Treatment and monitoring of patients with gastrointestinal stromal tumours using circulating tumour DNA

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The studies described in this thesis were performed at the Department of Medical Oncology of the University Medical Center Groningen in Groningen, The Netherlands.

Cover: P.A. Boonstra
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Printed by: Ipskamp printing

The work published in this thesis was performed with a KWF Alpe D'Huzes grant (RUG 2013-6355).

Printing of this thesis was financially supported by the Stichting Werkgroep Interne Oncologie, Rijksuniversiteit Groningen, Universitair Medisch Centrum Groningen, Bayer B.V., Pfizer Biopharmaceuticals Group and is gratefully acknowledged.

rijksuniversiteit groningen

Treatment and monitoring of patients with gastrointestinal stromal tumours using circulating tumour DNA

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

maandag 7 december 2020 om 14:30 uur

door

Pieter Aldert Boonstra

geboren op 9 mei 1984
te Naarden

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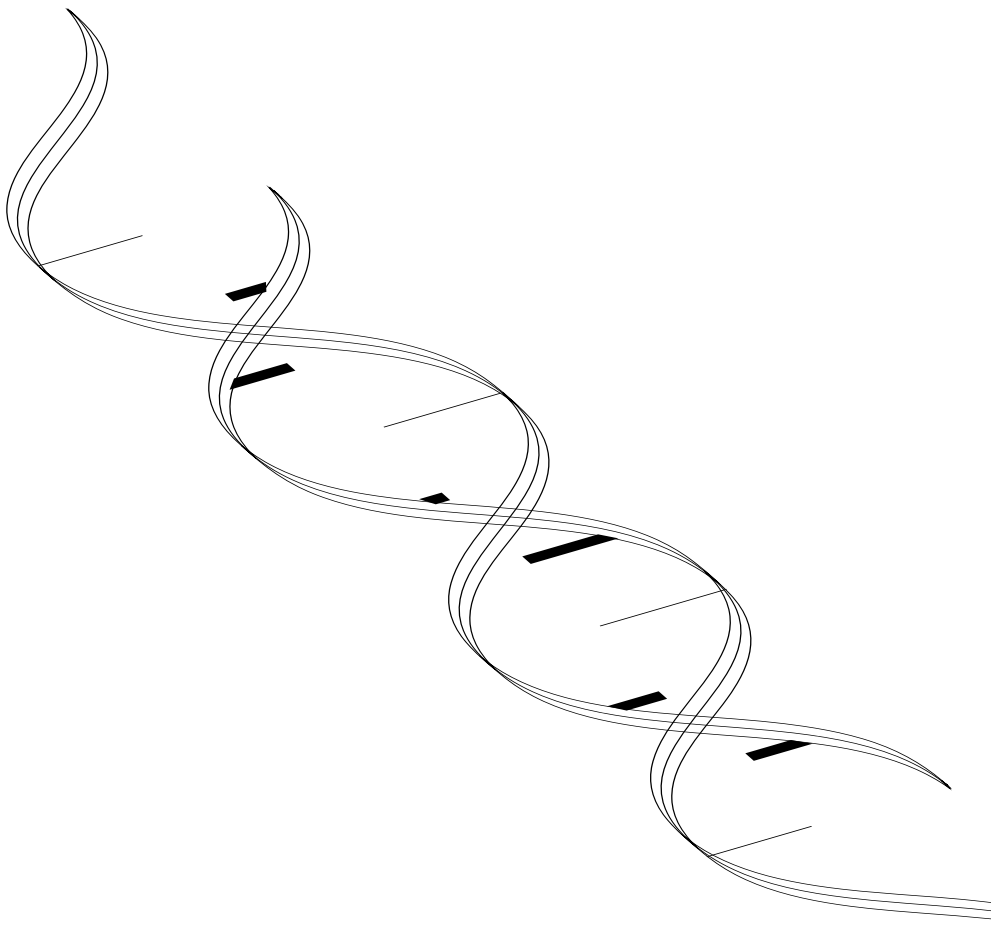

General introduction

Chapter 1

1. Gastrointestinal stomal tumours

Gastrointestinal stromal tumours (GISTs) are the most common mesenchymal neoplasms detected in the gastrointestinal tract.[1] GISTs can occur throughout the whole gastrointestinal system but most frequently originate from the stomach or small bowel in respectively 60% and 30% of the cases.[2] GISTs are soft tissue tumours and derive from the submucosal smooth muscle layers out of the interstitial cells of Cajal or their stem cell like precursor cells. These cells of Cajal are known as the pacemaker cells of the gut since they produce the electrical impulse that induces the peristaltic bowel movements.[2,3]
GIST as an entity is only known since the late 1990s when Hirota and colleagues discovered gain-of-function mutations in KIT as an unique character of GIST.[4] Before this breakthrough, GISTs were categorized as smooth muscle tumours; leiomyomas, leiomyosarcomas or leiomyoblastomas.[5]
Most GISTs have a similar genetic base. Gain-of-function mutations are found in the genes coding for KIT or platelet derived growth factor alpha (PDGFRα) in the majority of the tumours.[4] These genes encode for proteins that belong to the receptor tyrosine kinases. KIT is one of those receptor tyrosine kinases and is expressed on the cell surface in >95% of GISTs.[6] Antibodies to KIT (CD117) are therefore frequently used in immunohistochemistry to diagnose a gastrointestinal stromal tumour.
Normally, KIT and PDGFRα are activated by ligand binding, respectively stem cell factor and platelet derived growth factors (PDGFs). Ligand binding leads to dimerization of the receptor resulting in activation of the signalling pathway. This activation regulates essential cell functions including proliferation and apoptosis and is critical for the development and maintenance of the cells. Receptor tyrosine kinases with an oncogenic mutation are continuously activated independently of binding by its ligand (figure 1). This activation results in uncontrolled cell-growth and -proliferation.[7]

In the Netherlands, each year approximately 300 patients are diagnosed with a GIST.[8] This number only covers the clinically significant GISTs; a much higher incidence of small lesions is seen at autopsy or stomach resection specimens.[9] Since the rarity of the disease, patients with GIST are preferably treated in an expert centre in the Netherlands (Antoni van Leeuwenhoek Amsterdam, Leiden University Medical Centre, Erasmus Medical Centre Rotterdam, Radboud University Medical Centre Nijmegen and University Medical Centre Groningen) and registered in a national database, known as the Dutch GIST registry.
In this registry patient and clinical characteristics, pathology reports, as well as data on surgical procedures, systemic therapy, recurrence and survival are retrospectively and prospectively registered since 2009.
Most symptomatic patients present with abdominal pain, (acute) gastrointestinal blood loss or obstruction. Approximately 20% of the patients have asymptomatic

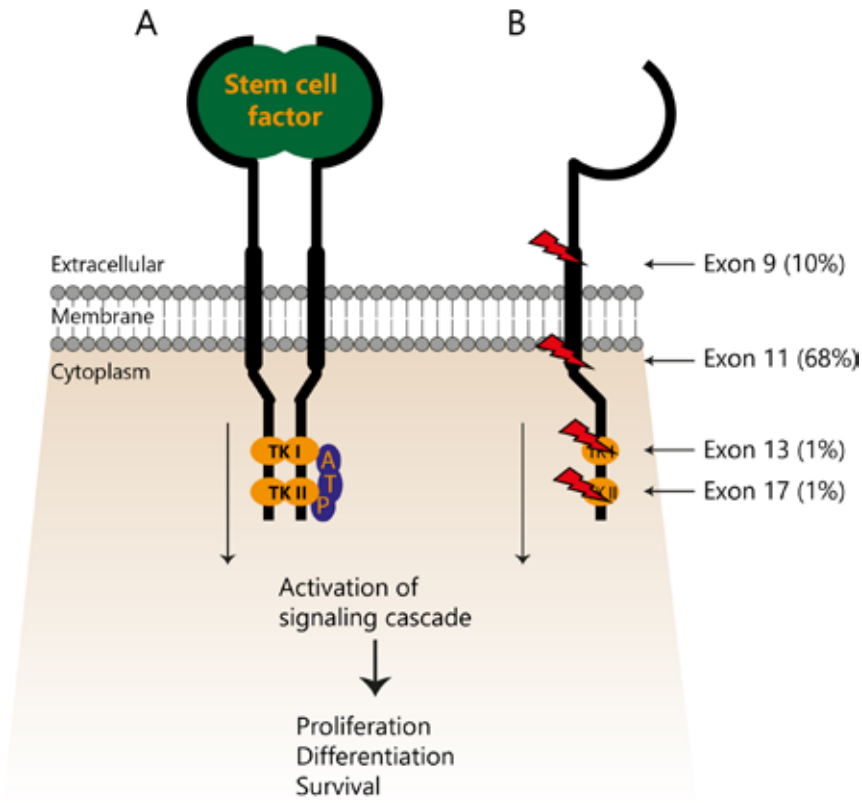

Figure 1. Activation of the KIT tyrosine kinase receptor in normal and malignant cells. **A.** The KIT tyrosine kinase receptor protein is activated by binding with its ligand, the stem cell factor. The ATP-driven activation of the intracellular signalling cascade results in cell proliferation, differentiation and survival. **B.** Specific mutations in the extracellular, juxta membrane and tyrosine kinase domains (TKI I and TKI II) of the KIT receptor encoded by respectively exon 9, 11, 13 or 17 observed in gastrointestinal stroma tumours result in activation without binding with its ligand and uncontrolled cell growth.[7] (Illustration adapted from Rubin et al.[1])

disease and are diagnosed by accident.[10]

Diagnosis is based on histopathological examination and relies on morphology and immunochemistry. As mentioned before, nearly all GISTs stain positive for KIT (CD-117) or the even more specific marker DOG-1 (discovered on gist-1).[11] Circa 55% of the patients present with localized disease.[12] Tumours preferably metastasize to the liver or to the peritoneum/abdominal cavity. Pulmonary and extra-abdominal metastases are very rare.

Treatment of patients with GIST consists of several steps. The standard curative treatment in patients with localized disease is radical surgical resection (R0) of the tumour. Imatinib, a specific KIT receptor tyrosine kinase inhibitor (TKI), is the standard first line treatment in patients with metastatic disease. It was the first effective therapy

for advanced GIST and has significantly increased the progression free survival (PFS), overall survival (OS) and quality of life.[13] When the tumour is locally advanced and radical surgical resection is not achievable, patients can be pre-treated with imatinib to downsize the tumour and become eligible for surgery.[14] After surgery, patients with high risk of recurrence are treated in an adjuvant setting with imatinib during three years at minimum (including neoadjuvant treatment) to reduce the risk of tumour recurrence.[15]

In a metastatic setting, median time to progression on imatinib treatment is approximately 23 months.[16] Second line therapy consists of sunitinib which is a multi-target TKI that inhibits several tyrosine kinase receptors such as VEGFR, PDGFR, FLT3, RET and KIT. Median time to tumour progression for sunitinib is 27 weeks compared to 6 weeks for placebo.[17] After confirmed progression on sunitinib, another placebo controlled randomized trial showed that regorafenib, a multikinase inhibitor (targeting VEGFR, TIE2, KIT, RET, RAF-1 and BRAF), significantly prolongs progression free survival.[18] Median PFS for patients treated with regorafenib was 4.8 months compared to 0.9 months for patients who received placebo.[19]

In daily practice, patients will have to regularly visit the outpatient clinic during treatment.[20] Assessments during these visits consists of the current clinical situation, laboratory testing (complete blood cell count, kidney and liver function) and imaging with MRI or CT scans (depending on tumour location). Imaging is usually performed every 3-6 months to evaluate whether the tumour is still sensitive for the treatment given. Not all GIST will respond equally to treatment with tyrosine kinase inhibitors. Patients with KIT exon 9 mutations will have a better change of treatment response when treated with imatinib 800 mg daily instead of 400 mg which is appropriate for patients with a KIT exon 11 mutation.[21] Specific mutations in KIT or PDGFRα (i.e. D842V) can impair the binding or decrease the sensitivity for imatinib, patients with these mutations have no or minimal treatment benefit of imatinib.

In the course of treatment with TKIs almost all GIST patients eventually will become resistant to the treatment, which is in most cases associated with the appearance of secondary mutations that are acquired during therapy.[22] After imatinib treatment in KIT exon 11 positive patients which was stopped due to disease progression mutations in KIT exon 13, 14, 17 or 18 are often detected that were not detectable in the primary tumour before imatinib treatment. In patients with these acquired secondary mutations, the median PFS is lower than in patients without secondary mutations treated with sunitinib, indicating a relation between secondary mutations and treatment resistance for sunitinib.[23] Evidence is available that suggests that the third line treatment with regorafenib enables effective inhibition of tumours with a secondary KIT exon 17 mutation. A median PFS could be reached of 22 months for patients with a secondary KIT exon 17 mutation compared to 13.2 months for the overall group.[24] This longer PFS for the overall group as compared to the phase 3 regorafenib registration trial is attributed by the authors to patient selection with a limited number of centers in the phase II trial compared to the larger multinational

phase III trial. In the phase III trial (GRID study) the response to treatment is not specifed for secondary mutations.[19]
Further knowledge regarding these acquired resistant mutations during therapy could point to new therapeutic targets and can guide as predictor of therapy response. At the current moment several new drugs are being developed to target standard therapy resistant mutations such as avapritinib (study name BLU-285, targeting secondary KIT mutations), crenolanib (PDGFRA D842V), larotrectinib (NTRK gene fusions), ripretinib (targeting several drug resistant KIT mutations, PDGFRA D842V) and cabozantinib (imatinib and sunitinib resistant KIT mutations).[25-29] Hence it is of great importance for proper treatment-decision-making to be informed about the presence of primary as well as secondary mutations on pre-treatment biopsy and during treatment.

2. Circulating tumour DNA

It has been known for a long time that DNA can be detected in the peripheral blood.[30] In the normal physiological process of cellular turnover DNA is shed into the circulation.[31] This circulating cell free DNA (ccfDNA) is found in blood plasma from healthy individuals and is elevated in case of inflammation or periods of intensive exercise.[32] Patients with malignant disease have higher levels of ccfDNA compared to healthy people.[33] In malignant disease, probably a higher cell turnover is present that results in higher levels of released DNA and thus genetic material of the tumour is present in the peripheral blood (referred to as circulating tumour DNA, ctDNA).[34] The exact mechanism by which this DNA is shed in the circulation is not entirely known.
Recent technological innovations enable the detection of these circulating DNA fragments in plasma (i.e. droplet digital PCR (ddPCR) and next generation sequencing (NGS)).[35] With these methods, tumour specific mutations can be detected and quantified in plasma of patients.
Until recently, the only method available for tumour mutational analysis was based on tumour tissue obtained with a biopsy or surgery. These are invasive methods with accompanying risks of perforation, bleeding and infection. Also, due to the anatomical localization or size of the tumours, metastases and recurrences a diagnostic biopsy cannot easily be performed in some cases. The detection of mutations in ctDNA in the peripheral blood takes away the necessity of performing tumour biopsies for mutational analysis.
Due to the invasive aspect of biopsy procedures, it is not always performed routinely in patients during the course of treatment. Routine mutation analysis could provide insights in the mechanisms of resistance that occur during treatment. This knowledge could guide the development of new treatments. This is for example seen in patients with EGFR mutated non-small cell lung cancer where testing and

monitoring for secondary and tertiary TKI-resistant mutations is common practice and specific drugs against those mutations are available.[36] Detection of mutations in plasma (also referred to as liquid biopsy) enables the analysis of the mutational status of the tumour at multiple time points during treatment. Another advantage of a liquid biopsy is that the circulating material is derived from all tumour locations and metastases in the patient. This is in contrast to a single tumour biopsy of which a profile might not adequately represent the tumour due to intra-tumour and inter -lesion heterogeneity. Furthermore, liquid biopsies are easy to perform and only slightly stressful for patients. Additionally, the quantitative assessment of mutations detected in plasma seems to correspond with clinical disease status.[37-39] After start of treatment the amount of detectable mutations will decrease in case of therapy response. When treatment resistance occurs, the level of primary mutation will rise and new acquired mutations that cause the therapy resistance might be detected in the plasma.[40] However, at the current moment there is insufficient evidence for the majority of the ctDNA assays, discordance has been shown between the results of various ctDNA assays and the clinical validity has yet to be proven. Further research is needed before clinical implementation is feasible. It is likely that in the near future evidence will emerge that confirms the high expectations of this new technique.[41]

3. Thesis

This thesis focuses on the treatment of patients with GIST, the detection of primary and secondary mutations in ctDNA of those patients and the application of ctDNA to monitor treatment response during treatment with a TKI.

To determine the optimal treatment strategy for patients with GIST it is necessary to be informed about the primary KIT/PDGFRα mutational status of the tumour. When progressive disease is observed during treatment, the status of resistant, secondary KIT/PDGFRα mutations is needed to decide on a possible therapy switch to another TKI. Mutations associated with acquired resistance to imatinib could also be associated with resistance to second line therapy. Furthermore, drugs that target specific (secondary) mutations will become available in the near future. For these reasons mutation analysis of a biopsy from progressive lesions is warranted for proper treatment decision making.

The use of liquid biopsies to detect ctDNA as treatment response marker has already been evaluated in several malignancies. **CHAPTER 2** provides an overview of current literature (until January 1st 2019) regarding the use of tumour-derived mutations in ccfDNA derived from plasma and the relation with therapy response monitoring. One of the pitfalls in analysing ccfDNA is the fact that ctDNA is generally present in very low concentrations and represents a very small fraction of the total amount of ccfDNA. This is due to the physiological process of cellular destruction and the high abundance of normal wild-type DNA. Highly analytical sensitive techniques

are therefore needed to detect ctDNA in liquid biopsies. Before performing the analysis an efficient extraction of the ccfDNA from the plasma is essential. An optimal method would select the fragments derived from tumour cells (ctDNA) and/or for the nucleosome-protected fragments. In **CHAPTER 3** we compared three different ccfDNA extraction methods using the same cell free plasma from cancer patients. The isolated ccfDNA was characterized for the integrity and total yield using the fragment bio-analyser and a β-actine droplet digital PCR (ddPCR) assay based on the assay reported by Norton and van Dessel which is able to detect DNA fragments of three different sizes characteristic for typical ctDNA fragments and larger DNA from healthy tissues.[42,43]

The design of a single-tube ddPCR drop-off assay to detect the most common KIT exon 11 mutations in patients with localized and advanced GIST is described in **CHAPTER 4**. The ddPCR assay is designed according a drop-off principle.[44] Since 70-80% of the known KIT exon 11 mutations occur in one of two hotspot regions within 80 base pairs (COSMIC), this assay consists of two probes that target each one of these two mutation hotspots.[45] With the designed primers the PCR will result in a product of 124 base pairs including these two hotspots. Since mutations occurring in both hotspots in the same tumour are rare, one probe acts as wild-type (control) probe while the loss of signal from the other probe represents the presence of a mutation, referred to as a drop-off. The performance of this assay is validated on both tissue biopsies and ccfDNA from a small subset of GIST patients.

In **CHAPTER 5** two patients with rare PDGFRα mutations are described. Both patients have a treatment response on various TKIs. Plasma samples were collected during treatment and in both patients the ctDNA levels were measured with specific designed ddPCR probes and correlated to the response on treatment.

It is known that the biological behaviour of GISTs is depending on the origin of the primary tumour. Patients with a small bowel GIST have for example a worse prognosis compared to patients with a GIST derived from the stomach. Most studies regarding treatment of patients with a GIST describe cohorts of patients with various anatomical origins together. We investigated the treatment of patients with a GIST originating from the small bowel who were registered between January 1st 2009 and December 31st 2016. In **CHAPTER 6** retro- and prospectively collected data of the Dutch GIST registry were described.

New applications of ctDNA detection with other techniques than ddPCR in patients with GIST are described in this thesis. In **CHAPTER 7**, ctDNA from plasma is analysed with next generation sequencing in our routine diagnostic laboratory. This because the patient presented with newly diagnosed pulmonary embolism and the use of anti-coagulants made it too hazardous to perform a tissue biopsy.

The main findings of this thesis are summarized and discussed in **CHAPTER 8**, followed by the future perspectives regarding the role of circulating tumour DNA in clinical practice. A Dutch translation of the summary of this thesis is provided in **CHAPTER 9**.

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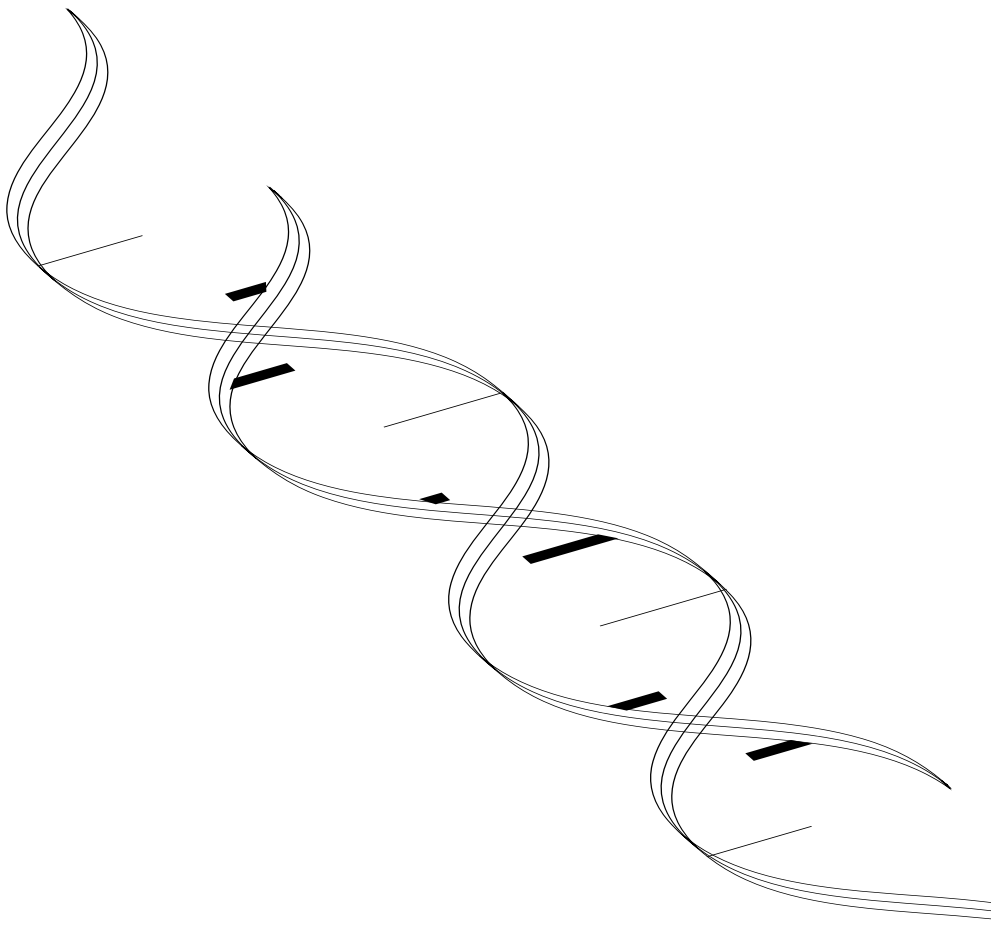

Circulating tumour DNA as a response and follow-up marker in cancer therapy

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Cancer Treatment Reviews, 2020

Abstract

Response evaluation for cancer treatment consists primarily of clinical and radiological assessments. In addition, a limited number of serum biomarkers that assess treatment response is available for a small subset of malignancies. Through recent technological innovations new methods for measuring tumour burden and treatment response are becoming available.

By utilization of highly sensitive techniques, tumour specific mutations in circulating DNA can be detected and circulating tumour DNA (ctDNA) can be quantified. These so called liquid biopsies provide both molecular information about the genomic composition of the tumour as well as opportunities to evaluate tumour response during therapy. Quantification of tumour specific mutations in plasma correlates well with tumour burden. Moreover, with liquid biopsies it is also possible to detect mutations causing secondary resistance during treatment.

This review focuses on the clinical utility of ctDNA as a response and follow-up marker in patients with non-small cell lung cancer, melanoma, colorectal cancer and breast cancer. Relevant studies were retrieved from a literature search using PubMed database. An overview of the available literature is provided and the relevance of ctDNA as a response marker in anti-cancer therapy for clinical practice is discussed. We conclude that the use of plasma derived ctDNA is a promising tool for treatment-decision making based on predictive testing, detection of resistance mechanisms, and monitoring tumour response. Necessary steps for translation to daily practice and future perspectives are discussed.

1. Introduction

Response evaluation during anti-cancer therapy and follow-up of patients with solid malignancies is currently primarily based on radiological assessments according to response evaluation criteria in solid tumours (RECIST).[1] Repeated radiologic assessments are however time consuming, costly, and increase the radiation burden for the patient. This is especially an issue in the context of the increasing number of long-term cancer-survivors due to new anti-cancer therapies. Moreover, response evaluation based on radiologic assessment is problematic with certain novel therapies. For example, immunotherapy can cause pseudoprogression on radiologic assessments as a result of influx of cytotoxic T-lymphocytes.[2] Irradiation of high grade glioma's can cause pseudoprogression on MRI in approximately one third of the patients.[3] And, anti-VEGF therapy in colorectal cancer can result in morphological changes such as altered delineation of the tumour, which predicts pathologic response and overall survival better than does standard radiologic assessment according to RECIST.[4] Finally, response assessment can be difficult in certain settings regardless the therapy given. In bone dominant disease such as prostate cancer and hormone-positive breast cancer, response assessment is hampered as bone lesions are considered non-evaluable by RECIST.[5]
Whereas novel therapies may not only cause difficulties with regard to radiologic response assessment, these new treatments often also aim at specific mutations (i.e. receptor tyrosine kinases that are in a continuously activated state due to genetic aberrations). Therefore, for treatment decision-making up to date information about the genomic composition of the tumour lesions is crucial. Frequently, archival tissue is used for genomic analysis of molecular aberrations. However, tumour characteristics can change during the course of disease, such as development of new mutations causing secondary resistance. Repeated biopsies may be obtained, but this is not always feasible, invasive, and not always representative of the whole tumour burden due to sampling error and tumour heterogeneity.[6]
To circumvent the above mentioned limitations regarding radiologic response assessment, as well as the need for up-to-date information about molecular characteristics, there is a clinical need for tumour-specific, highly sensitive, non-invasive assays to determine the genomic composition of tumours and to assess response accurately in solid malignancies.

2. Liquid biopsies

A potential method to obtain information about both the genomic composition of tumours as well as the tumour burden is through detection and quantification of tumour DNA in plasma. Tumour DNA can be identified by tumour-specific mutations that are derived from circulating tumour cells (CTCs), tumour derived

vesicles (exosomes) and nucleosome bound tumour DNA that is shed into the circulation during necrosis or apoptosis of tumour cells.[7-9] Various methods to analyse and quantify circulating tumour DNA (ctDNA) are available.[10-12] First generation sequencing methods are PCR-based techniques such as droplet digital PCR (ddPCR) and "breads, emulsification, amplification and magnetics (BEAMing). Although PCR-based techniques are limited by evaluating only a low number of pre-specified mutations, the costs are relatively low, an absolute number of aberrant copies per mL can be provided, turnaround time is short and sensitivity high. More recently next-generation sequencing (NGS) has been developed, which can cover larger panels of selected genes/ mutations, whole-exome or even whole-genome sequencing. Aside from its larger coverage when compared to ddPCR, NGS also has the advantage that mutations do not need to be pre-specified and therefore rare and novel mutations can be detected. However, NGS is more costly, turnaround time is longer, and sensitivity for mutations with low mutant allelic frequency can be lower than with ddPCR.[13]

As a method to quantify tumour burden, liquid biopsy has the advantage over radiologic assessments that it may differentiate between pseudoprogression and true progression, may be used to evaluate response in settings in which radiologic assessment is difficult (such as bone-dominant disease), and can reduce radiation burden. As a method to obtain molecular information, liquid biopsy has the advantage over biopsy-driven genomic analysis that it is non-invasive, can provide information about presence of various subclones, and gives the opportunity to evaluate for secondary resistance mutations during the course of disease. At this moment the evidence to support widespread use of ctDNA as a predictive or prognostic marker in patients with solid malignancies is limited.[14] In this review we summarize data on the application of ctDNA analysis as a treatment response and follow-up marker in patients with solid malignancies. We focus on non-small cell lung carcinoma (NSCLC), melanoma, colorectal carcinoma (CRC), and breast cancer, given the specific driver mutations that are often present and the availability of targeted drugs.

3. Search strategy and quality of the included studies

A PubMed search was performed on January 1, 2019 using the following syntax: (Oncology[tiab] OR Cancer* [tiab] OR malignant[tiab] OR malignanc*[tiab] OR tumor[tiab] OR tumour[tiab]) AND (DNA[tiab] OR " Deoxyribonucleic acid"[tiab] OR RNA[tiab] OR "Ribonucleic Acid"[tiab]) AND (Mutation*[tiab] OR Rearrange* [tiab]) AND (("circulating"[tiab] OR ctDNA[tiab] OR cfDNA[tiab] OR "liquid biopsy" OR "blood based" OR "Circulating tumor cells"[tiab] OR "Circulating tumour cells"[tiab] OR CTC[tiab] OR ("platelets"[tiab] OR Thrombocytes[tiab])) AND ("humans"[MeSH Terms] AND English[lang]). The search was limited to full articles, written in English. In total 1057 articles were identified. Articles were screened on title, abstract and full

text by PAB and TTW. Articles describing sequential ctDNA measurements in human patients with solid malignancies during systemic therapy were eligible. Studies regarding the use of CTCs, exosomes or other circulating markers were excluded. Studies that investigated detection of mutations in body fluids other than plasma were not within the scope of this review.
Finally, 82 articles were eligible for this review (table 1). Of these, 26 articles provided detailed descriptions of individual cases or case series. No randomized clinical trials were available. The remaining 56 articles consisted of studies that evaluated the association of plasma ctDNA levels with response rate (RR), progression free survival (PFS) and/or overall survival (OS). Relevant articles that not matched our search criteria were occasionally added. All papers were classified for level of evidence following the rules as depicted by the Oxford Centre for Evidence-Based Medicine.[15] Six studies were classified as exploratory cohort studies with good reference standards resulting in a score of 2b (2 melanoma and 4 CRC studies). Fifty studies were non-consecutive studies without consistently applied reference standards (3b) and 26 studies consisted of case-reports or small series without poor or non-independent reference standards (4, table 1). Although the largest study included 200 patients, most studies have low patient numbers (range 1-200, median 14 patients).

4.1 Non-small cell lung cancer

The mutations of interest in most studies regarding NSCLC are effecting the epidermal growth factor receptor (EGFR). Of all EGFR mutations described in this review, 99% is found in NSCLC. Other genes in which mutations were observed frequently in NSCLC were TP53 and KRAS. Detection rate of primary EGFR mutations in pre-treatment plasma ranged between 23-100%, highest detection was reached with PCR based methods compared to techniques based on (next-generation) sequencing (median 79% vs 66.6% respectively).
Thirty-three of the included 35 studies showed a positive relation between treatment response and a decline in mutant fraction after initiation of treatment. Disease progression could be detected with ctDNA in 28 studies, 6 studies did not have follow-up long enough for detection of progressive disease and in one study the decline in mutant ctDNA fragments did not correspond with clinical disease status (table 1).[16]
Prolonged PFS was observed for patients with undetectable levels of ctDNA during treatment versus patients with persistent detectable levels of ctDNA compared to baseline levels.[17-19] A decrease or even disappearance of mutant EGFR after start of treatment is a prognostic factor and indicator of response and is associated with longer OS.[20-24] An increase of the EGFR activating mutation is suggestive for therapy resistance and subsequent disease progression.[25-27] Smaller studies and case reports presented similar results.[28-30] The use of ctDNA as an early response

marker is implicated by a longer OS in patients with undetectable levels of ctDNA after 6 to 12 weeks of anti-EGFR therapy compared to patients with detectable levels of ctDNA after the same treatment period.[17-19,31,32]
In patients with acquired EGFR-tyrosine kinase inhibitor (TKI) resistant NSCLC, a rise of primary EGFR-mutated DNA occurred simultaneously with the detection of new mutations in the plasma in the majority of the tested patients during treatment. [21,33-35] Detection of the therapy resistant T790M mutation during treatment is suggestive for disease progression and a worse OS.[36-41] Secondary treatment resistant mutations can also be used for treatment monitoring but occur at lower frequencies than the primary mutation and are therefore less suitable for detection of disease progression.[42] Furthermore, these secondary mutations could almost only be detected in patients with a primary EGFR mutation.[43] New uncommon mutations that developed during treatment indicate clonal heterogeneity of the tumour and could be detected using sequencing; this is shown by the detection of a novel C797S or L747P mutation and EML4-ALK gene translocation additional to the primary EGFR exon 19 or T790M resistant mutation during treatment.[31,35,44,45]
Five studies reported an earlier detection of progressive disease by ctDNA assessment as detected with conventional radiological imaging.[19,21,42,46,47]
KRAS mutations can also be used as circulating marker in NSCLC patients treated with chemotherapy; patients with a detectable KRAS mutation had worse overall survival compared to patients with wild-type DNA (median 3.6 vs 8.4 months, respectively). [29] A detectable KRAS mutation also indicated resistance to treatment with EGFR-targeted therapy in those patients (i.e. erlotinib or pertuzumab).[48,49] Of interest is the recent development of a specific KRAS inhibitor that can target KRASG12C mutation.[50]
When treatment with novel agents as nivolumab (anti-PD-1) was initiated, a decrease in detectable specific mutations in plasma within eight weeks after start of therapy was observed in responders (n=11), while in non-responders (n=5) a stable or increasing level of plasma ctDNA was detected.[51,52]

4.2 Cutaneous melanoma

Mutations in cutaneous melanoma were primarily observed in v-Raf murine sarcoma viral oncogene homolog B (BRAF). Detection rate of primary mutations in plasma ranged between 37% and 100% (median 70%), only one study used a sequencing approach to detect mutations (table 1).
Two studies described a total of 31 patients with BRAF-mutated melanoma treated with BRAF-inhibitors (BRAF-i) alone or in combination with mitogen-activated protein kinase inhibitors (MEK-i).[53,54] A disease control rate (DCR) of 75% was found in patients in whom mutation copy levels in ctDNA decreased compared to a DCR of 18% in patients with a stable or increasing level of ctDNA after 8 days

of therapy.[54] Patients with undetectable ctDNA levels after a median of 13 days (range 6-40) of BRAF-i therapy had longer PFS compared to patients with persistent detectable ctDNA levels during therapy (n=36 in total).[53] Other studies in patients with metastatic melanoma treated with BRAF-i alone or in combination with MEK-i described similar observations.[55-59]

Seremet et al. described 7 patients treated with an immune checkpoint inhibitor (ICI) in which the course of treatment was reflected by changes in ctDNA in patients with BRAF or NRAS mutated disease.[60] After initiation of treatment the mutant BRAF/NRAS copy level decreased and remained low or undetectable during complete response and increased in case of progressive disease. However, another study in 15 patients reported no difference in ctDNA plasma levels after four to eight weeks of ICI therapy in 13 patients compared to pre-treatment levels although only four patients responded to treatment (of which two had a 10-fold reduction in ctDNA levels).[57]

Finally, in 20 patients treated with a combination of dacarbazine, cisplatin, vinblastine and tamoxifen, BRAF mutant copies were detected in plasma at baseline and could only be detected in the plasma of 1 out of 10 responders and in 7 out of 10 non-responders.[61] There were no studies reporting on the detection of new acquired mutations during treatment.

The introduction of BRAF-targeted and ICI therapy for patients with metastatic melanoma has led to an increase in OS.[62] In patients with irresectable cutaneous melanoma treated with ICI therapy, a major challenge is the differentiation between 'true' progression and pseudo progression (occurring in ~10% of patients) on radiological response evaluation. Although other markers, such as serum s100B, LDH and the immune-related response criteria for radiological response assessment provide some guidance, no marker is currently available. In a recent study, plasma samples obtained from 29 patients with cutaneous melanoma who showed progression of disease after 12 weeks of ICI therapy, all patients with pseudo progression (n=9) had undetectable or >10-fold decrease in ctDNA levels compared to pre-treatment levels.[63] Conversely, of the patients with 'true' progression (n=20), 90% had stable or increasing ctDNA levels compared to pre-treatment levels after 12 weeks of ICI therapy. Recent studies have shown an improvement of recurrence free survival in patients with stage III melanoma treated with surgery followed by adjuvant treatment with a ICI.[64] However, ICI therapy bears potential long-lasting risks such as immune-related adverse events, a proportion of patients will be treated in vain and therapy costs are high.[65,66] Therefore, selection of patients at risk for recurrence is of great importance.

4.3 Colorectal cancer

In colorectal cancer, most studies concern mutations in KRAS. The detection rate

of primary mutations in plasma was reported in 10 studies which all used PCR based techniques. The presence of KRAS mutations ranged between 18% and 100% (median 89%).
A higher response rate to chemotherapy and a longer PFS is described in patients in whom a decrease in ctDNA levels during therapy was observed compared to patients with stable or increasing ctDNA levels during treatment.[67,68] Although the studies showed a trend towards longer survival and better response rates in patients with decreasing or undetectable ctDNA levels upon treatment, no statistically significant association between ctDNA level, OS, PFS or radiological response has been described.[69-75] A decrease in total circulating cell free DNA (cfDNA) copies/ml and mutant KRAS/BRAF/TP53 levels after two cycles of therapy compared to baseline and a subsequent increase at the time of progression in patients with CRC was related to treatment response as well as resistance. The decrease after initiation of treatment was larger in responding than in non-responding patients.[76,77]
Resistance to EGFR targeted treatment can be caused due to amplification of the MET proto-oncogene and mutations in PIK3CA. This MET amplification is reported to be detected in ctDNA before relapse is clinically evident.[78,79] Mutations that are newly detected during treatment might reveal the rise of minor tumour clones that show resistance to the administered therapy.[80]
The emergence of KRAS mutations in KRAS wild type patients during anti-EGFR therapy is suggestive for disease progression and was in some studies detectable in the blood prior to radiographic detection of progressive disease.[81-84]
Three studies described differences in ctDNA levels in a total of 29 patients with CRC before and after surgery.[85-87] In all patients with a complete resection (n=26) a decline in ctDNA levels in plasma was observed. Three patients had tumour recurrence, which occurred simultaneously with recurrence of a KRAS mutation in ctDNA. In cases without complete resection (n=3), ctDNA levels decreased only slightly or even increased. Additionally, it was observed that in patients with disease recurrence an increase of plasma ctDNA levels occurred before or at the same moment the CEA-levels increased and 2-3 months before radiologic evaluation showed signs of recurrence.[87-89] The ctDNA status at postoperative day 30 could be indicative for disease recurrence. Of 94 patients, 10 patients had positive ctDNA samples at day 30 and had a significantly higher recurrence rate (70%) compared to patient without detectable ctDNA (11.9%) at day 30.[90]
Early detection of recurrence will increase the proportion of patients who are potentially eligible for curative therapy. A survival benefit from such an approach has been shown in several meta-analyses.[91]
Another study that used sequencing for analysis of ctDNA described an increase of 34% in the amount of different detectable mutations at the time of progression.[92] These mutations were not detectable at the time of primary disease, indicating clonal evolution of the disease. Furthermore, NGS can be used to detect new emerging mutations in the ALK kinase during treatment with the ALK inhibitor entrectinib.

[93] The emerged mutations are associated with treatment resistance and warrant treatment with second-generation ALK inhibitors.

4.4 Breast cancer

TP53-mutations (n=81), ESR1 (n=82), PIK3CA-mutations (n=53) and AKT-mutations (n=31) have most frequently been assessed to evaluate response to therapy using ctDNA in patients with breast cancer. As a large variety of mutations in breast cancer is present, NGS seems more feasible to detect mutations compared to ddPCR. Six of the 13 included studies used sequencing for the detection of mutations. The mutation detection rate ranged from 24% to 92% with a median of 50%.
Sequencing of PIK3CA and TP53 performed on ctDNA of 30 patients showed that changes in tumour burden correlated better with the height of plasma ctDNA levels compared to CA 15-3.[94] Detection of TP53 seems feasible to monitor treatment response as a decrease of TP53 after initiation of treatment corresponded with response and an increase was a sign of relapse.[95] Patients with undetectable levels of ctDNA after one cycle of neo-adjuvant chemotherapy had longer PFS and OS compared to patients in whom ctDNA remained detectable.[96,97] In 28 patients with estrogen receptor positive (ER+) and BCL-2 (estrogen responsive gene responsible for survival which is overexpressed in 80% of primary ER+ breast cancer) positive metastatic breast cancer (MBC) treated with tamoxifen and venetoclax (BCL-2 inhibitor) treatment responses were shown to correlate with serial changes in ctDNA in plasma. A significant reduction of both ESR1 and PIK3CA mutations was observed within 28 days of treatment in all patients and it appeared that radiological progression was preceded by a rise in ctDNA.[98] Changing allelic fractions of ctDNA for any given mutation reflected response to therapy and disease progression in 7 patients.[99] Similar results were described in smaller studies.[100-104]
Murtaza et al. described a patient with metastatic breast cancer (MBC) in which tumour-site specific mutations were identified implying heterogeneity of the tumour.[101] Sequencing of ctDNA showed that local progression of one tumour site coincided with an increase of the circulating abundance of mutations attributed to the lesion at that specific tumour site. This shows that ctDNA reflects dynamic alterations in size and activity of metastases at various tumour sites. This is supported by the findings of Page et al. which described rising cfDNA concentrations at the moment when PIK3CA/TP53/ESR1 mutations did not increase or resolved in the plasma.[105] The rise is probably caused by another clone that is shedding DNA into the blood that is not detected with the used ctDNA analysis method.
New mutations have been detected at the moment of progression which implicate acquired resistance to the treatment.[106,107] It was shown that patients with endocrine therapy resistant disease and detectable ESR1 mutations in ctDNA had longer PFS when treated with fulvestrant (n = 45) compared to patients treated with

exemestane (n = 18). Conversely, in patients with wild-type ESR1 no difference in PFS was observed between both treatment arms. This suggests that ctDNA may direct choice of treatment in patients with resistant disease. In line with these observations, a meta-analysis of a combined total of 1,530 patients with ER+ MBC showed shorter PFS for patients with a detectable ESR1 mutation in plasma ctDNA. Plasma ESR1 mutations were associated with shorter PFS after aromatase-inhibitor based therapy, but were not predictive of survival in patients treated with fulvestrant containing therapy.[108] Only three studies report data in comparison with the time of radiological assessment. In two of these studies the ctDNA preceded detection of recurrence with CT and in one study ctDNA analysis was as sensitive as the CT-scan. [100,107,109]
Several studies report the detection of novel mutations in PIK3CA and ESR1 during therapy in patients with MBC resistant to palbociclib and fulvestrant. These findings could also guide future treatment strategies to overcome resistance.[110-112]

5. Future perspectives

5.1 Liquid biopsies to guide targeted therapy

The studies discussed in this review show that various targets that directly affect treatment decision-making, such as EGFR mutation in NSCL, BRAF mutation in melanoma, and KRAS mutation in CRC can be detected by liquid biopsies. However, currently only one liquid biopsy assay to guide treatment decision-making is FDA approved; the Cobas EGFR v2, which can be used as a companion diagnostic for EGFR mutations associated with progression of EGFR-mutation-positive NSCLC.[113] Thus, translation towards clinical implementation of ctDNA testing as well as the availability of appropriate guidelines are urgently needed.[114] For EGFR mutation testing in NSCLC using plasma samples, External Quality Assessments (EQA) showed a need for quality improvements in clinical settings based on a high level of diagnostic errors.[113,115] Despite the promising results in the last few years (this review), disadvantages of current ctDNA testing include limited sensitivity, restricted clinical utility and loss of a direct link between a mutation and a given lesion.[116] Therefore ctDNA testing in clinical practice needs to be further investigated and international consensus has to be reached on standardized operating procedures.[14]
With regard to sensitivity of liquid biopsies, a broad range sensitivity for mutation detection is seen in the published studies. This could partly be related to the method of analysis since not all used methods have the same sensitivity or specificity. Moreover, the mutations in the reported studies are frequently solely detected in plasma and not necessarily compared to mutations detected in the tumour tissue. Therefore negative ctDNA results could in fact be true-negative due to absence of the given mutation. Since negative results can be either a result of detection limit as well as true negative results, it is questionable whether refrainment from treatment can

be based purely on the absence of a mutation in ctDNA, and tissue-based analysis will likely remain the golden standard. In contrast, positive ctDNA results have shown high specificity in the different studies and may well be used to guide therapy.
Ideally, either prospective evaluation or retrospective testing of ctDNA analysis and its relation with treatment outcome from randomized studies is needed to show that the predictive value of liquid biopsies is comparable to that of the current gold standard of tissue-based molecular analysis. For the FDA approved Cobas EGFR v2, for example, the observed benefit from erlotinib in the ENSURE trial was comparable for the patients that had a positive liquid biopsy when compared to tissue-positive patients.[117,118] In addition, in the phase III EURTAC trial positive, negative and overall agreement between liquid biopsy results and tissue-based analysis for EGFR mutation was very high (94.2%, 97.5% and 96.3% respectively), and it had similar predictive value for benefit from erlotinib over chemotherapy.[119] Finally, also in the phase II AURA2 trial it was shown that T790M positive patients by liquid biopsy had a high objective response rate to osimertinib.[120]
Comparable trials showing predictive value of liquid biopsies in other tumour types and for other treatments are needed before liquid biopsies can be considered as a replacement for repeated tumour biopsies. Currently, various liquid biopsy tests have been granted FDA breakthrough device designation, among which the FoundationOne Liquid, which captures 70 oncogenes in different tumour types, the Guardant360, which is a 73-gene panel to guide treatment decision in NSCLC, and Resolution HRD to determine aberrations in genes associated with homologous recombination deficiency.

5.2 Additional value of liquid biopsies for response evaluation

Currently, no liquid biopsy test is approved for response evaluation during treatment, but the studies discussed in this review indicate that this is a promising field. Detection of progressive disease with ctDNA before radiological progression is reported in twenty-one studies in this review. Since progression by ctDNA is detected simultaneously with radiological progression in the majority of the other studies it could possibly be used as a substitute for the latter. However, to reliably use ctDNA in daily practice instead of radiological imaging, a more consistent sensitivity has to be reached concerning the detection of predictive and resistant mutations in plasma. Especially cases were no mutations are detected in the plasma are unreliable and should be tested with more sensitive assays. Additionally, more studies are needed that correlate plasma mutations with radiologic data before replacing imaging with ctDNA can be considered. One of the most relevant settings in which ctDNA quantification may be of additional value is to differentiate between true progression and pseudoprogression in patients treated with immune checkpoint inhibitors. [121] Current studies are however limited by low patient numbers. Whether liquid biopsies can adequately result in refrainment from unnecessary treatment, costs, and potential side effects in patients with true progression on immunotherapy, while

treatment is continued and eventually results in response in patients with radiologic pseudoprogression should be addressed in future studies.

5.3 Liquid biopsies to evaluate mutations causing secondary resistance and tumour heterogeneity

Several studies describe the detection of new mutations during therapy implying progression on treatment and clonal heterogeneity of the tumours. In patients with NSCLC it has been demonstrated that mutations which potentially cause therapy resistance can be detected in ctDNA during treatment with EGFR-TKIs. For example, the well-known T790M mutation causing acquired resistance to EGFR inhibitors can be detected in ctDNA of lung cancer patients. Similarly, PIK3CA mutations causing endocrine therapy resistance in breast cancer patients can be detected in liquid biopsies.[122] Thus, ctDNA could be a promising technique to identify patients at risk for disease progression and select or adjust systemic therapy accordingly to improve patient-tailored therapy. Aside from known resistance mechanisms, liquid biopsies may also aid to detect new mutations and give insight in other mechanisms of secondary resistance. Whether these detected mutations during the course of disease have a role in acquired therapy resistance and whether they could be targeted to overcome such treatment resistance must be assessed in larger clinical studies. In particular, assessment of the association between the golden-standard (i.e. tumour biopsy) and detection of "new" mutations in plasma is essential.

5.4 Other promising applications of liquid biopsies

Although beyond the scope of this review, there are various other areas of interest which may show clinical utility of liquid biopsies. Among these are i) screening for early stage cancer, ii) to guide neoadjuvant therapy, iii) as a surveillance tool after curative treatment, iv) to assess recurrence risk after curative treatment and guide adjuvant therapy, v) liquid biopsies from other bodily fluids, such as urine or cerebrospinal fluid.[89,90]

6. Conclusion

The aim of this review was to evaluate the clinical utility of ctDNA as marker for treatment response and follow-up in patients with mutation driven solid malignancies during systemic therapy or after surgery. Although multiple studies show promising results for the utilization of ctDNA measurements in plasma to guide therapy decision-making and assess response in patients with solid tumours, larger prospective studies are needed. In order to be utilized as a blood-based marker, the association between ctDNA, tissue-based molecular analysis, tumour burden, radiologic response, and survival should be assessed for different tumour types, mutations, and targeted therapies individually.

Funding
PAB works on a grant provided by the Dutch Cancer Foundation (KWF, Alpe d'Huzes RUG 2013-6355).

Conflicts of interest
The authors have no conflicts of interest to declare.

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Author	Tumour type	Paper score	Gene of interest	Technique	Therapy	n	Disease status	Mutation detection rate in plasma	Predictive for disease progression	Predictive for response	Progression ctDNA before radiological
Alegre [25]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	8	Metastasized	65%	Yes	Yes	-
Arulananda [45]	NSCLC	4	EGFR	ddPCR	EGFR TKI	1	Metastasized	-	Yes	Yes	-
Demuth [43]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	144	Metastasized	100%	-	-	-
Guibert [49]	NSCLC	3b	KRAS	ddPCR	Multiple	16	Metastasized	78%	Yes	Yes	-
Guibert [52]	NSCLC	4	KRAS	ddPCR	Anti-PD-1	2	Metastasized	-	-	Yes	-
He [20]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	128	Metastasized	93%	Yes	Yes	-
Iijima [51]	NSCLC	3b	Various	NGS	Anti-PD-1	14	Metastasized	23%	-	Yes	-
Imamure [47]	NSCLC	3b	EGFR	NGS	EGFR TKI	38	Metastasized	73%	Yes	Yes	-
Imamure [22]	NSCLC	3b	EGFR	NGS	EGFR TKI	21	Metastasized	66,60%	-	Yes	-
Iwama [26]	NSCLC	3b	EGFR	ddPCR, NGS	EGFR TKI	32	Metastasized	81%	Yes	Yes	-
Jia [39]	NSCLC	3b	EGFR + KRAS	ddPCR	Not specified	150	Metastasized	89%	Yes	Yes	Unknown
Jiang [28]	NSCLC	3b	TP53	Seq	Chemotherapy	28	Metastasized	100%	Yes	Yes	-
Jovelet [34]	NSCLC	4	EGFR	ddPCR	EGFR TKI	7	Metastasized	62%	Yes	Yes	-
Knebel [46]	NSCLC	4	EGFR	ddPCR	EGFR TKI	1	Metastasized	-	Yes	Yes	Yes
Lee [19]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	40	Metastasized	74%	Yes	Yes	Yes
Liang [44]	NSCLC	4	EML4 - ALK, TP53	Seq	ALKi	1	Metastasized	-	Yes	Yes	-
Minari [27]	NSCLC	4	EGFR	ddPCR	EGFR TKI	5	Metastasized	100	-	Yes	-
Mok [18]	NSCLC	3b	EGFR	PCR	EGFR TKI	98	Metastasized	75%	Yes	Yes	-
Nakamura [37]	NSCLC	4	EGFR	PCR	EGFR-TKI	2	Metastasized	45%	Yes	Yes	-
Dowler Nygaard [29]	NSCLC	3b	KRAS	PCR	Chemotherapy	7	Metastasized	-	Yes	Yes	-
Oxnard [41]	NSCLC	4	EGFR, BRAF	PCR	EGFR TKI	4	Metastasized	50-81%	Yes	Yes	Yes
Pecuchet [17]	NSCLC	3b	EGFR, KRAS, BRAF	NGS, ddPCR	Multiple	85	Metastasized	71%	Yes	Yes	-
Piotrowska [33]	NSCLC	3b	EGFR	BEAMing	EGFR TKI	12	Metastasized	-	Yes	Yes	-

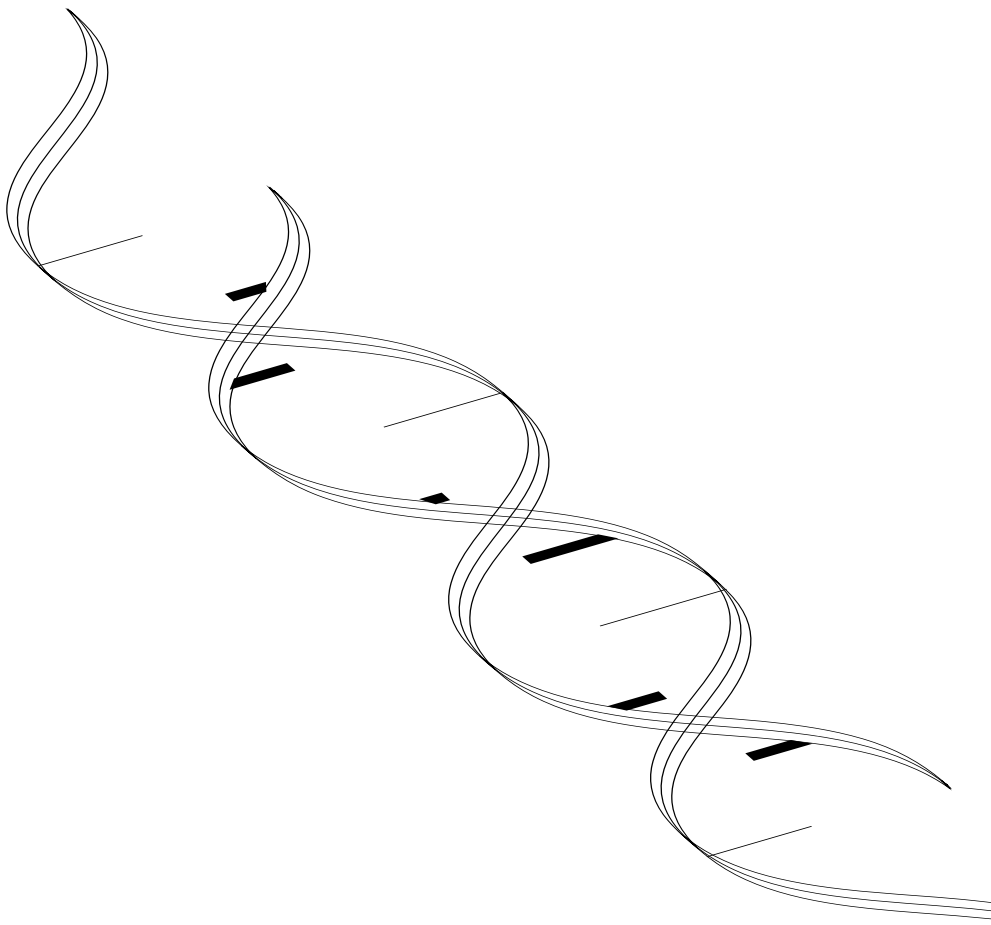
Punnoose [48]	NSCLC	3b	EGFR, KRAS, BRAF, PIK3CA	PCR	Pertuzumab, EGFR TKI	7	Recurrence	-	Yes	Yes	-
Riediger [42]	NSCLC	3b	EGFR + KRAS	ddPCR	EGFR TKI	16	Metastasized	93,70%	Yes	Yes	Yes
Seki [35]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	15	Metastasized	71%	Yes	No	-
Sueoka-Aragane [36]	NSCLC	3b	EGFR	MBP-QP	EGFR TKI	58	Metastasized	40%	Yes	Yes	-
Thress [31]	NSCLC	3b	EGFR	NGS, ddPCR	EGFR TKI	19	Metastasized	40%	Yes	Yes	-
Uchida [30]	NSCLC	3b	EGFR	MPS	EGFR TKI	10	Metastasized	75%	Yes	Yes	-
Watanabe [38]	NSCLC	3b	EGFR	PCR	EGFR-TKI	30	Metastasized	79%	Yes	Yes	-
Weber [32]	NSCLC	4	EGFR	PCR	EGFR TKI	1	Metastasized	-	-	Yes	-
Wei [23]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	200	Metastasized	84%	Yes	Yes	-
Yu [24]	NSCLC	3b	EGFR	BEAMing	EGFR TKI	46	Metastasized	86%	Yes	Yes	-
Zheng [40]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	55	Metastasized	81%	Yes	Yes	-
Zhou [16]	NSCLC	3b	EGFR	qPCR	EGFR TKI	80	Metastasized	-	No	Yes	-
Zhu [21]	NSCLC	3b	EGFR	ddPCR	EGFR TKI	48	Metastasized	81%	Yes	Yes	Yes
Ashida [58]	Mel	4	BRAF	castPCR	Multiple	6	Metastasized	50%	Yes	Yes	-
Casadevall [55]	Mel	4	BRAF	castPCR	BRAFi	1	Metastasized	-	Yes	Yes	-
Chen [54]	Mel	3b	BRAF	RT-PCR, WES	BRAFi	20	Metastasized	-	Yes	Yes	-
Gray [57]	Mel	3b	BRAF	ddPCR	MAPKi, BRAFi, Immuno	25	Metastasized	65%	Yes	Yes	Yes
Quereux [59]	Mel	4	BRAF	dPCR	BRAF, MEKi	1	Metastasized	100%	No	Yes	Yes
Sanmamed [56]	Mel	2b	BRAF	ddPCR	BRAFi	16	Metastasized	84%	Yes	Yes	-
Schreuer [53]	Mel	3b	BRAF	qPCR, ddPCR	BRAFi	36	Metastasized	70%	Yes	Yes	-
Seremet [60]	Mel	4	BRAF, NRAS	ddPCR	Multiple	7	Metastasized	100%	Yes	Yes	Yes
Shinozaki [61]	Mel	2b	BRAF	RT-PCR	Multiple	38	Various	37%	Yes	Yes	-
Arena [71]	CRC	3b	EGFR	ddPCR	Targeted therapy	2	Metastasized	18%	Yes	Yes	Yes
Bardelli [78]	CRC	4	KRAS, MET	PCR	EGFR TKI	1	Metastasized	-	Yes	No	Yes

Author	Tumour type	Paper score	Gene of interest	Technique	Therapy	N	Disease status	Mutation detection rate in plasma	Predictive for disease progression	Predictive for response	Progression ctDNA before radiological
Berger [69]	CRC	2b	KRAS	ddPCR	Chemotherapy	27	Metastasized	-	Yes	Yes	-
Carpinetta [86]	CRC	4	Various	NGS, ddPCR	Chemotherapy	4	Localized	-	Yes	Yes	Yes
Diehl [85]	CRC	3b	APC/KRAS/ PIK3CA/ TP53	BEAMing	Chemotherapy	11	Various	-	Yes	Yes	-
Garlan [77]	CRC	2b	BRAF/KRAS/ TP53	ddPCR	Chemotherapy	82	Metastasized	77%	No	Yes	No
Hong [70]	CRC	3b	BRAF	ddPCR	Multiple	12	Metastasized	-	Yes	Yes	-
Kakizawa [81]	CRC	3b	KRAS	ddPCR	Regorafenib	16	Metastasized	-	Yes	Yes	Yes
Khan [68]	CRC	3b	KRAS	ddPCR	Regorafenib	27	Metastasized	-	Yes	Yes	Yes
Oddo [75]	CRC	4	KRAS/BRAF/ NRAS/ EGFR/ MAP2K1,2	NGS	BRAFi, MEKi	1	Metastasized	-	Yes	No	-
Russo [74]	CRC	4	MEK1/KRAS	NGS, ddPCR	Panitumumab, Trametinib	1	Metastasized	-	Yes	Yes	No
Russo [73]	CRC	4	NTRK1,	NGS, ddPCR	Entrectinib	1	Metastasized	-	Yes	-	-
Siravegna [93]	CRC	4	CAD-ALK	PNA-PCR	ALK-inhibitor	1	Metastasized	-	Yes	No	Yes
Spindler [76]	CRC	3b	KRAS, BRAF	qPCR	Chemotherapy	35	Metastasized	85%	Yes	Yes	Yes
Sun [84]	CRC	3b	KRAS, BRAF, NRAS	ddPCR	EGFR TKI	140	Metastasized	97%	Yes	Yes	-
Thierry [88]	CRC	3b	KRAS/NRAS/ BRAF	qPCR	Folfox, Dasatinib, Cetuximab	42	Metastasized	88%	Yes	No	No
Tie [67]	CRC	3b	KRAS/APC/ BRAF/ TP53/ NRAS/ PIK3CA/ SMAD	MPS	Chemotherapy	48	Metastasized	92%	Yes	Yes	Yes

Toledo [82]	CRC	3b	BRAF/ PIK3CA	BEAMing	FOLFIRI-cetuximab	23	Metastasized	-	Yes	Yes	Yes
Vidal [83]	CRC	2b	KRAS	BEAMing	Chemotherapy, anti-EGFR	55	Metastasized	97%	Yes	Yes	Yes
Vietsch [92]	CRC	3b	Various	NGS	Chemotherapy	10	Various	28 - 47%	-	-	-
Wong [72]	CRC	3b	KRAS/ PIK3CA/ BRAF	BEAMing	Regorafenib	14	Metastasized	40%	Yes	Yes	-
Yamada [87]	CRC	3b	KRAS	ddPCR	EGFR TKI	24	Metastasized	90%	Yes	Yes	Yes
Yamauchi [80]	CRC	2b	Various	PCR	Anti-VEGF	21	Metastasized	100%	Yes	No	-
Zeng [79]	CRC	4	PIK3CA	PNA-PCR	FOLFOX	6	Metastasized	100%	No	No	No
Chenet al. [97]	BC	3b	TP53	RT-PCR	Chemotherapy	6	Localized	-	Yes	Yes	-
Garcia-Saenz [104]	BC	3b	PIK3CA	ddPCR	Not specified	8	Stage IIB - IV	55%	Yes	Yes	-
Guttery [110]	BC	3b	ESR1, TP53	NGS, ddPCR	Endocrine therapy	11	Metastasized	36%	Yes	-	-
Jansen [107]	BC	4	Various	NGS	Tamoxifen	1	Metastasized	-	Yes	-	Yes
Ma [109]	BC	3b	Various	NGS	TKI	18	Metastasized	50%	Yes	-	-
Murtaza [101]	BC	4	Various	Seq	Multiple	1	Metastasized	-	Yes	-	-
Nakagomi [95]	BC	4	TP53	NGS	Chemotherapy	1	Metastasized	-	Yes	Yes	-
Page [105]	BC	3b	ESR1, TP53, PIK3CA	NGS, ddPCR	Multiple	9	Metastasized	50%	Yes	Yes	-
Parsons [99]	BC	4	Various	NGS	Targeted treatment	26	Metastasized	92%	Yes	Yes	-
Riva [96]	BC	3b	TP53	ddPCR	Chemotherapy	36	Localized	75%	Yes	Yes	-
Sefrioui [100]	BC	4	ESR1	ddPCR	Endocrine therapy	2	Metastasized	67%	Yes	Yes	Yes
Takeshita [103]	BC	4	ESR1	ddPCR	Multiple	13	Metastasized	46.2%	-	-	-
Wang [102]	BC	3b	ESR1	ddPCR	Endocrine, chemotherapy	4	Metastasized	24%	Yes	Yes	-

BC = breast cancer	PCR = polymerase chain reaction	Seq = sequencing
Mel = melanoma	RT-PCR = real-time PCR	NGS = next generation sequencing
CRC = colorectal cancer	ddPCR = droplet digital PCR	WES = whole exome sequencing
NSCLC = non-small cell lung cancer	BEAMing = beads, emulsions, amplification, magnetics	MPS = massive parallel sequencing
	qPCR = quantitative PCR	
N = Number of patients for monitoring	MBP-QP = mutation based PCR - quenching probe	
- = Not reported	castPCR = competitive allele-specific Taqman PCR	
	PNA-PCR = peptide nucleic acid PCR	

Table 1. Overview of the papers retrieved by the search and included for this review. All papers were classified for level of evidence following the rules as depicted by the Oxford Centre for Evidence-Based Medicine.[11]



Comparison of circulating cell-free DNA extraction methods for downstream analysis in cancer patients

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Cancers, 2020

Abstract

Circulating cell-free DNA (ccfDNA) may contain DNA originating from the tumor in plasma of cancer patients (ctDNA) and enables noninvasive cancer diagnosis, treatment predictive testing, and response monitoring. A recent multicenter evaluation of workflows by the CANCER-ID consortium using artificial spiked-in plasma showed significant differences and consequently the importance of carefully selecting ccfDNA extraction methods. Here, the quantity and integrity of extracted ccfDNA from the plasma of cancer patients were assessed. Twenty-one cancer patient-derived cell-free plasma samples were selected to compare the Qiagen CNA, Maxwell RSC ccfDNA plasma, and Zymo manual quick ccfDNA kit. High-volume citrate plasma samples collected by diagnostic leukapheresis from six cancer patients were used to compare the Qiagen CNA (2 mL) and QIAamp MinElute ccfDNA kit (8 mL). This study revealed similar integrity and similar levels of amplified short-sized fragments and tumor-specific mutants comparing the CNA and RSC kits. However, the CNA kit consistently showed the highest yield of ccfDNA and short-sized fragments, while the RSC and ME kits showed higher variant allelic frequencies (VAFs). Our study pinpoints the importance of standardizing preanalytical conditions as well as consensus on defining the input of ccfDNA to accurately detect ctDNA and be able to compare results in a clinical routine practice, within and between clinical studies.

1. Introduction

Extensive research has been performed to utilize blood-based analytes for detection and monitoring of the disease in cancer patients. In many bodily fluids, including blood plasma, circulating cell-free DNA (ccfDNA) is present.[1] In healthy individuals, ccfDNA originates primarily from cell degradation through apoptosis or necrosis of cells of the hematopoietic lineage, resulting in shedding of genomic DNA into the circulation.[2,3] CcfDNA released through apoptosis consists of short fragments (<1000 bp), while ccfDNA shedded into circulation by exosomes or necrotic (tumor) cells is longer in size (>1000 bp).[4,5]

The application of ccfDNA for molecular profiling has recently taken a flight in the field of oncology. Elevated levels of ccfDNA can be detected in cancer patients of which a small fraction of the ccfDNA originates from tumor cells.[6] This so-called circulating tumor DNA (ctDNA) reflects the molecular characteristics of tumor tissue and promises to negate the limitations of conventional tissue biopsy (e.g., invasiveness, accessibility, and heterogeneity).[7] Similar to ccfDNA, ctDNA is shedded into the bloodstream through apoptosis, necrosis, or active excretion.[8] The majority of ctDNA in plasma has an apoptotic origin and in general their size corresponds to nucleosome-protected DNA, which ranges from 120 to 220 base pairs (bp) that peaks around 167 bp.[9] In addition to the apoptotic short-sized fragments, the ccfDNA in cell-free plasma also contains very long-sized fragments (~10,000 bp) resulting from (tumor) cell necrosis or derived from hemolysis during blood withdrawal and processing.[10–12] Altogether, the actual ctDNA fraction mostly represented in the shorter-sized fragments is often less than 1% of the total ccfDNA.[5,7] Thus, the extracted ctDNA is highly fragmented and has a short half-life, which can complicate subsequent analyses.[1] Therefore, efficient extraction of the short-sized fragments and highly sensitive techniques are required to detect these low abundant ctDNA fragments.[13] Nonetheless, ctDNA contains tumor-specific mutations that can be detected in the plasma using highly sensitive techniques such as droplet digital polymerase chain reaction (ddPCR) and highly-sensitive next-generation sequencing (NGS).[14,15] The detection of these short-sized ctDNA fragments in the plasma enables the (early) detection of new or recurrent predictive cancer biomarkers and is applicable for monitoring treatment response using a minimally invasive strategy.[16]

With the increasing interest for ccfDNA-based diagnostics, the number of ccfDNA extraction kits and methodologies has expanded drastically in recent years.[17] There is a great variety regarding the extraction method, plasma input, throughput, and price of the kits. To compare the performance of various ccfDNA extraction kits, total DNA yield is generally used as an outcome parameter. However, a substantial part of the ccfDNA originates from genomic DNA fragments of nontumor tissue released during blood withdrawal and processing of the samples due to hemolysis.[18] These increased levels of larger DNA fragments can interfere with the sensitivity of ctDNA

detection and could result in false negativity. In addition, no methods exist that enrich short-sized ccfDNA fragments or are able to discriminate ctDNA from regular ccfDNA. Altogether, it is important to evaluate the preanalytical conditions and integrity of the extracted ccfDNA when using quantitative approaches to accurately detect mutants during diagnostics or monitoring of the disease.

Many studies reported the comparison of different ccfDNA extraction methods.[19–26] At present, however, no collective international standardized protocols are available regarding the preanalytical ccfDNA extraction conditions. Most ccfDNA comparison studies were performed using reference samples, that typically consist of either artificial plasma or pooled plasma samples from healthy individuals both spiked with purified DNA.[19–22] Furthermore, most ccfDNA extraction methods do not provide any information regarding the preanalytical conditions of the obtained DNA.[17,27] A recent multicenter evaluation of workflows by the CANCER-ID consortium revealed considerable differences between various ccfDNA extraction methods regarding the quantity and integrity of extracted ccfDNA using artificial spiked-in plasma and showed the relevance of carefully selecting extraction methods and considering preanalytical conditions of the extracted ccfDNA.[7] In line with this CANCER-ID study, the aim was to evaluate the quantity and integrity of extracted ccfDNA from cancer patient-derived plasma samples using different ccfDNA extraction kits. Plasma samples from patients with either a gastrointestinal stromal tumor (GIST) or nonsmall cell lung carcinoma (NSCLC) were selected for the comparison of three different plasma ccfDNA extraction techniques (QIAamp Circulating Nucleic Acid Kit (CNA), Maxwell RSC ccfDNA Plasma Kit (RSC), and Zymo Quick ccfDNA Serum & Plasma Kit (Z)).

In the clinical setting, there is a rising demand for processing higher volumes of plasma in one run to minimize expenditure and generate highly concentrated eluates to enable subsequent analyses for diagnostic purposes such as NGS.[28,29] The commonly used CNA kit applying 2 mL of plasma served in our study as a reference to compare ccfDNA extraction with the QIAamp MinElute ccfDNA kit (ME) that preferably enables ccfDNA extraction from 8 mL of plasma. For this purpose, we collected a unique set of high-volume citrate plasma samples collected by diagnostic leukapheresis (DLA) from six patients to evaluate the recovery of ccfDNA using 8 mL of plasma, which is an average amount collected from two blood collection tubes (BCTs) compared to the CNA kit using 2 mL of plasma.

2. Results

2.1. Selection of plasma samples from cancer patients

Twenty-one plasma samples from eighteen cancer patients with metastatic disease were selected based on plasma availability in our plasma Biobank sufficient to be able to perform three ccfDNA extractions on the same sample (4 mL in total). Eight

samples from seven GIST patients and thirteen samples from eleven patients with NSCLC were used (table S1).

2.2. Quantitative comparison of DNA yield with different ccfDNA extraction methods

CcfDNA extraction using three different ccfDNA extraction kits showed a broad range of concentrations when measured with Qubit, varying from 1.53 ng ccfDNA per mL of plasma to 110 ng/mL (figure 1A,B). Overall, ccfDNA extraction using the CNA kit resulted in a significantly higher yield compared to the RSC ($p < 0.001$) and Z kits ($p < 0.01$) (figure 1A). Extraction using the CNA kit (Qiagen, Hilden, Germany) yielded the highest levels in eighteen out of twenty-one samples, whereas the lowest amount of ccfDNA was obtained in fourteen samples with the RSC kit (figure 1B).

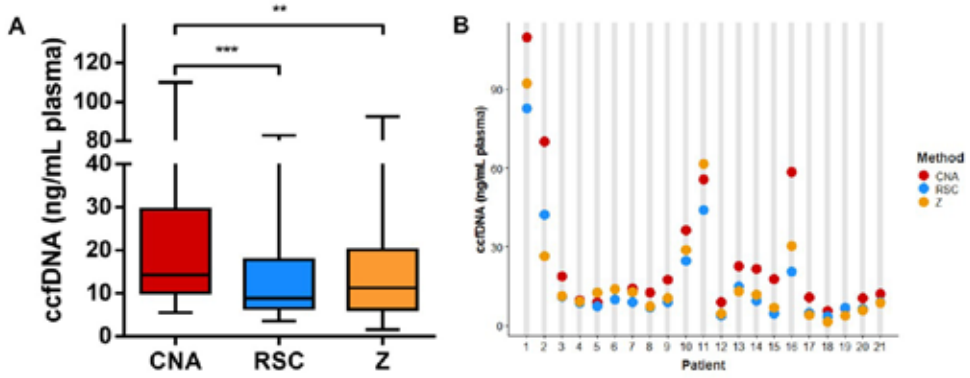

Figure 1. Comparison of the circulating cell-free DNA (ccfDNA) yield among different extraction methods. (**A**) Boxplot illustrating the yield as measured with Qubit per method. Horizontal lines represent the median, the boxes, and the interquartile range. (**B**) Individual patient ccfDNA yields for the different extraction methods are displayed for the plasma of twenty-one non-small cell lung carcinoma (NSCLC) patients. One-way ANOVA multiple comparison test, ** $p < 0.01$, *** $p < 0.001$.

2.3. CcfDNA integrity and mutation detection assessment

In order to validate the various fragment sizes in ccfDNA, a sample analysis using the Fragment Analyzer revealed no significant differences in the short-to-medium-sized fragment ratios (table S2). In order to determine the amplifiability assessment of fragment sizes, using the β-actin one-tube 3-size ddPCR assay resulted in a significantly higher number of copies per mL of plasma for the 137 and 420 bp fragment lengths in ccfDNA extracted with the CNA kit compared to both the RSC ($p < 0.05$ and $p < 0.01$, respectively) and Z kits ($p < 0.0001$ and $p < 0.001$, respectively) (figure 2A,B). For the long 1950 bp fragments, the CNA kit only extracted significantly more compared with the RSC kit (figure 2C). All median values and interquartile ranges are depicted in table S3. Extracted ccfDNA revealed no significantly different 137/420 bp fragment ratios, which is in agreement with the results from the Fragment Analyzer (table S2). The 137/1950 bp fragment ratios only showed a significant increase in the extraction

of long-sized ccfDNA fragments with the Z kit compared with the RSC kit (table S2). When comparing the number of copies of the 137 bp fragment per ccfDNA input (in ng), the mean number of copies per ng ccfDNA was similar between the CNA and RSC kits (p = 0.247) and also for each separate plasma sample using the CNA or RSC kit, no concordant pattern was observed (figure 2D).

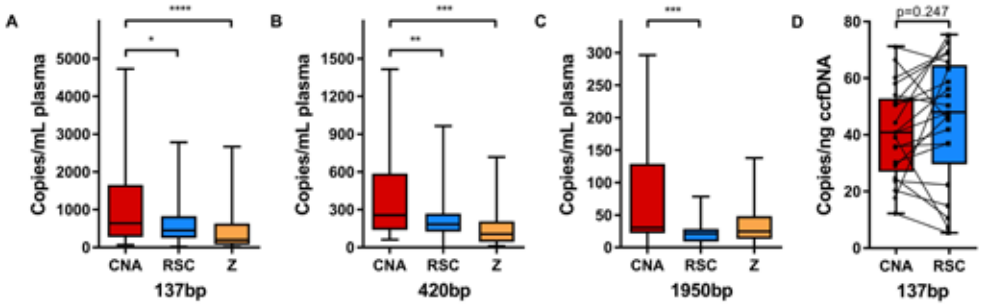

Figure 2. Comparison of β-actin fragment sizes among different ccfDNA extraction kits. Boxplots illustrating the number of copies per mL of plasma for the 137 (**A**), 420 (**B**), and 1950 bp (**C**) fragment sizes, as well as the 137 bp copies per ng of ccfDNA (**D**) as measured with the one-tube 3-sized β-actin ddPCR assay. Horizontal lines represent the median, the boxes, and the interquartile range. All median values and interquartile ranges are depicted in table S3. One-way ANOVA multiple comparison test, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Next, to compare the yield of the ctDNA fraction, mutation-specific ddPCR assays were performed on the extracted ccfDNA of multiple patients of which in four plasmas the mutations were detectable (table S1). Interestingly, ccfDNA extraction using the CNA kit resulted in more mutant copies per mL of plasma in two cases, while RSC-extracted ccfDNA showed more mutant copies per mL of plasma in the other two cases (figure 3A). In figure 3B, the detected number of mutant copies is plotted against the ccfDNA input in ng. Hereby, it can be determined whether the input amount affects the detected number of mutant copies per mL of plasma. The detected discrepancies are irrespective of the input amount of ccfDNA (figure 3B). In regard to variant allelic frequency (VAF), in three of the four samples RSC-extracted ccfDNA displayed a higher VAF compared with CNA-extracted ccfDNA (figure 3C).
Overall, this data using twenty-one different plasma samples of eighteen patients with cancer revealed a larger total yield of ccfDNA and a relatively higher number of short-sized (137 and 420 bp) fragments when using the CNA kit compared to the RSC kit, which is in agreement with data observed in spiked-in samples [7]. However, both methods provided the same number of short-sized fragments (n = 21) and mutant copies (n = 4) relative to the amount of ccfDNA input in ng.

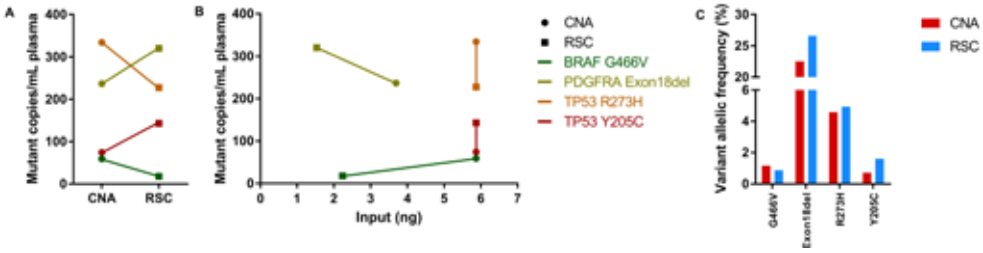

Figure 3. Mutation detection in ccfDNA extracted from plasma with the QIAamp Circulating Nucleic Acid (CNA) and Maxwell RSC ccfDNA Plasma (RSC) methods. (**A**) Before-after plot illustrating the number of mutant copies per mL of plasma for four paired CNA-extracted (dots) and RSC-extracted (squares) samples. (**B**) XY plot illustrating the detected mutant copies per mL of plasma plotted against the input in ng on the x-axis. Similarly colored results originate from the same patient harboring a detectable mutation according to the legend. (**C**) Bar graph illustrating the detected higher variant allelic frequency (VAF) in a percentage of four paired CNA-extracted (red) and RSC-extracted (blue) samples.

2.4. Comparing ccfDNA extraction kits using high-volume citrate plasma samples

The extracted ccfDNA is generally used for subsequent mutational analysis techniques such as ddPCR and NGS, which mostly require high quantities of ccfDNA derived from preferably 4 mL of plasma.[28,29] Therefore, processing high volumes of plasma in a single reaction is preferred. For this purpose, we compared the magnetic beads-based ME kit (8 mL plasma) specifically designed to process high volumes of plasma with the most frequently used ccfDNA CNA extraction kit (2 mL) (see table 1). In this analysis, ccfDNA extracted with the ME kit was compared with the CNA kit for yield, integrity, and amplifiability of short-sized fragments.

Kit	Manufacturer	Method	Input volume (mL)	Elution volume (µL)	Execution
QIAamp Circulating Nucleic Acid Kit (CNA)	Qiagen	Silica-based	1-4 (0.9-2)	20 – 150 (50)	Manual
Maxwell RSC ccfDNA Plasma Kit (RSC)	Promega	Magnetic beads	0.2-1 (0.7-0.9)	50 (50)	Automated
Zymo Quick ccfDNA Serum & Plasma Kit (Z)	Zymo research (BaseClear)	Silica-based	<10 (0.8-0.9)	>50 (47)	Manual
QIAamp MinElute ccfDNA midi kit (ME)	Qiagen	Magnetic beads	4-10 (8)	20-80 (47)	Manual

Table 1 Specifications of the ccfDNA extraction kits used in this study. The amounts that were used for this study are displayed within brackets. For detailed information regarding the kit specifications, we recommend to access the websites of the manufacturers.

DLA samples from six NSCLC patients with established mutations were selected based on plasma availability (>10 mL). With respect to yield, extraction using the CNA kit resulted in a 3-fold more ccfDNA per mL of plasma compared to the ME kit (figure 4A). Each CNA-extracted sample had a higher yield than the paired ME-extracted samples (figure 4B). A sample analysis with the Fragment Analyzer showed enrichment of short-sized fragments in ccfDNA extracted with the ME kit compared to the CNA kit, as demonstrated by an increased number of 50–250 bp fragments

and a higher short-to-medium-sized fragments ratio (table 2). The amplifiability of ccfDNA was assessed using the β-actin one-tube 3-size ddPCR. The median number of copies per mL of plasma of all three fragment lengths (137, 420, and 1950 bp) of the ME kit was slightly lower but not significantly different compared to the CNA kit (figure 5A–C). Extracted ccfDNA from the ME and CNA kits revealed no significant differences in the 137/420 bp and 137/1950 bp ratios, whereas the ME kit revealed a significantly higher short-to-medium-size ratio on the Fragment Analyzer (table 2). When considering the input amount of ccfDNA, a strong increase of 137 bp copies per ng ccfDNA (figure 5D) as well as for the 420 and 1950 bp fragment lengths (table S3) was observed in ME-extracted samples compared with the CNA kit. To validate the presumed augmented amplifiability of short-sized ctDNA fragments in ME-extracted ccfDNA, a mutation-specific ddPCR was performed on four ccfDNA samples that contained a mutation detectable with ddPCR (table S1). In all four cases, the number of mutant copies per mL of plasma was higher in ccfDNA extracted with the ME kit (figure 6A), which was irrespective of the input amount of ccfDNA (figure 6B). In addition, in all four samples a higher VAF was observed in ME-extracted ccfDNA compared with the CNA kit.

	Fragment Analyzer	β-actin one-tube 3-size ddPCR	
Kit	**Ratio S/M**	**Ratio 137/420bp**	**Ratio 137/1950bp**
CNA	1.81 (1.58 - 2.67)	1.64 (1.54–1.95)	6.56 (5.55–10.3)
RSC	3.10 (2.37 - 3.76)*	1.73 (1.48–1.89)	8.00 (6.47–9.31)

Table 2. Short- and medium-sized fragment percentages of citrate plasma as measured with the Fragment Analyzer. Measurements are displayed as a median percentage of retrieved fragment size with the interquartile range within brackets. Ratio S/M: Ratio between short-sized fragments (50–250 bp) and medium-sized fragments (250–450 bp). * p < 0.05 between the CNA and QIAamp MinElute ccfDNA (ME) extraction methods.

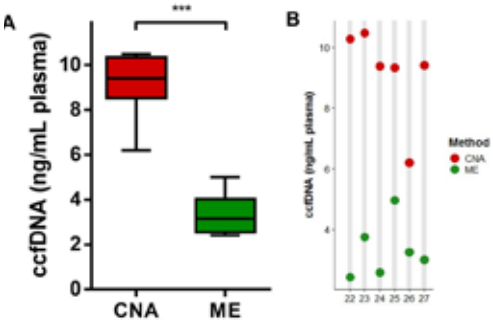

Figure 4. Comparison of the ccfDNA yield using the CNA and ME extraction methods in diagnostic leukapheresis (DLA) samples. (**A**) Boxplot illustrating the yield as measured with Qubit per method. Horizontal lines represent the median, the boxes, and the interquartile range. (**B**) Individual patient ccfDNA yields for the different extraction methods are displayed for the citrate plasma of six NSCLC patients. Generalized linear mixed model, *** p < 0.001.

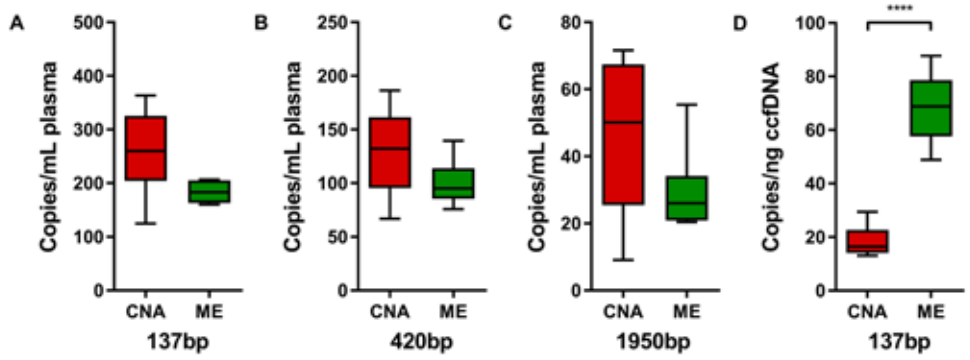

Figure 5. Comparison of β-actin fragment sizes among the CNA and ME extraction methods in DLA samples. Boxplots illustrating the number of copies per mL of plasma for the 137 (**A**), 420 (**B**), and 1950 bp (**C**) fragment sizes, as well as the 137 bp copies per ng of ccfDNA (**D**) as measured with the one-tube 3-sized β-actin ddPCR assay. Horizontal lines represent the median, the boxes, and the interquartile range. All median values and interquartile ranges are depicted in table S3. Generalized linear mixed model, * $p < 0.05$, *** $p < 0.001$.

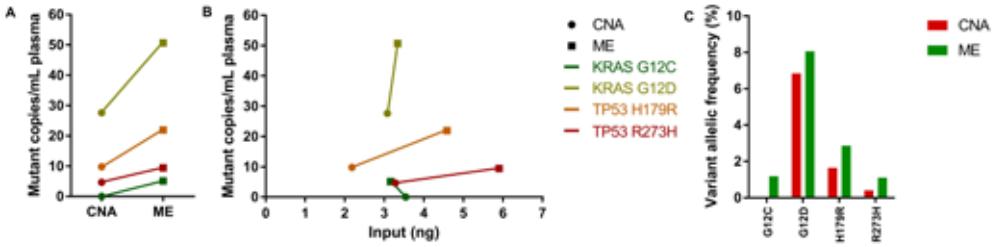

Figure 6. Mutation detection among the CNA and ME extraction methods in DLA samples. (**A**) Before-after plot illustrating the number of mutant copies per mL of plasma for four paired CNA-extracted (dots) and ME-extracted (squares) samples. (**B**) XY plot illustrating the detected mutant copies per mL of plasma plotted against the input in ng on the x-axis. Similarly colored results originate from the same patient harboring a detectable mutation according to the legend. (**C**) Bar graph illustrating the detected VAF in a percentage of four paired CNA-extracted (red) and ME-extracted (green) samples.

3. Discussion

In this study, the CNA kit served as the golden standard approach to evaluate the extraction of ccfDNA of other extraction methods regarding yield or integrity using plasmas of cancer patients, which eventually could lead to a higher sensitivity of variant detection. Based on our twenty-one plasma samples derived from cancer patients, both the integrity and levels of amplified short-sized fragments and tumor-specific mutants relative to the input amount of ccfDNA (in ng), as calculated for each reaction individually, revealed no differences between the RSC and CNA kits. However, when using the ccfDNA yield determined with DNA quantification methods such as Qubit or quantitative PCR approaches as reported in most other studies, the

yield of ccfDNA, as well as the yield of short-sized fragments, is significantly higher using the CNA kit in cancer patient-derived plasmas. The ME kit seems a suitable methodology when extraction from high amounts of plasma is favored since, despite a lower yield per mL of plasma, higher mutant copy numbers and VAFs were observed. Since the use of different extraction methods might introduce bias to the mutation detection rate, we highly recommend applying the same ccfDNA extraction method within studies, especially when monitoring the treatment response based on multiple plasma samples, to prevent variation in mutant levels due to technical factors. This is further supported by our previous data on spiked-in samples.[7]

The analysis of plasma-derived ccfDNA has recently become of importance in cancer diagnosis and treatment response monitoring. As ctDNA derives from the primary tumor or metastases, molecular characterization of the tumor is possible without the necessity to perform a tissue biopsy. Taking (multiple) biopsies is not always achievable and often accompanies health risks. With more targeted treatments available, the mutational status of the recurrent tumors or metastases has great implications for treatment decision making.[30] However, sample conditions should be optimal to be able to perform diagnostics on blood-based analytes and it is, therefore, key to critically analyze the plasma-derived material for accurate detection. Preanalytical procedures such as blood collection, transport, time before processing, and cell-free plasma processing have a major impact on the quality of the retrieved DNA and are the subject of several studies.[24,25,31] Furthermore, total ccfDNA concentrations can be influenced dramatically not only by technical factors such as hemolysis, but also by unrelated factors such as exercise and inflammation.[32,33] Finally, post-analytical determinants such as detection methods that differ in, e.g., sensitivity, complexity, and mutation coverage may also affect the clinical outcome.[17,27,34,35]

In recent years, many kits have become commercially available for ccfDNA extraction.[16,17] Yield is the major outcome parameter when comparing different extraction methods. This study determined yields as measured by Qubit between 1.5 and 110 ng/mL of plasma, which overlaps with the range of ccfDNA generally found in both healthy individuals and cancer patients, affirming that assessing ccfDNA quality solely based on yield is challenging.[10] When using different ccfDNA extraction methods, the highest yield was obtained using the CNA kit, whereas the RSC kit was the least efficient. These observations are consistent with previous reports and, therefore, the CNA kit is considered as the gold standard reference approach when yield is the primary criterium.[7,19,26]

To further analyze the integrity of the extraction product, the fragment size distribution and the amplifiability were tested. Two ALU1 assays that target different sizes of DNA fragment lengths of 187 and 60 bp (Alu-187 and Alu-60) were used to compare the integrity index as determined by the Alu-187/Alu-60 ratio as reported previously.[7] This analysis revealed that the integrity index of ccfDNA of RSC was lower than that of CNA (data not shown), similar as reported for the spiked-in plasma. However,

using the β-actin one-tube 3-size ddPCR assay, no significantly different 137/420 bp and 137/1950 fragments ratios were observed when comparing ccfDNA with the CNA and RSC kits. The Fragment Analyzer confirmed that the short-to-medium-sized fragments ratios are similar indicating that the integrity of the ccfDNA from the plasma of cancer patients does not differ between the CNA and RSC extraction methods.

In agreement with the higher yield of ccfDNA as determined with Qubit when using CNA, the amplifiability of the 137 and 420 bp fragments using the β-actin one-tube 3-size ddPCR assay and using the CNA kit showed significantly more copies per mL of plasma compared to the RSC and Z kits. Previous data showed a similar reduced recovery and decreased number of shorter fragments of RSC-extracted ccfDNA compared to CNA.[7,19] However, these differences diminish in perspective of the number of copies per ng of ccfDNA, implying that the amplifiability of the short-sized fragments is similar when corrected for the input amount for both CNA and RSC kits in plasmas of cancer patients. The Z kit seems to preferentially extract long-sized fragments and was outperformed in respect to yield and amplifiability.

The amplifiability of ctDNA was determined through the detection of tumor-specific mutations in ccfDNA in four samples. Interestingly, no differences could be detected regarding the number of mutant copies detected per mL of plasma, irrespective of ccfDNA input. However, RSC-extracted ccfDNA showed a higher VAF in three out of four cases. An increase in VAF when using the RSC kit for ccfDNA extraction is in agreement with the results we observed when using spiked-in plasma reported previously.[7]

For multigene predictive testing using NGS a high input of ccfDNA is required for optimal and sensitive detection of ctDNA. Thus, processing high volumes of plasma in highly concentrated eluates is required.[36] Using six cancer patient-derived DLA samples, the numbers of copies of 137, 420, and 1950 bp fragments per mL of plasma were slightly (not significant) higher for all three fragment sizes with the CNA kit, while a higher total ccfDNA yield per mL of plasma was detected using the CNA kit as determined by Qubit. The number of copies of the 137 bp fragments per ng of input ccfDNA revealed a 2.2-fold increase compared to the CNA kit. Despite ccfDNA extracted with the ME kit revealed a relatively higher short-to-medium-sized fragment ratio as determined by the Fragment Analyzer, no significantly different 137/420 bp and 137/1950 bp fragments ratios were observed using the β-actin one-tube 3-size ddPCR assay. This PCR-based analysis indicated that the integrity of the ccfDNA from citrate plasma from cancer patients does not differ between the CNA and ME extraction methods in agreement with our analysis using spiked-in plasma samples.[7] A mutation-specific ddPCR assay to quantify ctDNA (i.e., tumor-specific mutants) levels in ccfDNA on four DLA samples revealed an increase in mutant copies per mL of plasma and elevated VAF in all ME-extracted ccfDNA samples compared to the CNA kit, irrespective of ccfDNA input. Thus, despite the lower total ccfDNA yield using the ME kit, relatively more ctDNA is extracted. The overall lower levels of the

short-sized fragments recovered from citrate plasma samples compared to plasma samples collected in BCT tubes using the CNA kit (288 vs. 637 copies/mL of plasma, respectively) might be due to an abundance of long-sized fragments, as the short-to-medium fragment ratios determined with both the Fragment Analyzer and the β-actin one-tube 3-size ddPCR assay are lower for citrate plasma compared to the BCT plasma. However, as the citrate and BCT plasmas were not drawn from the same patients, factors such as stage of disease or response to therapy might explain these differences. Nevertheless, we have recently compared plasmas collected by DLA in citrate and from peripheral blood in Streck BCT-tubes from the same patients at the same time and using NGS analysis revealed a very high concordance between the VAFs of various tumor-specific variants.[36]

Altogether, this study using cancer patient-derived plasmas shows that ccfDNA extraction using different extraction methods resulted in similar integrity and similar levels of amplified small-sized fragments and tumor-specific mutants per ng of ccfDNA input, but significant differences in the yield of ccfDNA as well as of small-sized fragments per mL of plasma, which makes alternating the application of different ccfDNA extraction methods lead to inconsistent results. The CNA kit consistently showed the highest yield of ccfDNA and of small-sized fragments, however, in the RSC kit higher VAFs were found, implying a preferential extraction of the mutation harboring ctDNA similar as observed in the artificial spiked-in plasma samples.[7] Recent studies showed that fragmentation of DNA in cell-free plasma differs between cancer patients and healthy individuals.[5,37] The average fragment size of ccfDNA is around the size of nucleosome-protected DNA (160–170 bp), while ctDNA in many cancers was shown to be 20–30 bp smaller (130–150 bp). Interestingly, in a cohort of 344 plasmas from 200 cancer patients, the analysis of the smaller size-selected ccfDNA fragments revealed clinically actionable mutations and copy number alterations at high frequency.[37] Although none of the commercially available kits are designed to enrich specifically for the smaller nucleosome bound ccfDNA fragments, Kloten et al. reported that extraction methods based on magnetic beads more efficiently recover short ccfDNA fragments compared to silico-based methods.[23] Since the number of cancer patient-derived plasma samples and DLA samples with a tumor-specific mutation is relatively low, additional studies are needed to confirm our observations.

Overall, these data suggest that the use of different extraction methods might introduce differences in the levels of mutant copies per mL of plasma and VAF due to technical factors, which might represent inaccurate discrepancies in clinical-relevant mutant copies crucial for clinical application, especially in treatment response monitoring. Therefore, continuous use of the same ccfDNA extraction method based on validated standard operating procedures is recommended to obtain comparable results. As long as there is no harmonization and standardization of procedures using preanalytical and analytical methods for liquid biopsy testing (e.g., primary diagnosis, minimal residual disease (MRD), response monitoring), it cannot yet be routinely implemented in the clinical setting.[38]

4. Materials and methods

4.1 Sample collection and processing

Plasma samples were collected from twenty-one patients with metastatic disease who were treated in the University Medical Center Groningen (UMCG, Groningen, The Netherlands) for GIST or NSCLC. GIST samples were collected in EDTA tubes (vacutainer #367525, Becton Dickinson, Franklin Lakes, NJ, USA), whereas NSCLC samples were collected in cell-free DNA blood collection tubes (BCTs) (Streck, Omaha, NE, USA). EDTA samples were processed within 4 h after venipuncture following guidelines as reported previously.[39] Samples were centrifuged for 10 min at 820× g to separate the lymphocytes from the plasma. The supernatant was centrifuged at 16,000× g for another 10 min to separate plasma from the remaining debris. The supernatants were transferred in 1 mL fractions and stored at −80 °C until ccfDNA extraction. Cell-free DNA BCTs were processed within 24 h using the same protocol except for a first centrifugation step at 1600× g following the manufacturer's instructions.
The DLA procedure was performed as previously described.[40,41] In short, procedures were performed on six patients with the Spectra Optia® Apheresis System according to the standard continuous mononuclear cell (cMNC) protocol with a packing factor of 4.5 and the collection pump set to 1 mL per minute, hematocrit minus 3 percent points, and a flexible inlet flow and anticoagulation with anticoagulant citrate dextrose solution (starting concentration of 1:11). Following the cMNC protocol, up to 100 mL of plasma was collected with a packing factor of 20. The DLA samples were aliquoted and stored at −80 °C within 30 min after withdrawal. After thawing, DLA samples were centrifuged at 1600× g for 10 min to separate the plasma from the debris. All plasma processing was performed in a laboratory not used for any molecular testing to prevent contamination. For this validation study, the samples of patients with GIST were selected from a national GIST biobank study which is registered on ClinicalTrials.gov (NCT02331914), and the NSCLC samples from the lung plasma Biobank both at the UMCG. All patients gave written informed consent. Samples were selected irrespective of clinical and mutational status (table S1).

4.2 CcfDNA extraction techniques

In this study, four different methods for plasma ccfDNA extraction were used: CNA, RSC, Z, and ME (for specifications of each, see table 1). CcfDNA was extracted from the same plasma sample using the CNA (0.9–2 mL), RSC (0.7–0.9 mL), and Z (0.8–0.9 mL) kits according to the corresponding manufacturer's instructions. For the DLA samples, the same citrate plasma was used for ccfDNA extraction with CNA (2 mL) and ME (8 mL) according to the corresponding manufacturer's instructions.

4.3 Yield and integrity assessment of different extracted ccfDNA

CcfDNA was quantified using the Qubit dsDNA HS assay kit on a Qubit 2.0 fluorometer (Thermo Fischer Scientific, Waltham, MA, USA). As a measure of integrity, we

determined the fragment size distribution, integrity index, and amplifiability, similar as previously reported.[7] To determine fragment size distribution, the extracted ccfDNA samples were analyzed using the Fragment Analyzer. An amount of 2 µL of the ccfDNA samples was used for analysis according to the manufacturer's instructions (Agilent, Santa Clara, CA, USA). A smear analysis for the 50–250 bp fraction was used as a representation of the short-sized fragments and the 250–450 bp fraction to represent the medium-sized fragments. The ccfDNA integrity index was assessed using two different ALU1 PCR assays with lengths of 60 and 187 bp (TATAA, Göteborg, Sweden). The integrity index was calculated through the ratio between their quantitation cycle values (Alu-187/Alu-60) as previously reported [7]. The amplifiability of ccfDNA was evaluated using the β-actin one-tube 3-size ddPCR assay as described previously with minor adaptations.[12] In this multiplex assay, three different sized fragments of the β-actin gene are detected using the QX200™ Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Fragment sizes of 137 and 420 bp are detected with FAM- and HEX-labeled probes, respectively, and double positive droplets were counted as 1950 bp fragments. These primer and probe sequences were reported previously.[42] For mutation detection in ccfDNA extracted from plasma samples, mutation-specific ddPCR assays were performed. All applied primer and probe sequences are depicted in table S4. To demonstrate the ability to identify increased hemolysis, twelve plasma samples collected in EDTA tubes were stored for different times (4 h and five days) and at different temperatures (4 and 20 °C) prior to plasma processing (see figure S1).

DdPCR assays for the β-actin one-tube 3-size ddPCR, KRAS G12/G13 screening, BRAF G466V, PDGFRA M844_D846del, TP53 H179R, TP53 R273H, and TP53 Y205C were performed using the Bio-Rad QX200™ platform included positive, wild type, and no template controls. Performance of the PCR setup was according to the manufacturer's instructions. DdPCR analyses were performed on 5.0–8.8 µL of extracted plasma ccfDNA with different ccfDNA concentrations as measured by Qubit according to the manufacturer's instructions. Briefly, the appropriate amounts of primer mix and probes for the β-actin one-tube 3-size ddPCR assay (1.1 µL of 4 µM 137 bp primer mix, 1.1 µL of 4 µM 420 bp primer mix, 1.1 µL of 6 µM 137 bp FAM labeled probe, and 1.1 µL of 6 µM 420 bp HEX labeled probe) and the mutation-specific ddPCR assays (Bio-Rad: 1.1 µL of primers and probe mix; IDT: 1.1 µL of primer mix, and 1.1 µL of probes) were added to 11 µL ddPCR supermix and supplemented with water when necessary up to a volume of 22 µL. All used primer and probe sequences are shown in table S4. Data were analyzed with the QuantaSoftTM analytical software version 1.7.4.0917 and QuantaSoftTM Analysis Pro 1.0.596 (both Bio-Rad). Positive, wild type, and no template controls were used to establish cutoff levels. Droplet counts were used to calculate the number of copies per initial volume of plasma input as well as the VAF calculated by the QuantaSoftTM Analysis Pro 1.0.596 software. All molecular testing was performed in the ISO15189-accredited laboratory of molecular pathology at the UMCG. All standard precautions were taken to avoid contamination of amplification

products using separate laboratories for pre- and post-PCR handling.

4.4 Statistical analyses

Statistical analyses were performed using the IBM SPSS statistics version 25.0 (IBM, Armonk, NY, USA), R (version 3.6.0) and R Studio software (R Studio, Boston, MA, USA), and Prism 7.0 (GraphPad software, San Diego, CA, USA). For statistical assessment, a one-way ANOVA with repeated nonparametric measures (Friedman test) was performed followed by a Dunn's multiple comparison test. In case there were only two paired samples, a generalized linear mixed model was applied.

5. Conclusions

When using cancer patient-derived ccfDNA from blood plasma instead of artificial spiked-in reference samples, preanalytical conditions significantly influence the overall result of a ccfDNA extraction method. Inconsistent processing of the plasma and the use of different ccfDNA extraction kits might contribute to incorrect results, which eventually can lead to inappropriate variant calling or inaccurate VAF determination. Whatever ccfDNA extraction kit is selected will be up to personal preferences, however, it should not be changed within a cohort in order to preserve similar conditions in all cases. For biobanking of liquid biopsies, our findings also recommend the storage of cell-free plasma and not of extracted ccfDNA. Harmonization of procedures using preanalytical conditions will strongly improve interstudy similarity and compatibility and thereby contribute to the implementation of liquid biopsy approaches in the clinical practice.

Funding: This research received no external funding.

Authors E.S., H.J.M.G. and M.T. (Menno Tamminga) are part of the CANCER-ID consortium, which has received support from the Innovative Medicines Initiative (IMI) Joint Undertaking under grant agreement number 115749.

Acknowledgments: This work was supported by Qiagen GmbH (Hilden, Germany), Promega Benelux BV (Leiden, The Netherlands), BaseClear BV (Leiden, The Netherlands), and TATAA Biocenter Ab (Gothenburg, Sweden) through an in kind provision of the QIAamp MinElute ccfDNA kit, the Maxwell RSC ccfDNA plasma kit, the Zymo manual quick ccfDNA kit, and the TATAA ALU1 PCR assays, respectively. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Conflicts of Interest: E.S. has performed lectures for Bio-Rad, Novartis, Roche, Biocartis, Illumina, Pfizer, AstraZeneca, and Agena Bioscience; is a consultant in advisory boards for AstraZeneca, Roche, Pfizer, Novartis, Bayer, BMS, Amgen, Biocartis, Illumina, Agena Bioscience, and MSD/Merck; and received research grants from Pfizer, Biocartis, Agena Bioscience, BMS, Bio-Rad, Roche, and Boehringer Ingelheim. The other authors declare no conflicts of interest.

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Supplementary materials

Table S1. Sample details and malignancy status of included patients.

Sample ID	Source	Malignancy	Mutated gene	Amino acid change
1	BCT-plasma	NSCLC	TP53	p.Y205C[2]
2	BCT-plasma	NSCLC	TP53	p.R273H[2]
3	EDTA-plasma	GIST	PDGFRA	p.M844_D846del[1]
4	EDTA-plasma	GIST	KIT	p.Y503_F504insAY[1]
5	EDTA-plasma	GIST	PDGFRA	p.I843_D846del[1]
6	BCT-plasma	NSCLC	No	-
7	BCT-plasma	NSCLC	No	-
8	BCT-plasma	NSCLC	Unknown	-
9	BCT-plasma	NSCLC	No	-
10	BCT-plasma	NSCLC	No	-
11*	BCT-plasma	NSCLC	BRAF	p.V600E[1]
12*	BCT-plasma	NSCLC	BRAF	p.V600E[1]
13*	BCT-plasma	NSCLC	BRAF	p.V600E[1]
14	BCT-plasma	NSCLC	KRAS	p.G13C[1]
15	BCT-plasma	NSCLC	KRAS	p.G12V[1]
16	BCT-plasma	NSCLC	BRAF	p.G466V[2]
17	EDTA-plasma	GIST	KIT	p.A502_Y503dup[1]
18	EDTA-plasma	GIST	KIT	p.A502_Y503dup[1]
19	EDTA-plasma	GIST	KIT	p.A502_Y503dup[1]
20ǂ	EDTA-plasma	GIST	No	-
21ǂ	EDTA-plasma	GIST	No	-
22	Citrate-plasma	NSCLC	KRAS	p.G12C[2]
23	Citrate-plasma	NSCLC	EGFR	p.N771_H773dup[2]
24	Citrate-plasma	NSCLC	No	-
25	Citrate-plasma	NSCLC	TP53	p.R273H[2]
26	Citrate-plasma	NSCLC	TP53	p.H179R[2]
27	Citrate-plasma	NSCLC	KRAS	p.G12D[2]

Highlighted are the cases for which a mutation-specific ddPCR analysis has been performed in the twenty-one cancer patients plasma cohort (blue). In the ccfDNA of cases 11, 12 13 14 and 15, no mutant droplets were detected with ddPCR. In the high-volume citrate plasma samples of cases 22, 25, 26 and 27, a mutation has been determined with a mutation-specific ddPCR analysis (green). BCT-plasma: plasma from cell free BCT tubes, EDTA-plasma: plasma from EDTA tubes, Citrate-plasma: citrate plasma retrieved through diagnostic leukapheresis (DLA), NSCLC: non-small cell lung cancer, GIST: gastro-intestinal stromal tumor, [1]mutation detected with tissue NGS, [2]mutation detected with plasma NGS, *samples derived from the same patient at different timepoints during anticancer treatment, ǂsamples derived from the same patient.

Table S2. Short- and medium-sized fragment percentages as measured with the Fragment Analyzer.

	Fragment Analyzer	**β-actin one-tube 3-size ddPCR**	
Kit	**Ratio S/M**	**Ratio 137/420bp**	**Ratio 137/1950bp**
CNA	3.51 (2.88-4.43)	2.22 (1.84-3.02)	17.0 (10.9-22.6)
RSC	4.09 (3.24-5.20)	2.51 (1.92-3.63)	19.0 (13.1-51.7)
Z	3.54 (2.62-4.72)	3.00 (1.38-3.74)	6.51 (4.43-18.5)*

Measurements are displayed as median percentage of retrieved fragment size with the interquartile range within brackets. Ratio S/M: ratio between short-sized fragments (50–250bp) and medium-sized fragments (250-450bp). * $p < 0.05$ between RSC and Z.

Figure S1 Detection of plasma ccfDNA degradation after short or long storage at 4°C and 20°C. In order to evaluate the degree of ccfDNA degradation in plasma from EDTA-tubes, six samples were processed in duplicate, one within 4 hours (standard procedure) and the second after 5 days of venipuncture. Half of these samples were stored at 4°C and the other half at room temperature (20°C; three samples per temperature). All these samples were isolated with the QIAamp Circulating Nucleic Acid kit and evaluated with the β-actine one-tube 3-size ddPCR assay.
Bar graphs illustrating the degradation of plasma ccfDNA after 5 days of storage before extraction compared to extraction within 4 hours of venipuncture. Storage at 4°C for the 137bp **(A)**, 420bp **(B)**, and 1950bp **(C)** fragment sizes and the 137/1950bp ratio **(D)**, as well for storage at 20°C **(E-H)** are displayed. Two out of three plasma samples showed minimal ccfDNA degradation when stored at 4°C. All samples stored at 20°C showed an increase in total ccfDNA level. However, this is mainly in long-sized fragments, represented by the decreased short-to-long-ratio.

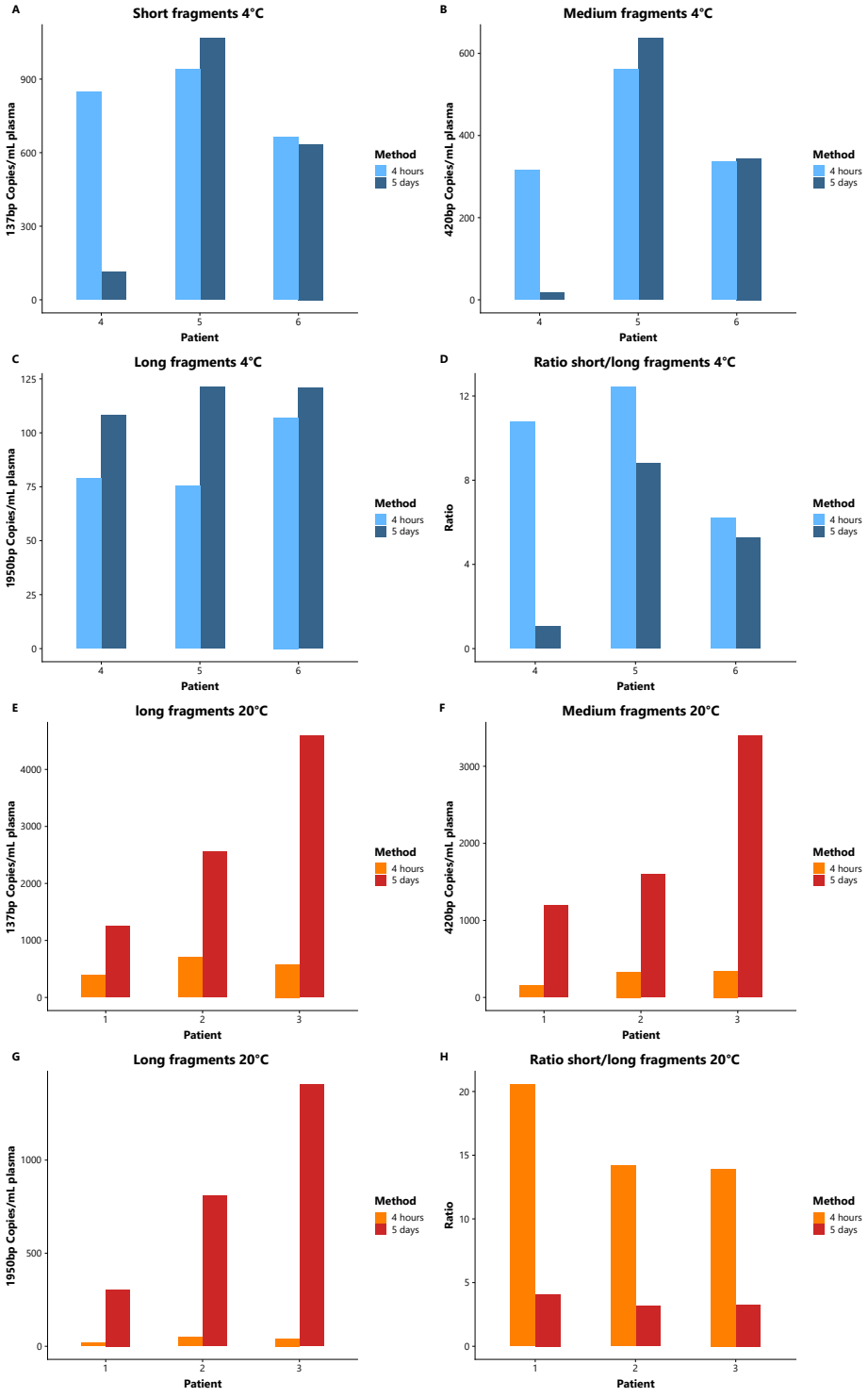

A
Short fragments 4°C
137bp Copies/mL plasma
0
300
600
900
4
5
6
Patient
Method
4 hours
5 days
B
Medium fragments 4°C
420bp Copies/mL plasma
0
200
400
600
4
5
6
Patient
Method
4 hours
5 days
C
Long fragments 4°C
1950bp Copies/mL plasma
0
25
50
75
100
125
4
5
6
Patient
Method
4 hours
5 days
D
Ratio short/long fragments 4°C
Ratio
0
4
8
12
4
5
6
Patient
Method
4 hours
5 days
E
long fragments 20°C
137bp Copies/mL plasma
0
1000
2000
3000
4000
1
2
3
Patient
Method
4 hours
5 days
F
Medium fragments 20°C
420bp Copies/mL plasma
0
1000
2000
3000
1
2
3
Patient
Method
4 hours
5 days
G
Long fragments 20°C
1950bp Copies/mL plasma
0
500
1000
1
2
3
Patient
Method
4 hours
5 days
H
Ratio short/long fragments 20°C
Ratio
0
5
10
15
20
1
2
3
Patient
Method
4 hours
5 days

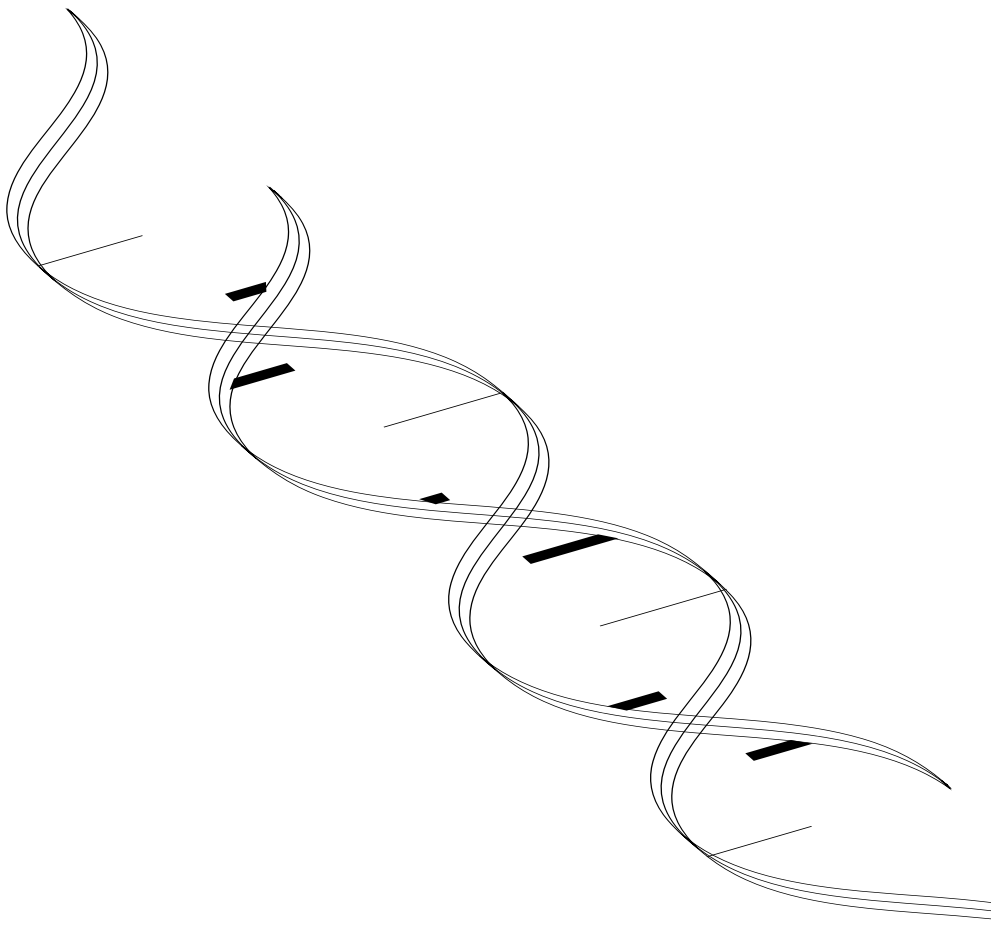
Table S3. Amplifiability of the extracted ccfDNA among different extraction kits.

Kit	137bp (copies/mL)	420bp (copies/mL)	1950bp (copies/mL)	137bp (copies/ng)	420bp (copies/ng)	1950bp (copies/ng)
CNA – blood plasma	637 (276-1658)	257 (140-587)	31 (22-129)	41 (27-53)	18 (13-22)	2.5 (1.6-3.2)
RSC – blood plasma	453 (254-832)	184 (125-269)	21 (9.2-29)	48 (30-65)	16 (12-23)	1.4 (0.4-2.8)
Z – blood plasma	203 (73-634)	106 (44-207)	25 (13-48)	21 (9.4-38)	10 (4.6-16)	2.4 (0.9-4.1)
CNA – blood plasma	288 (226-360)	146 (106-179)	55 (28-75)	35 (27-53)	20 (16-24)	5.7 (2.8-7.4)
ME – blood plasma	202 (181-227)	105 (95-126)	29 (23-38)	77 (61-81)	43 (38-50)	9.1 (7.0-12)

Highlighted are the cases results of the twenty-one cancer patients plasma cohort (blue) and of the six cancer patients high volume citrate plasma cohort (green). Measurements are displayed as median percentage of retrieved fragment size with the interquartile range within brackets. CNA – blood plasma: CNA-extracted blood plasma samples, RSC – blood plasma: RSC-extracted blood plasma samples, Z – blood plasma: Z-extracted blood plasma, CNA – citrate plasma: CNA-extracted blood citrate samples samples, ME – blood plasma: ME-extracted citrate plasma samples, copies/mL: copies per initial mL of plasma input, copies/ng: copies per ng of ccfDNA.

Table S4. Sequences of the applied primers and probes.

Assay	Forward primer	Reverse primer	Probe	Assay ID
137bp	5′-GCGCCGTTCCGAAAGTT-3′	5′-CGGCGGATCGGCAAA-3′	5′-ACCGCCGA-GACCGCGTC-3′	-
420bp	5′-CCGCTACCTCTTCTGGTG-3′	5′-GATACACCATGTCACACT-3′	5′-CCTCCCTC-CTTCCTGGCCTC-3′	-
KRAS G12/G13 screening	-	-	-	1863506
BRAF p.G466V	-	-	-	dHsaMDS2510966
TP53 p.H179R	-	-	-	dHsaMDV2510520
PDGFRA p.M844_D846del	5′-CTCCTGGCACAAGGAAA-3′	5′-AAAGGCAGTGTACGTCC-3′	5′-TGGCCAGAGA-CATCAACTATGTGTCG-3′	-
TP53 p.R273H	-	-	-	dHsaMDV2010109
TP53 p.Y205C	-	-	-	dHsaMDV2516922



A single digital droplet PCR assay to detect multiple *KIT* exon 11 mutations in tumor and plasma from patients with gastrointestinal stromal tumors

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Oncotarget, 2018

Abstract

Background: Gastrointestinal stromal tumors (GISTs) are characterized by oncogenic KIT mutations that cluster in two exon 11 hotspots. The aim of this study was to develop a single, sensitive, quantitative digital droplet PCR (ddPCR) assay for the detection of common exon 11 mutations in both GIST tumor tissue and in circulating tumor DNA (ctDNA) isolated from GIST patients' plasma.
Methods: A ddPCR assay was designed using two probes that cover both hotspots. Available archival FFPE tumor tissue from 27 consecutive patients with known KIT exon 11 mutations and 9 randomly selected patients without exon 11 mutations were tested. Plasma samples were prospectively collected in a multicenter bio-databank from December 2014. CtDNA was analyzed of 22 patients with an exon 11 mutation and a baseline plasma sample.
Results: The ddPCR assay detected the exon 11 mutation in 21 of 22 tumors with exon 11 mutations covered by the assay. Mutations in ctDNA were detected at baseline in 13 of 14 metastasized patients, but in only 1 of 8 patients with localized disease. In serial plasma samples from 11 patients with metastasized GIST, a decrease in mutant droplets was detected during treatment. According to RECIST 1.1, 10 patients had radiological treatment response and one patient stable disease.
Conclusion: A single ddPCR assay for the detection of multiple exon 11 mutations in ctDNA is a feasible, promising tool for monitoring treatment response in patients with metastasized GIST and should be further evaluated in a larger cohort.

1. Introduction

Gastrointestinal stromal tumors (GISTs) are rare malignancies of the gastrointestinal tract.[1] GIST is known to have driver single nucleotide variants, deletions and insertions (further referred to as mutations) in genes encoding the tyrosine kinase receptors KIT and PDGFRα. These occur in respectively 80% and 10% of GIST patients .[2, 3] In untreated GIST patients, the most frequent mutations are in KIT exon 11 (70%) coding for the juxta- membrane domain, and KIT exon 9 (10% of patients), coding for the extracellular domain of the receptor.[4] Around 80% of the mutations in KIT exon 11 cluster in two hotspot regions of approximately 25 base pairs within a 100 base-pair range of each other.[5]

Therapy consists of surgery and/or treatment with one of several selective tyrosine kinase inhibitors (TKIs). Adjuvant treatment with a TKI is based on Miettinen's risk classification which takes the size of the tumor, location and mitotic index into account.[6] Independently of the Miettinen classification, there is also a difference in recurrence risk between GISTs with different KIT and PDGFRα mutations.[7] First line treatment for locally advanced or metastatic GISTs consists of imatinib (400 mg daily), a selective inhibitor of the KIT tyrosine kinase.[8] Almost all patients with exon 11 mutated GIST respond to imatinib treatment, whereas exon 9 mutated GISTs have lower response rates. A large meta-analysis of 1,640 patients showed that bi-daily imatinib 400 mg is more effective than once daily dosing in KIT exon 9 mutated patients.[9] Resistance to imatinib treatment is usually the result of one or multiple secondary mutations that develop during treatment.[10, 11]

Second and third line treatment, with respectively sunitinib and regorafenib, also showed differential response rates that correlated with the primary mutational status of the tumor.[12–14]

Molecular diagnostic testing of relevant predictive biomarkers, including KIT and PDGFRα, is becoming routine practice in clinical decision-making. Mutation detection is routinely performed on pre-treatment tumor biopsies or resection specimens. For the detection of mutations a variety of methods are used, including as Sanger sequencing, pyro-sequencing, next generation sequencing (NGS) and high-resolution-melting (HRM) analysis with reflex sequencing.[15] These techniques are expensive, time consuming and require sufficient amounts of DNA (>100 ng) and a sufficient percentage of neoplastic cells (>5–20%). In some cases, no representative tumor material is available for molecular testing. Alternative methods for mutation detection, ideally also allowing serial non-invasive measurements, are urgently needed.

Interestingly, recent advantages in molecular pathology enable the detection of tumor specific mutations in circulating tumor DNA (ctDNA) extracted from blood plasma.[16] CtDNA can be used to define targets for selective therapy in both untreated and TKI-resistant non- small-cell-lung-cancer (NSCLC) tumors.[17, 18] The

detection of mutations in ctDNA as a predictive biomarker has been reported in both metastatic breast cancer (MBC) [19] and metastatic colorectal cancer (CRC). [20] Finally, mutation testing in ctDNA allows monitoring of TKI treatment response, where an increase in mutations could predict recurrence or disease progression.[18] Sporadic reports describe the use of ctDNA to detect mutations in GIST patients. [21–24] CtDNA is present in low amounts in plasma within a much more abundant background of non-tumor DNA (wild type) and is varying based on tumor type. [25] Highly analytical sensitive methods are used to detect ctDNA in plasma, these include BEAMing [26] and digital droplet PCR (ddPCR).[26, 27] Both BEAMing and ddPCR assays require the use of a separate assay for each tumor specific mutation. In general practice, based on the mutation detected in the tumor sample, a unique assay for the specific mutation is designed. Recently, the use of a single ddPCR assay to simultaneously detect various EGFR-exon 19 deletions in the plasma of NSCLC patients was reported.[28]
Given the long disease course of GIST patients and the multiple therapeutic options depending on mutational status of the tumor, a non-invasive test that can easily assess the presence of mutations is especially interesting for this patient group. Therefore, the aim of this exploratory study was to develop a ddPCR assay to detect most common exon 11 KIT mutations in. For the validation of this drop-off ddPCR assay we tested 36 formalin fixed paraffin embedded (FFPE) pre-treatment biopsies of patients with GIST previously tested for mutations using sequencing. To investigate the utility of this assay for detecting exon 11 mutations in ctDNA, plasma from 22 GIST patients was analyzed at baseline and at various time points during TKI treatment.

2. Results

2.1 Mutations in KIT exon 11 in GIST FFPE tumor tissue detected using the ddPCR drop-off assay

27 tumors with KIT exon 11 mutations were included, 17 tumors had a deletion, 1 a duplication, 4 a deletion/insertion and 5 had single nucleotide variants (SNV) as previously identified by sanger sequencing or NGS. Seventeen mutations cluster in hotspot 1, 6 in hotspot 2, one tumor had a deletion affecting both hotspots (sample 12) and in 3 tumors the deletion did not occur within the hotspots (samples 25, 26, 27, figure 1). Using the drop-off assay, a KIT mutation was detected in 21/27 tumors (see examples in supplementary figure 1). Tumor 18 had a duplication and was considered negative in the drop-off assay, however a typical pattern of droplet distribution was seen (supplementary figure 2). In 4 of the 5 negative tumors (20, 25, 26 and 27) the deletion did not allow annealing of the PCR-primer and therefore a PCR- product could not be generated (true negative tumors). Tumor 21 carried a SNV within the detection range of probe 2 and was the only true false-negative tumor.

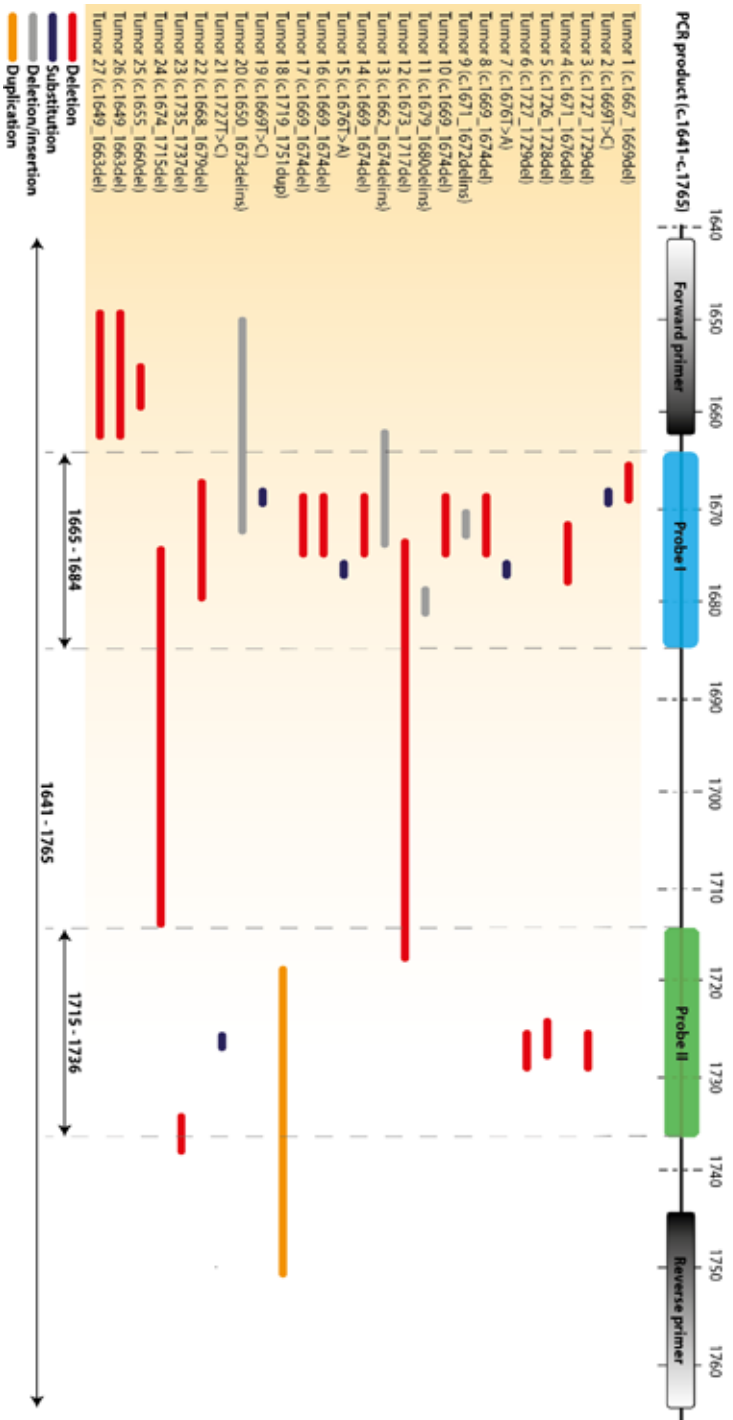

Figure 1 Location of KIT exon 11 mutations in GIST tumour samples as tested with Sanger sequencing or NGS.
The mutations are displayed relative to the actual position of the Forward and Reverse primers, the two probes (I and II) and PCR product. Type of mutations: red = deletion, grey = substitution, blue = deletion/insertion, orange = duplication.

Table 1 Tested patients with drop-off assay in relation to NGS results

	Allelic frequency				
Patient	**Mutant ddPCR**	**Mutant NGS**	**Mutation in probe area**	**Mutation**	
1	44,00%	38%	**1**	KIT Exon 11	c.1667_1669del
2	58,00%	61%	**1**	KIT Exon 11	c.1669T>C
3	53,00%	59%	**2**	KIT Exon 11	c.1727_1729del
4	41,00%	41%	**1**	KIT Exon 11	c.1671_1676del
5	30,00%	27%	**2**	KIT Exon 11	c.1726_1728del
6	34,00%	38%	**2**	KIT Exon 11	c.1727-1729delTTC
7	42,00%	38%	**1**	KIT Exon 11	c.1676T>A
8	45,00%	45%	**1**	KIT Exon 11	c.1669_1674del
9	54,00%	55%	**1**	KIT Exon 11	c.1671_1672delinsTG
10	40,00%	38%	**1**	KIT Exon 11	c.1669_1674del
11	83,00%	80%	**1**	KIT Exon 11	c.1679_1680delinsAG
12*	22,00%	28%	**1,2**	KIT Exon 11	c.1673_1717del
13	24,00%	45%	**1**	KIT Exon 11	c.1662_1674delinsGGAAGAA
14	88,00%	91%	**1**	KIT Exon 11	c.1669_1674del
15	35,00%	34%	**1**	KIT Exon 11	c.1676T>A
16	35,70%	57%	**1**	KIT Exon 11	c.1669_1674delTGGAAG
17	22,30%	25%	**1**	KIT Exon 11	c.1669_1674del
18$	0,00%	11%	**2**	KIT Exon 11	c.1719_ 1751dup
19	43,60%	44%	**1**	KIT Exon 11	c.1669T>C
20&	0,00%	79%	**1**	KIT Exon 11	c.1650_1673delinsCCTTCG
21	0,00%	Sanger	**2**	KIT Exon 11	c.1727 T>C
22	95,00%	86%	**1**	KIT Exon 11	c.1668_1679del
23	41,00%	Sanger	**2**	KIT Exon 11	c.1735_1737del
24	47,00%	Sanger	**1**	KIT Exon 11	c.1674_1715del
25&	0,00%	41%	**0**	KIT Exon 11	c.1655_1660delTGTATG
26&	0,00%	56%	**0**	KIT Exon 11	c.1649_1663del
27&	0,00%	44%	**0**	KIT Exon 11	c.1649_1663del

Detection of mutations in KIT with the ddPCR assay of pre-treatment tumor samples compared to the mutant allelic frequency as determined with NGS.
*despite a deletion partially overlapping with both hotspot areas, a signal was observed with probe 2; &deletion located in the forward primer annealing site precluding amplification of the mutated allele; $duplication considered negative, however a typical pattern of droplet distribution was seen. For representative examples see supplementary figure 1.

Of 22 tumors with mutations in KIT exon 11 covered by the drop- off assay, 21 tumors were positive resulting in a sensitivity of 95%. Analysis of the allelic frequency of mutant alleles versus wild type alleles of ddPCR corroborated the NGS results (table 1). As a negative control, 9 tumors without KIT exon 11 mutations were analyzed. These consisted of 4 tumors with a PDGFRα mutation, 2 with a KIT exon 9 mutation and 3 without any KIT/PDGFRα mutations.
All control GIST samples were negative resulting in a specificity of 100% (supplementary figure 3).

2.2 Tumor and treatment characteristics

Plasma samples taken before start of TKI treatment (baseline) of 22 patients with GIST were analyzed. Of these 22 patients, 14 had metastatic disease and 8 localized disease. Four patients with localized disease were planned to start with imatinib 400mg treatment in a neo-adjuvant setting (patient 7, 9, 13 and 17) and four patients underwent primary surgery. Samples of patients with metastatic disease were taken before start of a new line of TKI treatment (table 2).

Table 2 Plasma ctDNA analysis with drop-off ddPCR assay of GIST patients with metastasized disease

Patient	Primary GIST location	Prior treatment	New treatment	Mutation		Fractional abundance
3	Stomach	Imatinib	Sunitinib	KIT exon 11	c.1727_1729del	12,00%
4	Stomach	-	Imatinib	KIT exon 11	c.1671_1676del	0,40%
6	Small bowel	Imatinib Sunitinib	Regorafenib	KIT exon 11	c.1727-1729del	0,00%
11	Small bowel	-	Imatinib	KIT exon 11	c.1679_1680delinsAG	0,10%
14	Stomach	-	Imatinib	KIT exon 11	c.1669_1674del	14,20%
15	Small bowel	-	Imatinib	KIT exon 11	c.1676T>A	1,00%
16	Stomach	-	Imatinib	KIT exon 11	c.1669_1674del	1,40%
39	Small bowel	Imatinib Sunitinib Regorafenib	-	KIT exon 11	c.1676_1684del	3,00%
40	Small bowel	-	Imatinib	KIT exon 11	c.1668_1717delinsACCTT	7,00%
41	Stomach	-	Imatinib	KIT exon 11	c.1671_1715del	8,70%
42	Stomach	Imatinib	Masitinib	KIT exon 11	c.1670_1675del	0,90%
43	Small bowel	-	Imatinib	KIT exon 11	c.1676T>A	0,40%
44	Small bowel	-	Imatinib	KIT exon 11	c.1665_1676del	0,90%
45	Stomach	Imatinib	Sunitinib	KIT exon 11	c.1674_1695del	3,10%

Plasma samples were collected before start of a new line of TKI treatment. Pre-treatment primary tumors of 12 patients tested positive with the drop-off assay (patient 3–16 see table 1, patient 39–43 data not shown, patient 44 and 45 were not tested due to lack of tumors tissue). The fractional abundance was determined using DNA input representing 4 ml plasma.

2.3 Detection of exon 11 mutations in ctDNA with the drop-off ddPCR assay

KIT exon 11 mutations were detected in the baseline plasma ctDNA from 13/14 patients with metastasized disease (table 2). Pre-treatment tumor DNA available for 12 of these patients tested positive using the drop-off ddPCR assay. Plasma from one patient (patient 6) with metastasized disease had no detectable mutant ctDNA while a KIT mutation was detected in the pre-treatment tumor biopsy. In plasma ctDNA collected before start of treatment in eight patients with localized disease and a tumor KIT exon 11 mutation, only one patient (sample 7) had a detectable mutation in the ctDNA (table 3).

Table 3 Plasma ctDNA analysis of GIST patients with localized/locally advanced disease

Patient	Primary GIST location	Disease status	Mutation		Mutant allelic frequency
7	Rectum	Localized	KIT exon 11	c.1676T>A	1,95%
9	Stomach	Localized	KIT exon 11	c.1671_1672delinsTG	0,00%
10	Stomach	Localized	KIT exon 11	c.1669_1674del	0,00%
12	Stomach	Post-surgery	KIT exon 11	c.1673_1717del	0,00%
13	Stomach	Localized	KIT exon 11	c.1662_1674delinsGGAAGAA;	0,00%
17	Stomach	Localized	KIT exon 11	c.1669_1674del	0,00%
19	Small bowel	Localized	KIT exon 11	c.1669T>C	0,00%
37	Stomach	Localized	KIT exon 11	c.1679 T>A	0,00%

Primary tumors of 7 of these patients were tested positive with the assay (patient 7–19 see table 1, patient 37 data not shown, tumor of patient 38 was not positive). Samples were taken before start of any treatment.

To exclude that the lack of detectable ctDNA mutations was due to low sensitivity of the drop-off ddPCR assay, tumor and plasma samples collected from two patients with metastasized disease (patient 3 and 15) during treatment with a TKI were also tested with a specific ddPCR mutation assay. As shown in table 4, the mutant fractional abundance is comparable between the ddPCR and the specific assay. In addition, plasma samples from three different patients were tested using the highly sensitive analytical L-PCR technique for the detection of specific KIT exon 11 mutations earlier described [22] (table 5). For this analysis, we selected eight plasma samples from three patients with different KIT exon 11 mutations tested with the drop-off ddPCR assay. Low level mutant frequencies detected with the L-PCR technique (<0.1%) were also positive in the drop- off samples. Of the samples that tested negative using the drop-off assay, 4/5 were also negative using the L-PCR technique.

Table 4 Correlation between the ddPCR with a mutation-specific assay and the drop-off assay

Patient	Type	Fractional abundance drop-off probe	Fractional abundance mutant specific probe	Mutation
3	Tumor	53%	48%	c.1727_1729del
3A	Plasma	12,02%	11,60%	c.1727_1729del
3B	Plasma	8,70%	7,20%	c.1727_1729del
3C	Plasma	0,70%	0,72%	c.1727_1729del
15	Tumor	35%	33%	c.1676T>A
15A	Plasma	0,90%	1,02%	c.1676T>A
15B	Plasma	5,50%	4,90%	c.1676T>A
15C	Plasma	0,00%	0,00%	c.1676T>A

Two tumor samples and six plasma samples of two patients with metastasized disease were tested with a probe specifically designed for the mutation.
*A = before start of treatment, *B = after two weeks of treatment, *C = after 6 weeks of treatment.

Table 5 Comparison of L-PCR with ddPCR

Patient	Mutation	L-PCR Mutation/ wild type %	ddPCR Mutation/ wild type %	Disease status	
7A	c.1676T>A	0,0019	1,95	Localized	Before start of treatment
7B	c.1676T>A	0,0024	0	Localized	1 week treatment imatinib
7C	c.1676T>A	0	0	Localized	4 week treatment imatinib
10A	c.1669_1674del	0	0	Localized	Before surgical treatment
10B	c.1669_1674del	0	0	Localized	3 days after surgery
15A	c.1676T>A	0,0015	0,94	Metastasized	Before start of treatment
15B	c.1676T>A	0,0012	5,60	Metastasized	2 weeks treatment with imatinib
15C	c.1676T>A	0	0	Metastasized	6 weeks treatment with imatinib

To evaluate the sensitivity of our assay, multiple samples of three patients were analysed with the earlier described L-PCR technique. Quantitative L-PCR analysis was performed on 1 ml plasma as reported previously.[22] Four samples were scored low-level positive (<0,1% mutant/wild type ratio). When looked at positive/negative samples the results where –except for sample 7B- comparable with the ddPCR assay.

2.4 The detection of mutations in plasma ctDNA at different time points during treatment

In order to monitor the presence of mutations after start of TKI treatment compared to baseline samples, serial plasma samples of 11 patients with metastatic disease using the ddPCR drop-off assay were available and analyzed. The analysis of plasma samples at 2–3 weeks after start therapy revealed an increase in fractional abundance in 5 out of 11 patients (figure 2, supplementary table 1). In all available plasma samples obtained six weeks after start of treatment the fractional abundance decreased below

the levels observed at baseline or the 2–3 weeks after start of treatment sample. In agreement with the loss of detection of mutant DNA, ten patients showed a tumor response and one patient stable disease (no. 45) on treatment with TKI according to the first radiological evaluation performed approximately 3 months after start of therapy.

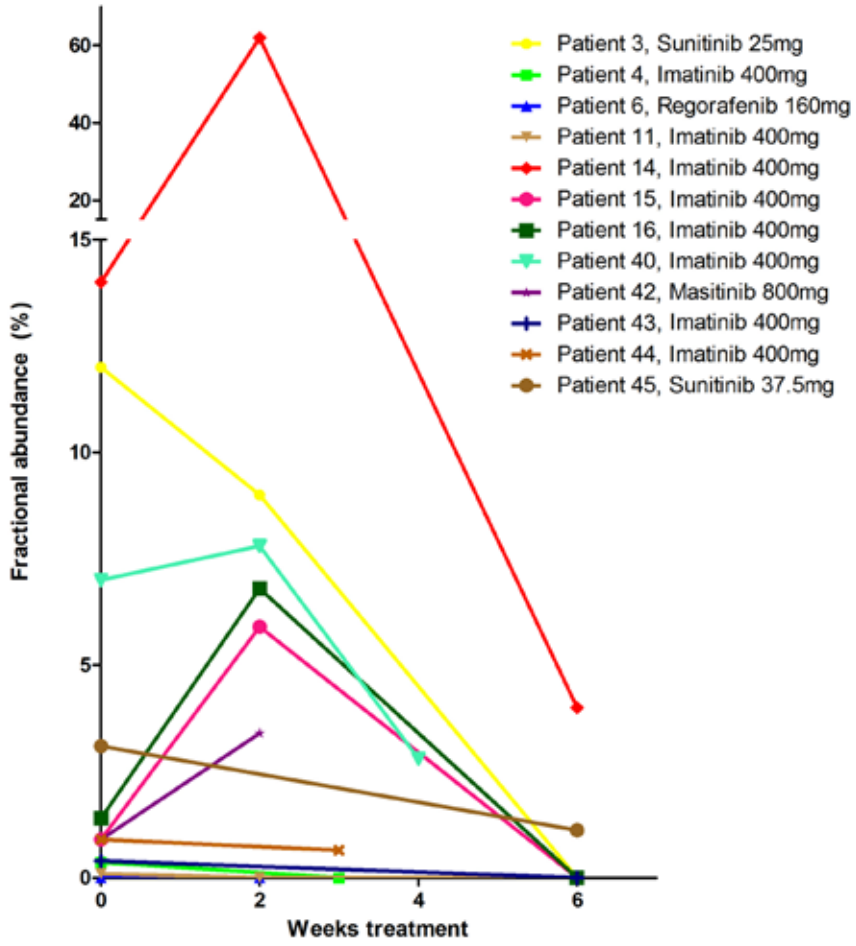

Figure 2 Detection of KIT exon 11 mutations using the ddPCR drop-off assay in ctDNA in patients with metastasized GIST at baseline (before start TKI-treatment) and 2–6 weeks after start of treatment. Mutation frequency is expressed as fractional abundance in % (see supplementary table 1). Twelve patients with metastasized GIST with both a baseline plasma sample as well as at least one sample collected 2–6 weeks after staring TKI treatment were selected. Both pre-treatment FFPE DNA (table 1) and baseline plasma samples (table 2) were tested with the same ddPCR. Patient 39 and 41 (table 3) were not included since no follow-up plasma samples were available.

3. Discussion

In this study, an in-house designed single ddPCR assay was able to detect multiple mutations in KIT exon 11 with high sensitivity (95%) and specificity (100%) in tumor biopsies of patients with GIST. Sensitivity of the assay for all known KIT exon 11 mutations in GIST is lower than 95% since the designed assay covers 80% of the described KIT exon 11 mutations (in the described cohort 21/27 mutations were detected resulting in a sensitivity of 77%). For LOB analysis 5 plasma samples from healthy individuals and 5 normal FFPE samples were analyzed. No false positive droplets were detected in the ddPCR analysis of these samples. As expected, due to the quality of the FFPE material, highly damaged DNA as well as artifactual C>T transitions the ddPCR resulted in a reduced separation of wild type droplets.[29] This should be taken into account when interpreting test results. However, for mutation screening in freshly-processed cell- free plasma DNA, the separation between drop-off and wild type droplets was excellent in all samples tested in this study. Despite a very good LOB and a high sensitivity of 0.1%, the maximum sensitivity that can be obtained is limited by the input of the total number of copies of a genome. As in plasma samples the total amount of DNA is often close to 2ng, this input of DNA would result in a sensitivity of 1%.

This assay enabled the detection of low-level copy mutations and the identification of mutations in 12 of 13 cell-free plasma samples of patients with metastatic disease at baseline. DdPCR is relatively cheap and has a short turn-around time. Since the probe does not detect specific mutations in exon 11 of the KIT gene, the drop-off ddPCR assay is especially suitable for predictive testing of GIST in case not enough tissue or neoplastic cells are available for NGS analysis or for monitoring treatment response in ctDNA.

Mutation testing in ctDNA might be an alternative source for tissue biopsies particular when no biopsies or biopsies with insufficient neoplastic cells percentages for molecular profiling are available.[30] In addition, mutation analysis of ctDNA during treatment has been reported as a new tool for monitoring treatment response since the amount of ctDNA correlates with the volume of vital tumor tissue.[31] Circulating DNA in the cell- free plasma fraction originates from many different cells including lymphocytes and neoplastic cells.[32] Their nuclear and mitochondrial DNA is released into the circulation in the process of cellular destruction by apoptosis or necrosis.[25] Therefore, ctDNA in cell- free plasma is a very low fraction of the total amount of circulating DNA. For the detection of mutations in ctDNA in a high background of total plasma DNA, various detection assays with high analytical sensitivity have been reported including digital PCR, BEAMing, sequencing based methods, Ligand PCR, ARMS-PCR and PNA- clamping PCR.[33] Because the analytical sensitivity of NGS is around 1–5% and also requires high amounts of input DNA, NGS is at present not suitable for mutation screening in ctDNA from plasma in malignancies with low abundance of ctDNA. On the other hand, the ddPCR has been reported as a

quantitative, accurate assay with high analytical sensitivity.[34] Sensitivities of 0.005–0.1% for EGFR-T790M (own unreported data, [28]), 0,1% and 0,5% for ALK-C1156Y and ALK-G1269A in lung cancer [35] and 0.025% for KRAS in CRC [36] are reached.
To the best of our knowledge, this is the first time a single ddPCR assay to detect multiple KIT exon 11 mutations in tumor tissue and ctDNA of patients with GIST has been reported. Few other studies have described the use of mutational analysis of ctDNA in GIST. In a recent study, using a NGS platform after enrichment PCR with PNA probes, KIT mutations were detected in the plasma of 13 out of 18 patients with localized gastric GIST.[37] With the allele specific ligation PCR assay KIT mutations were found in 9 out of 18 patients with active disease, furthermore mutations at low levels were detected in 6 out of 20 patients in complete remission.[22] Another study using BEAMing detected primary mutations in 5 out of 30 patients with TKI-refractory GIST (17%).[23] Both BEAMing and the allele specific ligation PCR assay require the generation of specific primers/probes for each genomic KIT mutation. In GIST patients with localized disease and proven KIT mutations in the pre-treatment biopsy, our assay detected the mutations in the baseline plasma DNA in only 1 of 8 cases. An explanation for this discrepancy is that localized tumors may not actively shed tumor DNA into the circulation. In other malignancies an association was reported with the detection of mutations in plasma and advanced stage disease.[38] The ddPCR drop-off assay was previously described for the detection of various clinical-relevant deletions in exon 19 of the EGFR gene in lung cancer.[28] This ddPCR drop-off del 19 assay showed a sensitivity of 5–50 mutant copies in a background of 10,000 wild type copies which is similar to our observed sensitivity for the ddPCR drop-off assay for KIT exon 11 mutations.
In the analysis of serial ctDNA samples, an evident rise of fractional abundance was seen after initiation of treatment. We hypothesized that the rise of mutational level could be due to increased cell death induced therapy initiation. This early response is not reported in other malignancies treated with TKI.[39] Our result implies that treatment response can be monitored by using this ddPCR assay in cell-free plasma. Similar observations were also reported using quantitative L-PCR in 5 patients with advanced GIST.[22] Monitoring of treatment response has also been reported in anti-EGFR treated CRC using KRAS mutations [40], TKI-treated lung cancer for EGFR del19/L858R [41], BRAF mutated melanoma [42] and gynecologic malignancies [43] and detection of progression on primary TKI in ctDNA has been reported in EGFR mutated NSCLC [44] and CRC.[45]
Since tumors evolve during treatment and secondary mutations can cause therapeutic resistance, a new biopsy can be required during treatment to define the actual mutational status.[46] This has recently been demonstrated in patients with NSCLC during treatment with EGFR-TKI. The EGFR TKI-resistance mutation T790M was detected in ~70% of plasma ctDNA of patients with advanced disease who had acquired TKI- resistance.[47] These resistant mutations could be missed by conventional tissue biopsy due to tumor heterogeneity.[48] In addition, repeated

tumor biopsies have risks e.g. bleeding, perforation and infection. Thus, there is a need for less invasive techniques that provide information about mutational status of tumors and that can be easily performed at different time points during treatment. The detection of primary and secondary resistant mutations in ctDNA cannot be used only to monitor recurrences before clinical manifestation, but might also warrant a different therapeutic approach. Recently, the FDA approved the detection of the EGFR TKI resistant T790M mutation in plasma (June 1, 2016) as a marker for a second generation EGFR TKI specifically inhibiting the T790M mutation.[49] Similarly, in GIST, resistance develops during imatinib treatment. In 50% of patients with progressive disease, a secondary mutation, besides the primary KIT mutation, is detected.[50] Treatment response to standard second line therapy, sunitinib, differs between patients with secondary KIT exon 13/14 or exon 17/18 mutations.[51] The detection of secondary mutations in plasma was reported in 4 patients using pre-amplification and NGS. Mutant alleles were detected in a range of 0.010–9.385%.[24] In a study using BEAMing secondary mutations were detected in 11 out of 30 patients (41%).[23] Therefore, the implementation of ddPCR (or other sensitive) detection assays to identify resistant KIT mutations in plasma ctDNA is warranted for the development of more optimal treatment strategies in patients with GIST treated with TKIs.

The detection of multiple KIT exon 11 mutations with a single ddPCR assay has high sensitivity and specificity. It is suitable for predictive testing of GIST in case not enough tissue or neoplastic cells are available for routine NGS analysis in FFPE tissue. This technique can be easily performed, is cost-effective and has a short turn-around-time. Therefore, this ddPCR assay might be especially suitable for treatment response monitoring by ctDNA analysis in plasma. Our study will be extended to include the monitoring of early progression based on ctDNA, which may guide early treatment adaptations.

4. Materials and methods

4.1 Study design

The reported work is part of an open-label, non- randomized, non-interventional, explorative multicenter study aiming to detect the most frequently occurring KIT exon 11 mutations using a single ddPCR assay. The assay was first tested on archival formalin-fixed paraffin-embedded (FFPE) tumor tissue stored at the University Medical Centre Groningen (UMCG). After validation with tumor tissue, this assay is tested in prospectively collected plasma samples from 22 GIST patients before and during treatment with a TKI. These 22 patients were treated in one of the five hospitals in the Dutch GIST consortium (Antoni van Leeuwenhoek, Amsterdam; Leiden University Medical Centre, Leiden; Erasmus University Medical Centre, Rotterdam; Radboud University Medical Centre, Nijmegen; University Medical Centre Groningen,

Groningen). 405 plasma samples of 140 GIST patients before or during treatment with a TKI have been prospectively collected (Dec 2014 - Sept 2016). Treatment, follow-up and response evaluation by CT according to RECIST 1.1, were performed according to (inter)national guidelines. Plasma samples were available before start and at multiple time points after start of a TKI for 8 patients with localized GIST and 14 patients with metastasized GIST. All patients had measurable disease before collection of the first plasma sample and received systemic treatment during the study period. Plasma samples were collected at every visit to the outpatient clinic. Disease evaluation was performed by CT-scans performed approximately every 3 months. Response evaluation was performed using RECIST version 1.1 criteria by a radiologist, unaware of obtained ctDNA results.
All patients gave written informed consent. The Medical Ethical Committee approved the study and it is registered on ClinicalTrials.gov (NCT02331914).

4.2 Tumor sample collection and DNA extraction
Pre-treatment FFPE tumor biopsies for 27 consecutive patients diagnosed with exon 11 mutated GIST between 2012 and 2015 were retrieved from the local pathology archive at the UMCG . Nine GIST tumors with mutations outside KIT exon 11 from the same period were randomly selected as controls. FFPE samples of healthy controls were obtained from the pathology department of UMCG. Tumor-specific mutations were determined by routine diagnostic NGS of a gene panel with relevant predictive markers (version PGMv001; www.moloncopath.nl) on the IonTorrent platform (Thermo Fisher Scientific, Waltham, MA, USA). The analysis of some older tumors was performed using Sanger sequencing as reported previously.[52]
In brief, two to four 10 µm thick sections were cut from the original FFPE blocks preceded and followed by a 4 µm section. After haematoxylin and eosin staining, the 4 µm slides were evaluated by an experienced pathologist for the presence of an area with sufficient tumor cells (>20%). Genomic DNA from FFPE slides was extracted using the Cobas extraction kit (Roche, Basel, Switzerland) and quantified using Qubit (Thermo Fisher Scientific). All molecular testing was performed in the CCKL/ ISO15189- accredited laboratory of molecular pathology at the UMCG. All standard precautions were taken to avoid contamination of amplification products using separate laboratories for pre- and post-PCR handling. To avoid cross-contamination, a new microtome blade was used each time a new sample was sectioned.

4.3 Next generation sequencing using IonTorrent
Libraries were generated using an in-house panel (version PGMv001) using the IonTorrent platform. This panel consists of 30 primer pairs covering 11 clinically relevant genes including hotspots in exon 9, 11, 13 and 17 of KIT and exon 12, 14 and 18 of PDGFRα (http:// www.moloncopath.nl). 10 ng of DNA from each sample was used to prepare barcoded libraries using IonXpress barcoded adapters (Thermo Fisher Scientific). Libraries were combined to a final concentration of 100 pmol using

the Ion Library Quantification Kit (Thermo Fisher Scientific), and emulsion PCR was performed using the IonTorrent OneTouch TM2 system. Samples were sequenced on the IonTorrent semi-conductor sequencer using Ion 316 or 318 chips. Sequence reads were aligned to the 11 genes based on the Human Genome version 19 using Sequence Pilot v4.2.0 (JSI Medical Systems GmbH, Ettenheim, Germany). Also read depth and uniformity of coverage across individual amplicons were assessed. In data analysis the cut-off was set at mutations found in > 5% of the reads. Only non-synonymous and non-sense variations in coding regions were included.

4.4 Drop-off ddPCR assay

Since in 80% of the cases exon 11 mutations occur in one of the two hotspot regions, one probe serves as a wild type probe while the loss of signal from the second probe represents the presence of a mutation (figure 3).

A ddPCR assay consisting of a single set of PCR primers and two TaqMan probes (FAM or HEX) was designed using PrimerQuest (http://eu.idtdna.com/Primerquest) and purchased from IDT (Coralville, IA, USA). The primer sequences are Fwd. 5′-CCACAGAAACCCATGTATGAAG-3′ (position c.1641-c.1662) and Rev. 5′-GAGTTTCCC AGAAACAGGC-3′ (position c.1746-c.1765) resulting in a PCR product of 124 base pairs covering both hotspots in KIT exon 11 (position c.1641-c.1765, figure 3). The sequence of probe I (FAM) is 5′-ACAGT GGAAGGTTGTTGAGGAGAT-3′ and probe II (HEX) 5′-ACCCAACACAACTTCCTTATGATCACA-3′. Temperature gradient PCRs of the primers and probes were performed to detect the optimal annealing temperature and resulted in an optimal PCR temperature of 60 °C.

4.5 Specific ddPCR assays

For the detection of the c.1676T>A mutation, a commercially available assay was purchased (dHsaCP2506828 and dHsaCP2506829, Bio-Rad, Hercules, CA, USA). The specific c.1727_1729del assay was designed in-house and purchased from IDT. The primer sequences are Fwd. 5-′CCACAGAAACCCATGTATG-3′ (position c.1643-c.1661) and Rev. 5′-GCCTGTTTC TGGGAAAC-3′ (position c.1750-c.1766). The sequence of wild type-probe I (FAM) is 5′-ACCCAACACAACC TTATGATCACAAATG-3′ and mutant-probe II (HEX) 5′-ACAGTGGAAGGTTGTTGAGGAG-3′.

4.6 DdPCR analysis of DNA of pretreatment tumor biopsies

DdPCR on tumor tissue was performed on 2 ng of genomic DNA as measured by Qubit according to the manufacturer's instructions. Briefly, 11 µl ddPCR Supermix for probes, 1 µl of the ddPCR assay (wild type and mutation primer/probes) and genomic DNA were mixed in a final volume of 22 µl. Droplets were generated from 20 µl of the suspension using the QX100 Droplet generator after addition of 70 µl droplet generation oil (Bio-Rad). The PCR was performed on a T100 Thermal Cycler (Bio-Rad) using the following cycling conditions: 10 minutes at 95 °C, 40 cycles of 95 °C for 30 seconds, 60 °C for 1 minute followed by 98 °C for 10 minutes (ramp

rate 2.5 °C/sec). Samples were transferred to the QX200 Droplet Reader (Bio- Rad) for fluorescent measurement of FAM and HEX probes and data were analyzed using Quantasoft software version 1.6.6. Samples were considered positive when 3 or more FAM/HEX positive droplets were detected, while no FAM/HEX positive droplets in the no-template and no single positive droplets in the wild type controls were observed. The fractional abundance is based on the ratio between mutant and wild type droplets after correction using the Poisson distribution (calculated by the Quantasoft software).

4.7 Circulating tumor DNA analysis

Plasma samples from patients treated at the UMCG were collected in EDTA tubes (vacutainer #367525, Becton Dickinson, Franklin Lakes, NJ, USA) and processed within 4 hours after venipuncture. Samples from patients from other centers were collected in cell free DNA BCT tubes (Streck, Omaha, NE, USA), which stabilizes blood samples for a minimum of 7 days at room temperature.[53] The cell free BCT tubes were sent by regular mail to the UMCG and processed on the day of arrival. For quantitative validation of the assay, plasma samples of five anonymous healthy controls were collected in the same cell free BCT tubes.

EDTA samples were first centrifuged for 10 minutes at 820 g to separate the lymphocytes from the plasma. The supernatant was transferred to a new Eppendorf tube and centrifuged at 16,000 g for 10 minutes to separate plasma from the remaining debris. After the last centrifugation step the supernatant was transferred and stored at −80° C until analysis. Cell free DNA BCT tubes were processed identically but with a different first centrifugation step (1,600 g). Plasma processing was performed in a laboratory not used for any molecular testing.

For ctDNA isolation, samples were thawed after storage at –80 °C and centrifuged for 5 minutes at 16,000 g. DNA was extracted from plasma using the QIAamp Circulating Nucleic Acid kit. (QIAgen, Hilden, Germany) following manufacturers protocols. DNA from 4 ml of plasma was isolated and eluted in 2 × 250 µl of elution buffer. This eluate was concentrated using an Amicon filter column (Merck, Darmstadt, Germany). The final amount of eluate was 15–20 µl. After isolation the eluate was stored at 4 °C until experiments were performed.

The designed drop-off ddPCR assay was used for analysis of ctDNA. Experimental conditions were identical to those for analysis of tumor tissue except for the input per reaction. For analysis of ctDNA the maximum input (9 µl) of isolated DNA per reaction was used, each run included wild type (WT) and no template controls (NTC). The presence of a mutation was calculated as the fractional abundance.

Quantitative L-PCR using 1 ml of cell-free plasma was performed as reported in detail previously.[22] The laboratory technicians who performed the ddPCR experiments were not aware of the mutational and clinical status of the tested patient samples.

4.8 Quantitative performance of the drop-off ddPCR assay

The sensitivity of the assay was determined using DNA from FFPE pretreatment biopsies with mutations in exon 11 hotspot 1 (c.1669T>C and c. 1671_1676del) and hotspot 2 (c.1727_1729del) with known mutation allelic frequency (MAF) determined by NGS diluted with wild type DNA. A significant correlation was observed between tumor DNA input as measured by NGS-MAF and mutated droplet detection in three different samples (supplementary figure 4). The limit of detection (LOD) of the drop-off ddPCR assay on DNA extracted from FFPE- tissue with 30 ng DNA input was

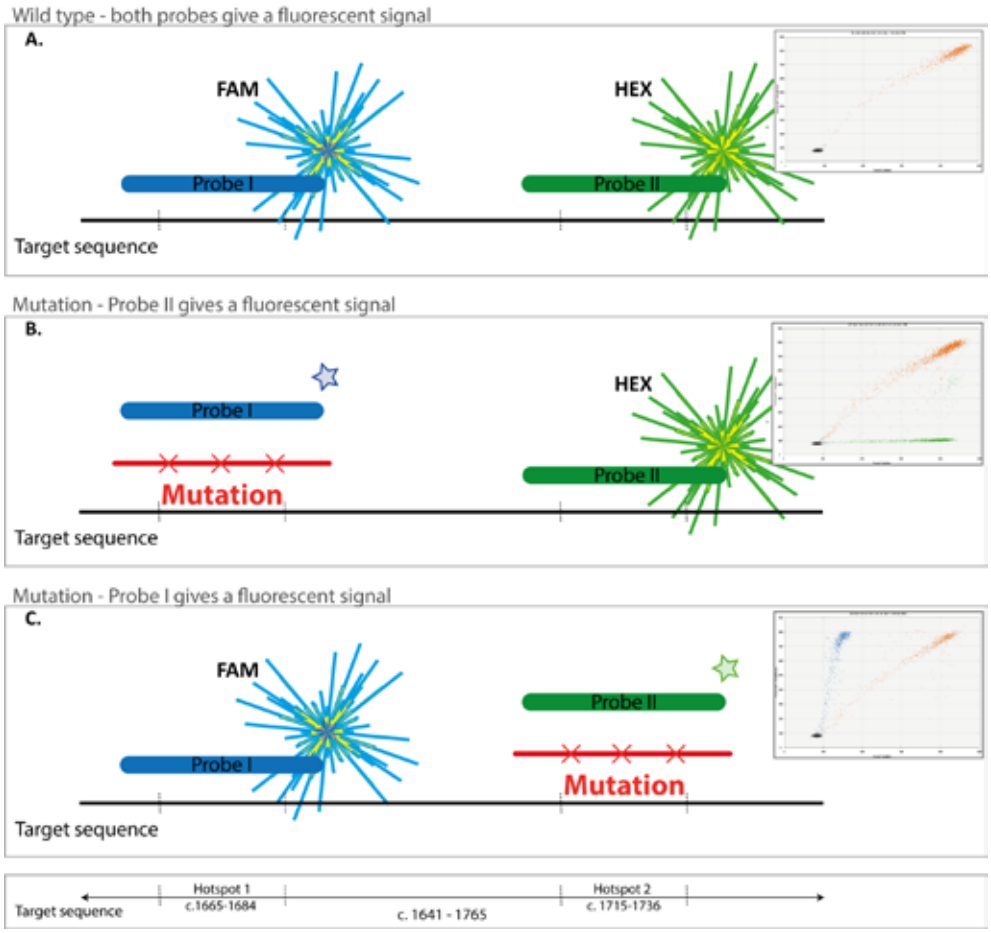

Figure 3 KIT exon 11 mutation/deletion detection assay.
A When no mutation is present, both probes (FAM and HEX) will anneal and droplets with a dual fluorescent signal will be detected (coloured orange in the figure).
B In cases with a mutation in hotspot region I, only droplets with WT region II are detected (HEX, green signal). Also wild type fragments are detected (orange droplets) in the graph.
C Example of a case with a mutation in hotspot region II, only droplets with WT region I are detected (FAM, blue signal). Wild type fragments are also detected (orange droplets).

0.11% (not shown) and with a lower DNA input (2 ng) a maximum of 1% mutant alleles was still detected (see example in supplementary figure 5).
The limit of blank (LOB, false mutation rate) as reported earlier [54] of the ddPCR assays was estimated using five FFPE healthy tissue. FFPE samples were tested with an input of 2ng and 30ng resulting in respectively in a mean of 545 and 5345 wild type droplets and 0 false- positive droplets per sample (LOB = 0% for tissue DNA). To determine the LOB on plasma-derived ctDNA, cell free DNA was isolated from five plasma samples of healthy controls following the study extraction protocol and maximum input was used resulting in a mean of 791 wild type droplets and 0 false-positive droplets (LOB = 0% for normal plasma DNA, supplementary figure 6).

Funding
This project is funded by the Dutch Cancer Foundation (KWF), Alpe d'HuZes research grant RUG 2013-6355.

Conflicts of interest
The authors declare no conflicts of interest.

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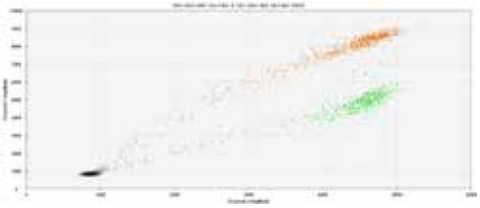
Supplementary data

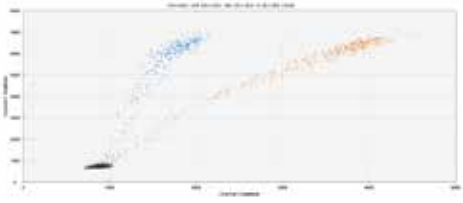
S1

A. Mutation in probe area 1 (c.1674_1715del)



B. Mutation in probe area 1 (c.1676T>A)

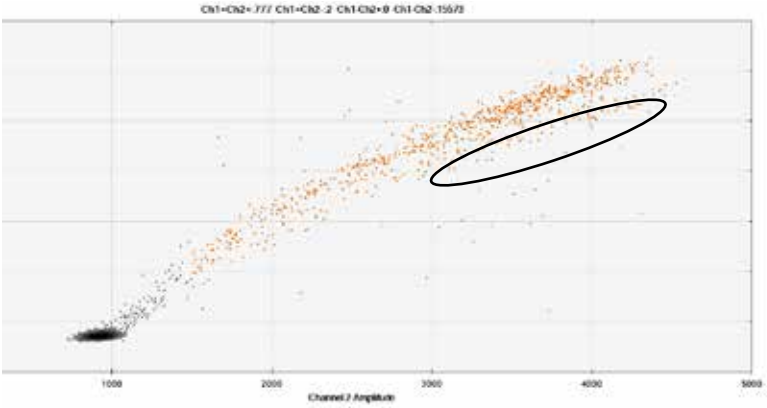
C. Mutation in probe area 2 (c.1727_1729del)



D. Mutation in probe area 2 (c.1735_1737del)

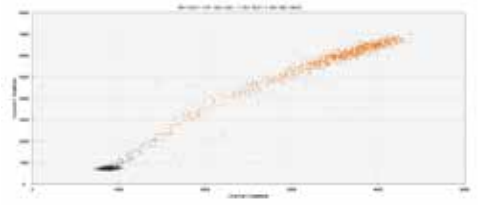
Supplementary figure 1
Examples of ddPCR drop-off results of FFPE-samples with a KIT exon 11 mutation.
A Tumour 24 contains a deletion in probe area 1. It shows a common pattern observed in most cases with mutant (green) and wild-type (orange) droplets. **B** Tumour 7 with a point mutation in area 1 with mutant (green) and wild-type (orange) droplets. **C** Tumour 6 with mutation in the second probe area shows mutant droplets (blue) and wild-type droplets (orange). **D** Tumour 23, with deletion in the second probe area, shows mutant droplets (blue) and wild-type droplets (orange).

S2

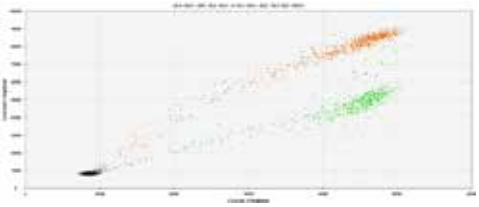


Supplementary figure 2
Tumor 18 carries a duplication (c.1719_1751dup). The ddPCR drop-off assay did not result in a common drop-off pattern seen in other cases with mutations and deletions (see supplementary figure 1). However, within the wild-type cluster region defined by the orange droplets, in addition to the prominent wild-type cluster, a separate cluster with slightly lower fluorescence intensity can be distinguished marked by the black oval. The number of signals of these clusters is similar to the mutant allelic frequency of 11% as determined with NGS on the same DNA and strongly suggests that this cluster represents the mutant droplets.

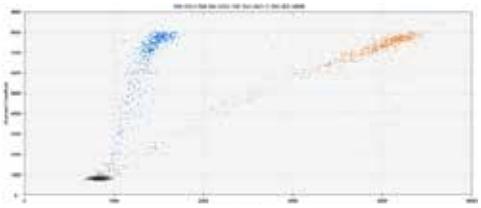
S3



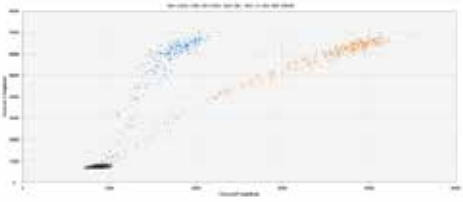
A. FFPE DNA of GIST with PDGFRA exon 18 c.2525A>T



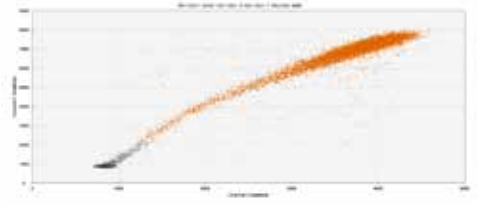
B. FFPE DNA of GIST with PDGFRA exon 18 c.2531_1542del



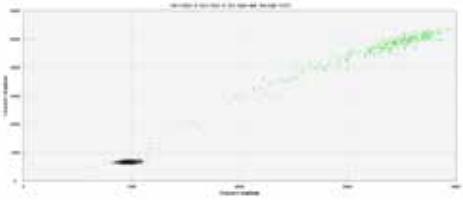
C. FFPE DNA of GIST with KIT exon 9 c.1502_1503insTGCCTA



D. FFPE DNA of GIST with KIT exon 9 c.1502_1503insTGCCTA



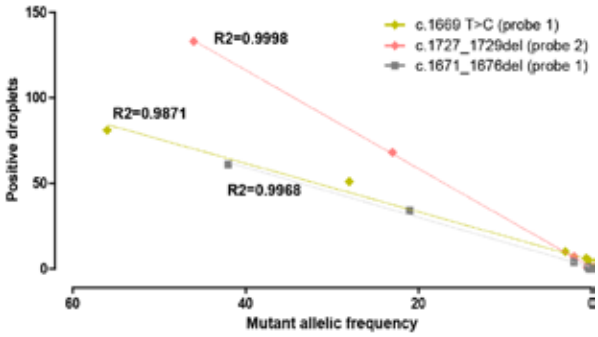
E. FFPE DNA of GIST without any mutation tested with NGS



F. Plasma derived DNA of patient with GIST without any known mutations tested with NGS

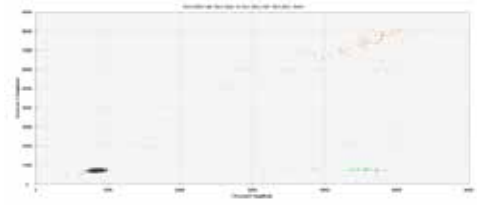
Supplementary figure 3
Examples of tumours without KIT exon 11 mutations and considered as negative controls.
A–D The ddPCR drop-off assay was performed on 2 ng input DNA extracted from FFPE tissue of GIST with mutations other than KIT exon 11. **E** DdPCR analysis of plasma derived DNA (input 10 ng). According to our criteria these samples are considered negative although few scattered positive droplets were observed outside the clusters commonly detected for HEX only, FAM only or double HEX/FAM droplets (see supplementary figure 6). Using fluorescent measurement of FAM and HEX probes and data analysis with Quantasoft software version 1.6.6, in general we define only droplets above channel amplitude 1000 for HEX and above 2000 for FAM as true positive. The amount of these scattered droplets is associated with the quality of DNA and therefore sometime observed in DNA from old-FFPE tissue blocks and very rarely in plasma derived DNA(**F**).

S4

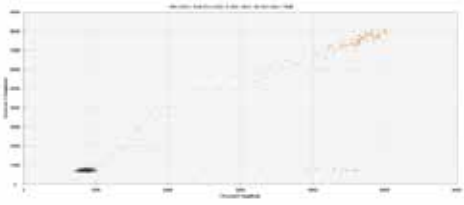


Supplementary figure 4
Correlation between mutant allelic frequencies as determined with NGS and positive droplets detected by ddPCR of three different tumour FFPE samples. DNA input is 2 ng. Two samples have a KIT exon 11 mutation in hotspot 1 and one sample a mutation in hotspot 2.

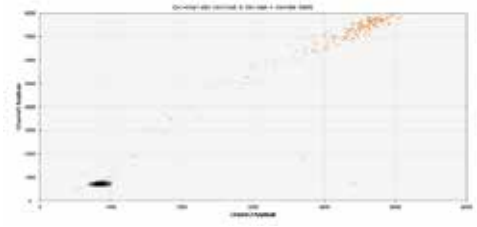
S5



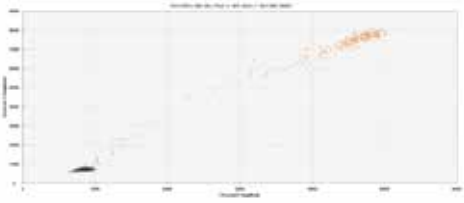
A. FFPE DNA with 100% neoplastic cells (undiluted) with a KIT c.1671_1676del mutation.



B. FFPE DNA with 50% neoplastic cells with a KIT c.1671_1676del mutation.



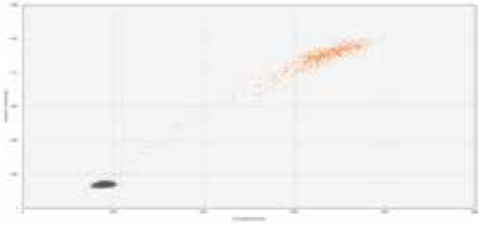
C. FFPE DNA with 5% neoplastic cells with a KIT c.1671_1676del mutation.



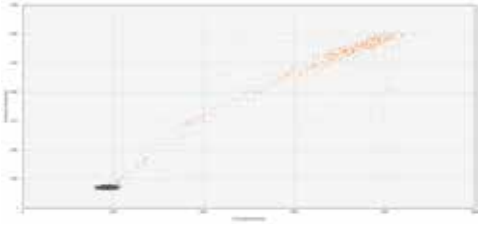
D. FFPE DNA with 1% neoplastic cells with a KIT c.1671_1676del mutation.

Supplementary figure 5
DNA dilution series of a FFPE tumour sample with a KITc.1671_1676del mutation using 2 ng total DNA input. **A** ddPCR analysis of undiluted tumour DNA (100% neoplastic cells) detected 61 positive droplets and a fractional abundance of 40%. NGS revealed a similar mutant allelic frequency of 42%. **B** ddPCR of 50% dilution results in 34 positive droplets and a fractional abundance of 16%. **C** ddPCR of 5% tumour dilution results in 4 positive droplets and fractional abundance of 1%. **D** ddPCR of 1% tumour dilution resulted in 1 detectable droplet according to our criteria considered as negative (no deletion/mutation is present).

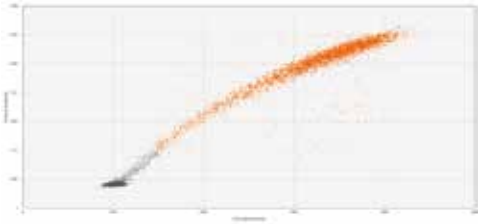
S6



A. ddPCR of plasma sample from healthy donor, input is 9.72ng.



B. FFPE sample of a tonsil, input of 2ng.



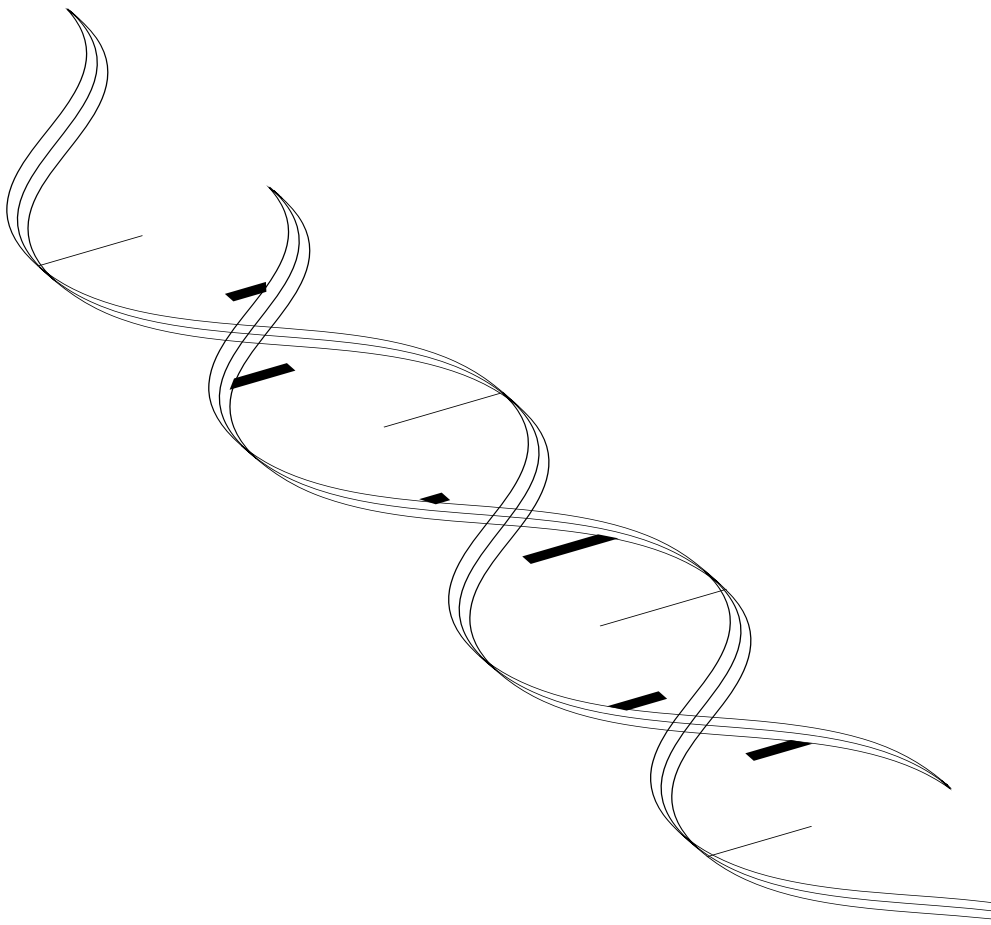
C. ddPCR of same FFPE sample as S6B but with input of 30ng.

Supplementary figure 6
LOB detection of FFPE and plasma samples.
A Plasma sample of a healthy donor, input is 9.72 ng. **B** ddPCR of a FFPE tonsil sample with 2 ng input, a broader range of wild type is detected compared to the plasma sample. **C** Same FFPE tonsil sample as S6B with input of 30 ng. A broader range on both sides of the wild type droplets are detected but none were positive for a single probe considered as drop-off (also see legend in supplementary figure 3).

Supplementary table 1

Patient	Fractional abundance at baseline	Fractional abundance at 2–3 weeks treatment	Fractional abundance at 4–6 weeks treatment
3	12,0%	9,0%	0,0%
4	0,4%	0,0%	0,0%
6	0,0%	0,0%	–
11	0,1%	0,0%	0,0%
14	14,0%	62,0%	4,0%
15	0,9%	5,9%	0,0%
16	1,4%	6,8%	0,0%
40	7,0%	7,8%	2,8%
42	0,9%	3,4%	-
43	0,4%	0,0%	0,0%
44	0,9%	0,7%	–
45	3,1%	–	1,1%

Supplementary table 1
Fractional abundance of mutant alleles at baseline and after 2–3 weeks and 4–6 weeks after start of treatment (also see figure 2)



Tyrosine kinase inhibitor sensitive PDGFRA mutations in GIST. Two cases and review of literature

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Oncotarget, 2017

Abstract

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal malignancies of the gastrointestinal tract. Most GISTs harbor a c-KIT (80%) or a PDGFRα (10%) mutation that leads to constitutive activation of the tyrosine kinase receptor. Response to treatment with tyrosine kinase inhibitors (TKIs) is dependent on mutational status of the tumor. The most common mutation in PDGFRα, D842V, is known to be imatinib resistant. Almost all other PDGFRα mutations are imatinib sensitive. We describe two patients with a PDGFRα exon 18 mutated GIST responding to treatment with TKIs. One of these patients has a p.M844_S847 deletion, not previously described in relation with TKI treatment response. Mutations in circulating tumor DNA were detectable with digital droplet PCR in serial plasma samples taken during treatment and correlated with treatment response of both patients. Computer 3D-modeling of the PDGFRα kinase domain of these two variants revealed no direct interference in imatinib or sunitinib binding and no effect in its activity in contrast to the reported structure of the imatinib resistant D842V mutation.

An overview is given of the literature regarding the evidence of patients with different PDGFRα mutated GISTs on response to TKIs. The findings emphasize the use of mutational analysis in GIST to provide patients personalized treatment. Detection of mutations in plasma is feasible and can provide real-time information concerning treatment response. We suggest to register GIST patients with these uncommon mutations in a prospective international database to understand the tumor biology and obtain more evidence of such mutations to predict treatment response.

1. Introduction

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal malignancies of the gastrointestinal tract with an incidence of 10 cases per million people.[1] About 50% of GIST arises in the stomach, 30% in the small intestine and 20% in other parts of the gastrointestinal tract.[2] Tumors originate from the interstitial cells of Cajal (or its precursor cells), the smooth muscle pacemaker cells. Constitutively activating mutations in the genes coding for the tyrosine kinase receptors KIT or platelet derived growth factor alpha (PDGFRα) play a crucial role in the biology of these tumors.[3] Approximately 80% of GIST harbor mutations in KIT, 10% in PDGFRα. The remaining part are wild type, has a BRAF mutation or inactivation of the SDH complex. KIT exon 11 mutant tumors can occur anywhere in the gastrointestinal tract, whereas PDGFRα-mutant tumors arise primarily in the stomach, mesentery and omentum. KIT exon 9 mutant tumors are mostly found in the small intestine.[4]
Surgery is the only curative treatment and treatment of choice when feasible. Patients with irresectable tumors due to local advancement or metastatic disease can be treated with imatinib mesylate, a KIT selective tyrosine kinase inhibitor (TKI) in neo-adjuvant and palliative setting. Response on systemic treatment is strongly dependent on mutational status of the tumor. Patients with an imatinib-sensitive mutation have a response or stable disease for a median time of 27 months.[5] When imatinib treatment fails, second line treatment with sunitinib and third line treatment with regorafenib is available.[6] Resistance in patients who have an imatinib sensitive primary mutation occurs often as a result of secondary mutations in the tumor that develop during treatment.[7] After potentially curative surgery, patients with PDGFRα mutations and those with wild-type GIST have a lower risk of recurrence than patients with KIT mutations.[8] Once recurrences occur, the most common PDGFRα mutation in exon 18 (D842V) is known to be resistant to imatinib treatment. But not all GISTs with a mutation in exon 18 of the PDGFRα gene are resistant to treatment with a TKI. Since the introduction of mutation analysis in biopsies of GIST tumors, it is known that specific PDGFRα mutations appear to be imatinib sensitive.[9] Response to therapy is generally evaluated by radiological imaging. Recent advances in molecular biology enable the detection of tumor mutations in circulating tumor DNA (ctDNA) in plasma. This plasma mutational load can reflect the treatment response and current disease state.[10]
Two patients with various PDGFRα deletions who responded on TKI treatment are described and serial plasma samples of both patients were analyzed with digital droplet PCR (ddPCR). Finally, an overview of literature concerning PDGFRα mutations in GIST is presented.

2. Materials and methods

2.1 Mutational analysis

For mutational analysis, DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue using the Cobas DNA extraction kit (Roche, Basel, Switzerland). Next generation sequencing (NGS) analysis using the University Medical Center Groningen onco-panel on the Ion-Torrent platform (Thermo Fischer Scientific, Waltham, MA, USA) was performed. Torrent Suite Software was used to pre-process the raw data, and base calling, alignment, coverage analysis and variant calling was performed using SeqNext software (JSI medical systems GmbH) as reported previously.[11] According to international guidelines for clinical NGS panels [12], the minimal depth of coverage was set at 250 reads per tested amplicon. This to confidently identify also low frequency relevant variants in clinical tissues resulting from heterogeneity due to admixture of non-neoplastic cells, intratumoral variations (different clones) and viability of tumor cells. Relevant exons that are tested with this panel included KIT exons 8, 9, 11, 13, 14, 17 and PDGFRα exons 12, 14, 18 and BRAF codon 594, 599, 600 (www.moloncopath.nl).

2.2 Analysis of circulating tumor DNA

Blood samples were collected in EDTA tubes (vacutainer #367525, Becton Dickinson, Franklin Lakes, NJ, USA) and processed within 4 hours after vena puncture. Samples were processed and isolation of DNA were performed as described elsewhere.[17] Digital droplet PCR (ddPCR) primers and probes were in-house designed and ordered at IDT (Coralville, IA, USA). The primer sequences for patient 1 (p.M844_S847del; c.2531_2542del) were Fwd. 5′-CTCCTGGCACAAGGAAA-3′ (c.2473-c.2489) and Rev. 5′-GGACGTACACTGCCTTT-3′ (c.2554-c.2570) resulting in a PCR product of 98 base pairs. The sequence of probe I (FAM) was 5′-GCCAGAGACATCAACTATGTGTCG-3′ and probe II (HEX) 5′-CATGCATGATTCGAACTATGTGTCG-3′. For patient 2 (p.I843_D846del; c.2527_2538del) the primer sequences were Fwd. 5′-ATTGTGAAGATCTGTGACTTTG-3′ (position c.2491-c.2512) and Rev. 5′-AGTGAGGGAAGTGAGGA-3′ (position c.2568-c.2584) resulting in a PCR product of 94 base pairs. The sequence of probe I (FAM) was 5′-GCCAGAGACTCGAACTATGTGTCG-3′ and probe II (HEX) 5′-TGCATGATTCGAACTATGTGTCGAA-3′. Temperature gradient PCR of the primers and probes were performed to detect the optimal annealing temperature and resulted in an optimal PCR temperature of 55 ˚C for both assays. The specific assays were validated on available tumor tissue. DdPCR was performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) and samples were transported to the QX200 Droplet Reader (Bio-Rad) for fluorescent measurement of FAM and HEX probes, data was analyzed with Quantasoft software version 1.6.6.

3. Results

3.1 Clinical summary patient 1

A 76-year old man was referred with a large abdominal tumor suspected for GIST. His WHO-performance score at first presentation was 3, being bed bound for the majority of the day. CT and FDG-PET scan showed a 29 cm large, irresectable tumor without evidence of metastases (figure 1A). Mutation analysis on the performed biopsy specimen confirmed the diagnosis GIST with a mutation in exon 18 of PDGFRα (NM_006206.5: c.2531_2542del; p.(M844-S847del)). Treatment with imatinib 400 mg once daily was initiated in a neo-adjuvant setting. After one week he reported a clinical relevant benefit by disappearance of nausea and increasing energy levels. On the CT-scans performed every three months, stable disease was seen during one year of treatment. Based on a growing nodule (figure 1B), progression was suspected and surgical resection of the tumor was considered. However, progression with peritoneal metastasis was seen on the following CT scan three months thereafter. Surgery with curative intent was no longer feasible and 15 months after start of imatinib, treatment was switched to second line treatment with sunitinib 37.5mg daily. The patient responded during one year on sunitinib (figure 1C), until he was admitted to the hospital with malaise and ascites based on disease progression (figure 1D). He recovered after drainage of ascites.

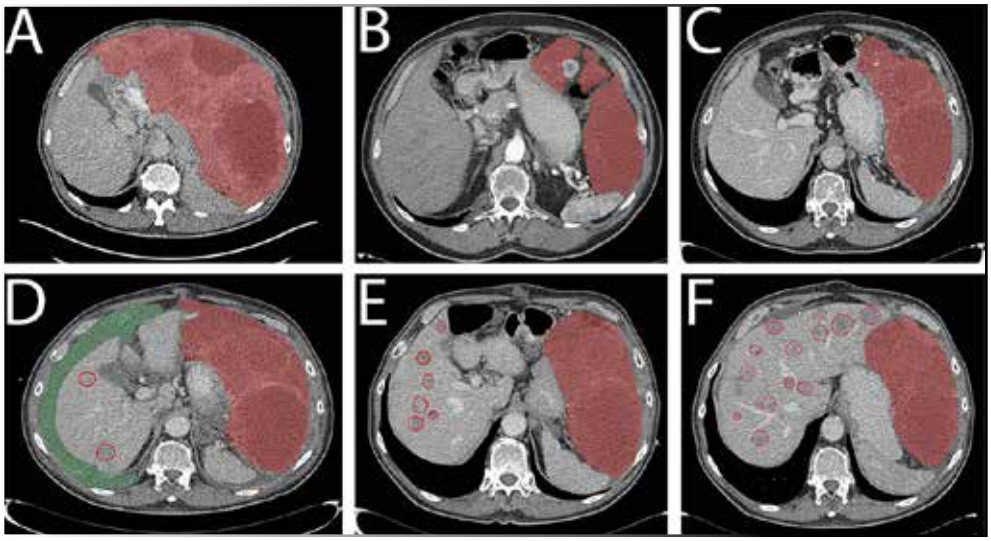

Figure 1 CT images of patient 1. Primary GIST (red area), liver metastases (red circles) and ascites (green) are indicated. A. Pretreatment scan. B. After 12 months imatinib treatment, growing nodule. Start sunitinib. C. 6 months on sunitinib, stable disease. D. One year sunitinib. Progression. Ascites, liver metastasis. Start regorafenib. E. 3 months regorafenib, stable disease. Demarquation of the liver metastasis is seen as response on therapy. F. 6 months regorafenib, progression, more liver metastases are seen.

A biopsy of a progressive nodule was performed and treatment with regorafenib (160mg daily for 3 out of 4 weeks) was started. With this regimen stable disease during 5 months was obtained (figure 1E). He died one month after stopping treatment with regorafenib due to progressive disease (figure 1F), no clinical benefit was reached with a re-challenge of imatinib. Plasma samples were available during treatment with regorafenib and 4th line imatinib. An increase in mutational copies/ml is seen between stable disease (figure 1E) and progression (figure 1F) of 180 to 850 mutant copies/ml (figure 3A). At the last visit to the outpatient clinic (two weeks before death), mutant copies were 4767 copies/ml (month 33).
In total, this patient responded or had stable disease according to RECIST criteria for 32 months on several tyrosine kinase inhibitors. Mutation analysis performed on the progressive tumor nodule before start of regorafenib showed the same p.(M844_S847del) (c.2531_2542del) mutation in exon 18 of PDGFRα as detected in the primary tumor, whereas no additional mutations were found in exon 12 and 14 of PDGFRA nor in exon 9, 11, 13, 14, 17 of KIT (the average coverage is ~2000 reads in tumor DNA with neoplastic content of 80%). Detection of the primary mutation in plasma reflected the clinical course of the disease in this patient.

3.2 Cinical summary patient 2

A 76-year old woman was referred for analysis of abdominal pain. A CT scan showed a large abdominal tumor (diameter 25 cm) with multiple liver metastases (figure 2A and 2B). Biopsy of a liver metastasis showed a CD117 positive tumor, characteristic for GIST. Mutational analysis showed no mutation in KIT, PDGFRα analysis was not performed at the time.
Treatment with imatinib 400 mg daily was initiated and after ten days she had clinical benefit. She tolerated the treatment well and a partial response was seen on the CT-scan performed every 3 months (figure 2C and 2D). After 30 months of treatment progression of the primary tumor as well as the liver metastases was seen (figure 2E and 2F). Additional mutation analysis was performed on the biopsy taken at diagnosis and revealed a PDGFRα exon 18 (NM_006206.5: c.2527_2538del; p.(I843_D846del)) mutation (the average coverage is ~2500 reads in tumor DNA with neoplastic content of 95%). Treatment was continued with increased dosage of imatinib 400 mg twice daily and the patient tolerated this dosage, yet no clinical and radiological response was seen (progressive lesions on CT-scan after 3 months treatment, figure 2G and 2H). Two months after the treatment was switched to sunitinib 37.5 mg daily the patient continued to deteriorate and she died 36 months after the initial diagnosis. A tissue biopsy of a progressive lesion was not available to evaluate the secondary mutational status. Blood samples for ctDNA analysis (figure 3B) were first drawn after 16,5 months of treatment with imatinib 400 mg (6 mutant copies/ml). The mutant copies level remained stable until progressive disease was detected at the CT scan at 30 months. After initiation of imatinib 400mg twice daily an increase in mutant copies (5 to 275 /ml) was detected.

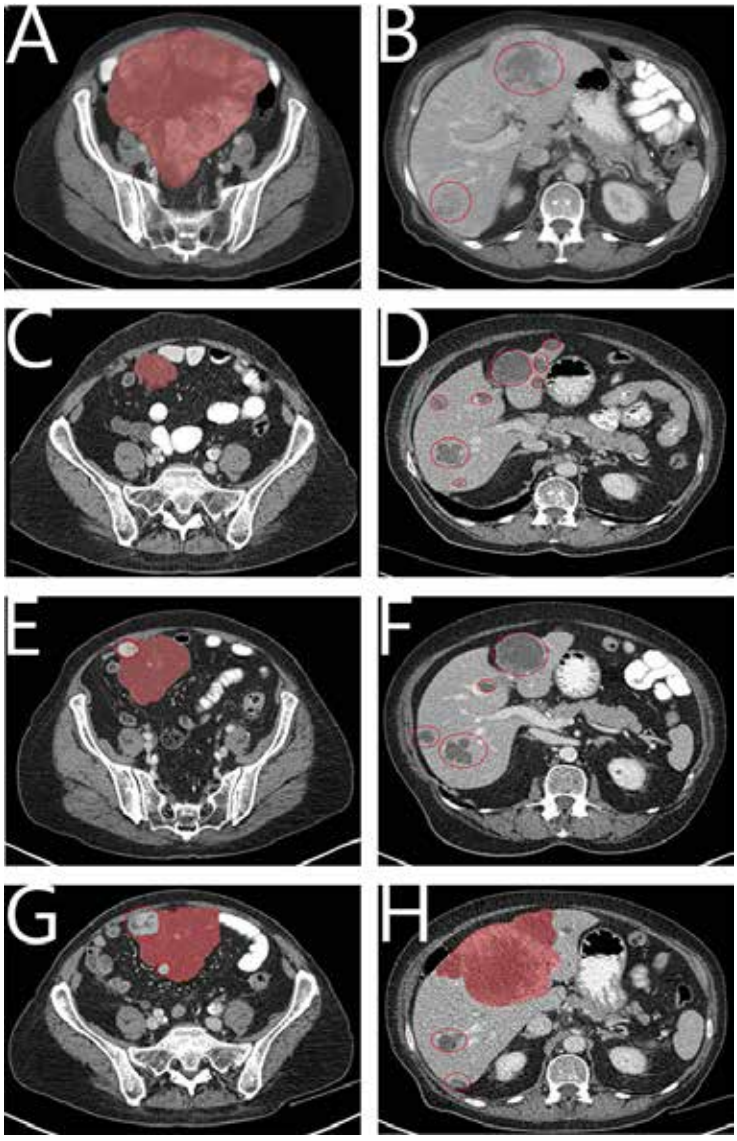

Figure 2 CT images of patient 2, left column images of pelvis, right column images of liver at the same time. Primary tumor (red area), metastases (red circles).
A. Pretreatment, large tumor in the lower abdomen.
B. Pretreatment, multiple liver metastases.
C. 24 months imatinib, treatment response.
D. 24 months imatinib, demarquation of metastases.
E. 30 months imatinib, progressive tumor nodule (red circle).
F. 30 months imatinib, progression liver metastases.
G. 33 months, multiple progressive nodules (red circle).
H. 33 months, progression of liver metastases.

The mutational level continued to rise to 852 mutant copies/ml corresponding with progressive disease on the CT scan performed at 33 months.
After initiation of sunitinib treatment, a decrease in mutant copies (208 mutant copies/ml) was measured. Unfortunately, no further samples were available for analysis since the patient deceased after 2 months.

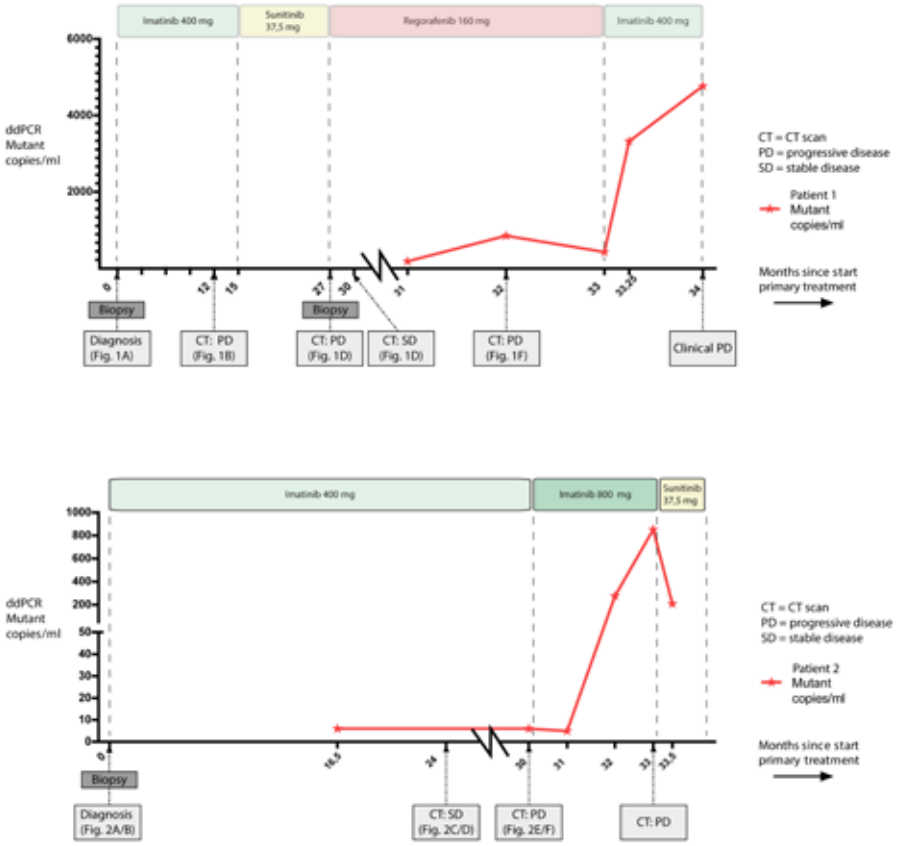

Figure 3 Serial plasma samples were analyzed during treatment. The PDGFRα mutant copies/ml level as tested with ddPCR are shown.
A. Patient 1 with the p.M844-S847del; c.2531_2542del variant. First plasma samples was collected 31 months after start of treatment, disease progression as determined with CT after 32 months corresponded with a rise in mutant copies/ml. Patient died 33 months after start of first treatment.
B. Patient 2 with the p.I843_D846del; c.2527_2538del variant. First plasma sample was collected 16,5 months after start of treatment, disease progression at 30 months was not detected in plasma ctDNA, however after switch of therapy an increase in mutant copies/ml is detected corresponding with disease progression on CT at 32 months. After initiation of sunitinib, a decrease in mutant copies ml is seen. Patient died 36 months after initial diagnosis.

3.3 Computer 3D-modeling of the PDGFRα kinase domain

Computer 3D-modeling of the activation loop in the kinase domain of PDGFRα was recently reported to stabilize the kinase in the inactive state and to facilitate the binding of imatinib. The crystal structure with the D842V mutation suggested activation of the kinase and kinetic data confirmed an increased affinity for ATP both in agreement with the observed drug resistance in patients with the D842V mutation. [13] To evaluate the possible consequences of the I843_D846del and M844_S847del variants on the activation loop of PDGFRα, three 3D-models (PDGFRα-D842V,

PDGFRα-M844_S847del and PDGFRα- I843_D846del) were built (figure 4). The predicted orientation of D842, described to be essential for the auto-inhibited state of the tyrosine kinase domain [9], was conserved. The proposed hydrogen bond between D842 and H845(PDB; 4BKJ [14]) is almost certainly not formed, as the geometry of the interaction does not fall close to experimentally determined angular distributions.[15] Our 3D-modeling indicates that the PDGFRα M844_S847del and I843_D846del variants would play no role in activation.

In order to evaluate whether these 2 variants affect residues of PDGFRα that specifically interact with imatinib, the reported structure of the tyrosine kinase domain of DDR1 bound to imatinib (PDB; 4BKJ [14]) was used as template for homology modeling (35% sequence identity).[16] Our model predicts that imatinib interacts with the same amino acids of DDR1 (E672, T701, V763, H764, D784) that are conserved in PDGFRα (E644, T674, V815, H816, D836). Considering that the PDGFRα M844_S847del and I843_D846del variants do not affect these 5 residues, it is highly likely that these variants will not directly affect binding of imatinib or sunitinib.

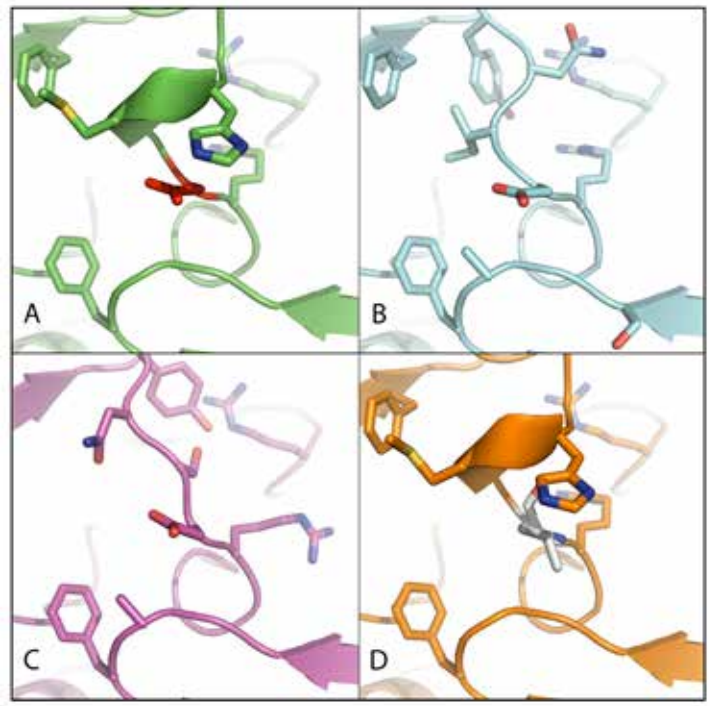

Figure 4 Residue 842 of human PDGFRα.
A Residue D842 (in red) in the structure of wild type PDGFRα (pdb 5K5X).
B Residue D842 in the structure of wild type PDGFRα modeled with variant M844_S847del.
C Residue D842 in the structure of wild type PDGFRα modeled with the I843_D846del variant.
D Residue V842 (in grey) in the structure of wild type PDGFRα modeled with mutation D842V.

4. Review

4.1 PDGFRα mutations and response on imatinib

PubMed was searched for articles concerning GIST patients with PDGFRα mutations and response to treatment with imatinib. Data of 14 papers and a total of 102 patients with PDGFRα exon 18 mutated GIST were retrieved by our search strategy (figure 5). Five of the fourteen papers describe data of progression free survival (PFS) or overall survival (OS) of patients with a PDGFRα mutated advanced GIST treated with imatinib (table 1). A 10-fold increase in PFS is seen in patients with non-D842V mutated GIST compared with D842V mutated who were treated with imatinib. Recently, a series of 823 GIST patients including 13 patients with a PDGFRα exon 18 mutated GIST who were treated with first line imatinib is described.[18] The OS of patients with a D842V mutation was 25.2 months compared to 59.8 months for patients with a non-D842V PDGFRα mutated GIST (p=0.02). At least two other studies showed a better median OS in patients with a non-D842V PDGFRα mutation compared to patients with a D842V mutation.[18, 19]

In COSMIC (catalogue of somatic mutations in cancer; www.cancer.sanger.ac.uk/cosmic) the p.M844_S847del mutation is described in eight patients in five studies. All patients were treated surgically and had no evidence of disease or recurrence afterwards, so no information was reported regarding the response on treatment (table 2).

A few patients with a GIST with a p.I843_D846del mutation have been described, although information regarding the response to TKI treatment is scarce.[20, 37-47]

4.2 Response on sunitinib as second line treatment

Two papers report on response to second-line treatment with sunitinib. In nine patients, no objective response was seen.[18] Patients with the D842V mutation tended to show poorer PFS than those with non-D842V PDGFRα mutations (median PFS 1.9 months for D842V mutant vs. 7.3 months for non-D842V PDGFRα mutations; p = 0.26). Another cohort consisted of 11 patients with a PDGFRα mutated GIST.[20] Of those patients, three had disease stabilization for more than 6 months. No significant difference could be shown between the different PDGFRα mutations (PFS 2.1 months for D842V mutant and 7.8 months for other mutations, p = 0.2489).

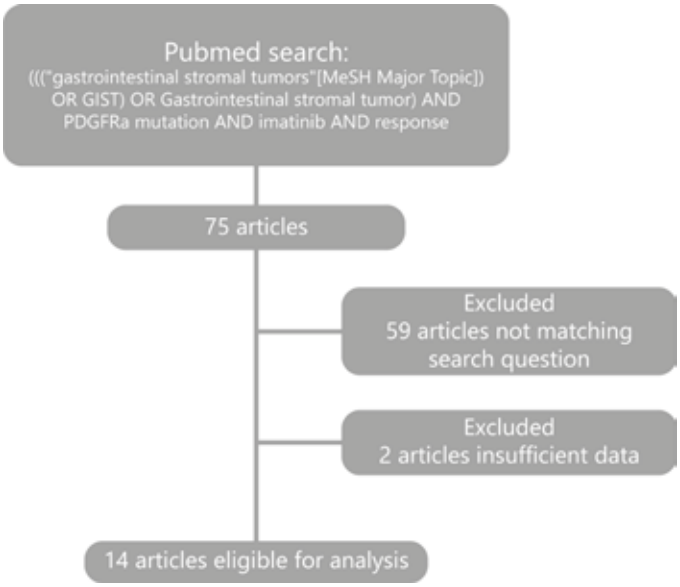

Figure 5 Flow-chart of search strategy. In total 61 articles were excluded because of other study questions or insufficient data, resulting in 14 papers eligible for analysis.

5. Discussion

Our two case patients with a non-D842V PDGFRα mutation responded or had prolonged periods of non-progressive disease to various tyrosine kinase inhibitors. Although patients with advanced disease and a PDGFRα mutation can respond to treatment with imatinib, the overall survival of these patients is worse than that of patients with a KIT mutation bearing GIST. This is reported in studies with advanced GIST patients where a median survival of 57 months for patients expressing KIT compared to a median overall survival of 23.7 months for patients with a PDGFRα mutation is reached.[20, 48] Imatinib resistance was reported most frequently in GIST patients with PDGFRα mutations and wild type GISTs. However, the PDGFRα mutations were not specified.[49] In contrast to the mutation distribution described in literature of 10% PDGFRα mutations in GIST, most articles report a much lower fraction of patients with PDGFRα mutations, suggesting underreporting.

To our knowledge, this is the first report regarding a p.M844_S847del variant in relation to TKI treatment. We show the clinical importance of mutation detection as patients with specific PDGFRα mutations respond well on imatinib treatment. The published reports that were found in the PubMed search provide limited PFS and OS data. However, patients with a non-D842V mutation have a favorable prognosis when treated with imatinib compared to patients with a D842V PDGFRα mutated GIST. A recent report showed a better overall survival in patients with non-D842V when treated with imatinib compared to patients with D842V mutated tumors.[18] Due to the latter it is of great importance to differentiate between those.

The frequency of recurrence after surgery is lower in patients with gastric versus non-gastric PDGFRα mutated GIST, similar to patients with other mutations.[50] In metastatic D842V mutated GIST the role of TKI is very limited and the prognosis is clearly far inferior to other mutated GISTs.[51, 52] Several new therapies are being investigated for patients with a PDGFRα (D842V) mutation [27, 53], however none of them are already available for daily clinical practice.

TKI sensitive mutations are mostly located near the imatinib resistant D842V domain. It has been implied that primary resistance to imatinib correlates specifically with substitution mutations that affect residue D842 of the kinase activation loop.[9] Modifications of this domain are interfering with a swinging movement of the activation loop. This movement is linked to a conformational shift of the ATP binding pocket from an "open" or active set-up to a "closed" or inactive set-up. Since imatinib is an ATP competitor and binds exclusively to the closed form of the kinase, substitutions of PDGFRα D842 reduce the accessibility of the ATP pocket and thereby give relative resistance to the drug.[13] An increased affinity for ATP of the mutated tyrosine kinase domain has also been reported to contribute to the acquired resistance to imatinib.[54] In contrast to D842V, our 3D-modeling of the kinase activation loop indicates that the M844_S847del and I843_D846del variants would play no role in activation. In addition, homology modeling predicted that imatinib interacts with 5 residues in PDGFRα (E644, T674, V815, H816, D836) conserved with those in DDR1. Because the PDGFRα M844_S847del and I843_D846del variants do not affect these 5 residues, it is highly likely that these variants will not directly affect binding of imatinib or sunitinib. In summary, our 3D-modeling analysis indicates that PDGFRα proteins harboring the M844_S847del and I843_D846del variants would not directly interfere in imatinib or sunitinib binding and would not affect activity. Consequently, both variants would still allow binding and inhibition of imatinib and sunitinib. This is in good agreement with the observed response to imatinib in these two patients. However, to assess sensitivity to tyrosine kinase inhibitors of these particular mutations, cell line-based drug sensitivity analysis would be of added value.

Secondary resistance usually occurs between 6-24 months after start of imatinib treatment. Secondary mutations as cause of TKI insensitivity have been found in patients with primary KIT mutations and rarely in patients with primary PDGFRα mutations.[5] Alternative pathways for secondary resistance are activation of other growth pathways and loss of the remaining wild-type PDGFRα and overexpression of PDGFRα or other tyrosine kinase receptors.[55] In patient 1, a biopsy at progression was taken. However, in addition to the M844_S847del variant in exon 18 of PDGFRα, no secondary treatment resistant mutations were detected with NGS.

The detection of mutations in circulating tumor DNA from cell free plasma (ctDNA) of patients with GIST has been reported before.[56-60] In this study we report on the correlation between clinical course/treatment response and the detection of the tumor-specific PDGFRA mutations in ctDNA of serially taken blood samples of these

2 patients with GIST, showing the use of mutation detection in ctDNA in plasma to monitor treatment response. In patient 1, the clinical course correlated well with the ctDNA level. However, in patient 2 the moment of progression on first line treatment was not detected in the ctDNA. According to the CT scan at 30 months (figure 2E/F) there is one progressive nodule detected implying treatment resistance. Some lesions became larger, but have a cystic aspect what in GIST could match with treatment response. Since there is little active tumor tissue, we suggest the limited DNA shed of the progressive nodule in this phase is below the detection level of ddPCR. The following CT scan (figure 2G/H) shows massive progression which is preceded by a detectable rise in mutant ctDNA copies. Further research has to reveal the clinical value of detectable mutant ctDNA copies.
In conclusion, the p.M844_S847del and p.I843_D846del mutations are rare but have clinical importance since these specific mutations are associated by a response to treatment with TKIs. This report emphasizes the importance of mutational analysis of tumors and is exemplary for the implementation of personalized medicine. Mutational analysis should be performed of each primary and resistant tumor to increase the knowledge of primary and secondary resistant mutations. Mutation detection in ctDNA to assess treatment response seems feasible. We suggest to register patients with very uncommon genetic aberrations in a prospective international database to understand the tumor biology and obtain more evidence to predict treatment response and eventually contributing to the development of new targeted therapies.

Grant support
PAB works on a research grant provided by the Dutch Cancer Society (KWF, Alpe D'HuZes research grant RUG 2013-6355).

Conflicts of interest
The authors declared no conflicts of interest.

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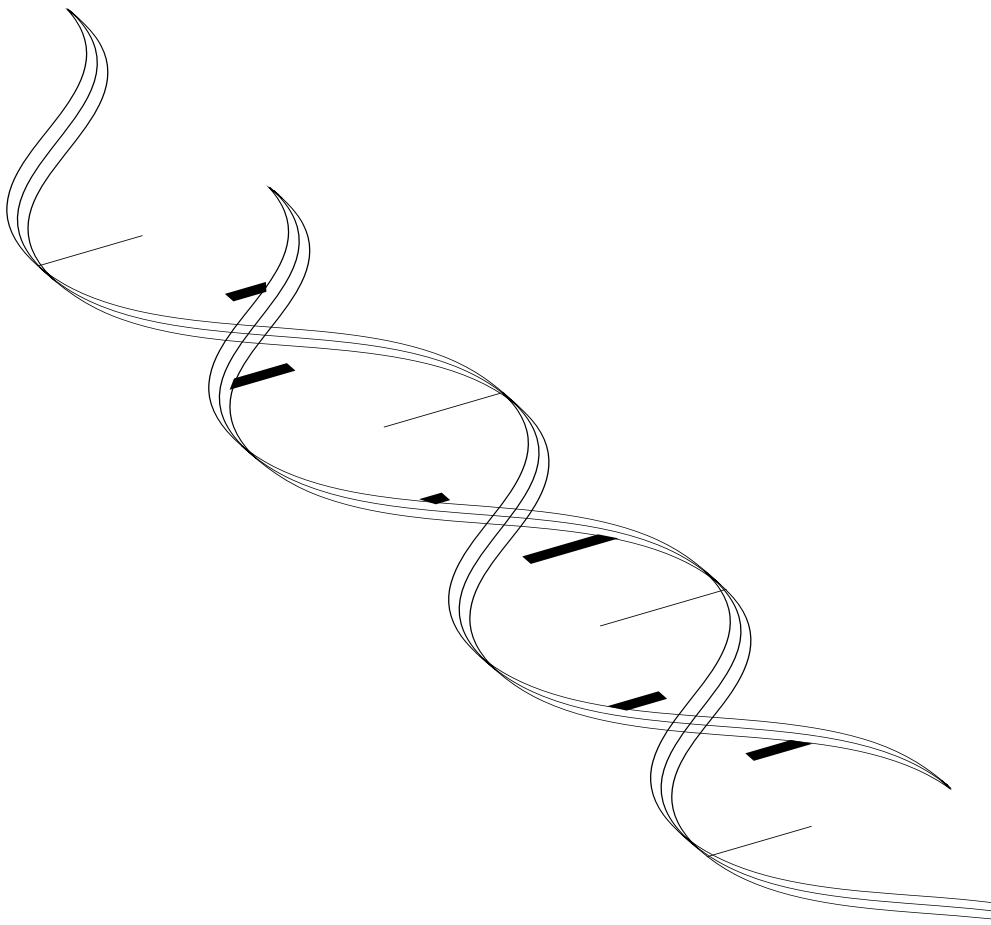

Surgical and medical management of small bowel gastrointestinal stromal tumors: a report of the Dutch GIST registry

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European Journal of Surgical Oncology, 2018

Abstract

Background: A cohort of 201 patients with small bowel gastrointestinal stromal tumors (GIST) treated between January 1st, 2009 and December 31st, 2016 in five GIST expertise centers in the Netherlands was analyzed. Goal of this study was to describe the clinical, surgical and pathological characteristics of this rare subpopulation of GIST patients, registered in the Dutch GIST registry.
Methods: Clinical outcomes and risk factors of patients with small bowel GIST who underwent surgery or treated with systemic therapy were analyzed. A classification was made based on disease status at diagnosis (localized vs. metastasized).
Results: 201 patients with small bowel GIST were registered of which 138 patients (69%) were diagnosed with localized disease and 63 patients (31%) with metastatic disease. Approximately 19% of the patients had emergency surgery, and in 22% GIST was an accidental finding. In patients with high risk localized disease, recurrence occurred less often in patients who received adjuvant treatment (4/32) compared to patients who did not (20/31, $p < 0.01$). Disease progression during palliative imatinib treatment occurred in 23 patients (28%) after a median of 20.7 (range 1.8-47.1) months. Ongoing response was established in 52/82 patients on first line palliative treatment with imatinib after a median treatment time of 30.6 (range 2.5-155.3) months.
Conclusion: Patients with small-bowel GIST more frequently present with metastatic disease when compared to patients with gastric GIST in literature. We advocate for prospective registration of these patients and investigate the use of surgery in patients with limited metastatic disease.

1. Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal malignancies of the gastrointestinal tract.[1] The annual incidence of GIST is between 11 and 20 cases per million people.[2] GISTs originate from the interstitial cells of Cajal, known as the smooth muscle pacemaker cells of the gastrointestinal tract.[3] Most frequently, GISTs originate in the stomach (60%) or small bowel (30%).[4] GISTs develop due to driver mutations in genes coding for the tyrosine kinase receptor c-KIT (80%) or PDGFRa (10%), both expressed on the cell surface, 10% consist of wild-type tumors or rare occurring mutations in other genes.[2,5]
Surgery is the only curative treatment in patients with localized disease or oligo-metastases. GISTs generally metastasize primary to the liver or within the abdominal cavity. Patients with locally advanced GIST who are not suitable for primary surgery can be treated in a neo-adjuvant setting with imatinib, a tyrosine kinase inhibitor, targeting KIT and PDGFRa.[6] If volume reduction is achieved, patients may become eligible for surgery. Patients with metastatic disease respond on average for 24 months on first line therapy with imatinib, but this is largely dependent on mutational status of the tumor.[7] Second line therapy consists of sunitinib, and third line of regorafenib.[8,9]
Not all GISTs have the same clinical behavior.[10,11] GISTs with PDGFRa mutations generally originate in the stomach. Despite the fact that these GISTs are relatively often imatinib insensitive, they have a good prognosis since they are often detected as localized disease. Gastric GISTs have other biological and clinical properties than GISTs originating from the small bowel or duodenum as seen in the different rates in risk of recurrent disease.[12]
Many papers have been published describing the treatment and characteristics of patients with GIST, however only few are available regarding the subpopulation of patients with small bowel GIST.
The clinical, surgical and pathological characteristics of a Dutch cohort of patients with small bowel GIST in the era of targeted therapy are described. The knowledge obtained from a large cohort of small bowel GISTs treated in expert centers may facilitate future clinical decision making.

2. Materials and methods

Patients with GIST treated in or referred to one of the five GIST centers of expertise in the Netherlands (Antoni van Leeuwenhoek Amsterdam, Leiden University Medical Center, Erasmus Medical Center Rotterdam, Radboud University Medical Center Nijmegen, University Medical Center Groningen) are retrospectively and prospectively registered in a national database since 2009 (Dutch GIST registry).[13]
Patient and clinical characteristics, pathology reports, as well as data on surgical

procedures, systemic therapy, recurrence and survival are registered. Patients with a small bowel GIST treated between January 1st, 2009 and December 31st, 2016 were identified and included in this retrospective study. Patients were categorized based on having localized or metastatic disease on CT or PET-CT scanning at time of diagnosis. After surgery, patients received adjuvant systemic treatment based on the primary tumor characteristics using Miettinen or Joensuu risk classification and follow up was performed according to international guidelines.[12,14,15] Response evaluation during systemic therapy was performed every 3, 6 or 12 months using CT scans that were reviewed by a trained radiologist in one of the expertise centers following RECIST and/or CHOI criteria.Staining of tumor samples (CD-117 and/or DOG-1) was done according to local pathology protocols. Mutational analysis of the primary tumors was performed using Sanger sequencing and next generation sequencing. Genes tested included the most common mutated genes in GIST, KIT and PDGFRa. Statistical analysis was performed using SPSS statistical software, version 23 and R software for statistical computing and graphics.

3. Results

3.1 Patient characteristics

In total, 201 patients were identified in the registry (containing 878 patients) with a newly diagnosed GIST originating from the small bowel (table 1). Patients who did not present in an emergency setting generally not experienced pain or discomfort from the tumor. Anemia was frequently diagnosed, in 72 patients (44%) at diagnosis. 31% of the patients presented with metastasized disease, all patients had intra-abdominal metastases but four patients (2%) were also diagnosed with lung metastases.

		n	
Patients		**201**	
Male / female		**98 / 103**	
Age at diagnosis		**60.8**	Range 18,5 - 87,5
WHO performance score at baseline			
	0-1	**124**	
	2-3	**9**	
	Unknown	**68**	
Hb level at diagnosis		**7.8**	Range 4,4 - 10,5
Tumor status at diagnosis			
	Localized	**138**	69%
	Metastasized	**63**	31%
Primary median tumor size		**80**	Range 8 -250

Table 1 Baseline characteristics at time of entry in the registry. Besides primary tumor size, no significant differences were present between patients with localized and metastasized disease at diagnosis (data not shown).

3.2 Pathology

Pathology reports were available of 195 patients (table 2). Staining for CD-117 was reported of 177 samples (91%) of which 174 were positive (98%). Three primary tumors that were CD-117 negative, were DOG-1 positive. DOG-1 staining was negative in 1 of the 118 tested samples. None of the tested samples were negative for both CD-117 and DOG-1 staining. Mutational analysis was performed for 156 (80%) primary tumors. Data from diagnostic biopsies was available of 65 patients and adequate risk classification could be made in 57 of these patients (based on tumor location, tumor size and mitotic index).

		n	%
Available reports		**195**	97%
Histology			
	Spindle cell	**140**	72%
	Epitheloid	**13**	7%
	Mixed type	**24**	12%
	Unknown	**18**	9%
Risk category (Miettinen)			
	Low/medium	**93**	48%
	High risk	**97**	50%
	Unknown	**5**	3%
Immunohistochemistry			
	CD-117 positive	**174**	89%
	CD-117 negative	**3**	2%
	Unknown	**18**	9%
	DOG-1 positive	**117**	60%
	DOG-1 negative	**1**	1%
	Unknown	**77**	39%
Mutational analysis			
	KIT exon 9	**26**	13%
	KIT exon 11	**106**	54%
	KIT exon 13	**4**	2%
	KIT exon 17	**2**	1%
	PDGFRα exon 18	**0**	0%
	Wild type	**18**	9%
	Unknown	**39**	20%

Table 2 Pathology characteristics of 201 patients. Of 175 patients a tissue sample of the primary tumor (biopsy and/or resection) was available.

3.3 Surgery

Hundred and forty five patients underwent surgery for resection of the primary tumor. Fifteen patients (10%) underwent more than one operation for primary tumor resection and resection of metastases (table 3). Twenty-seven patients (19%) with metastatic disease underwent surgery. Indications for patients with metastatic

disease who underwent surgery (n = 27, 35 surgeries) were resection of the primary tumor or to reach a minimal residual disease volume (n = 20), emergency setting (n = 8) or incidental diagnosis during surgery for other indications (n = 7). Surgery in emergency setting for the primary tumor (i.e. ileus, perforation, gastro-intestinal blood loss) occurred in 30 patients which resulted in a R1 or R2 resection in 7 patients (23%). Thirty-two patients (22%) had per-operative tumor spill, of which the vast majority (n = 22, 69%) were operated for other indications or in an emergency setting. Recurrent disease occurred in 22% of the patients (7/32) with tumor spill per-operatively with a median time to recurrence of 26.3 (range 9.7 - 52.4) months.

	No of procedures	**Referral center**	**Expertise center**
Surgeries performed	**161**	**91**	**70**
Resection primary tumor	**145**	**89**	**56**
Resection recurrence or metastases	**16**	**2**	**14**
Median age at surgery (range)	**60 (18-84)**	**60 (18-83)**	**63 (29-84)**
Reason for surgery			
Planned	**90**	**41**	**49**
Planned because of other disease	**36**	**26**	**10**
Emergency	**30**	**20**	**10**
Unknown	**5**	**4**	**1**
Type of surgery			
Laparotomy	**143**	**77**	**66**
Laparoscopy	**11**	**9**	**2**
Unknown	**7**	**5**	**2**
Type of resection			
Local	**141**	**79**	**62**
Multivisceral	**14**	**8**	**6**
Unknown	**6**	**4**	**2**
Surgery result			
R0	**121**	**71**	**50**
R1	**13**	**8**	**5**
R2	**17**	**8**	**9**
Unknown	**10**	**4**	**6**
Tumor spill	**32**	**21**	**11**

Table 3 Surgery characteristics. Local resection involved a typical small bowel segment or wedge resection.

3.4 Systemic treatment

In total, 141 patients received systemic treatment, 23 in neoadjuvant, 49 in adjuvant and 82 patients in palliative setting. First line systemic treatment consisted in 140 patients of imatinib (neoadjuvant/adjuvant and palliative). Two patients switched early to sunitinib due to adverse events related to imatinib (nausea and orbital cellulitis with neutropenia). Twenty-two patients were treated with sunitinib as second line treatment and 12 patients were treated with regorafenib as third line treatment.

3.5 Patients treated with curative intention

Of patients with localized disease (n = 138), 110 patients (80%) directly underwent surgery for resection of the primary tumor. Fourteen patients underwent surgery following neo-adjuvant treatment (n = 23) after a median of 7 (range 1-11.5) months. Nine patients did not undergo surgery. No progressive disease was detected during neo-adjuvant treatment (partial response = 10, stable disease = 13). Three patients had to interrupt neo-adjuvant treatment because of nausea, and restarted at a lower dose.

Median primary tumor diameter decreased from 99 mm (18-250 mm) to a median of 78 mm (range 18-190 mm) during neo-adjuvant treatment.

Disease recurrence was detected in 31 patients after surgery with curative intention after a median time of 20.6 (range 0-210) months. Eight patients underwent second surgery for resection of the recurrent tumor, 26 patients received also palliative systemic treatment (five patients did not receive any treatment).

In patients with high risk tumors (n = 63) adjuvant treatment was administered to 32 patients. Recurrent disease was detected in four (13%) patients treated with adjuvant treatment and in 20 patients without adjuvant imatinib (65%, $p < 0.01$). Six patients received neo-adjuvant treatment as well as adjuvant treatment.

Of 138 patients treated with curative intention, 126 patients are alive after a median follow up of 48.6 (range 24.5-414.5) months. Twelve patients died after a median time of 26.3 (range 1.3-69.9) months. Five of those patients died due to not GIST related causes.

3.6 Patients treated with curative intention

Twenty-one out of 63 patients with metastatic disease at time of diagnosis underwent surgery for resection of the primary tumor and/or metastases. Six patients underwent surgery for metastatic disease at the time of disease recurrence after intentionally curative treatment. Palliative systemic treatment was followed by surgery in five patients (after a median time of 10 (range 7-30) months).

In total, 82 patients received palliative systemic therapy. All but one (patient on study medication) received imatinib 400 mg daily as first line therapy. Fifty-two patients have an ongoing response after a median follow up of 30.6 (range 2.5-155.2) months. Progression occurred in 23 patients after a median of 20.7 (range 1.8-47.1) months and 7 patients had to stop imatinib treatment due to adverse events after a median of 3.5 (range 0.9-36.1) months.

Second line therapy with sunitinib was administered in the majority of the patients with disease progression on imatinib (85%). Thirteen out of 22 patients treated with sunitinib had disease progression after a median of 5.8 (range 2.2-20.3) months, ongoing response was seen in 5 patients with a median follow up of 37.2 (range 33.9-49.6) months. Regorafenib was started in 12 patients. Two patients have an ongoing response at 30 and 31 months, disease recurrence was detected in 8 patients after a median treatment of 4.5 (range 0.5-11.5) months and two patients had to stop within

one month due to adverse events.
Of all patients who were diagnosed with metastatic disease, 52 patients (83%) are alive after a median follow up of 34.2 (range 0-107.1) months and 11 patients (17%) died after a median follow up of 29.2 (range 0.7-61.3) months.

3.7 Overall survival

Median overall survival was not reached for patients in both groups. Patients with metastatic disease who underwent surgery for the primary tumor and resection of metastases (n = 9) combined with systemic therapy did not have a better overall survival than patients who only received systemic therapy in the described cohort (Fig. 1).

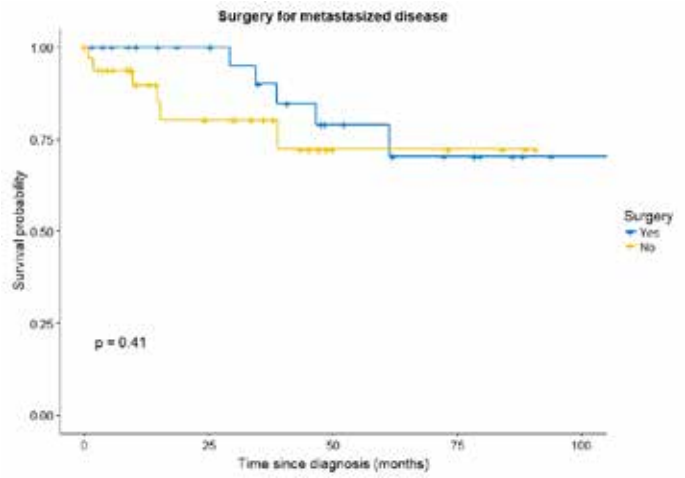

Figure 1 Survival of patients with metastatic disease who underwent surgical resection of the primary tumor and resection of metastases.

4. Discussion

To date, limited data is available regarding the pathological features and clinical management of small bowel GIST. The largest available cohort is from 2006 and describes 906 patients with GIST of the jejunum and ileum. However, this cohort is from the preimatinib era and consists of all type of GISTs.[11]
In our cohort 44% of the patients had anemia at diagnosis. Next to this, the most reported symptom at diagnosis was pain or a palpable mass in the abdomen. Regrettably the exact reason for presentation and the performed imaging before presentation is not registered. At first presentation, 31% of patients had metastatic disease which is relatively high as compared to GIST originating from the stomach. [16] Recent analysis of the SEER database showed 15% of gastric GIST patients having

metastatic disease at diagnosis and 20% metastatic disease in small bowel GIST.[17] Unfortunately, the exact anatomical location (proximal/distal) of the tumor is not described in the registry, so epidemiological and prognostic differences between these variants cannot be made.

According to the most recent ESMO guidelines on GIST, contrast enhanced abdominal and pelvic CT scan is the investigation of choice for patients with small bowel GIST.[18] Since GISTs derive from the outer muscular layers they usually show exophytic growth. This is in contrast to the most common malignancy in the small bowel which is adenocarcinoma that usually appears as an annular lesion in the proximal small intestine.[19] For response evaluation during treatment, two different methods could be used, following the RECIST or CHOI's criteria. The assessment of lesion density is important since response to therapy is commonly reflected by a decrease in lesion attenuation due to myxoid degeneration.[20]

The quality of accuracy of the diagnosis in this registry is exemplified by the fact that the combination of CD-117 and DOG-1 were positive in 100% of cases. Mutational analysis was performed mostly in primary tumor samples. Mutational analysis of metastasis or recurrent disease could be very informative with respect to tumor heterogeneity and the development of TKI resistant disease. Remarkable is the low number of wild-type tumors, where in other large series these tumors specifically occur in the small bowel.[10,11]

Neo-adjuvant treatment can be safely administered to downsize the tumor size before surgery. No patients in our cohort treated with neo-adjuvant imatinib showed tumor progression or had sideeffects warranting emergency surgery. Median time between start of neo-adjuvant treatment and surgery was 7.4 months, which is in accordance with international consensus that surgery should be performed within 6-12 months when maximal tumor response is reached.[14] Despite the proven effect and safety of neo-adjuvant therapy, the absolute number of patients who were treated with neo-adjuvant treatment in our cohort is relatively low.[21] This could be due to the fact that small bowel GIST occurs mainly in the large central abdominal space and primary radical resection can be more easily achieved in comparison with other anatomical locations (i.e. stomach, rectum). Neo-adjuvant treatment should be considered in patients with larger primarily resectable tumors to reduce the risk for R1/R2 resections.

Noteworthy, 61% of the patients operated with curative intent were operated in a non-expert center and afterwards referred to an expert center for further treatment. Laparotomy was the surgical treatment of choice, as GISTs of the small bowel can be large and vulnerable. So far, nine percent (3 out of 32) of the patients with pre- or per-operative tumor spill had recurrent disease, but the relatively short follow up period of the registry could have affected this favorable outcome. Most patients with tumor spill were operated in an emergency setting or for other indications therefore optimal pre-treatment diagnostics has not been performed. Chiguchi et al. report a recurrence rate of 49%-74% within two years depending on the

timing of tumor spill (pre-vs preoperatively).[22] The relatively high number of emergency surgery in the described cohort is probably due to advanced disease since early stage GISTs are known for their limited clinical symptoms. Adjuvant treatment is indicated when the risk of recurrence is high according to Miettinen's criteria.[14,23] Of 62 patients with an indication for adjuvant treatment, only 31 patients received this. The majority of patients that did not receive adjuvant treatment had in hindsight an indication before adjuvant treatment was commonplace. According to current guidelines these patients should all be discussed with an expert center and referred for adjuvant treatment and optimal oncological follow-up. Ideally, all patients should be discussed with expert centers before surgery to optimize staging, neo-adjuvant treatment options and surgical results.

A median PFS of 20.7 months of patients on first line palliative treatment is comparable to literature where a median PFS of 20 months is reported in patients with advanced GIST treated with imatinib.[24] A recent analysis showed no significant difference in overall survival between gastric and small bowel GIST.[25] Most detected mutations in patients with small bowel GIST is in KIT exon 11. Only a few patients with KIT exon 9 or wild type received systemic treatment. Conclusions of outcome based on mutational analysis can therefore not be made. From larger studies it is known that patients with KIT exon 9 mutations have a longer PFS when treated with imatinib 400mg twice daily.[26]

The overall survival of selected patients treated with surgery for metastasized disease was not better than from patients treated with systemic therapy alone. According to earlier published patients with limited metastatic disease could be referred for surgical evaluation to increase overall survival, however this is not substantiated with our data.[27] However, these studies describe only a limited number of patients and are not specifically about patients with small bowel GIST.

To conclude, this report summarizes the clinical management and pathological characteristics of patients with localized and metastasized small bowel GIST from 2009 until 2017 in the Netherlands. Remarkable is the high number of patients who underwent emergency surgery for resection of the primary tumor. Patients with small-bowel GIST more frequently present with metastatic disease when compared to patients with gastric GIST in literature. Adjuvant treatment decreased the recurrence rate in patient with high risk tumors. We suggest to prospectively register patients with small bowel GIST in a large international database and investigate the use of surgery in patients with (limited) metastatic disease.

Financial support

The Dutch GIST registry is financially supported by unconditional research grants from Bayer, Novartis and Pfizer. PAB works on a research grant provided by the Dutch Cancer Society (KWF, Alpe D'HuZes research grant RUG 2013-6355).

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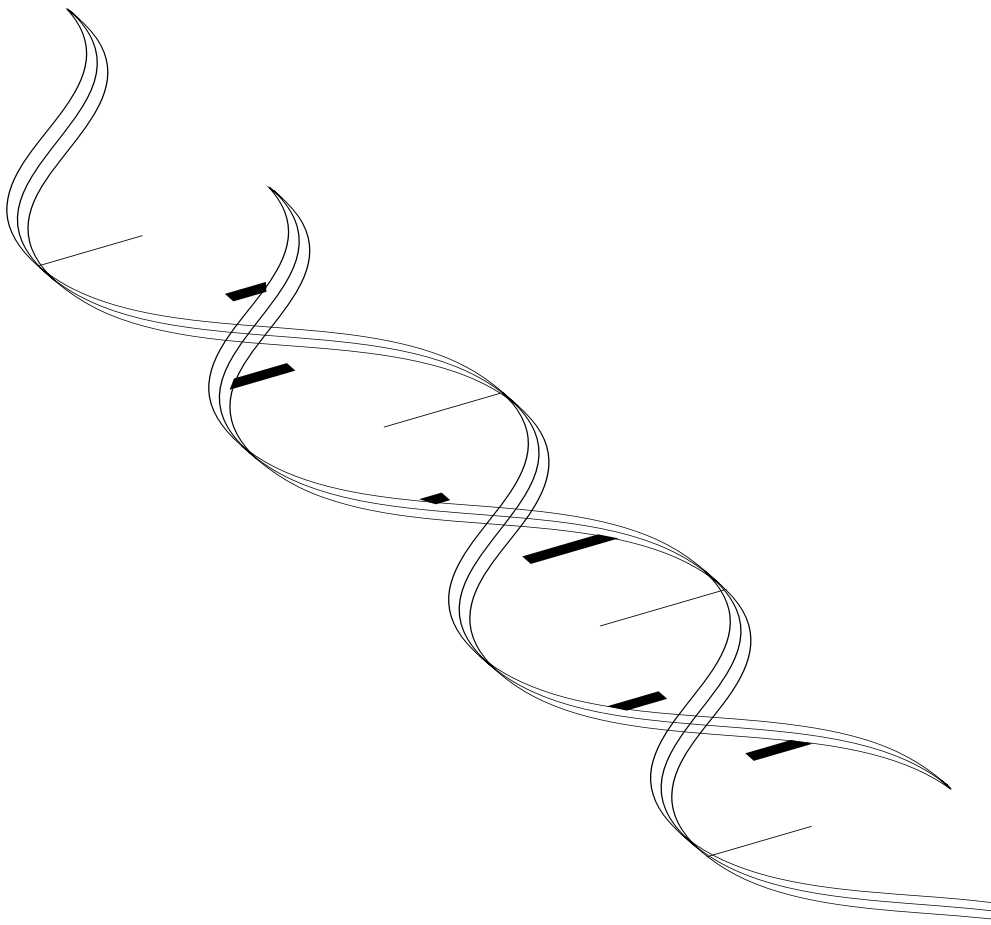

Diagnosis and treatment monitoring of a patient with a gastrointestinal stromal tumour by next-generation sequencing and droplet digital polymerase chain reaction of a PDGFRA mutation in plasma-derived cell-free tumour DNA

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The Oncologist, 2019

Chapter 7

Abstract

In patients with a suspected malignancy, standard-of care management currently includes histopathologic examination and analysis of tumor-specific molecular abnormalities. Herein, we present a 77-year-old patient with an abdominal mass suspected to be a gastrointestinal stromal tumor (GIST) but without the possibility to collect a tumor biopsy. Cell-free DNA extracted from a blood sample was analyzed for the presence of mutations in GIST-specific genes using next generation sequencing. Furthermore, liquid biopsies were used to monitor the levels of mutant DNA copies during treatment with a tumor-specific mutation droplet digital PCR assay that correlated with the clinical and radiological response. Blood-based testing is a good alternative for biopsy-based testing. However, it should only be applied when biopsies are not available or possible to obtain because overall, in only 50%–85% of the cell-free plasma samples is the known tumor mutation detected.

1. Report

In patients with a suspected malignancy, standard-of-care management currently includes histopathologic examination and analysis of tumor-specific molecular abnormalities. For malignancies that are characterized by known serum tumor markers (i.e., prostate-specific antigen in prostate cancer, carcinoembryonic antigen in colorectal cancer), analysis of blood could be indicative for diagnosis, treatment response, and detection of recurrence.[1, 2] Unfortunately, such biomarkers are not available for all malignancies.

Herein, we present a patient with an abdominal mass sus- pected to be a gastrointestinal stromal tumor (GIST) but without the possibility of collecting a tumor biopsy. Because GISTs are characterized by the presence of activating mutations in the tyro- sine kinase domain of the KIT or PDGFRα gene in more than 80% of the cases [3], cell-free DNA (cfDNA) extracted from a blood sample was analyzed for the presence of mutations in these genes using next-generation sequencing (NGS). Liquid biopsies were used to monitor the levels of mutant DNA copies during treatment with a tumor-specific mutation droplet digital polymerase chain reaction assay (ddPCR) to predict clinical response.

A 77-year-old woman presented at the hospital with pain and a palpable mass in the abdominal region; she also had anemia (Hb 5.4 mmol/l) for which she received a blood transfusion. A computed tomography (CT) scan showed a mass of 14 × 12 × 16 cm with loco-regional tumor depositions. The pri- mary mass was suspect to be a GIST (Fig. 1A). The largest lesion seemed to originate from the stomach, and additional liver and intra-abdominal metastases were seen. Gastroscopy showed, besides chronic gastritis, no abnormalities. A radiologic biopsy of the mass was planned, but the patient deteriorated and a new CT scan showed massive pulmonary embolism, warranting therapeutic anticoagulation. The planned biopsy had to be canceled because of the high risk of bleeding.

The patient gave permission to participate in a study to perform mutation analysis on cell-free DNA extracted from plasma of patients with advanced GIST (KWF research grant RUG 2013-6355, ClinicalTrials.gov NCT02331914). cfDNA was extracted and sequenced using NGS as reported previously.[4] The analysis revealed a mutation in PDGFRα (NM_006206.5: c.2524_2532del; p.D842_M844del), with a variant allelic fre- quency (VAF) of 4%. This PDGFRα c.2524_2532del; p.D842_ M844del mutation was reported in GIST once in the COSMIC database.[5] Validation with an in house designed mutation- specific ddPCR assay confirmed the presence of this mutation in cfDNA with a VAF of 2.9%. Imatinib sensitivity of a patient with this mutation was reported in 2015.[6] Standard first-line treatment with the tyrosine kinase inhibitor imatinib 400 mg was initiated. Shortly after initiation of therapy, the patient needed fewer blood transfusions and was able to leave the hospital. Positron emission tomography (PET) and CT scans performed before and 1 week after start of treatment showed a decrease in metabolic activity (Fig. 1C, 1D). A CT scan performed 3 months

after start of treatment showed a large cystic lesion with only minimal active tumor lesions, indicating response to treatment (Fig. 1B).

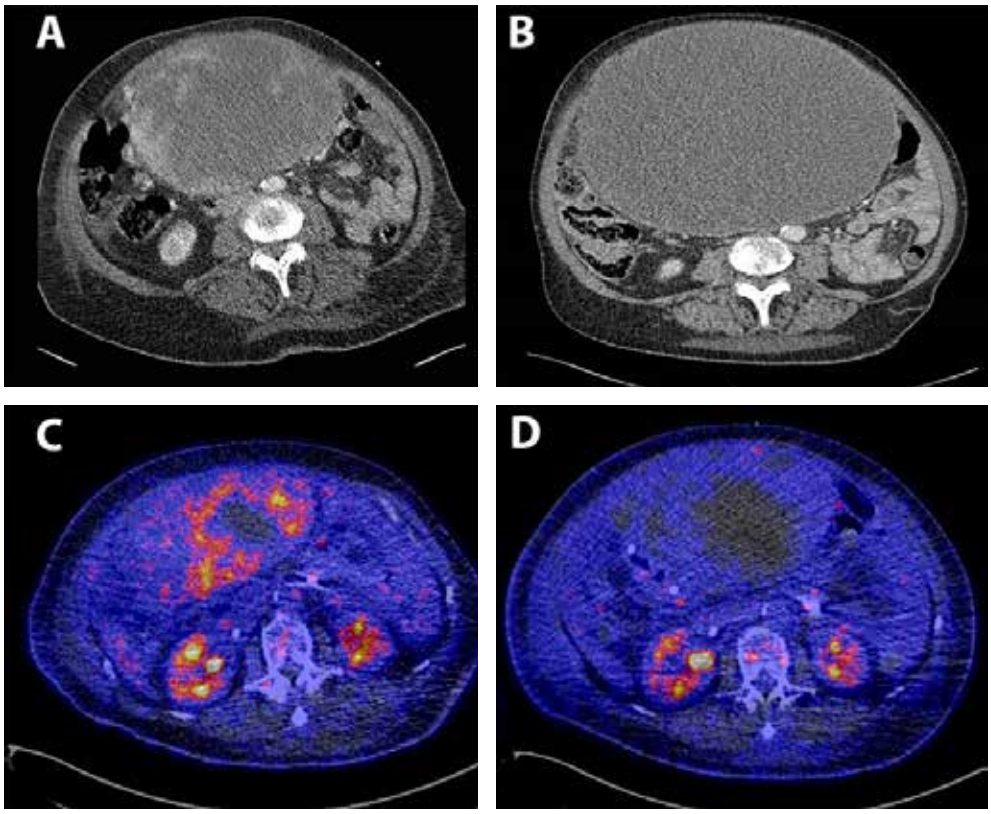

Figure 1. A. CT-scan at the moment of presentations. An abdominal tumor of 11x14 cm with solid and necrotic parts is seen, suspected for GIST. B. CT scan after three months of treatment. A large homogeneous cyst of approximately 20cm is seen with almost no active tumor parts. C. PET-CT scan before start of treatment, uptake of FDG is clearly seen in the tumor. D. PET-CT scan one week after start of treatment with imatinib 400mg, almost no uptake is present anymore in the primary tumor, physiological uptake at both kidneys is still present.

During treatment, the mutation-specific ddPCR assay was used at baseline and 1, 3, 4, 8, and 16 weeks after start of targeted therapy for cfDNA analysis. This showed a gradual decrease in mutant copies per mL of plasma, suggesting treatment response (Fig. 2). Because the cfDNA results based on levels of mutants per mL of plasma are in very good agreement with the observed response as determined with PET/CT scanning, our data sug- gest that the minimal invasive cfDNA assay might be an interesting alternative to monitor response. With an average turnaround time of 1–2 and 5–7 days, respectively, for ddPCR and NGS in a diagnostic setting, it is applicable in daily clinical practice.[7]

The course of the mutant allelic fraction in the cfDNA as measured with ddPCR

correlates with the clinical and radio- logical response; however, after 5 days of treatment, a flare is detected in the cfDNA mutational level. We observed a similar flare 1–2 weeks after start with imatinib in 5 of 11 patients with GIST with a detectable KIT-exon11 mutation and/or deletion in the baseline sample.[8] We hypothesize that this increase is caused by massive DNA shed by dying tumor cells caused by the initiation of treatment. This phenomenon has been described earlier in patients with colorectal cancer (CRC) shortly after start of treatment and could be used as a marker for early response.[9]

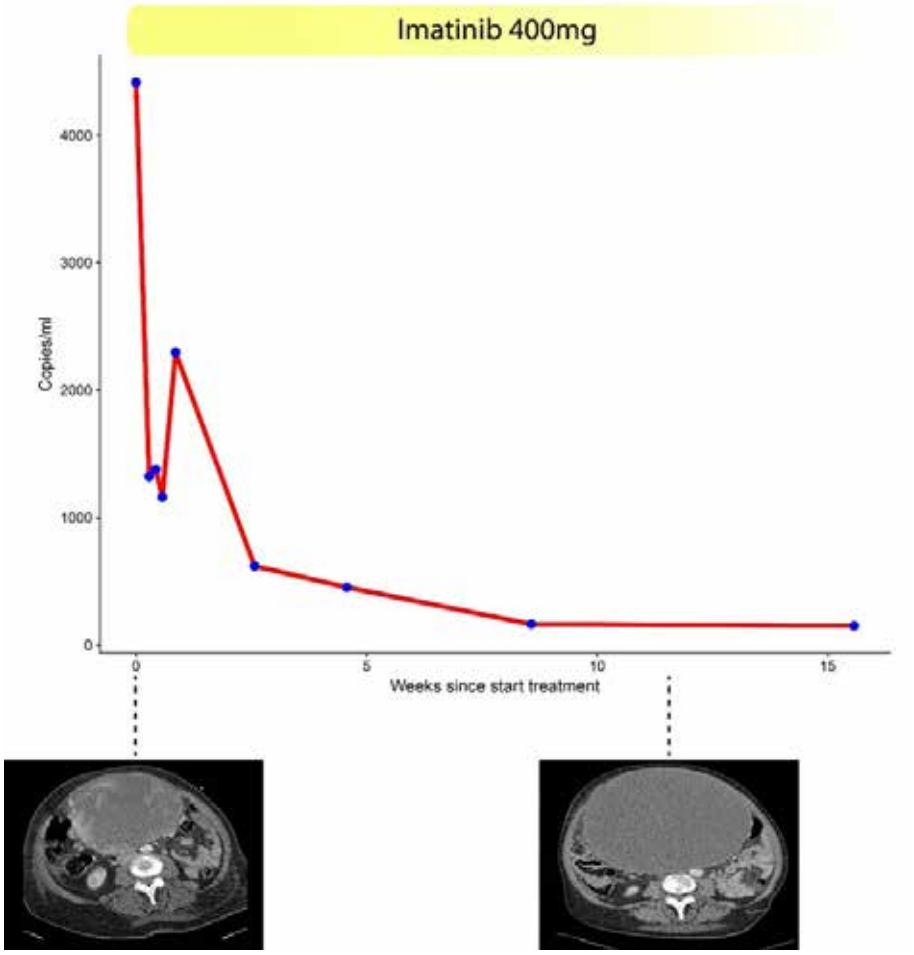

Figure 2. Detection of mutant copies/ml using ddPCR analysis to detect the PDGFRα c.2524_2532del as measured before start and during treatment. In the weeks after start of treatment, the mutant copies/ml decreased further, this in good concordance with the tumor response as seen on PET and at CT scan after three months were no active tumor lesions are seen (figure 1C/D). Procedures for DNA extraction and ddPCR analysis are reported previously4, 6. Upon request, details on primers and probes could be provided.

Blood-based testingis a good alternative for biopsy- based testing. However, it should only be applied when biopsies are not available or not possible to obtain because overall in only 50%–85% of the cell-free plasma samples (depending on disease status) the known tumor mutation is detected.[10] Recently, we reported on the feasibility of detecting KIT/PDGFRα mutations in baseline-pretreatment plasma samples in a series of patients with GIST with paral- lel analysis of a pretreatment biopsy with NGS. This study showed that the sensitivity of detecting a relevant KIT muta- tion in baseline plasma is ~90% in metastasized GIST.[8] Furthermore, a tissue biopsy provides important information on histology, diagnostic, and tumor-specific immunophenoty- pic markers and mostly a higher amount of neoplastic cells for molecular testing.

Detection of mutations in patients with GIST using liquid biopsy was reported previously.[11–15] These studies used sequencing as well as polymerase chain reaction- based techniques for detection of mutations in tumor and used the known tumor mutation for monitoring therapy in plasma.

As the circulating tumor DNA (ctDNA) in plasma might originate from different lesions (the primary tumor and its metastases), liquid biopsies can provide information regarding the spatial and temporal heterogeneity of tumors. Monitoring the mutational levels in plasma in time can guide personalized treatment and could possibly improve treatment outcome.

The use of liquid biopsies has been extensively validated in non-small cell lung cancer, melanoma, and CRC.[16–18] Practically all applications of liquid biopsy are regarding the monitoring of treatment response to (targeted) treatment and detection of secondary treatment resistant mutations. In 2016, the Food and Drug Administration approved ctDNA analysis in patients with lung cancer for initial genotyping of tumors when insufficient tissue is available for molecular characterization. This is exemplary of a shift in the use of liquid biopsies to broader applications, also in a diagnostic setting.

This case highlights the use of liquid biopsies for treatment response monitoring. The application of plasma-based mutation analysis in a diagnostic and response monitoring setting seems promising and has several advantages when compared with traditional methods. However, more and larger prospective studies are needed to confirm the use and justify the implementation of this technique into daily practice.

Acknowledgements

This project is funded by the Dutch Cancer Foundation (KWF), Alpe d'HuZes research grant RUG 2013-6355.

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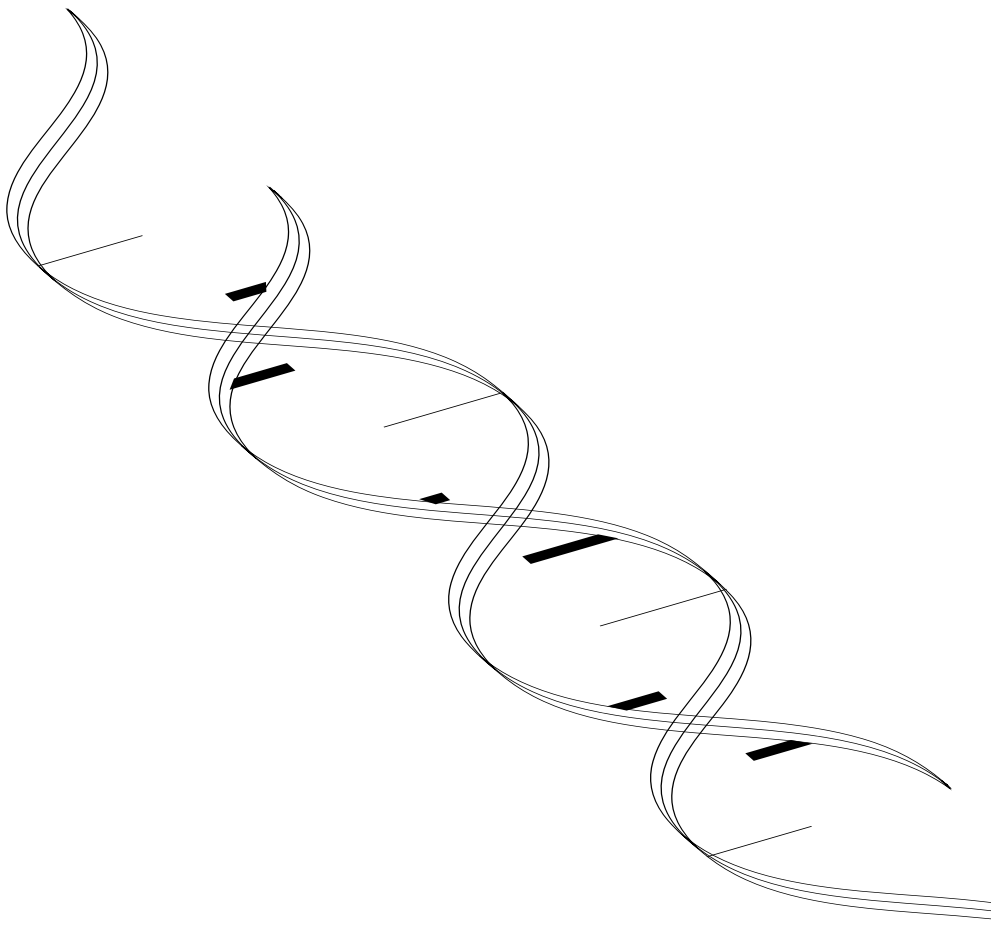

Summary
Discussion
Future perspectives

Chapter 8

1. Summary

Gastrointestinal stromal tumours (GISTs) are rare but the most common mesenchymal malignancies of the gastrointestinal tract. GISTs can occur throughout the complete gastrointestinal system but most frequently originate from the stomach or small bowel in respectively 60% and 30% of the cases.[1] Despite clinical and pathological differences, GISTs share a similar genetic profile. This includes, in most cases, oncogenic insertions, deletions and mutations (further referred to as mutations) in KIT or platelet-derived growth factor-alpha (PDGFRα).[2] Both genes encode for tyrosine kinase receptors and the gain-of-function mutations will lead to disruption of essential cell functions regarding proliferation, apoptosis, chemotaxis and adhesion. Since almost all GISTs have such a mutation, detection of this mutation in tumour tissue or peripheral blood is suggestive for a patient having the disease.
It is known that in the physiological process of cellular destruction and apoptosis cells shed DNA into the circulation. This includes cells from normal healthy tissue, but also from malignant tumours. It has already been proven that oncogenic mutations from tumour cells can be detected in the peripheral blood of patients with a malignancy. [3] This thesis focuses on the detection of the GIST specific mutations in plasma of patients with localized and metastatic disease. Furthermore, this thesis regards the application of monitoring circulating tumour DNA (ctDNA), referred to as liquid biopsy, during the treatment of patients with GIST (and other malignancies).

CHAPTER 2 provides a review of current literature concerning the use of mutation detection in circulating cell-free DNA and the relation with therapy response monitoring in a variety of solid tumours. More than 82 studies regarding solid malignancies with an oncogenic driver mutation, like non-small-cell lung cancer (NSCLC) with epidermal growth factor receptor mutations (EGFR), melanoma with mutations in BRAF, colorectal cancer (CRC) associated with KRAS mutations and breast cancer with TP53, ESR1, PIK3CA and AKT mutations were included in this review. Other sources for tumour DNA (i.e. circulating tumour cells, exosomes, urine, liquor) were not within the scope of this review. Most papers present clinical case studies, whereas larger randomized studies to evaluate the treatment response monitoring using ctDNA are scarce. A correlation between the detection of mutations and clinical disease status is reported frequently. In the majority of the included cases, a decrease in mutant load compared to the baseline level after start of therapy was found to be related to tumour response. Therefore, ctDNA can be used to detect early treatment response. This initial decrease is seen in almost all patients with detectable baseline ctDNA. Shortly after start of treatment (days/weeks) an initial rise of mutant allelic frequency can be detected, possibly due to massive cell decay as sign of treatment response. Detection of progressive disease is not in all patients correlated with an increase of the primary oncogenic mutation. Detection of secondary treatment resistant mutations is also feasible, but these mutations

are usually present in lower concentrations than the primary driver mutation and therefore more difficult to detect. For now, the primary driver mutation level in plasma seems the most appropriate to use for treatment response monitoring. This is currently only performed in trial settings and further research has to be performed before it can be implemented in clinical practice.

One of the pitfalls in analysing circulating tumour DNA is the fact that mutant DNA is generally present in a very low concentration. The normal physiological process of cellular destruction of healthy tissue will result in an always-present amount of wild type DNA that is much more abundant than the tumour derived DNA. Highly sensitive techniques are therefore needed to detect the low abundant mutant DNA. Before performing ctDNA analysis, an efficient extraction of the ccfDNA from the blood plasma is essential. An optimal method would isolate preferably mutant fragments and lose the wild type genomic DNA (derived from i.e. healthy tissue, leucocytes). Therefore, we compared three different plasma DNA isolation methods in **CHAPTER 3**. Moreover, the integrity of the extracted isolated ccfDNA was analysed with a fragment analyser and an in-house designed β-actine droplet digital PCR (ddPCR) assay. Three commonly-used ccfDNA extraction methods: Maxwell RSC cfDNA plasma kit, Zymo manual quick cfDNA kit and the standard kit (Qiagen CNA) used in our laboratory, were compared. The highest yield was obtained using the CNA kit, whereas the RSC kit was the least efficient. The fragment analyser revealed that cfDNA extraction using the RSC kit showed the highest short-to-medium-sized fragments ratio. The CNA kit showed consistently the highest yield and amplifiability of cfDNA, however, in the magnetic beads-based RSC kit augmented variant-allelic-frequencies (VAFs) were found, implying a preferential extraction of the ctDNA. We advocate harmonization and standardization of procedures within and between laboratories before implementation is feasible in the clinical setting. Furthermore we advise to use the same extraction method when monitoring tumor response using changes in ctDNA plasma levels within an individual patient.

To analyse the fractional abundance of ctDNA in extracted DNA from plasma samples different techniques are available. We chose to use ddPCR because of the high sensitivity (between 0,0005% and 10% depending on the amount of input DNA) and the relatively low costs per experiment. To evaluate the occurrence of the most-common KIT exon 11 mutations in GIST, a specific assay was designed. The design of this assay and the first analyses of plasma samples are described in **CHAPTER 4.** The ddPCR assay is based on the drop-off principle. Two different probes are able to anneal on two hotspot sequences in KIT exon 11 (short fragment of DNA in which mutations frequently occur). If a mutation is present in one of the hotspots, the probe will not anneal and no signal is detected in the ddPCR. Since mutations in these two hotspots are mutually exclusive in GIST, one probe will always anneal and function as a control. In theory, this assay should be able to detect approximately 70% of the known KIT exon 11 mutations. The assay was tested on selected tumour tissue samples and compared to next generation sequencing results. The KIT mutation

could be detected in 21 out of 22 samples with a theoretically detectable mutation using our ddPCR. When tested on baseline plasma samples of the same patients with GIST, we detected mutations in 13/14 patients with metastasized disease and only in 1/8 patients with localized disease. After initiation of treatment, a rise of mutant copies/ml was seen in 5/11 patients, possibly due to massive cell decay as sign of treatment response. After initiation of treatment the ctDNA mutant fractional abundance declined eventually in all patients with therapy response based on CT-scan analysis.

In **CHAPTER 5**, two patients with rare PDGFRα mutations are described. Both patients have a mutation near the known tyrosine kinase inhibitor (TKI) resistant D842 domain (M844_S847del and I843_D846del). The patients were treated with various TKIs and showed a treatment response. Specific probes were designed to detect these mutations in plasma. During treatment, plasma was longitudinally collected and analysed with the mutation-specific ddPCR probes. With these ctDNA analyses, the course of treatment could be monitored in the patients. When progressive disease occurred, a rise in level of the primary mutation was detected and after initiation of second or third line of treatment the level decreased in correspondence with radiological treatment response. To investigate the observed TKI sensitivity of the tumours with these 2 mutations, computer 3D-modeling of this PDGFRα kinase domain of these two variants revealed no direct interference in imatinib or sunitinib binding and no effect in its activity in contrast to the reported structure of the imatinib resistant D842V mutation. This modelling showed that both mutations do not affect the binding of imatinib and confirms the response to treatment with imatinib.

In the Netherlands, patients with GIST who are treated in an expert centre are registered in a national database known as the Dutch GIST registry. In **CHAPTER 6** this registry was used to identify patients diagnosed with small bowel GIST and to describe the clinical, surgical and pathological characteristics of these patients. Most studies about treatment of patients with GIST are describing cohorts of patients with different anatomical origins together. Between 2009 and 2016, 201 patients with small bowel GIST were registered of which 138 patients (69%) were diagnosed with localized disease and 63 patients (31%) with metastatic disease. Approximately 19% of the patients had emergency surgery, and in 22% GIST was an accidental finding. In patients with high risk localized disease (based on the risk classification as depicted by Miettinen et al.), recurrence occurred less often in patients who received adjuvant treatment with imatinib (4/32) compared to patients who did not (20/31, $p<0.01$). The majority of patients that did not receive adjuvant treatment had in hindsight an indication before adjuvant treatment was commonplace. Disease progression during palliative imatinib treatment occurred in 23 patients (28%) after a median of 20.7 (range 1.8-47.1) months. Ongoing response was established in 52/82 patients on first line palliative treatment with imatinib after a median treatment time of 30.6 (range 2.5 -155.3) months. Overall, patients with small-bowel GIST more frequently present with metastatic disease when compared to patients with gastric GIST according to

data in literature. Mutational analysis was performed for 156 (80%) primary tumours. Most detected mutations in patients with small bowel GIST were in KIT exon 11 (54%). Only a few patients with KIT exon 9 or wild type received systemic treatment with imatinib. Conclusions of outcome based on mutational analysis can therefore not be made. The overall survival of selected patients treated with surgery for metastasized disease was not better than from patients treated with systemic therapy alone. This is in contrast with earlier published studies where overall survival was increased with surgery for limited metastatic disease. We advocate a prospective registration of these patients and further investigation of the use of surgery in patients with limited metastatic disease.

In **CHAPTER 7**, a patient with a suspected GIST is described. A radiologic biopsy of the mass was planned, but the patient deteriorated and a new CT-scan showed massive pulmonary embolism, warranting therapeutic anticoagulation. The planned biopsy had to be cancelled because of the high risk of bleeding. CcfDNA of plasma was extracted and analysed using next generation sequencing. This revealed a mutation in PDGFRα (NM_006206.5: c.2524_2532del; p.D842_M844del), with a VAF of 4%. This PDGFRα mutation was previously reported in GIST once in the COSMIC database.[4] Validation with an in house designed mutation specific ddPCR assay confirmed the presence of this mutation in ccfDNA with a VAF of 2.9% in the baseline plasma. During treatment, the mutation-specific ddPCR assay was used to assess the number of mutant copies at baseline and 1, 3, 4, 8, and 16 weeks after start of targeted therapy. This showed a gradual decrease in mutant copies per mL of plasma, suggesting treatment response. Because the cfDNA results based on levels of mutants per mL of plasma were in very good agreement with the observed response as determined with PET/CT scanning, our data suggest that the minimal invasive cfDNA assay might be an interesting alternative to monitor response.

To conclude, this thesis showed the promising use of the analysis of circulating tumour DNA in patients with GIST. However, before implementation in daily practice, the benefits with respect to PFS and OS have to be proven in future studies.

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2. Discussion

2.1 Clinical utility of liquid biopsies

The use of liquid biopsies in the field of oncology has gained more attention in recent years. This is emphasized by the increasing number of published articles regarding the analysis of circulating tumour DNA (ctDNA) (figure 1). Liquid biopsies offer a non-invasive method for detection of mutations representative for primary tumours or metastases with several clinical applications.[1] The analysis of ctDNA can be used in a diagnostic setting, for monitoring response during systemic therapy, detection of specific drug-resistant mutations and for follow-up after initially curative treatment.

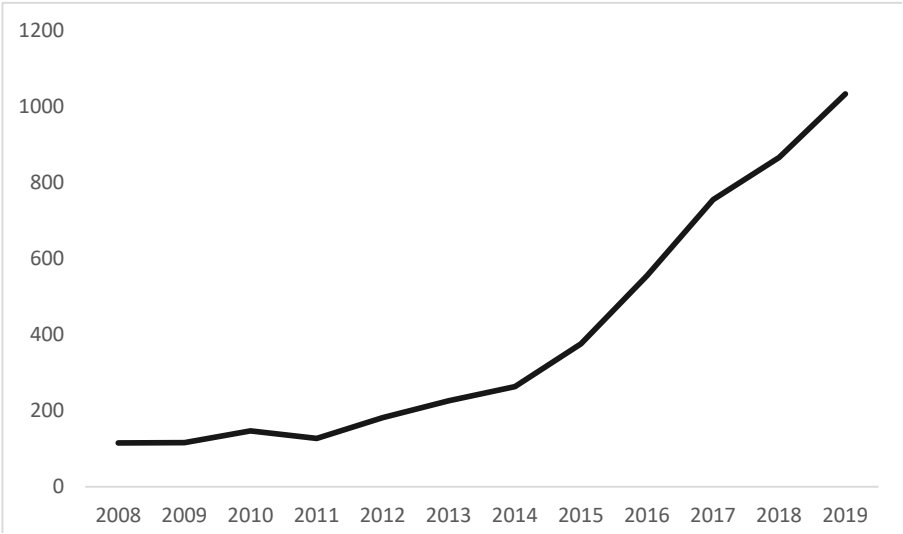

Figure 1. The number of papers published each year in the past decade with 'circulating tumour DNA' mentioned in the title.

The detection of tumour specific mutations in plasma or other bodily fluids before, during or after treatment could guide treatment decision making.[2, 3] This is illustrated in patients with non-small cell lung cancer (NSCLC) where in 2016 the FDA approved the Cobas EGFR mutation test v2 (Roche Diagnostics) assay for the detection of 42 EGFR mutations including the EGFR therapy resistant T790M mutation in ctDNA.[4] The detection of this mutation is a predictive marker to treat patients with a second-generation EGFR tyrosine kinase inhibitor (osimertinib) against the T790M mutation.[5] This is at the moment, next to the promotor methylation-based test of SEPT9 (Epi proColon assay, Epigenomics AG) in patients with colorectal cancer, the only FDA/EMA approved and commercially available test for the detection of mutations in plasma ctDNA in routine clinical practice.[6]

In this thesis the development of a new droplet digital PCR (ddPCR) assay which is able to detect the most frequent occurring mutations in patients with gastrointestinal stromal tumours (GIST) is described. In patients with GIST, 70-80% of the driver

mutations are found in exon 11 of the KIT domain. Approximately 80% of these mutations occur within two hotspot regions (figure 2 in chapter 4). The designed ddPCR 'drop-off assay' detects mutations in both of these hotspot regions. With this assay it is feasible to monitor the effect of treatment with tyrosine kinase inhibitors targeting the KIT receptor with a single blood test based on changes in the quantities of mutant fragments in plasma during treatment. The development of this assay and its use is described in chapter 4 in a limited number of patients. The results are promising, however, before this assay can be implemented in daily practice several hurdles have to be taken. For example, more knowledge regarding the dynamics of circulating tumour DNA is warranted, the detection of mutations using the ddPCR assay should be compared to an independent assay, alternatives for ctDNA as liquid biopsy have to be evaluated (i.e. RNA, exosomes, tumour educated platelets) and universal standard operating procedures for storage and analysis of samples have to be implemented. Since the results of ctDNA testing can have critical clinical implications, ideally the sensitivity as well as the specificity have to be high. Furthermore, the test has to be cost-effective compared to current daily practice and provide results within a reasonable time frame.

Before the ddPCR assay could be used as a suitable biomarker, further studies have to be performed following the standards of study design as stated by Pepe et al. in 2008.[7] This should include prospective sample collection from a cohort of patients that represent the target population for clinical application of the biomarker. The outcomes should be clearly defined. The purpose of the biomarker (increase of the primary mutation) is to distinguish patients with a bad outcome (progressive disease, case patients), from patients with a good outcome (stable disease/partial response, control subjects). Random selections of the relevant case patients and control subjects can be achieved only with a prospective cohort of subjects from the target population.

Performance criteria of the ddPCR assay have to be developed to assess the success or failure of the test. The true-positive rate and the false-positive rate are the typical performance measures of interest. Minimal acceptable values for both the true-positive rate and false-positive rate must be agreed upon in the design of the study. Procedures, collection, processing, and storage have to be defined and must be detailed in the study protocol. The study has to be performed blinded to avoid any bias. The assessment of biomarker performance must be separated from biomarker discovery since the performance of the samples in a discovery study is biased in an overoptimistic direction.[7] When such a study is successfully completed, a broader application of the assay in daily clinical practice is possible.

2.2 Dynamics of circulating DNA

More understanding is needed about the origin and dynamics of cell free DNA in plasma. Even in healthy persons circulating DNA is present in the peripheral blood. In the normal physiological process of cellular apoptosis and necrosis DNA is shed

into the circulation. Dead cells are rapidly cleared by macrophages which then release the DNA into the blood.[8] In healthy individuals this DNA is believed to be mainly derived from cells of the hematopoietic lineage. Certain conditions such as trauma, extensive exercise, stroke, inflammation or a malignancy result in higher concentrations of cell free DNA by mechanisms that are not well understood.[1, 9, 10] Hypothesized is the contribution of NETosis to the increase of cell free DNA. This is a rapid process consisting of nuclear disintegration and cell death leading to the extrusion of neutrophil extracellular traps (NETs) which are a network of extra cellular fibres primary composed of DNA from neutrophils that binds to pathogens and has a central role in the immune defence.[11, 12] Accordingly, a large variety in circulating DNA levels is measured between different individuals and within one individual at different time points. Therefore, the quantification of cell free DNA concentration itself is not useful in a diagnostic setting. There is an overlap in levels of cell free DNA in healthy people and those with an infection or malignancy.[13]

DNA can also enter the circulation by active secretion of DNA fragments. In studies with cultured cell lines of different origins this active DNA release is reported and hypothetically this could be executed by cancer cells to affect transformation of receptive cells at distant sites.[14] In the nucleus of the cell, DNA is bound to nucleosomes that consist of eight histone protein cores on which the DNA is wound in parts of approximately 147 base pairs, including the linker DNA the histone-complex is named a chromatosome and ~166 base pairs in size.[15] These protein bound DNA fragments survive digestion during apoptosis and phagocytosis and end up in the circulation.[16] The unbound DNA is sensitive to plasma nucleases such as DNase 1 which result in rapid clearance from the circulation.[17] The half-life of cfDNA is thought to be between minutes and several hours and some forms might survive longer than others (double stranded DNA is longer detectable in the circulation than single stranded DNA).[13, 18, 19] Although the mechanism is not well understood, it appears that DNA is cleared by the spleen, liver and kidneys but the influence of other factors such as circadian rhythms, inflammation or particular therapies is still unknown.

2.3 Sensitivity of various detection methods

Several techniques are available to test the presence of mutations in ctDNA from plasma samples.[20] These can largely be divided into two major groups, particularly methods based on (next-generation) sequencing aiming for a broader coverage or (digital) polymerase chain reactions (PCR) which are targeted for a single or small number of variants.

DdPCR for mutation detection has a higher sensitivity compared to sequencing techniques in samples with a low concentration of DNA such as plasma.[21] The droplet digital PCR which partitions the sample in 20.000 picoliter sized droplets has been reported as a quantitative, accurate assay with high analytical sensitivity.[22] Sensitivities of 0.005-0.1% for EGFR-T790M, 0,1% and 0,5% for ALK-C1156Y and

ALK-G1269A in lung cancer and 0.025% for KRAS in colorectal cancer are reached. [23-26] It is possible to detect mutant target fragments with a high sensitivity, but only specific mutations can be detected. These targeted assays are useful for detection of specific known mutations that occur frequently at very low levels and could be used to identify variants that are associated with response to specific drugs in specific tumour types. These PCR based techniques can also be used as treatment response marker in patients with known mutations as also has been described (this thesis). In contrast to the targeted assays, next generation sequencing (NGS) based approaches have the capability of detecting a large number of variants in multiple genes. Therefore, in patients with unknown mutations, a sequencing approach is recommended. However, a limitation of NGS is the high frequency in which bases are scored incorrectly due to artefacts that are introduced during the sample preparation and sequencing. In several steps of the sequencing process such as with the polymerase phase and during cluster amplification errors arise. This results in approximately 0.1-1% of bases being called inaccurately. As described earlier, for ctDNA analysis in which rare mutant fragments occur in a large wild type background a level of detection below 1 in 10.000 is desirable. This level cannot be reached with conventional NGS where due to the frequency of base errors a detection limit is reached of around 1 in 100.[27] Fortunately, due to recent technological innovations such as the development of barcode sequencing the analytical sensitivity of next generation sequencing has increased and the use thereof could be useful for the reliable detection of low frequent predictive and resistant mutations in cfDNA.[28]

2.4 Alternatives for ctDNA as liquid biopsy

To enable a broad utilization of liquid biopsies, alternatives for ctDNA should be evaluated. As mentioned before, tumours are frequently associated with genomic aberrations of which some act as relevant markers for diagnosis and treatment decision making. The use of liquid biopsies is based on the detection of such genetic abnormalities (chapter 2). Some abnormalities such as gene-fusion (ALK-EML4) or MET-skipping can be efficiently detected with RNA based methods. In addition, expression profiles have been reported to be associated with diagnosis (tumour typing) and prognosis. An example is the MammaPrint for detection of high-risk breast cancer using tumour tissue.[29] RNA originating from the tumour can also be detected in plasma. At least three plasma sources have been reported: cell free RNA in plasma, RNA in thrombocytes and RNA in extracellular vesicles (or exosomes). [30] Tumour cells can impose changes on RNA and proteins present in platelets. As a result, these tumour-educated platelets can in various ways promote tumour cell survival due to their altered function.[31] Platelets are capable of taking up proteins and nucleotides. Extracellular vesicles can harbour tumour specific RNA. With PCR based techniques this circulating tumour RNA (ctRNA) can be analysed and tumour specific aberrations detected. Specific transcripts have been detected in thrombocyte RNA such as the biomarkers EGFR, EML4-ALK, KRAS and PIK3CA mutants.[32]

RNA molecules are relative unstable and susceptible to many degrading factors, the half-time of naked RNA in the circulation is approximately 15 seconds. mRNAs have also been detected in peripheral blood samples in patients with prostate cancer where the plasma human telomerase reverse transcriptase (hTERT) mRNA levels showed a correlation with progression free and overall survival.[33] Furthermore, also other forms of RNA such as non-coding RNAs, long noncoding RNAs, piRNAs, snRNAs and snoRNAs are of interest for application as liquid biopsy.[34]

Another source for RNA/DNA are circulating tumour cells (CTCs). These cells are considered to attribute to the development of metastases.[35] For instance in breast cancer the number of CTCs in blood correlates negatively with progression free and overall survival.[36] At this moment the difficulty is to effectively and reliable isolate these cells from the plasma and to discriminate CTCs from other non-malignant cells. CTCs have a low occurrence of approximately 1-10 in 7.5 mL blood in patients with breast and lung cancer compared to 106 – 108 white blood cells.[37] This low number and difficult detection of CTCs prevents a rapid implementation of CTC testing in diagnostics today.

Blood plasma is not the only source for ctDNA/ctRNA since ctDNA/ctRNA has been detected as well in other bodily fluids like urine, sputum, saliva, liquor and faeces. Detection and surveillance of bladder cancer and colorectal cancer is achievable using urine tumour DNA, and KRAS mutations were detected in urine cell free DNA of patients with colorectal cancer.[38-40] KRAS mutations have also been detected in stools of patients with colorectal carcinoma.[41] In patients with brain metastases of EGFR mutated adenocarcinoma of the lung, mutations can be detected in the cerebrospinal fluid.[42]

In conclusion, many possibilities of liquid biopsies can be explored in the near future to develop methods for a non- or minimal invasive way to detect tumour-specific mutations for treatment decision making and treatment monitoring.

2.5 Standard operating procedures

Currently, a broad spectrum in DNA extraction kits and ctDNA detection methods is available.[43] Each assay has its own (dis)advantages. To have universal comparable tests it is essential that various (pre-) analytical conditions are harmonized between the separate laboratories. The first step in this process is the collection, processing and storage of the samples. Since the concentration of total DNA in blood which is collected in regular EDTA tubes is not stable, a quick sample workup is essential to prohibit lysis of white blood cells contaminating the sample with wild type non-malignant DNA.[44] To decrease the effect of haemolysis, the use of special blood collection tubes that stabilize the blood sample for at least a couple of days enables transportation of the samples and a more flexible workup flow.[45-47] Other factors affecting haemolysis are processing of plasma by centrifugation and storage/ shipping temperature (chapter 3).[48, 49] Therefore, it is also recommended to use strict standardized operation procedures. A few centrifugation steps have to be

performed to collect the supernatant with the DNA fragments and lose all cells and debris. The supernatant has to be stored at -80 °C to prohibit further degradation of the RNA/DNA molecules. Limited data is available concerning the effect of blood draw procedures, storage temperature and patient related factors (inflammation, exercise, smoking etc.) on the quality and levels of cell-free DNA. Various efforts are being made to establish methods to determine the optimal collection and pre-analytical work up.

Recently a large consortium was established consisting of 36 partners from 13 countries to establish standard protocols for and clinical validation of blood-based biomarkers (www.cancer-id.eu). In the Netherlands the COIN project (ctDNA on the way to implementation in the Netherlands) in which participate several centres (Antoni van Leeuwenhoek, Amsterdam, UMC Groningen, Radboudumc Nijmegen, Universitair Medisch Centrum Utrecht, Erasmus MC Rotterdam, Amsterdam UMC, IKNL and PALGA) is established to standardize the way of implementation of ctDNA in clinical practice.[50] At this moment no standardized operating procedures are available for the most efficient and effective methods to extract circulating DNA from the plasma. It is recommended when using regular EDTA anti-coagulant tubes to store the samples at 4 °C and process the sample within 6 hours. After centrifugation, the resultant plasma layer can be used for testing directly or stored at -20 °C for a maximum of 5 days or at -80 °C for a not well specified longer storage.

To compare and reproduce experiments for reliable quantification of ctDNA it is essential to have easy-to-use, cost-effective, robust and reproducible work flows with a short turn-around-time. At this moment, comparison between different assays is difficult since each assay has its own specifications and studies comparing cfDNA extraction methods showed a large variety in total DNA yield.[43]

For the near future standardized operation procedures have to be developed with regard to blood collection (using certified blood collection tubes), handling and storage of the samples. Furthermore, validation studies have to be performed to show the ability of laboratories to reach the sensitivity and specificity as described for the specific assays when using these data in clinical practice. This is highlighted by a recently performed European external quality assessment schema which was organised by the IQN Path organization. The pilot scheme consisted of a set of eight samples containing mutations in EGFR, KRAS, NRAS and two wild type samples where participants were asked to isolate and genotype the samples. Interestingly, a high rate of genotype errors (20.1%) was observed.[21] Especially in the samples with a low variant allelic frequency errors occurred, a fraction of the false-negative reports occurred because the variant was detected at a frequency below the limit of detection.

3. Future perspectives

Liquid biopsies can provide information regarding the spatial and temporal heterogeneity of tumours. Monitoring the mutational levels in plasma over time can guide personalized treatment and could improve treatment outcome.
The use of liquid biopsies has been extensively validated in NSCLC, melanoma, breast cancer and CRC (chapter 2).[18, 23, 51, 52] At the current moment, liquid biopsies are mostly used for the monitoring of treatment response to (targeted) treatment and detection of new, treatment resistant mutations.
The successful detection of mutations in patients with a known malignant disease can guide further development of DNA extraction and analytical techniques and cause a shift in the use of liquid biopsies to broader applications in a diagnostic setting.

3.1 Liquid biopsies in diagnostic setting

Mutation testing in ctDNA might be an alternative source for tissue biopsies particular when no biopsies or biopsies with insufficient neoplastic cell percentages for molecular profiling are available.[53] This can be of use in patients who have a suspected malignancy too. An example is shown in chapter 7 were a patient with a suspected diagnose of GIST was confirmed using NGS on ctDNA. This could be used in other patients when a tissue biopsy is not feasible and there is a high suspicion for GIST or other tumours were driver mutations frequently occur. Since liquid biopsies are minimally invasive and have the potential to detect a large variety in mutations with NGS based methods, it could theoretically be used for screening purposes. Ideally, a screening programme should detect early stage cancer with a high sensitivity and specificity. Since there are still limitations in the sensitivity of NGS and general mutational profiles of many tumours are not available this has until recently not led to an implementation in clinical practice. ctDNA analysis could be a value addition to existing screening programmes and this should be investigated in future studies.

3.2 Treatment response monitoring

The quantification of mutations in plasma seems to correlate well with treatment response. A decline in mutational load during treatment response has been reported in many studies (this thesis).[54, 55] In case of treatment resistance, the primary mutation will rise again along with new mutations that confer treatment resistance.[56] In addition, mutation analysis of ctDNA during treatment is already been reported as a new tool for monitoring treatment response in several malignancies since the amount of ctDNA correlates with the volume of vital tumour tissue.[18]
However, not all patients with progression are detected, especially when progression is caused by the development of new unknown treatment resistant mutations. Another application of the use of liquid biopsies as treatment response marker might be the (partial) replacement of routine CT-scans to evaluate response to treatment. This will

possibly reduce costs, radiation exposure and stress for patients. Additionally, there is evidence that disease progression could be detected earlier with rising ctDNA levels as compared to conventional imaging (chapter 2). This replacement of CT-scans might potentially work for GIST and will be investigated in a future study. This study has an observational multicentre design in continuation to the GALLOP study in which a large biobank is established containing hundreds of plasma samples of patients with GIST treated in the Netherlands.[57] In this follow-up study the technical validity of the designed ddPCR KIT exon 11 assay (chapter 4) will be assessed where the assay will be implemented in other participating laboratories. Furthermore, the clinical validity will be investigated of the KIT exon 11 assay in an observational cohort study. In this part, all patients with a proven KIT exon 11 mutated GIST within the range of the designed assay and an indication to have CT-scans (once in 3-12 months) in the follow-up of treatment will have real time information on the fractional abundance of the KIT exon 11 mutation in plasma. Whether an increase in fractional abundance is present before progression is seen on CT scan is one of the goals of the study. Moreover, the validity of the ddPCR assay will be compared to an independent ctDNA mutation assay. Next to detection of the primary mutation, also detection of secondary mutations in progressive patients is subject of the study. The benefits of this study could be substantial. When ctDNA can replace one or more CT-scans the radiation load is reduced and patients would experience probably less stress due to the minimal invasive way of ctDNA analysis compared to a CT-scan. Nonetheless, the most important benefit of this study would be if the ctDNA analysis could detect progression of disease earlier than CT-scans. An earlier treatment switch based on the early progression or detection of secondary mutations could contribute to an increase in progression free- or overall survival (PFS/OS). At this moment most published studies regarding the monitoring of patients with malignancies are of retrospective nature and did not demonstrate convincingly any improved patient outcomes when compared to standard monitoring approaches. As the field rapidly expands, this might be likely proven in the next few years.

3.3 Detection of treatment resistant mutations

In 2016, a kit for the detection of the EGFR TKI resistant T790M mutation in plasma has been approved for use in daily practice.5 Since tumours evolve during treatment and secondary mutations can cause therapeutic resistance, a new biopsy is required during treatment to define the actual mutational status.[58] The occurrence of secondary mutations has been demonstrated in patients with NSCLC during treatment with EGFR-TKIs. The EGFR TKI-resistance mutation T790M was detected in ~70% of plasma ctDNA of patients with advanced disease who had acquired TKI-resistance.[59] Similarly, in GIST, resistance develops during imatinib treatment. In 50% of patients with progressive disease, a secondary mutation, besides the primary KIT mutation, is detected.[60] Treatment response to standard second line therapy, sunitinib, differs between patients with secondary mutations in KIT exon 13/14 or

exon 17 where especially tumours with mutations that reside in the activation loop (D816H/V in exon 17) remain resistant.[61] These resistant mutations could be missed by conventional tissue biopsy due to tumour heterogeneity.[62] In addition, repeated tumour biopsies have risks (e.g. bleeding, perforation and infection). Several new drugs are currently in development or in a clinical trial phase with promising results. These drugs will target tumours with specific first or second line treatment resistant mutations.[63-67] Thus, the detection of primary and secondary resistant mutations in ctDNA cannot be used only to monitor recurrences before clinical manifestation, but also provide information for changes of therapy.

To conclude, in this thesis we report on a new approach for the detection of KIT-exon 11 mutations in both tumour biopsies and cell free plasma in patients with GIST. However, for proper implementation of this assay into daily practice more understanding regarding origin and dynamics of ctDNA is needed such as improving the sensitivity of mutation detection and universal standard operating procedures for collection, storage, work-up and analysis of the samples. Further developments in sequencing approaches could reduce costs for testing and increase the sensitivity which will enable a broader application and provide new opportunities. Before widespread application of the designed ddPCR KIT exon 11 assay in patients with GIST is feasible, the analytical and clinical validity and utility of the assay has to be proven in a future study. The application of liquid biopsies in general is a promising development, however the benefit in progression free and overall survival that it theoretically carries has still to be proven in future studies.

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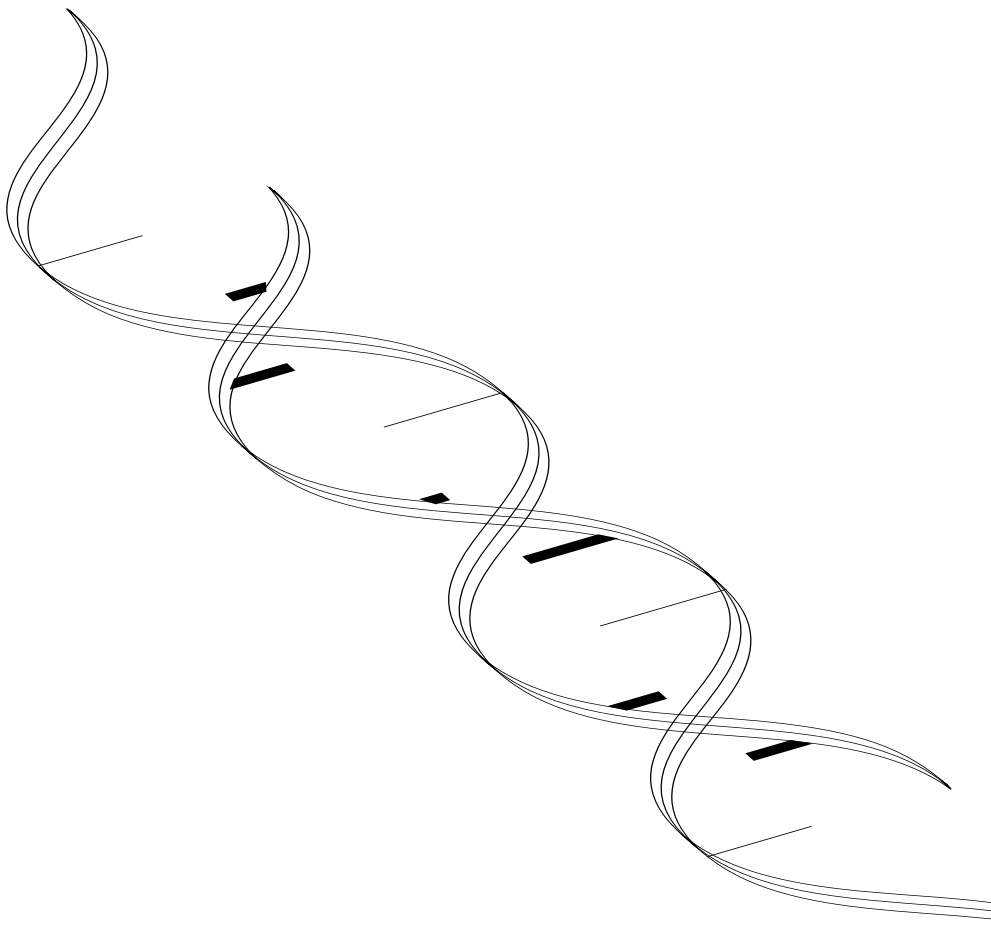

Nederlandse samenvatting

Dankwoord

Appendix

A

Nederlandse samenvatting

Gastro-intestinale stromacel tumoren (GIST) zijn zeldzame tumoren die voorkomen in het hele maagdarmstelsel. De meeste GIST komen voor in de maag (60%) en dunne darm (30%) en hebben een vergelijkbare genetische oorzaak. Dit betreft een of meerdere veranderingen (mutaties) in de genen die coderen voor de tyrosine kinase receptoren KIT of de platelet-derived growth factor-alpha (PDGFRα). Deze receptoren hebben invloed op verschillende processen in de cel en kunnen leiden tot een ongecontroleerde groei en differentiatie. Aangezien vrijwel alle GIST een van deze veranderingen in het DNA hebben is het aantonen in tumorweefsel of bloed van een patiënt zeer suggestief voor het hebben van de ziekte. Patiënten met deze specifieke mutaties kunnen gericht behandeld worden met een therapie welke specifiek aangrijpt op tumorcellen met deze mutatie en zo de groei hiervan kan remmen.
In het normale proces van verval en apoptose (geprogrammeerde dood) van de cel komt er DNA vrij in het bloed. Dit DNA wordt circulerend celvrij DNA genoemd (ccfDNA) en kan afkomstig zijn van zowel gezonde als van kwaadaardige cellen (en wordt dan circulerend tumor DNA ofwel ctDNA genoemd). In eerder onderzoek is aangetoond dat tumor DNA aangetoond kan worden in het bloed van patiënten met een kwaadaardige tumor. Dit proefschrift focust zich op de detectie van GIST-specifieke mutaties in het bloed van patiënten met zowel lokale als gemetastaseerde ziekte. Daarnaast is gebruik van ctDNA in het algemeen en meer specifiek voor monitoring tijdens de behandeling van patiënten met GIST, onderwerp van dit proefschrift.

HOOFDSTUK 2 geeft een overzicht van recente literatuur over het gebruik van ctDNA voor het monitoren van de respons op behandeling bij patiënten met een kwaadaardige tumor. Voor deze analyse zijn 82 studies gevonden met name over patiënten met longkanker met EGFR-mutaties, melanoom met BRAF-mutatie, darmkanker met een KRAS-mutatie en borstkanker met verschillende mutaties. De meeste publicaties betreffen relatief kleine patiëntenseries en er zijn vrijwel geen grote gerandomiseerde studies gepubliceerd. In de meerderheid van de studies wordt een duidelijke relatie gezien tussen de kwantitatieve detectie van mutaties en de respons op therapie van patiënten. CtDNA kan gebruikt worden voor het vaststellen van een vroege respons op de therapie. Dit is zichtbaar in een duidelijke daling van de hoeveelheid ctDNA na het starten van de behandeling gericht op de aanwezige mutatie. Dit is mogelijk in vrijwel alle patiënten met detecteerbaar ctDNA voor start van de behandeling (baseline). In sommige gevallen wordt er enkele dagen na start van de behandeling een kortdurende sterke stijging gezien van de hoeveelheid ctDNA. Dit kan worden veroorzaakt door massale afbraak van cellen en lijkt een positief effect meteen na start van de behandeling. Ondanks veelbelovende resultaten is progressie van ziekte tijdens de behandeling niet bij alle patiënten aan te tonen met behulp van ctDNA.

Gedurende de behandeling raken de meeste patiënten resistent voor therapie, dit wordt onder andere veroorzaakt door secundaire mutaties die de tumor ongevoelig maken voor de initiële doelgerichte therapie. Deze resistente mutaties ontstaan tijdens de behandeling en kunnen ook gedetecteerd worden in ctDNA. Secundaire mutaties zijn echter vaak in lagere concentraties aanwezig dan de primaire mutatie en daardoor moeilijker te detecteren. Vooralsnog lijkt daarom de primaire mutatie het meest geschikt om de behandeling te monitoren.
Een van de problemen bij het analyseren van ctDNA is de vaak lage concentratie van het gemuteerde tumor DNA in cel vrij plasma. Het normale proces van cel-verval en -vernieuwing zorgt voor een altijd aanwezige achtergrond van niet gemuteerd DNA (wild-type) in het bloed afkomstig van normale weefsels. De fractie ctDNA in het plasma afkomstig van de tumor is in algemeen lager dan 0.1%. Daarom zijn gevoelige mutatie-detectie-methoden nodig om het ctDNA te kunnen detecteren.
Voordat het DNA geanalyseerd kan worden moet het uit bloed geïsoleerd worden. Idealiter zou men alleen de kortere fragmenten isoleren die van de tumor afkomstig zijn en de wild-type fragmenten (afkomstig van gezond weefsel en witte bloedcellen) niet meenemen. In **HOOFDSTUK 3** zijn drie verschillende methoden om ccfDNA uit het bloed te isoleren met elkaar vergeleken (Qiagen CNA kit (standaard in UMCG-laboratorium), Maxwell RSC cfDNA plasma kit en de Zymo manual quick cfDNA kit). Het geïsoleerde ccfDNA werd getest op integriteit met een door ons zelf ontwikkelde β-actine droplet digital PCR (ddPCR) assay en een DNA fragment analyzer. Dit liet zien dat de techniek met de hoogste opbrengst niet het beste scoort met betrekking tot de integriteit van het DNA. In de samples met de hoogste opbrengst werden veel langere wild-type fragmenten gevonden. Dit kan het gevolg zijn van witte bloedcellen die kapotgaan tijdens de procedure van het opwerken van het bloed. De hoogste opbrengst werd verkregen met de CNA kit, maar met de op magnetic beads gebaseerde RSC kit werden hogere aantallen gemuteerd DNA gevonden. Mogelijk dat de RSC kit bij voorkeur ctDNA isoleert. De verschillen in opbrengst en integriteit van het ccfDNA uit hetzelfde plasma tonen aan dat verschillende ccfDNA technieken de uitkomsten beïnvloeden zeker wanneer in opeenvolgende plasma samples de hoeveelheid ctDNA gebruikt wordt voor therapie adviezen. Wij zijn groot voorstander van (inter)nationale harmonisatie en standaardisatie van de isolatieprocedures uit plasma voordat de techniek breed ingezet kan worden in de kliniek.
Verschillende technieken zijn beschikbaar voor het analyseren van geïsoleerd DNA. Wij hebben gekozen voor een droplet digital PCR-techniek (ddPCR). Met deze techniek wordt het DNA verdeeld over +/- 20.000 minuscule druppeltjes die elk één DNA-fragment bevatten en vervolgens per druppel met een PCR geanalyseerd kunnen worden: dus we doen in één test 20.000 afzonderlijke PCRs wat resulteert in een hoge gevoeligheid (mutanten in een achtergrond van wildtype DNA kunnen worden gedetecteerd tot 0,05% afhankelijk van de hoeveelheid input DNA) en de relatief lage kosten per experiment. Ongeveer 60-70% van de patiënten met GIST heeft een mutatie in exon 11 van het KIT-gen.

Om de meest voorkomende KIT exon 11 mutaties in patiënten met GIST te kunnen detecteren, hebben wij een specifieke assay ontwikkeld. Het ontwerp van deze assay en de eerste resultaten staan beschreven in **HOOFDSTUK 4**. Deze assay is ontwikkeld volgens het drop-off principe. Twee gelabelde probes (groen en blauw) kunnen binden aan het niet-gemuteerde (wild-type) DNA elk in een van twee mutatie 'hotspot' gebieden in KIT exon 11. In geval dat de ddPCR op normaal DNA wordt uitgevoerd, zullen beide probes aan het DNA binden en zullen in alle druppels zowel het groene als blauwe signaal zichtbaar zijn. Wanneer een mutatie aanwezig is in een van deze hotspot regio's zal de probe die in het gebied met de mutatie ligt niet meer kunnen binden en wordt in de druppels dus maar 1 probe zichtbaar; de probe die niet kan binden (drop-off genoemd in het Engels) betekent dus dat er een mutatie aanwezig is in het DNA. Omdat mutaties in beide hotspots tegelijkertijd niet beschreven zijn functioneert 1 probe altijd als controle voor de hoeveel DNA die in de PCR zit. Met deze assay is het mogelijk om 70% van de bekende KIT exon 11 mutaties te detecteren. De assay is eerst getest op tumorweefsel en vergeleken met de resultaten van een andere techniek (next generation sequencing), in 21 van de 22 samples werd de mutatie gedetecteerd. Vervolgens is er getest op plasma samples van patiënten met een GIST. De mutatie in het ccfDNA kon gedetecteerd worden in 13 van de 14 patiënten met uitgezaaide ziekte en in 1 van de 8 patiënten met gelokaliseerde ziekte. Na het starten van de behandeling werd in eerste instantie een stijging van het aantal mutaties gezien in 5 van 11 patiënten wat mogelijk veroorzaakt wordt door een snel therapie effect en het hierbij behorende cel verval. Na het starten van de behandeling daalde het aantal gemuteerde fragmenten uiteindelijk in alle patiënten met een radiologische therapie respons (gebaseerd op CT-uitslagen).
In **HOOFDSTUK 5** zijn twee patiënten beschreven met een zeldzame PDGFRα mutatie. Beide patiënten hebben een mutatie die dichtbij het D842 domein ligt waarvan we weten dat het resistentie voor behandeling met tyrosine kinase remmers (TKI) veroorzaakt. De patiënten werden behandeld met verschillende TKI's en hadden hierop een respons. Probes voor de detectie van patiënt-specifieke mutaties werden ontwikkeld om het verloop van de hoeveelheid van het ctDNA te kunnen volgen in het bloed. Tijdens de behandeling werd op verschillende momenten bloed verzameld en de mutaties werden gemeten om het beloop van de behandeling te monitoren. Bij progressie van ziekte werd een stijging gezien van het aantal mutanten in het plasma en na het starten van tweede en derde lijn behandeling daalde dit aantal weer, in overeenstemming met de radiologische respons. Om de gevoeligheid van de tumoren met deze zeldzame PDGFRα mutaties voor de behandeling met imatinib te verklaren, werd een 3D-eiwit model met het kinase domein van PDGFRα met de verschillende mutaties gemaakt. Dit 3D-model liet zien dat beide mutaties geen structurele veranderingen ten gevolge hebben in de eiwitten waarop imatinib aangrijpt en daarmee suggereert dat deze mutaties geen resistentie voor de behandeling als gevolg hebben.

In Nederland worden patiënten met een GIST die behandeld worden in een expertisecentrum geregistreerd in een landelijke database (GIST registry). In **HOOFDSTUK 6** is gebruikt gemaakt van deze database om de klinische, chirurgische en pathologische eigenschappen te beschrijven van patiënten die een GIST hebben uitgaande van de dunne darm. Er zijn recent geen studies gepubliceerd over deze specifieke zeldzame patiëntengroep. De meeste studies die patiënten met GIST beschrijven doen dit aan de hand van cohorten die bestaan uit patiënten met verschillende lokalisaties van GIST. Tussen 2009 en 2016 werden 201 patiënten met een GIST uitgaande van de dunne darm geregistreerd in de database. Hiervan waren 138 (69%) patiënten gediagnosticeerd met lokale ziekte en 63 (31%) met uitgezaaide ziekte. Ongeveer 19% van de patiënten onderging een spoedoperatie. In 22% van de gevallen werd de tumor bij toeval ontdekt. Patiënten die een hoog risico hadden op een recidief bij gelokaliseerde ziekte bleken een duidelijke lagere kans op recidief te hebben (4 uit 31 patiënten kreeg een recidief) met aanvullende imatinib behandeling na de operatie (adjuvant) dan patiënten die dit niet hadden gekregen (20 uit 31, $p<0.01$). De meerderheid van de patiënten die deze aanvullende behandeling niet kreeg zou dat nu wel krijgen, maar toentertijd was dit nog geen standaard dagelijkse praktijk. Progressie van ziekte werd gezien tijdens palliatieve behandeling (gericht op stabiliseren van de ziekte) met imatinib in 23 (28%) patiënten na een mediane behandelduur van 20.7 (range 1.8-47.1) maanden. Een aanhoudende respons werd bereikt in 52 van 82 patiënten met een mediane behandelduur van 30.6 (range 2.5 – 155.3) maanden met de palliatieve behandeling met imatinib. Uit deze studie blijkt dat patiënten met een dunne darm GIST zich vaker presenteren met uitgezaaide ziekte dan patiënten met een GIST uitgaande van de maag. Mogelijk is er een voordeel voor patiënten met uitgebreide ziekte die chirurgische resectie ondergaan, echter onze data zijn hierin niet conclusief. Wij zouden graag zien dat alle patiënten met een dunne darm GIST prospectief geregistreerd worden om onder andere de rol van chirurgie in patiënten met beperkte gemetastaseerde ziekte te kunnen beoordelen.

In **HOOFDSTUK 7** is een patiënt die verdacht werd van een GIST beschreven. Er was reeds een biopsie van de tumor afgesproken echter verslechterde de toestand van de patiënt acuut. Een CT-scan liet massale longembolieën zien waarvoor bloedverdunners gestart moesten worden. Hierdoor kon een biopsie niet veilig meer worden verricht. Uit het afgenomen bloed van de patiënt werd DNA geïsoleerd en geanalyseerd met next generation sequencing. Dit liet een mutatie zien in PDGFRα (NM_006206.5: c.2524_2532del; p.D842_M844del). Deze mutatie was eenmaal eerder gerapporteerd in de COSMIC database.4 Voor deze mutatie werd een specifieke ddPCR assay ontwikkeld en gebruikt voor het monitoren van de respons tijdens behandeling. Samples afgenomen op baseline, week 1, 3, 4, 8 en 16 na start van de behandeling werden geanalyseerd. Dit liet een geleidelijke afname van het aantal fragmenten met de mutatie zien. De resultaten van ctDNA analyse in het plasma van deze patiënt kwamen sterk overeen met de respons zoals die werd gemeten met een PET/CT-scan. Mede door deze resultaten denken wij dat de detectie en kwantificatie

van ctDNA een mogelijk alternatief is voor het monitoren van de respons op behandeling en tevens gebruikt zou kunnen worden in een diagnostische setting.

Concluderend laat dit proefschrift zien dat het gebruik van ctDNA in patiënten met GIST maar ook in het algemeen veelbelovend is. Echter, voordat het in de dagelijkse praktijk gebruikt kan worden zullen de voordelen met betrekking tot de progressie vrije – en algehele overleving aangetoond moeten worden in toekomstige studies.

A

Dankwoord

Dit proefschrift had nooit tot stand kunnen komen zonder de hulp van een groot aantal mensen, een aantal daarvan wil ik hierbij graag persoonlijk bedanken.

Uiteraard was zonder deelname van de patiënten, die vaak in een vergevorderd stadium van de ziekte nog openstonden voor een studie waar ze zelf niks meer aan zouden hebben, dit hele onderzoek op niks uitgelopen. Voor deze medewerking mijn grootse dank. Samen hebben we zo de wereld een klein stukje beter gemaakt.

Geachte **prof. dr. A.K.L. Reyners**. Beste An, Jij nam mij aan voor deze studie vanuit Amsterdam omdat je iemand nodig had die het een en ander kon regelen. Ik hoop dat je uiteindelijk niet teleurgesteld bent, maar ik heb goede hoop dat het meevalt. Het was heel prettig om samen met jou aan dit onderzoek te werken. Je was vrijwel altijd bereikbaar voor overleg, even een kopje koffie of zomaar wat smalltalk. Heel erg bedankt voor je begeleiding de afgelopen jaren, vaak goed gemutst. Tenslotte bedankt voor de vrijheid die je mij hebt gegeven ten aanzien van het thuiswerken in Amsterdam. Ik hoop dat je nog lang van de tuinkabouters zal genieten en wellicht werken we in de toekomst nog eens samen.

Geachte **prof. dr. E. Schuuring**, beste Ed, jouw scherpe commentaar en goede tips hebben mij vaak op weg geholpen met de papers. Overdag was het soms lastig om een afspraak met je te maken, maar na 17 uur wanneer het 'productiewerk' gedaan was, had je alle tijd, maar dan ook echt alle tijd om te praten over de papers, nieuwe onderzoeken en in welke nieuwe commissies, stuurgroepen, of onderzoeken je weer betrokken was geraakt. Ik heb er erg veel respect voor hoe jij een miljoen ballen tegelijk de lucht in kan houden, daarnaast nog wetenschap op hoog niveau bedrijft, praatjes houdt, deelneemt aan projecten en ook nog een gezin runt. Gelukkig lijdt je nachtrust er niet onder (want daar doe je niet aan :-)). Het samen bier drinken in New Orleans ergens in een megadruk jazzcafe op Bourbon Street is voor mij een mooie herinnering.

Neeltje, bedankt voor je hulp bij het opzetten van de logistiek van de studie in het AvL. Fijn dat je mijn co-promotor wilde zijn en bedankt voor de opbouwende kritiek op mijn stukken. Daarnaast was het voor mij heel prettig dat ik frequent op vrijdag een werkplek kon krijgen in het AvL zodat ik niet naar Groningen hoefde te reizen.

Graag dank ik de leden van de leescommissie; prof.dr. R.K. Weersma, prof.dr. J.W.M. Martens en prof.dr. P. Schöffski voor het beoordelen van dit proefschrift.

Roomies van Y3 (Lars, Thijs, Lotte, Maaike, Kirsten, Iris), een mooie tijd hebben we gehad. Als eerste dank voor de vissen, wat hebben we daar een hoop lol mee gehad. Lars, ik zal jouw gezeur over pietepeuterige kleine dingen missen, daarnaast is jouw statistische kennis groots, zonder jouw hulp en het ontwikkelen van de BCS-score (pas na de verdediging hier vragen over stellen svp) was mijn promotie op een mislukking uitgelopen. Iris en Thijs, mijn kennis over drugsgebruik heeft een vlucht genomen de afgelopen jaren dankzij jullie. Slammend het weekend door werd het nieuwe binge drinken. Thijs, weekendje Ameland, wat was dat goed geregeld. Lotte, goed dat jij er was om ons ook nog een beetje aan het werk te houden, de koffiemomenten liepen soms wat uit de hand, maar gelukkig was jij daar om de tijd te bewaken. Kirsten, onze gezamenlijke randstedelijke afkomst was altijd wel voer voor een beetje niet-werkgerelateerde praat. Ideaal voor als het schrijven weer eens niet wilde lukken. Uiteraard wil ik ook de collega's van het rivaliserende U4 bedanken voor de leuke tijd (de 'melding' over de vissen zal ik nooit vergeten), activiteiten, weekendjes weg en bier+worst feesten.

Arja, bedankt voor de hulp en het geduld bij het uitleggen van de NGS, gelukkig kon je mij altijd wel weer op weg helpen hiermee. De dinsdagmiddag meetings waren altijd nuttig en de weddenschappen om chocola waren een goed idee.

Marco, Anneke, Lisette en Jill, dank voor alle experimenten die jullie voor mij gedaan hebben. Ook de uitleg van voor jullie normale technieken deden jullie altijd rustig en duidelijk, ook als ik voor de 100e keer vroeg hoe het ook alweer zat met die ddPCR probes en wat nou precies een quencher was. Dank voor jullie inzet en de nauwkeurigheid waarmee jullie werken, als ik het allemaal zelf had moeten doen dan was er helemaal niets van terecht gekomen. Succes met jullie verdere (wetenschappelijke) carrieres!

Verder dank ik iedereen met wie ik in het UMCG heb gewerkt, Gerry, gelukkig was jij vrijwel altijd beschikbaar als ik er weer eens een paar dagen tussenuit was. Het blijft bijzonder dat precies altijd dan alles uit de hand liep. Ik vergeet niet meer dat ik in de rij stond voor het vliegtuig naar Nepal en ineens een mail kreeg dat het AvL mee wilde doen aan de studie, wat nu? Gelukkig is alles goed gekomen. Ik hoop dat de M&M's je zullen smaken!

Ik dank ook de mensen uit de andere centra (AvL (Sheima), LUMC (prof. Gelderblom), ErasmusMC (prof. Matthijssen), RadboudUMC (dr. Desar)) voor de prettige samenwerking.

Paul van der Leest en Michel van Kruchten bedankt voor de hulp bij het afmaken van hoofdstuk 2 en 3. Het heeft geresulteerd in mooie papers!

Koos, we kwamen elkaar al aan het begin tegen, samen de verplichte vinkjes halen bij de pathologie introductie. We hebben regelmatig naast het werk afgesproken en vaak koffie gedronken. Ik heb dat altijd erg gewaardeerd. Dank voor de keren dat ik bij jullie mocht slapen, dat maakte het voor mij een stuk beter te doen. Daarnaast vond ik het ook leuk om je te leren hoe je PES moest spelen… Mooi dat we samen begonnen en ongeveer tegelijk onze promotie afronden. Succes met je verdere opleiding tot MKA-chirurg.

Graag wil ik al mijn collega's in het Isala van de maag-darm-leverziekten en interne geneeskunde noemen. Jullie zijn een groot deel van mijn werkplezier en maken dat ik elke dag met een glimlach naar het ziekenhuis ga.

Wasemkapf, vrienden voor altijd. De dingen die we samen hebben meegemaakt zal ik nooit meer vergeten. Wat vind ik het fijn om jullie nog regelmatig te spreken en laten we ondanks de inmiddels forse afstanden en drukke gezinslevens proberen om jaarlijkse een bijkomweekendje te organiseren.

Heeren van S.T.A.N.D. dank voor de vriendschap die we in de afgelopen jaren hebben opgebouwd. Het blijft mooi om met al onze verschillende achtergronden te discussiëren over diverse onderwerpen onder het genot van een corona (…) ergens in de jacuzzi of aan de mooie Amsterdamse grachten. Ik hoop dat we de traditie nog lang kunnen voortzetten.

Pieter van der Kolk, waar moet ik beginnen. Je maakt uiteraard deel uit van Wasemkapf, maar toch wil ik je als paranimf nog speciaal noemen. Al vanaf het begin van de studententijd kruisen onze wegen; na al geruime tijd samengewoond te hebben dan toch nog even samen op 'kamers' in Groningen. Het troosteloze appartement wat wij daar bewoonden was nog enigszins te doen door elkaars aanwezigheid. Lekker op de kringloopwinkel bank gebakken aardappeltjes met boomstammetjes eten achter de TV of een beetje ouwehoeren, gelukkig voor ons beiden (en onze partners) heeft dit niet al te lang geduurd.

Lars, ook al was het afgelopen jaar een rare tijd voor je, ik vind het fijn dat je ook mijn paranimf wil zijn. We hebben een hoop gelachen op de kamer en ik heb ook veel van je geleerd. Structuur, structuur en nog eens structuur. Als bij jou de paperclips niet op de juiste plek lagen, kon je hele dag mislukken. Jij was mijn leermeester in R en als ik weer eens zat te klungelen had je het meestal vrij vlot opgelost. Mooi dat ik mijn auto bij jou kon droppen naast het ziekenhuis. Ik hoop dat je je promotie ondanks alles ook snel kan afronden en door kan gaan knallen voor de volgende titel….

Papa, mama, Mark, Hans, Jeroen. Bedankt voor jullie altijd aanwezige steun en hulp. Het maakt niet uit waarmee, maar als er iets moet gebeuren zijn jullie er. Bij jullie

kan ik altijd terecht en jullie zijn erg waardevol voor mij, ik hoop dat we nog lang van elkaar mogen genieten.

Dorothée, je begrijpt nog niet wat er allemaal gebeurt en je mag er ook niet bij zijn vandaag, maar jij bent een van de grootste drijfveren om dit proefschrift dan eindelijk af te maken. Ik kijk er naar uit om samen met jouw en je nieuwe broertje dingen te gaan ondernemen.

Als allerlaatste en meest belangrijke; Lilian, super bedankt voor alle steun de afgelopen jaren. De ontbijtjes die je voor mij maakte als ik om half zes onder de douche sprong om de trein te halen zorgden ervoor dat ik weer met frisse moed de reis naar het hoge noorden ondernam. Ik was veel van huis en had niet altijd het beste humeur als ik thuis kwam maar je bleef en blijft mij altijd steunen. Ik hou van jou, je bent een topper en ik hoop nog lang samen met jou, Dorothée en de nieuwe spruit van elkaar en de wereld te kunnen genieten. Liefs.