from patient records (Supplementary materials and methods, clinical and pathological data) [10,22]. The mismatch repair status of PMs was not tested in any of the patients included in the study. The outcome of the CRS was determined according to the maximal size of residual tumor tissue and was classified as: R1) when no macroscopically visible tumor remained in situ (complete resection), R2a) when the residual tumor was smaller than 2.5 mm or R2b) when it was larger than 2.5 mm [36]. CRS-HIPEC was performed according to a standard protocol [11,37]. If a complete CRS was achieved, HIPEC was performed using the open coliseum technique with mitomycin C. If residual tumor tissue remained after CRS, HIPEC was not performed [6,34].

2.2. Ethics Approval and Consent to Participate

This study was registered with the Dutch Trial Registry [38] and was conducted in accordance with the Declaration of Helsinki with the approval of the Amsterdam UMC, VU University Medical Ethical Testing Committee (2016.254-NL57226.029.16 and 2017-302(A2018). All patients provided written informed consent to participate in the study.

2.3. Blood and Tumor Tissue Collection

PMs previously removed alongside primary tumor resection were retrieved from the Biobank at Amsterdam University Medical Centers (UMC) -location VUmc, and retrospectively analyzed to identify mutations to test in cfDNA. Preoperative blood samples were collected after patients were placed under general anesthesia but immediately before surgical incision for the intended CRS-HIPEC procedure. Blood was kept at room temperature until plasma separation within 8 h of collection (Supplementary materials and methods, blood processing). At least one postoperative blood sample was taken by venipuncture from all CRS-HIPEC patients,

typically within 2–4 weeks, but no later than 3 months after the procedure. Further samples were taken during routine follow-up every 3 months, up to 25 months after CRS-HIPEC. If a recurrence was diagnosed during follow-up by physical assessments and (PET-) CT imaging, an additional blood sample was taken at diagnosis or within 1 month. No postoperative blood samples were obtained from patients who did not undergo the complete CRS-HIPEC procedure because the presence of residual tumor excluded them from postoperative ctDNA analysis.

2.4. DNA Isolation and Mutation Analysis

Formalin-fixed paraffin-embedded (FFPE) tumor tissue of PMs was processed as previously described [39,40]. Genomic DNA was subsequently isolated using a Qiagen QIAamp DNA FFPE Tissue kit according to the instructions of the manufacturer (Qiagen, Venlo, the Netherlands). Cell-free DNA was isolated from up to 3 mL aliquots of plasma using the Qiagen QIAsymphony Circulating DNA Kit. A plasma sample of known cfDNA concentration was included in each isolation run to ensure consistent performance of the isolation kit. Genomic DNA from PMs underwent NGS-based mutation analysis using the TruSeq Amplicon Cancer Panel (TSACP; Illumina Inc., San Diego, CA, USA) or a High-Resolution Melting assay followed by Sanger sequencing (HRM-sequencing) (Supplementary materials and methods, DNA isolation and mutation analysis) [40,41]. Only genomic variants of known oncogenic significance identified by HRM-sequencing, or by TSACP sequencing with a Variant Allele Frequency (VAF) of ≥3% in the PMs were included (Table S1A). These mutations were targeted in cfDNA samples using specific mutant and wild-type ddPCR primer and probe combination kits (BioRad, California, USA) (Table S1B). Results for each assay were used to calculate the VAF and estimate the concentration of cfDNA (Table S1C). All cfDNA samples were tested using the *KRAS* G12/13 screening kit (cat. #1863506, BioRad, Hercules, CA, USA) regardless of the *KRAS* mutation status of the PMs. The performance of all kits was verified using gBlocks when available (Integrated DNA Technologies, Iowa, USA); 191–230nt fragments of synthetic double-stranded DNA containing the nucleotide change of interest (Supplementary materials and methods, gBlocks and ddPCR; Table S1D).

2.5. Statistical Analysis

The association between clinico-pathological variables and ctDNA detection was tested using the Fisher's exact test for two dichotomous variables or the Mann–Whitney U test for a continuous variable combined with a dichotomous variable. Comparison of preoperative cfDNA input to ddPCR reactions was performed using the Mann–Whitney U test. Comparison of preoperative cfDNA input with follow-up samples was tested using the Kruskal–Wallis test for a continuous variable in multiple groups. Statistical significance was defined as a p-value < 0.05 (two-sided test). Univariate associations between DFS and clinico-pathological variables

and ctDNA detection were tested using the log-rank test (Kaplan–Meier method). A cox regression analysis was performed to generate hazard ratios and 95% confidence intervals (95% CI). No correlation between OS and clinico-pathological variables was calculated as the number of events within the follow-up period was insufficient for statistical analysis. Dichotomization was performed on the basis of mean values for continuous variables. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 23 for Windows (IBM Corporation, Armonk, NY, USA) and GraphPad Prism v7.02 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Patient Baseline Characteristics

Thirty patients eligible to receive CRS-HIPEC were included in the study, following the exclusion of fourteen patients for the reasons outlined in Figure 1. Baseline characteristics of all patients are presented in Table 1. Of these patients, 24 underwent CRS-HIPEC after intraoperative assessment determined them to have resectable metastases. None of the patients had received neo-adjuvant chemotherapy prior to CRS-HIPEC. To account for the effect of liver metastases on ctDNA levels, CRS-HIPEC patients were postoperatively sub-classified into those with isolated PMs (n = 22) or PMs with resectable liver metastases (n = 2) (Figure 1). The remaining six patients were intraoperatively disqualified from CRS-HIPEC so instead underwent an open–close procedure due to a PCI higher than 20 (n = 4), or because irresectable liver metastases (n = 1) or para-aortic lymph nodes (n = 1) were discovered.

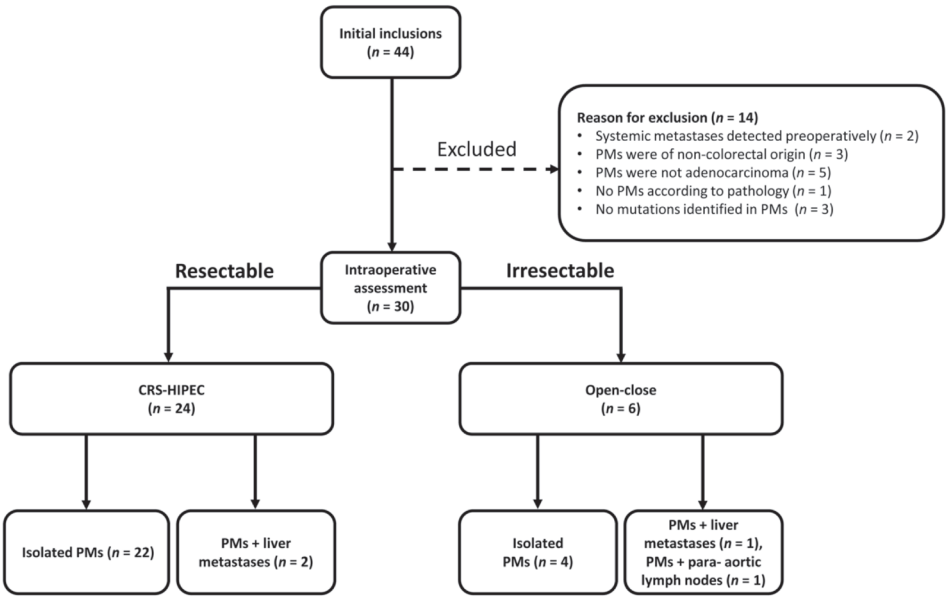


Figure 1. Flowchart of patient classification after initial inclusion in the study. The reasons for patient exclusion prior to intraoperative assessment are described. After the intraoperative assessment, patients either underwent Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy (CRS-HIPEC) or received an open–close procedure. PMs: Peritoneal Metastases.

Table 1. Baseline characteristics of all patients in the study and the selection of patients that underwent Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy (CRS-HIPEC).

		All Patients (*n* = 30)		Underwent CRS-HIPEC (*n* = 24)	
Characteristics					
General					
Age in years	Mean/SD	65.1	9.4	65.2	9.6
	Median/range	67	37–81	66.5	36–81
Male gender	(*n*/%)	17	56.7	14	58.3
BMI	Mean/SD-all	27.1	5.2	27	5.5
	male	25.6	3.1	25.4	2.7
	female	29.1	6.7	29.2	7.6
	Median/range-all	26.8	21.3–49	26.8	21.3–49
	male	24.4	21.3–32.1	24.3	21.3–29.8
	female	28.4	22.0–49.0	27.9	22.0–49.0
ASA classification	I-II (*n*/%)	22	73.3	17	70.8
	III (*n*/%)	8	26.7	7	29.2
Primary Tumor		*n*	%	*n*	%
Location	Colon	27	90	22	91.7
	Rectum	3	10	2	8.3
TNM-stage at diagnosis	II	8	26.7	7	29.2
	III	10	33.3	8	33.3
	IV	12	40	9	37.5
Differentiation grade	Good/moderate	24	92.3	19	90.5
	Poor	1	3.8	1	4.8
	Signet cell	1	3.8	1	4.8
Lymph invasion		7	25.9	5	22.7
Venous invasion		10	37	8	36.4
Tumor type	Adenocarcinoma	21	70	17	70.8
	Mucinous adenocarcinoma	8	26.7	6	25
	Signet cell type	1	3.3	1	4.2
Treatment					
Adjuvant chemotherapy primary	(*n*/%)	11	36.7	9	37.5
Primary tumor in situ at intended CRS-HIPEC	(*n*/%)	8	26.7	7	29.2
Liver metastases at intended CRS-HIPEC	(*n*/%)	3	10	2	8.3
Lymph node metastases at intended CRS-HIPEC	(*n*/%)	7	24.1	6	25

SD: Standard Deviation. BMI: Body Mass Index. ASA: American Society of Anesthesiologists Physical Status Classification System. TNM: TNM classification of malignant tumors. PCI: Peritoneal Cancer Index.

3.2. Accuracy of ctDNA Analysis for Detection of PMs

Between one and four mutations (median 1.5) were identified in the tumor tissue of PMs from each of the 30 patients in the study. The three most frequently mutated genes were: *KRAS* (18/30 patients; 60%); *TP53* (13/30 patients; 43%) and *APC* (8/30 patients; 27%). Mutations in these genes are typically found in 43%, 60% and 81% of non-hypermutated CRC tumors, respectively [42]. A detailed list of mutations is described in Table S1A. By targeting these tissue-guided mutations by ddPCR analysis, ctDNA was detected preoperatively in 10/30 (33%) patients (Figure 2A). In the subgroup of patients who underwent CRS-HIPEC, the rate of detection was the same (8/24; 33%) and was marginally lower when the two patients with liver metastases were excluded (6/22; 27%). To estimate the relative amounts of ctDNA shed into the plasma, a median VAF of 1.8% (range 0.6–10) was determined for the eight patients who had detectable ctDNA. Interestingly, the VAF was marginally higher in patients who were later diagnosed with a systemic recurrence (median 2.8%, range 2.1–10, n = 4) compared to a loco-regional recurrence (median 0.6%, range 0.6–1.4, n = 3, p = 0.057). In comparison, patients in the open–close subgroup had the same detection rate as the overall study group (2/6; 33%), with a median VAF of 1.75% (range 0.9–2.6, n = 2). This suggests that there is no correlation between the preoperative PCI and the likelihood of ctDNA detection in the circulation.

Detection of ctDNA did not correlate significantly with any of the tested clinical variables used to assess eligibility for CRS-HIPEC, including PCI (Table S2). When the total cfDNA concentration was calculated for all 30 patients, there was no significant difference between samples that contained detectable ctDNA and those that did not (p = 0.422), or between samples from patients who underwent CRS-HIPEC (median 8.7ng ml−1, range 4.3–70.4) or an open–close procedure (median 7.2 ng ml−1, range 4.4–9.9; p = 0.174) (Figure 2C). If a cfDNA sample contained multiple mutations, their proportions closely mirrored those observed in PMs (Figure 2B), which suggests a faithful representation of tumor DNA in the circulation. All preoperative plasma samples had 100% concordance with the *KRAS* mutation status in PMs when tested with the *KRAS* G12/13 screening kit, which further indicates that the PMs were the source of the ctDNA.

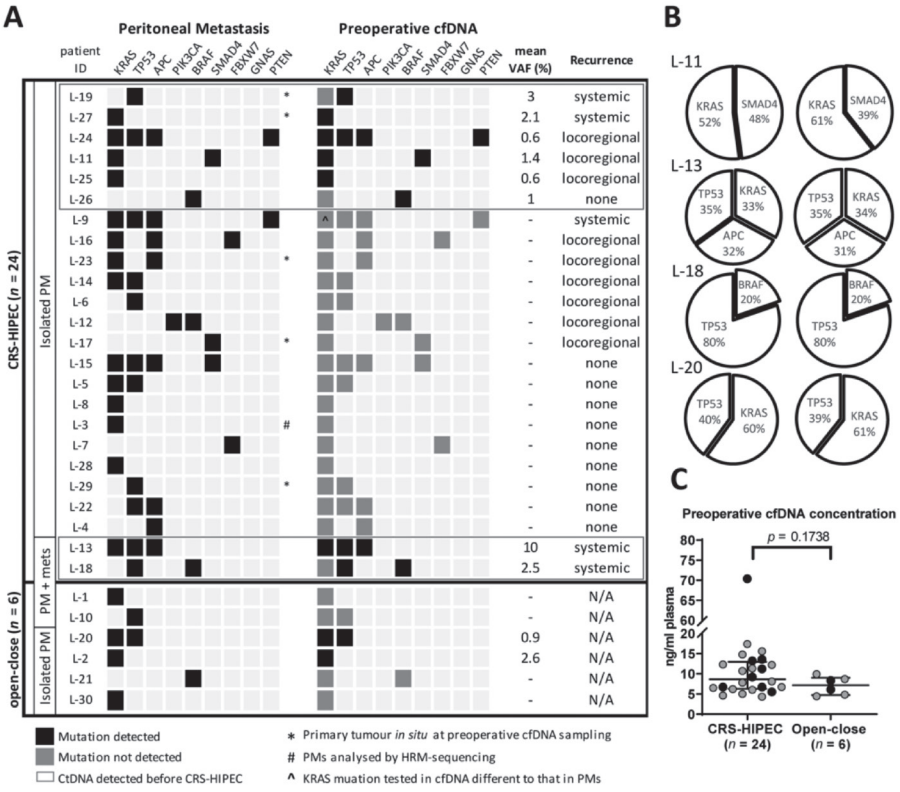


Figure 2. (A) An overview of the genes in which a somatic mutation was identified in peritoneal metastases (PMs) and tested for in the corresponding preoperative cfDNA sample. A black square indicates that the mutation was identified in both PMs and cfDNA; a grey square indicates a negative result for the corresponding mutations in the cfDNA. All cfDNA samples were tested using the *KRAS* G12/13 screening kit. (B) Comparison of the relative proportion of mutations detected in PMs and preoperative cfDNA samples. (C) Concentration of cfDNA samples. A black dot denotes that ctDNA was detected; lines indicate the median and the interquartile range. HRM-sequencing: High-Resolution Melting assay followed by Sanger sequencing.

3.3. Preoperative ctDNA as a Prognostic Marker of Recurrence

The association between preoperative ctDNA detection and recurrence was tested in 14/24 patients who were diagnosed with a recurrence during the follow-up period (median DFS 17 months, range 6–25). The proportion of these patients who had detectable ctDNA preoperatively was higher for those with a systemic- (4/5, 80%) compared to a loco-regional recurrence (3/9, 33%). Regardless of the type of recurrence, preoperative ctDNA detection was associated with a median DFS of 6.0 months (95%-CI 1.8–10.2), significantly worse compared to patients without ctDNA

detection (median DFS not reached, p = 0.016; HR 3.454, 95% CI 1.145–10.423) (Figure 3A). When the two patients with resectable liver metastases were excluded from the survival analysis, both of whom had detectable ctDNA, a trend was still observed, but the difference was no longer significant (median DFS 7.0 months vs median DFS not reached, p = 0.086; HR 2.673, 95% CI 0.806–8.857) (Figure 3B). A univariate analysis was performed to test the association between other clinically relevant clinico-pathological variables and DFS after CRS-HIPEC. A PCI higher than 10 (5.0 months versus median DFS not reached, p = 0.035) and the presence of liver metastases (2.0 months versus 12.0 months, $p < 0.001$) were found to have a significant association (Table S3).

3.4. ctDNA to Support Recurrence Diagnosis During Follow-up

None of the samples taken initially after CRS-HIPEC had detectable levels of ctDNA, except for patient L-27. In this case, recurrences to the lungs and the spleen were diagnosed 7 months after CRS-HIPEC, which suggests the potential presence of systemic micro-metastases at the time of CRS-HIPEC. Due to the exploratory approach of this study, only 19 of the 24 patients provided additional samples during the follow-up period. Of the five patients who had a systemic recurrence, ctDNA was detected in four out of four patients who provided a follow-up sample (Figure 4). Of the nine patients who had a loco-regional recurrence, ctDNA was detected in one out of eight patients who provided a follow-up sample. Detection of ctDNA in these samples either occurred at or after diagnosis of a recurrence. Circulating tumor DNA was not detectable in any of the seven patients who provided a sample and did not have a recurrence during the follow-up period, which suggests a negative predictive value. Notably, levels of background cfDNA were significantly higher during the 2 months following CRS-HIPEC ($p \leq 0.001$) and returned to preoperative levels by 7 months, commensurate with tissue damage associated with the procedure (Figure S1).

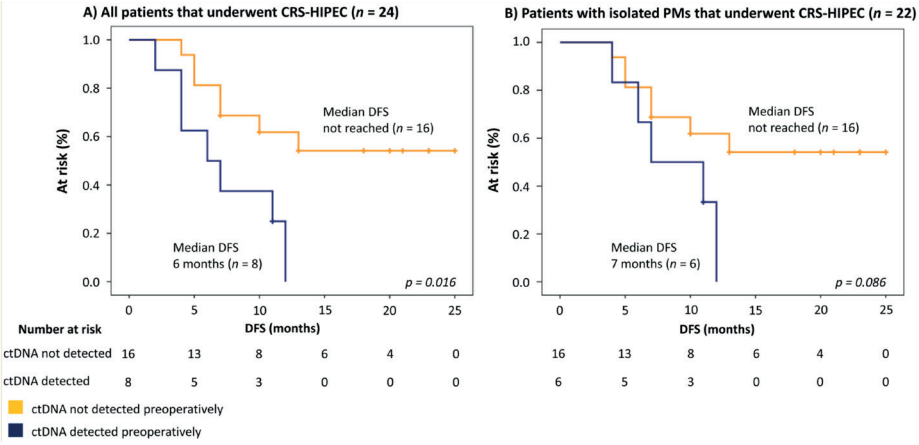


Figure 3. Survival analysis of patients who received CRS-HIPEC. (A) All patients (n = 24). (B) All patients except those diagnosed with preoperative liver metastases (n = 22). DFS: Disease-free Survival.

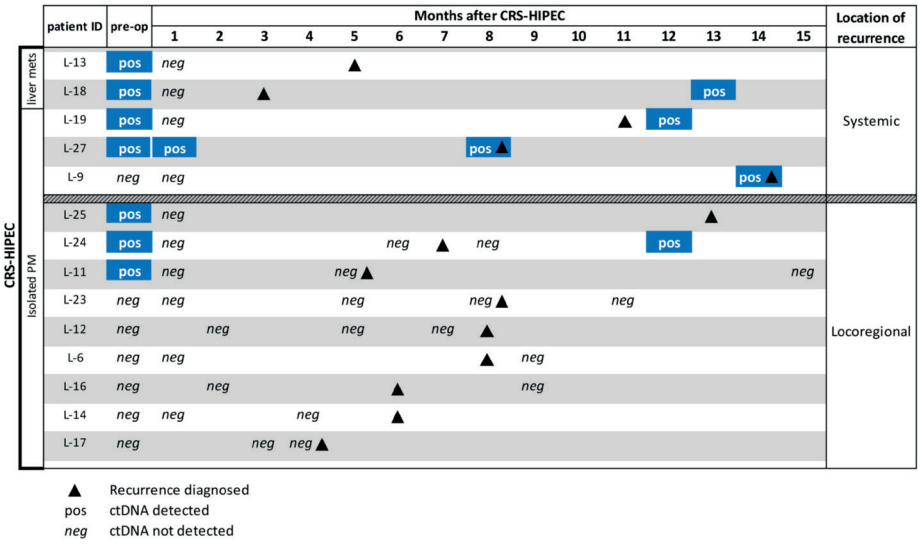


Figure 4. Tissue-guided ctDNA analysis of plasma samples taken from 14/24 patients during follow-up after CRS-HIPEC. Ten patients did not provide a sample. Patients are categorized by the location of metastases before or during CRS-HIPEC and the location of recurrence(s) during follow-up. The blue color is to highlight a positive result.

4. Discussion

This pilot study demonstrates the promising feasibility of ctDNA as a prognostic marker of recurrence in CRC patients with PMs who are eligible for CRS-HIPEC. Preoperative detection of ctDNA could influence the decision to undergo CRS-HIPEC but larger studies are required to validate the clinical utility of this approach. In this study, ctDNA was detected preoperatively in 33% of patients and there was a trend between detection and reduced DFS. However, it was not possible to quantify the extent of PMs based on ctDNA detection and cfDNA quantification. This is the first study to apply preoperative tissue-guided ctDNA analysis exclusively to patients selected for CRS-HIPEC to treat PMs of CRC.

A comparable study by Baumgartner et al. (2018) used an NGS-based approach to investigate preoperative ctDNA in patients who underwent surgery to treat PMs of various cancer types. This study reported an overall ctDNA detection rate of 39% across all the included cancer types, with a solid-tissue concordance of 35.3% when comparison was possible [30]. In our investigation, the ctDNA detection rate in patients with isolated PMs was similar at 33%. Despite patients having a form of metastasis, these ctDNA detection rates are more in line with those seen in stage I CRC (40%) [43]. There is little evidence that ctDNA detection in early-stage CRC has prognostic value [28], but interestingly in our study it was found to be predictive of a reduced DFS, despite a comparatively low detection rate. PMs are understood to spread to the peritoneum through a local form of dissemination [2,44], rather than through the circulation [45]. The metastatic site is an important determinant of ctDNA detection, as CRC metastases to the lung, for example, have a lower VAF than to liver or lymph nodes [46]. If CRC patients have isolated PMs, the VAF tends to be lower compared to patients with no PMs or PMs with involvement from other metastatic sites [45]. We hypothesize that ctDNA from isolated PMs is usually poorly shed into the plasma compared to primary or systemically-spread CRC, which could explain the comparatively low preoperative ctDNA detection rate and lack of a significant relationship to the PCI score. Preoperative detection of ctDNA could occur because these patient's PMs shed greater than usual quantities of ctDNA and are more prone to systemic spread. This might also explain why seven out of eight patients who had detectable ctDNA preoperatively were later diagnosed with a recurrence, despite the intended removal of all PMs by CRS-HIPEC. Alternatively, there may have been an additional ctDNA contribution from un-diagnosed systemic metastases that already existed below the detection threshold of imaging techniques at preoperative assessment. CtDNA from these metastases may have been masked in the weeks following CRS-HIPEC, due to increased background cfDNA from tissue damage and inflammation [47], then become detectable again at diagnosis of recurrence. Another reason for postoperative detection could have been the presence of minimal residual

disease after CRS-HIPEC. Recent studies have shown patients with localized CRC to be at a higher risk of recurrence if ctDNA was detected after resection [48]. Similarly, locally advanced rectal cancer patients could be stratified into groups at high or low risk of recurrence based on postoperative ctDNA detection [49].

If preoperative ctDNA detection is validated as a marker of a more invasive tumor type or of undiagnosed metastases, the use of a local treatment such as CRS-HIPEC as a stand-alone therapy may have limited curative potential in this situation. A more appropriate treatment for these patients would be (neoadjuvant) systemic therapy in addition to CRS-HIPEC, an approach used in the PRODIGE-7 trial [14] and is currently under investigation in the CAIRO-6 study [50]. It may even be appropriate to withhold CRS-HIPEC, as the median DFS of 6 months in patients with detectable preoperative ctDNA is similar to the typical physical and quality of life recovery time [51].

Our results with a limited number of available follow-up blood samples hints at the potential of ctDNA analysis to support recurrence monitoring after CRS-HIPEC. Detection of ctDNA in samples from all four patients with a systemic- and one out of eight with a loco-regional recurrence, either at or after the diagnosis of recurrence by standard techniques, suggests a confirmatory role for ctDNA. If validated by a larger study, this finding could lead to an earlier start of palliative treatments to reduce disease-related symptoms, or indicate a repeat HIPEC with curative intent in patients with oligo- and loco-regional recurrence [18].

Despite the limited number of tissue-guided mutations tested in cfDNA by ddPCR, the detection rate was comparable to Baumgartner et al. (2018) who screened for mutations with an NGS panel. We identified a median of 1.5 mutations per patient (range 1–4, n = 30) in PMs, with all tested mutations found back in samples that contained ctDNA, except for in one follow-up sample from patient L-18. Baumgartner et al. (2018) detected a median of 2 mutations (range 1–6, n = 7) in ctDNA of CRC patients with PMs [30]. A comparable study of ctDNA in patients with metastatic or recurrent CRC had a similar detection rate (median 2 mutations, range 0–25, n = 74) [52]. An explanation for our slightly lower detection rate could be that the TSACP panel was limited to 48 genes, so may have missed less common mutations. This, plus the low sample numbers, could also explain the difference between the mutations detection in our study and those detected in frequently mutated genes described in the literature. A limitation of our tissue-guided approach is that patients who had not undergone a primary tissue resection before CRS-HIPEC—in this case 5/30 patients—would need a biopsy of PMs to enable ctDNA analysis. However, available biopsied tissue does not guarantee that mutations will be identified, as three patients were excluded from the study due to a lack of targetable mutations in their PMs. If biopsied tissue is not available

or mutations are not detected, an NGS panel of commonly mutated genes should be considered to screen for ctDNA, provided a sensitivity comparable to ddPCR can be reached.

An alternative approach to non-invasive PM assessment is through improved imaging modalities. Two recent studies showed the feasibility of DW-MRI to improve the detection and quantification of colorectal PMs compared to conventional CT imaging [25,53]. However, DW-MRI could underestimate the extent of metastases, especially of signet ring cell or mucinous adenocarcinomas [25,53]. In our study, 9/30 (30%) patients had these sub-types so may not have benefitted from DW-MRI alone. Interestingly, a study by Vidal et al. 2017 showed patients with mucinous tumors to have a lower than expected ctDNA VAF [45]. This poses an additional challenge for the detection and quantification of tumors with mucinous histology. Molecular characterization of the tumor through a solid or liquid biopsy, as demonstrated in our study, would still be required to enable the use of targeted therapies, for example. Confirmatory studies are necessary before DW-MRI can be implemented as a standard technique for analysis of PMs.

The clinical variability of patients in our pilot study supports the feasibility of our approach. Inclusion of patients with a primary tumor in situ or liver metastases reflects the clinical reality of CRS-HIPEC candidates, even though these factors may have increased the chance of ctDNA detection. To the best of our knowledge, this is the first study to analyze the mutation status of PMs and both pre- and postoperative plasma samples in a cohort of CRC patients eligible to receive CRS-HIPEC. Investigations are needed to understand the biological factors affecting ctDNA representation in plasma, particularly in cases of localized dissemination such as PMs. Although a pilot study, the findings presented here are potentially practice changing and should be validated by larger clinical studies.

5. Conclusions

This pilot study demonstrates the feasibility of ctDNA as a prognostic marker in the clinical management of CRC patients with PMs. If ctDNA is detected preoperatively, patients may experience greater benefit from chemotherapy in addition to, or instead of, CRS-HIPEC. Additionally, the approach outlined here could support the detection of recurrences along-side conventional diagnostic methods during follow-up. To allow clinical implementation, these results require confirmation by larger trials and ultimately, by prospective studies in which treatment decisions are based on ctDNA analysis.

Author Contributions

Conceptualization, N.C.T.v.G., B.Y. and J.B.T.; Data Curation, J.J.B. and N.R.S.; Formal analysis, J.J.B. and N.R.S.; Funding acquisition, G.K., N.C.T.v.G., B.Y. and J.B.T.; Investigation, J.J.B., N.R.S., S.B., P.P.E. and D.A.M.H.; Methodology, D.A.M.H., M.K., D.M.P., N.C.T.v.G., B.Y. and J.B.T.; Project administration, J.J.B., N.R.S., B.Y. and J.B.T.; Resources, B.Y.; Supervision, B.Y. and J.B.T.; Validation, J.J.B. and N.R.S.; Visualization, J.J.B., N.R.S. and S.B.; Writing—Original Draft, J.J.B. and N.R.S.; Writing—review & editing, S.B., P.E., S.L.V., D.A.M.H., M.K., D.M.P., G.K., N.C.T.v.G., B.Y. and J.B.T. All authors agreed to the manuscript in its published version and contributed to the Review & Editing. All authors have read and agreed to the published version of the manuscript.

Funding

This project was supported by the Vrije Universiteit Medical Center, Cancer Center Amsterdam Foundation. The funding body had no role in the study design, execution of the research activities, or the decision to submit the manuscript for publication.

Conflicts of Interest

DAMH has been on the speakers' bureau of QIAGEN, serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb, and is minority shareholder of Self-screen B.V., a spin-off company of Amsterdam UMC, Vrije Universiteit Amsterdam. No other authors declare a conflict of interest

References

1. Bray F., Ferlay J., Soerjomataram I., Siegel R.L., Torre L.A., Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 2018;68:394–424.

2. Koppe M.J., Boerman O.C., Oyen W.J., Bleichrodt R.P. Peritoneal carcinomatosis of colorectal origin: Incidence and current treatment strategies. Ann. Surg. 2006;243:212–222.

3. Jayne D.G., Fook S., Loi C., Seow-Choen F. Peritoneal carcinomatosis from colorectal cancer. Br. J. Surg. 2002;89:1545–1550.

4. Hugen N., van de Velde C.J., de Wilt J.H., Nagtegaal I.D. Metastatic pattern in colorectal cancer is strongly influenced by histological subtype. Ann. Oncol. 2014;25:651–657.

5. Franko J., Shi Q., Meyers J.P., Maughan T.S., Adams R.A., Seymour M.T., Saltz L., Punt C.J., Koopman M., Tournigand C., et al. Prognosis of patients with peritoneal metastatic colorectal cancer given systemic therapy: An analysis of individual patient data from prospective randomised trials from the Analysis and Research in Cancers of the Digestive System (ARCAD) database. Lancet Oncol. 2016;17:1709–1719.

6. Verwaal V.J., Bruin S., Boot H., van Slooten G., van Tinteren H. 8-year follow-up of randomized trial: Cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy in patients with peritoneal carcinomatosis of colorectal cancer. Ann. Surg. Oncol. 2008;15:2426–2432.

7. Franko J., Ibrahim Z., Gusani N.J., Holtzman M.P., Bartlett D.L., Zeh H.J., 3rd Cytoreductive surgery and hyperthermic intraperitoneal chemoperfusion versus systemic chemotherapy alone for colorectal peritoneal carcinomatosis. Cancer. 2010;116:3756–3762.

8. Cashin P.H., Mahteme H., Spang N., Syk I., Frodin J.E., Torkzad M., Glimelius B., Graf W. Cytoreductive surgery and intraperitoneal chemotherapy versus systemic chemotherapy for colorectal peritoneal metastases: A randomised trial. Eur. J. Cancer. 2016;53:155–162.

9. Esquivel J. Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for colorectal cancer: Survival outcomes and patient selection. J. Gastrointest. Oncol. 2016;7:72–78.

10. Elias D., Gilly F., Boutitie F., Quenet F., Bereder J.M., Mansvelt B., Lorimier G., Dube P., Glehen O. Peritoneal colorectal carcinomatosis treated with surgery and perioperative intraperitoneal chemotherapy: Retrospective analysis of 523 patients from a multicentric French study. J. Clin. Oncol. 2010;28:63–68.

11. Kuijpers A.M., Mirck B., Aalbers A.G., Nienhuijs S.W., de Hingh I.H., Wiezer M.J., van Ramshorst B., van Ginkel R.J., Havenga K., Bremers A.J., et al. Cytoreduction and HIPEC in the Netherlands: Nationwide long-term outcome following the Dutch protocol. Ann. Surg. Oncol. 2013;20:4224–4230.

12. Mirnezami R., Mehta A.M., Chandrakumaran K., Cecil T., Moran B.J., Carr N., Verwaal V.J., Mohamed F., Mirnezami A.H. Cytoreductive surgery in combination with hyperthermic intraperitoneal chemotherapy improves survival in patients with colorectal peritoneal metastases compared with systemic chemotherapy alone. Br. J. Cancer. 2014;111:1500–1508.

13. Sluiter N.R., Rovers K.P., Salhi Y., Vlek S.L., Coupe V.M.H., Verheul H.M.W., Kazemier G., de Hingh I., Tuynman J.B. Metachronous Peritoneal Metastases After Adjuvant Chemotherapy are Associated with Poor Outcome After Cytoreduction and HIPEC. Ann. Surg. Oncol. 2018;25:2347–2356.

14. Quenet F., Elias D., Roca L., Goere D., Ghouti L., Pocard M., Facy O., Arvieux C., Lorimier G., Pezet D., et al. A UNICANCER phase III trial of hyperthermic intra-peritoneal chemotherapy (HIPEC) for colorectal peritoneal carcinomatosis (PC): PRODIGE 7. J. Clin. Oncol. 2018;36:LBA3503.

15. Feferman Y., Solomon D., Bhagwandin S., Kim J., Aycart S.N., Feingold D., Sarpel U., Labow D.M. Sites of Recurrence After Complete Cytoreduction and Hyperthermic Intraperitoneal Chemotherapy for Patients with Peritoneal Carcinomatosis from Colorectal and Appendiceal Adenocarcinoma: A Tertiary Center Experience. Ann. Surg. Oncol. 2019;26:482–489.

16. Braam H.J., van Oudheusden T.R., de Hingh I.H., Nienhuijs S.W., Boerma D., Wiezer M.J., van Ramshorst B. Patterns of recurrence following complete cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in patients with peritoneal carcinomatosis of colorectal cancer. J. Surg. Oncol. 2014;109:841–847.

17. Eng O.S., Dumitra S., O'Leary M., Raoof M., Wakabayashi M., Dellinger T.H., Han E.S., Lee S.J., Paz I.B., Lee B. Association of Fluid Administration With Morbidity in Cytoreductive Surgery With Hyperthermic Intraperitoneal Chemotherapy. JAMA Surg. 2017;152:1156–1160.

18. van Eden W.J., Elekonawo F.M.K., Starremans B.J., Kok N.F.M., Bremers A.J.A., de Wilt J.H.W., Aalbers A.G.J. Treatment of Isolated Peritoneal Recurrences in Patients with Colorectal Peritoneal Metastases Previously Treated with Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy. Ann. Surg. Oncol. 2018;25:1992–2001.

19. Koh J.L., Yan T.D., Glenn D., Morris D.L. Evaluation of preoperative computed tomography in estimating peritoneal cancer index in colorectal peritoneal carcinomatosis. Ann. Surg. Oncol. 2009;16:327–333.

20. Duffy M.J. Carcinoembryonic antigen as a marker for colorectal cancer: Is it clinically useful? Clin. Chem. 2001;47:624–630.

21. Verwaal V.J., Zoetmulder F.A. Follow-up of patients treated by cytoreduction and chemotherapy for peritoneal carcinomatosis of colorectal origin. Eur. J. Surg. Oncol. 2004;30:280–285.

22. Sugarbaker P.H. Intraperitoneal chemotherapy and cytoreductive surgery for the prevention and treatment of peritoneal carcinomatosis and sarcomatosis. Semin. Surg. Oncol. 1998;14:254–261.

23. Passot G., Dumont F., Goere D., Arvieux C., Rousset P., Regimbeau J.M., Elias D., Villeneuve L., Glehen O., Group B.-R.S.W. Multicentre study of laparoscopic or open assessment of the peritoneal cancer index (BIG-RENAPE) Br. J. Surg. 2018;105:663–667.

24. Elias D., Goere D., Dumont F., Honore C., Dartigues P., Stoclin A., Malka D., Boige V., Ducreux M. Role of hyperthermic intraoperative peritoneal chemotherapy in the management of peritoneal metastases. Eur. J. Cancer. 2014;50:332–340.

25. Dresen R.C., De Vuysere S., De Keyzer F., Van Cutsem E., Prenen H., Vanslembrouck R., De Hertogh G., Wolthuis A., D'Hoore A., Vandecaveye V. Whole-body diffusion-weighted MRI for operability assessment in patients with colorectal cancer and peritoneal metastases. Cancer Imaging. 2019;19:1.

26. Macintyre G., Ylstra B., Brenton J.D. Sequencing Structural Variants in Cancer for Precision Therapeutics. Trends Genet. TIG. 2016;32:530–542.

27. Van Helden E.J., Angus L., Menke-van der Houven van Oordt C.W., Heideman D.A.M., Boon E., van Es S.C., Radema S.A., van Herpen C.M.L., de Groot D.J.A., de Vries E.G.E., et al. RAS and BRAF mutations in cell-free DNA are predictive for outcome of cetuximab monotherapy in patients with tissue-tested RAS wild-type advanced colorectal cancer. Mol. Oncol. 2019;13:2361–2374.

28. Bach S., Sluiter N.R., Beagan J.J., Mekke J.M., Ket J.C.F., van Grieken N.C.T., Steenbergen R.D.M., Ylstra B., Kazemier G., Tuynman J.B. Circulating Tumor DNA Analysis: Clinical Implications for Colorectal Cancer Patients. A Systematic Review. JNCI Cancer Spectr. 2019;3:pkz042.

29. Bettegowda C., Sausen M., Leary R.J., Kinde I., Wang Y., Agrawal N., Bartlett B.R., Wang H., Luber B., Alani R.M., et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci. Transl. Med. 2014;6:224ra24.

30. Baumgartner J.M., Raymond V.M., Lanman R.B., Tran L., Kelly K.J., Lowy A.M., Kurzrock R. Preoperative Circulating Tumor DNA in Patients with Peritoneal Carcinomatosis is an Independent Predictor of Progression-Free Survival. Ann. Surg. Oncol. 2018;25:2400–2408.

31. Demuth C., Spindler K.G., Johansen J.S., Pallisgaard N., Nielsen D., Hogdall E., Vittrup B., Sorensen B.S. Measuring *KRAS* Mutations in Circulating Tumor DNA by Droplet Digital PCR and Next-Generation Sequencing. Transl. Oncol. 2018;11:1220–1224.

32. Reinert T., Scholer L.V., Thomsen R., Tobiasen H., Vang S., Nordentoft I., Lamy P., Kannerup A.S., Mortensen F.V., Stribolt K., et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2016;65:625–634.

33. Sato K.A., Hachiya T., Iwaya T., Kume K., Matsuo T., Kawasaki K., Abiko Y., Akasaka R., Matsumoto T., Otsuka K., et al. Individualized Mutation Detection in Circulating Tumor DNA for Monitoring Colorectal Tumor Burden Using a Cancer-Associated Gene Sequencing Panel. PLoS ONE. 2016;11:e0146275.

34. Verwaal V.J., van Ruth S., de Bree E., van Sloothen G.W., van Tinteren H., Boot H., Zoetmulder F.A. Randomized trial of cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy and palliative surgery in patients with peritoneal carcinomatosis of colorectal cancer. J. Clin. Oncol. 2003;21:3737–3743.

35. De Cuba E.M., Kwakman R., Knol D.L., Bonjer H.J., Meijer G.A., Te Velde E.A. Cytoreductive surgery and HIPEC for peritoneal metastases combined with curative treatment of colorectal liver metastases: Systematic review of all literature and meta-analysis of observational studies. Cancer Treat. Rev. 2013;39:321–327.

36. Verwaal V.J., van Ruth S., Witkamp A., Boot H., van Slooten G., Zoetmulder F.A. Long-term survival of peritoneal carcinomatosis of colorectal origin. Ann. Surg. Oncol. 2005;12:65–71.

37. Sugarbaker P.H., Jablonski K.A. Prognostic features of 51 colorectal and 130 appendiceal cancer patients with peritoneal carcinomatosis treated by cytoreductive surgery and intraperitoneal chemotherapy. Ann. Surg. 1995;221:124–132.

38. Sluiter N.R. Netherlands Trial Register, Trial NL6960 (NTR7148) [(accessed on 2 June 2020)]; Available online: https://www.trialregister.nl/trial/6960.

39. Van Essen H.F., Ylstra B. High-resolution copy number profiling by array CGH using DNA isolated from formalin-fixed, paraffin-embedded tissues. Methods Mol. Biol. 2012;838:329–341.

40. Sie D., Snijders P.J., Meijer G.A., Doeleman M.W., van Moorsel M.I., van Essen H.F., Eijk P.P., Grunberg K., van Grieken N.C., Thunnissen E., et al. Performance of amplicon-based next generation DNA sequencing for diagnostic gene mutation profiling in oncopathology. Cell. Oncol. 2014;37:353–361.

41. Heideman D.A., Thunnissen F.B., Doeleman M., Kramer D., Verheul H.M., Smit E.F., Postmus P.E., Meijer C.J., Meijer G.A., Snijders P.J. A panel of high resolution melting (HRM) technology-based assays with direct sequencing possibility for effective mutation screening of EGFR and K-ras genes. Cell. Oncol. 2009;31:329–333.

42. Cancer Genome Atlas Network Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487:330–337.

43. Reinert T., Henriksen T.V., Christensen E., Sharma S., Salari R., Sethi H., Knudsen M., Nordentoft I., Wu H.T., Tin A.S., et al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. JAMA Oncol. 2019;5:1124–1131.

44. Ceelen W.P., Bracke M.E. Peritoneal minimal residual disease in colorectal cancer: Mechanisms, prevention, and treatment. Lancet Oncol. 2009;10:72–79.

45. Vidal J., Muinelo L., Dalmases A., Jones F., Edelstein D., Iglesias M., Orrillo M., Abalo A., Rodriguez C., Brozos E., et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Ann. Oncol. 2017;28:1325–1332.

46. Bando H., Kagawa Y., Kato T., Akagi K., Denda T., Nishina T., Komatsu Y., Oki E., Kudo T., Kumamoto H., et al. A multicentre, prospective study of plasma circulating tumour DNA test for detecting RAS mutation in patients with metastatic colorectal cancer. Br. J. Cancer. 2019;120:982–986.

47. Ehlert T., Tug S., Brahmer A., Neef V., Heid F., Werner C., Jansen-Winkeln B., Kneist W., Lang H., Gockel I., et al. Establishing PNB-qPCR for quantifying minimal ctDNA concentrations during tumour resection. Sci. Rep. 2017;7:8876.

48. Tarazona N., Gimeno-Valiente F., Gambardella V., Zuñiga S., Rentero-Garrido P., Huerta M., Roselló S., Martinez-Ciarpaglini C., Carbonell-Asins J.A., Carrasco F., et al. Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer. Ann. Oncol. 2019;30:1804–1812.

49. Tie J., Cohen J.D., Wang Y., Li L., Christie M., Simons K., Elsaleh H., Kosmider S., Wong R., Yip D., et al. Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: A prospective biomarker study. Gut. 2019;68:663–671.

50. Rovers K.P., Bakkers C., Simkens G., Burger J.W.A., Nienhuijs S.W., Creemers G.M., Thijs A.M.J., Brandt-Kerkhof A.R.M., Madsen E.V.E., Ayez N., et al. Perioperative systemic therapy and cytoreductive surgery with HIPEC versus upfront cytoreductive surgery with HIPEC alone for isolated resectable colorectal peritoneal metastases: Protocol of a multicentre, open-label, parralel-group, phase II-III, randomised, superiority study (CAIRO6) BMC Cancer. 2019;19:390.

51. Dodson R.M., McQuellon R.P., Mogal H.D., Duckworth K.E., Russell G.B., Votanopoulos K.I., Shen P., Levine E.A. Quality-of-Life Evaluation After Cytoreductive Surgery with Hyperthermic Intraperitoneal Chemotherapy. Ann. Surg. Oncol. 2016;23:772–783.

52. Kato S., Schwaederle M.C., Fanta P.T., Okamura R., Leichman L., Lippman S.M., Lanman R.B., Raymond V.M., Talasaz A., Kurzrock R. Genomic Assessment of Blood-Derived Circulating Tumor DNA in Patients With Colorectal Cancers: Correlation With Tissue Sequencing, Therapeutic Response, and Survival. JCO Precis. Oncol. 2019;3:1–16.

53. Van't Sant I., van Eden W.J., Engbersen M.P., Kok N.F.M., Woensdregt K., Lambregts D.M.J., Shanmuganathan S., Beets-Tan R.G.H., Aalbers A.G.J., Lahaye M.J. Diffusion-weighted MRI assessment of the peritoneal cancer index before cytoreductive surgery. Br. J. Surg. 2019;106:491–498.

Supplementary material

Materials and methods

Clinical and pathological data

The following clinical and pathological data were obtained from the patient's records: age, sex, body-mass index, ASA-classification, primary tumour location, TNM-stage and tumour histology. Information relating to prior treatment was obtained, including: previous administration of systemic chemotherapy; previous resection of the primary tumour; the presence of lymph node or haematogenous metastases at the time of CRS-HIPEC; PCI scores from zero to 39, measured by DLS.[1,2]

Blood processing

Preoperative blood was collected through a central venous or arterial line into two 10ml Cell-free DNA Blood Collection Tubes (Streck Inc, Nebraska, USA). If Streck tubes were unavailable, three 6ml EDTA tubes (BD Vacutainer, NJ, USA). All samples were centrifuged within eight hours of collection at 820g for 10 minutes, the plasma layer transferred to a 50ml tube without disturbing the buffy coat, then transferred to 1.5ml tubes and spun at 16 000g for 10 minutes. The plasma was subsequently transferred into 1.8ml Nunc CryoTubes (Merck KGaA, Darmstadt, Germany) without disturbing the pellet and stored at -80˚C.

DNA isolation and mutation analysis

Biopsied PMs were processed according to a routine protocol in which the formalin-fixed paraffin-embedded tumour tissue was manually macro-dissected from serial sections guided by a haematoxylin and eosin stained tissue section on which the tumour region was marked by a pathologist. [3,4] Genomic DNA from PMs were analysed using the 48-gene, 212-amplicon TruSeq Amplicon Cancer Panel (TSACP) of commonly mutated onco- and tumour suppressor genes of solid malignancies Illumina Inc., CA, USA) as previously described. [4] One sample could not be successfully analysed using TSACP, so underwent testing with High Resolution Melting assay follow by Sanger sequencing (HRM-sequencing) for *KRAS* and *NRAS* exon 2-4, *BRAF* exon 15 and *PIK3CA* exon 9 and 20 as previously described. [5]

gBlocks and ddPCR

All gBlocks were individually diluted to a concentration of 1% in 8ng of pooled wild-type human DNA (Megapool Reference DNA, Leica Biosystems, Wetzlar, Germany), which had been sheared to an average length of 150 base-pairs by ultrasonification (Covaris Focussed-ultrasonicator M220, MA, USA) to mimic the properties of cfDNA fragmentation. Droplet digital PCR was performed on a

QX200 (BioRad) using 8ul undiluted cfDNA elution under the following thermo-cycler conditions: 95˚c for 10 minutes, 40 cycles of 94˚c for 30 seconds then 53˚c (55˚c for *KRAS* screening kit) for 1 minute, followed by 98˚c for 10 minutes and an overnight hold at 12˚c. Thresholds to determine positive droplets were set individually for each kit based primarily on the performance of the gBlock analysis (Supp. table 1A). Results were deemed mutant-positive if four or more FAM-dye positive droplets were observed.

References

1. Elias D, Gilly F, Boutitie F, et al. Peritoneal colorectal carcinomatosis treated with surgery and perioperative intraperitoneal chemotherapy: retrospective analysis of 523 patients from a multicentric French study. J Clin Oncol. 2010;28(1):63-68.
2. Sugarbaker PH. Intraperitoneal chemotherapy and cytoreductive surgery for the prevention and treatment of peritoneal carcinomatosis and sarcomatosis. Seminars in surgical oncology. 1998;14(3):254-261.
3. van Essen HF, Ylstra B. High-resolution copy number profiling by array CGH using DNA isolated from formalin-fixed, paraffin-embedded tissues. Methods in molecular biology (Clifton, NJ). 2012;838:329-341.
4. Sie D, Snijders PJ, Meijer GA, et al. Performance of amplicon-based next generation DNA sequencing for diagnostic gene mutation profiling in oncopathology. Cell Oncol. 2014;37(5):353-361.
5. Heideman DA, Thunnissen FB, Doeleman M, et al. A panel of high resolution melting (HRM) technology-based assays with direct sequencing possibility for effective mutation screening of EGFR and K-ras genes. Cell Oncol. 2009;31(5):329-333.

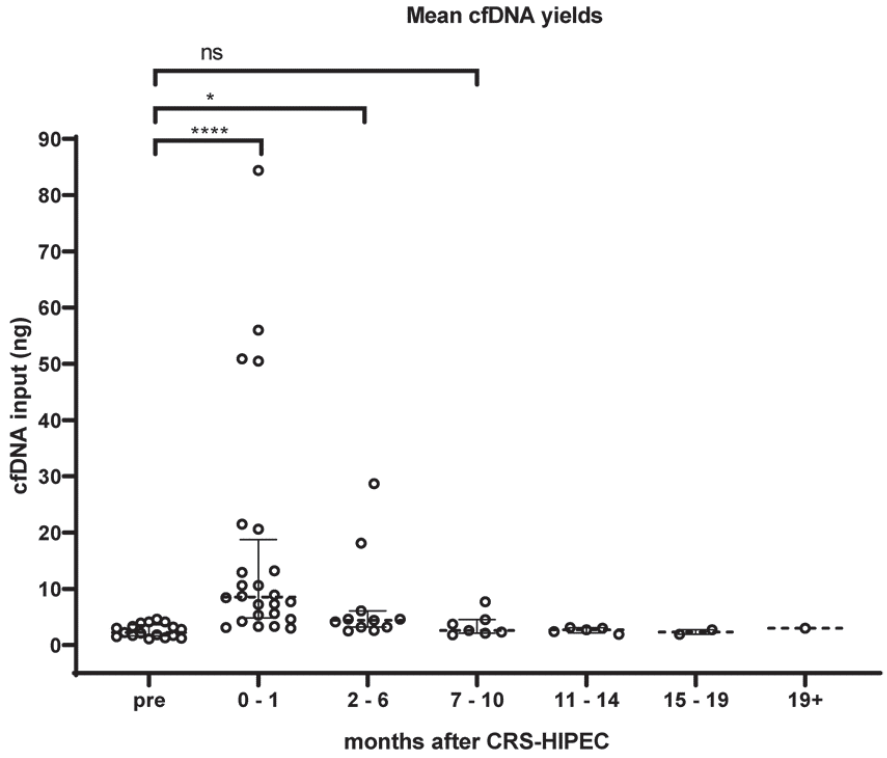


Figure S1. Mean cfDNA yields of plasma samples taken preoperatively and during follow-up. All samples were negative for ctDNA.

Table S2. Correlations between preoperative cfDNA detection (positive versus negative for ctDNA) and clinical and pathological variables. * Mann-Whitney U test. ** Fisher's exact test. BMI: Body Mass Index. PCI: Peritoneal Cancer Index.

		Preoperative ctDNA analysis		
Characteristic		**ctDNA pos (n=10)**	**ctDNA neg (n=20)**	**p-value**
		Mean (SD)	**Mean (SD)**	
BMI		26.8 (3.9)	27.3 (5.8)	0.801*
PCI		10.1 (8.7)	10.2 (8.2)	0.910*
		n (%)	**n (%)**	
Primary tumour location	**Colon**	10 (100)	17 (85)	0.532**
	Rectum	0 (0)	3 (15)	
Differentiation grade primary	**Good/moderate**	9 (100)	14 (88)	0.520**
	Poor/signet cell	0 (0)	2 (13)	
Mucinous tumour	**Yes**	2 (20)	6 (30)	0.682**
	No	8 (80)	14 (70)	
Previous chemotherapy	**Yes**	4 (40)	7 (35)	1.000**
	No	6 (60)	13 (65)	
Primary tumour *in situ*	**Yes**	3 (30)	5 (25)	1.000**
at CRS-HIPEC	**No**	7 (70)	15 (75)	
Lymph node metastases	**Yes**	3 (30)	4 (21)	0.665**
at CRS-HIPEC	**No**	7 (70)	15 (79)	
Liver metastases	**Yes**	2 (20)	1 (5)	0.251**
at CRS-HIPEC	**No**	8 (80)	19 (95)	
Procedure	**CRS-HIPEC**	8 (80)	16 (80)	1.000**
	Open-close	2 (20)	4 (20)	

Table S3. Correlations between clinical and pathological characteristics and Disease Free Survival (DFS) in months. *log-rank test. NR: Not Reached. BMI: Body Mass Index. ASA: American Society of Anesthesiologists Physical Status Classification System. PCI: Peritoneal Cancer Index

		Disease-free survival		
Characteristic		**Number of patients**	**Median DFS (95% CI)**	**p-value***
General				
Age in years	**<66**	12	7.0 (0.2-13.8)	0.122
	≥66	12	NR	
Gender	**Male**	14	7.0 (5.2-8.8)	0.282
	Female	10	13.0 (9.2-16.8)	
BMI	**≤27**	13	11.0 (4.2-17.8)	0.359
	>27	11	NR	
ASA	**I-II**	17	12.0 (4.6-19.4)	0.923
	III	7	10.0 (2.3-17.7)	
Preoperative ctDNA	**Yes**	8	6.0 (1.8-10.2)	0.016
	No	16	NR	
Primary tumour				
T-stage	**T1-3**	9	NR	0.056
	T4	15	7.0 (2.2-11.8)	
N-stage	**N0**	8	NR	0.594
	N1-2	16	11.0 (3.1-18.9)	
Differentiation grade	**Good/moderate**	19	13.0 (-)	0.051
	Poor/signet cell	2	4.0 (-)	
Angio-invasion	**Yes**	8	7.0 (1.5-12.5)	0.632
	No	14	10.0 (2.6-17.4)	
Lymphatic invasion	**Yes**	5	6.0 (3.9-8.1)	0.218
	No	17	12.0 (8.8-15.2)	
Mucinous tumour	**Yes**	6	7.0 (1.0-13.0)	0.243
	No	18	13.0 (-)	
Treatment				
Primary tumour *in situ* at CRS-HIPEC	**Yes**	7	7.0 (3.2-10.8)	0.266
	No	17	13.0 (7.9-18.1)	
PCI	**≤10**	16	NR	0.035
	>10	8	5.0 (0.8-9.2)	
Liver metastases	**Yes**	2	2.0 (-)	<0.001
	No	22	12.0 (8.2-15.8)	
Lymph node metastases at CRS-HIPEC	**Yes**	6	7.0 (0.3-13.7)	0.407
	No	18	12.0 (6.9-17.1)	

Chapter 4

Circulating tumor DNA analysis of *EGFR*-mutant non-small cell lung cancer patients receiving osimertinib following previous tyrosine kinase inhibitor treatment

Jamie J Beagan*
Sander Bach*
Robert A van Boerdonk
Erik van Dijk
Erik Thunnissen
Daan van den Broek
Janneke Weiss
Geert Kazemier
D Michiel Pegtel
Idris Bahce
Bauke Ylstra*
Daniëlle A M Heideman*

*Contributed equally as first and senior authors, respectively

Lung Cancer. 2020 Jul;145:173-180.

Highlights

- ctDNA testing of NSCLC patients with complex treatment history in clinical setting.
- A trend between ctDNA detection and progression under osimertinib in 2nd / 3rd line.
- Monitoring response with ctDNA may benefit patients following previous EGFR-TKIs.

Abstract

Objectives

Circulating tumor (ct)DNA analysis is rapidly gaining acceptance as a diagnostic tool to guide clinical management of advanced non-small cell lung cancer (NSCLC). Clinically-actionable *EGFR* mutations can be detected in ctDNA before or after first-line *EGFR*-Tyrosine Kinase Inhibitor (TKI) treatment, but data are limited for patients with a complex treatment history. This study aimed to explore the feasibility of ctDNA testing in a clinical setting of NSCLC patients receiving osimertinib as a second or third line EGFR-TKI.

Materials and methods

Twenty *EGFR* T790M-positive NSCLC patients, who had received osimertinib as a second or third line EGFR-TKI and had donated blood samples while attending routine follow-up consultations between April and November 2016, were retrospectively selected to test plasma cfDNA for tumor-guided *EGFR* mutations. We used *EGFR* mutations previously identified in tumor-tissue to retrospectively test plasma ctDNA from 20 patients who had received osimertinib as a second or third line EGFR-TKI. Both EGFR-TKI sensitising and T790 M resistance mutations were analysed by droplet digital PCR (ddPCR) in plasma taken alongside routine consultations and ctDNA detection was correlated with response under osimertinib. Follow-up solid-tissue biopsies were obtained after disease progression.

Results

CtDNA was detected under osimertinib treatment in four out of the eight patients (50 %) who showed no response, two out of the seven (29 %) who showed an initial response and none of the five patients (0 %) who showed an ongoing response. The fraction of *EGFR*-mutant ctDNA in plasma tended to be higher in non-responders (0.1-68 %), compared to the initial responders (0.2-1.1 %). Blood samples were donated up to 34, 27 and 49 weeks after the start of osimertinib for the non-, initial and ongoing responders, respectively.

Conclusions

These findings support a potential role for ctDNA analysis in response monitoring of NSCLC patients with a complex EGFR-TKI treatment history. The weak trend between ctDNA detection and disease progression warrants larger studies to further investigate potential clinical utility.

Keywords

Circulating tumor DNA; EGFR; EGFR-TKI; Monitoring; NSCLC; Osimertinib.

1. Introduction

Mutations in the epidermal growth factor receptor (*EGFR*) gene are among the most clinically relevant biomarkers in non-small cell lung cancer (NSCLC) [1]. Molecular analysis usually requires a cytological sample or a solid-tissue biopsy, which is invasive and not always feasible when the tumor cannot be safely biopsied or material is insufficient for molecular testing [2]. A more accessible bio-source may be circulating tumor (ct)DNA, the portion of cell-free (cf)DNA that originates from tumor cells [3]. In studies of NSCLC patients, ctDNA was shown to give a good representation of tumor *EGFR* status before EGFR-Tyrosine Kinase Inhibitor (TKI) treatment [4],[5]. A significant correlation was found between pre-treatment ctDNA quantity and tumor volume, which can serve as a dynamic measure of tumor response under EGFR-TKIs [6, 7, 8]. Crucially, the detection of emerging mutations in ctDNA can provide early signs of resistance, even before clinical confirmation using standard radiographic techniques [6],[9].

As a result, ctDNA analysis is recommended to guide therapy decisions based on the detection of *EGFR* mutations that confer sensitivity or resistance to initial lines of EGFR-TKI treatment [10, 11, 12]. This recommendation awaits evidence before expansion to include additional markers and/or applications, such as post-osimertinib progression evaluation to guide next line therapy. For example, ctDNA *MET* amplifications may serve as a predictive marker for a combination of osimertinib and savolitinib [13]. Furthermore, the clinical value of *EGFR* ctDNA analysis from NSCLC patients with a complex treatment history and previously established resistance to first or second-line EGFR-TKIs remains unclear. Recent case studies have revealed an increase in the complexity of the mutational landscape in tumors from patients exposed to EGFR-TKIs [14, 15, 16], which raises questions about the interpretation and clinical validity of ctDNA analysis in this situation. This study set out to explore the feasibility of ctDNA testing in a clinical setting of NSCLC patients with a history of EGFR-TKI treatment. Using the highly sensitive droplet digital PCR (ddPCR) technique, we aimed to investigate the relationship between the detection of tumor-guided *EGFR* mutations in the ctDNA of patients receiving osimertinib as a second or third line EGFR-TKI and to explore the relationship with disease progression.

2. Materials and methods

2.1 Patients

NSCLC patients gave written informed consent and donated extra blood samples during routine consultations between April and November 2016, for storage in the Liquid Biopsy Center at the Cancer Center Amsterdam, Amsterdam University Medical Center, The Netherlands. Only patients with histologically confirmed

adenocarcinoma and a tumor sample positive for *EGFR* T790 M and a concurrent EGFR-TKI sensitising mutation – either an *EGFR* exon 19 deletion (n = 14) or exon 21 L858R (n = 6) – were retrospectively selected. Follow-up solid-tissue biopsies were taken during or after osimertinib treatment if progression was diagnosed, to identify potentially new targetable mutations. For this study, only patients who had provided at least one blood sample while receiving osimertinib were ultimately included. Clinical, pathological and radiographic data were retrieved from patient records, including the site of metastases at the time of first blood sampling (Table 1). None of the patients received chemotherapy and osimertinib concurrently. Response under osimertinib treatment was recorded at the time of blood sampling according to Response Evaluation Criteria in Solid Tumors (RECIST) analysis [17]. An estimation of overall tumor burden was not feasible because exact measures were not documented in patient records during routine clinical practice.

Table 1. Baseline characteristics of all patients included in the study.

Characteristic	Non-responder (%)	Responder (%)		Total (%)
		Initial	Ongoing	
No. of patients	8 (40)	7 (35)	5 (25)	20
Age at first blood sampling (years) Mean (SD) Median (range)	63 (8) 65 (54−79)	68 (7.8) 70 (57−81)	50 (10.9) 45 (41−68)	61 (11) 64 (41−81)
Sex				
Male	0	5 (71)	1 (20)	6 (30)
Female	8 (100)	2 (29)	4 (80)	14 (70)
Smoking status				
Never	1 (13)	0	4 (80)	5 (25)
Former	3 (38)	3 (43)	0	6 (30)
Current	0	0	1 (20)	1 (5)
Unknown	4 (50)	4 (57)	0	8 (40)
WHO performance status				
0	0	1 (14)	1 (20)	2 (10)
1	6 (75)	4 (57)	3 (60)	13 (65)
2	2 (25)	2 (29)	0	4 (20)
3	0	0	1 (20)	1 (5)
Number of sites of metastasis				
1	1 (13)	1 (14)	0	2 (10)
2	1 (13)	4 (57)	5 (100)	10 (50)
3	5 (63)	2 (29)	0	7 (35)

Characteristic	Non-responder (%)	Responder (%)		Total (%)
		Initial	Ongoing	
4	1 (13)	0	0	1 (5)
Site(s) of metastasis				
Lung	5 (63)	6 (86)	1 (20)	12 (60)
CNS	4 (50)	1 (14)	1 (20)	6 (30)
Leptomeningeal	1 (13)	0	1 (20)	2 (10)
Bone	3 (38)	5 (71)	3 (60)	11 (55)
Liver	2 (25)	1 (14)	1 (20)	4 (20)
Pleura	2 (25)	2 (29)	3 (60)	7 (35)
Other	3 (38)	0	0	3 (15)
Prior EGFR-TKI treatment				
gefitinib	1 (13)	3 (43)	4 (80)	8 (40)
erlotinib	7 (88)	4 (57)	1 (20)	12 (60)
afatinib	1 (13)	0	0	1 (5)
rociletinib	3 (38)	0	1 (20)	4 (20)
ALK-TKI				
ceritinib	1 (13)	0	0	1 (5)
crizotinib	2 (25)	0	0	2 (10)
Other targeted therapies	3 (38)	1 (14)	2 (40)	6 (30)
Chemotherapy	6 (75)	2 (29)	2 (40)	10 (50)
EGFR mutation				
L858R	2 (25)	3 (43)	1 (20)	6 (30)
Exon 19 deletion	6 (75)	4 (57)	4 (80)	14 (70)
T790M	8 (100)	7 (100)	5 (100)	20 (100)

Patients were classified into three groups for response evaluation: 1) non-responders (n = 8), if they had progressive disease at the time of their last blood sample under osimertinib (up to 34 weeks), 2) initial responders (n = 7), if they had partial response or stable disease by the time of their last blood sample (up to 27 weeks) followed by progression after this period, or 3) ongoing responders (n = 5), if they had partial response or stable disease during sampling (up to 49 weeks) and no progression was detected at the time of review. The study was approved by the Institutional Review Board (IRB) / Independent Ethics Committee (IEC) according to GCP and to Dutch regulatory and legal requirements.

2.2 Tumor tissue mutation status

Mutation analysis of formalin-fixed paraffin-embedded (FFPE) tumor tissue biopsies was performed as part of ISO certified routine diagnostic procedures at the Department of Pathology, Amsterdam UMC, location VUmc, with the Illumina TruSeq Amplicon Cancer Panel (TSACP) as previously described [18]. This next generation sequencing (NGS) panel is designed to sequence mutational hotspots, including single nucleotide variants and small indels, targeting 212 amplicons in 48 cancer-related genes [18]. Amplifications in *EGFR*, *ERBB2* and *MET* were inferred from NGS coverage data [19]. If NGS was unsuccessful, High Resolution Melting assay followed by Sanger sequencing (HRM-sequencing) was performed as previously described [18],[20]. If DNA analysis had already been performed in routine diagnostics at an external institute, the *EGFR* status was extracted from the clinical records or pathology reports.

2.3 Blood collection

Blood was collected through venepuncture into three 6 mL EDTA tubes. Whole blood was centrifuged at 900 g for 7 min, typically within six hours of collection, the plasma layer carefully transferred to a clean 10 mL tube without disturbing the buffy coat (interphase layer containing leukocytes and platelets), then centrifuged again at 2500 g for 10 min. Avoiding the pellet at the bottom of the tube, plasma was transferred again to fresh 10 mL tube and centrifuged at 500 g for 10 min. Following a final transfer to a clean 10 mL tube, 1.5 mL aliquots of plasma were transferred to Nunc CryoTubes (Merck KGaA, Darmstadt, Germany) and stored at −80 °C until further use.

2.4 Plasma cell-free (cf)DNA isolation

Three millilitres of plasma were used as input for the QIAsymphony Circulating DNA Kit according to the manufacturer's instructions (QIAgen, Hilden, Germany). To control for the consistent performance of isolations, a plasma pool of controls from individuals without NSCLC was established from which an aliquot was included in each run. All cfDNA concentrations were measured using the Qubit Double Stranded DNA High Sensitivity Assay Kit (ThermoFisher Scientific, MA, USA). To estimate the cfDNA concentration per mL of plasma, the Qubit measurement was extrapolated based upon a plasma volume of 3 mL and the cfDNA elution yield of each sample.

2.5 Droplet Digital (dd)PCR assays

Isolated cfDNA was tested using ddPCR for the specific *EGFR* exon 19 deletion or L858R mutation identified in the osimertinib baseline biopsy, in addition to T790 M (Sup. Table 1, sup. Materials). Reactions were set-up according to the

manufacturer's protocol, except for an annealing temperature of 54˚c (BioRad, California, USA). The results from the mutant and wild-type probe for each assay (number of copies/well) were used to calculate the Variant Allele Frequency (VAF %) and estimate the concentration of cfDNA in each plasma sample. All kits were verified using serial dilutions of mutant DNA in a background of wild-type DNA (0.02 %–50 %) prepared from genomic DNA isolated from cell-lines: NCI-H1975 (T790 M and L858R), NCI-H1650 (E746_A750del), SiHa and HCT-116 (wild-type for assayed *EGFR* mutations) that was fragmented to an average length of 150bp using an S220 Focussed-ultrasonicator (COVARIS, MA, USA). Each assay demonstrated an analytical sensitivity of 0.5 % at a total DNA input of 5 ng (approximately 1500 template copies). Furthermore, the workflow was verified by inter-laboratory evaluation and by successful participation in European Society of Pathology Lung External Quality Assessment Scheme for cfDNA [21].

2.6 Data and statistical analysis

Data from ddPCR were analysed using QuantaSoft software (version 1.7.4.0917; Bio-Rad Laboratories, Inc.) with quality-dependent thresholds for mutation detection set manually per run for the exon 19 deletion multiplex kit (Ch1 3487–5400, Ch2 4281–5250) or with an R-script for all other kits (code available upon reasonable request). The R-script removed outlier empty droplets (amplitude <0) and defined clusters based on the relative number of droplets at each amplitude. The mean amplitude of the clusters was calculated for each channel and thresholds fixed at three standard deviations (thresholds: L858R Ch1 1985, Ch2 1935; exon 19 deletion Ch1 2588, Ch2 3645; T790 M Ch1 2692, Ch2 2042). A sample was considered mutant positive when five or more FAM (fluorescent dye) positive droplets were detected. The result was considered valid if no more than two FAM positive droplets were observed in the no-template or the wild-type controls included in each run. The ddPCR input was based on a standard volume of 8 μL, rather than on a fixed concentration. To account for the effect of cfDNA input on the limit of detection, the theoretical VAF necessary to produce a positive result was calculated for each reaction using the method described by Heitzer et al. (2019) [22]. Differences in cfDNA concentration between response groups was tested using the Kruskal-Wallis one-way analysis of variance by ranks (GraphPad Prism v7.02) with statistical significance based upon a p-value <0.05.

3. Results

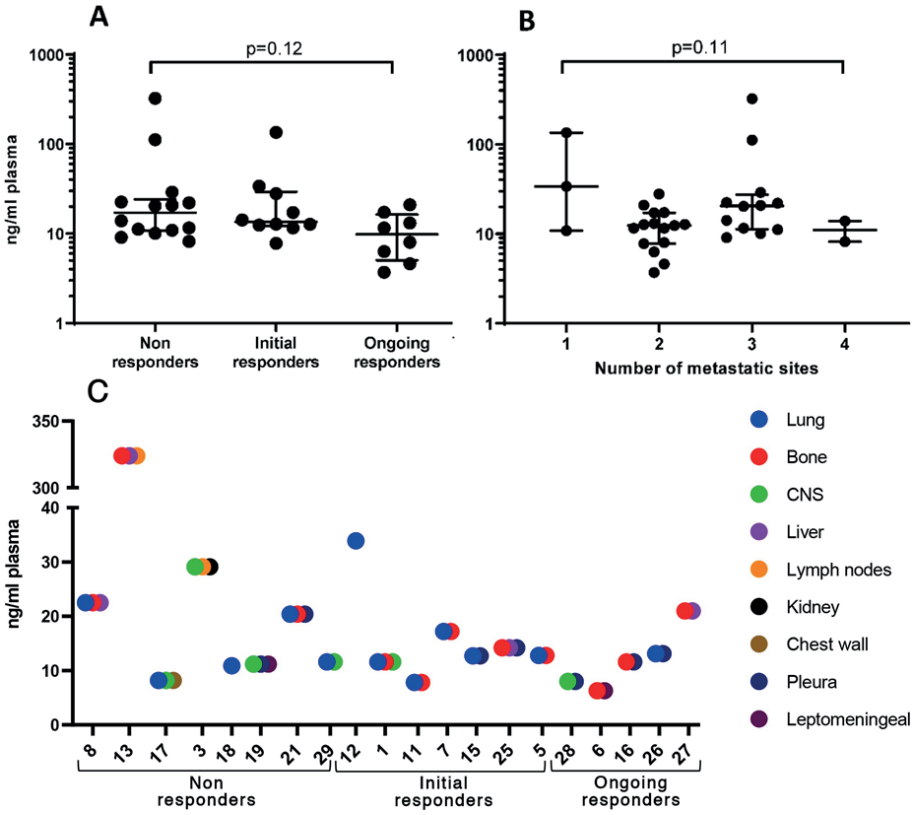
3.1 Study cohort

Table 1 reports baseline characteristics of the twenty T790M-positive NSCLC patients receiving osimertinib as a second or third line EGFR-TKI, stratified for clinical treatment response (non-responders (n = 8), initial responders (n = 7), and ongoing responders (n = 5)). The timing of blood sampling was heterogeneous as it coincided with routine consultations which occurred up to 34, 27 and 49 weeks after the start of osimertinib for the non-, initial and ongoing responders, respectively.

3.2 Plasma analysis

3.2.1 cfDNA concentrations

A median cfDNA concentration of 13 ng mL−1 plasma (range, 3–324) was measured across all samples by Qubit analysis, with concentrations of 17.1 (8–324), 13.5 (7–135) and 8.0 (3–21) ng mL−1 from non-responders, initial responders and ongoing responders, respectively. Although concentrations tended to be higher in non-responders, there was no statistically significant difference between response groups (Fig. 1A). There was also no difference between the cfDNA concentration and the number of detected metastatic sites at the time of sampling (Fig. 1B) nor the particular site of metastasis (Fig. 1C). The cfDNA concentration estimated by ddPCR showed a strong correlation with Qubit analysis (R2 = 0.967) (Fig. 2A).



Figre 1A. Concentration of each cfDNA sample estimated from Qubit analysis (y-axis) and categorized by patient response to osimertinib (x-axis). B: Concentration of each sample according to the number of detectable metastatic sites identified in each patient at the time of sampling. Horizontal long lines indicate the median and the shorter lines the inter-quartile range. The Kruskal-Wallis one-way analysis of variance by ranks was used to test difference between groups (p-values are displayed on top of the figures). C: Concentration of cfDNA in the first sample from each patient, categorized by response. The colors represent the particular metastatic site at the time of sampling.

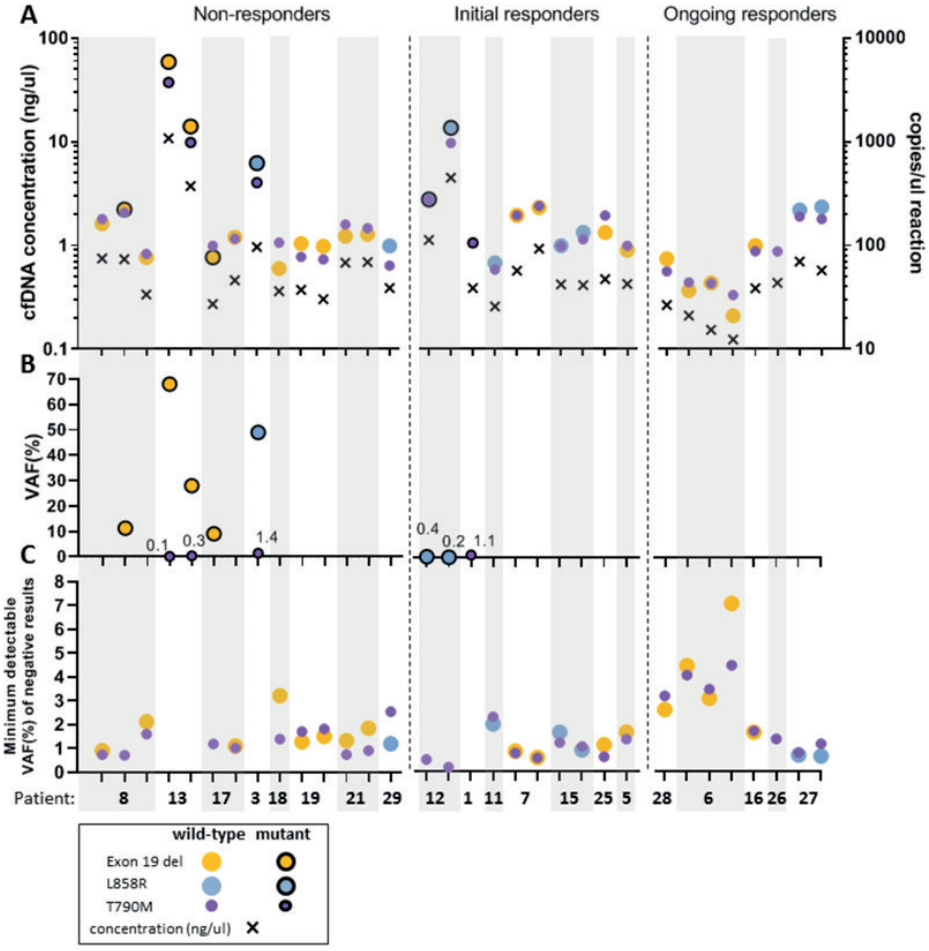


Figure 2A. The concentration of each cfDNA sample measured by Qubit (ng/uL, left y-axis) and ddPCR (copies/ul, right y-axis). Each mark on the x-axis indicates a patient sample. Each patient provided between one and three samples, separated by grey shading. If a patient provided multiple samples, the sampling time-points run from left to right. Dashed lines indicate the divide between response categories which are labelled on top of the figure. Colors indicate the result of the tumor-guided assays used (see box); a black border around a point indicates that ctDNA was detected. B: VAF (%) in samples that contained ctDNA. C: The theoretical upper bound of ctDNA detection given the cfDNA input and the requirement of five positive droplets for a positive result.

3.2.2 EGFR ctDNA detection and variant allele frequency (VAF)

The ddPCR kits detect ctDNA when a probe binds to a fragment that contains a specific *EGFR* mutation. A second probe targets the wild-type sequence to allow calculation of the VAF in the sample. In this manner, *EGFR* ctDNA was detected in at least one of the plasma samples from 6/20 (30 %) patients overall. There was no clear link between ctDNA detection and the number of metastatic sites nor the particular site of metastasis (supplementary Fig. 1). Stratified by response, detection occurred in 4/8 (50 %) non-responders, 2/7 (29 %) initial responders and 0/5 (0 %) ongoing responders. If *EGFR* ctDNA was not detected in a ddPCR reaction, the minimum detectable fraction of ctDNA was predicted given the variable cfDNA input to each reaction (median 3.4 ng; range 1 –86 ng). The detectable fraction ranged from 0.2 % for the highest input to 7.0 % for the lowest input of cfDNA (Fig. 2C). This dynamic threshold is specific to each reaction so should be considered in the interpretation of any negative result reported here.

A greater proportion of non-responders had detectable ctDNA and it was usually at a higher VAF compared to initial responders (Fig. 2A&B). None of the five ongoing responders had detectable levels of ctDNA (Fig. 3C). In samples from the non-responders, the co-occurring sensitising and T790 M *EGFR* mutations were both detected in patients P3 and P13; while only the sensitising *EGFR* exon 19 deletion was detected in patients P8 and 17. The VAF ranged from 9 to 68 % for the sensitising mutation and between 0.1–1.4 % for T790 M (Fig. 2B).

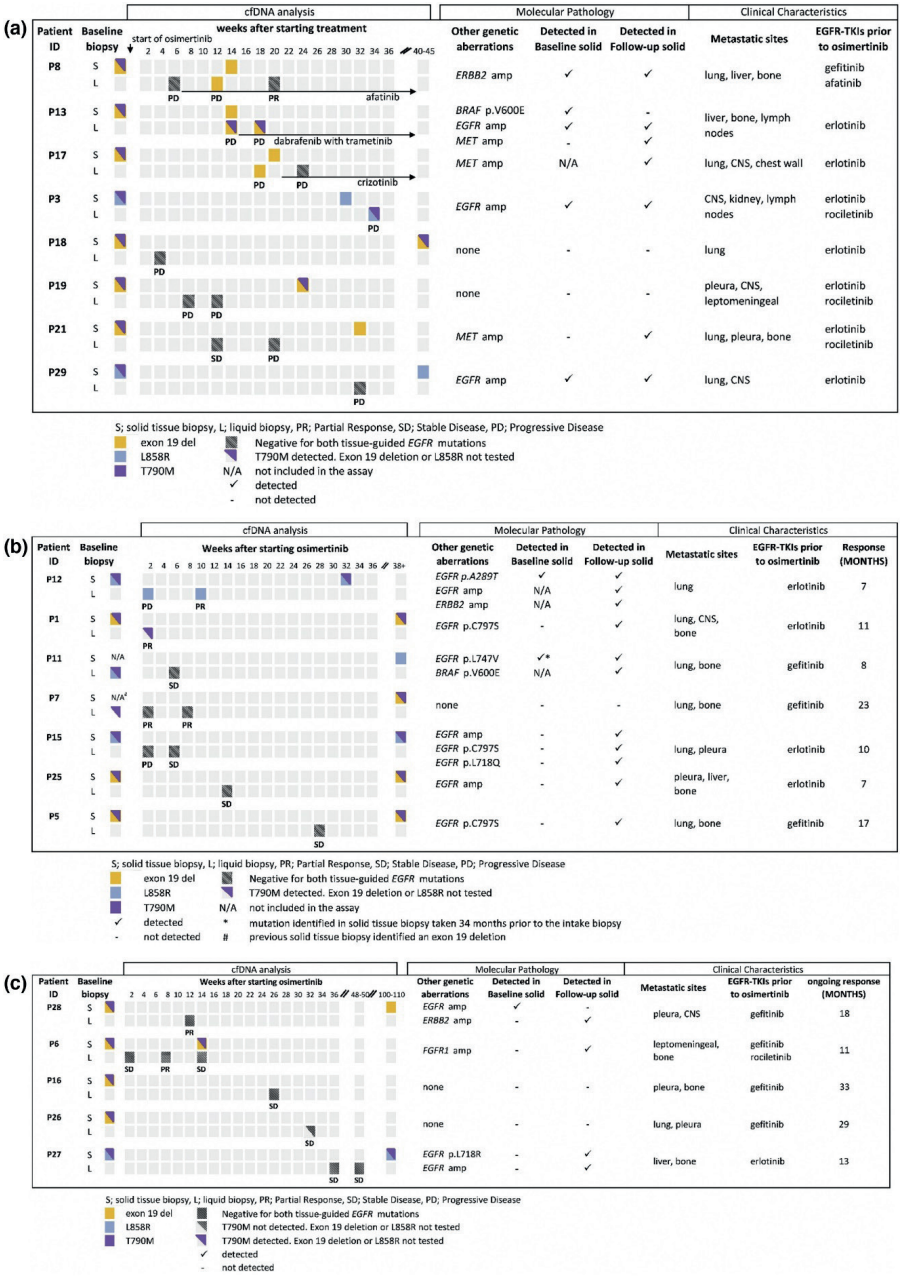


Figure 3. Pathological and clinical details of A: non-responders, B: initial responders and C: ongoing responders to osimertinib. PR; Partial Response, SD; Stable Disease, PD; Progressive Disease, S; solid tissue biopsy, L; liquid biopsy, N/A; mutation not included in assay used, - ; mutation not detected, ✓; mutation detected.

Two (29 %) of the seven initial responders (P1 and 12) had detectable levels of ctDNA (Fig. 3B). The L858R mutation was detected in patient P12 across two time points under osimertinib, with a VAF ranging from 0.2 – 0.4 %. The T790 M mutation was detected in patient P1 at a VAF of 1.1 %, however the sensitising mutation could not be tested as it was not targeted by the ddPCR assay (Sup. Table 1, sup. Methods).

3.3 Solid tissue analysis

Data regarding genetic alterations in both the osimertinib baseline and follow-up solid tumor biopsies were considered in relation to treatment response and EGFR-TKI treatment history. All patients had received erlotinib or gefitinib as a first-line EGFR-TKI and five had subsequently received afatinib (n = 1) or rociletinib (n = 4) as a second line prior to starting osimertinib treatment.

Of the non-responders, half had received two previous lines of EGFR-TKI treatment prior to starting osimertinib treatment, which is the highest proportion across the three response groups (Table 1). Data regarding genetic alterations in both the osimertinib baseline and follow-up solid tumor biopsies were available for seven out of eight patients. Amplifications in *EGFR* or *ERBB2* detected in the baseline biopsy of four patients (P3, 8, 13 and 29; Fig. 3A, Sup. Table 1) were confirmed in the follow-up. A *MET* amplification was detected in the follow-up of three patients (P13, 17 and 21) but was not detected in the baseline biopsy of P13 and P21 and was not tested for in the baseline of P17. Interestingly, *EGFR* T790 M was not detected in the follow-up solid-tissue biopsy of six (75 %) out of eight non-responders.

All initial responders had received one previous line of EGFR-TKI, either gefitinib or erlotinib, before starting osimertinib treatment. In three patients (P1, 5 and 15; Fig. 3B, Sup. Table 1), *EGFR* mutations L718Q and/or C797S were detected in the follow-up but not in the osimertinib baseline biopsy. Other mutations such as *BRAF* V600E and amplifications in *ERBB2* and *EGFR* were also identified in follow-up biopsies but were not tested in the baseline. The T790 M mutation was not detected in the follow-up solid-tissue biopsy of one (14 %) out of seven initial responders (P11).

The majority of ongoing responders (four out of five) had received one line of EGFR-TKI before starting osimertinib treatment. In three of the five patients, the follow-up tumor-tissue biopsy had an amplification in either *ERBB2*, *FGFR1* or *EGFR* and one patient had an L718R *EGFR* mutation, none of which were detected in the baseline biopsy (Fig. 3C, Sup. Table 1). *EGFR* T790 M was not detected in one patient and was maintained in two. For the remaining two patients it could not be determined as no follow-up biopsy was available.

4. Discussion

This study shows the feasibility of *EGFR*-mutant ctDNA detection in patients treated with osimertinib after previous lines of EGFR-TKI. CtDNA was most frequently detected in plasma from non-responders to osimertinib, although negative results were observed among all three response groups. In line with clinical recommendations, a negative result should prompt additional analysis of either ctDNA or DNA from a tumor biopsy, preferably with an NGS-based test [12].

Liquid biopsy has the potential to monitor response during EGFR-TKI treatment without the need for repeat solid-tissue sampling. Despite the implementation of technically valid approaches, important questions remain over the clinical validity and utility of ctDNA analysis [23]. Our results build on previous studies of advanced NSCLC patients, although these are not always directly comparable due to differences in pre-analytical workflows, analytical techniques or patient populations. Despite this, the median cfDNA concentration of 13 (3–324) ng mL–1 plasma in the current study is in line with earlier reports of NSCLC patients under systemic therapy [24]. Plasma cfDNA concentration, regardless of mutation status, can be a prognostic biomarker in treatment-naive patients [25]. However, in the current study this measure alone could not distinguish between the response groups, which suggests a reduced clinical utility in patients with a complex treatment history.

For response monitoring, the detection of *EGFR*-mutant ctDNA in 50 % of non-responders in our study is similar to the 42 % detection rate in 26 stage IV adenocarcinoma patients after progression under osimertinib [26]. Three (12 %) of the 26 osimertinib resistant patients in the aforementioned study acquired *EGFR* C797S and maintained both the T790 M and the initial *EGFR* sensitising mutation [[26]]. We observed this in the follow-up biopsy from 20 % of patients in our study, all of whom were initial responders prior to developing resistance to osimertinib. The loss of T790 M is associated with earlier resistance to osimertinib and was seen in 68 % of cases in a separate study after re-sequencing of solid tumor material at the time of resistance [27]. In our study, we report the apparent loss of T790 M in the solid-tissue follow-up biopsy in 75 % of non-responders, 14 % on initial- and 20 % of ongoing responders, for whom we have comparable data. We could also support earlier reports of amplifications in *MET*, *ERBB2* and the well-studied *EGFR* C797S mutation as potential mediators of resistance in some patients [28, 29, 30, 31]. Differences in solid-tumor mutation profiles between osimertinib response groups could be related to the number of previous TKIs a patient received - as non-responders in our study typically received more previous lines than responders - a finding that warrants further investigation.

A strength of our study was the use of ddPCR for tumor-guided detection of ctDNA in plasma. *EGFR* driver mutations were selected to maximise the likelihood of ctDNA detection, as they are theoretically present in each of the tumor cells. Although other targets were identified in solid-tissues, the limited cfDNA material available meant that only the *EGFR* driver and T790 M mutations could be tested. The highly sensitive ddPCR technique was chosen because plasma ctDNA concentration can be very low under EGFR-TKI treatment, often less than 1 % VAF in the case of T790 M [32]. The minimum detectable VAF was included to highlight the effect of low cfDNA input to reduce the sensitivity of the test, as previously established [[22]]. Targeted testing with ddPCR offers a good chance of detecting ctDNA, but is limited by the small number of mutations that can be tested in each sample given the limited quantity of available cfDNA. An NGS-based approach would increase the number of genomic targets that can be tested in cfDNA [7], an important consideration given the heterogeneity of osimertinib resistance mechanisms in NSCLC patients after progression under previous lines of EGFR-TKI [[27],[29]], but tumor-guided ddPCR offered the most sensitive and convenient approach for this study. Future validation studies could include cfDNA testing by NGS provided the sensitivity is equal to ddPCR.

The sampling and molecular testing of solid-tissue and plasma at routine consultations was subject to the clinical and practical considerations of a hospital diagnostic workflow, which was irregular, but placed our findings in the context of clinical practice. However, the inconsistent interrogation of genetic alterations between tumor-tissue samples and patients limited direct comparison and restricted the potential to draw conclusions about possible osimertinib resistance mechanisms in our cohort. Liquid biopsy has fewer practical restrictions than solid-tissue biopsy, but the analytical sensitivity is limited by the low cfDNA quantities typically obtained from a routine blood draw. This is compounded by an incomplete understanding of the biological processes behind ctDNA representation in the circulation, which still limits meaningful interpretation of results in routine clinical practice. Despite the utility of positive results, false negatives have been reported in advanced cancer patients across many cancer types [33]. These factors require a conservative approach to testing, which currently restrains the clinical utility of ctDNA as a biomarker.

At the time of our study, osimertinib was approved only to treat NSCLC patients with a confirmed T790 M mutation and progression following earlier lines of EGFR-TKI. Recent clinical trials have since led to the adoption of osimertinib as a first-line treatment [[34]]. Despite this, osimertinib may remain a second or third line treatment in a sub-set of patients. The utility of ctDNA analysis following previous lines of EGFR-TKI and a more complex mutational landscape therefore remains pertinent and warrants validation towards clinical implementation.

In this study, we demonstrate the feasibility of ctDNA analysis for response monitoring in patients receiving osimertinib after progression on previous lines of EGFR-TKI. Despite the small number of inclusions and a highly heterogeneous patient group, we identified a weak trend between ctDNA detection and response. This finding could support the current gold standard of treatment monitoring by tumor-tissue biopsy in patients who received multiple lines of EGFR-TKI. Further evaluation of ctDNA detection in a larger cohort of *EGFR*-mutant NSCLC patients with a complex treatment history is now warranted to address its utility as a reliable treatment monitoring modality in this setting.

CRediT authorship contribution statement

Jamie J. Beagan: Project administration, Validation, Data curation, Formal analysis, Writing - original draft, Visualization. Sander Bach: Investigation, Formal analysis, Writing - original draft, Visualization. Robert A. van Boerdonk: Investigation, Writing - review & editing. Erik van Dijk: Software, Formal analysis, Writing - review & editing. Erik Thunnissen: Resources, Writing - review & editing. Daan van den Broek: Validation, Resources, Writing - review & editing. Janneke Weiss: Resources, Writing - review & editing. Geert Kazemier: Funding acquisition, Writing - review & editing. D Michiel Pegtel: Conceptualization, Writing - review & editing. Idris Bahce: Resources, Conceptualization, Writing - review & editing. Bauke Ylstra: Supervision, Conceptualization, Methodology, Funding acquisition, Writing - review & editing. Daniëlle A.M. Heideman: Supervision, Conceptualization, Methodology, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

DAMH has been on the speakers' bureau of QIAGEN, serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb, and is minority shareholder of Self-screen B.V., a spin-off company of VUmc. All remaining authors have declared no conflicts of interest.

Acknowledgements

The authors would like to thank all patients for their consent to donate blood and medical history details. In addition we like to thank Sandra A W M Veldt-Verkuijlen, Marije W Doeleman, Marinda I H van Moorsel, Marjolein Bekker-Lettink, Paul P Eijk, Hendrik F B van Essen, Sandra Mongera, Zuzana Stachová Rychnavská, Monique van Eijndhoven, Lars Nooi, Stefan Strijbis, Cees Oudejans, Allerdien Visser, Ron van Schooten and Ingrid Bakker for their help and advice in DNA isolations, ddPCR optimizations and experiments.

References

[1] Y.-L. Zhang, J.-Q. Yuan, K.-F. Wang, X.-H. Fu, X.-R. Han, D. Threapleton, Z.-Y. Yang, C. Mao, J.-L. Tang, The prevalence of EGFR mutation in patients with non-small cell lung cancer: a systematic review and meta-analysis, Oncotarget 7 (48) (2016) 78985–78993.

[2] C. Rolfo, P.C. Mack, G.V. Scagliotti, P. Baas, F. Barlesi, T.G. Bivona, R.S. Herbst, T.S. Mok, N. Peled, R. Pirker, L.E. Raez, M. Reck, J.W. Riess, L.V. Sequist, F.A. Shepherd, L.M. Sholl, D.S.W. Tan, H.A. Wakelee, I.I. Wistuba, M.W. Wynes, D.P. Carbone, F.R. Hirsch, D.R. Gandara, Liquid biopsy for advanced non-small cell lung Cancer (NSCLC): a statement paper from the IASLC, J. Thorac. Oncol. 13 (9) (2018) 1248–1268.

[3] G. Siravegna, S. Marsoni, S. Siena, A. Bardelli, Integrating liquid biopsies into the management of cancer, Nat. Rev. Clin. Oncol. 14 (2017) 531.

[4] J.Y. Douillard, G. Ostoros, M. Cobo, T. Ciuleanu, R. Cole, G. McWalter, J. Walker, S. Dearden, A. Webster, T. Milenkova, R. McCormack, Gefitinib treatment in EGFR mutated caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status, J. Thorac. Oncol. 9 (9) (2014) 1345–1353.

[5] G. Zhu, X. Ye, Z. Dong, Y.C. Lu, Y. Sun, Y. Liu, R. McCormack, Y. Gu, X. Liu, Highly sensitive droplet digital PCR method for detection of EGFR-Activating mutations in plasma cell-free DNA from patients with advanced non-small cell lung Cancer, J. Mol. Diagn. 17 (3) (2015) 265–272.

[6] B.S. Sorensen, L. Wu, W. Wei, J. Tsai, B. Weber, E. Nexo, P. Meldgaard, Monitoring of epidermal growth factor receptor tyrosine kinase inhibitor-sensitizing and resistance mutations in the plasma DNA of patients with advanced non-small cell lung cancer during treatment with erlotinib, Cancer 120 (24) (2014) 3896–3901.

[7] A.M. Newman, S.V. Bratman, J. To, J.F. Wynne, N.C. Eclov, L.A. Modlin, C.L. Liu, J.W. Neal, H.A. Wakelee, R.E. Merritt, J.B. Shrager, B.W. Loo Jr., A.A. Alizadeh, M. Diehn, An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage, Nat. Med. 20 (5) (2014) 548–554.

[8] C. Abbosh, N.J. Birkbak, G.A. Wilson, M. Jamal-Hanjani, T. Constantin, R. Salari, J. Le Quesne, D.A. Moore, S. Veeriah, R. Rosenthal, T. Marafioti, E. Kirkizlar, T.B.K. Watkins, N. McGranahan, S. Ward, L. Martinson, J. Riley, F. Fraioli, M. Al Bakir, E. Gronroos, F. Zambrana, R. Endozo, W.L. Bi, F.M. Fennessy, N. Sponer, D. Johnson, J. Laycock, S. Shafi, J. Czyzewska-Khan, A. Rowan, T. Chambers, N. Matthews, S. Turajlic, C. Hiley, S.M. Lee, M.D. Forster, T. Ahmad, M. Falzon, E. Borg, D. Lawrence, M. Hayward, S. Kolvekar, N. Panagiotopoulos, S.M. Janes, R. Thakrar, A. Ahmed, F. Blackhall, Y. Summers, D. Hafez, A. Naik, A. Ganguly, S. Kareht, R. Shah, L. Joseph, A. Marie Quinn, P.A. Crosbie, B. Naidu, G. Middleton, G. Langman, S. Trotter, M. Nicolson, H. Remmen, K. Kerr, M. Chetty, L. Gomersall, D.A. Fennell, A. Nakas, S. Rathinam, G. Anand, S. Khan, P. Russell, V. Ezhil, B. Ismail, M. Irvin-Sellers, V. Prakash, J.F. Lester, M. Kornaszewska, R. Attanoos, H. Adams, H. Davies, D. Oukrif, A.U. Akarca, J.A. Hartley, H.L. Lowe, S. Lock, N. Iles, H. Bell, Y. Ngai, G. Elgar, Z. Szallasi, R.F. Schwarz, J. Herrero, A. Stewart, S.A. Quezada, K.S. Peggs, P. Van Loo, C. Dive, C.J. Lin, M. Rabinowitz, H. Aerts, A. Hackshaw, J.A. Shaw, B.G. Zimmermann, C. Swanton, Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution, Nature 545 (7655) (2017) 446–451.

[9] G.R. Oxnard, C.P. Paweletz, Y. Kuang, S.L. Mach, A. O'Connell, M.M. Messineo, J.J. Luke, M. Butaney, P. Kirschmeier, D.M. Jackman, P.A. Janne, Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA, Clin. Cancer Res. 20 (6) (2014) 1698–1705.

[10] C. Mayo-de-Las-Casas, N. Jordana-Ariza, M. Garzon-Ibanez, A. Balada-Bel, J. Bertran-Alamillo, S. Viteri-Ramirez, N. Reguart, M.A. Munoz-Quintana, P. LianesBarragan, C. Camps, E. Jantus, J. Remon-Massip, S. Calabuig, D. Aguiar, M.L. Gil, N. Vinolas, A.K. Santos-Rodriguez, M. Majem, B. Garcia-Pelaez, S. Villatoro, A. Perez-Rosado, J.C. Monasterio, E. Ovalle, M.J. Catalan, R. Campos, D. MoralesEspinosa, A. Martinez-Bueno, M. Gonzalez-Cao, X. Gonzalez, I. Moya-Horno, A.E. Sosa, N. Karachaliou, R. Rosell, M.A. Molina-Vila, Large scale, prospective screening of EGFR mutations in the blood of advanced NSCLC patients to guide treatment decisions, Ann. Oncol. 28 (9) (2017) 2248–2255.

[11] S. Laufer-Geva, A.B. Rozenblum, T. Twito, R. Grinberg, A. Dvir, L. Soussan-Gutman, M. Ilouze, L.C. Roisman, E. Dudnik, A. Zer, O. Rotem, R.B. Lanman, N. Peled, The clinical impact of comprehensive genomic testing of circulating cell-free DNA in advanced lung Cancer, J. Thorac. Oncol. 13 (11) (2018) 1705–1716.

[12] N.I. Lindeman, P.T. Cagle, D.L. Aisner, M.E. Arcila, M.B. Beasley, E.H. Bernicker, C. Colasacco, S. Dacic, F.R. Hirsch, K. Kerr, D.J. Kwiatkowski, M. Ladanyi, J.A. Nowak, L. Sholl, R. Temple-Smolkin, B. Solomon, L.H. Souter, E. Thunnissen, M.S. Tsao, C.B. Ventura, M.W. Wynes, Y. Yatabe, Updated molecular testing guideline for the selection of lung Cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the college of american pathologists, the international association for the study of lung Cancer, and the association for molecular pathology, Arch. Pathol. Lab. Med. 142 (3) (2018) 321–346.

[13] L.V. Sequist, J.-Y. Han, M.-J. Ahn, B.C. Cho, H. Yu, S.-W. Kim, J.C.-H. Yang, J.S. Lee, W.-C. Su, D. Kowalski, S. Orlov, M. Cantarini, R.B. Verheijen, A. Mellemgaard, L. Ottesen, P. Frewer, X. Ou, G. Oxnard, Osimertinib plus savolitinib in patients with < em > EGFR < /em > mutation-positive, < em > *MET* < /em > -amplified, non-small-cell lung cancer after progression on EGFR tyrosine kinase inhibitors: interim results from a multicentre, open-label, phase 1b study, Lancet Oncol. 21 (3) (2020) 373–386.

[14] P.L. Chia, H. Do, A. Morey, P. Mitchell, A. Dobrovic, T. John, Temporal changes of EGFR mutations and T790M levels in tumour and plasma DNA following AZD9291 treatment, Lung Cancer 98 (2016) 29–32.

[15] J.J. Chabon, A.D. Simmons, A.F. Lovejoy, M.S. Esfahani, A.M. Newman, H.J. Haringsma, D.M. Kurtz, H. Stehr, F. Scherer, C.A. Karlovich, T.C. Harding, K.A. Durkin, G.A. Otterson, W.T. Purcell, D.R. Camidge, J.W. Goldman, L.V. Sequist, Z. Piotrowska, H.A. Wakelee, J.W. Neal, A.A. Alizadeh, M. Diehn, Circulating tumour DNA profiling reveals heterogeneity of EGFR inhibitor resistance mechanisms in lung cancer patients, Nat. Commun. 7 (2016) 11815.

[16] C.M. Blakely, T.B.K. Watkins, W. Wu, B. Gini, J.J. Chabon, C.E. McCoach, N. McGranahan, G.A. Wilson, N.J. Birkbak, V.R. Olivas, J. Rotow, A. Maynard, V. Wang, M.A. Gubens, K.C. Banks, R.B. Lanman, A.F. Caulin, J. St John, A.R. Cordero, P. Giannikopoulos, A.D. Simmons, P.C. Mack, D.R. Gandara, H. Husain, R.C. Doebele, J.W. Riess, M. Diehn, C. Swanton, T.G. Bivona, Evolution and clinical impact of co-occurring genetic alterations in advanced-stage EGFRmutant lung cancers, Nat. Genet. 49 (12) (2017) 1693–1704.

[17] E.A. Eisenhauer, P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe, J. Verweij, New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1), Eur. J. Cancer 45 (2) (2009) 228–247.

[18] D. Sie, P.J. Snijders, G.A. Meijer, M.W. Doeleman, M.I. van Moorsel, H.F. van Essen, P.P. Eijk, K. Grunberg, N.C. van Grieken, E. Thunnissen, H.M. Verheul, E.F. Smit, B. Ylstra, D.A. Heideman, Performance of amplicon-based next generation DNA sequencing for diagnostic gene mutation profiling in oncopathology, Cell. Oncol. 37 (5) (2014) 353–361.

[19] A. Eijkelenboom, B.B.J. Tops, A. van den Berg, A.J.C. van den Brule, W.N.M. Dinjens, H.J. Dubbink, A. ter Elst, W.R.R. Geurts-Giele, P.J.T.A. Groenen, F.H. Groenendijk, D.A.M. Heideman, M.M.H. Huibers, C.J.J. Huijsmans, J.W.M. Jeuken, L.C. van Kempen, E. Korpershoek, L.I. Kroeze, W.W.J. de Leng, C.J.M. van Noesel, E.-J.M. Speel, M.J. Vogel, T. van Wezel, P.M. Nederlof, E. Schuuring, M.J.L. Ligtenberg, Recommendations for the clinical interpretation and reporting of copy number gains using gene panel NGS analysis in routine diagnostics, Virchows Arch. 474 (6) (2019) 673–680.

[20] D.A. Heideman, F.B. Thunnissen, M. Doeleman, D. Kramer, H.M. Verheul, E.F. Smit, P.E. Postmus, C.J. Meijer, G.A. Meijer, P.J. Snijders, A panel of high resolution melting (HRM) technology-based assays with direct sequencing possibility for effective mutation screening of EGFR and K-ras genes, Cell. Oncol. 31 (5) (2009) 329–333.

[21] C. Keppens, E.M.C. Dequeker, S.J. Patton, N. Normanno, F. Fenizia, R. Butler, M. Cheetham, J.A. Fairley, H. Williams, J.A. Hall, E. Schuuring, Z.C. Deans, I.Q.N.P.A, On behalf of, International pilot external quality assessment scheme for analysis and reporting of circulating tumour DNA, BMC Cancer 18 (1) (2018) 804.

[22] E. Heitzer, I.S. Haque, C.E.S. Roberts, M.R. Speicher, Current and future perspectives of liquid biopsies in genomics-driven oncology, Nat. Rev. Genet. 20 (2) (2019) 71–88.

[23] J.D. Merker, G.R. Oxnard, C. Compton, M. Diehn, P. Hurley, A.J. Lazar, N. Lindeman, C.M. Lockwood, A.J. Rai, R.L. Schilsky, A.M. Tsimberidou, P. Vasalos, B.L. Billman, T.K. Oliver, S.S. Bruinooge, D.F. Hayes, N.C. Turner, Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review, J. Clin. Oncol. 36 (16) (2018) 1631–1641.

[24] B.T. Li, A. Drilon, M.L. Johnson, M. Hsu, C.S. Sima, C. McGinn, H. Sugita, M.G. Kris, C.G. Azzoli, A prospective study of total plasma cell-free DNA as a predictive biomarker for response to systemic therapy in patients with advanced non-small-cell lung cancers, Ann. Oncol. 27 (1) (2016) 154–159.

[25] M.A. van der Drift, B.E.A. Hol, C.H.W. Klaassen, C.F.M. Prinsen, Y.A.W.G. van Aarssen, R. Donders, J.W.J. van der Stappen, P.N.R. Dekhuijzen, H.F.M. van der Heijden, F.B.J.M. Thunnissen, Circulating DNA is a non-invasive prognostic factor for survival in non-small cell lung cancer, Lung Cancer 68 (2) (2010) 283–287.

[26] A. Buder, M.J. Hochmair, S. Schwab, T. Bundalo, P. Schenk, P. Errhalt, R.E. Mikes, G. Absenger, K. Patocka, B. Baumgartner, U. Setinek, O.C. Burghuber, H. Prosch, R. Pirker, M. Filipits, Cell-free plasma DNA-Guided treatment with osimertinib in patients with advanced EGFR-Mutated NSCLC, J. Thorac. Oncol. 13 (6) (2018) 821–830.

[27] G.R. Oxnard, Y. Hu, K.F. Mileham, H. Husain, D.B. Costa, P. Tracy, N. Feeney, L.M. Sholl, S.E. Dahlberg, A.J. Redig, D.J. Kwiatkowski, M.S. Rabin, C.P. Paweletz, K.S. Thress, P.A. Janne, Assessment of resistance mechanisms and clinical implications in patients with EGFR T790M-Positive lung Cancer and acquired resistance to osimertinib, JAMA Oncol. 4 (11) (2018) 1527–1534.

[28] S.I. Ou, N. Agarwal, S.M. Ali, High *MET* amplification level as a resistance mechanism to osimertinib (AZD9291) in a patient that symptomatically responded to crizotinib treatment post-osimertinib progression, Lung Cancer 98 (2016) 59–61.

[29] Z. Yang, N. Yang, Q. Ou, Y. Xiang, T. Jiang, X. Wu, H. Bao, X. Tong, X. Wang, Y.W. Shao, Y. Liu, Y. Wang, C. Zhou, Investigating Novel Resistance Mechanisms to Third-Generation EGFR Tyrosine Kinase Inhibitor Osimertinib in Non-Small Cell Lung Cancer Patients, Clin. Cancer Res. 24 (13) (2018) 3097–3107.

[30] K.S. Thress, C.P. Paweletz, E. Felip, B.C. Cho, D. Stetson, B. Dougherty, Z. Lai, A. Markovets, A. Vivancos, Y. Kuang, D. Ercan, S.E. Matthews, M. Cantarini, J.C. Barrett, P.A. Janne, G.R. Oxnard, Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M, Nat. Med. 21 (6) (2015) 560–562.

[31] X. Le, S. Puri, M.V. Negrao, M. Nilsson, J.P. Robichaux, T.A. Boyle, J.K. Hicks, K. Lovinger, E.B. Roarty, W. Rinsurongkawong, M. Tang, H. Sun, Y.Y. Elamin, L. Lacerda, J. Lewis, J.J. Lee, J.A. Roth, S.G. Swisher, J. Zhang, W.N. William, B.S. Glisson, V.A. Papadimitrakopoulou, J.E. Gray, J.V. Heymach, Landscape of EGFR -dependent and -independent resistance mechanisms to osimertinib and continuation therapy post-progression in EGFR-mutant NSCLC, Clin. Cancer Res. (2018) clincanres.1542.2018..

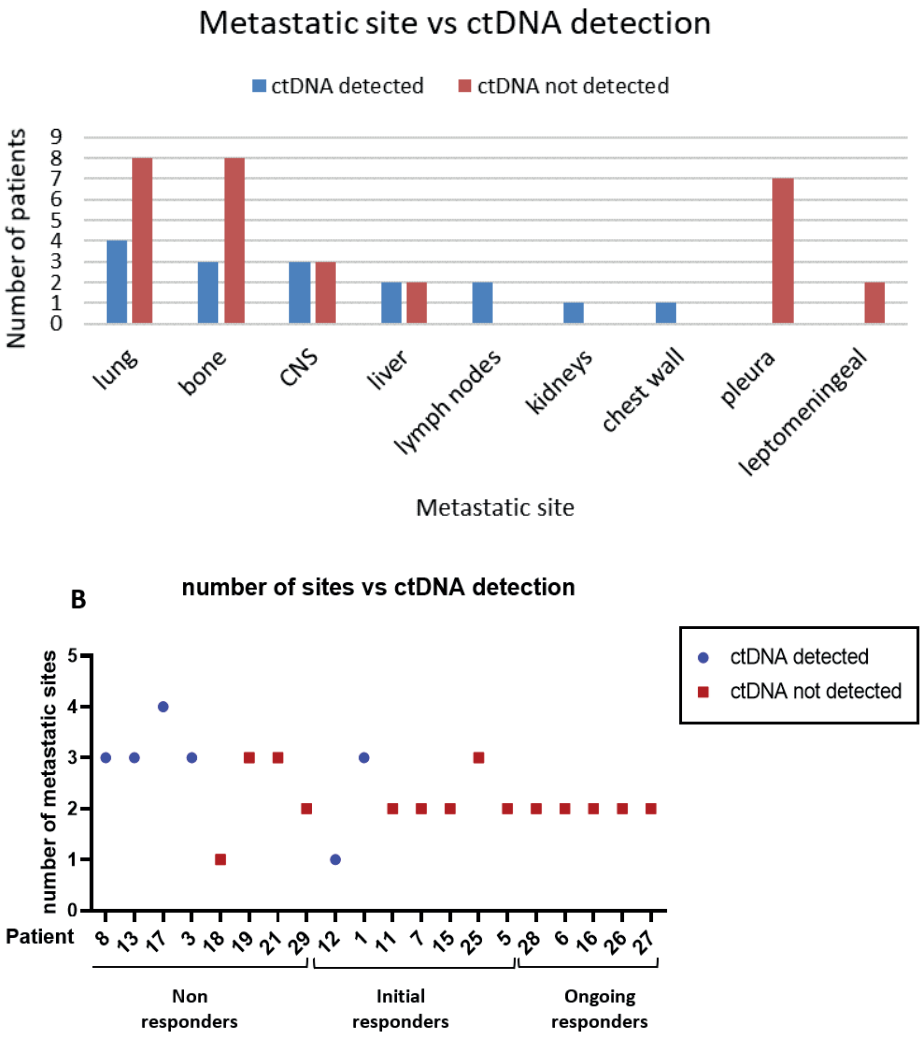
[32] J. Remon, C. Caramella, C. Jovelet, L. Lacroix, A. Lawson, S. Smalley, K. Howarth, D. Gale, E. Green, V. Plagnol, N. Rosenfeld, D. Planchard, M.V. Bluthgen, A. Gazzah, C. Pannet, C. Nicotra, E. Auclin, J.C. Soria, B. Besse, Osimertinib benefit in EGFRmutant NSCLC patients with T790M-mutation detected by circulating tumour DNA, Ann. Oncol. 28 (4) (2017) 784–790.

[33] C. Bettegowda, M. Sausen, R.J. Leary, I. Kinde, Y. Wang, N. Agrawal, B.R. Bartlett, H. Wang, B. Luber, R.M. Alani, E.S. Antonarakis, N.S. Azad, A. Bardelli, H. Brem, J.L. Cameron, C.C. Lee, L.A. Fecher, G.L. Gallia, P. Gibbs, D. Le, R.L. Giuntoli, M. Goggins, M.D. Hogarty, M. Holdhoff, S.M. Hong, Y. Jiao, H.H. Juhl, J.J. Kim, G. Siravegna, D.A. Laheru, C. Lauricella, M. Lim, E.J. Lipson, S.K. Marie, G.J. Netto, K.S. Oliner, A. Olivi, L. Olsson, G.J. Riggins, A. Sartore-Bianchi, K. Schmidt, M. Shih l, S.M. Oba-Shinjo, S. Siena, D. Theodorescu, J. Tie, T.T. Harkins, S. Veronese, T.L. Wang, J.D. Weingart, C.L. Wolfgang, L.D. Wood, D. Xing, R.H. Hruban, J. Wu, P.J. Allen, C.M. Schmidt, M.A. Choti, V.E. Velculescu, K.W. Kinzler, B. Vogelstein, N. Papadopoulos, L.A. Diaz Jr., Detection of circulating tumor DNA in early- and late-stage human malignancies, Sci. Transl. Med. 6 (224) (2014) 224ra24.

[34] J.C. Soria, Y. Ohe, J. Vansteenkiste, T. Reungwetwattana, B. Chewaskulyong, K.H. Lee, A. Dechaphunkul, F. Imamura, N. Nogami, T. Kurata, I. Okamoto, C. Zhou, B.C. Cho, Y. Cheng, E.K. Cho, P.J. Voon, D. Planchard, W.C. Su, J.E. Gray, S.M. Lee, R. Hodge, M. Marotti, Y. Rukazenkov, S.S. Ramalingam, Osimertinib in untreated EGFR-Mutated advanced non-small-Cell lung Cancer, N. Engl. J. Med. 378 (2) (2018) 113–125. J

Supplementary data

Supplementary Figure 1



Supplementary table 1. Pathological data relating to the intake and follow-up biopsy of each patient in the study. Patients are grouped by response category. Missing data, or a negative result in the case of gene amplification, are indicated by a dash. VAF, Variant Allele Frequency; NGS, Next Generation Sequencing; HRM, High Resolution Melting; ddPCR, droplet digital PCR.

Non-responders										
P-number	**Biopsy**	**Analysis technique**	**Tumour cell percentage**	**EGFR-TKI sensitising mutation**	**VAF (%)**	**EGFR-TKI resistance mutation**	**VAF(%)**	**Other mutations**	**VAF (%)**	**Gene amplification**
8	Intake	NGS	-	Exon 19 del (not specified)	-	c.2369C>T; p.(Thr790Met)	0.32	-	-	*ERBB2*
	Follow-up	NGS	>50	c.2240_2257del; p.(Leu747_Pro753delinsSer)	44	-	-	-	-	*ERBB2*
13	Intake	NGS	75	c.2235_2249del; p.(Glu746_Ala750del)	-	c.2369C>T; p.(Thr790Met)	-	*BRAF* c.1799T>A; p.(Val600Glu)*	-	*EGFR*
	Follow-up	NGS	20	c.2235_2249del; p.(Glu746_Ala750del)	95	-	-	-	-	*EGFR* *MET*
17	Intake	HRM	10-20	c.2239_2253del; p.(Leu747_Thr751del)	-	c.2369C>T; p.(Thr790Met)	-	-	-	-
	Follow-up	NGS	20	c.2239_2253del; p.(Leu747_Thr751del)	54	-	-	-	-	*MET*
3	Intake	NGS	80	c.2573T>G; p.(Leu858Arg)	66	c.2369C>T; p.(Thr790Met)	7	-	-	*EGFR*
	Follow-up	NGS	>40	c.2573T>G; p.(Leu858Arg)	70	-	-	-	-	*EGFR*
18	Intake	NGS	30	c.2240_2257del; p.(Leu747_Pro753delinsSer)	47	c.2369C>T; p.(Thr790Met)	13	-	-	-
	Follow-up	NGS	50	c.2240_2257del; p.(Leu747_Pro753delinsSer)	38	c.2369C>T; p.(Thr790Met)	16	-	-	-

Non-responders

P-number	Biopsy	Analysis technique	Tumour cell percentage	EGFR-TKI sensitising mutation	VAF (%)	EGFR-TKI resistance mutation	VAF(%)	Other mutations	VAF (%)	Gene amplification
19	Intake	NGS	-	c.2235_2249del; p.(E746_A750del)	-	c.2369C>T; p.(Thr790Met)	-	-	-	-
	Follow-up	HRM	40	c.2235_2249del; p.(Glu746_Ala750del)	-	c.2369C>T; p.(Thr790Met)	-	-	-	-
21	Intake	NGS	70	c.2240_2254del; p.(Leu747_Thr751del)	22	c.2369C>T; p.(Thr790Met)	8	-	-	-
	Follow-up	NGS	40	c.2240_2254del; p.(Leu747_Thr751del)	58	-	-	-	-	*MET*
29	Intake	NGS	60	c.2573T>G; p.(Leu858Arg)	91	c.2369C>T; p.(Thr790Met)	63	-	-	*EGFR*
	Follow-up	NGS	10	c.2573T>G; p.(Leu858Arg)	75	-	-	-	-	*EGFR*

Initial-responders

P-number	Biopsy	Analysis technique	Tumour cell percentage	EGFR-TKI sensitising mutation	VAF (%)	Other EGFR mutation	VAF (%)	Other mutations	VAF (%)	Gene amplification
12	Intake	NGS	70	c.2573T>G; p.(Leu858Arg)	-	c.2369C>T; p.(Thr790Met)	-	*EGFR* c.865G>A; p.(Ala289Thr)*	-	-
	Follow-up	NGS	>20	c.2573T>G; p.(Leu858Arg)	92	c.2369C>T; p.(Thr790Met)	4	*EGFR* c.865G>A; p.(Ala289Thr)*	91	*EGFR* *ERBB2*
1	Intake	NGS	10-30	c.2237_2257delinsTCT; p.(Glu746_ Pro753delinsValSer)*	38	c.2369C>T; p.(Thr790Met)	4	-	-	-
	Follow-up	NGS	20	c.2237_2257delinsTCT; p.(Glu746_ Pro753delinsValSer)	57	c.2369C>T; p.(Thr790Met)	7	*EGFR* c.2390G>C; p.(Cys797Ser) *in cis**	7	-
11	Intake	cfDNA ddPCR	-	c.2573T>G; p.(Leu858Arg)	-	c.2369C>T; p.(Thr790Met)	-	-	-	-

Initial-responders										
P-number	**Biopsy**	**Analysis technique**	**Tumour cell percentage**	**EGFR-TKI sensitising mutation**	**VAF (%)**	**Other EGFR mutation**	**VAF (%)**	**Other mutations**	**VAF (%)**	**Gene amplification**
	Follow-up	NGS	35	c.2573T>G; p.(Leu858Arg)	42	c.2239T>G; p.(Leu747Val)*	42	*BRAF* c.1799_1800delinsAA; p.(Val600Glu)*	16	-
7	Intake 1	HRM	50	c.2240_2254del; p.(Leu747_Thr751del)	-	-	-	-	-	-
	Intake 2	cfDNA ddPCR	-	-	-	c.2369C>T; p.(Thr790Met)	-	-	-	-
	Follow-up	NGS	40	c.2240_2254del; p.(Leu747_Thr751del)	24	c.2369C>T; p.(Thr790Met)	13	-	-	-
15	Intake	HRM	50	c.2573T>G; p.(Leu858Arg)	-	c.2369C>T; p.(Thr790Met)	-	-	-	-
	Follow-up	NGS	67	c.2573T>G; p.(Leu858Arg)	87	c.2369C>T; p.(Thr790Met)	19	*EGFR* c.2389T>A; p.(Cys797Ser) *in cis**	5	*EGFR*
								EGFR c.2153T>A; p.(Leu718Gln)*	6	
25	Intake	NGS	>20	c.2236_2250del; p.(Glu746_Ala750del)	70	c.2369C>T; p.(Thr790Met)	11	-	-	-
	Follow-up	NGS	50	c.2236_2250del; p.(Glu746_Ala750del)	-	c.2369C>T; p.(Thr790Met)	-	-	-	*EGFR*
5	Intake	NGS	70	c.2235_2249del; p.(Glu746_Ala750del)	-	c.2369C>T; p.(Thr790Met)	-	-	-	-
	Follow-up	NGS	30	c.2235_2249del; p.(Glu746_Ala750del)	51	c.2369C>T; p.(Thr790Met)	23	*EGFR* c.2389T>A; p.(Cys797Ser) *in cis**	12	-

Ongoing-responders										
P-number	**Biopsy**	**Analysis technique**	**Tumour cell percentage**	**EGFR-TKI sensitising mutation**	**VAF (%)**	**Other EGFR mutation**	**VAF (%)**	**Other mutations identified**	**VAF (%)**	**Gene amplification**
28	Intake	NGS	40	c.2235_2249del; p.(Glu746_Ala750del)	83	c.2369C>T; p.(Thr790Met)	10	-	-	*EGFR*
	Follow-up	NGS	50	c.2235_2249del; p.(Glu746_Ala750del)	51	-	-	-	-	*ERBB2*
6	Intake	NGS	40	c.2236_2250del; p.(Glu746_Ala750del)	18	c.2369C>T; p.(Thr790Met)	9	-	-	-
	Follow-up	NGS	20	c.2236_2250del; p.(Glu746_Ala750del)	32	c.2369C>T; p.(Thr790Met)	7	-	-	*FGFR1*
16	Intake	NGS	10	c.2235_2249del; p.(Glu746_Ala750del)	21	c.2369C>T; p.(Thr790Met)	7	-	-	-
	Follow-up	NA	NA	NA	NA	NA	NA	NA	NA	NA
26	Intake	NGS	30	c.2237_2251; p.(Glu746_Thr751delinsAla)*	34	c.2369C>T; p.(Thr790Met)	11	-	-	-
	Follow-up	NA	NA	NA	NA	NA	NA	NA	NA	NA
27	Intake	NGS	20-40	c.2573T>G; p.(Leu858Arg)	46	c.2369C>T; p.(Thr790Met)	44	-	-	-
	Follow-up	NGS	20	c.2573T>G; p.(Leu858Arg)	-	c.2369C>T; p.(Thr790Met)	-	*EGFR* c.2153T>G; p.(Leu718Arg)*	-	*EGFR*

***** Mutation not tested in cfDNA

Supplementary materials

ddPCR kits used in the investigation

EGFR c.2369C>T; p.(Thr790Met)
Reference: dHsaCP2000019
EGFR c.2573T>G; p.(Leu858Arg)
Reference: dHsaCP2000021
EGFR c.2235_2249del; p.(Glu746_Ala750del)
Reference: dHsaCP2000039
EGFR Exon 19 Deletions Screening Kit
Reference: 12002392

Contains probes for the following mutations in exon 19:
2235_2252>AAT (dHsaMDS391737271)
2235_2249del15 (dHsaMDV2010039)
2236_2250del15 (dHsaMDS542127747)
2238_2252>GCA (dHsaMDS435546404)
2238_2255del18 (dHsaMDS529140399)
2239_2253>CAA (dHsaMDS703176693)
2239_2251>C (dHsaMDV2516890)
2239_2258>CA (dHsaMDS196056967)
2239_2252>CA (dHsaMDS651224081)
2239_2256del18 (dHsaMDV2516758)
2239_2248TTAAGAGAAG>C (dHsaMDV2516748)
2239_2253del15 (dHsaMDV2516752)
2239_2247delTTAAGAGAA (dHsaMDS88236242)
2240_2254del15 (dHsaMDS778667043)
2240_2257del18 (dHsaMDV2510546)

Chapter 5

PCR-Free Shallow Whole Genome Sequencing for Chromosomal Copy Number Detection from Plasma of Cancer Patients Is an Efficient Alternative to the Conventional PCR-Based Approach

Jamie J Beagan
Esther E E Drees
Phylicia Stathi
Paul P Eijk
Laura Meulenbroeks
Floortje Kessler
Jaap M Middeldorp
D Michiel Pegtel
Josée M Zijlstra
Daoud Sie
Daniëlle A M Heideman
Erik Thunnissen
Linda Smit
Daphne de Jong
Florent Mouliere
Bauke Ylstra
Margaretha G M Roemer*
Erik van Dijk*

**Contributed equally as senior authors*

J Mol Diagn. 2021 Nov;23(11):1553-1563.

Abstract

Somatic copy number alterations can be detected in cell-free DNA (cfDNA) by shallow whole genome sequencing (sWGS). PCR is typically included in library preparations, but a PCR-free method could serve as a high-throughput alternative. To evaluate a PCR-free method for research and diagnostics, archival peripheral blood or bone marrow plasma samples, collected in EDTA- or lithium-heparin-containing tubes, were collected from patients with non-small-cell lung cancer (n = 10 longitudinal samples; 4 patients), B-cell lymphoma (n = 31), and acute myeloid leukemia (n = 15), or from healthy donors (n = 14). sWGS was performed on PCR-free and PCR library preparations, and the mapping quality, percentage of unique reads, genome coverage, fragment lengths, and copy number profiles were compared. The percentage of unique reads was significantly higher for PCR-free method compared with PCR method, independent of the type of collection tube: EDTA PCR-free method, 96.4% (n = 35); EDTA PCR method, 85.1% (n = 32); heparin PCR-free method, 94.5% (n = 25); and heparin PCR method, 89.4% (n = 10). All other evaluated metrics were highly comparable for PCR-free and PCR library preparations. These results demonstrate the feasibility of somatic copy number alteration detection by PCR-free sWGS using cfDNA from plasma collected in EDTA- or lithium-heparin-containing tubes and pave the way for an automated cfDNA analysis workflow for samples from cancer patients.

Introduction

Somatic copy number alterations (SCNAs) drive a wide range of cancer types and are utilized as molecular markers.[1,2] Next to mutation analysis, specific chromosomal SCNAs are used in patient selection for targeted therapies, such as an amplification of chromosome *17q/ERBB2* for trastuzumab/pertuzumab combination therapy in breast cancer.[3] More recently, amplification of chromosome *9p/PDL1* has become a potential marker for responses to immune checkpoint inhibition,[4-6] and loss of chromosome *18q* was suggested as a marker for anti-angiogenic treatment in metastatic colorectal cancer patients.[7] Other SCNAs offer diagnostic or prognostic value, including loss of chromosome *20q* to diagnose breast implant–associated anaplastic large cell lymphoma,[8] or amplification of chromosome *2p/MYCN*, which is associated with poor prognosis in neuroblastoma.[9]

Genome-wide SCNA analysis is typically performed on DNA from tumor tissue biopsies using microarrays or shallow whole genome sequencing (sWGS).[10,11] Unfortunately, tumor tissue biopsies are invasive and carry risks associated with surgical procedures. For patients with a hematological malignancy, an alternative source of tumor DNA is bone marrow plasma, obtained by aspiration. Peripheral blood can offer a more accessible source of tumor DNA, and collection greatly reduces the burden on the patient.

A major challenge to reliably detect SCNAs from cell-free DNA (cfDNA) is the fraction of tumor DNA in the blood plasma of cancer patients.[12] Unlike solid tissue analysis, where the tumor cell fraction can be enriched before DNA isolation,[13] tumor and nontumor cfDNA fractions in blood are intermixed and highly variable, often with a low yield.[12,14] As circulating tumor DNA (ctDNA) in blood tends to be shorter (median length, 134 to 144 bp) than cfDNA from healthy cells (median length, 166 bp),[15] selection of 90- to 150-bp fragments has the potential to increase the sensitivity and specificity of SCNA detection.[16] Size selection can be performed either physically before sWGS, using *in vitro* gel electrophoresis to separate fragments by length, or after sWGS, using *in silico* selection of sequencing reads.[16]

The consensus approach to blood collection for cfDNA analysis is to use standard EDTA-containing tubes, or tubes containing a specialized preservative. Plasma is separated from the blood and stored at –80°C before cfDNA isolation.[17] The subsequent sequencing library preparation typically includes a PCR step to increase the quantity of DNA for sWGS.[18] Blood tubes containing lithium or sodium-heparin are also widely used to collect samples for molecular diagnostic assays but are generally considered unsuitable for sWGS because of an inhibitory effect of heparin on PCR.[19] Additional pre-analytical processing or selection of specific polymerases can be required to overcome this inhibition.[19,20] It has been suggested that PCR could be omitted from library preparation while maintaining the capability to perform sWGS in cancer samples.[21,22]

A PCR-free sWGS workflow has recently been implemented nationwide in the Netherlands to screen for fetal copy number alternations in the peripheral blood of pregnant women.[23] This approach, referred to as noninvasive prenatal testing (NIPT), can determine if a fetus has specific germline copy number alterations. This has led to the incidental detection of SCNAs and a subsequent diagnosis of cancer in the mother.[24] Application of the NIPT workflow using blood from cancer patients could theoretically serve as a sensitive method for earlier cancer detection, treatment response monitoring, and residual disease detection.[25-28] Moreover, sWGS can detect and estimate the fraction of SCNAs in cfDNA.[29] The feasibility of PCR-free sWGS has yet to be evaluated in the oncology setting, where patients could benefit from timely SCNA detection to inform treatment decisions.

In this study, the performance of PCR and PCR-free sWGS library preparations of cfDNA from peripheral blood was compared to evaluate the applicability of PCR-free preparations in the diagnostic setting. In addition, the feasibility of PCR-free preparations to detect SCNAs for research purposes was evaluated on archived plasma collected in EDTA- or heparin-containing tubes.

Materials and Methods

Patient Samples

Plasma samples were selected on the basis of availability in the biobank. A total of 10 longitudinal plasma samples from peripheral blood of four non–small-cell lung cancer (NSCLC) patients, collected in EDTA-containing tubes at various time points over the course of their treatment, were obtained from the biobank of Amsterdam University Medical Center, location Vrije Universiteit medical center. One sample per subject was collected in all other cases. Blood samples from seven healthy donors were collected, pooled, and then split to yield sufficient material for four technical replicates. Samples from 10 other healthy donors were obtained from an external blood bank and analyzed individually. In addition, plasma samples from 25 B-cell lymphoma patients were collected: 15 in EDTA-containing tubes and 10 in heparin-containing tubes. Matched formalin-fixed, paraffin-embedded (FFPE) tissue was collected from all 4 NSCLC patients and 6 of 25 B-cell lymphoma patients. Finally, 15 plasma samples derived from bone marrow (n = 13) or peripheral blood (n = 2) were collected from patients with acute myeloid leukemia (AML) (Figure 1A).

All patients provided informed consent for their samples and clinical data to be used for research purposes. B-cell lymphoma samples collected in EDTA were part of the BioLymph study (VUmc *MET*c registration number 2017.008; Dutch CCMO registration NL60245.029.17), performed in accordance with the Declaration of Helsinki (seventh revision, October 2013) and the Medical Research Involving Human Subjects Act. B-cell lymphoma samples in heparin were collected in accordance with the Declaration of Helsinki (fifth revision, 2000) as part of the biobank, Amsterdam UMC, location VUmc. Informed consent from the 4 NSCLC patients and the 7 healthy donors who donated samples to the biobank was provided through the Liquid Biopsy Center, Amsterdam UMC, location VUmc, and from the 10 healthy blood donors through the national blood bank. Surplus diagnostic material from AML patients was provided by the Hematology Department, Amsterdam UMC, location VUmc, in accordance with the Human Tissue and Medical Research Code of Conduct for Responsible Use (2011).

Blood Collection

Blood in EDTA Tubes

Peripheral blood was collected in clinical-grade EDTA-containing blood tubes at unspecified quantities (Becton Dickinson, Franklin Lakes, NJ). Plasma was separated from the cellular components of whole blood on the day of collection by centrifugation at 900 × g for 7 minutes at room temperature; the upper layer was transferred to a clean tube, centrifuged at 2500× g for 10 minutes, transferred again, and then centrifuged at 500 × g for 10 minutes. The final centrifugation step

was omitted for the B-cell lymphoma samples, as this step did not affect yield and sequencing results. Plasma was stored in aliquots of up to 1.5 mL at -80°C for up to a year from healthy donors, up to 3 years from B-cell lymphoma patients, or up to 4 years from NSCLC patients. All plasma samples from blood collected in EDTA tubes were thawed and centrifuged at 16,000 × g for 10 minutes before cfDNA isolation.

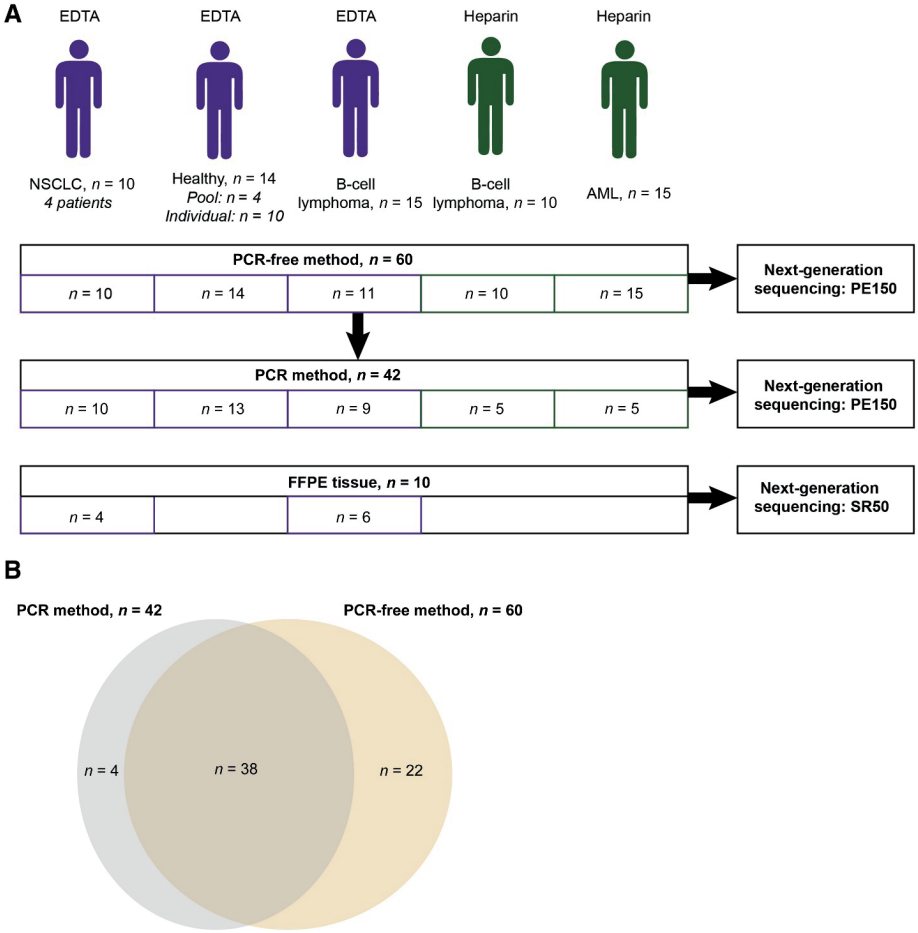


Figure 1. Summary of samples and analysis methods. A: Overview of samples and subjects in the study categorized by cancer type and including sample number, anticoagulant in blood collection tube, PCR or PCR-free library preparation method, next-generation sequencing method, and whether formalin-fixed, paraffin-embedded (FFPE) sample was also analyzed. B: Venn diagram, indicating the overlap between PCR and PCR-free samples. Thirty-eight samples underwent both PCR and PCR-free workflows. AML, acute myeloid leukemia; CCMO, Central Committee on Research Involving Human Subjects; *METc*, Medical Ethical Testing Committee; NSCLC, non–small-cell lung cancer; PE, paired end; SR, single read.

Blood and Bone Marrow in Lithium-Heparin Tubes

Peripheral blood or bone marrow was collected from B-cell lymphoma and AML patients in clinical-grade lithium-heparin–containing blood tubes (Becton Dickinson) at unspecified quantities. Bone marrow was collected by aspiration. Plasma was separated from whole blood or bone marrow by centrifugation at 900 × g for 7 minutes at room temperature, transferred to a clean tube, and centrifuged again at 2500 × g for 15 minutes, then split in to 1-mL aliquots and stored between 4 and 9 years at –80°C. Peripheral blood was collected from B-cell lymphoma patients in clinical-grade collection tubes and initially diluted in a 1:1 ratio with phosphate-buffered saline, then centrifuged with Lymphoprep density gradient media (Stemcell Technologies, Vancouver, BC, Canada) for 15 minutes at 1000 × g at room temperature. The diluted plasma was transferred to 10- or 50-mL tubes and stored between 12 and 15 years at –20°C.

cfDNA Isolation and Quantification

Samples Collected in EDTA-Containing Tubes

Plasma from blood collected in EDTA-containing tubes underwent cfDNA isolation using the QIAsymphony Circulating DNA Kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). For the healthy controls and NSCLC samples, 3 mL of plasma was used. For B-cell lymphoma samples, between 1 and 2 mL of plasma was diluted up to a volume of 3 mL with phosphate-buffered saline before cfDNA isolation.

Samples Collected in Lithium-Heparin–Containing Tubes

AML plasma samples were thawed and diluted with phosphate-buffered saline to 3 mL from a starting volume of 1 to 3 mL. For B-cell lymphoma samples, between 3 and 5.5 mL of the 1:1 plasma/phosphate-buffered saline was used. Because of larger storage volumes, cfDNA was isolated using the QIAamp Circulating nucleic acid kit (Qiagen), according to the manufacturer's protocol. To compare the performance of this column-based protocol to the bead-based protocol described above, two B-cell lymphoma samples were isolated and subsequently sequenced after both isolation methods (Supplemental Figure S1A).

FFPE Tissue

Genomic DNA from FFPE tissue samples was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen), following the manufacturer's protocol. Isolated DNA was measured by fluorometric quantification using the Qubit Double Stranded DNA High Sensitivity Assay Kit (ThermoFisher Scientific, Waltham, MA).

Sequencing Library Preparation

The concentration of all cfDNA samples was measured using the Qubit system before sequencing library preparation. Two frequently used library preparation methods were employed: TruSeq DNA PCR-Free Low Throughput Library Prep Kit (Illumina, San Diego, CA) and KAPA HTP Library Preparation Kit (Roche, Basel, Switzerland), following manufacturer's protocols. A direct comparison of both protocols was performed on a healthy pool and two B-cell lymphoma samples to confirm similar performance (Supplemental Figure S1B). For all 38 samples that were analyzed with both PCR and PCR-free methods (Figure 1B), a single library preparation was performed and then split. One part of the library was sequenced directly, and the other part was sequenced following a PCR step, whereby 1 to 2 ng of the preparation was subjected to seven cycles of PCR. An additional 22 samples underwent only a PCR-free preparation, followed directly by sequencing. For another four samples, only sequencing of the PCR library was performed (Figure 1B). A median of 20 ng (SD, 9.4 ng; range, 3.7 to 40.5 ng) cfDNA was used as input for all library preparations. The cfDNA input and library preparation protocol for each sample are specified in Supplemental Table S1.

PCR library preparations were measured using the Qubit system and the Agilent High Sensitivity D1000 ScreenTape System (Agilent, Santa Clara, CA) to determine the concentration and approximate cfDNA fragment length. The concentration and proportion of cfDNA fragments with ligated adapters in PCR-free library preparations were measured using the qubit system and the droplet digital PCR library quantification kit for Illumina TruSeq (catalog number 1863040; BioRad, Hercules, CA), following the manufacturer's protocol.

Equimolar sequencing pools of 8 to 15 PCR-free library preparations were generated and diluted to 10 μL in a final target concentration of 3 nmol/L (range, 1 to 13 nmol/L). The PCR products were combined into 25-μL, 6 to 10 nmol/L, pools of 13 to 27 samples. All pools were measured with the Qubit system and underwent a paired-end 150 sequencing protocol on a separate flow cell lane of an Illumina HiSeq4000 sequencer (Figure 1A).

Isolated DNA from FFPE material was sheared on a Covaris ME220 Focused-Ultrasonicator (Covaris Inc., Woburn, MA) and then measured on a Tapestation (Agilent Technologies, Santa Clara, CA). Library preparation was performed using TruSeq or KAPA library preparation kits, as described above, and subjected to a single-read 50 sequencing protocol on an Illumina HiSeq4000 sequencer (Figure 1A).

Data Analysis

Sequencing read adapter trimming was performed using Cutadapt version 1.9.1. Reads were then aligned to genome build hg19 using BWA mem version 0.7.10, and duplicates were marked using Picardtools version 1.111. The percentage of unique and aligned reads was generated with Samtools version 0.1.19. The following sequencing quality metrics were assessed with FastQC version 0.11.2 and MultiQC version 1.7: i) Q37, a measure of mapping quality representing the percentage of reads correctly mapped to the genome with a probability of 99.98% estimated by BWA; ii) G-C base pair content, the percentage of the total base calls across all reads that are either a G or C; iii) adapter content, the proportion of reads that match known adapter sequences; and iv) overrepresented sequences, those that make up >0.1% of the total.

Copy number profiles were generated using the default version of QDNAseq version 1.1.[12,11] including all unique reads with mapping quality of at least 37. Size selected profiles were generated *in silico* using an adapted version of QDNAseq (https://github.com/edk360/QDNAseq, last accessed June 10, 2021) that contained additional code to select only reads mapped to the genome in the correct orientation relative to each other (properly mapped), with an insert size from 90 to 150 bp. The fragment length plots used the same sequencing read inclusion criteria as the size-selected copy number profiles. Sequencing noise was calculated in QDNAseq as the SD of the difference between log2 values between neighboring bins. Downstream analysis and visualization of the following were performed with R (https://www.r-project.org/v3.6.1, last accessed July 5, 2019): unique versus total sequencing reads, G-C base pair content, fragment length distribution, and plots of the sequencing noise. Comparison of the percentage of the total reads that were unique (ie, not sequencing duplicates), between PCR and PCR-free samples, was performed in R using a two-sided Wilcoxon test.

Quantification of Detection Limit of SCNAs

The minimal detectable ctDNA fraction with PCR and PCR-free library preparations was quantified by *in silico* dilution of two liquid biopsy samples with a high ctDNA fraction (Lung_Cancer_1 and B-Cell Lymphoma_20). The ploidy of these two malignancies was estimated by fitting copy number profiles from matching FFPE samples with ACE version 1.10.0. For the B-cell lymphoma sample, fluorescence *in situ* hybridization analysis was performed to verify the results.30 The ctDNA fraction of the liquid biopsy samples was estimated by the function squaremodel with the following parameter settings: penalty, 0.5; penploidy, 0.5; and default settings for all other parameters. The ploidies obtained from the FFPE samples were used to select the correct fit for the ctDNA fraction.

Reads from these samples were mixed with reads from age-matched healthy donors to generate simulated samples with varying proportions of ctDNA (1% to 20%). The total number of reads of the simulated samples were matched to the tumor samples. Variability of the normalized segment value was quantified by analyzing all healthy donors. A linear fit was performed of the normalized read counts of chromosomes 7 and 12 of the B-cell lymphoma sample and chromosomes 10 and 14 of the lung cancer sample, as whole-arm SCNAs in these regions were also identified in matched FFPE samples. Z-scores were calculated for each ctDNA fraction. An alteration was called if the Z-score was >5. This conservative cutoff was used to account for our relatively small number of healthy samples.[31]

Results

PCR-Free Method Offers Similar Quality and More Unique Reads than PCR Method

The mapping quality of sequencing data was highly comparable between the 32 EDTA samples that underwent PCR and the 35 samples that underwent PCR-free library preparations, as demonstrated by the Q37 scores (PCR method, 92.5%; SD, 0.36%; PCR-free method, 93.1%; SD, 0.30%), and the percentage of reads aligned to the genome (PCR method, 99.1%; SD, 0.36%; PCR-free method, 99.1%; SD, 0.27%). After alignment of sequencing reads to the genome, the percentage of unique reads was significantly higher using the PCR-free method (96.4%) compared with the PCR approach (85.1%; $P < 0.001$) (Figure 2A). To generate the required number of reads, a higher input of cfDNA molecules may be required for the PCR-free approach. However, it was possible to obtain sufficient sequencing results with a cfDNA library preparation input of only 3.7 ng (median, 9.9 ng; range, 3.7 to 40.5 ng) for the PCR-free protocol (Supplemental Table S1).

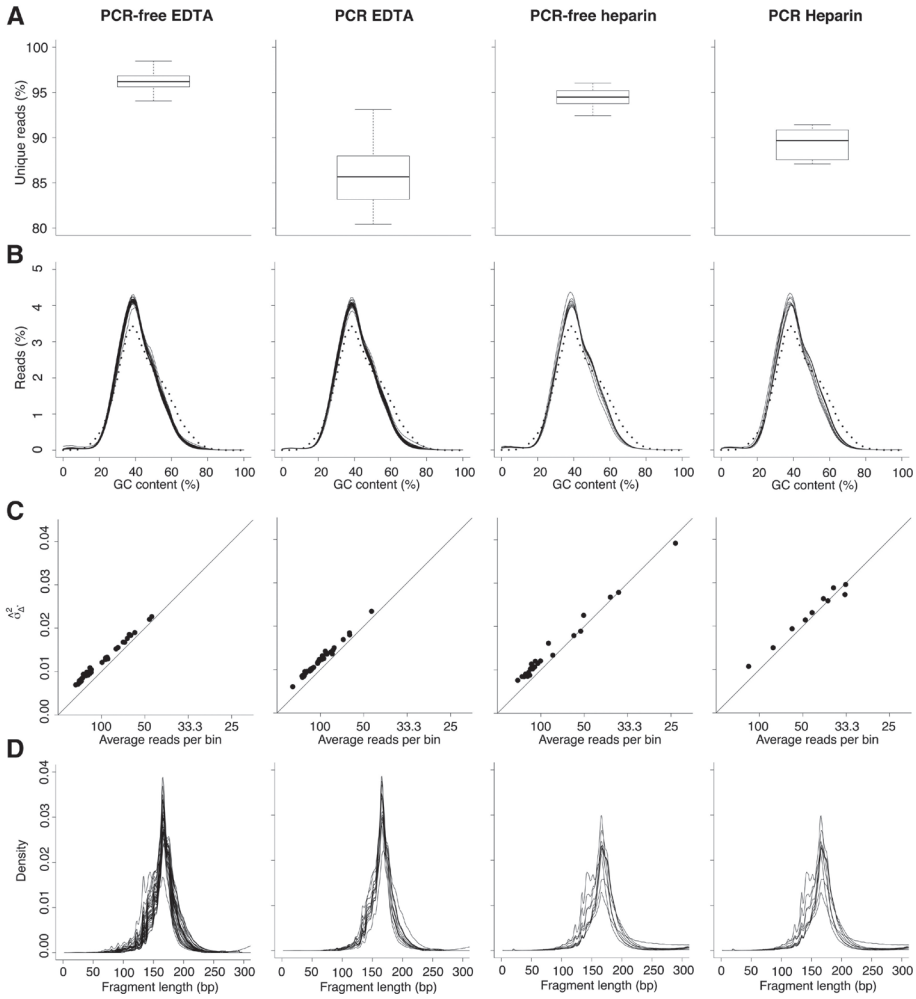


Figure 2. Quality control metrics for each sample group. A: Percentage of unique reads. B: GC content: the percentage of reads (y axis) that contain a given GC content (x axis) per sample (individual lines). The dotted line represents the expected GC content based on reference data. C: Sequencing noise: the SD of the difference between log2 values between neighboring bins (y axis; σˆ2Δ) relative to the number of reads per bin (x axis). D: Fragmentation: overlays of the density (y axis) of sequencing reads of a given length (x axis).

Genome-Wide Representation of PCR-Free Preparation Is Highly Comparable to PCR Library Preparations

The G-C base pair content of aligned sequencing reads was highly comparable between the 32 EDTA samples that underwent PCR library preparations (mean, 41.4%; SD, 0.32%) and the 35 samples that underwent PCR-free library preparations (mean, 41.1%; SD, 0.40%), which indicates that the omission of PCR does not affect the genome-wide base pair representation (Figure 2B). This is an important consideration because sequences with a high G-C content can be underrepresented in PCR-based library preparations. The higher energy input required to break the bonds between G-C pairs during PCR can result in less efficient amplification compared with A-T.32 Furthermore, no significant difference in sequencing noise was observed between the PCR and PCR-free EDTA samples [median observed noise (σˆ2Δ) was 0.0104 and 0.0103, respectively; P = 0.54, Wilcoxon test] (Figure 2C), a further indication of an even post-alignment genome coverage.

There was no discernible difference in the sequencing fragment size between EDTA samples that underwent a PCR- or PCR-free library preparation (Figure 2D). This is relevant to cfDNA analysis because size selection could allow enrichment of the tumor fraction based on its shorter length compared with background cfDNA.[16] PCR has been shown to preferentially amplify shorter fragments,[32] potentially overrepresenting this fraction in a sequencing library. Our results show that fragment size analysis, the majority of which has been conducted using a PCR-based approach, can be performed equally well on data from PCR-free sequencing without any corrections to the analysis methods.

PCR-Free Copy Number Profiles Show Similar Deflection Compared with PCR

SCNAs are inferred by significant deflection of segmented values from the zero line in a copy number profile. A high deflection indicates a high ctDNA fraction in the sample. As deflection is influenced by noise and biases in the sequencing, differences between the relative amount of deflection between identical samples analyzed by PCR or PCR-free method can be used to assess the quality of copy number profiles. The deflection observed in a cfDNA copy number profile from sample Lung_Cancer_1 was comparable between PCR and PCR-free methods (Figure 3, A and B). *In silico* selection of shorter reads (90 to 150 bp) increased the deflection of the segmented values from the zero line for the PCR-free method in cfDNA from Lung_Cancer_1 (Figure 3C), an effect previously reported for a PCR method.[16] A comparison of other samples evaluated with both PCR and PCR-free methods showed a similar effect in some but not all samples. Size selection could also be applied to heparin samples. However, *in silico* size selection did not always

increase deflection but always reduced the number of sequencing reads available to produce a copy number profile. Thus, although a higher deflection increases the statistical power to call alterations, the reduced number of reads limits this advantage.

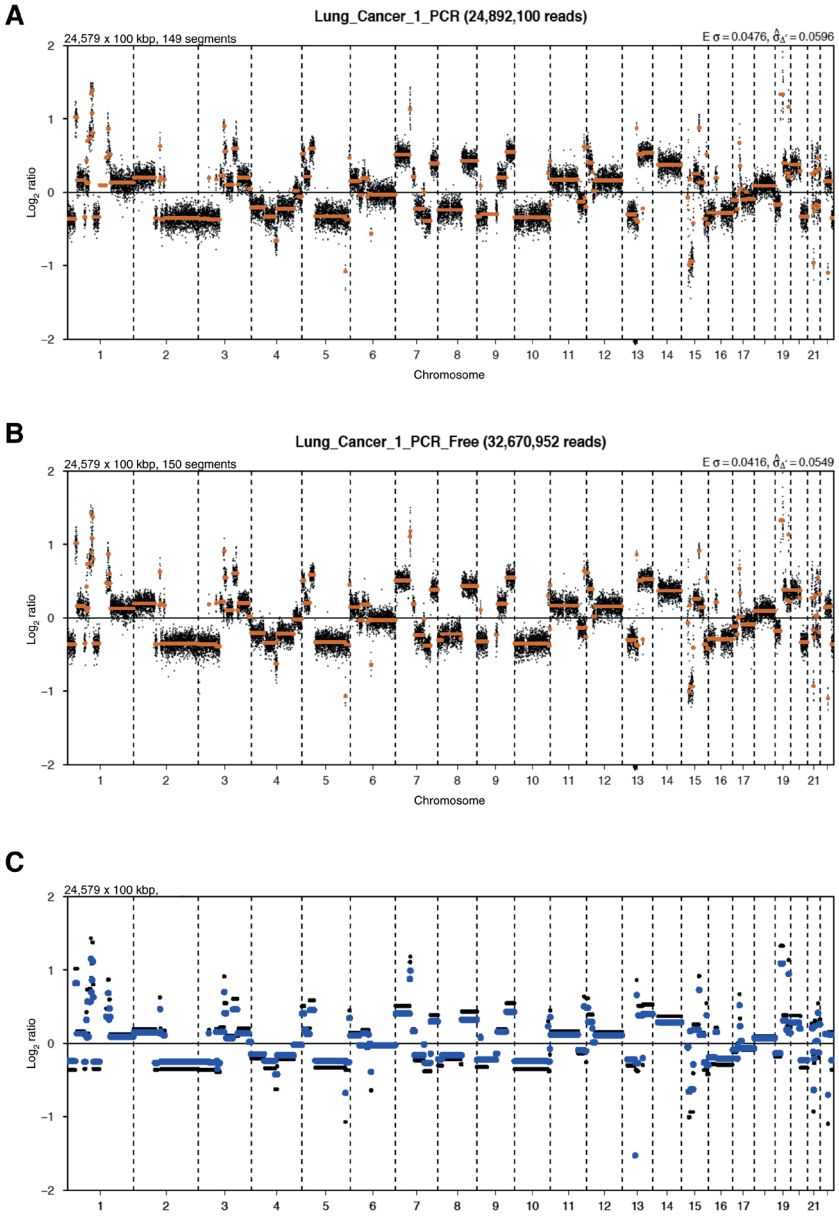


Figure 3. Copy number profiles from PCR and PCR-free methods. A and B: Size-selected copy number profiles from a cell-free DNA sample from a lung cancer patient (Lung_Cancer_1) sequenced following either the PCR method (A) or PCR-free method (B). Black dots represent the median-normalized log2-transformed read counts per bin. The log2 tumor/normal ratio (y axis) is plotted in relation to the chromosomal position (x axis). Sequencing reads are in 24,579 100-kbp bins indicated in the top left corner of the profile, followed by the number of segments per profile. Segment values are represented by horizontal orange lines. C: Overlay of copy number profile segment values generated without size selection (blue) and with size selection (black). σˆ2Δ , the sequencing noise defined as the SD of the difference between log2 values between neighboring bins; Eσ, the expected value of the sequencing noise assuming no biases.

Similar Copy Number Profiles Were Observed in FFPE Tissue

To compare the performance of SCNA detection in cfDNA and FFPE tissue, sequencing of DNA from matched FFPE tissue material from 10 patients was performed. Comparable copy number profiles were observed in most FFPE samples, although the median observed noise was much higher compared with cfDNA samples (0.10 versus 0.06, respectively). Representative examples are provided in Figure 4, A–C.

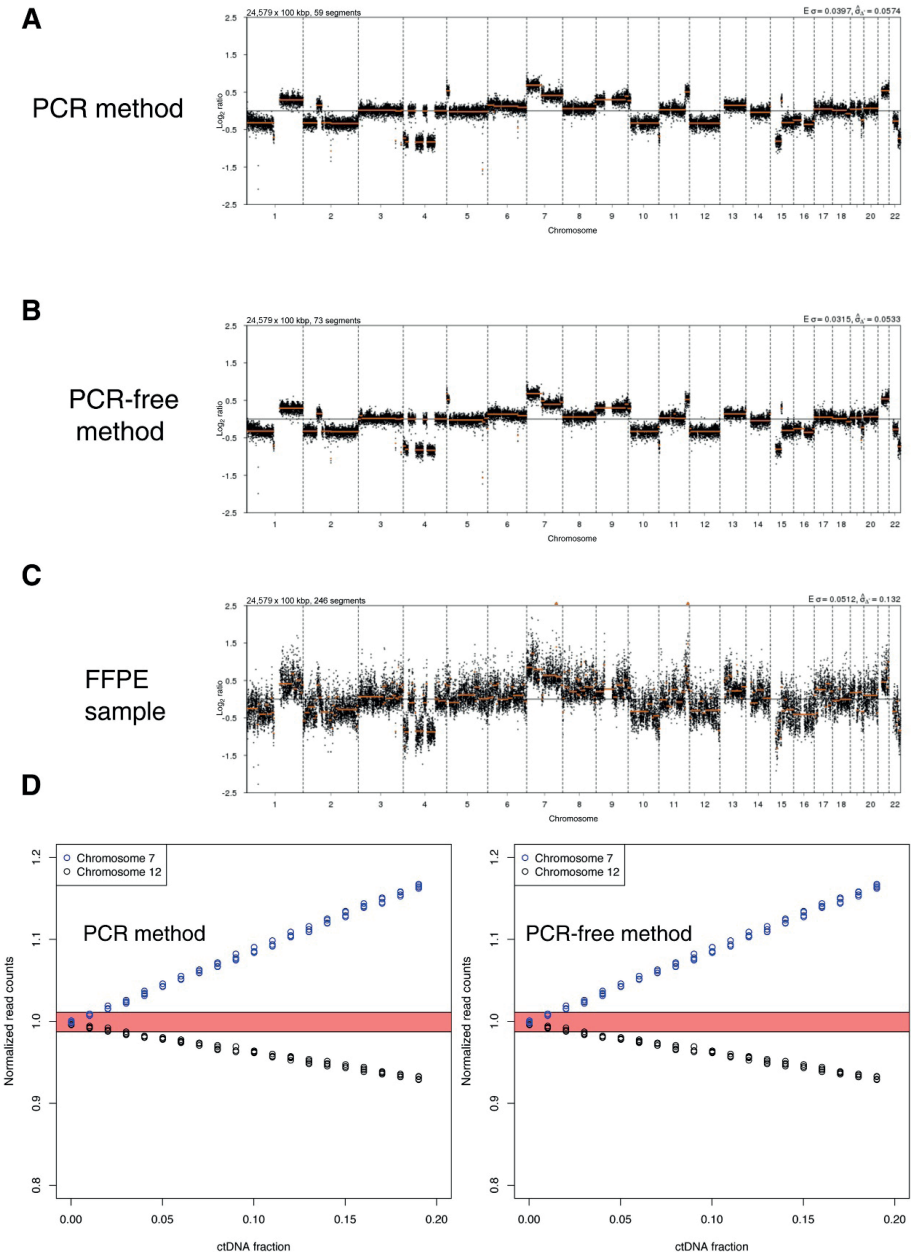


Figure 4. Detection limit of somatic copy number alterations with PCR and PCR-free methods. A and B: Copy number profile of B-Cell Lymphoma_20 cell-free DNA (cfDNA), sequenced with the PCR method (A) and PCR-free method (B). C: Copy number profile for B-cell lymphoma_20, from formalin-fixed, paraffin-embedded (FFPE) tissue. D: Dilution series of cfDNA sample with the PCR method (left panel) and PCR-free

method (right panel). Dilution was performed by mixing reads from B-cell lymphoma_20 with four healthy donors *in silico*. The x axis shows the circulating tumor DNA (ctDNA) fraction of the dilution, and the y axis shows the normalized read counts for chromosome 7 in blue (a gain) and chromosome 12 in black (a loss). The red area between the horizontal black lines indicates the limits of detection. σˆ2Δ , the sequencing noise defined as the SD of the difference between log2 values between neighboring bins; Eσ, the expected value of the sequencing noise assuming no biases.

Detection Limit of SCNAs in PCR and PCR-Free Sequencing Methods

An *in silico* dilution series was performed for a B-cell lymphoma and a lung cancer cfDNA sample to establish the SCNA detection limit. For the B-cell lymphoma sample (B-Cell Lymphoma_20: ctDNA fraction, 55%), the detection limit ranged between a ctDNA fraction of 3% and 4% for both the PCR and PCR-free methods (Figure 4D). This limit was determined by assessment of the gain on chromosome 7 and loss on chromosome 12 identified in FFPE tumor tissue material. The lung cancer sample (Lung_Cancer_1: ctDNA fraction, 24%) showed a similar detection limit (4% to 6%) (Supplemental Figure S2). Overall, the detection limit did not differ significantly between PCR and PCR-free methods.

PCR and PCR-Free Methods Are Compatible with cfDNA Collected in Heparin-Containing Tubes

Archived plasma samples collected in lithium-heparin–containing tubes present numerous research opportunities, but typically require additional processing to enable molecular analysis.[20] To address this, 25 plasma samples (distinct from the EDTA cases) collected in lithium-heparin–containing tubes were tested using the PCR-free sWGS method. Ten of these samples were also tested with the PCR method (Figure 1A). The sequencing metrics from the heparin-containing samples that underwent PCR (Q37, 92.0%; SD, 0.65%; aligned reads, 99.6%; SD, 0.07%) and PCR-free (Q37, 91.9%; SD, 0.65%; aligned reads, 99.3%; SD, 0.29%) methods were highly comparable to the EDTA samples that also underwent PCR and PCR-free library preparations. As was observed for the EDTA samples, the percentage of unique reads was significantly higher using the PCR-free (94.5%) compared with the PCR approach (89.4%; $P \leq 0.001$) in the heparin samples (Figure 2A). The G-C base pair content and sequencing noise of the 25 PCR-free and 10 PCR heparin samples was also highly concordant (Figure 2, B and C), as was the quality of the copy number profiles. Of interest was a comparable fragmentation pattern observed in blood samples collected in heparin and EDTA (Figure 2D). Also notable was the sequencing metrics and genome representation of the 13 AML bone marrow plasma samples (Q37, 91.9%; SD, 0.57%; percentage aligned, 99.1%; SD, 0.25%; percentage unique reads, 94.2%; SD, 0.97%; G-C base pair content, 40.4%; SD, 0.73%), which were comparable to the peripheral blood plasma samples in this study. These results demonstrate that high-quality sequencing data can be

obtained from cfDNA extracted from archival blood samples collected in either EDTA or heparin-containing tubes using both PCR and PCR-free methods.

Discussion

Fast and efficient automated cfDNA analysis holds huge potential to broaden the clinical utility of SCNAs as cancer biomarkers. This study shows that a PCR-free method is a viable alternative to a widely used PCR-based approach to analyze freshly collected and archived plasma samples. A PCR-free method is already in clinical use under International Organization for Standardization accreditation as part of the NIPT workflow,[23] having recently transitioned from a PCR-based approach. NIPT throughput can be high with several sequencing runs weekly, particularly in large institutions. A PCR-free method for ctDNA can now be tested using this automated workflow, which should enable a sufficiently quick turnaround time for clinical implementation.

A clear benefit of the PCR-free workflow is the slightly higher percentage of unique sequencing reads. The obvious explanation is that PCR amplification inherently produces duplicate amplicons that will be sequenced. PCR-free method directly sequences the original DNA templates, and the few duplicates detected are designated as optical duplicates, a technical phenomenon consequential of Illumina sequencing technology. The PCR-free workflow can theoretically allow multiplexing of more samples on a sequencing lane, further improving cost-effectiveness. However, equimolar pooling requires accurate quantification of cfDNA, which is challenging with conventional electrophoresis and fluorometric techniques because of interference from the fork-shaped adapters in PCR-free libraries. To overcome this, a droplet digital PCR workflow was adapted to accurately determine the concentration of cfDNA fragments with ligated adapters. Although droplet digital PCR provides highly accurate results, the workflow is time-consuming and uses precious library material, which is problematic when the cfDNA yield from a sample is low. In a high-throughput setting, when repeated sequencing of a sample is reasonable, such as with NIPT, fluorometric analysis alone would be sufficient for library quantification. In a low-throughput setting, which is typically the case in research laboratories, droplet digital PCR is recommended as a more accurate method of quantification to reduce the necessity for time-consuming and expensive repeated sequencing. PCR-free analysis of cancer samples is therefore likely to be more cost-effective for laboratories that already have a high-throughput NIPT workflow in place.

The omission of amplification in PCR-free library preparations generally requires higher quantities of cfDNA to generate a chromosomal copy number profile. If the cfDNA quantity in a sample is low, or other applications, such as mutation

detection, are required, a PCR library preparation may be a more efficient use of limited material. However, this study shows that SCNAs are detectable from an input of only 3.7 ng cfDNA, a quantity easily available in plasma as the average cfDNA concentration is 5.25 ng/mL from healthy individuals and significantly higher in cancer patients.[14] Furthermore, it was found that whole-arm SCNAs can be detected with both PCR and PCR-free methods in samples using a simple Z-score–based method for a ctDNA fraction as low as 3% to 6%. This detection limit is in line with previous studies of cfDNA SCNA analysis in cancer patients who demonstrated detection limits of 2.5% and 3% ctDNA.[16,29] We stress that our Z-score–based method was included to compare the detection limit of the PCR with the PCR-free method and provides a conservative estimate that can be improved by increasing the number of healthy controls, refining the computational analysis and/or incorporating cfDNA fragmentation analysis.

An important and well-documented consideration for cfDNA analysis is pre-analytical sample processing. To enable a fair comparison between PCR and PCR-free library preparations, 38 samples were subjected to the same pre-analytical conditions before both PCR and PCR-free library preparations. In this way, any observed variation would most likely come from the library preparation method (PCR or PCR-free method) and not the pre-analytical processing. The samples included in this study were initially collected for various clinical investigations that employed a variety of plasma separation, storage, and cfDNA isolation protocols. These samples were also selected to assess the applicability of the PCR and PCR-free methods to material previously overlooked for SCNA analysis. The prevailing opinion is that pre-analytical conditions must be highly controlled to allow reliable molecular analysis, specifically that samples should be collected in EDTA-containing, or specialized cfDNA-preserving, blood tubes and stored at –80°C.[17] Heparin is believed to interfere with PCR amplification[20] and to disrupt the nucleosome structure in plasma, causing greater cfDNA fragmentation compared with samples collected in EDTA.[33] This study shows that both the PCR and PCR-free workflows presented herein are robust enough to generate high-quality data from cancer samples despite collection in heparin-containing tubes and variation in pre-analytical processing.

Conclusion

We show that PCR-free library preparation for sWGS is at least as effective as the standard PCR-based approach for cfDNA SCNA detection in cancer samples. The PCR-free workflow generates copy number profiles with similarly low sequencing noise to PCR. We also show that archival plasma cfDNA from peripheral blood and bone marrow taken in lithium-heparin tubes and stored for up to 15 years at -20°C can be used to generate raw and processed data of comparable quality to samples

taken in EDTA blood tubes, the standard for sWGS. PCR-free method is a viable alternative for routine tumor cfDNA detection, such as monitoring of treatment response or testing for recurrence. Our results pave the way for a cost-efficient and time-efficient automated cfDNA screening workflow for SCNA detection in cancer patients.[23]

Acknowledgment

We thank Ted Bradley (Amsterdam University Medical Center Core Facility Genomics) for technical advice during the project.

References

1. ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium: Pan-cancer analysis of whole genomes. Nature 2020, 578:82-93

2. Macintyre G, Ylstra B, Brenton JD: Sequencing structural variants in cancer for precision therapeutics. Trends Genet 2016, 32:530-542

3. Swain SM, Baselga J, Kim S-B, Ro J, Semiglazov V, Campone M, Ciruelos E, Ferrero J-M, Schneeweiss A, Heeson S, Clark E, Ross G, Benyunes MC, Cortés J: Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med 2015, 372:724-734

4. Goodman AM, Piccioni D, Kato S, Boichard A, Wang H-Y, Frampton G, Lippman SM, Connelly C, Fabrizio D, Miller V, Sicklick JK, Kurzrock R: Prevalence of PDL1 amplification and preliminary response to immune checkpoint blockade in solid tumors. JAMA Oncol 2018, 4:1237-1244

5. Roemer MG, Advani RH, Ligon AH, Natkunam Y, Redd RA, Homer H, Connelly CF, Sun HH, Daadi SE, Freeman GJ, Armand P, Chapuy B, de Jong D, Hoppe RT, Neuberg DS, Rodig SJ, Shipp MA: PD-L1 and PD-L2 genetic alterations define classical Hodgkin lymphoma and predict outcome. J Clin Oncol 2016, 34:2690-2697

6. Roemer MGM, Redd RA, Cader FZ, Pak CJ, Abdelrahman S, Ouyang J, Sasse S, Younes A, Fanale M, Santoro A, Zinzani PL, Timmerman J, Collins GP, Ramchandren R, Cohen JB, De Boer JP, Kuruvilla J, Savage KJ, Trneny M, Ansell S, Kato K, Farsaci B, Sumbul A, Armand P, Neuberg DS, Pinkus GS, Ligon AH, Rodig SJ, Shipp MA: Major histocompatibility complex class II and programmed death ligand 1 expression predict outcome after programmed death 1 blockade in classic Hodgkin lymphoma. J Clin Oncol 2018, 36:942-950

7. van Dijk E, Biesma HD, Cordes M, Smeets D, Neerincx M, Das S, Eijk PP, Murphy V, Barat A, Bacon O, Prehn JHM, Betge J, Gaiser T, Fender B, Meijer GA, McNamara DA, Klinger R, Koopman M, Ebert MPA, Kay EW, Hennessey BT, Verheul HMW, Gallagher WM, O'Connor DP, Punt CJA, Loupakis F, Lambrechts D, Byrne AT, van Grieken NCT, Ylstra B: Loss of chromosome 18q11.2-q12.1 is predictive for survival in patients with metastatic colorectal cancer treated with bevacizumab. J Clin Oncol 2018, 36:2052-2060

8. Los-de Vries GT, De Boer M, van Dijk E, Stathi P, Hijmering NJ, Roemer MGM, Mendeville M, Miedema DM, de Boer JP, Rakhorst HA, van Leeuwen FE, van der Hulst RR, Ylstra B, De Jong D: Chromosome 20 loss is characteristic for breast implant-associated anaplastic large cell lymphoma. Blood 2020, 136:2927-2932

9. Huang M, Weiss WA: Neuroblastoma and MYCN. Cold Spring Harbor Perspect Med 2013, 3:a014415

10. Michels E, De Preter K, Van Roy N, Speleman F: Detection of DNA copy number alterations in cancer by array comparative genomic hybridization. Genet Med 2007, 9:574-584

11. Scheinin I, Sie D, Bengtsson H, van de Wiel MA, Olshen AB, van Thuijl HF, van Essen HF, Eijk PP, Rustenburg F, Meijer GA, Reijneveld JC, Wesseling P, Pinkel D, Albertson DG, Ylstra B: DNA copy number analysis of fresh and formalin-fixed specimens by shallow whole-genome sequencing with identification and exclusion of problematic regions in the genome assembly. Genome Res 2014, 24: 2022-2032

12. Heitzer E, Haque IS, Roberts CES, Speicher MR: Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev 2019, 20:71-88

13. van Essen HF, Ylstra B: High-resolution copy number profiling by array CGH using DNA isolated from formalin-fixed, paraffin-embedded tissues. Methods Mol Biol 2012, 838:329-341

14. van der Pol Y, Mouliere F: Toward the early detection of cancer by decoding the epigenetic and environmental fingerprints of cell-free DNA. Cancer Cell 2019, 36:350-368

15. Wan JC, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N: Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer 2017, 17:223-238

16. Mouliere F, Chandrananda D, Piskorz AM, Moore EK, Morris J, Ahlborn LB, et al: Enhanced detection of circulating tumor DNA by fragment size analysis. Sci Transl Med 2018, 10:eaat4921

17. Markus H, Contente-Cuomo T, Farooq M, Liang WS, Borad MJ, Sivakumar S, Gollins S, Tran NL, Dhruv HD, Berens ME, Bryce A, Sekulic A, Ribas A, Trent JM, LoRusso PM, Murtaza M: Evaluation of pre-analytical factors affecting plasma DNA analysis. Sci Rep 2018, 8:7375

18. Heitzer E, Ulz P, Belic J, Gutschi S, Quehenberger F, Fischereder K, Benezeder T, Auer M, Pischler C, Mannweiler S, Pichler M, Eisner F, Haeusler M, Riethdorf S, Pantel K, Samonigg H, Hoefler G, Augustin H, Geigl JB, Speicher MR: Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 2013, 5:30

19. Satsangi J, Jewell DP, Welsh K, Bunce M, Bell JI: Effect of heparin on polymerase chain reaction. Lancet 1994, 343:1509-1510

20. Vaughan-Shaw PG, Walker M, Ooi L, Gilbert N, Farrington SM, Dunlop MG: A simple method to overcome the inhibitory effect of heparin on DNA amplification. Cell Oncol (Dordr) 2015, 38:493-495

21. Curnow KJ, Sanderson RK, Beruti S: Noninvasive detection of fetal aneuploidy using next generation sequencing. Methods Mol Biol 2019, 1885:325-345

22. Karlsson K, Sahlin E, Iwarsson E, Westgren M, Nordenskjold M, Linnarsson S: Amplification-free sequencing of cell-free DNA for prenatal non-invasive diagnosis of chromosomal aberrations. Genomics 2015, 105:150-158

23. van der Meij KRM, Sistermans EA, Macville MVE, Stevens SJC, Bax CJ, Bekker MN, et al: TRIDENT-2: national implementation of genome-wide non-invasive prenatal testing as a first-tier screening test in the Netherlands. Am J Hum Genet 2019, 105:1091-1101

24. Amant F, Verheecke M, Wlodarska I, Dehaspe L, Brady P, Brison N, Van Den Bogaert K, Dierickx D, Vandecaveye V, Tousseyn T, Moerman P, Vanderstichele A, Vergote I, Neven P, Berteloot P, Putseys K, Danneels L, Vandenberghe P, Legius E, Vermeesch JR: Presymptomatic identification of cancers in pregnant women during noninvasive prenatal testing. JAMA Oncol 2015, 1:814-819

25. Lenaerts L, Vandenberghe P, Brison N, Che H, Neofytou M, Verheecke M, Leemans L, Maggen C, Dewaele B, Dehaspe L, Vanderschueren S, Dierickx D, Vandecaveye V, Amant F, Vermeesch JR: Genomewide copy number alteration screening of circulating plasma DNA: potential for the detection of incipient tumors. Ann Oncol 2019, 30:85-95

26. Bach S, Sluiter NR, Beagan JJ, Mekke JM, Ket JCF, van Grieken NCT, Steenbergen RDM, Ylstra B, Kazemier G, Tuynman JB: Circulating tumor DNA analysis: clinical implications for colorectal cancer patients: a systematic review. JNCI Cancer Spectr 2019, 3:pkz042

27. Chen X, Chang C-W, Spoerke JM, Yoh KE, Kapoor V, Baudo C, Aimi J, Yu M, Liang-Chu MMY, Suttmann R, Huw L-Y, Gendreau S, Cummings C, Lackner MR: Low-pass whole-genome sequencing of circulating cell-free DNA demonstrates dynamic changes in genomic copy number in a squamous lung cancer clinical cohort. Clin Cancer Res 2019, 25:2254-2263

28. Silva S, Danson S, Teare D, Taylor F, Bradford J, McDonagh AJG, Salawu A, Wells G, Burghel GJ, Brock I, Connley D, Cramp H, Hughes D, Tiffin N, Cox A: Genome-wide analysis of circulating cell-free DNA copy number detects active melanoma and predicts survival. Clin Chem 2018, 64:1338-1346

29. Adalsteinsson VA, Ha G, Freeman SS, Choudhury AD, Stover DG, Parsons HA, et al: Scalable whole-exome sequencing of cell-free DNA reveals high concordance with metastatic tumors. Nat Commun 2017, 8:1324

30. Poell JB, Mendeville M, Sie D, Brink A, Brakenhoff RH, Ylstra B: ACE: absolute copy number estimation from low-coverage whole-genome sequencing data. Bioinformatics 2019, 35:2847-2849

31. Armbruster DA, Pry T: Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 2008, 29(Suppl 1):S49-S52

32. McDowell DG, Burns NA, Parkes HC: Localised sequence regions possessing high melting temperatures prevent the amplification of a DNA mimic in competitive PCR. Nucleic Acids Res 1998, 26: 3340-3347

33. Han DSC, Ni M, Chan RWY, Chan VWH, Lui KO, Chiu RWK, Lo YMD: The biology of cell-free DNA fragmentation and the roles of DNASE1, DNASE1L3, and DFFB. Am J Hum Genet 2020, 106: 202-214

Supplementary data

Supplementary data are available online

Chapter 6

Summary, Discussion and Future Perspectives

Summary of Thesis

The aim of this thesis is to improve the pre-analytical and analytical conditions for ctDNA detection and develop assays to assess the feasibility of implementation in a clinical setting.

In **Chapter 1** the field of liquid biopsy in general is introduced. We outline challenges facing ctDNA detection in clinical practice. The chapter concludes by setting out the specific aims of the thesis and how we intended to achieve them.

In **Chapter 2** the clinical implications of ctDNA analysis for colorectal cancer (CRC) patients are reviewed [1]. A brief introduction to ctDNA analysis is followed by an in-depth investigation into the most promising biomarkers for diagnosis, prognostication and monitoring of CRC. The broad scope of the review covers DNA methylation and copy number aberrations in addition to point mutations. The review identified *SEPT9* methylation status of ctDNA as the most promising marker for CRC detection and found mutations in *KRAS* to be the most useful for prognostication and monitoring.

Chapter 3 is a study of the feasibility of ctDNA detection to select CRC patients for Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy (CRS-HIPEC). CRS-HIPEC is a highly invasive procedure with curative intent but a long recovery time and a high morbidity rate [2]. This is the first chapter in the thesis to apply a tumour tissue-guided approach to detect mutations in ctDNA using droplet digital PCR, one of the most sensitive and specific techniques available. The study shows that ctDNA detection prior to CRS-HIPEC could be a marker of disease recurrence following the procedure. We furthermore show that this liquid biopsy approach has the potential, pending validation in an independent cohort, to be used to exclude patients from unnecessary CRS-HIPEC.

Chapter 4 assesses the utility of ctDNA detection to monitor treatment response in non-small cell lung cancer (NSCLS) patients receiving osimertinib, an *EGFR* targeted therapy [3]. A tissue-guided approach like that used in **Chapter 3**, revealed a trend between ctDNA detection and a lack of response to osimertinib treatment. This liquid biopsy application has since been validated and implemented at Amsterdam UMC to detect ctDNA in plasma from advanced NSCLC patients if a solid tissue biopsy is unavailable.

Chapter 5 is a technical investigation to assess ctDNA detection using PCR-free shallow whole genome sequencing (sWGS) [4]. Instead of interrogating mutations previously identified in solid tissue like in **Chapters 3 and 4**, a genome-wide approach is used to screen for copy number aberrations in cfDNA. The PCR-free

workflow produced highly comparable data to the standard PCR-based approach and lends itself to high-throughput applications. We also show that material processed under various pre-analytical conditions, including blood collection in heparin-containing tubes, can be used to generate high quality data.

Overall conclusion

This thesis bridges the gap between solid tissue and liquid biopsies and demonstrates the utility of ctDNA detection in an academic hospital. We show that routine mutation analysis of ctDNA is technically feasible and can inform clinical decisions in diverse subsets of cancer patients. However, the utility of ctDNA detection is hindered by poorly understood biological variables, which will be discussed in this chapter. Until these gaps in our knowledge are filled, ctDNA detection will be mostly restricted to a test of last resort or to support solid tissue biopsies.

Discussion

Relevance of findings to the field of ctDNA analysis

This thesis offers both a proof of principle and a blueprint for the translation of ctDNA detection from research to the clinic. **Chapter 2** condenses a large body of ctDNA research into a useful format, serving as an accessible guide for colorectal cancer clinicians and molecular pathologists looking to implement liquid biopsy in routine patient care. **Chapters 3 and 4** support previously identified trends between ctDNA detection and clinical characteristics, providing an incremental increase in our understanding. Both chapters also offer technical instructions to implement tissue-guided ctDNA detection within the capabilities of an academic medical centre. Finally, **Chapter 5** paves the way for high-throughput screening of CNAs in cancer samples, a similar approach to that used in non-invasive prenatal testing (NIPT).

A detailed critique of the relevance of each finding is provided in the discussion sections of each chapter. To draw direct comparisons between the work in this thesis as a whole and other studies is problematic due to wide variation in pre-analytical and analytical techniques and highly selective patient inclusion criteria. However, it is interesting to note that the tissue-guided approach employed in **Chapters 3 and 4**, which uses ddPCR, could be as effective as NGS panel-based screening to detect ctDNA and associated clinical trends. As solid tissue is routinely collected in pathology departments, molecular analysis can provide a set of targets for limited but highly informative ctDNA analysis with quantitative or digital PCR methods. Such affordable and user-friendly techniques offer a low bar to entry for clinical laboratories looking to add liquid biopsy to their diagnostic repertoire. Methods of ctDNA detection have developed rapidly since the start of this PhD

project. Technical improvements have brought the sensitivity of NGS in-line with digital PCR techniques, the gold standard of ctDNA detection [5]. It is now possible to detect dozens of cancer-associated mutations simultaneously using large NGS panels [6], albeit at a much higher cost per sample than digital PCR. Interrogating a broader spectrum of mutation targets makes NGS an attractive alternative when solid tissue is unavailable or when screening for unknown mutations. However, when looking for previously identified mutations guided by tissue, or new mutations frequently associated with treatment resistance, a narrowly targeted analysis by ddPCR remains highly effective.

As our biological understanding has grown, applications beyond the detection of mutations and CNAs have been developed to exploit the physical characteristics of cfDNA [7]. For example, genome-wide methylation analysis by microarrays has enabled the identification of tissue-specific methylation signatures in cfDNA that can indicate the cell-type of origin [8]. An over-representation of these characteristic signatures from a particular cell-type can suggest new tissue growth and the presence of cancer, independent of mutation or copy number status. However, the low quantity of cfDNA in a single sample can be insufficient to perform both genome-wide methylation microarrays and NGS for mutation analysis [9]. Other physical characteristics of cfDNA, including fragment length and break-point, have become important aspects of detection that can be applied to NGS data produced for mutation or copy number analysis [10–12]. The combination of these characteristics presents a powerful set of tools to exploit the wealth of biological information in cfDNA.

Issues still facing ctDNA detection

Despite the rapid technical advances, fundamental challenges still hamper the utility of ctDNA detection as a clinical tool. Highly variable and unpredictable yields of cfDNA in plasma present the greatest obstacle to reliable analysis. A recent study of cfDNA yields in healthy individuals and lung cancer patients showed variation of 25% within- and 30% between subjects over three days [13]. In the same study, a significant decrease in the cfDNA yield from individual subjects was observed over the course of a single day [13]. This issue is exacerbated by variability in the quantity of ctDNA in cancer patient samples, which also showed wide variation between samples. Intriguingly, a complete lack of ctDNA detection is sometimes observed in samples from advanced cancer patients, despite a high tumour burden and the presence of metastases [14]. The subsequently low ctDNA input from these samples is more likely to return a false negative result because the quantity is insufficient to reach the detection threshold of even the most sensitive molecular assays.

Little is currently understood of the factors that influence the quantity of cf- and ctDNA in plasma. Outside of oncology, raised levels of cfDNA have also been observed after trauma or exercise and in patients with chronic inflammatory diseases [13,15]. The physical and biological processes known to release cf- and ctDNA into the circulation such as apoptosis, necrosis and active secretion [16] require further investigation to improve the likelihood of ctDNA detection in a blood sample. In cancer patients, it is likely that a number of factors have a cumulative effect on ctDNA levels in plasma, including but not limited to tumour burden, stage, location, tumour micro-environment, response to treatment and genomic landscape. The variability in yield caused by these factors currently feeds into the weak relationship between ctDNA detection and clinical characteristics, challenging the ability of liquid biopsy analysis to aid in clinical decision making.

Furthermore, confident detection of alterations in ctDNA does not necessarily mean a tumour is present. Cancer-associated mutations frequently arise in non-cancer cells, occurring at a higher frequency in cell types with a high proliferation rate or increased exposure to environmental mutagens [17]. These cells are probably either eliminated by the immune system at an early stage or at the end of their healthy life, releasing cancer-associated mutations into the plasma. The effect is amplified when mutations accumulate in otherwise-healthy haematopoietic stem cells. This phenomenon, known as clonal haematopoietic mutations of indeterminate potential (CHIP), can lead to sustained over-representation of cancer-associated mutations, copy number aberrations or translocations in plasma [18], causing false positive results that would have serious implications for a patient's treatment [19]. More research is needed to quantify the effect of these factors, which are difficult to isolate due to variations in treatment and the underlying complexity of cancer as a disease.

To what extent were the aims formulated in the introduction of the thesis met?

Aim 1. Set up and validate workflows for point mutation and copy number aberration (CNA) analysis to detect ctDNA

Technical issues, outlined in the introduction **Chapter 1**, were overcome. Pre-analytical conditions including blood collection, plasma separation and cfDNA isolation were tested and optimised. The sensitivity and specificity of ddPCR was tested using positive controls to ensure reliable ctDNA detection within the scope of the assays. The cfDNA input to each ddPCR reaction was identified as a sensitivity-limiting factor and considered when calling a positive result. Finally, a PCR-free sWGS workflow was developed and optimised to enable CNA analysis of cfDNA.

Aim 2. Detect point mutations in ctDNA that were previously identified in solid tissue

We effectively targeted mutations previously identified in solid tissue material to detect ctDNA from plasma. This approach was applied most effectively to samples from the NSCLC patient cohort in **Chapter 4**, where various clonal and sub-clonal *EGFR* mutations were previously identified in one or more solid tissue samples [3]. The chances of detecting a mutation in ctDNA were maximised because a sub-set of the *EGFR* mutations were under selection pressure to arise, due to Tyrosine Kinase Inhibitor treatments received by the patients. The tissue-guided approach was less suitable to CRC patients considered for CRS-HIPEC in **Chapter 3**, who were not receiving targeted therapies [2]. Specific mutations in their ctDNA were therefore more difficult to predict and subsequently target. Targeted sequencing of solid tissue samples from three CRC patients identified no mutations at all, so ctDNA detection relied upon screening with a ddPCR kit that targets a limited number of mutations in *KRAS*. In this CRC patient cohort, a larger NGS-panel may have identified mutations in other genes, thereby improving ctDNA detection.

Aim 3. Assess the utility of ctDNA detection to address real-world clinical needs

The use of tissue-guided ctDNA detection with ddPCR showed clear utility in two of the clinical situations evaluated in this thesis: 1) treatment monitoring in NSCLC patients and 2) treatment selection in CRC patients. Detailed clinical and pathological annotation enabled the characteristics of the individual patient's tumour to be correlated with ctDNA detection, despite the small number of patients included. If validated in larger trials, the workflow may be implemented in the clinical setting to better inform patient management.

Future Perspectives

The use of genetic liquid biopsy testing is becoming a routine part of cancer patient care. The adaptation of pre-existing molecular techniques to plasma ctDNA analysis has quickly led to highly sensitive, clinically valid tests. The further application of these techniques to liquids such as cervical smears, saliva, cerebrospinal fluid and urine promises to reduce the burden on patients in specific clinical situations, such as tumour detection and treatment monitoring. Cost barriers, particularly associated with NGS, will continue to decrease allowing all clinically informative mutations to be interrogated in a single routine ctDNA sample.

However, even with improved detection and a better understanding of the biology of ctDNA in the circulation, it is likely that there is a limit to the information that ctDNA analysis can yield. Other liquid biopsy biomarkers such as miRNAs in

exosomes, RNA in tumour-educated platelets and analysis of tumour proteins, offer different and alternative information about an individual patient's cancer. In the coming years, a repertoire of genetic analyses will become available for each cancer type for a range of clinical circumstances. Such a repertoire will allow analysis of the most appropriate cancer biomarker to inform clinical decision making, resulting in a better treatment and outcome for the patient.

References

1. Bach S, Sluiter NR, Beagan JJ, et al. Circulating tumor DNA analysis: Clinical implications for colorectal cancer patients. A systematic review. *JNCI Cancer Spectr*. 2019;3(3):1-14. doi:10.1093/jncics/pkz042
2. Beagan JJ, Sluiter NR, Bach S, et al. Circulating Tumor DNA as a Preoperative Marker of Recurrence in Patients with Peritoneal Metastases of Colorectal Cancer: A Clinical Feasibility Study. *J Clin Med*. 2020;9(6). doi:10.3390/jcm9061738
3. Beagan JJ, Bach S, van Boerdonk RA, et al. Circulating tumor DNA analysis of EGFR-mutant non-small cell lung cancer patients receiving osimertinib following previous tyrosine kinase inhibitor treatment. *Lung Cancer*. 2020;145(April):173-180. doi:10.1016/j.lungcan.2020.04.039
4. Beagan JJ, Drees EEE, Stathi P, et al. PCR-Free Shallow Whole Genome Sequencing for Chromosomal Copy Number Detection from Plasma of Cancer Patients Is an Efficient Alternative to the Conventional PCR-Based Approach. *J Mol Diagnostics*. Published online October 9, 2021. doi:10.1016/j.jmoldx.2021.08.008
5. Li BT, Janku F, Jung B, et al. Ultra-deep next-generation sequencing of plasma cell-free DNA in patients with advanced lung cancers: results from the Actionable Genome Consortium. *Ann Oncol*. 2019;30(4):597-603. doi:10.1093/annonc/mdz046
6. Yaung SJ, Fuhlbrück F, Peterson M, et al. Clonal Hematopoiesis in Late-Stage Non-Small-Cell Lung Cancer and Its Impact on Targeted Panel Next-Generation Sequencing. *JCO Precis Oncol*. 2020;(4):1271-1279. doi:10.1200/PO.20.00046
7. van der Pol Y, Mouliere F. Toward the Early Detection of Cancer by Decoding the Epigenetic and Environmental Fingerprints of Cell-Free DNA. *Cancer Cell*. 2019;36(4):350-368. doi:10.1016/j.ccell.2019.09.003
8. Moss J, Magenheim J, Neiman D, et al. Comprehensive human cell-type methylation atlas reveals origins of circulating cell-free DNA in health and disease. *Nat Commun*. 2018;9(1):5068. doi:10.1038/s41467-018-07466-6
9. Keller L, Belloum Y, Wikman H, Pantel K. Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. *Br J Cancer*. 2021;124(2):345-358. doi:10.1038/s41416-020-01047-5
10. Underhill HR, Kitzman JO, Hellwig S, et al. Fragment Length of Circulating Tumor DNA. *PLoS Genet*. 2016;12(7):1-24. doi:10.1371/journal.pgen.1006162
11. Mouliere F, Piskorz AM, Chandrananda D, et al. Selecting short DNA fragments in plasma improves detection of circulating tumour DNA. *bioRxiv*. Published online 2017. doi:10.1101/134437
12. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell*. 2016;164(1):57-68. doi:https://doi.org/10.1016/j.cell.2015.11.050
13. Madsen AT, Hojbjerg JA, Sorensen BS, Winther-Larsen A. Day-to-day and within-day biological variation of cell-free DNA. *EBioMedicine*. 2019;49:284-290. doi:10.1016/j.ebiom.2019.10.008
14. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224):224ra24-224ra24. doi:10.1126/scitranslmed.3007094
15. Atamaniuk J, Vidotto C, Tschan H, Bachl N, Stuhlmeier KM, Müller MM. Increased Concentrations of Cell-Free Plasma DNA after Exhaustive Exercise. *Clin Chem*. 2004;50(9):1668-1670. doi:10.1373/clinchem.2004.034553

16. Hu Z, Chen H, Long Y, Li P, Gu Y. The main sources of circulating cell-free DNA: Apoptosis, necrosis and active secretion. *Crit Rev Oncol Hematol*. 2021;157:103166. doi:https://doi.org/10.1016/j.critrevonc.2020.103166

17. Yizhak K, Aguet F, Kim J, et al. RNA sequence analysis reveals macroscopic somatic clonal expansion across normal tissues. *Science (80-)*. 2019;364(6444):eaaw0726. doi:10.1126/science.aaw0726

18. Abbosh C, Swanton C, Birkbak NJ. Clonal haematopoiesis: a source of biological noise in cell-free DNA analyses. *Ann Oncol*. 2019;30(3):358-359. doi:10.1093/annonc/mdy552

19. Hu Y, Ulrich BC, Supplee J, et al. False-positive plasma genotyping due to clonal hematopoiesis. *Clin Cancer Res*. 2018;24(18):4437-4443. doi:10.1158/1078-0432.CCR-18-0143

Chapter 7

Lay Summary

A tumour is a mass of mutated cells that can replicate uncontrollably. During tumour growth, some cells die because they have become too old or damaged, requiring their removal from the body. Circulating tumour (ct)DNA is the DNA from these dead cells that is found free in the blood after cellular breakdown. Like other biological waste products, ctDNA is temporarily carried in the circulation until it is filtered out by the kidneys or taken up by other organs and broken down further.

The ability to analyse ctDNA is truly revolutionary and could have an enormous impact on the way cancer patients are treated. The value to clinicians, patients, researchers and companies is that ctDNA contains an accessible and comprehensive record of the mutations in a tumour. Usually, the only way to obtain this information is to perform a biopsy of the tumour mass. This is a surgical procedure that is burdensome to the patient, provides only limited insight and is not always possible to perform. Analysis of ctDNA in a routine blood draw, a so-called 'liquid biopsy', can therefore make it easier to monitor tumour growth, identify new mutations and select the most effective treatments. If ctDNA analysis can be implemented, it could become routine in every hospital in the developed world.

The aim of this thesis was to develop tests for ctDNA to be used in a hospital setting. The existence of ctDNA has been known for decades, but techniques that can accurately and reproducibly detect it with high sensitivity, thus enabling detection of small tumors and those with less blood flow, have only recently become available. CtDNA is typically a small fraction of the total (tumour and non-tumour) circulating DNA in the blood, the majority of which comes from healthy cells that have died and broken down. One of the techniques used extensively in this thesis can detect specific tumour mutations in this background of non-tumour DNA with a high degree of sensitivity. In this thesis, we sought to develop this technique and provide a blueprint for effective and reliable ctDNA detection for a variety of cancer types, thereby enabling more effective treatment.

Chapter 1 introduces the aims of the thesis, outlines our approach and provides background to the field of ctDNA analysis. This is followed in **chapter 2** by a scientific literature review of the most useful ctDNA markers - specific types of ctDNA - which may help clinicians make treatment decisions for colorectal cancer.

In this thesis, the central approach to ctDNA detection in blood targeted mutations previously identified in a biopsied or surgically removed tumor. This tissue-guided approach maximised the chance of detecting ctDNA by allowing the effective use of a highly sensitive technique that targets only a small number of mutations. We used the tissue-guided approach to detect ctDNA in blood from patients with lung or colorectal cancer and linked this to certain clinical outcomes, such as survival time after treatment. The alternative approach, screening several commonly

mutated genes for potential mutations, would have been less sensitive and considerably more expensive. In **chapter 3** we used the tissue-guided approach to show that ctDNA was more likely to be detected in lung cancer patients who were not responding to a specific cancer medicine (Osimertinib), by testing for a mutation that often occurs in recipients of this drug. In **chapter 4** we showed that for colorectal cancer patients who had surgical removal of local tumour metastases followed by local chemotherapy, detection of ctDNA before the procedure could mean an increased chance of the tumor returning.

DNA in cancer cells can be unstable, resulting in duplication or deletion of genes when the cell replicates. Every cell should have two copies of each gene; changes in the number of copies can have a negative, neutral or positive effect on tumour growth. DNA copy number changes in blood can therefore indicate the presence of a tumour. In **Chapter 5** we used a technique to count gene copies in DNA in the blood. This technique was adapted from a recently introduced blood test for pregnant women to detect fetal copy number changes, the cause of diseases like down's syndrome. We modified this to analyse samples from lung cancer, lymphoma and leukemia patients and went on demonstrate how this approach could be implemented in a hospital, especially if the necessary testing infrastructure was already in place.

With these chapters, we offer improved techniques to enable ctDNA detection in a hospital setting, thereby achieving the aims of the thesis. We successfully applied these techniques to sample sets with detailed clinical annotation, such as cancer type, stage and treatment history, to combine ctDNA analysis results with better treatment options. The test for mutations in lung cancer patients has already been implemented in the VUmc to enable better treatment decisions. Following additional clinical validation, the techniques used in this thesis may be further implemented to provide improved care for an expanded pool of cancer patients.

Chapter 8

Nederlandse Samenvatting

Een tumor is een cluster gemuteerde cellen die zich ongecontroleerd kunnen vermenigvuldigen. Tijdens tumorgroei sterven sommige cellen omdat ze te oud of beschadigd zijn, waardoor ze uit het lichaam moeten worden verwijderd. Circulerend tumor (ct)DNA is het DNA van deze dode cellen dat na celafbraak vrij in het bloed wordt aangetroffen. Net als andere biologische afvalproducten wordt ctDNA tijdelijk in de circulatie gebracht totdat het door de nieren wordt uitgefilterd of door andere organen wordt opgenomen en verder wordt afgebroken.

De mogelijkheid om ctDNA te analyseren is zeer revolutionair en zou een enorme impact kunnen hebben op de manier waarop kankerpatiënten worden behandeld. De waarde voor clinici, patiënten, onderzoekers en bedrijven is dat ctDNA een toegankelijke en uitgebreide informatiebron is van de mutaties in een tumor. Meestal is de enige manier om deze informatie te verkrijgen het nemen van een biopt van de tumormassa. Dit is een chirurgische ingreep die belastend is voor de patiënt, slechts beperkt inzicht geeft en niet altijd mogelijk is. Analyse van ctDNA via een routinematige bloedafname, het nemen van een zogenaamd 'vloeibaar biopt', kan het daarom makkelijker maken om tumorgroei te volgen, nieuwe mutaties te identificeren en de meest effectieve behandelingen te selecteren. Als ctDNA analyse kan worden geïmplementeerd, zou dit routine kunnen worden in elke ziekenhuisomgeving in de ontwikkelde wereld.

Het doel van dit proefschrift was het ontwikkelen van ctDNA tests voor gebruik in een ziekenhuisomgeving. Het bestaan van ctDNA is al tientallen jaren bekend. Echter, technieken die het ctDNA nauwkeurig en reproduceerbaar met hoge gevoeligheid kunnen detecteren, waardoor kleine tumoren en tumoren met een verminderde bloedstroom kunnen worden opgespoord, zijn pas sinds kort beschikbaar. CtDNA is normaal gesproken een kleine fractie van het totale (tumor en non-tumor) vrij circulerende DNA in het bloed: het merendeel van dit DNA is afkomstig van gezonde cellen die afgestorven en afgebroken zijn. Een van de technieken die veelvuldig in dit proefschrift wordt gebruikt, kan met een hoge mate van gevoeligheid specifieke tumormutaties in deze achtergrond van non-tumor DNA detecteren. In dit proefschrift hebben we geprobeerd deze techniek zo goed mogelijk te ontwikkelen en een blauwdruk te bieden voor effectieve en betrouwbare ctDNA detectie voor een verscheidenheid aan kankertypes om zo de behandeling tegen kanker te verbeteren.

Hoofdstuk 1 introduceert de doelstellingen van het proefschrift, schetst onze aanpak en geeft achtergrondinformatie over het gebied van ctDNA analyse. Dit wordt in **hoofdstuk 2** gevolgd door een wetenschappelijke literatuurstudie van de meest bruikbare ctDNA markers, indicatoren van specifieke soorten ctDNA. Deze informatie kan door clinici gebruikt worden bij het nemen van behandelingsbeslissingen voor colorectale kanker.

In dit proefschrift is de centrale benadering van ctDNA detectie in bloed toegepast op kennis van mutaties die eerder zijn geïdentificeerd in tumoren die tijdens operatie zijn verwijderd. Deze weefselgerichte benadering maximaliseerde de kans op het detecteren van ctDNA door gebruik te maken van een zeer gevoelige techniek die zich slechts op een klein aantal mutaties richt. We gebruikten de weefselgerichte aanpak om ctDNA op te sporen in bloed van patiënten met long- of darmkanker en koppelden dit aan bepaalde klinische behandelingsresultaten, zoals bijvoorbeeld overlevingstijd na behandeling. De alternatieve benadering, het screenen van verschillende vaak gemuteerde genen op mogelijke mutaties, zou minder gevoelig en aanzienlijk duurder zijn geweest. In **hoofdstuk 3** hebben we deze weefselgerichte aanpak gebruikt om aan te tonen dat ctDNA eerder werd gedetecteerd bij longkankerpatiënten die niet reageerden op een specifiek kankermedicijn (Osimertinib), door te testen op een mutatie die vaak voorkomt bij ontvangers van dit medicijn. In **hoofdstuk 4** hebben we laten zien dat voor colorectale kankerpatiënten die in aanmerking kwamen voor chirurgische verwijdering van lokale metastasen door middel van plaatselijke toediening van chemotherapie, detectie van ctDNA vóór deze procedure een verhoogde kans op terugkeer van de tumor zou kunnen betekenen.

DNA in kankercellen kan onstabiel zijn, wat resulteert in duplicatie of deletie van genen wanneer de cel repliceert. Elke cel zou twee exemplaren van elk gen moeten hebben. Veranderingen in het aantal kopieën kunnen een negatief, neutraal of positief effect hebben op de tumorgroei. Wanneer bij een patiënt meer of minder dan twee kopieën van een gen in het bloed aangenomen wordt, kan dit wijzen op de aanwezigheid van een tumor. In **hoofdstuk 5** is een techniek gebruikt die de hoeveelheid DNA van elk chromosoom in het bloed telt om hiermee de aanwezigheid van ctDNA in het bloed aan te tonen. Deze techniek is overgenomen van een onlangs geïntroduceerde bloedtest voor zwangere vrouwen om veranderingen in het aantal kopieën van de foetus op te sporen, de oorzaak van ziekten zoals het syndroom van Down. We hebben we deze methode aangepast om monsters van longkanker-, lymfoom- en leukemiepatiënten te analyseren en we hebben laten zien hoe deze aanpak kan worden geïmplementeerd in een ziekenhuisomgeving, vooral als de benodigde testinfrastructuur al aanwezig is.

Met deze hoofdstukken bieden we verbeterde tools om ctDNA detectie in een ziekenhuisomgeving mogelijk te maken, waardoor de doelstellingen van het proefschrift worden bereikt. We hebben met succes deze technieken toegepast door belangrijke patientinformatie, zoals bijvoorbeeld ziekte type, ziekte stadium en behandelingsgeschiedenis, te koppelen aan ctDNA analyse resultaten, om zo een betere indicatie te krijgen van behandelingsmogelijkheden. De test voor mutaties bij longkankerpatiënten is al geïmplementeerd in het VUmc om behandelbeslissingen te verbeteren. Na verdere klinische validatie kunnen de technieken die in dit proefschrift worden gebruikt geïmplementeerd worden om betere zorg te bieden aan een grotere groep kankerpatiënten.

Chapter 9

Publication List

Curriculum Vitae

Cover Art Caption

Publication List

Bach S*, Sluiter NR*, **Beagan JJ***, Mekke JM, Ket JCF, van Grieken NCT, et al. *Circulating Tumor DNA Analysis: Clinical Implications for Colorectal Cancer Patients. A Systematic Review*. JNCI Cancer Spectrum. 2019;3(3), DOI:10.1093/jncics/pkz042.

Beagan JJ*, Sluiter NR*, Bach S, Eijk P, Vlek SL, Heideman DAM, et al. *Circulating tumor DNA as a preoperative marker of recurrence in patients with peritoneal metastases of colorectal cancer: a clinical feasibility study.* Journal of Clinical Medicine. 2020; 9(6) 1738, DOI:10.3390/jcm9061738.

Beagan JJ*, Bach S*, van Boerdonk RA, van Dijk E, Thunnissen E, van den Broek D, et al. *Circulating tumor DNA analysis of EGFR-mutant non-small cell lung cancer patients receiving osimertinib following previous tyrosine kinase inhibitor treatment*. Lung Cancer. 2020, DOI:10.1016/j.lungcan.2020.04.039.

Beagan JJ, Drees EEE, Stathi P, Eijk PP, Meulenbroeks L, Kessler F, et al. *PCR-Free Shallow Whole Genome Sequencing for Chromosomal Copy Number Detection from Plasma of Cancer Patients Is an Efficient Alternative to the Conventional PCR-Based Approach*. J Mol Diagn. 2021 Nov;23(11):1553-1563. doi: 10.1016/j.jmoldx.2021.08.008.

Strijker M, Soer EC, de Pastena M, Creemers A, Balduzzi A, **Beagan JJ**, et al. *Circulating tumor DNA quantity is related to tumor volume and both predict survival in metastatic pancreatic ductal adenocarcinoma*. Int J Cancer. 2020 Mar 1;146(5):1445-1456. doi: 10.1002/ijc.32586.

*Authors contributed equally

Curriculum Vitae

Jamie Beagan was born in Southampton, England on 17th October 1988, the youngest of three brothers. He received secondary education at Brookfield Community School (2000-2005), leaving with good grades but no idea about his future career. He went on to Barton Peveril College (2005-2007) where a teacher persuaded him to add Human Biology to his list of courses, a decision that ignited a passion for the subject. He later took a place at the University of Nottingham (2007-2011) to follow a Bachelors in Biology, which he extended to a Masters with a specialisation in Molecular Genetics. Following graduation, he carried out a short placement with the Influenza Group of Public Health England to learn more about molecular genetics in healthcare. This led him to a job as a technician in the Genetics Department at Guy's Hospital in London (2012-2016) where he honed his lab skills and became deeply interested in healthcare genetics. Deciding that he wanted to improve the diagnostic options available to patients, he took up a PhD in the Cancer Centre Amsterdam with the Department of Pathology at the VUmc. Through this programme, he learned how to translate molecular genetic techniques to the hospital setting for the benefit of cancer patients. Partly in response to the Covid-19 pandemic, in 2020 he took a job at Viroclinics Biosciences in Rotterdam, where he works as the Research and Development Manager in the Molecular Virology Services Department. Through this role, he applies his knowledge and experience to develop and improve molecular genetic assays to support clinical trials of new vaccines and anti-viral treatments.

Cover Art Caption

Panning for gold shares several similarities with a liquid biopsy.

A hopeful prospector will travel to a valley where gold has previously been discovered or its presence is suspected. Instead of digging haphazardly in the ground, he will begin his search at a river where the flowing water has already cut a deep fissure in the landscape. Using a simple pan, he will scoop up a sample of sediment from the riverbed and gently sieve through it in search of tiny, sparkling flecks. Often, he will find nothing. When a glint of gold finally emerges from the dirt, his excitement is dampened by knowledge of the work that must follow. The tiny fragments in his pan possess little value in themselves but hint at a much larger deposit of gold nearby, gradually eroded by the relentless flow of the river. Before claiming his fortune, the prospector must first locate and extract the deposit using different and altogether more arduous techniques.

In a liquid biopsy, traces of tumour DNA carried by the flowing blood are easily collected then analysed using widely available laboratory techniques. Non-tumour DNA must be sieved out to reveal fragments from a tumour. Often no trace is found, but like fragments of gold in the dirt, detection can betray the presence of a valuable goal - a tumour somewhere in the body. Like for the prospector, detection is merely the first step and other techniques must be used to locate the tumour so it can be biopsied, removed or targeted with therapies.

9

10

Chapter 10

Dankwoord (Acknowledgements)

Finally, I would like to thank the many people who have helped get me to this point. A PhD is a demanding undertaking that I certainly could not have completed alone. I would like to make special mention of the following:

First, I would like to thank **Bauke**, my promotor. I will always remember your phone call after my interview, as I waited at Schiphol for my flight back to London. The offer you made to take me on as a PhD candidate marked the start of a new chapter in my career and the beginning of a life in a new country. You helped me move, get set up in Amsterdam and welcomed me into your group. Throughout my time in TGAC you gave me the freedom to pursue my own ideas, provided crucial guidance and insightful suggestions when I needed it. You were always generous with your comments and gave me encouragement when things weren't going my way. I admire your enthusiasm, optimism and drive to get difficult things done. I would not be where I am today without your continuous support.

Michiel (co-promotor), thank you for your guidance. It was a pleasure to be a guest member of the Exosomes Group, especially for the great borrels and uitjes! Your knowledge, ideas and insightful observations were crucial to guide this project to completion. Your boundless energy and enthusiasm are infectious and helped to keep me going.

Paul (paranymph), always friendly and willing to help. Thanks for helping me find my way in a new group and a new country. I didn't know many people during my early days at the CCA so you said I could call you if I needed anything - that was very kind. Not only did you help me in the lab but you also improved my Dutch though our lively chats. Nu ben ik lekker ingeburgerd! I enjoyed our borrels, lab uitjes and trips to the cinema.

Sander (paranymph), I'm very pleased that I got to work with you throughout my time at the CCA. Not only did you add the all-important clinical perspective, but you made the work fun. You are the personification of the 'can-do' attitude and you never shy away from a challenge. I enjoyed the banter and jokes about anything and everything. I'll remember the fun times at the PhD retreat and drinking whiskey sours!

To the promotion committee: Thanks to **Ed Schuuring** and **Remond Fijneman** for agreeing to be committee members and for you quick assessment of my thesis. I'm looking forward to meeting you at my defence to thank you in person. **Jacqueline Cloos,** I enjoyed our collaboration and appreciate your involvement in the committee. **Jurriaan Tuynman,** your thoughtful insights and calm manner made for a highly productive collaboration. I appreciate your contribution to my thesis, your membership of my committee and your excellent cooking! **Renske**

Steenbergen, you always made such sharp and insightful comments and suggestions after my presentations in the CCA. I'm looking forward to more of the same at my defence. **Nick Gilbert,** my fellow Brit, thank you for being part of the committee. I enjoyed our brainstorming sessions and your valuable contribution to the conceptual design of chapter five of my thesis.

Thanks to all the members of TGAC I've had the pleasure of working with. **Erik**, it was great to work closely with you on our manuscript and to share an office. As you had recently finished your own PhD, you gave me encouragement and helped me keep things in perspective with good humour. I enjoyed our chats about everything from Brexit to tennis. **Marit**, one of the most knowledgeable and driven biologists I know. It was a pleasure to work on our manuscript and have a good laugh together in our gezellige office. **Daoud**, thanks for the good advice, interesting discussions and for your dry humour. **Jos**, your enthusiasm was something to behold. I enjoyed our chats about liquid biopsy and how frustrated you got when your computer didn't obey your commands. Thanks to **Yongsoo** for always wanting to help and for being so polite and thoughtful. Thanks also to **Irsan, Jeroen, Thomas** and **Saskia** for your support and useful feedback.

Hedde, who started in TGAC on the very same day. It has been quite a journey to this point, but you made it fun by sharing the ups and downs with a good dose of dark humour. I'll remember the fun times at the PhD retreat and catch-ups over a beer (or two). **Matias**, thanks for always being willing to help and for your positive attitude. **Irene**, you added a lot of cheerful energy to the group and were a fun office mate. **Tjitske**, thanks for your useful feedback and nice chats by the coffee machine. **Tanya** and **Barbara**, I enjoyed our lunch chats.

Dirk, thanks for your help in the lab, your coding skills and constant willingness to help. **Phylicia**, I admire your efficiency, organisation and ability to remember exactly what we did with each plasma sample – something I was no good at! **Reno**, thanks for helping me get set up at the start and for your useful suggestions. **Sandra**, you showed me the ropes and helped me get settled in. Thanks for your help and the borrels during in the early days. **Jacqueline,** I enjoyed working with you and appreciate your cheerful attitude. **Danielle**, it was nice to collaborate with you. **Ruben**, my first student. Thanks for your enthusiastic approach to lab work. It was a pleasure to see you develop and grow over the course of your internship. I also learned a lot from our collaboration! **Laura**, thanks for all the work you did towards our publication. It was a pleasure to have you as a student and I'm pleased that you have continued to work in the lab after your Masters.

To Renske's group, with whom I shared labs, offices and fun times. **Barbara**, it was nice to share an office with you. I enjoyed sharing the ups and downs of PhD

life and the near constant supply of M&Ms when you were around. **Wina, Iris** and **Desiree,** thanks for the chats and fun times in the Star Wars room. You made me feel welcome from the start. **Maarten**, we always had good banter in the corridor and at the occasional borrel. I also enjoyed working with you in Rotterdam and hope that we stay in touch. **Annelieke**, **Anina** and **Sylvia**, thanks for your help in the lab and for organising great events! **Chris**, it was always nice to chat to you by the coffee machine. **Angelina** and **Putri**, thanks for your guidance and for being such friendly office mates.

To the Exosomes Group, thanks for treating me as one of your own. **Rubina**, it was always a pleasure to chat to you and share our experiences of living and working in a foreign country. **Florent**, your knowledge of cfDNA is truly enviable. Our discussions about the latest research paper were always stimulating and led to new ideas. Thanks for your generous and crucial contribution to our manuscript. **Esther**, you always asked how I was doing whenever our paths crossed. Your knowledge of clinical procedure was vital to the success of my thesis and I greatly appreciate how generous you were with your time and energy. **Maarten**, your enthusiasm and love of your work are visible from space! Talking to you was always engaging and enjoyable. **Monique**, you were a huge help with getting my projects going. Your all-encompassing knowledge of the lab and everything in it made it much easier to get great results. **Sandra** and **Nils**, thanks for the help and advice you gave me throughout my project, it was a pleasure to work with you both.

Daniëlle H, you played a crucial role in the set-up and validation of the ddPCR workflow and guided my projects towards clinical implementation. Thanks for the lively discussions, your valuable insights, comments on our manuscripts and for making the time for me. **Robert**, thanks for supporting my projects and taking the *EGFR* test through to clinical implementation. **Cees**, **Lars** and **Stefan**, thanks for your help with the cfDNA isolations. **Nicole**, I really appreciate your contribution as a Pathologist and highly collaborative researcher. It was a pleasure to work with you. **Pieter and Daphne,** thank you for supporting my thesis and providing guidance when I needed it. **Erik**, thank you for providing the all-important blood samples and for your guidance at the start.

Nina, I enjoyed our cappuccino-fueled discussions about LIBEC in the topwerkplein. I learned so much about the clinical side from you, it is clear that you love being a surgeon. Our complementary knowledge and shared passion for the subject made it an excellent collaboration. **Idris**, your friendly, approachable manner always made working with you enjoyable. Your perspective as a senior clinician was crucial to chapter four of my thesis. **Tom**, your advice at the start of my project helped steer me in the right direction. **Hans**, **Dennis, Maxime, Nik** and **Piotr**, we had some great borrels! **Frank** and **Tim**, it was always fun to go for a beer with you two.

To my high school teacher **Jane**, thanks for sparking my passion for biology seventeen years ago and setting me on the path to where I am now. To my secondary school headteacher **Mr. Roe†**, thank you for taking the time and effort to personally teach me to aim high. Thanks to the lads back in the UK for great weekends in London and epic ski trips. Thanks to **Jan**, **Jeannette**, **Robbert**, **Michiel** and **Marjo** for the fun days out and very enjoyable visits to Zeeland. To my parents and stepparents, **Mum**, **Dad**, **Nick** and **Pascale** and to my brothers **Tom** and **Nick**, thanks for all your support and encouragement over the years.

Eline, to you I am the most grateful. Your loving support and encouragement have kept me going through the failed experiments, rejected manuscripts and weeks of Covid lockdowns. It has been wonderful getting to know you these past five years while exploring our new city together. I am truly lucky to have such a kind, thoughtful and generous partner. I'm looking forward to many more exciting adventures with you, I love you.