PERSONALIZED CANCER MEDICINE GUIDED BY LIQUID BIOPSIES

NICK BEIJE

The studies described in this thesis were performed within the framework of the Erasmus MC Molecular Medicine (MolMed) Graduate School at the department of Medical Oncology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands.

The work in this thesis was financially supported by Cancer Genomics Netherlands (CGC.nl)/Netherlands Organization for Scientific Research (NWO), the Dutch Cancer Society (KWF; grant numbers DDHK 2010-4678, EMCR 2012-5390, EMCR 2014-6340), Stichting Coolsingel (project #319), Janssen Diagnostics, Roche Netherlands and Pink Ribbon (project WO 61).

Financial support for this thesis was generously provided by the Department of Medical Oncology of the Erasmus MC Cancer Institute, Erasmus University Rotterdam, Pfizer, AstraZeneca, Boehringer Ingelheim, Kyowa Kirin and Thermo Fisher Scientific.

ISBN 978-94-028-0449-2

Cover design: Mirjam Leppers, Persoonlijkproefschrift.nl

Layout: Mirjam Leppers, Persoonlijkproefschrift.nl

Printing: Ipskamp Printing, Enschede

Copyright © 2016, N.Beije, Rotterdam, The Netherlands.

All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording or any other information storage or retrieval system, without prior written permission of the copyright owner.

PERSONALIZED CANCER MEDICINE GUIDED BY LIQUID BIOPSIES

Een op maat behandeling van kanker op basis van "vloeibare biopsies"

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de

rector magnificus

prof. dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 1 februari 2017 om 15.30 uur

Nick Beije

geboren te Rotterdam

Erasmus University Rotterdam

Erasmus

Promotiecommissie:

Promotor:	**Prof. dr. S. Sleijfer**
Overige leden:	**Prof. dr. J.G.J.V. Aerts**
	Prof. dr. M.J. van den Bent
	Prof. dr. H.M. Verheul
Copromotor:	**Dr. J.W.M. Martens**

CONTENTS

CHAPTER 1	**Introduction**	11
CHAPTER 2	**Circulating tumor cell enumeration by the CellSearch system: The clinician's guide to breast cancer treatment?** *Cancer Treat Rev. 2015 Feb;41(2):144-50*	23
CHAPTER 3	**Prevalence and prognostic impact of HER2 and ER status of circulating tumor cells in metastatic breast cancer patients with a HER2-negative primary tumor** *Neoplasia. 2016 Oct 17;18(11):647-653*	43
CHAPTER 4	**ESR1 mutations: moving towards guiding treatment decision-making in metastatic breast cancer patients** *Cancer Treat Rev. 2016 in press*	63
CHAPTER 5	**Estrogen receptor mutations and splice variants determined in liquid biopsies from metastatic breast cancer patients** *Submitted*	87
CHAPTER 6	**Application of circulating tumor DNA in prospective clinical oncology trials: standardization of pre-analytical conditions** *Mol Oncol. 2016 in press*	113
CHAPTER 7	**Somatic mutation detection using various targeted detection assays in paired samples of circulating tumor DNA, primary tumor and metastases from patients undergoing resection of colorectal liver metastases** *Mol Oncol. 2016 in press*	135

CHAPTER 8 Exploration of biomarkers for diagnosis and prognostication in pleural effusion and peripheral blood from patients with pleural malignant mesothelioma
Submitted 167

CHAPTER 9 Prognostic value and kinetics of circulating endothelial cells in patients with recurrent glioblastoma randomised to bevacizumab plus lomustine, bevacizumab single agent or lomustine single agent
Br J Cancer. 2015 Jul 14;113(2):226-31 189

CHAPTER 10 Circulating endothelial cell enumeration demonstrates prolonged endothelial damage in recipients of myeloablative allogeneic stem cell transplantation
Haematologica. 2015 Jun;100(6):e246-9 203

CHAPTER 11 Summary and general discussion 219

APPENDICES Nederlandse samenvatting 243

Curriculum vitae 245

PhD portfolio 255

List of publications 259

Dankwoord 267

CHAPTER 1

General introduction and outline of the thesis

INTRODUCTION

THE WAR ON CANCER

Cancer is the leading cause of death in the Netherlands (1), and amongst the leading causes of death in the world (2). Now 45 years ago, the importance of performing cancer research in a collective and collaborative way to beat cancer was first recognized. In 1971, President Nixon of the United States of America signed the National Cancer Act, allocating more funds towards cancer research, which was dubbed as the start of "the war on cancer". Since then numerous success stories in the treatment of cancer have been described, for example in the cure of acute lymphocytic leukemia and other childhood cancers, Hodgkin's lymphoma, and testicular cancer. Meaningful improvements have also been achieved by the early detection and adjuvant therapy for a variety of cancers. These success stories have led to significant improvements in 5-year overall survival for patients with cancer (3, 4). However, despite all these improvements and an enormous progress of our understanding of cancer, cancer remains to be a major cause of death.

LESSONS LEARNED

Our increased understanding of cancer has led to important observations that explain why it is so difficult to adequately treat cancer. First of all, it is important to realize that cancer mortality is generally not caused by the primary tumor, but by the formation of distant metastases. Current treatment of patients with metastatic cancer is generally driven by the characteristics of the primary tumor. However, an important lesson was that tumors are plastic: their characteristics change over time and under treatment pressure. This is for example reflected by comparing clinically actionable targets in breast cancer such as ER and HER2 between the primary tumor and the metastases. These targets for treatment differ between the primary tumor and metastases in 10-20% of the patients, leading to a change in patient management (5, 6). Another example of tumor plasticity is the fact that tumor cells acquire resistance against anti-tumor agents, for example due to resistance mutations. This was for example demonstrated in patients with lung cancer progressing on first-line tyrosine kinase inhibitors, in whom a mutation in the *EGFR* gene (T790M mutation) contributes to resistance to these therapies (7). These observations underlined that it is of utmost importance to evaluate the tumor cells, not only at diagnosis or before treatment, but also during treatment and at treatment progression.

Another important lesson we learned was that tumors are highly heterogeneous, even down to the single cell level (8-13). This implies that targeting cancer cells with a specific "magic bullet" (targeted agents, such as trastuzumab and vemurafinib) is unlikely to eradicate all the tumor cells. Indeed, this is what is generally observed in clinical practice: in the best case scenarios, an initial response is observed in a large portion of the patients treated with targeted agent, however, resistance to therapy will eventually almost always arise. An example is the use of vemurafinib in patients with *BRAF* V600E-mutated metastatic melanoma, for which in the first study a spectacular objective response rate (ORR) of 48% was seen (14), compared to 5% ORR in patients treated with traditional chemotherapy. However, after 9 months of treatment virtually all patients in this study had progressed on vemurafinib.

All these data stress that there is an urgent need for markers to improve diagnosis, prognostication, and prediction for patients with cancer, and these markers should preferentially be available sequentially under therapy. An obvious way to gain insight into the problem of plasticity of tumor cells in patients with metastatic cancer could be to obtain biopsies from metastatic tumor tissue. However, not only is this often a cumbersome and patient-unfriendly procedure and impossible in some patients due to inaccessible metastatic lesions, it is also a procedure that cannot realistically be performed sequentially during therapy. In addition, intra-tumor and inter-metastatic heterogeneity may be missed by performing single biopsies, as elegantly demonstrated by recent next-generation sequencing efforts (8, 13, 15-17). If tumor heterogeneity and plasticity could be assessed in a minimally invasive way, during the course of treatment, this could prove to be a huge step forward in oncology. The use of *liquid biopsies* sampled from the blood of patients with cancer is therefore a promising way to evaluate tumor characteristics and response to therapy repeatedly during therapy.

LIQUID BIOPSIES OF CANCER

Several types of liquid biopsies of solid tumors have been described in the past decades. All of them have distinct characteristics, and they may be used alone or in combination. The purpose of these liquid biopsies generally are to sample the tumor cells themselves, however also endothelial cells that surround the tumor cells can be shed into the circulation and sampled. This thesis will focus on three important kinds of liquid biopsies: circulating tumor cells (CTCs), circulating endothelial cells (CECs) and circulating tumor DNA (ctDNA). While these liquid biopsies can all be measured in the blood, CTCs and ctDNA may be measured in other bodily fluids as well, such as pleural effusions, ascites or cerebrospinal fluid. Of note is that there are other liquid biopsies that are used in oncology, for example circulating exosomes, tumor-educated blood platelets and serum-derived biomarkers (such as CEA, CA15.3, etcetera), but these biomarkers will be not be discussed in this thesis. The liquid biopsies discussed in this thesis are presented in **Figure 1**, and explained in more depth below.

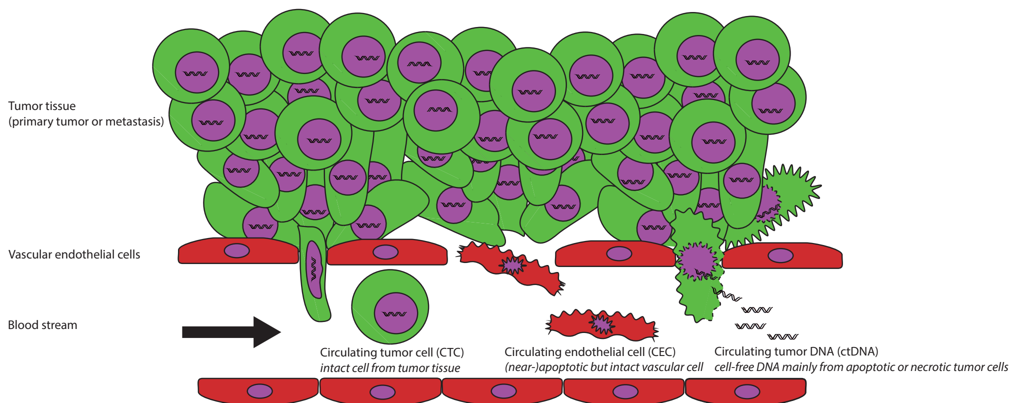


FIGURE 1. LIQUID BIOPSIES DISCUSSED IN THIS THESIS

CIRCULATING TUMOR CELLS (CTCS)

CTCs are tumor cells derived from solid tumors detectable in the peripheral blood of cancer patients. It is thought that in patients with metastatic disease, CTCs represent characteristics of the metastases (18, 19). While a plethora of assays are currently commercially available to isolate CTCs (as reviewed by my colleagues (20, 21)), the CellSearch system (Janssen Diagnostics, Raritan, NJ, USA) is the only system that is approved by the Food and Drug Administration (FDA) for diagnostic purposes in breast cancer, prostate cancer and colorectal cancer. This system relies on the enrichment of EpCAM-positive tumor cells by using magnetic beads coupled to an anti-EpCAM antibody. Using the CellSearch system, the enumeration of CTCs before the start of treatment has robust prognostic value as has been demonstrated for numerous tumor types in the non-metastatic and the metastatic setting (22-25). Also changes during treatment, especially in the metastatic setting, have strong prognostic value (22-25). However, prognostic value and clinical validity of CTC enumeration do not necessarily translate into clinical utility. This is also reflected by the fact that the use of CTC enumeration is currently not advised in any of the ASCO or ESMO guidelines for any clinical decision in any tumor type. However, counting of CTCs only touches upon the surface of the possibilities with CTCs, as CTCs are bona fide tumor cells and thus express proteins and contain DNA and RNA. Therefore, CTCs have great promise to be used as a tool to characterize (metastatic) tumor cells multidimensionally during the course of treatment. While this is an attractive premise, characterization of CTCs is however hampered by the rarity of CTCs (median of 5 cells in patients with metastatic breast cancer) and the lack of purity of CTCs caused by the high background of leukocytes after using CellSearch-based enrichment (ratio of CTCs to leukocytes approximately 1:1000 (26)). Our group has previously demonstrated that regardless of these limitations, characterization of CTCs is feasible at the RNA (26-29) and DNA level (30). While CTC characterization is considered to be of huge interest to characterize tumor cells sequentially under therapy, its clinical relevance as a prognostic, predictive or response marker is currently still limited to the androgen receptor (AR) splice variant V7 (31-34).

CIRCULATING TUMOR DNA (CTDNA)

Tumor cells undergoing apoptosis release DNA fragments, while viable tumor cells may actively secrete DNA fragments. This DNA can then be detected in the blood stream as cell-free DNA (cfDNA). Cell-free DNA circulates in the blood stream and can be acquired by isolating DNA from plasma or serum. Subsequently, downstream analyses are needed to detect ctDNA. Nowadays, next generation sequencing(NGS)-based methods and digital PCR(dPCR)-based methods are most frequently used to detect ctDNA in a background of wildtype DNA. While CTCs have the great advantage of multidimensional characterization (protein, DNA, RNA level), the advantage of ctDNA over CTCs is the ease of collection and processing of blood for analysis, in addition to better options for high-throughput analysis (35). Also ctDNA seems to be more frequently present than CTCs in blood (36). This is also reflected by results obtained with techniques based on dPCR; these techniques are able to detect mutant ctDNA in about 47-69% of the patients with stage I-III cancers, while mutant ctDNA can be detected in about 82% of the patients with metastatic stage IV cancer (37). The capture frequency for CTCs across all tumor types is typically much lower. While the first years of ctDNA research have yielded highly promising results, standardization of pre-analytical conditions, the

choice of the optimal test to answer specific research questions, the exact diagnostic, prognostic or predictive value and optimal clinical utility of ctDNA are important matters that have not been crystallized as of yet.

CIRCULATING ENDOTHELIAL CELLS (CECS)

CECs are cells that are present in peripheral blood and are thought to originate from the vessel wall, reflecting the extent of angiogenesis or endothelial damage. CEC levels are increased in patients with different types of malignancies as opposed to healthy donors (38) and are thought to reflect damage coming from normal as well as tumor vasculature (38, 39). As CECs only possess endothelial characteristics (and not tumor characteristics, even if they originate from tumor vasculature), they cannot be used to assess tumor plasticity or heterogeneity. However, they may be an interesting surrogate marker for tumor response to therapy, especially in patients with tumors that are highly vascularized, or in patients receiving drugs with anti-angiogenic activities (e.g. bevacizumab, sunitinib, everolimus). In addition, they may be a compelling marker for vascular damage in general, also outside of the field of oncology, for example in cardiology or transplantation medicine. Because CECs in patients with cancer are derived from two compartments (the normal endothelium and tumor endothelium compartment), interpretation of CEC enumeration before treatment and changes during treatment is hampered by a lack of information on how these compartments change relative to each other. Recently, we identified a CEC marker (CD276) that is able to detect CECs that are shed from the tumor vasculature in a number of human tumors (40). The use of this marker has great potential to increase the specificity of the CEC test to identify true tumor-associated CECs. However, clinical data on the true clinical relevance of CD276-positive CECs is currently lacking.

CHALLENGES WITH LIQUID BIOPSIES

For liquid biopsies to be truly used to personalize treatment of cancer patients, what is needed are technically sound assays to measure these biomarkers with acceptable sensitivity and specificity, and these assays should at least have the potential to improve clinical decision-making. The three liquid biopsies as discussed in this thesis are all in different stages of development and implementation, meaning that various research questions emerge in this thesis. CTC enumeration is as of now the most developed biomarker: enumeration of CTCs has high reproducibility (41), high inter-reader agreement (42) and clear prognostic value and clinical validity (25). However, the exact clinical utility of enumerating CTCs remains to be established. CTC characterization has been shown to be feasible, but its clinical relevance is still limited to AR-V7 (31-34). For CECs, reports on their prognostic value have been inconsistent, probably due to different assays used to detect CECs (38) and presence of CECs from non-tumorous vasculature. Data on tumor-specific CEC markers have not been published as of yet. Therefore, prospective trials evaluating CECs and tumor-associated CECs using a robust assay are of utmost importance to further evaluate the role of CECs as a prognostic marker in oncology. Circulating tumor DNA is the marker that has most recently been widely introduced, yielding exiting data but still a lot of unanswered questions are present regarding the validity of ctDNA tests, their optimal use, clinical relevance and clinical utility.

FOCUS OF THIS THESIS

This thesis focusses on several technical and clinical aspects of liquid biopsies, all with the ultimate aim to allow personalized medicine based on these liquid biopsies that are able to markedly improve the clinical outcome of cancer patients.

In **Chapter 2**, current evidence on the clinical validity of CTC enumeration using CellSearch in primary and metastatic breast cancer is reviewed, and recommendations for the use of CTCs are provided.

These CTCs enumerated by the CellSearch system can be directly analyzed for clinically relevant markers such as HER2. Metastatic breast cancer patients who have a HER2-positive primary tumor, have poorer outcome on endocrine therapy. In **Chapter 3**, the prognostic relevance of HER2-positive CTCs in patients with an HER2-negative primary tumor is investigated, with a special interest in those patients receiving endocrine therapy. In addition, CTC characterization on the mRNA level is performed to assess the ER status of the CTCs and to explore its clinical relevance.

Beyond classical predictive factors in breast cancer such as HER2 and ER, recently the occurrence of resistance mutations in the gene coding for the estrogen receptor, *ESR1*, has sparked a lot of interest. **Chapter 4** reviews data on *ESR1* mutations in metastatic breast cancer patients, with a particular interest in the techniques to detect them, their functional role and clinical relevance.

Chapter 5 further examines the role of *ESR1* mutations in patients with metastatic breast cancer. The concept of CTC characterization is taken a step further, with molecular characterization of CTCs not only at the mRNA level, but also at the DNA level. *ESR1* mutations are assessed in CTCs and cfDNA in a cohort of patients starting first-line endocrine therapy and in a cohort of patients progressing on endocrine therapy. Also *ESR1* splice variants are assessed in both cohorts.

ESR1 mutations can be assessed in cfDNA. But before large prospective multicenter studies can be initiated on the predictive value of *ESR1* mutations using cfDNA, it is of utmost importance to determine the right pre-analytical conditions under which cfDNA is collected. In **Chapter 6**, different time intervals to plasma collection and different blood tubes are tested to optimize and standardize blood collection for cfDNA analysis.

When cfDNA is optimally collected, assessing mutations in cfDNA can provide information on primary and acquired resistance to therapies. However, several techniques to assess these mutations in ctDNA have been described, all with their own specific pros and cons. In **Chapter 7**, three targeted techniques to assess mutations in ctDNA are described and compared in colorectal cancer patients undergoing a resection of liver metastases.

Malignant pleural mesothelioma is a highly treatment-resistant malignancy, leading to a very poor prognosis. Its diagnosis is challenging, and prognostic markers are lacking. In **Chapter 8**, efforts are undertaken to improve the diagnosis of mesothelioma by using an alternative (MCAM-based) approach to enumerate CTCs. In addition, MCAM-positive CTCs and CECs are explored as novel biomarkers to improve prognostication of patients with mesothelioma.

In **Chapter 9**, another treatment-resistant malignancy, malignant glioblastoma, is investigated. As glioblastomas are highly vascularized, and anti-angiogenic treatments are given in patients with glioblastoma, the enumeration of CECs as a prognostic or response marker is evaluated in this chapter.

In **Chapter 10**, an application of CECs outside of the field of oncology is explored. As vascular damage may be related to complications after allogeneic stem cell transplantation, the use of CECs as a marker for these complications was evaluated.

Finally, **Chapter 11** discusses on current state and future of liquid biopsies and provides a summary of the thesis.

REFERENCES

1. Overledenen; belangrijke doodsoorzaken. Centraal Bureau voor de Statistiek. 2014.

2. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012 Dec 15;380(9859):2095-128.

3. Howe HL, Wingo PA, Thun MJ, Ries LA, Rosenberg HM, Feigal EG, et al. Annual report to the nation on the status of cancer (1973 through 1998), featuring cancers with recent increasing trends. J Natl Cancer Inst. 2001 Jun 6;93(11):824-42.

4. Verdecchia A, Francisci S, Brenner H, Gatta G, Micheli A, Mangone L, et al. Recent cancer survival in Europe: a 2000-02 period analysis of EUROCARE-4 data. Lancet Oncol. 2007 Sep;8(9):784-96.

5. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol. 2012 Feb 20;30(6):587-92.

6. Chia S. Testing for discordance at metastatic relapse: does it matter? J Clin Oncol. 2012 Feb 20;30(6):575-6.

7. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med. 2005 Feb 24;352(8):786-92.

8. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012 Mar 8;366(10):883-92.

9. Shah SP, Roth A, Goya R, Oloumi A, Ha G, Zhao Y, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature. 2012 Jun 21;486(7403):395-9.

10. Baca SC, Prandi D, Lawrence MS, Mosquera JM, Romanel A, Drier Y, et al. Punctuated evolution of prostate cancer genomes. Cell. 2013 Apr 25;153(3):666-77.

11. Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. Proc Natl Acad Sci U S A. 2013 Mar 5;110(10):4009-14.

12. Wang Y, Waters J, Leung ML, Unruh A, Roh W, Shi X, et al. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature. 2014 Aug 14;512(7513):155-60.

13. Yates LR, Gerstung M, Knappskog S, Desmedt C, Gundem G, Van Loo P, et al. Subclonal diversification of primary breast cancer revealed by multiregion sequencing. Nat Med. 2015 Jul;21(7):751-9.

14. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011 Jun 30;364(26):2507-16.

15. Nik-Zainal S, Van Loo P, Wedge DC, Alexandrov LB, Greenman CD, Lau KW, et al. The life history of 21 breast cancers. Cell. 2012 May 25;149(5):994-1007.

16. Gundem G, Van Loo P, Kremeyer B, Alexandrov LB, Tubio JM, Papaemmanuil E, et al. The evolutionary history of lethal metastatic prostate cancer. Nature. 2015 Apr 16;520(7547):353-7.

17. Lipinski KA, Barber LJ, Davies MN, Ashenden M, Sottoriva A, Gerlinger M. Cancer Evolution and the Limits of Predictability in Precision Cancer Medicine. Trends Cancer. 2016 Jan;2(1):49-63.

18. Lohr JG, Adalsteinsson VA, Cibulskis K, Choudhury AD, Rosenberg M, Cruz-Gordillo P, et al. Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. Nat Biotechnol. 2014 May;32(5):479-84.

19. Onstenk W, Sieuwerts AM, Mostert B, Lalmahomed Z, Bolt-de Vries JB, van Galen A, et al. Molecular characteristics of circulating tumor cells resemble the liver metastasis more closely than the primary tumor in metastatic colorectal cancer. Oncotarget. 2016 Jun 20.

20. Mostert B, Sleijfer S, Foekens JA, Gratama JW. Circulating tumor cells (CTCs): detection methods and their clinical relevance in breast cancer. Cancer Treat Rev. 2009 Aug;35(5):463-74.

21. Onstenk W, Gratama J, Foekens J, Sleijfer S. Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. Cancer Treat Rev. 2013;39(7):691-700.

22. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004 Aug 19;351(8):781-91.

23. Cohen S, Punt C, Iannotti N, Saidman B, Sabbath K, Gabrail N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26(19):3213-21.

24. de Bono J, Scher H, Montgomery R, Parker C, Miller M, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(19):6302-9.

25. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014 Apr;15(4):406-14.

26. Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, Mostert B, Martens JW, et al. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. Breast Cancer Res Treat. 2009 Dec;118(3):455-68.

27. Mostert B, Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, van Galen A, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. Ann Oncol. 2015 Mar;26(3):510-6.

28. Onstenk W, Sieuwerts AM, Weekhout M, Mostert B, Reijm EA, van Deurzen CH, et al. Gene expression profiles of circulating tumor cells versus primary tumors in metastatic breast cancer. Cancer Lett. 2015 Jun 28;362(1):36-44.

29. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res. 2011 Jun 1;17(11):3600-18.

30. Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-de Vries J, Biermann K, et al. KRAS and BRAF mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue. Int J Cancer. 2013 Jul;133(1):130-41.

31. Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Nakazawa M, et al. Androgen Receptor Splice Variant 7 and Efficacy of Taxane Chemotherapy in Patients With Metastatic Castration-Resistant Prostate Cancer. JAMA Oncol. 2015 Aug;1(5):582-91.

32. Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med. 2014 Sep 11;371(11):1028-38.

33. Onstenk W, Sieuwerts AM, Kraan J, Van M, Nieuweboer AJM, Mathijssen RHJ, et al. Efficacy of Cabazitaxel in Castration-resistant Prostate Cancer Is Independent of the Presence of AR-V7 in Circulating Tumor Cells. European urology. 2015 Dec;68(6):939-45.

34. Scher HI, Lu D, Schreiber NA, Louw J, Graf RP, Vargas HA, et al. Association of AR-V7 on Circulating Tumor Cells as a Treatment-Specific Biomarker With Outcomes and Survival in Castration-Resistant Prostate Cancer. JAMA Oncol. 2016 Jun 4.

35. Haber DA, Velculescu VE. Blood-Based Analyses of Cancer: Circulating Tumor Cells and Circulating Tumor DNA. Cancer discovery. 2014;4(6):650-61.

36. Dawson SJ, Rosenfeld N, Caldas C. Circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013 Jul 4;369(1):93-4.

37. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014 Feb 19;6(224):224ra24.

38. Kraan J, Sleijfer S, Foekens JA, Gratama JW. Clinical value of circulating endothelial cell detection in oncology. Drug Discov Today. 2012 Jul;17(13-14):710-7.

39. Strijbos MH, Gratama JW, Kraan J, Lamers CH, den Bakker MA, Sleijfer S. Circulating endothelial cells in oncology: pitfalls and promises. Br J Cancer. 2008 Jun 3;98(11):1731-5.

40. Kraan J, van den Broek P, Verhoef C, Grunhagen DJ, Taal W, Gratama JW, et al. Endothelial CD276 (B7-H3) expression is increased in human malignancies and distinguishes between normal and tumour-derived circulating endothelial cells. Br J Cancer. 2014 Jul 8;111(1):149-56.

41. Kraan J, Sleijfer S, Strijbos MH, Ignatiadis M, Peeters D, Pierga JY, et al. External quality assurance of circulating tumor cell enumeration using the CellSearch((R)) system: a feasibility study. Cytometry B Clin Cytom. 2011 Mar;80(2):112-8.

42. Ignatiadis M, Riethdorf S, Bidard FC, Vaucher I, Khazour M, Rothe F, et al. International study on inter-reader variability for circulating tumor cells in breast cancer. Breast Cancer Res. 2014;16(2):R43.

CHAPTER 2

Circulating tumor cell enumeration by the CellSearch system: the clinician's guide to breast cancer treatment?

Nick Beije, Agnes Jager
and Stefan Sleijfer

Cancer Treat Rev. 2015 Feb;41(2):144-50

ABSTRACT

Circulating tumor cells (CTCs) are cancer cells that are present in the blood of patients with solid cancers and are shed from existing tumor lesions into the blood stream. The enumeration of CTCs has long been considered to hold great promise in guiding treatment decision-making in breast cancer patients. However, guidelines on how to use CTC enumeration in clinical decision-making in primary breast cancer and metastatic breast cancer are lacking. Here, we set out to review the most relevant literature to date, to ultimately come to general recommendations regarding the use of CTC enumeration in primary breast cancer and metastatic breast cancer.

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed malignancy in women and the leading cause of cancer deaths in the world (1). Evidently, almost all BC-related deaths are caused by distant metastases and not by the primary breast tumor itself. Treatment strategies in primary BC are focused on preventing recurrence and preventing the development of distant metastatic disease. However, once BC has metastasized the treatment aims are to relieve or delay cancer related symptoms, thereby improving overall survival (OS) and quality of life. Multiple clinical and pathological features currently guide treatment decision making in BC. But disappointingly, the lack of reliable prognostic and predictive factors and imperfect tools to evaluate treatment success both in the primary and metastatic setting still results in serious overtreatment of patients. Novel strategies for determining prognosis and adequately monitoring treatment success in both primary and metastatic BC are therefore urgently needed.

Circulating tumor cells (CTCs) are tumor cells that circulate in the peripheral blood of patients with solid malignancies and are shed from an existing primary tumor or from metastatic lesions into the blood stream. Since its first description, CTC enumeration was envisioned to establish prognosis, to evaluate treatment success and to guide treatment decision making in BC. However, although CTC enumeration is increasingly used by some medical oncologists, clear guidelines as to the optimal use of CTC enumeration are still lacking. Here, we will review the most relevant published literature to date and discuss the current possibilities for clinicians to use CTC enumeration into daily clinical practice, at different stages of BC treatment. Additionally, we make recommendations regarding the use of CTC enumeration in primary and metastatic BC. Since most of the non-interventional studies have used CellSearch for CTC enumeration, the only FDA approved CTC enumeration method to be used for clinical purposes, only publications in which CellSearch enumeration was employed will be discussed.

CELLSEARCH ASSAY FOR CTC ENUMERATION

The CellSearch method **(Figure 1)** relies on the automatized immunomagnetic enrichment of epithelial tumor cells expressing EpCAM by adding magnetic ferrofluids coupled to anti-EpCAM antibodies to 7.5 mL of whole blood. The enriched sample is subsequently immunocytochemically stained for nucleated (DAPI positive) cells expressing cytokeratin 8/18/19, while contaminating leukocytes are excluded by using the pan-leukocyte marker CD45. Finally, a trained user identifies and counts all cells meeting the criteria for CTCs according to consensus guidelines (2) using an automatized fluorescence microscope.

The CellSearch method has been demonstrated to be highly specific and its performance is still robust at CTC counts as low as 1 CTC/7.5 mL of blood when made sure there is minimal inter-reader variability (3, 4). Consequently, minimal inter-(2) and intra-reader (5) variability were demonstrated using this system. If blood is collected in a CellSave tube which contains a mild fixative, CTCs are stabilized for up to 96 hours following blood draw and can be shipped by room temperature, which renders it feasible to ship a blood sample to remote locations.

In contrast to these clear advantages, also several disadvantages are associated with the use of CellSearch. Since the system relies on EpCAM-enrichment, tumor cells not

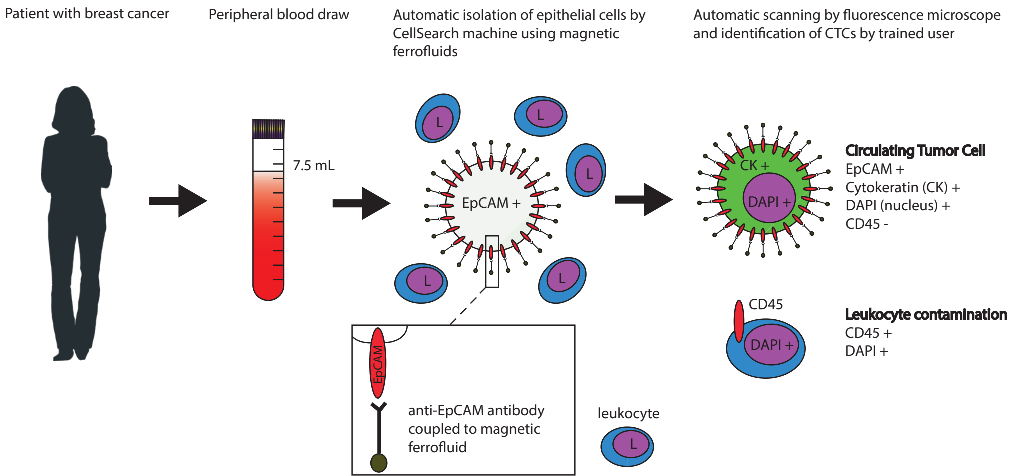


FIGURE 1. WORKFLOW OF CTC ENUMERATION BY CELLSEARCH

expressing EpCAM, in particular breast tumor cells exhibiting stem cell features (6), go uncaptured by using CellSearch. In addition, tumor cells may undergo epithelial-to-mesenchymal transition, which might lead to a decrease or loss of EpCAM expression (7). Consequently, these “mesenchymal CTCs” are probably also not captured by CellSearch based isolation of CTCs. CTCs lacking cytokeratin 8/18/19 expression may also go uncaptured when using the CellSearch system (8).

CTCS IN PRIMARY BREAST CANCER

Additionally to local treatment for the primary tumor, management of an individual primary BC patient is determined by the risk of distant metastases. Patients with primary BC who harbor a high risk for developing distant metastases are treated with neo-adjuvant or adjuvant systemic treatment. The risk estimate for developing distant metastases is currently based on multiple classical clinicopathological factors including patient characteristics such as younger age; as well as primary tumor characteristics such as large tumor size, high Bloom-Richardson differentiation grade and the presence of lymph node metastases. Additionally, predictive factors determined on the primary tumor like the presence of estrogen receptor (ER) expression and HER2 amplification are of key importance. In those patients with BC expressing the ER and HER2 receptor adjuvant treatment with endocrine treatment and anti-HER2 treatment, respectively, have been demonstrated to decrease BC recurrence rates substantially (9-11). However, following adjuvant therapies the absolute improvement in 15-year breast cancer mortality is relatively modest at 3-10% (9). These findings illustrate that many patients have to be treated to benefit a few and that many are unnecessarily exposed to significant toxicity impairing quality of life both in the short and long-term. These observations clearly stress the high need for robust prognostic factors in primary BC to prevent overtreatment.

Over the last years, commercially available molecular tests on primary breast tumor material determining the prognosis have become available and have been implemented in daily clinical practice to advice about the usefulness of adjuvant chemotherapy treatment (12, 13). CTC enumeration also has the potential to guide treatment decision making in primary BC. In primary BC, potential applications for CTC enumeration may include the identification of patients with a low probability of developing distant metastases in whom adjuvant systemic therapy may be omitted or patients with high probability of developing distant metastases in whom peri-operative systemic therapy may be intensified. In addition, CTC count changes could be used to evaluate treatment response, thereby possibly identifying non-responding patients at an early stage who could switch to non-cross-resistant treatment options.

NEOADJUVANT TREATMENT

Next to reducing BC recurrences, neoadjuvant chemotherapy is considered in patients who are diagnosed with primary BC in whom the goal is to shrink the tumor, enabling breast conserving surgery instead of a mastectomy or enabling a mastectomy in case of a large tumor (14). In addition, in patients in whom the indication to receive adjuvant chemotherapy is already present before surgery, neoadjuvant chemotherapy can be considered and is increasingly becoming the standard of care.

The clinical validity of CTC enumeration in neoadjuvant chemotherapy for BC was assessed by three studies. The REMAGUS02 trial (15) investigated 118 patients in whom CTCs were enumerated before and after neoadjuvant chemotherapy. At least one CTC was detected in 23% of all patients before treatment, and in 17% of all patients after treatment. While the occurrence of CTCs before and/or after chemotherapy was not associated with pathological complete response (pCR), tumor size, nodal status and tumor grade, patients without CTCs before and after treatment had a relative higher chance of 4.15 (95% CI 1.29-13.3, p=0.017) to maintain free from distant metastases in multivariate analysis, compared to patients in whom CTCs were present before and/or after treatment. Recently, a 70 month follow-up of the REMAGUS02 trial was published (16) demonstrating that the presence of CTCs before treatment was only borderline associated with OS (HR 3.0, 95% CI 1.0-9.5, p=0.05). Since the observed hazard ratios in this study decreased over time, it was suggested that the prognostic impact of CTC before treatment in the neoadjuvant setting seems to be limited to the first 3-4 years after treatment, limiting its potential clinical utility. It should however be noted that the REMAGUS02 analysis should be interpreted as purely exploratory since only 95 patients were analyzed and the occurrence of relapses and deaths were thus relatively rare (17% and 12%, respectively). After 70 months of follow-up CTC counts after neoadjuvant treatment were not associated with distant metastases-free survival or OS.

The GeparQuattro trial (17) comprised 213 patients with HER2-positive primary BC undergoing neoadjuvant chemotherapy. At least one CTC was present in 22% patients before treatment and in 11% of patients after treatment. In accordance with the REMAGUS02 trial was the absence of a correlation between CTC counts and pCR. However, in contrast to the REMAGUS02 study, the GeparQuattro trial did not observe any correlations between CTC counts and early relapse or OS. An explanation for the lack of correlation between CTC counts and clinical outcome could be that only patients with a HER2-positive tumor were included in this study. CTC enumeration might have

less prognostic value in patients with HER2-positive disease receiving trastuzumab (18-20). The HER2-positive subtype is however relatively rare compared to other BC subtypes and therefore all studies evaluating the prognostic value of HER2-positive disease suffer from subgroup analyses on small numbers.

The BEVERLY-2 study evaluated 52 patients with HER2-positive inflammatory breast cancer starting a bevacizumab-based neoadjuvant regimen (21). At baseline, 35% of patients had at least one detectable CTC , while at least one CTC was present in 13% of patients during treatment. As in the other neoadjuvant studies, CTC count was not correlated with pCR. A recent abstract including three-year follow-up data however revealed that the presence of CTCs at baseline was an independent prognostic factor for poor disease-free survival (DFS) (22) (p-value or HR not reported).

In conclusion, current literature in the neoadjuvant setting of BC regarding the prognostic power of CTC enumeration before or after neoadjuvant chemotherapy is not convincing. Although the REMAGUS02 study suggested that the presence of CTC might be a prognostic factor for a worse OS and unpublished data of the BEVERLY-2 suggested the same for worse DFS in patients with HER2-positive inflammatory breast cancer, large, appropriately powered studies with a longer follow-up are needed to further explore whether such an association is indeed present; and if so, whether it holds clinical value and should be implemented in treatment decision-making. Also it may be worthwhile for such studies to explore the use of other markers than EpCAM, for example MCAM, which has been reported to improve CTC capture rate in the neoadjuvant setting when used together with EpCAM (23). In conclusion, at this point there does not seem to be a role for CTC enumeration in the neoadjuvant setting.

ADJUVANT TREATMENT

Multiple, relatively small studies investigated the occurrence and prognostic power of CTCs before or after surgery for primary BC, all suggesting that the occurrence of CTCs before surgery is associated with early relapse or decreased OS (24-28). By far the largest study is the SUCCESS study that accrued 2026 patients and which assessed the prognostic value of CTC before and after adjuvant treatment (29). In this study, since CTCs were expected to be present at a low frequency, 30 mL instead of 7.5 mL of peripheral blood was drawn and following Ficoll cell separation processed on CellSearch. After the resection of the primary tumor and before the start of adjuvant chemotherapy, CTCs were detected in 435 of 2026 patients (21.5%) in 30 mL of blood.
In the SUCCESS trial, the occurrence of at least one CTC before starting adjuvant chemotherapy was associated with poor DFS (HR 2.11, 95% CI 1.49-2.99) and impaired OS (HR 2.18, 95% CI 1.32-3.59). Patients who had lymph node metastases were more frequently CTC positive. Looking at subgroups, the presence of CTCs before adjuvant treatment was not prognostic for DFS in node-negative patients, a finding which was in contrast to node-positive patients, in whom the occurrence of CTCs was associated with poor prognosis. After receiving adjuvant chemotherapy, CTCs were detected in 330 of 1493 patients (22.1%). Patients in whom no CTCs were detected before and after chemotherapy had a good 3-year DFS of 94.2% and 3-year OS of 97.6%. The persistence of CTCs after chemotherapy was associated with shorter 3-year DFS (85.9%), however, only a trend towards reduced OS (HR 1.16, 95% CI 0.99-1.37) was observed when comparing this group to other subgroups in which CTC were not present before and/

or after chemotherapy. Of note was that in 17% of patients in this study CTCs switched from absent prior to chemotherapy to present after chemotherapy. In these patients a 3-year DFS rate of 94.9% was demonstrated which was similar to patients who were stable negative, which suggests that a switch towards the presence of CTCs after chemotherapy was not associated with worse outcome. Disappointingly, patients in whom CTCs switched from present before chemotherapy to absent after chemotherapy had a worse 3-year DFS (91.1%) than the group who switched from absent before to present after chemotherapy (3-year DFS 94.9%).

The SUCCESS study certainly provided valuable data regarding the prognostic value of CTCs in the adjuvant setting. However, at this point we can only speculate how this prognostic value may be relevant for the clinic. Since the presence of ≥1 CTC before and after adjuvant chemotherapy was associated with poor DFS and a trend towards poor OS, one might consider studies to explore additional treatments in this subset of patients. Since this study had a relatively short follow-up of 3 years it will certainly be interesting to see some more mature data regarding these patients with seemingly resistant tumor cells.

Disappointing was that the switch of CTCs from absent before treatment and present after treatment and vice versa did not seem to render particular prognostic effects in this study, raising doubts on the value of CTC enumeration as a marker for response in this setting. Importantly, the use of 30 mL of blood and an additional Ficoll separation to allow CellSearch enumeration may have led to reduced reproducibility, which might have been caused by variation in CTC recovery following Ficoll separation. While the use of 30 mL of blood probably had an statistical background, meaning that the use of more blood would simply improve the chances of catching a CTC (30), we are not aware of any studies evaluating the occurrence of CTCs in BC in 7.5 mL of blood versus 30 mL of blood. Of note is that the CTC capture rate in the SUCCESS study was comparable with studies in the neoadjuvant setting and peri-operative setting using only 7.5 mL of blood for CTC enumeration, rendering CTC capture rates of 22 to 24% (15, 17, 26). Since conclusions are drawn based on the occurrence of only one CTC in an individual's blood sample, matters regarding reproducibility should be evaluated before any firm conclusions about the true clinical value and place of CTC enumeration in the adjuvant setting can be established. In addition, if true clinical prognostic value exists, it still has to be investigated how CTC enumeration compares to and/or adds to other already available methods such as Mammaprint (31) or Oncotype DX (32) in stratifying primary BC patients into good and poor prognosis groups.

PROGNOSTIC VALUE OF CTCS IN METASTATIC BREAST CANCER

In 2004, Cristofanilli and colleagues published their landmark paper on CTC enumeration in metastatic breast cancer (MBC) patients starting a new line of therapy for MBC (33). In 61% of all patients, at least 2 CTCs were observed. Thresholds between 1 and 10000 CTCs prior to a new line of treatment for MBC were systematically correlated with progression free survival in a training set of 102 patients, to find the optimal cut-off to identify patients with poor prognosis. Following this correlation, a cut-off of ≥5 CTCs for poor prognosis was established, which was subsequently prospectively validated in a validation set of 75 patients. In patients who had ≥5 CTCs before starting a new line of

therapy, progression occurred after a median of 2.7 months, while this was 7 months in patients who had <5 CTS. Patients who had ≥5 CTCs also had a shorter median OS than patients who had <5 CTCs (10.1 months versus >18 months, respectively).

Ever since this study has demonstrated that a subset of patients with poor prognosis could be identified using CTC enumeration, the robustness and clinical validity of CTC enumeration in MBC have been important subjects of investigation. Especially the monitoring of CTC changes during treatment was considered to have great promise as an early response marker. Other clinical consequences of stratifying MBC patients according to CTC counts that can be thought of are treating patients with a good prognosis with less intensive treatment regimens (for example endocrine therapy) than patients with a poor prognosis.

Ten years after the landmark paper of Cristofanilli and colleagues, the first pooled analysis of individual patient data evaluating the clinical validity of CTC enumeration at baseline and during treatment was recently performed by Bidard et al. (34). This analysis involved patients with MBC starting a new line of therapy, for whom at least a CTC baseline value and follow-up regarding progression-free survival (PFS) and OS was available. 17 centers in Europe provided patient data from 1944 patients with a median follow-up of 23 months. This study confirmed the findings of the study by Cristofanilli and colleagues. Patients who had ≥5 CTCs at baseline had a significantly shorter median PFS (6.5 vs 11.4 months, respectively) and OS (15.5 vs 37.1 months, respectively). In addition to baseline CTC counts, also changes in CTC counts after 3-5 weeks of treatment were evaluated as a potential marker for treatment response. Patients with <5 CTCs at baseline and in whom CTCs were stable (i.e., <5 CTCs) during treatment demonstrated the best prognosis with a median OS of 41.5 months. Importantly, patients who had ≥5 CTCs prior to treatment while CTCs decreased <5 during treatment, demonstrated much better OS than the patients in whom CTCs did not decrease <5 during treatment (13.1 months vs 27.0 months, respectively). Notably, only in 2.5% of patients CTCs increased from <5 to ≥5 CTCs during treatment. This last observation suggests that is not worthwhile to investigate CTC changes in patients with <5 CTCs at baseline.

In conclusion, level A clinical evidence for the clinical validity of CTC enumeration as a prognostic marker in each treatment line of MBC was presented by this large analysis.

CTCS VERSUS SERUM BIOMARKERS

Besides the focus on the prognostic impact of CTCs, a lot of work has been performed to further evaluate and establish the role of CTC enumeration as opposed to other frequently used methods to monitor tumor burden and treatment efficiency, like radiographic imaging studies and serum biomarkers. Serum biomarkers are frequently used by medical oncologists to monitor tumor burden and treatment success in MBC patients. The 2007 ASCO guideline recommendations (35) and 2013 ESO guidelines (36) advocate to only use the serum biomarkers cancer antigen 15-3 (CA 15-3), carcinoembryonic antigen (CEA) and cancer antigen 27.29 (CA 27.29) in conjunction with imaging and patient characteristics to contribute to clinical decision making regarding MBC therapy. The use of these biomarkers as the sole parameter to alter therapy for MBC is only recommended in case metastatic disease is not adequately measurable, for example in those patients who have bone metastases only.

As a side-study of their large pooled CTC data analysis, Bidard and colleagues (34) compared the clinical value of serum biomarkers CEA and CA15-3 with the clinical value of CTC enumeration. It was demonstrated that a model with clinicopathological parameters harboring primary tumor characteristics, previous treatments, metastases free-interval, metastatic sites, age and performance status combined with the CTC count before the start of MBC treatment was the strongest model associated with OS. A model of these clinicopathological parameters combined with either baseline CEA or CA15-3 without CTC count was also associated with OS, albeit less strongly than the model including CTC count. Remarkably, the addition of CEA or CA15-3 changes to the strongest baseline model did not result in a significantly better prognostic model for OS, while the addition of CTC changes during changes to the model did contribute to a significantly better prognostic model for OS. These results clearly suggest that the use of CTC enumeration prior to and during treatment in MBC is superior to serum biomarker assessment. Since serum biomarkers are now the clinical standard to evaluate treatment success in patients with poorly evaluable metastatic disease, for example those with bone metastasis only, the monitoring of CTC changes instead of serum biomarkers should be considered especially in these patients.

CTCS VERSUS IMAGING

Radiographic response evaluation is one of the cornerstones of measuring treatment success in MBC patients. In addition, response evaluation using the RECIST (37) is a commonly used endpoint in clinical trials. However, the absence of progression as assessed by RECIST does not necessarily translate into clinical benefit for an individual patient (38).

Budd et al. (39) compared CTC enumeration in MBC patients to CT-scan with respect to its association with OS. CTCs were enumerated at baseline and following 4 weeks of therapy, while radiographic response evaluation was performed every 9 to 12 weeks during therapy. CTC counts were always reviewed at a local laboratory and a central laboratory and two radiologists centrally reviewed all radiologic responses. There was excellent inter-reader agreement for CTC counts for which 0.7% variability was demonstrated, while radiologic responses showed 15.2% inter-reader variability. Patients with <5 CTCs after four weeks of treatment and who had stable disease or partial response on radiographic response evaluation demonstrated the best median OS at 26.9 months. Strikingly, patients who had radiographic progressive disease (PD) had largely differing prognoses according to their CTC counts at first follow-up: for patients who had radiographic PD and <5 CTCs a median OS of 19.9 months was demonstrated, while for patients who had radiographic PD and ≥5 CTCs an abysmal OS of 6.4 months was reported. A study performed in MBC patients comparing CTCs with *18F-fluorodeoxyglucose positron emission tomography* combined with *computed tomography* (FDG-PET/*CT*) for radiographic response evaluation demonstrated similar results as Budd et al. (40). CTC counts at mid-therapy in this study were demonstrated to be the strongest factor associated with OS in this study, independent of mid-therapy FDG-PET/CT response. These studies indicate that CTCs can be used as an early endpoint for clinical response evaluation instead of, or in addition to radiographic evaluations. In addition, these studies suggest that it might be better to switch treatment based on CTC enumeration rather than imaging.

TREATMENT SWITCH BASED ON CTC CHANGES

While it is appreciated that CTC changes following therapy for MBC have prognostic relevance, it was unknown whether a therapy switch in patients in whom the CTC count did not decrease <5 during treatment, thus suggesting treatment failure, would be beneficial. In 2014 the first interventional trial using CTC enumeration and addressing this question was published by Smerage et al. (41). The primary objective of this SWOG S0500 study was to demonstrate an OS benefit in CTC non-responding patients switching from first-line therapy to second-line therapy for MBC, as opposed to patients in whom first-line therapy was maintained and switched at the time of progression as shown by conventional means such as CT-scanning. The study was powered to demonstrate a 70% increase in median OS in the patients in whom therapy was switched early on the basis of a lack of CTC response compared to patients in whom therapy was maintained. Following an initial screening of 595 patients, 288 MBC patients who had ≥5 CTCs starting treatment were evaluated after 3 weeks of first-line chemotherapeutic treatment. A total of 123 patients who had persistently ≥5 CTCs were then randomized between arm C1, in which first-line therapy was maintained, and arm C2, in which therapy was switched. Disappointingly, no benefit in PFS or OS was demonstrated between the randomized arms, with median OS being a poor 10.7 months in arm C1 and 12.5 months in arm C2. Although this study did not have sufficient power to demonstrate smaller differences between the two randomization arms that can be considered clinically relevant, while furthermore OS benefits can be diluted in the first line setting due to subsequent salvage treatments, the least we can conclude is that a therapy switch in these poor prognosis patients does not seem to yield large benefits. At this point, it can only be speculated why a therapy switch in these poor prognosis patients did not lead to significantly improved outcomes. Perhaps the presence of some sort of general chemo-resistance or cross-resistance between commonly used first- and second-line therapies for MBC might be present and future research should further unravel the potential underlying mechanism.

CONCLUSIONS AND RECOMMENDATIONS

Ever since the landmark paper by Cristofanilli and colleagues in 2004 (33), CTC enumeration by using the CellSearch machine has been considered to have great promise as a tool to individualize BC treatment. Over the past ten years, lots of efforts have been made to further validate and incorporate the findings of the original seminal paper, which has further added to the robustness of CTC enumeration. At this point, CTC enumeration in primary BC has not yet proved to hold true clinical value in today's clinical oncology practice. In contrast, the recent milestone paper with a large pooled CTC data analysis by Bidard et al. (34) has provided level A evidence for the clinical validity of CTC enumeration in MBC. However, it has become clear that the presence of clinical validity in this case does not necessarily translate into clinical utility. The SWOG S0500 trial demonstrated that a therapy switch in MBC patients who had a persistence of ≥5 CTC during treatment did not result in better clinical outcome. Importantly, although this study was powered to reveal a 70% increase in median OS, which would be huge in a first-line setting, switching treatment early on the basis of CTC changes is unlikely to results in a much better outcome. For the group of patients with persistence

of ≥5 CTC during treatment, supposedly not responding to traditional first- and second line chemotherapeutics, alternative treatment options, preferably guided by other individualized treatment approached will have to be explored in the near future.

The results of the SWOG interventional trial were undeniably disappointing. However, it is not all bad news. There is robust evidence to recommend the use of CTC enumeration in two clinical situations (**Table 1**). First, there is a superiority of CTCs over serum biomarkers in MBC, clearly suggesting that CTCs should be used more often to monitor tumor aggressiveness and response to treatment in patients who have poorly evaluable disease such as those with bone metastases only. Second, CTC enumeration is a robust way to evaluate treatment response in MBC and early changes in CTC counts, already 4 to 6 weeks after start of treatment, are strongly correlated with clinically relevant endpoints including PFS and OS. Therefore, the use of CTCs as an early response marker in early clinical trials such as phase I or II studies can be advocated instead of or in addition to radiographic response evaluation.

In addition to already available clinical uses of CTC enumeration, promising interventional studies using CTC enumeration to guide treatment decision making using different angles than the SWOG0500 trial are ongoing (42). For example, in the STIC CTC METABREAST study, therapy-naive first-line hormone-receptor positive MBC patients are randomized between a clinicians choice arm and a CTC count-driven choice to guide the choice of endocrine therapy versus chemotherapy (43). In the CTC-count driven arm patients who have less than 5 CTCs, who have a relatively good prognosis and therefore a more indolent tumor, will receive endocrine therapy, while patients who have ≥5 CTC and a more aggressive tumor will receive chemotherapy.

Another interesting trial with a similar design as the SWOG S0500 study is the CirCe01 trial, which evaluates a therapy switch in the third- and subsequent lines of MBC treatments until a CTC response <5 is achieved (44). Since the SWOG S0500 study suggested that the metastases in patients in whom CTCs were persistently increased ≥5 harbored some sort of general chemo-resistance, the repeated use of CTC enumeration to evaluate treatment response might be suitable to identify the right drug for the right patient. Besides OS, a co-primary endpoint involves a medico-economic study to

Table 1. Recommendations on the use of CTC CellSearch enumeration in breast cancer

Stage of disease	Recommendation
Primary breast cancer (neoadjuvant and adjuvant setting)	Present data is insufficient to recommend the use of CTC enumeration for risk stratification and treatment response evaluation
Metastatic breast cancer	- In patients with poorly evaluable disease, for example those with bone metastases only, CTC enumeration should be used over serum biomarkers when the clinician wishes to evaluate tumor aggressiveness and response to treatment - CTC enumeration may be used as an early response marker in clinical trials to allow shorter follow-up - Present data is insufficient to recommend the use of CTC enumeration to perform early switches based on CTC counts

evaluate whether CTC-based early treatment switches used in this study are able to minimize toxicity and costs of inefficient treatments. We believe that this may prove to be another important and valid use of CTC enumeration, which might ultimately benefit patients and health care in general.

Set aside these current clinical applications and the ongoing promising studies for CTC enumeration, one could argue that just enumerating CTCs is merely a one-dimensional use of these cells. Fortunately, despite lots of technical challenges, for example the presence of leukocyte contamination after CTC isolation (45), other strategies using information on CTCs on a molecular level are on the way (**Figure 2**). For example, the molecular characterization of CTCs to identify patients suitable for a certain drug treatment is promising and a clinical trial evaluating a gene profile determined on CTCs, tentatively able to predict sensitivity for cisplatin chemotherapy, has recently started (46). In addition, HER2 immunocytochemical characterization, which can be performed directly in the CellSearch machine, identifies patients with a HER2-negative primary tumor but HER2-positive CTCs who may benefit from trastuzumab treatment, which is now tested in several clinical trials (42). Particularly interesting is that in one of the trials investigating this, subsequent to CellSearch isolation also multiplex *in situ* characterization of phosphorylated HER2, ER and mutations in PIK3CA is performed, possibly allowing even better identification of subsets of patients responding to trastuzumab treatment (47). Finally, CTCs were recently isolated out of peripheral blood and cultured ex vivo, allowing drug sensitivity screening on cultured CTCs (48). All these relatively new techniques are exciting and we believe that in addition to CTC enumeration, great potential is present for CTC molecular characterization to eventually make it to the clinic which may then prove to be the next step towards more individualized BC treatment.

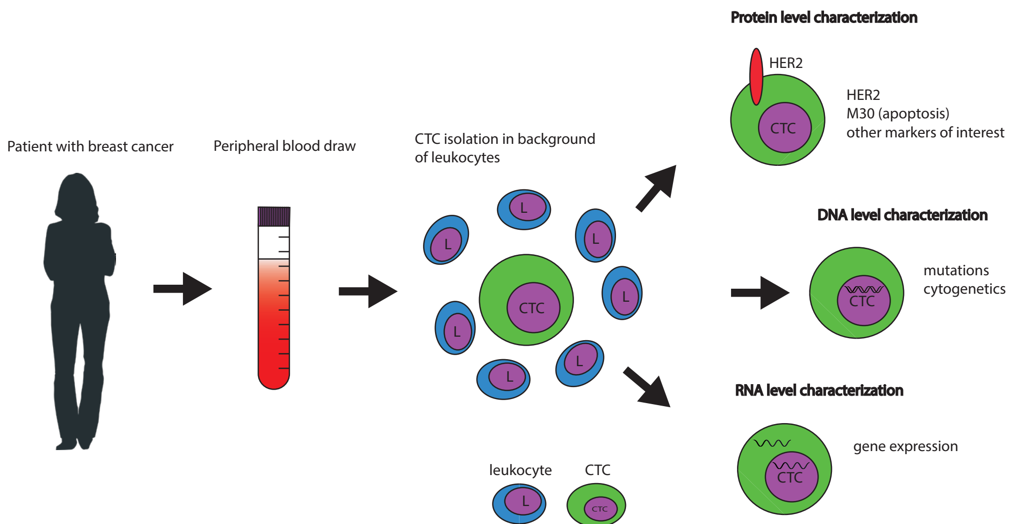


FIGURE 2. POSSIBILITIES FOR MOLECULAR CHARACTERIZATION OF CTCS

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.

2. Ignatiadis M, Riethdorf S, Bidard FC, Vaucher I, Khazour M, Rothe F, et al. International study on inter-reader variability for circulating tumor cells in breast cancer. Breast Cancer Res. 2014;16(2):R43.

3. Tibbe AG, Miller MC, Terstappen LW. Statistical considerations for enumeration of circulating tumor cells. Cytometry A. 2007 Mar;71(3):154-62.

4. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res. 2004 Oct 15;10(20):6897-904.

5. Kraan J, Sleijfer S, Strijbos MH, Ignatiadis M, Peeters D, Pierga JY, et al. External quality assurance of circulating tumor cell enumeration using the CellSearch((R)) system: a feasibility study. Cytometry B Clin Cytom. 2011 Mar;80(2):112-8.

6. Sieuwerts AM, Kraan J, Bolt J, van der Spoel P, Elstrodt F, Schutte M, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. J Natl Cancer Inst. 2009 Jan 7;101(1):61-6.

7. Bednarz-Knoll N, Alix-Panabieres C, Pantel K. Plasticity of disseminating cancer cells in patients with epithelial malignancies. Cancer Metastasis Rev. 2012 Dec;31(3-4):673-87.

8. Mostert B, Kraan J, Sieuwerts AM, van der Spoel P, Bolt-de Vries J, Prager-van der Smissen WJ, et al. CD49f-based selection of circulating tumor cells (CTCs) improves detection across breast cancer subtypes. Cancer Lett. 2012 Jun 1;319(1):49-55.

9. Early Breast Cancer Trialists' Collaborative G. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005 May 14-20;365(9472):1687-717.

10. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005 Oct 20;353(16):1659-72.

11. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005 Oct 20;353(16):1673-84.

12. Arpino G, Generali D, Sapino A, Del Matro L, Frassoldati A, de Laurentis M, et al. Gene expression profiling in breast cancer: a clinical perspective. Breast. 2013 Apr;22(2):109-20.

13. Carlson JJ, Roth JA. The impact of the Oncotype Dx breast cancer assay in clinical practice: a systematic review and meta-analysis. Breast Cancer Res Treat. 2013 Aug;141(1):13-22.

14. Kaufmann M, von Minckwitz G, Mamounas EP, Cameron D, Carey LA, Cristofanilli M, et al. Recommendations from an international consensus conference on the current status and future of neoadjuvant systemic therapy in primary breast cancer. Ann Surg Oncol. 2012 May;19(5):1508-16.

15. Pierga J-YY, Bidard F-CC, Mathiot C, Brain E, Delaloge S, Giachetti S, et al. Circulating tumor cell detection predicts early metastatic relapse after neoadjuvant chemotherapy in large operable and locally advanced breast cancer in a phase II randomized trial. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(21):7004-10.

16. Bidard FC, Belin L, Delaloge S, Lerebours F, Ngo C, Reyal F, et al. Time-Dependent Prognostic Impact of Circulating Tumor Cells Detection in Non-Metastatic Breast Cancer: 70-Month Analysis of the REMAGUS02 Study. Int J Breast Cancer. 2013;2013:130470.

17. Riethdorf S, Muller V, Zhang L, Rau T, Loibl S, Komor M, et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res. 2010 May 1;16(9):2634-45.

18. Giordano A, Giuliano M, De Laurentiis M, Arpino G, Jackson S, Handy BC, et al. Circulating tumor cells in immunohistochemical subtypes of metastatic breast cancer: lack of prediction in HER2-positive disease treated with targeted therapy. Ann Oncol. 2012 May;23(5):1144-50.

19. Peeters DJ, van Dam PJ, Van den Eynden GG, Rutten A, Wuyts H, Pouillon L, et al. Detection and prognostic significance of circulating tumour cells in patients with metastatic breast cancer according to immunohistochemical subtypes. Br J Cancer. 2014 Jan 21;110(2):375-83.

20. Wallwiener M, Hartkopf AD, Baccelli I, Riethdorf S, Schott S, Pantel K, et al. The prognostic impact of circulating tumor cells in subtypes of metastatic breast cancer. Breast Cancer Res Treat. 2013 Jan;137(2):503-10.

21. Pierga JY, Petit T, Delozier T, Ferrero JM, Campone M, Gligorov J, et al. Neoadjuvant bevacizumab, trastuzumab, and chemotherapy for primary inflammatory HER2-positive breast cancer (BEVERLY-2): an open-label, single-arm phase 2 study. Lancet Oncol. 2012 Apr;13(4):375-84.

22. Pierga JY, Petit T, Levy C, Ferrero JM, Campone M, Gligorov J, et al. Circulating tumor cell count at baseline is an independent prognostic value from pathological complete response among patients treated for primary inflammatory HER2-positive breast cancer: survival results of the BEVERLY-2 study. Annals of Oncology 25 (Supplement 4): iv110–iv115, 2014 doi:101093/annonc/mdu3282.

23. Onstenk W, Kraan J, Charehbili A, Mostert B, Smit VTHBM, Kroep JR, et al. A combined EpCAM and MCAM circulating tumor cell (CTC) CellSearch enrichment to improve CTC capture rate in stage II/III breast cancer: A Dutch Breast Cancer Trialists' Group (BOOG) side study. J Clin Oncol 31, 2013 (suppl; abstr e22106).

24. Sandri MT, Zorzino L, Cassatella MC, Bassi F, Luini A, Casadio C, et al. Changes in circulating tumor cell detection in patients with localized breast cancer before and after surgery. Ann Surg Oncol. 2010 Jun;17(6):1539-45.

25. Biggers B, Knox S, Grant M, Kuhn J, Nemunatitis J, Fisher T, et al. Circulating tumor cells in patients undergoing surgery for primary breast cancer: preliminary results of a pilot study. Ann Surg Oncol. 2009 Apr;16(4):969-71.

26. Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, et al. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol. 2012 Jul;13(7):688-95.

27. Franken B, de Groot MR, Mastboom WJ, Vermes I, van der Palen J, Tibbe AG, et al. Circulating tumor cells, disease recurrence and survival in newly diagnosed breast cancer. Breast Cancer Res. 2012;14(5):R133.

28. Karhade M, Hall C, Mishra P, Anderson A, Kuerer H, Bedrosian I, et al. Circulating tumor cells in non-metastatic triple-negative breast cancer. Breast Cancer Res Treat. 2014 Sep;147(2):325-33.

29. Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, et al. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. Journal of the National Cancer Institute. 2014;106(5).

30. Coumans FA, Ligthart ST, Uhr JW, Terstappen LW. Challenges in the Enumeration and Phenotyping of CTC. Clin Cancer Res. 2012 Oct 15;18(20):5711-8.

31. Buyse M, Loi S, van't Veer L, Viale G, Delorenzi M, Glas AM, et al. Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. J Natl Cancer Inst. 2006 Sep 6;98(17):1183-92.

32. Paik S, Tang G, Shak S, Kim C, Baker J, Kim W, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Oncol. 2006 Aug 10;24(23):3726-34.

33. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004 Aug 19;351(8):781-91.

34. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014 Apr;15(4):406-14.

35. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol. 2007 Nov 20;25(33):5287-312.

36. Lin NU, Thomssen C, Cardoso F, Cameron D, Cufer T, Fallowfield L, et al. International guidelines for management of metastatic breast cancer (MBC) from the European School of Oncology (ESO)-MBC Task Force: Surveillance, staging, and evaluation of patients with early-stage and metastatic breast cancer. Breast. 2013 Jun;22(3):203-10.

37. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009 Jan;45(2):228-47.

38. Robinson AG, Booth CM, Eisenhauer EA. Progression-free survival as an end-point in solid tumours - Perspectives from clinical trials and clinical practice. Eur J Cancer. 2014 Sep;50(13):2303-8.

39. Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, et al. Circulating tumor cells versus imaging--predicting overall survival in metastatic breast cancer. Clin Cancer Res. 2006 Nov 1;12(21):6403-9.

40. De Giorgi U, Valero V, Rohren E, Dawood S, Ueno NT, Miller MC, et al. Circulating tumor cells and [18F]fluorodeoxyglucose positron emission tomography/computed tomography for outcome prediction in metastatic breast cancer. J Clin Oncol. 2009 Jul 10;27(20):3303-11.

41. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol. 2014 Nov 1;32(31):3483-9.

42. Bidard F-CC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabières C, Janni W, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer metastasis reviews. 2013;32(1-2):179-88.

43. Bidard FC, Baffert S, Hajage D, Brain E, Armanet S, Simondi C, et al. Circulating tumor cells to guide the choice between chemotherapy and hormone therapy as first line treatment for hormone receptors positive metastatic breast cancer patients: the STIC CTC METABREAST trial. Cancer Res. 2012;72 ((24 Suppl): Abstract nr OT3-4-06).

44. Bidard FC, Asselain B, Baffert S, Brain E, Delaloge S, Bachelot T, et al. Use of early CIRculating tumor CElls count changes to guide the use of chemotherapy in advanced metastatic breast cancer patients: the CirCe01 randomized trial. Cancer Res. 2012;72((24 Suppl): Abstract nr OT3-4-05).

45. Onstenk W, Gratama J, Foekens J, Sleijfer S. Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. Cancer Treat Rev. 2013;39(7):691-700.

46. Dutch Trial Registry: “CTC-cDDP study” http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=4046.

47. CareMoreCTC studies. http://www.caremorectc.eu/.

48. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science. 2014 Jul 11;345(6193):216-20.

02

CHAPTER 3

Prognostic impact of HER2 and ER status of circulating tumor cells in metastatic breast cancer patients with a HER2-negative primary tumor

Nick Beije, Wendy Onstenk, Jaco Kraan,
Anieta M. Sieuwerts, Paul Hamberg,
Luc Y. Dirix, Anja Brouwer,
Felix E. de Jongh, Agnes Jager,
Caroline M. Seynaeve, Ngoc M. Van,
John A. Foekens, John W.M. Martens,
Stefan Sleijfer

Neoplasia. 2016 Oct 17;18(11):647-653

ABSTRACT

BACKGROUND Preclinical and clinical studies have reported that human epidermal growth factor receptor 2 (HER2) overexpression yields resistance to endocrine therapies. Here the prevalence and prognostic impact of HER2-positive circulating tumor cells (CTCs) was investigated retrospectively in metastatic breast cancer (MBC) patients with a HER2-negative primary tumor receiving endocrine therapy. Additionally, the prevalence and prognostic significance of HER2-positive CTCs was explored in a chemotherapy cohort, as well as the prognostic impact of the ER (estrogen receptor)-CTC status in both cohorts.

METHODS Included were MBC patients with a HER2-negative primary tumor, with ≥1 detectable CTC, starting a new line of treatment. CTCs were enumerated using the CellSearch system, characterized for HER2 with the CellSearch anti-HER2 phenotyping reagent, and characterized for ER mRNA expression. Primary endpoint was progression-free-rate after 6 months (PFR6months) of endocrine treatment in HER2-positive versus HER2-negative CTC patients.

RESULTS HER2-positive CTCs were present in 29% of all patients. In the endocrine cohort (n=72) the PFR6months was 53% for HER2-positive, versus 68% for HER2-negative CTC patients (P=0.23). In the chemotherapy cohort (n=82) no prognostic value of HER2-positive CTCs on PFR6months was observed either. Discordances in ER status between the primary tumor and CTCs occurred in 25% of all patients, but had no prognostic value in exploratory survival analyses.

CONCLUSION Discordances regarding HER2 status and ER status between CTCs and the primary tumor occurred frequently, but had no prognostic impact in our MBC patient cohorts.

INTRODUCTION

Treatment of metastatic breast cancer (MBC) is currently mainly driven by the characteristics of the primary tumor (PT). However, clinically relevant discordances between the PT and the metastases with respect to the estrogen receptor (ER)-status and the human epidermal growth factor receptor 2 (HER2)-status occur and influence systemic therapy choices and patient management (1). As a result, both European and American guidelines (2, 3) recommend to perform biopsies of metastatic lesions to assess their receptor status and decide on subsequent systemic therapy. Biopsies of metastases are, however, often not performed since this is regarded a cumbersome procedure for patients, and it is sometimes even impossible due to inaccessibility of the metastases. Another limitation of taking single metastatic biopsies is that intra-tumor and inter-metastatic heterogeneity is missed. To assess the characteristics of metastatic tumor cells in a minimally invasive way, it is attractive to obtain circulating tumor cells (CTCs) as a liquid biopsy from peripheral blood. Discrepancies between the ER-status and the HER2-status of the PT and the CTCs in MBC have been demonstrated by numerous groups (4-18). Some of these groups have also reported on the prognostic value of receptor discrepancies between PTs and CTCs, but such reports have been scarce (17, 18). Further, previous studies have been performed in rather small and heterogeneous groups of patients, making it difficult to draw firm conclusions on the exact clinical relevance of ER and HER2 expression in CTCs.

It has been suggested that the HER2-status of the tumor impacts response to endocrine therapy. Several pre-clinical studies demonstrated that the introduction of the HER2 transcript in ER-positive breast cancer cell lines confers endocrine resistance (19-21). In addition, a meta-analysis in 2,379 MBC patients demonstrated that patients with a HER2-positive PT were less sensitive to endocrine treatment than patients with a HER2-negative PT (22). In the current study we investigated whether or not MBC patients with a HER2-negative PT, but HER2-positive CTCs, have a worse outcome to endocrine treatment compared to patients with HER2-negative CTCs. In addition, a separate control group of MBC patients with a HER2-negative PT receiving first-line chemotherapy was included in which the prognostic impact of HER2-positive CTCs was explored. Also explored in both cohorts were the clinical impact of switches in ER expression between the PT and CTCs.

METHODS

PATIENTS AND TREATMENT

MBC patients with a HER2-negative PT and the presence of at least one detectable CTC by the CellSearch system (see below), who started a new line of systemic treatment for MBC, were eligible for this study. We used data from our CTC studies with patients starting first line chemotherapy (study 06-248 (9, 17, 23)) or starting a new line of endocrine therapy (study 09-405 (24)) for MBC. All patients had been included between February 2008 and March 2015 in six centers the Netherlands and Belgium. From all patients 2 x 7.5 mL of blood was drawn for CTC enumeration and CTC characterization. The local institutional review board of each participating center approved the study protocols (Erasmus MC ID MEC-06-248 & MEC-09-405). All patients provided written informed consent.

ENUMERATION OF CTCS AND HER2 STAINING

Before the start of a new line of systemic treatment for MBC, 7.5 mL of blood was drawn in CellSave tubes (Janssen Diagnostics, Raritan, NJ, USA). CTC enumerations were performed within 96 hours of the blood draw using the CellSearch system (Janssen Diagnostics). CTCs were characterized for HER2 expression directly in the CellSearch system using the anti-HER2 antibody as described by the manufacturer (CellSearch tumor phenotyping reagent HER2, Janssen Diagnostics). We used the cut-off for HER2-positivity in CTCs as described by Riethdorf and colleagues (25), a gallery of representative images for scoring is presented in **Supplementary Figure 1**. When at least one CTC immunofluorescently stained 2+ or 3+ for HER2, the patient was considered as having HER2-positive CTCs. The results of the enumeration were always checked by a second certified reviewer. The results of the HER2 staining were reviewed in a HER2 consensus meeting involving 2 investigators (NB & JK).

CTC ER ASSAY

Simultaneously with the blood draw for CTC enumeration, 7.5 mL of blood was drawn in EDTA tubes and enriched for CTCs using the CellSearch profile kit (Janssen Diagnostics). Samples were processed within 24 hours and subsequently frozen at -80 °C for RNA isolation and analysis. Larger than 200 bp RNA was isolated using the AllPrep DNA/RNA Micro Kit (Qiagen, Germantown, MD, USA). Generation of cDNA, pre-amplification and reverse transcription quantitative polymerase chain reaction (RT-qPCR) to quantify the expression of *ESR1* were performed as described in detail before, using a validated *ESR1* Taqman assay (Hs00174860 m1, Applied Biosystems, San Francisco, CA, USA) (9). ER-positivity in CTCs was defined as an *ESR1* mRNA ΔCq level higher than -3.89 corrected for background healthy donor blood signal, which we previously demonstrated to be a reliable cut-off for *ESR1* with excellent sensitivity and specificity (17).

STATISTICAL CONSIDERATIONS

The primary endpoint of this study was the progression-free rate after six months of treatment (PFR6months) in patients receiving endocrine therapy. A small survey amongst medical oncologists revealed that a PFR6months of 20% for endocrine therapy alone in MBC patients with HER2-positive CTCs, would be convincing enough for medical oncologist not to treat a MBC patient with an ER-positive PT with endocrine therapy alone. Given that the expected PFR6months for endocrine therapy in unselected MBC patients is around 70% (and certainly not lower than 50%), and the prevalence of HER2-positive CTCs was expected to be around 25%, we calculated that 60 patients would render 15 patients with HER2-positive CTCs to detect a PFR6months of 20% with a 95% confidence interval (CI) not higher than 50% (4-48%), with a type I error probability (α) of 0.05 & a type II error probability (β) of 0.2.

Secondary objectives were 1) to explore the association between the HER2-status of CTCs and the PFR6months on chemotherapy (as a control cohort), 2) to establish the incidence of ER-positive CTCs in the endocrine and chemotherapy cohort, 3) to establish discrepancy rates of the ER and HER2 status in CTCs compared to PT characteristics in both cohorts, and 4) to explore whether an ER-status switch between the primary breast tumor and the CTCs was associated with outcome in both cohorts. The date of progression was established by the treating physician and was defined as radiological

progression according to RECIST version 1.1 (26). In case of poorly evaluable disease, the treating physician was allowed to use other techniques considered to be appropriate (e.g. bone scan, serum biomarkers, CTC counts) to assess progressive disease. Data on the HER2 and ER-status (including HercepTest (Dako, Glostrup, Denmark) scoring and the percentage of ER-positive tumor cells) of the PT were collected from the pathology report. A HER2-negative PT was defined as having 0 or 1+ scoring according to the HercepTest (scored according to the manufacturer's instructions) on the PT, or 2+ scoring in combination with negative HER2 in situ hybridization. An ER-negative PT was defined as having <10% of the primary tumor cells staining for ER using immunohistochemistry. Differences in the PFR6months between patients with HER2-positive versus HER2-negative CTCs were analyzed using the chi-square test. Univariate Cox regression was used to evaluate whether the presence of at least one HER2-positive CTC (as a dichotomous variable) was associated with progression-free survival (PFS) or overall survival (OS). The HER2/CTC ratio was calculated by dividing the number of HER2-positive CTCs by the total number of CTCs. No statistics were performed in the ER-CTC-related analyses, as this study was not appropriately powered to evaluate the prognostic power of the ER-CTC status. Instead Kaplan-Meier curves were constructed to explore the potential prognostic power of the ER-CTC status. Associations between the HercepTest score and the HER2-CTC status were investigated with the chi-square test, while associations between the percentage of ER-positive tumor cells in the PT and ER-switches were compared with the Kruskall-Wallis test. Reported *P*-values are two-sided, and a significance level $\alpha = 0.05$ was used. REMARK criteria (27) were taken into account for this report. Analyses were done using Stata/SE version 12 (StataCorp LP, College Station, TX, USA).

RESULTS

PATIENT CHARACTERISTICS

A total of 154 MBC patients were included in the current analysis **(Table 1)**; 72 patients were treated with endocrine therapy, and 82 patients with first-line chemotherapy for MBC. Patients treated with endocrine therapy mainly started this as first-line therapy (69%) for MBC, and mostly received an aromatase inhibitor (64%). The patients in the chemotherapy cohort predominantly received taxane-based (48%) or anthracycline-based chemotherapy (35%). No patients in either cohort received targeted therapies such as trastuzumab or everolimus. Median follow-up of all patients was 15 months.57%, *P*=0.48). In patients in whom HER2-positive CTCs were present, the median HER2 to CTC ratio (total number of HER2-positive CTCs divided by total number of CTCs) was 0.08 (Q1 0.03 – Q3 0.22).

TABLE 1. Baseline characteristics

	Endocrine therapy (n=72)	Chemotherapy (n=82)
Age at inclusion, median (range)	67 (37-88)	61 (33-85)
Primary tumor ER-positive	72 (100%)	57 (70%)
Previous chemotherapy lines for MBC		
0	68 (94%)	82 (100%)
1	4 (6%)	
Previous endocrine therapy lines for MBC		
0	50 (69%)	78 (96%)
1	17 (24%)	2 (2%)
2	5 (7%)	2 (2%)
Chemotherapy regimen received after inclusion		
Taxane-based		39 (48%)
Anthracycline-based		29 (35%)
Other		14 (17%)
Endocrine therapy regimen received after inclusion		
AI-based	46 (64%)	
Tamoxifen-based	17 (24%)	
Other	9 (12%)	
CTC count at baseline		
1-4 CTCs/7.5 mL	30 (42%)	24 (29%)
≥5 CTCs/7.5 mL	42 (58%)	58 (71%)
Follow-up, median days (range)	511 (30 - 1436)	406 (8 - 1430)

TABLE 2. Prevalence and PRF6 months in relation to the HER2-CTC status

	Endocrine therapy (n=72)	Chemotherapy (n=82)
HER2-positive CTCs (2+ or 3+ HER2 staining) present	19 (26%)	26 (32%)
HER2-positive CTCs (3+ HER2 staining) present	6 (8%)	9 (11%)
PFR 6 months		
Absent HER2-positive CTCs (2+ or 3+ HER2 staining)	68%	57%
≥1 HER2-positive CTCs (2+ or 3+ HER2 staining) present	53%	65%
Chi-square PFR 6 months *P*-value (absent vs present)	0.23	0.48

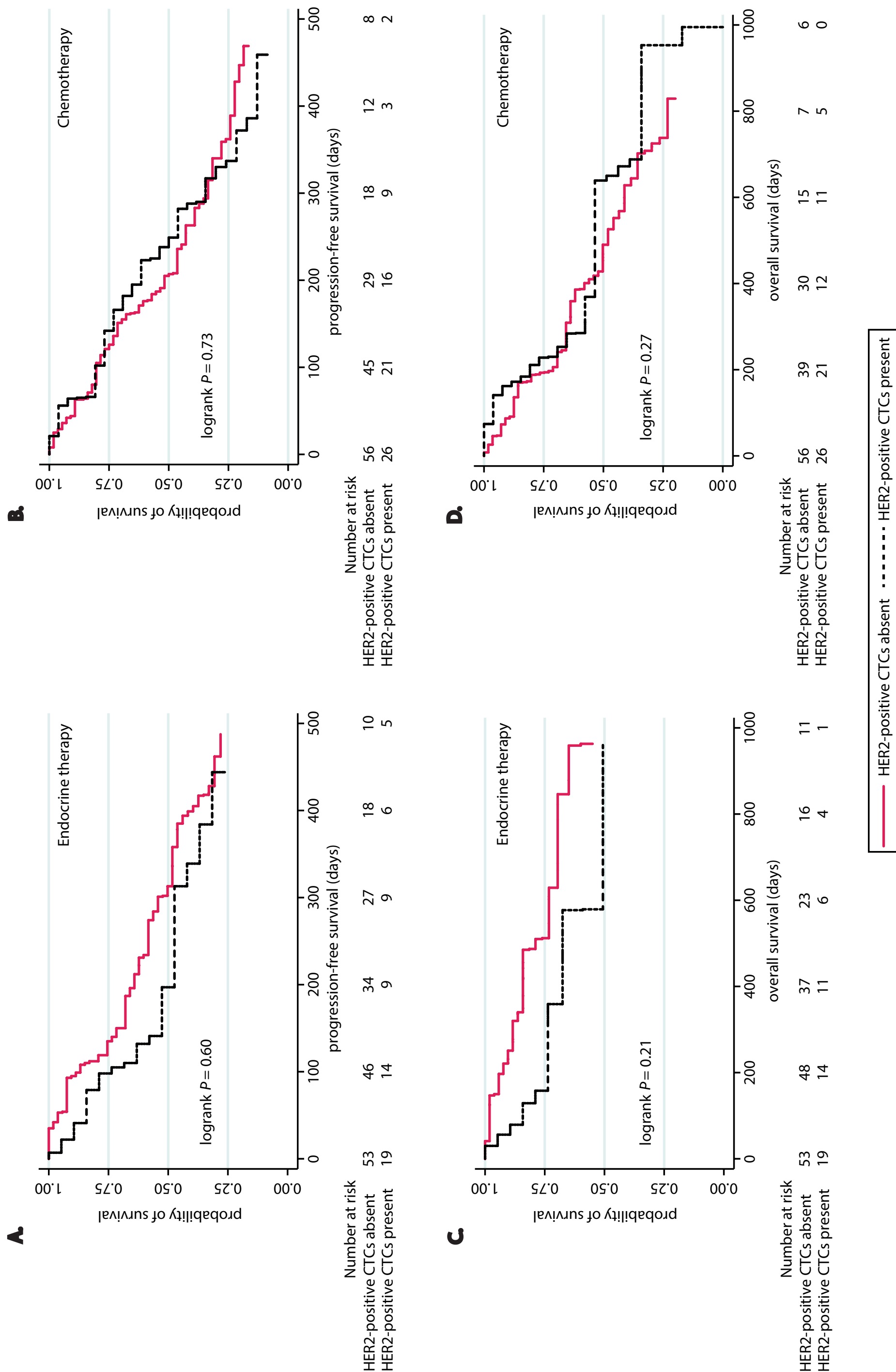


FIGURE 1. PFS AND OS ACCORDING TO HER2-CTC STATUS. Panel A and C show PFS and OS, respectively, for the endocrine therapy cohort, Panel B and D show PFS and OS, respectively, for chemotherapy cohort.

Because we had ample follow-up time for the included patients, we also explored whether the presence of HER2-positive CTCs was associated with PFS or OS in a univariate Cox regression model. The presence of at least one HER2-positive CTC in the endocrine therapy cohort was not associated with a difference in PFS (HR 1.17, 95% CI 0.65-2.09) or OS (1.72, 95% CI 0.73-4.03) (**Figure 1 A&C**). Similarly, in the chemotherapy cohort no association of HER2-positive CTCs with change in PFS (HR 1.09, 95% CI 0.67-1.78) or OS (HR 0.93, 95% CI 0.53-1.63) was observed (**Figure 1 B&D**).

When the cut-off for HER2-positivity was shifted to only the CTCs that had a 3+ immunofluorescent staining, HER2-positive-3+ CTCs were observed in 6 patients (8%) in the endocrine therapy cohort and in 9 patients (11%) in the chemotherapy cohort. As the number of patients with HER2-positive-3+ CTCs was very limited, we did not perform formal statistics on differences in PFR6months.

INCIDENCE AND PROGNOSTIC VALUE OF SWITCHES IN ER STATUS BETWEEN PT AND CTCS

The ER-status of CTCs was assessed on the mRNA level and determined using our pre-defined cut-off for *ESR1*-positivity as described before (17). We compared the ER-status of the CTCs with the ER-status of the PT as reported by the pathologist. The ER-CTC status could not be determined in 38 patients (25%): in 9 patients no sample for mRNA analysis was available, in 29 patients the mRNA was of poor quality or the epithelial mRNA signal was too low, the latter being indicative of a CTC count too low for a reliable mRNA analysis. We were thus able to determine the ER-status on CTCs in 116 patients (75%) **(Table 3)**. In the endocrine therapy cohort, consisting solely of patients with ER-positive PTs, 10 patients (14%) had ER-negative CTCs. In the chemotherapy cohort, 31% of the patients had a discordant ER-status between the PT and the CTCs. Interestingly, out of 19 patients who had an ER-negative PT, 13 patients (68%) had ER-positive CTCs. In addition, in 7 out of 46 patients (15%) with an ER-positive PT, the CTCs were negative for ER.

TABLE 3. Discordances between primary tumor and CTC regarding the ER status

	***ESR1* status CTCs negative**	***ESR1* status CTCs positive**	**Total**
ENDOCRINE THERAPY			
ER status primary tumor			
Positive	10	41	51
CHEMOTHERAPY			
ER status primary tumor			
Negative	6	13	19
Positive	7	39	46
Total	13	52	65

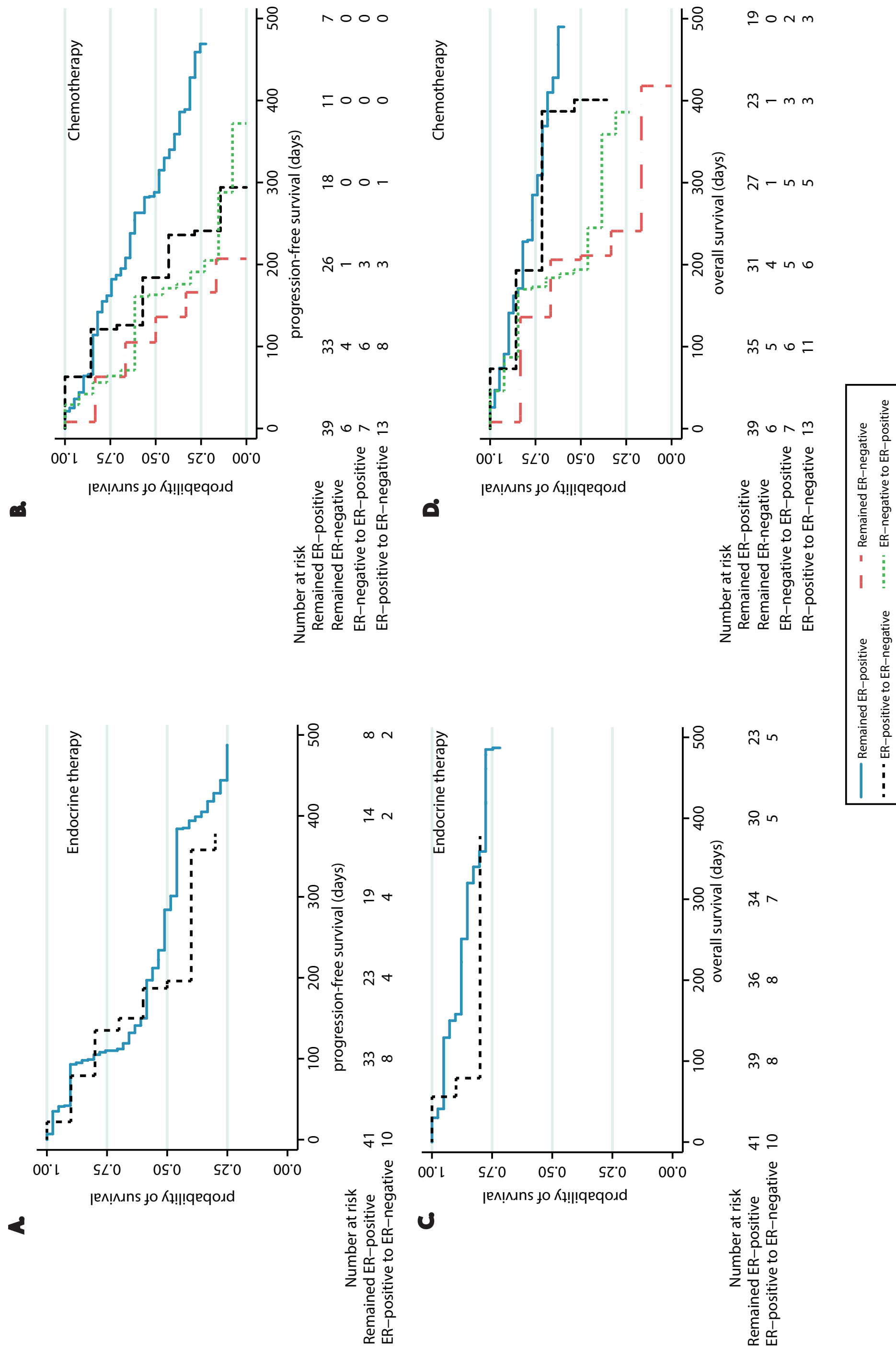


FIGURE 2. PFS AND OS ACCORDING TO ER-CTC STATUS. Panel A and C show PFS and OS, respectively, for the endocrine therapy cohort, Panel B and D show PFS and OS, respectively, for chemotherapy cohort.

Exploratory Kaplan-Meier curves for the prognostic impact of ER-switches between the PT and the CTCs were constructed as planned. As depicted in **Figure 2**, no clear prognostic impact of ER-switch appeared to be present in either the endocrine therapy cohort or the chemotherapy cohort.

ASSOCIATIONS BETWEEN HER2 AND ER-STATUS OF CTCS AND THE PT

We explored whether HER2-positive CTCs were related to the HER2 HercepTest score as assessed on the PT by the pathologist in the context of standard clinical care. For 42 patients, no data on the PT HercepTest score was available. In the remaining 112 patients, the HercepTest score (0, 1+ or 2+) was not found to be associated with the presence or absence of HER2-positive CTCs (p=0.24, **Supplementary Table 1**). We also explored whether switches in patients with an ER-positive PT to ER-negative CTCs were associated with the percentage of ER-positive tumor cells in the PT. Patients who had an ER-positive PT, but ER-negative CTCs, had lower percentages of ER-positive tumor cells in the PT than patients with an ER-positive PT in whom the CTCs remained ER-positive (p=0.03; **Supplementary Figure 2**). No data on the percentage of ER-positive tumor cells was available for patients with an ER-negative PT.

DISCUSSION

CTCs are an attractive alternative to solid biopsies, and may give insight into intra-tumor and inter-metastatic heterogeneity (28, 29). Previous studies demonstrated discrepancies in the HER2 and ER status between the PT and the metastases, as well as discordances of these markers between the PT and the CTCs. However, appropriately powered studies evaluating the prognostic impact of HER2-positive CTCs have been scarce. We set out to evaluate the prevalence and prognostic value of HER2-positive CTCs in MBC patients with HER2-negative PTs. We hypothesized that MBC patients with HER2-positive CTCs would have a lower PFS after six months of endocrine therapy compared to patients with HER2-negative CTCs. While discordance in the HER2-status between the PT and CTCs was frequently present, no prognostic value of HER2-positive CTCs was observed in MBC patients treated with endocrine therapy.

Our findings that HER2-positive CTCs occur in a relatively large subset of HER2-negative MBC patients (45/154 patients, 29%) is in accordance with previous reports. Wallwiener and colleagues (18) used the same method as we did to score HER2-positive CTCs, and found HER2-positive CTCs in 30% of their 107 MBC patients with ≥5 CTCs and HER2-negative PTs. In contrast to our results, this study observed that patients with ≥5 CTCs and HER2-positive CTCs had a longer PFS than patients with ≥5 CTCs without HER2-positive CTCs. However, since also patients with HER2-positive PTs who were treated with HER2-targeted treatments were included in that study, the results cannot directly be compared with our results. Slightly higher numbers of HER2-positive CTCs than in our study were observed by Fehm et al. (30), whom used an immunofluorescent staining on CellSearch for HER2 of 3+, and observed HER2-positive CTCs in 25 of 76 (33%) MBC patients with HER2-negative PTs. Although other studies have investigated the prognostic impact of HER2-CTC status in MBC (4, 8, 12), these studies did either not use a FDA-cleared method to enumerate CTCs followed by characterization for HER2 expression, or they did not sufficiently describe their technique to score HER2-positive

CTCs. For these reasons, direct comparison of the results from these studies with our study is not possible.

While HER2-positive CTCs were frequently present in our MBC patients, their presence was not of prognostic value. Our study was powered to detect a 20% PFR6months in patients with HER2-positive CTCs receiving endocrine therapy versus 70% PFR6months in patients with HER2-negative CTCs, which was a difference considered clinically relevant after a small survey amongst medical oncologists. We believe it is justified to have powered this study for these sorts of large differences in PFR, because only such large differences will have a clear clinical impact and affect clinical decision making. Our data at least suggests that the presence of HER2-positive CTCs in patients with HER2-negative PTs does not have a major impact on their prognosis. There are several explanations for the lack of prognostic value of HER2-positive CTCs. First, it is possible that there is indeed no association between HER2 overexpression in CTCs of MBC patients and outcome to endocrine treatment. Second, specificity issues may have occurred when using the CellSearch Tumor Phenotyping reagent HER2. We found a moderate but significant correlation between the number of CTCs and the number of HER2-positive CTCs (Spearman r= 0.31, $P<0.001$). This suggests that when a higher number of CTCs would be present, there would be larger chance of finding at least one HER2-positive CTC. This may be in line with the observed heterogeneity for HER2 expression in CTCs as seen in the median HER2 to CTC ratio, or indicate specificity issues. Third, a limitation of our study is that we did not perform FISH analysis on CTCs to confirm amplification of HER2, which may have further improved the specificity of the HER2-CTC-assay. Fourth, a subset of patients in the endocrine therapy cohort had already received prior endocrine therapy for MBC, which may have impacted the analyses regarding PFS in this cohort. However, in a subgroup analysis of patients receiving first-line endocrine therapy which also met our power calculation, no prognostic value of HER2-positive CTCs was observed either. Fifth, the fact that HER2 is overexpressed, does not necessarily mean that it is also an active driver of tumor growth in that particular patient. The determination of phosphorylated HER2 or markers downstream of HER2 in CTCs may provide better insight into the activity of the HER2-signaling pathway in CTCs (31). Lastly, there is currently no consensus on the optimal cut-off for HER2-positivity. We chose CTCs immunofluorescently staining 2+ or 3+ as HER2-positive, given that this was the cut-off used in the CellSearch/Veridex inter-reader variability study (32), and good agreement for this cut-off was demonstrated between academic readers and Veridex consensus. However, other cut-offs for HER2 positivity on CTCs might yield different results regarding the prognostic impact of HER2-positive CTCs. Consensus on the optimal cut-off for HER2-positive CTCs is needed, and should be driven by the prognostic power and clinical utility of such a cut-off (for example for response to anti-HER2 targeted agents).

With regard to ER-status, we found discordance between the PT and CTCs in 26% of our patients, which is in line with our previous reports (9, 17) and reports by others (6, 13, 33-35), describing heterogeneity of CTCs for ER and discordances in ER-status in 24-45% of the MBC cases. Especially of interest is that ER-positive CTCs were observed in 68% of the patients with an ER-negative PT, which might indicate that a subset of patients with an ER-negative PT might benefit from endocrine therapy. Also worth noting is the finding that patients in whom the PT was ER-positive, but the CTCs ER-negative,

the number of ER-positive tumor cells in the primary was significantly lower than in patients in whom the CTCs remained ER-positive. This suggests that heterogeneity in ER expression in the PT may give a higher chance of clonal evolution of an ER-negative clone. While heterogeneity and discordances between PTs and CTCs for ER expression have frequently been described, little is known about the prognostic impact of the ER-CTC status. We previously explored the prognostic value of the ER-CTC status and found patients with ER-negative PTs but ER-positive CTCs to have a longer time-to-treatment-switch than patients who remained ER-negative (17). However, in the present study (comprising 30 of the patients who were also included in our previous report), we were unable to confirm these findings. This could be due to the facts that our previous cohort was smaller (n=62), and that included patients were treated with either endocrine therapy or chemotherapy. In addition, the applied cut-off for ER-positivity of CTCs in our previous study was based on *ESR1* mRNA levels in the PTs. Although this cut-off was demonstrated to have excellent sensitivity and specificity (17), it was not feasible to validate that cut-off value for the current study since the PT tissue was not available for all patients. The exploratory analyses here indicating lack of prognostic value should however be interpreted with caution, as the number of patients who had a switch in ER status was limited. Larger studies are required to evaluate the prognostic value of ER discordances between the PT and CTCs, preferably also evaluating ER-expression in CTCs at the single cell level to enable the evaluation of heterogeneity in ER expression between single CTCs. We have recently started a study in which we are evaluating heterogeneity of ER-positive-CTCs and their prognostic impact using a proximity-ligation-assay technique (CareMore-Trastuzumab study & CareMore-AI study; NTR5121 (36, 37)), while others have reported on immunofluorescent ER staining directly in the CellSearch machine, similar to HER2 in this study (35).

In conclusion, a lack of prognostic value was observed for HER2-positive CTCs and ER-positive CTCs with respect to outcome to endocrine therapy or chemotherapy in MBC patients. Future research should focus on ER characterization of CTCs in a larger patient cohort, but may also focus on looking beyond classical predictive factors (such as HER2 and ER expression) that are related to endocrine resistance. This could for example be done by determining resistant mutations in *ESR1* (34) or measuring gene expression panels associated with resistance to anti-tumor therapy (23, 24). Such characterization of several prognostic and predictive markers on CTCs correlated with resistance to either endocrine therapy or chemotherapy may eventually lead to improved prognostication and prediction of therapeutic response in MBC patients.

REFERENCES

1. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol. 2012 Feb 20;30(6):587-92.

2. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E, Group EGW. Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2012 Oct;23 Suppl 7:vii11-9.

3. Van Poznak C, Somerfield MR, Bast RC, Cristofanilli M, Goetz MP, Gonzalez-Angulo AM, et al. Use of Biomarkers to Guide Decisions on Systemic Therapy for Women With Metastatic Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline. J Clin Oncol. 2015 Aug 20;33(24):2695-704.

4. Meng S, Tripathy D, Shete S, Ashfaq R, Haley B, Perkins S, et al. HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci U S A. 2004 Jun 22;101(25):9393-8.

5. Fehm T, Becker S, Duerr-Stoerzer S, Sotlar K, Mueller V, Wallwiener D, et al. Determination of HER2 status using both serum HER2 levels and circulating tumor cells in patients with recurrent breast cancer whose primary tumor was HER2 negative or of unknown HER2 status. Breast Cancer Res. 2007;9(5):R74.

6. Tewes M, Aktas B, Welt A, Mueller S, Hauch S, Kimmig R, et al. Molecular profiling and predictive value of circulating tumor cells in patients with metastatic breast cancer: an option for monitoring response to breast cancer related therapies. Breast Cancer Res Treat. 2009 Jun;115(3):581-90.

7. Pestrin M, Bessi S, Galardi F, Truglia M, Biggeri A, Biagioni C, et al. Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients. Breast Cancer Res Treat. 2009 Dec;118(3):523-30.

8. Munzone E, Nole F, Goldhirsch A, Botteri E, Esposito A, Zorzino L, et al. Changes of HER2 status in circulating tumor cells compared with the primary tumor during treatment for advanced breast cancer. Clin Breast Cancer. 2010 Oct 1;10(5):392-7.

9. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res. 2011 Jun 1;17(11):3600-18.

10. Ignatiadis M, Rothe F, Chaboteaux C, Durbecq V, Rouas G, Criscitiello C, et al. HER2-positive circulating tumor cells in breast cancer. PloS one. 2011;6(1):e15624.

11. Pestrin M, Bessi S, Puglisi F, Minisini AM, Masci G, Battelli N, et al. Final results of a multicenter phase II clinical trial evaluating the activity of single-agent lapatinib in patients with HER2-negative metastatic breast cancer and HER2-positive circulating tumor cells. A proof-of-concept study. Breast Cancer Res Treat. 2012 Jul;134(1):283-9.

12. Hayashi N, Nakamura S, Tokuda Y, Shimoda Y, Yagata H, Yoshida A, et al. Prognostic value of HER2-positive circulating tumor cells in patients with metastatic breast cancer. Int J Clin Oncol. 2012 Apr;17(2):96-104.

13. Nadal R, Fernandez A, Sanchez-Rovira P, Salido M, Rodriguez M, Garcia-Puche JL, et al. Biomarkers characterization of circulating tumour cells in breast cancer patients. Breast Cancer Res. 2012;14(3):R71.

14. Onstenk W, Gratama J, Foekens J, Sleijfer S. Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. Cancer Treat Rev. 2013;39(7):691-700.

15. Liu Y, Liu Q, Wang T, Bian L, Zhang S, Hu H, et al. Circulating tumor cells in HER2-positive metastatic breast cancer patients: a valuable prognostic and predictive biomarker. BMC Cancer. 2013;13:202.

16. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, et al. Development of Circulating Tumor Cell-Endocrine Therapy Index in Patients with Hormone Receptor Positive Breast Cancer. Clin Cancer Res. 2014 Nov 7.

17. Onstenk W, Sieuwerts AM, Weekhout M, Mostert B, Reijm EA, van Deurzen CH, et al. Gene expression profiles of circulating tumor cells versus primary tumors in metastatic breast cancer. Cancer Lett. 2015 Jun 28;362(1):36-44.

18. Wallwiener M, Hartkopf AD, Riethdorf S, Nees J, Sprick MR, Schonfisch B, et al. The impact of HER2 phenotype of circulating tumor cells in metastatic breast cancer: a retrospective study in 107 patients. BMC Cancer. 2015;15(1):403.

19. Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, et al. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene. 1995 Jun 15;10(12):2435-46.

20. Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, et al. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat. 1992;24(2):85-95.

21. Liu Y, el-Ashry D, Chen D, Ding IY, Kern FG. MCF-7 breast cancer cells overexpressing transfected c-erbB-2 have an in vitro growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity in vivo. Breast Cancer Res Treat. 1995 May;34(2):97-117.

22. De Laurentiis M, Arpino G, Massarelli E, Ruggiero A, Carlomagno C, Ciardiello F, et al. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. Clin Cancer Res. 2005 Jul 1;11(13):4741-8.

23. Mostert B, Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, van Galen A, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. Ann Oncol. 2015 Mar;26(3):510-6.

24. Reijm EA, Sieuwerts AM, Smid M, Vries JB, Mostert B, Onstenk W, et al. An 8-gene mRNA expression profile in circulating tumor cells predicts response to aromatase inhibitors in metastatic breast cancer patients. BMC Cancer. 2016;16(1):123.

25. Riethdorf S, Muller V, Zhang L, Rau T, Loibl S, Komor M, et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res. 2010 May 1;16(9):2634-45.

26. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009 Jan;45(2):228-47.

27. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Treat. 2006 Nov;100(2):229-35.

28. Lohr JG, Adalsteinsson VA, Cibulskis K, Choudhury AD, Rosenberg M, Cruz-Gordillo P, et al. Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. Nat Biotechnol. 2014 May;32(5):479-84.

29. Onstenk W, Sieuwerts AM, Mostert B, Lalmahomed Z, Bolt-de Vries JB, van Galen A, et al. Molecular characteristics of circulating tumor cells resemble the liver metastasis more closely than the primary tumor in metastatic colorectal cancer. Oncotarget. 2016 Jun 20.

30. Fehm T, Muller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat. 2010 Nov;124(2):403-12.

31. Frogne T, Laenkholm AV, Lyng MB, Henriksen KL, Lykkesfeldt AE. Determination of HER2 phosphorylation at tyrosine 1221/1222 improves prediction of poor survival for breast cancer patients with hormone receptor-positive tumors. Breast Cancer Res. 2009;11(1):R11.

32. Ignatiadis M, Riethdorf S, Bidard FC, Vaucher I, Khazour M, Rothe F, et al. International study on inter-reader variability for circulating tumor cells in breast cancer. Breast Cancer Res. 2014;16(2):R43.

33. Aktas B, Muller V, Tewes M, Zeitz J, Kasimir-Bauer S, Loehberg CR, et al. Comparison of estrogen and progesterone receptor status of circulating tumor cells and the primary tumor in metastatic breast cancer patients. Gynecol Oncol. 2011 Aug;122(2):356-60.

34. Babayan A, Hannemann J, Spotter J, Muller V, Pantel K, Joosse SA. Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients. PloS one. 2013;8(9):e75038.

35. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer. Clin Cancer Res. 2015 Jun 1;21(11):2487-98.

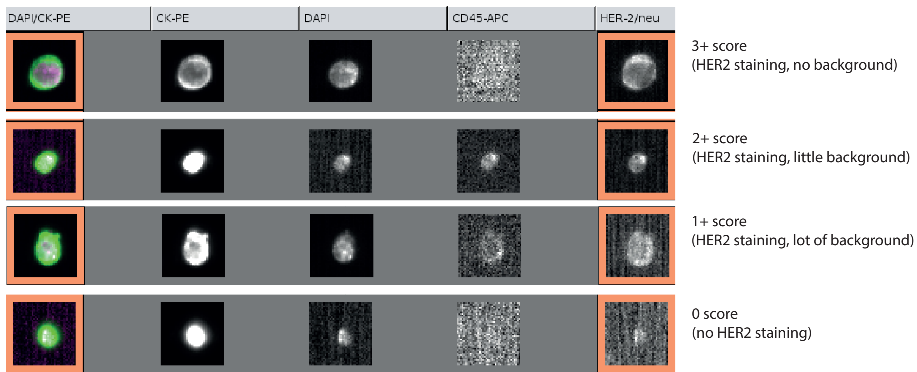
36. CareMoreCTC studies. http://www.caremorectc.eu/.

37. Beije N, Jager A, Sleijfer S. Circulating tumor cell enumeration by the CellSearch system: the clinician's guide to breast cancer treatment? Cancer Treat Rev. 2015 Feb;41(2):144-50.

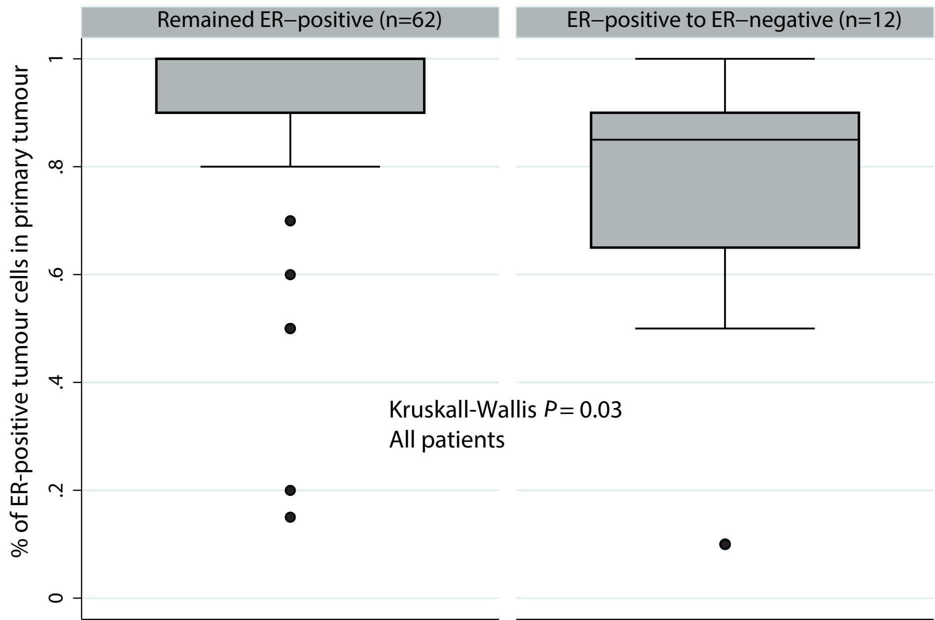
SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE 1. HercepTest score in the primary tumor and association with HER2-positive CTCs		
HER2 status of primary tumor	**No HER2-positive CTCs (n=76)**	**HER2-positive CTCs (n=36)**
HercepTest score 0	34	12
HercepTest score 1+	29	13
HercepTest score 2+	13	11

Chi-square P-value = 0.24



SUPPLEMENTARY FIGURE 1. HER2 scoring in CTCs. According to the Riethdorf criteria (Clinical Cancer Research 2010)



SUPPLEMENTARY FIGURE 2. ER-switches according to the percentage of ER-positive tumor cells in the primary tumor

CHAPTER 4

ESR1 mutations: moving towards guiding treatment decision-making in metastatic breast cancer patients

Nick Beije*, Lindsay Angus*, Agnes Jager,
John W.M. Martens, Stefan Sleijfer

Cancer Treat Rev. 2016 in press

* These authors contributed equally to
this manuscript

ABSTRACT

Mutations in the gene coding for the estrogen receptor (ER), *ESR1*, have been associated with acquired endocrine resistance in patients with ER-positive metastatic breast cancer (MBC). Functional studies revealed that these *ESR1* mutations lead to constitutive activity of the ER, meaning that the receptor is active in absence of its ligand estrogen, conferring resistance against several endocrine agents. While recent clinical studies reported that the occurrence of *ESR1* mutations is rare in primary breast cancer tumors, these mutations are more frequently observed in metastatic tissue and circulating cell-free DNA of MBC patients pretreated with endocrine therapy. Given the assumed impact that the presence of *ESR1* mutations has on outcome to endocrine therapy, assessing *ESR1* mutations in MBC patients is likely to be of significant interest to further individualize treatment for MBC patients. Here, *ESR1* mutation detection methods and the most relevant pre-clinical and clinical studies on *ESR1* mutations regarding endocrine resistance are reviewed, with particular interest in the ultimate goal of guiding treatment decision-making based on *ESR1* mutations.

INTRODUCTION

Endocrine therapy with selective estrogen receptor modulators/downregulators (SERMs/SERDs) or by estrogen deprivation using aromatase inhibitors (AIs), is the most important treatment modality for estrogen receptor (ER)-positive metastatic breast cancer (MBC) patients (1). Unfortunately, 40% of patients do not benefit from first-line endocrine therapy due to intrinsic resistance, and the remainder of patients initially responding will eventually develop resistance during therapy (1). Several mechanisms have been linked to endocrine resistance, however, no marker for resistance has reached wide clinical use yet (2-4). Recently, mutations in the gene encoding ERα, *ESR1*, have attracted particular interest as a mechanism for endocrine resistance in MBC. Large-scale next-generation sequencing (NGS) efforts on MBC tissues revealed that these mutations are enriched in MBC patients treated with endocrine agents while these variants are not or only at very low frequencies present in primary tumor tissue (5, 6). Importantly, this implies that their presence has to be assessed in metastatic lesions, or in “liquid biopsies” such as circulating cell-free DNA (cfDNA) as a representative of metastatic tumor cells. Here we review the pros and cons of current detection methods for *ESR1* mutations, the pre-clinical and clinical studies investigating *ESR1* mutations and highlight its potential role in treatment decision-making in MBC patients.

FUNCTIONAL STUDIES ON ESR1 MUTATIONS

The ER belongs to the nuclear hormone receptor superfamily (7) and consists of two activation function (AF)-1/2 domains, DNA binding and hinge domains, and a ligand binding domain (LBD) (**Figure 1**). The ER functions as a ligand-dependent transcription factor. Binding of estradiol to the LBD leads to a conformational change of helix 12, resulting in recruitment of coregulatory proteins (8). This eventually yields transcription of genes important in normal physiological processes but also for breast tumorigenesis and breast cancer (BC) progression (9).

Recent NGS efforts revealed that somatic *ESR1* mutations in the LBD were more frequently present in metastatic lesions than previously thought. In preclinical models to evaluate the role of *ESR1* mutations in endocrine resistance, it was demonstrated that cell lines transfected with a D538G, Y537S, L536Q, Y537N, Y537C, S463P or E380Q *ESR1* mutation exert activity in the absence of estrogen (6, 10-15) (**Figure 1**)**.** This constitutive activity suggests that estrogen-depriving therapies such as AIs are not or less effective in patients with activating *ESR1* mutations. Cell lines transfected with mutant *ESR1* variants were however still responsive to treatment with tamoxifen and fulvestrant, though sensitivity to these drugs was relatively impaired compared to *ESR1* wildtype transfected cell lines (5, 6, 12, 13). Similar observations were recently made for novel SERM/SERD hybrid endocrine therapies pipendoxifene and bazedoxifene (16).

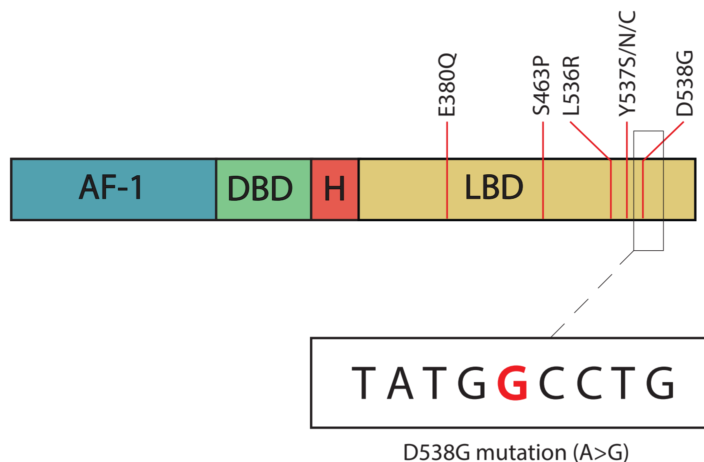


FIGURE 1. SCHEMATIC OVERVIEW OF THE DIFFERENT DOMAINS OF THE ER. Activation function (AF) domain-1 present at the N-terminus acts in a ligand-independent manner, whereas, the AF-2 within the ligand binding domain (LBD) is dependent on estradiol for its activation (53). The DNA binding domain encodes two zinc finger molecules, playing an important role in receptor dimerization and binding of the ER to specific DNA sequences: the estrogen response element (ERE) (54). H=hinge region. *ESR1* mutations, some hotspot mutations shown as vertical red lines, mainly occur in the C-terminal domain of the receptor encoding for the LBD of the ER.

TECHNIQUES TO DETECT ESR1 MUTATIONS

Several techniques can be used to assess *ESR1* mutations in tissue or cfDNA (**Figure 2**), all having their own advantages and disadvantages. Importantly, these techniques widely vary in their sensitivity. NGS can be performed either in the context of whole genome sequencing, as part of a whole exome panel, or as part of a targeted *ESR1* panel. While NGS is an established and widely used approach for mutation detection in tumor tissue, mutation detection in cfDNA is more challenging, as the relative number of mutant to wildtype DNA alleles has to be taken into account. Frequencies of circulating tumor DNA (ctDNA) vary largely between patients, frequently being below 1% of the total cfDNA (17), which is beyond the sensitivity of conventional NGS. Therefore, techniques based on digital PCR (dPCR) have been introduced enabling the detection of ctDNA in frequencies as low as 0.001% (18, 19). In dPCR-based techniques, each individual DNA molecule, within its own partition, is able to react with a specific probe for wildtype *ESR1* and another probe for a specific *ESR1* mutant. There are several commercially available dPCR-based assays (e.g. digital PCR, droplet digital PCR (ddPCR), BEAMing), differing in used reagents and sample readouts, but generally having similar sensitivity (17, 20). In a study comparing conventional targeted NGS with dPCR to detect mutations in cfDNA, threefold more D538G *ESR1* mutations in cfDNA were observed using dPCR than with NGS (21). One disadvantage of dPCR assays is however that only a subset of hotspot mutations can be evaluated. Other assays, using some sort of target-enrichment prior to analysis, can be used to detect multiple hotspot mutations (OnTarget assay (22, 23)) or multiple frequently mutated genes (e.g. SafeSeqS (24), CAPP-Seq (25)), however to date these assays have not yet been reported to be used to detect *ESR1* mutations.

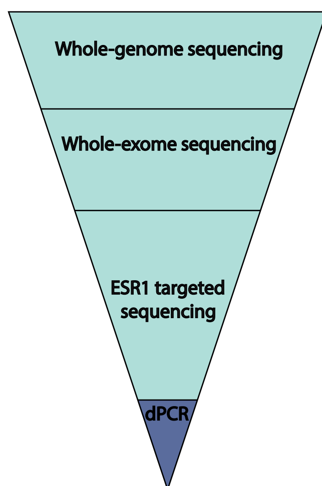


FIGURE 2. VARIOUS TECHNIQUES FOR *ESR1* MUTATION DETECTION. The pyramid represents the range in which the genome is investigated. *ESR1* mutations can be detected by large-scale NGS efforts such as whole-genome sequencing or whole-exome sequencing, or by more targeted methods as targeted sequencing of the *ESR1* gene only, or by the interrogation of individual mutations in *ESR1* by digital PCR.

CLINICAL STUDIES ON THE SIGNIFICANCE OF *ESR1* MUTATIONS

ESR1 MUTATIONS IN PRIMARY AND METASTATIC TUMOR TISSUE

Although already described anecdotally in the 90s (11, 26, 27), *ESR1* mutations were thought to be rare in BC. They occur only in up to 3% of primary tumors using NGS (**Supplementary table 1**) (5, 6, 12, 13). Using more sensitive dPCR-based techniques, the *ESR1* mutation rate in primary BC tumors may mildly increase (28, 29), however, only at very low variant allele frequencies (VAF; 0.07-0.2%) (29).

In contrast to mutation rates in primary BC, the landmark papers of Toy *et al*. (6) and Robinson *et al.* (13) showed much higher *ESR1* mutation rates in metastatic lesions (**Supplementary table 2**). Toy and colleagues (6) found *ESR1* mutations (predominantly D538G and Y537S) in metastatic tissues in 9/36 ER-positive MBC patients who had received at least 3 months of endocrine therapy. All patients with an *ESR1* mutation were at least treated with two lines of endocrine therapy; all containing an AI. In an independent cohort of 44 metastatic tumors from patients pretreated with endocrine therapy, 5 metastases (11%) harbored an *ESR1* mutation.

Likewise, Robinson *et al.* (13) demonstrated *ESR1* mutations in 6/11 (55%) evaluated metastatic biopsies of ER-positive MBC patients. All patients with an *ESR1* mutation were pretreated with AIs and SERMs or SERDs. None of three available matched primary tumors of patients with a metastatic *ESR1* mutation harbored an *ESR1* mutation. Based on these findings and the accompanied functional studies, both groups hypothesized that *ESR1* mutations are a common mechanism underlying endocrine resistance, developing during estrogen deprivation, especially in the context of AI treatment.

Prompted by these findings, several studies investigated *ESR1* mutations in metastatic tissue of MBC patients. In 5/13 (38%) ER-positive MBC patients, who failed on multiple lines of endocrine treatment, a D538G *ESR1* mutation was reported (12). Furthermore, Jeselsohn *et al.* (5) detected in 9/76 (12%) ER-positive metastatic tumors *ESR1* mutations (Y537N/C/S and D538G) using NGS, whereas none of the 115 ER-negative tumors they

assessed had such mutations. In both studies no *ESR1* mutations in matched primary tumors were detected (5, 12).

In a study using dPCR, an *ESR1* mutation was revealed in metastatic tissue of 11/55 ER-positive MBC patients (28). Notably, polyclonal *ESR1* mutations (multiple *ESR1* mutations in one sample) were observed in 4/11 (36%) patients. Also of particular interest was that two patients with *ESR1* mutations were not pretreated with any therapies at all and 4/11 only received prior treatment with tamoxifen, supporting a previous observation (5) that *ESR1* mutations are not exclusively found following AI treatment. In another study (29) applying dPCR, *ESR1* mutations were found in 3/43 primary tumors, 1/12 bone metastasis tissues and 3/38 brain metastasis tissues in ER-positive MBC patients. The prevalence of *ESR1* mutations and their VAF were higher in bone (1.4% VAF) and especially in brain metastases (34.3-44.9% VAF) compared to primary tumors (0.07-0.2% VAF), suggesting an enrichment of *ESR1*-mutant subclones in metastatic tissue.

All these tissue-based studies provided important insights into the prevalence of *ESR1* mutations and the population of patients in which they occur. However, the biggest disadvantage of these studies is that they concerned mostly small, heterogeneously treated, and retrospectively selected patient cohorts. Furthermore, of note is that biopsies were usually taken at various time points and therefore the evidence at which moment *ESR1* mutations emerge, which is suggested to be mainly after AI treatment, is indirect. The majority of the above mentioned drawbacks are mainly driven by the fact that taking metastatic biopsies is a cumbersome procedure and even impossible in some patients, not easily allowing the assessment of *ESR1* mutations over time. In addition, taking metastatic biopsies may lead to sample bias due to tumor heterogeneity (30). Therefore, recent studies have focused on *ESR1* mutation detection in "liquid biopsies" as a patient-friendly alternative to taking biopsies from metastatic lesions.

ESR1 MUTATIONS IN 'LIQUID BIOPSIES'

Circulating blood biomarkers such as circulating tumor cells (CTCs) and cfDNA are increasingly used as non-invasive surrogate "liquid biopsies", and are thought to represent the most important metastatic tumor sites (31, 32). Both these types of liquid biopsies can be measured in peripheral blood, with CTCs being intact tumor cells and cfDNA being DNA mainly derived from apoptotic tumor cells. Recently, several studies investigating the presence of *ESR1* mutations in liquid biopsies, particularly in cfDNA, have been published (**Table 1**).

To evaluate NGS and dPCR techniques to detect *ESR1* mutations in plasma, Guttery *et al.* examined cfDNA of 48 ER-positive MBC patients (21). In 3/48 patients (6%), they observed an *ESR1* mutation in cfDNA using NGS. In one patient with a D538G mutation also CTCs, isolated by the CellSearch system, were sequenced, and the same mutation was detected in CTCs. When dPCR was performed in the same cohort for the D538G mutation only, the D538G mutation was found in 6 additional patients (15%) at VAF below 1%, underlining the limited sensitivity of NGS to detect low frequent mutations. In eleven patients, serial plasma samples were available. Interestingly, in one patient an *ESR1* mutation was present at baseline and was further enriched (0.4% VAF to 13.6% 3 months later) while treated with chemotherapy (docetaxel/vinorelbine).

Table 1. Overview of *ESR1* mutation analysis in "liquid biopsies" of metastatic breast cancer patients

Patients	Method	*ESR1* mutation	Substrate	Number of patients with *ESR1* mutations	D538G	Y537S	Y537N	Y537C	Other	Ref
6 pts with ER- positive MBC either off or progressing on therapy	RNA sequencing	whole transcriptome	Cultured CTCs	3/6 (50%)	1/6 (17%)	1/6 (17%)	-	-	1/6 (17%) L536P	(44)
48 pts with ER-positive MBC receiving endocrine therapy	NGS	E380Q, V392I, P535H, Y537C/N/S, D538G	cfDNA	3/48 (6%)	1/48 (2%)	1/48 (2%)	-	-	1/48 (2%) E380Q	(21)
48 pts with ER-positive MBC receiving endocrine therapy	ddPCR	D538G	cfDNA	9/48 (19%)	9/48 (19%)	NP	NP	NP	NP	
3 pts with *ESR1* mutation detected in cfDNA by NGS	NGS	E380Q, V392I, P535H, Y537C/N/S, D538G	CTCs	1/3 (33%)	1/3 (33%)	-	-	-	-	
128 pts with ER-positive MBC, progression on therapy	ddPCR	D538G, Y537C/N/S, L536R	cfDNA	18/128 (14%)	14/128 (11%)	3/128 (2%)	4/128 (3%)	2/128 (2%)	2/128 (2%) L536R	(34)
11 pts with ER-positive MBC (8 with known *ESR1* mutation in metastatic biopsy by NGS)	ddPCR	D538G, Y537N/S	cfDNA	9/11 (82%)	6/11 (55%)	3/11 (27%)	1/11 (9%)	NP	NP	(33)
8 pts with ER-positive MBC	ddPCR	D538G, Y537N/S	cfDNA	6/8 (75%)	4/8 (50%)	2/8 (25%)	1/8 (13%)	NP	NP	
29 pts with MBC	ddPCR	K303R, S463P, Y537C/N/S, D538G	cfDNA	7/29 (24%)	6/29 (21%)	2/29 (7%)	-	1/29 (3%)	-	(29)
161 pts ER-positive MBC with prior sensitivity to nonsteroidal AI (SoFEA)	ddPCR	E380Q, L536R, Y537C/N/S, D538G, S463P	cfDNA	63/161 (39%)	29/161 (18%)	16/161 (10%)	23/161 (14%)	3/161 (2%)	6/161 (4%) E380Q, 6/161 (4%) S463P, 2/161(1%) L536R	(38)
360 pts with ER-positive MBC with progression on endocrine therapy (PALOMA3)	ddPCR	E380Q, L536R, Y537C/N/S, D538G, S463P	cfDNA	91/360 (25%)	51/360 (14%)	23/360 (6%)	14/360 (4%)	5/360 (1%)	22/360 (6%) E380Q, 4/360 (1%) S463P, 1/360 (1%) L536R	
153 pts with ER-positive MBC pre-treated with AI (FERGI)	BEAMing	E380Q, S463P, V524E, P535H, L536H/P/Q/R, Y537C/N/S, D538G	cfDNA	57/153 (37%)	31/153 (20%)	19/153 (12%)	10/143 (7%)	6/143 (4%)	15/153 (26%) E380Q, 5/143 (3%) S463P, 7/143 (5%) L536P	(36)
5 pts with MBC (4 ER+, 1 TN), with ≥100 CTCs	NGS	*ESR1* exome	40 single CTCs	10/40 (25%)	7/40 (18%)	-	-	-	3/40 (8%) E380Q	(45)
5 pts with MBC (4 ER+, 1 TN), with ≥100 CTCs	NGS	*ESR1* exome	cfDNA	3/5 (60%)	2/5 (40%)			1/5 (20%)	1/5 (20%) E380Q	
541 pts with ER-positive MBC with progression after nonsteroidal AI (BOLERO-2)	ddPCR	D538G, Y537S	cfDNA	156/541 (29%)	113/541 (21%)	72/541 (13%)	-	-	-	(40)

ddPCR= droplet digital PCR, NP= Not performed. Number of patients with a *ESR1* mutation in different study cohorts are listed. The specific mutations observed in these patients are also shown; in case of polyclonality, these numbers may exceed the total number of patients with a *ESR1* mutation.

To further explore whether *ESR1* mutations present in metastases are also represented in the cfDNA, Chu *et al*. (33) assessed *ESR1* mutations in plasma cfDNA in 11 ER-positive MBC patients in whom the *ESR1* mutation status in a metastatic lesion was assessed by NGS. All *ESR1* mutations (8/8) observed in the metastatic lesions were also observed in the cfDNA using dPCR. In one patient with an *ESR1* wildtype metastatic lesion, a low frequency *ESR1* mutation was observed in the cfDNA. It should however be noted that the cfDNA was obtained two months after the biopsy, meaning that changes in *ESR1* mutation status could also be due to therapy-related effects emerging after the initial biopsy. In an independent cohort consisting of 8 ER-positive patients, dPCR was once more demonstrated to be able to detect *ESR1* mutations in cfDNA, and in two more patients an *ESR1* mutation was observed in the cfDNA but not in the metastatic lesion. This study further underscored that dPCR is able to readily detect *ESR1* mutations in the cfDNA and that cfDNA seems to represent *ESR1* mutations in the metastatic lesions. Also, strikingly, *ESR1* mutations were detected in cfDNA but not in metastatic lesions, which may be indicative of heterogeneity within the metastatic lesion or between multiple metastases.

Another study only used dPCR to detect *ESR1* mutations (29), and *ESR1* mutations were detected in 7/29 MBC patients (24%), with one patient having polyclonal *ESR1* mutations. All patients with an *ESR1* mutation in cfDNA received at least one line of endocrine treatment, mainly AIs or tamoxifen. In this series, also an *ESR1* mutation was seen in a patient who had only received prior treatment with fulvestrant. Of particular interest were the serial blood draws in the patient with the polyclonal *ESR1* mutations, which revealed that two mutations were enriched during AI treatment and chemotherapy, while one mutation was absent after treatment. This may suggest that different mutations react differently to different treatments.

Schiavon and colleagues (34) were the first to present a study in which *ESR1* mutations were assessed in a relatively large cohort of MBC patients. With dPCR to examine cfDNA from MBC patients at the time of progression under endocrine therapy, *ESR1* mutations were observed in 18/128 patients (14%), with D538G mutations comprising 56% of all observed *ESR1* mutations. Polyclonality of *ESR1* mutations was observed in 21% of the patients. All patients in whom *ESR1* mutations were observed had received prior AI treatment, while no *ESR1* mutations were observed in a subset of 22 patients who had only received tamoxifen treatment. Interestingly, *ESR1* mutations were mainly detected in patients who received AIs only in the metastatic setting (36%), and not in patients who received AIs only in the adjuvant setting (4%) or in the adjuvant and metastatic setting (8%). In accordance were observations in two relatively small independent cohorts, in which no *ESR1* mutations were observed in 32 BC biopsies taken at recurrence after adjuvant AI treatment or in 7 cfDNA samples of MBC patients who received adjuvant AI treatment only. Regarding the outcome of patients with *ESR1* mutations, subgroup analyses in *ESR1* mutant versus wildtype patients revealed a significantly poorer progression-free survival (PFS) on subsequent AI treatment in patients harboring an *ESR1* mutation, although these analyses should be seen as exploratory given the small number of patients eligible for such analyses.

The observations by Schiavon *et al.* suggests that AI treatment in the metastatic setting, but not in adjuvant setting, causes *ESR1* mutations. This may suggest selection of subclones already present in the primary tumor, or in the metastases when the tumor load is increased and the probability of acquiring mutations increases (35). This first observation could be in line with the previously mentioned findings by Wang *et al.* whom found *ESR1* mutations at extremely low VAF in primary tumors of MBC patients with *ESR1* mutations. While the study by Schiavon and colleagues also provided evidence for an impaired response to AI treatment, larger studies were needed to confirm these findings and to examine whether MBC patients with *ESR1* mutations will have improved responses on alternative therapie

ESR1 MUTATIONS AND OUTCOME ON ENDOCRINE THERAPIES

In the randomized phase II FERGI trial, baseline plasma samples of patients failing to AI treatment randomized either to fulvestrant combined with the pan-PI3K inhibitor pictilisib or to the combination of fulvestrant and placebo, were examined for *ESR1* and *PIK3CA* mutations in tissue and cfDNA using BEAMing (36). They detected *ESR1* mutations in cfDNA in 57/153 (37%) of patients at baseline; 13 patients (23%) harbored polyclonal mutations. Surprisingly, the prevalence of the E380Q mutation was rather high (26%), while this mutation was previously not often observed. No *ESR1* mutations were detected in 42 matched primary tumors of patients with *ESR1* mutations in cfDNA. *PIK3CA* mutations were observed in the cfDNA of 40% of the patients and were generally concordant with findings in matched metastatic tissue. For the *ESR1* mutations, discordances between the cfDNA and metastatic biopsies occurred more frequently and cfDNA sometimes harbored more *ESR1* mutations than the metastatic biopsies. These analyses were however limited by the fact that metastatic tissue and cfDNA were generally not collected on the same day. Of note was that the median VAF of *PIK3CA* mutations was markedly higher than for *ESR1* mutations (3.6% versus 0.45%). The higher VAFs and concordance with tissue probably reflect that *PIK3CA* mutations usually occur in earlier stages of BC (37), in contrast to *ESR1* mutations. Similar to Wang and colleagues (29), it was observed in multiple longitudinal samples in patients with polyclonal *ESR1* mutations that different *ESR1* mutations reacted differently under treatment.

The clinical analyses in the fulvestrant/placebo arm of the FERGI study revealed that patients with an *ESR1* mutation in ctDNA had no impaired PFS on fulvestrant compared to *ESR1* wildtype. When the analyses were further restricted to those patients with polyclonal *ESR1* mutations or *ESR1* mutation with VAF above the median, also no effect on PFS was observed. Also no differences in PFS were observed in patients with and without *ESR1* mutations receiving fulvestrant and pictilisib.

The data from the FERGI study suggested that fulvestrant does not have reduced activity in patients with *ESR1* mutations. However, data on the impact of *ESR1* mutations on outcome to fulvestrant versus AI treatment and the addition of other agents to fulvestrant treatment were still missing. These gaps were filled by data from two phase III randomized trials, reported by Fribbens *et al.* whom assessed *ESR1* mutations in

cfDNA by dPCR (38). In the SoFEA study, patients who had previously benefited from a non-steroidal AI were randomly assigned to fulvestrant combined with anastrozole, fulvestrant with placebo, or exemestane alone. Mutations were detected at baseline in 63/161 (39%) patients; 27/55 (49%) patients evaluable for polyclonal mutations had such mutations. Patients with an *ESR1* mutation had an improved PFS after taking a fulvestrant-containing regimen versus exemestane (median PFS fulvestrant-containing 5.7 versus exemestane 2.6 months, *P*=0.02), in contrast to *ESR1* wildtype patients in whom a similar PFS was found (5.4 months versus 8.0 months, *P*=0.77). Within the exemestane-treated patients, patients with *ESR1* mutations (n=18) had a worse PFS compared to patients having an *ESR1* wildtype (n=39), (median PFS 2.6 versus 8.0 months *P*=0.01).

In the PALOMA3 study, patients who failed on prior endocrine therapy were randomized to fulvestrant in combination with the CDK4/6-inhibitor palbociclib or to fulvestrant and placebo. In 91/360 patients (25%), *ESR1* mutations were detected with polyclonal mutations observed in 26/91 (29%). The main study revealed a significant PFS benefit in patients receiving fulvestrant/palbociclib versus patients receiving fulvestrant alone (median 9.5 versus 4.6 months, *P*=0.0001) (39). This PFS benefit was maintained in patients with *ESR1* mutations (median 9.4 versus 3.6 months, *P*=0.002), while no PFS difference was observed between *ESR1* mutants and wildtype in patients treated with fulvestrant/palbociclib (median 9.4 versus 9.5 months, respectively). Although PFS seemed to be slightly worse in the *ESR1* mutated patients treated with fulvestrant alone (3.6 months 95% CI, 2.0-5.5) compared to *ESR1* wildtype (5.4 months 95% CI 3.5-7.4), this was not statistically significant, which is in line with the results of the FERGI study (36).

So far, the only large study providing overall survival (OS) data with respect to *ESR1* mutations is the phase III BOLERO-2 study (40). In this study, postmenopausal women who progressed on an AI were randomized to the AI exemestane combined with everolimus, or exemestane and placebo. Overall, 156/541 (28.8%) of evaluable patients had either a D583G and/or Y537S *ESR1* mutation detected in their cfDNA, with double-mutations detected in 30/541 (5.5%) patients. *ESR1* mutations were more prevalent in patients who had previously received AI treatment for metastatic disease (33%) than in patients who had received AIs as adjuvant therapy (11%), supporting previous data from Schiavon et al (34). The results of the main study revealed that PFS was significantly improved in patients treated with everolimus and exemestane compared to exemestane and placebo (7.8 months versus 3.2 months), though the combination therapy did not result in improved OS (41, 42). In the *ESR1* mutation-driven subgroup analyses for PFS in the exemestane arm, patients with a mutation in D538G had a shorter PFS than *ESR1* wildtype patients (2.7 versus 3.9 months), which is in accordance with the findings of the SoFEA study (40, 43). When the analysis was restricted to patients with an Y537S mutation only, this association was not observed, which may be related to the limited sample size for these subgroup analyses. Of note is that the PFS of *ESR1* wildtype patients was 3.9 months in this study, while in the SoFEA study this was 8 months. This discrepancy in PFS might be due to differences in selection criteria of both studies. In the SoFEA trial only patients who received a non-steroidal AI as adjuvant therapy or as first-line therapy for MBC were included whereas patients in the BOLERO-2 trial were also included after receiving more lines of

therapy for MBC representing a more advanced disease stage. When everolimus was added to exemestane this resulted in an improved PFS in both D538G mutated (5.8 months; HR 0.34, 95% CI 0.02-0.6) and wildtype patients (8.5 months; HR 0.4, 95% CI 0.3-0.5), suggesting that *ESR1* mutated patients could still benefit from the addition of everolimus. Of note is that benefit of the addition of everolimus was not demonstrated for patients with an Y537S mutation alone (4.2 months; HR 0.98, 95% CI 0.5-1.9), or with both an Y537S and D538G mutation (5.4 months; HR 0.53, 95% CI 0.2-1.3). Again, one should keep in mind that these analyses may have suffered from the limited sample size of patients with only an Y537S mutation or a polyclonal *ESR1* mutation. If larger future studies confirm that patients with an Y537S indeed do not benefit from the addition of everolimus, this mutation might be used to select for patients who should be treated with other treatment modalities. Overall, the absolute median PFS interval seemed to be shorter in patients with an *ESR1* mutation than in *ESR1* wildtype patients, however, no formal analyses on these potential differences were observed. In this context, it was intriguing that OS analyses according to *ESR1* mutation status showed that patients with an *ESR1* mutation had a worse OS compared to wildtype patients (median OS 22 versus 32 months). Noteworthy, the type of individual mutations was also suggested to influence OS, with a median OS of 26 months for patients with a D538G mutation only and 20 months for the Y537S mutation alone. In patients harboring both mutations the OS was even worse with a median OS of 15 months. Overall, these results may indicate that *ESR1* mutations are associated with more aggressive disease biology.

DISCUSSION

The putative role of *ESR1* mutations in endocrine resistance has sparked a wide interest in techniques enabling their detection, the conditions under which they appear, and whether their detection can ultimately assist treatment decision-making in MBC patients. Regarding the best substrate for *ESR1* mutation detection, data from multiple studies suggests that the cfDNA compartment sometimes provides additional mutations compared to matched metastatic tumor material. This may indicate that cfDNA is more representative of the whole somatic tumor landscape. Another obvious advantage of cfDNA over metastatic biopsies is that it can easily be obtained repeatedly during treatment. Therefore, future studies on the clinical relevance of *ESR1* mutations should preferably be performed using cfDNA, measuring mutations not only at baseline but also sequentially during treatment. Of note, *ESR1* mutations can also be detected in CTCs (21, 44, 45), but at this point it is unclear how *ESR1* mutation detection in CTCs relates to *ESR1* mutation detection in cfDNA, and if this adds anything to *ESR1* mutation analyses in cfDNA.

Assessing *ESR1* mutations in tissue and cfDNA provided clues as to how these *ESR1* mutations are enriched in MBC patients. Very strong indirect evidence exists for the enrichment of these *ESR1* mutations during treatment with AIs in the metastatic setting. However, to date no direct evidence for the enrichment of *ESR1* mutations under AI treatment has been presented yet. In this context it is also of note that several studies observed *ESR1* mutations in metastases or cfDNA from patients treated with SERMs or SERDs only, or from patients not treated with endocrine therapy at all (5, 21, 28, 29). This further underlines that the understanding on how *ESR1* mutations exactly occur is still

limited. *ESR1* mutations are present at very low frequencies in primary BC tumors using dPCR (29), supporting the hypothesis that *ESR1* mutations may already be subclonally present in the primary tumor, and because of growth advantages, become the more prominent clone under treatment pressure (34). *ESR1* mutations might also occur as a result of mutational processes such as initiated by the APOBEC enzymes, however the mutational pattern of the hotspot *ESR1* mutations (T>A/C/G) does not follow an APOBEC pattern or the pattern of any other mutational signature known to date (46, 47). While the exact mechanism behind the enrichment of *ESR1* mutations in MBC is still unknown, the clinical relevance of *ESR1* mutations being present in cfDNA becomes evident. PFS after treatment with the AI exemestane was impaired in the patients harboring an *ESR1* mutation (38, 40), while fulvestrant had similar efficacy in patients with an *ESR1* mutation versus patients without an *ESR1* mutation. Given these results with fulvestrant, efficacy of tamoxifen may also be unaffected in patients harboring *ESR1* mutations, however, no clinical data on this is present as of yet. For the addition of other agents to endocrine treatment, for example palbociclib or everolimus, the question remains whether the presence of *ESR1* mutations is of any predictive significance for the efficacy of these agents. While the *ESR1* mutation status did not seem to impact median PFS in patients receiving fulvestrant and palbociclib, the presence of an *ESR1* mutation in patients treated with exemestane and everolimus might be associated with decreased PFS compared to *ESR1* wildtype patients.

Since a raise in *ESR1* mutation ratio during the course of treatment may be indicative of progressive disease (48) and *ESR1* mutations in general are associated with poor outcome (40), it will be of particular interest to see whether certain treatments (for example fulvestrant combined with palbociclib or specific chemotherapeutic regimen) are able to select against *ESR1* mutant subclones. Recently, it was shown that upon the discontinuation of anti-epidermal growth factor receptor (EGFR) antibodies, resistant KRAS mutant clones decay, allowing re-challenges with anti-EGFR antibodies in particular patients (49). If *ESR1* mutations are lost with certain treatment regimen, this could potentially allow re-challenges with AIs in a subset of patients.

Also currently unknown is whether the different *ESR1* mutations result in distinctive phenotypes. Functional studies on *ESR1* mutations did not specifically focus on differences between various *ESR1* mutations, and for some *ESR1* mutations that have been measured in clinical studies (e.g. K303R, V524E, P535H, L536H/P/R), very little functional evaluation of its constitutive activity and potential role in endocrine resistance has been performed at all. In addition, clinical studies to date have generally been underpowered for subgroup analyses evaluating differential effects of different *ESR1* mutations. Even further complicating such analyses is the described polyclonality of *ESR1* mutations. Multiple studies with anecdotal longitudinal sampling data suggested that in patients with polyclonal *ESR1* mutations there are differential effects of therapy on different *ESR1* mutations. This suggests that *ESR1* mutations are present in different subclones, and not in the same cell. Theoretically, this may mean that patients with polyclonal mutations are more difficult to treat given the wider repertoire of resistance mutations. However, in rather small analyzed groups of patients with polyclonal *ESR1* mutations treated with fulvestrant such effects were not observed. Given that some *ESR1* mutations are rarer than others, the most pragmatic way to evaluate the prognostic value of these rare *ESR1* mutations will likely be in the form of a meta-analysis in due time, as it is virtually impossible to evaluate the prognostic value of these mutations in single

studies. In addition, functional studies evaluating all LBD *ESR1* mutations described in patients to date, validating their constitutive activity and exploring potential differential effects of different *ESR1* mutations are of interest.

The current evidence on *ESR1* mutations warrants prospective studies in which patients are randomized and treated according to the *ESR1* mutation status in cfDNA. Therefore, standardized methods to process plasma, to isolate cfDNA and to prepare and analyze the dPCR chips are needed. A lot of the recent *ESR1* mutation research was performed on cfDNA samples that were suboptimally collected. For example, in the SoFEA trial, plasma was collected in EDTA tubes and processed up to 9 days after sample collection which may have consequences for the sensitivity to detect *ESR1* mutations, especially in the context of longitudinal sampling (50, 51). Recently, it was demonstrated that blood collected in CellSave or BCT blood tubes assures optimal quality of cfDNA for dPCR or NGS analyses for up to 96 hours after the blood draw (50-52), providing opportunities to send blood samples to remote locations for plasma isolation. In addition, it is of utmost importance to assess variables such as intra-assay, inter-assay, inter-lab and inter-observer variability when using dPCR, which are currently not only poorly studied for *ESR1* mutations, but also for cfDNA analyses in general.

In conclusion, the presence of *ESR1* mutations in patients with ER-positive MBC has high potential for clinical validity and utility. Prospective studies in which the exact role of how *ESR1* mutations can be used to guide treatment decision-making have to be initiated, but firstly standardization of protocols to assess these mutations will be necessary to eventually allow clinical implementation.

REFERENCES

1. Pritchard KI. Endocrine therapy: is the first generation of targeted drugs the last? J Intern Med. 2013 Aug;274(2):144-52.

2. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med. 2011;62:233-47.

3. De Laurentiis M, Arpino G, Massarelli E, Ruggiero A, Carlomagno C, Ciardiello F, et al. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. Clin Cancer Res. 2005 Jul 1;11(13):4741-8.

4. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol. 2012 Feb 20;30(6):587-92.

5. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res. 2014 Apr 1;20(7):1757-67.

6. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet. 2013 Dec;45(12):1439-45.

7. Olefsky JM. Nuclear receptor minireview series. J. Biol. Chem. 2001 Oct 5;276(40):36863-4.

8. Kojetin DJ, Burris TP, Jensen EV, Khan SA. Implications of the binding of tamoxifen to the coactivator recognition site of the estrogen receptor. Endocr Relat Cancer. 2008 Dec;15(4):851-70.

9. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, et al. Estrogen receptors: how do they signal and what are their targets. Physiol Rev. 2007 Jul;87(3):905-31.

10. Weis KE, Ekena K, Thomas JA, Lazennec G, Katzenellenbogen BS. Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. Mol Endocrinol. 1996 Nov;10(11):1388-98.

11. Zhang QX, Borg A, Wolf DM, Oesterreich S, Fuqua SA. An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. Cancer Res. 1997 Apr 1;57(7):1244-9.

12. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, et al. D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. Cancer Res. 2013 Dec 1;73(23):6856-64.

13. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet. 2013 Dec;45(12):1446-51.

14. Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, et al. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. Cell Rep. 2013 Sep 26;4(6):1116-30.

15. Pakdel F, Reese JC, Katzenellenbogen BS. Identification of charged residues in an N-terminal portion of the hormone-binding domain of the human estrogen receptor important in transcriptional activity of the receptor. Mol Endocrinol. 1993 Nov;7(11):1408-17.

16. Wardell SE, Ellis MJ, Alley HM, Eisele K, VanArsdale T, Dann SG, et al. Efficacy of SERD/SERM Hybrid-CDK4/6 Inhibitor Combinations in Models of Endocrine Therapy-Resistant Breast Cancer. Clin Cancer Res. 2015 Nov 15;21(22):5121-30.

17. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008 Sep;14(9):985-90.

18. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods. 2006 Jul;3(7):551-9.

19. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A. 1999 Aug 3;96(16):9236-41.

20. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem. 2011 Nov 15;83(22):8604-10.

21. Guttery DS, Page K, Hills A, Woodley L, Marchese SD, Rghebi B, et al. Noninvasive detection of activating estrogen receptor 1 (ESR1) mutations in estrogen receptor-positive metastatic breast cancer. Clin Chem. 2015 Jul;61(7):974-82.

22. Kidess E, Heirich K, Wiggin M, Vysotskaia V, Visser BC, Marziali A, et al. Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget. 2015 Feb 10;6(4):2549-61.

23. Thompson JD, Shibahara G, Rajan S, Pel J, Marziali A. Winnowing DNA for rare sequences: highly specific sequence and methylation based enrichment. PloS one. 2012;7(2):e31597.

24. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011 Jun 7;108(23):9530-5.

25. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014 May;20(5):548-54.

26. Karnik PS, Kulkarni S, Liu XP, Budd GT, Bukowski RM. Estrogen receptor mutations in tamoxifen-resistant breast cancer. Cancer Res. 1994 Jan 15;54(2):349-53.
27. Roodi N, Bailey LR, Kao WY, Verrier CS, Yee CJ, Dupont WD, et al. Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. J Natl Cancer Inst. 1995 Mar 15;87(6):446-51.

28. Takeshita T, Yamamoto Y, Yamamoto-Ibusuki M, Inao T, Sueta A, Fujiwara S, et al. Droplet digital polymerase chain reaction assay for screening of ESR1 mutations in 325 breast cancer specimens. Transl Res. 2015 Dec;166(6):540-53 e2.

29. Wang P, Bahreini A, Gyanchandani R, Lucas PC, Hartmaier RJ, Watters RJ, et al. Sensitive Detection of Mono- and Polyclonal ESR1 Mutations in Primary Tumors, Metastatic Lesions, and Cell-Free DNA of Breast Cancer Patients. Clin Cancer Res. 2016 Mar 1;22(5):1130-7.

30. Yates LR, Gerstung M, Knappskog S, Desmedt C, Gundem G, Van Loo P, et al. Subclonal diversification of primary breast cancer revealed by multiregion sequencing. Nat Med. 2015 Jul;21(7):751-9.

31. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer Discov. 2014 Jun;4(6):650-61.

32. Onstenk W, Sieuwerts AM, Mostert B, Lalmahomed Z, Bolt-de Vries JB, van Galen A, et al. Molecular characteristics of circulating tumor cells resemble the liver metastasis more closely than the primary tumor in metastatic colorectal cancer. Oncotarget. 2016 Jun 20.

33. Chu D, Paoletti C, Gersch C, VanDenBerg DA, Zabransky DJ, Cochran RL, et al. ESR1 Mutations in Circulating Plasma Tumor DNA from Metastatic Breast Cancer Patients. Clin Cancer Res. 2016 Feb 15;22(4):993-9.

34. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med. 2015 Nov 11;7(313):313ra182.
35. Lipinski KA, Barber LJ, Davies MN, Ashenden M, Sottoriva A, Gerlinger M. Cancer Evolution and the Limits of Predictability in Precision Cancer Medicine. Trends Cancer. 2016 Jan;2(1):49-63.

36. Spoerke JM, Gendreau S, Walter K, Qiu J, Wilson TR, Savage H, et al. Heterogeneity and clinical significance of ESR1 mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. Nat Commun. 2016;7:11579.

37. Nik-Zainal S, Alexandrov LB, Wedge DC, Van Loo P, Greenman CD, Raine K, et al. Mutational processes molding the genomes of 21 breast cancers. Cell. 2012 May 25;149(5):979-93.

38. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, et al. Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. J Clin Oncol. 2016 Jun 6.

39. Cristofanilli M, Turner NC, Bondarenko I, Ro J, Im SA, Masuda N, et al. Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. Lancet Oncol. 2016 Apr;17(4):425-39.

40. Chandarlapaty S, Chen D, He W, Sung P, Samoila A, You D, et al. Prevalence of ESR1 Mutations in Cell-Free DNA and Outcomes in Metastatic Breast Cancer: A Secondary Analysis of the BOLERO-2 Clinical Trial. JAMA Oncol. 2016 Aug 11.

41. Yardley DA, Noguchi S, Pritchard KI, Burris HA, 3rd, Baselga J, Gnant M, et al. Everolimus plus exemestane in postmenopausal patients with HR(+) breast cancer: BOLERO-2 final progression-free survival analysis. Adv Ther. 2013 Oct;30(10):870-84.

42. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Overlies plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2. Ann Oncol. 2014 Dec;25(12):2357-62.

43. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, et al. Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. J Clin Oncol. 2016 Sep 1;34(25):2961-8.

44. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science. 2014 Jul 11;345(6193):216-20.

45. Shaw JA, Guttery DS, Hills A, Fernandez-Garcia D, Page K, Rosales BM, et al. Mutation analysis of cell-free DNA and single circulating tumor cells in metastatic breast cancer patients with high CTC counts. Clin Cancer Res. 2016 Jun 22.

46. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature. 2013 Aug 22;500(7463):415-21.

47. Morganella S, Alexandrov LB, Glodzik D, Zou X, Davies H, Staaf J, et al. The topography of mutational processes in breast cancer genomes. Nat Commun. 2016;7:11383.

48. Takeshita T, Yamamoto Y, Yamamoto-Ibusuki M, Inao T, Sueta A, Fujiwara S, et al. Clinical significance of monitoring ESR1 mutations in circulating cell-free DNA in estrogen receptor positive breast cancer patients. Oncotarget. 2016 May 31;7(22):32504-18.

49. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med. 2015 Jul;21(7):827.

50. Rothwell DG, Smith N, Morris D, Leong HS, Li Y, Hollebecque A, et al. Genetic profiling of tumours using both circulating free DNA and circulating tumour cells isolated from the same preserved whole blood sample. Mol Oncol. 2016 Apr;10(4):566-74.

51. Van Dessel LF, Beije N, Helmijr J, Vitale S, Kraan J, Look MP, et al. Application of circulating tumor DNA in prospective clinical oncology trials: standardization of pre-analytical conditions. Mol Oncol. 2016 in press.

52. Kang Q, Henry NL, Paoletti C, Jiang H, Vats P, Chinnaiyan AM, et al. Comparative analysis of circulating tumor DNA stability In K3EDTA, Streck, and CellSave blood collection tubes. Clin Biochem. 2016 Apr 27.

53. Ruff M, Gangloff M, Wurtz JM, Moras D. Estrogen receptor transcription and transactivation: Structure-function relationship in DNA- and ligand-binding domains of estrogen receptors. Breast Cancer Res. 2000;2(5):353-9.

54. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. Physiol Rev. 2001 Oct;81(4):1535-65.

55. Sefrioui D, Perdrix A, Sarafan-Vasseur N, Dolfus C, Dujon A, Picquenot JM, et al. Short report: Monitoring ESR1 mutations by circulating tumor DNA in aromatase inhibitor resistant metastatic breast cancer. Int J Cancer. 2015 Nov 15;137(10):2513-9.

SUPPLEMENTARY DATA

Supplementary Table 1. Overview of *ESR1* mutation analysis in primary breast cancer

Patients	Method	*ESR1* mutation	No. *ESR1* mutations	Ref
183 pts with ER-positive MBC participating in BOLERO-2	NGS	*ESR1* exome	6/183 (3%)	(6)
390 ER-positive tumors resected before endocrine therapy (TCGA sequenced tumors)	NGS		0/390	(13)
80 ER-negative tumors			0/80	
134 pts with ER-positive/HER2-negative BC	NGS	*ESR1* exome	0/58	(5)
104 pts with ER-negative BC			0/115	
270 pts with ER-positive BC	ddPCR	*ESR1* D538G, Y537S/N	7/270 (0.03%)	(28)
43 ER-positive primary tumors	ddPCR	D538G, Y537S/N/C, S463P, K303R	3/43 (7%)	(29)

Supplementary Table 2. Overview of *ESR1* mutation analysis in metastatic breast cancer

Patients	Method	*ESR1* mutation	Samples with *ESR1* mutations	D538G	Y537S	Y537N	Y537C	Other	Ref
11 ER-positive MBC patients	NGS	*ESR1* exome	6/11 (55%)	2/11 (18%)	3/11 (27%)	-	-	1/11 (9%) L536Q	(13)
36 ER-positive MBC patients, with progressive disease while at least 3 months treated with endocrine treatment	NGS	*ESR1* exome	9/36 (25%)	3/36 (8%)	4/36 (11%)	1/36 (3%)	-	3/36 (8%) S463P/L536R/ V534E	(6)
44 ER-positive MBC patients, participating in the BOLERO-2, with progressive disease on an AI			5/44 (11%)	1/44 (2%)	1/44 (2%)	1/44 (2%)	1/44 (2%)	2/44 (5%) S463P/P535H	
13 ER-positive MBC patients, failing several lines of treatment	NGS	*ESR1* exome	5/13 (38%)	5/13 (38%)	-	-	-	-	(12)
76 ER-positive MBC patients	NGS	*ESR1* exome	9/76 (12%)	3/76 (4%)	2/76 (3%)	3/76 (4%)	1/76 (1%)	-	(5)
31 ER-positive MBC patients with progression on therapy	ddPCR	D538G, Y537S/ N/C, L536R	4/31 (13%)	1/31 (3%)	1/31 (3%)	2/31 (6%)	1/31 (3%)	-	(34)
55 ER-positive MBC patients	ddPCR	D538G, Y537S/ N/C, L536R	11/55 (20%)	4/55 (7%)	5/55 (9%)	4/55 (7%)	4/55 (7%)	-	(28)
11 bone metastasis of MBC patients	ddPCR	D538G, Y537S/ N/C, S463P, K303R	1/12 (8%)	1/12 (8%)	-	-	-	-	(29)
38 brain metastasis of MBC patients			3/38 (8%)	3/38 (8%)	1/38 (3%)	-	-	-	
7 metastases of ER-positive MBC patients	Sanger sequencing		4/7 (57%)	2/7 (29%)	1/7 (14%)	1/7 (14%)	-	-	(55)
	dPCR	Y537N, Y537S, D538G	6/7 (71%)	3/7 (29%)	2/7 (29%)	2/7 (29%)	-	-	

CHAPTER 5

Estrogen receptor mutations and splice variants determined in liquid biopsies from metastatic breast cancer patients

Nick Beije*, Anieta M. Sieuwerts*, Jaco Kraan, Ngoc M. Van, Wendy Onstenk, Silvia R. Vitale, Michelle van der Vlugt-Daane, Luc Y. Dirix, Anja Brouwer, Paul Hamberg, Felix E. de Jongh, Agnes Jager, Caroline M. Seynaeve, Maurice P.H.M. Jansen, John A. Foekens, John W.M. Martens, Stefan Sleijfer

* These authors contributed equally to this manuscript

Submitted

ABSTRACT

Mutations and splice variants in the estrogen receptor (ER) gene, *ESR1*, may yield endocrine resistance in metastatic breast cancer (MBC) patients. This research aimed to determine if these putative resistance mechanisms occur more frequently in circulating tumor cells (CTCs) of MBC patients progressing on endocrine treatment and in matched cell-free DNA (cfDNA). Two MBC cohorts were evaluated: 1) patients starting first-line endocrine therapy (n=43, baseline cohort) and 2) patients progressing on endocrine therapy (n=40, progressing cohort). *ESR1* splice variants and *ESR1* hotspot mutations were evaluated in CTC-enriched fractions and for *ESR1* mutations compared with matched cfDNA (n=18 baseline cohort; n=26 progressing cohort). Only the Δ5 splice variant was CTC-specific expressed, but not enriched in the progressing cohort. Sensitivity for detecting *ESR1* mutations in CTC-enriched fractions was markedly lower than for cfDNA. *ESR1* mutations detected in the cfDNA were enriched upon progression, suggesting a role in conferring endocrine resistance in MBC.

INTRODUCTION

Endocrine therapy is the mainstay of treatment for estrogen receptor (ER)-positive metastatic breast cancer (MBC) patients. However, 40% of these patients obtain no clinical benefit from first-line endocrine therapy, and virtually all of the patients in whom the tumor initially responds will eventually develop resistance (1). Several mechanisms have been linked to endocrine resistance (2), but none of these have been implemented in daily clinical practice because their clinical value could not be confirmed, or was not strong enough. One recently revealed mechanism for acquired resistance is the emergence of mutations in the gene coding for ER, *ESR1*, yielding a constitutively activated ER. Functional studies have suggested that tumor cells with these mutations are less responsive to estrogen deprivation as induced by aromatase-inhibitors (AIs) (3, 4), but may still experience growth inhibition by ER blocking agents such as tamoxifen and fulvestrant (3-5). This was recently supported in a retrospective clinical analysis, in which a modest progression-free survival benefit was observed for MBC patients with an *ESR1* mutation who were treated with fulvestrant, when compared to the AI exemestane (6). These results have further emphasized the potential for the determination of *ESR1* mutations to guide treatment-decision making in ER-positive MBC.

Another mechanism that potentially contributes to acquired endocrine therapy resistance is the occurrence of *ESR1* mRNA splice variants. *ESR1* splice variants have been described as having various effects on the transcriptional activity of the ER (7), and are heterogeneously expressed in primary breast cancers (8). The ERαΔ5 splice variant is of particular interest, since preclinical experiments have reported that this variant exerts constitutional transcriptional activity (9, 10). However, to date, the putative role of *ESR1* splice variants with regard to endocrine resistance in MBC has not been assessed.

ESR1 mutations and mRNA splice variants are likely to emerge during treatment, and can therefore only be observed in tumor cells obtained during or after treatment. Thus, these investigations require metastatic tumor tissue obtained through biopsies, which can be technically challenging, or even impossible.

Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) are alternative and minimally-invasive means for assessing the characteristics of metastatic cancer cells. Theoretically, each are different substrates for DNA, with DNA from CTCs coming from intact cancer cells, and ctDNA (which is part of the total cell-free DNA (cfDNA)) is thought to originate mainly from apoptotic tumor cells (11). The introduction of very sensitive digital polymerase chain reaction (dPCR) assays has opened new avenues to determine the presence of mutations in ctDNA and in CTC-derived DNA of cancer patients. Although promising results have been achieved with the detection of *ESR1* mutations in cfDNA using dPCR (6, 12-17), the important advantage of using CTCs over cfDNA is that multiple parameters in multiple dimensions (DNA, RNA and protein) can be measured in the same sample, and can be associated with, for example, endocrine resistance. This implies that besides assessing mutations in CTC-derived DNA, the characterization of RNA from CTCs permits the assessment of splice variants.

The current study set out to evaluate *ESR1* mutations and splice variants in CellSearch-enriched CTCs of MBC patients before the start of first-line endocrine therapy, and during progression under any line of endocrine therapy. The main objective was to determine whether these putative mechanisms for endocrine resistance are enriched in patients progressing on endocrine therapy. To this end, a cohort of MBC patients before the beginning of first-line endocrine therapy for MBC was defined, as well as a cohort of MBC patients progressing under any line of endocrine therapy. Additionally, in a subgroup of these patients, the *ESR1* mutation status in CTCs was compared with patient-matched cfDNA.

METHODS

PATIENTS AND TREATMENT

The patients evaluated in this study were selected from two CTC studies comprised of patients receiving endocrine therapy (study 06-248 (18-20) and study 09-405 (21)). Six centers in the Netherlands and Belgium participated in these studies from February 2008 through March 2015. The patients were included in these studies if they had MBC, and a new line of endocrine therapy was begun. Blood was sampled before the start of endocrine therapy and/or at the time of progression to palliative endocrine treatment. At both of these time points, 10 mL of blood was drawn for CTC enumeration, and another 10 mL of blood was drawn for CTC characterization. In each participating center, the institutional board approved the study protocols (Erasmus MC ID MEC-06-248 & MEC-09-405). All patients provided written informed consent.

Two cohorts of patients were defined for the current study: a cohort starting first-line endocrine therapy for MBC, and a separate cohort progressing under any line of palliative endocrine therapy. Further eligibility criteria required that the patient had ≥5 CTCs/7.5 mL of blood at the time of the blood draw, to allow for the characterization of CTCs.

ENUMERATION AND ISOLATION OF DNA AND RNA FROM CTCS AND CFDNA AND ESR1 MUTATION DETERMINATION

Details regarding the CTC enumeration and isolation of DNA/RNA from CTCs have been reported previously (18-21). Briefly, in each patient, 10 mL of blood was drawn in CellSave tubes (Janssen Diagnostics, Raritan, NJ, USA) for CTC enumeration, which was performed on 7.5 mL of blood within 96 hours of the blood draw using the CellSearch system (Janssen Diagnostics). Another 10 mL of blood was drawn in EDTA tubes for CTC characterization, and CTCs were isolated from 7.5 mL of blood within 24 hours using the CellSearch system with the CellSearch profile kit (Janssen Diagnostics). Subsequently, DNA and RNA were isolated from enriched CTCs using the AllPrep DNA/RNA Micro Kit (Qiagen, Germantown, MD, USA) (20). For cfDNA analyses, the remainder of the EDTA blood (maximum of 2.5 mL) was centrifuged to isolate plasma within 24 hours after the blood draw. Cell-free DNA (cfDNA) was isolated from a total of 200 µL of plasma using the QIAamp circulating nucleic acid kit (Qiagen).

DNA from the CellSearch-enriched CTC fractions and cfDNA from plasma were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific,

Waltham, MA, USA). The DNA (0.1 to 1 ng/µL) was subjected to an *ESR1* target-specific amplification of 15 cycles with TaqMan PreAmp Master Mix (Thermo Fisher Scientific), as recommended by the manufacturer, using the *ESR1* PreAmp primer combination (**Supplementary Table 1**) at a final concentration of 400 nM each. The resulting pre-amplified 136 base pair product covering the positions of all 4 *ESR1* hotspot mutation sites (D538G and Y537S/C/N) was diluted 10-fold, and quantified via regular quantitative PCR (qPCR) for wild type (WT) *ESR1* using the same primers. The resulting Cq value was used to control the number of WT copies to be loaded onto the chips for dPCR analyses. The variant allele frequencies (VAF) of the studied mutations for *ESR1* were evaluated with mutation-specific TaqMan assays (the primer and probe sequences are given in **Supplementary Table 1**, and the reproducibility of these assessments in **Supplementary Figure 1**) via chip-based dPCR (QuantStudio 3D, Thermo Fisher Scientific) according to the manufacturer's instructions. Positive and negative control DNA was always included in each dPCR run, and all of the analyzed DNA samples (CTC and cfDNA) were evaluated in duplicate.

Digital PCR was performed for 4 *ESR1* hotspot mutation sites (D538G and Y537S/C/N). Ten healthy blood donors were used to specify the cut-offs for the presence of *ESR1* mutations in CellSearch-enriched samples. Seven of them had sufficient plasma available, and these samples were used to specify the cut-offs for the presence of *ESR1* mutations in cfDNA. The cut-off for the positivity for each individual assay was set at the highest VAF in the healthy blood donors plus 2.58 standard deviations (SD) (99% confidence interval) (**Supplementary Figure 2 & 3**). The cut-offs were: D538G = 0.6% (CTCs) and 1.0% (cfDNA), Y537S = 0.3% (for both CTCs and cfDNA), Y537N = 0.3% (CTCs) and 1.65% (cfDNA), Y537C = 0.5% (CTCs) and 0.65% (cfDNA). Both of the duplicate *ESR1* mutation measurements had to be above the cut-offs for a sample to be considered positive for a specific *ESR1* mutation.

SHORT TANDEM REPEAT ANALYSIS ON PATIENT-MATCHED CTC-DNA AND CFDNA

In a subset of samples, a short tandem repeat (STR) analysis was performed to confirm that the CellSearch-enriched DNA and cfDNA were indeed from the same donor. The PowerPlex 16 System (Promega, Madison, WI, USA) in combination with an ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific) and GeneMarker v1.91 software (Softgenetics LLC, State College, PA, USA)), was used to genotype the DNA, as recommended by the manufacturer's instructions.

ESR1 SPLICE VARIANTS AND EXPRESSION IN RNA FROM ENRICHED CTCS

The measured "splice variant gene panel" consisted of full-length (FL) *ESR1* and *ESR1* splice variants Δ5, Δ7, 36KD and 46KD. In addition, reference genes and epithelial genes were evaluated. Two µL of complementary DNA was pre-amplified in 15 cycles with TaqMan assays and TaqMan PreAmp Master Mix (Thermo Fisher Scientific), as recommended by the manufacturer, using the gene panel combination given in **Supplementary Table 1**. After pre-amplification, each gene was individually measured via qPCR with the same TaqMan assay used in the pre-amplification. Positive and negative controls were included in each individual experiment to monitor the reproducibility of the measurements (for reproducibility, see also **Supplementary Figure 4**).

The splice variants were assessed in CellSearch-enriched fractions of 10 healthy blood donors to evaluate the possible leukocyte expression of FL *ESR1* and splice variants. The splice variant gene panel was always evaluated in duplicate, and the averages of the duplicate measurements were used for further calculation. Only those samples with sufficient mRNA signal (reference genes average ΔCq<26.5) and epithelial signal (*KRT19/EPCAM* average ΔCq<26.5), as described previously (20, 22, 23), were used for further evaluation of splice variants. The ΔCq values for the splice variants were calculated relative to the FL *ESR1*. In those cases where no expression could be measured for both the splice variant and the FL *ESR1*, the sample was excluded from the analysis.

STATISTICAL CONSIDERATIONS

The primary objective of this research was to investigate whether *ESR1* mutations were more frequently observed in CTCs of MBC patients progressing on endocrine therapy, than in those patients starting first-line endocrine therapy. Based on data from the literature (3, 4), it was hypothesized that *ESR1* mutations in CTCs would be detectable in 30% of MBC patients experiencing progressive disease (PD) during palliative endocrine therapy, and that *ESR1* mutations in CTCs would be present in 5% of those patients beginning palliative first-line endocrine therapy. In order to detect this difference ($\alpha=0.05$ and $\beta=0.2$), 44 MBC patients progressing on palliative endocrine therapy, and 44 MBC control patients initiating first-line endocrine therapy were needed.

Secondary objectives included 1) an assessment of *ESR1* mutations in cfDNA samples, and a comparison between the detection of *ESR1* mutations in cfDNA versus CTC; 2) an exploration of whether *ESR1* mutations measured in cfDNA are enriched under endocrine therapy; 3) an exploration of whether *ESR1* splice variants are more prevalent in those patients experiencing PD than in patients beginning first-line endocrine therapy for MBC; and 4) an exploration of whether certain clinical factors are associated with the presence of *ESR1* mutations and/or splice variants.

Differences in the prevalence of *ESR1* mutation and splice variants between the baseline cohort and the progressing cohort were calculated using Fisher's exact test (2-sided), while those patients with matched samples in the baseline and the progressing cohort were excluded from this analysis. Correlations were tested using Kendall's tau correlation coefficient, and the differences of splice variant ΔCq values between groups were tested using the Kruskal-Wallis test. All of the analyses were performed using Stata/SE version 12 (StataCorp LP, College Station, TX, USA).

RESULTS

PATIENT CHARACTERISTICS

For the baseline cohort, a total of 43 patient samples was included, while the progressing cohort contained a total of 40 patient samples (**Table 1**). Most of the patients in the baseline cohort were not treated with any adjuvant chemotherapy (79%); however, 17 patients (40%) had been treated with adjuvant endocrine therapy. Samples in the progressing cohort originated mainly from patients progressing on first-line (55%) or second-line (30%) palliative endocrine therapy. Prior to the PD sample, 37 patients (93%) had received at least one line of AI treatment. Most patients (81%) in the baseline

TABLE 1. Baseline characteristics		
Parameter	**Baseline cohort (n=43)**	**PD cohort (n=40)**
Age at sample draw		
Median age (range)	72 (37 - 83)	63 (35 - 88)
Adjuvant endocrine therapy		
No	26 (60%)	26 (65%)
Yes, tamoxifen only	10 (23%)	9 (23%)
Yes, tamoxifen + AI	5 (12%)	4 (10%)
Yes, AI only	2 (5%)	1 (2%)
Adjuvant chemotherapy		
No	34 (79%)	28 (70%)
Yes	9 (21%)	12 (30%)
Neoadjuvant therapies		
No	43 (100%)	40 (100%)
Number of previous lines endocrine therapy lines for MBC		
0	43 (100%)	
1		22 (55%)
2		12 (30%)
≥3		6 (15%)
Endocrine therapy after start (BL cohort) or before PD (PD cohort)		
AI	30 (70%)	25 (63%)
Tamoxifen	13 (30%)	7 (17%)
Fulvestrant		8 (20%)
Previous endocrine therapy lines for MBC (in case of inclusion at PD on ≥2nd-line endocrine therapy)		
Yes, AI only		9 (23%)
Yes, AI + tamoxifen		6 (15%)
Yes, tamoxifen only		3 (7%)
Progression on the current line		
Yes	35 (81%)	40 (100%)
CTC count		
Median count (range)	81 (6 – 32492)	21 (5 – 2837)

cohort experienced PD on endocrine therapy during the time of follow-up. For 6 of these patients, matched samples from the baseline cohort and progressing cohort were available; however, for the other 29 patients, no PD sample was available, mainly because it was not collected. The median CTC count was higher in the baseline cohort (81 CTCs/7.5 mL) than in the progressing cohort (21 CTCs/7.5 mL).

ESR1 MUTATIONS IN CTCS AND MATCHED CFDNA

In the 6 matched samples from the baseline and progressing cohorts, no *ESR1* mutations were detected. *ESR1* mutations were observed in the CTCs of 2 (5%) baseline cohort samples (2x Y537N), and 3 (8%) progressing cohort samples (2x D538G, 1x Y537S) (*P*=0.66) (**Table 2**). One of the patients in the baseline cohort with an *ESR1* mutation had received prior adjuvant treatment with tamoxifen, while the other patient had not received any prior adjuvant therapy. Two of the *ESR1* mutations in CTCs from patients in the progressing cohort, occurring after palliative first-line therapy, were observed in one patient who had been treated with an AI, and one patient who had been treated with tamoxifen. The third *ESR1* mutation was observed in a patient progressing on fulvestrant as second-line palliative endocrine therapy, who had received an AI as her first-line treatment.

Matched cfDNA and CTCs from the same time point were available from a subset of the patients in the baseline cohort (n=18) and the progressing cohort (n=26) (**Supplementary Table 2**). Two *ESR1* mutations (1x D538G and 1x Y537S) (11%) were observed in cfDNA of the baseline cohort, and 12 *ESR1* mutations were observed in 11 patients (42%) in cfDNA of the progressing cohort (8x D538G, 2x Y537S, 1x Y537N, 1x Y537C) (*P*=0.04) (**Table 2**). In the 4 matched cfDNA samples from the baseline and progressing cohorts, no *ESR1* mutations were detected. Neither of the mutations found in cfDNA from the baseline cohort were observed in the CTCs (**Table 2**). In one of these patients, however, an Y537N mutation was observed in CTCs but not in cfDNA. Neither of the patients with *ESR1* mutations in cfDNA from the baseline cohort had received any adjuvant therapy.

When the mutations in cfDNA from the progressing cohort samples were compared with the mutation status of the CTCs, 3 out of 3 mutations observed in CTCs were confirmed in cfDNA. With one exception, variant allele frequencies (VAFs) of the mutations were much higher in cfDNA than in CTCs (**Table 2**). In addition, 9 mutations in 8 patients were observed in the cfDNA, but not in the CTCs. The mutations found in cfDNA of the progressing cohort occurred after first-line endocrine therapies (n=6) including AIs (n=5) and tamoxifen (n=1), and after second-line endocrine therapies (n=5) including fulvestrant (n=3) and tamoxifen (n=2). All of these latter patients had received an AI as first-line palliative endocrine treatment.

From 4 patients with matched CTC-cfDNA samples and discordant CTC versus cfDNA *ESR1* mutation results, unamplified DNA was available to perform STR analyses (**Table 2**). These analyses showed that both of the DNA fractions originated from the same patient, and thus excluded sample swapping.

Table 2. Observed *ESR1* mutations in CTC and cfDNA samples

CTC code	baseline CTCs	baseline cfDNA	Adjuvant therapy	PD CTCs	PD cfDNA	Progression on therapy	Prior therapies for MBC
CTC798**	D538G (0.14%)	**D538G (1.93%)**	none	not available	not available		
CTC1581	Y537S (0.39%) * **Y537N (0.42%)**	**Y537S (0.47%)** Y537N (0.05%)	none	not available	not available		
CTC1571	**Y537N (3.77%)**	not available	tamoxifen	not available	not available		
CTC1007**	not available	not available	none	Y537S (0.01%)	**Y537S (9.26%)**	fulvestrant	AI
CTC1364**	not available	not available	none	D538G (0.25%)	**D538G (40.05%)**	tamoxifen	AI
CTC1565**	not available	not available	tamoxifen + AI	D538G (0.14%)	**D538G (5.14%)**	fulvestrant	AI
CTC1569	not available	not available	none	Y537N (0.25%)	**Y537N (1.96%)**	AI	
CTC1352	not available	not available	none	D538G (0.47%)	**D538G (20.93%)**	AI	tamoxifen
CTC1567	not available	not available	none	**Y537S (1.98%)**	**Y537S (1.21%)**	tamoxifen	
CTC1360	not available	not available	none	D538G (0.52%)	**D538G (2.86%)**	AI	
CTC1587	not available	not available	tamoxifen	**D538G (0.84%)**	**D538G (15.98%)**	fulvestrant	AI
CTC1406	not available	not available	tamoxifen	**D538G (1.13%)**	**D538G (10.18%)**	AI	
CTC1393	not available	not available	none	D538G (0.18%) Y537C (0.23%)	**D538G (27.1%)** **Y537C (12.96%)**	AI	
CTC1410	not available	not available	tamoxifen	D538G (0.37%)	**D538G (23.84%)**	AI	

* average VAF positive, but negative in duplicate analysis
** STR analysis confirmed that the CTC DNA and cfDNA samples were from the same patient. For other samples not enough DNA available for STR analysis.

All patients in whom a mutation was called in either CTCs or cfDNA, along with clinical information. Shown percentages are variant allele frequencies. Called mutations are depicted in bold.

ESR1 SPLICE VARIANTS IN CTCS

In order to assess the presence of *ESR1* splice variants in CTCs, RNA was extracted from CellSearch-enriched CTCs, and analyzed for the expression of 4 *ESR1* splice variants relative to full-length *ESR1*. In the baseline cohort, 10 of the 43 (23%) samples were excluded from further analysis, because of insufficient quality of mRNA (n=4) or lack of an epithelial signal (n=6). In the progressing cohort, 17 out of 40 (43%) samples had to be excluded because of insufficient quality of the mRNA (n=2), lack of an epithelial signal (n=6), or unavailable RNA (n=9).

ESR1 splice variant ΔCq values relative to full-length *ESR1* were not correlated with CTC counts (**Supplementary Figure 5**). ΔCq values of the Δ5 splice variant relative to full-length *ESR1* were significantly higher in patients than in healthy blood donors (HBDs) (**Figure 1A**), but the Δ5 splice variant was not enriched in the progressing cohort, when compared to the baseline cohort (*P*=0.39). When 4 matched samples, taken from the baseline and progressing cohorts, were analyzed from patients receiving first-line AI treatment, the Δ5 splice variant was enriched at PD in two of the patients (**Supplementary Figure 6**). The Δ7 and 36KD splice variants were similarly expressed in patient samples and HBDs (**Figure 1B-C**). Nevertheless, for the 4 matched samples from the baseline and progressing cohorts, the Δ7 and 36kD splice variants were enriched at PD in 1 and 3 patients, respectively (**Supplementary Figure 6**). The 46KD splice variant was only observed in patient samples and not in HBDs; however, this did not reach statistical significance (**Figure 1D**).

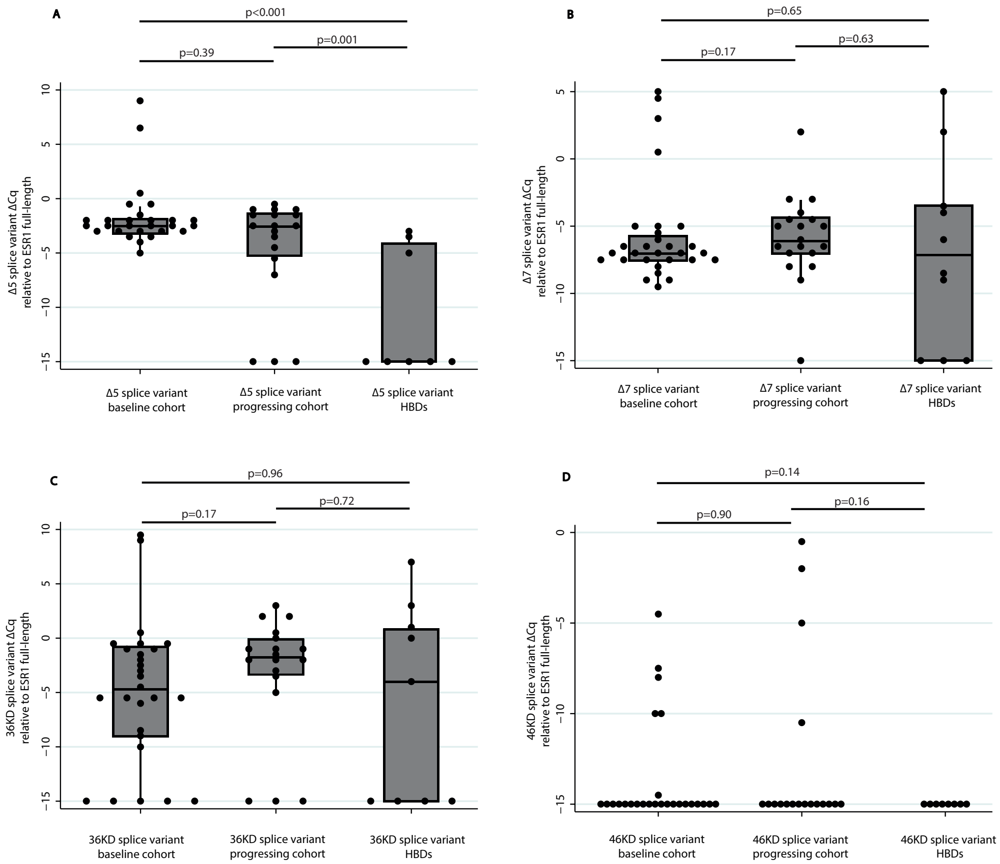


FIGURE 1. OCCURRENCE OF SPLICE VARIANTS IN THE BASELINE COHORT, THE PROGRESSING COHORT AND HEALTHY BLOOD DONORS (HBDS). Boxes demonstrate median and IQR, lines represent adjacent values (1.5*IQR). Observations were binned at ΔCq of 0.5.

DISCUSSION

The current study evaluated whether *ESR1* mutations and splice variants were enriched in CTCs from MBC patients progressing under endocrine therapy. No enrichment of any of these putative resistance mechanisms in CTCs was observed after endocrine therapy. However, cfDNA analyses did reveal an enrichment of *ESR1* mutations at the time of progression on endocrine therapy, when compared to before the initiation of first-line endocrine treatment.

The observation that *ESR1* mutations were more frequently observed in cfDNA than in CTCs suggests that cfDNA is a more sensitive substrate for the analysis of *ESR1* mutations than CTCs enriched by the FDA-approved CellSearch system. This is also reflected by the VAFs in the CTCs, which were generally low (range: up to 3.8%), as opposed to the VAFs in the cfDNA, which were generally much higher (range: up to 40%). One explanation for this difference could be the presence of contaminating leukocytes following the CellSearch enrichment of CTCs, which has been reported to be around 1,000 leukocytes (23), thereby decreasing the sensitivity for the detection of *ESR1* mutations in CTCs. Although cfDNA analysis is also challenged by contamination of wildtype DNA, our results suggest that this is less of an issue in cfDNA than in CTCs.

The stringency of the cut-offs for *ESR1* mutations, now arbitrarily set at the highest VAF observed in HBDs plus 2.58xSD (representing the 99% confidence interval), could have played a role in the limited sensitivity of *ESR1* mutation detection in CTCs. When less stringent cut-offs based on the highest VAF in HBDs were explored (data not shown), slightly more *ESR1* mutations were observed in CTCs; however, the majority of these mutations were not observed in cfDNA, suggesting that relaxing the cut-offs for *ESR1* mutation positivity may lead to false-positive findings. This stresses the need to include HBDs, and to be stringent with setting the cut-off value for *ESR1* mutation positivity. This also seems to apply to cfDNA: while most reports using dPCR have used the presence of at least 2 mutant signals as a cut-off for *ESR1* mutation positivity in cfDNA, we observed that some HBDs harbor more than 2 mutant signals (**Supplementary Figure 7**). However, it should be noted that all studies to date have used the BioRad droplet dPCR system, whereas we used the Quantstudio 3D dPCR system. Interestingly, the current study observed one *ESR1* mutation exclusively present in CTCs and not in cfDNA. This finding suggests that some *ESR1* mutations may be missed by cfDNA analysis only, albeit this observation may be merely anecdotal.

The current study is among the first to assess *ESR1* mutations in a cohort of patients beginning first-line endocrine treatment for MBC. While it has already been recognized that primary breast cancers rarely harbor *ESR1* mutations (3, 5), most studies thus far have evaluated patients who had been pre-treated with palliative endocrine therapy, suggesting that these mutations become enriched during treatment with AIs (14). Here, it has been confirmed that *ESR1* mutations are not frequently present in MBC patients before first-line endocrine therapy, and are enriched in MBC patients progressing under endocrine therapy.

Most of the patients in this study having an *ESR1* mutation progressed on AI treatment, or had previously been treated with an AI. In three of the patients, *ESR1* mutations were observed after progression on fulvestrant, suggesting that although it has been reported that fulvestrant is more effective than AIs in *ESR1* mutant patients (6, 24), mutant subclones can still be observed at PD on fulvestrant therapy. Of further note is the fact that in the current study the observed mutations in the baseline cohort occurred in those patients who were not pre-treated with AIs, or who received no pre-treatment with endocrine therapy at all. In addition, an *ESR1* mutation was observed in CTCs and cfDNA of one patient progressing on first-line palliative tamoxifen therapy, but who had not received any AI treatment, also not in the adjuvant setting. These findings are in line with the observations of multiple groups (5, 12, 15), who reported *ESR1* mutations in

metastatic biopsies or cfDNA of patients who had only received tamoxifen, or no pre-treatment at all. This could also fit with the observations by Wang and colleagues (16), who reported that *ESR1* mutations were sometimes present in primary breast cancers of patients at extremely low VAFs.

In the current study the *ESR1* splice variant Δ5 was expressed at higher levels in the CellSearch-enriched samples from MBC patients than in HBD samples; however, we found no enrichment of this splice variant during endocrine therapy for MBC. The Δ7, 36KD and 46KD splice variants were not significantly more highly expressed in patients versus HBDs. The fact that full-length *ESR1* and splice variants were also measured in a subset of HBDs suggests that leukocytes, which are known to express *ESR1* (25), may also express these splice variants. This clearly complicates the analysis of *ESR1* splice variants measured in CellSearch-enriched CTC fractions, where one thousand-fold of leukocytes is still present. In metastatic prostate cancer, the presence of the androgen receptor (AR) splice variant V7 in CTCs was previously demonstrated to be strongly associated with resistance to endocrine agents (26), but not to chemotherapy (22, 27, 28). It should, however, be noted that splice variants of *ESR1* in breast cancer differ importantly from splice variants of the AR, since *ESR1* splice variants are also expressed in healthy breast tissue (8), and full-length AR and splice variants are typically absent in CellSearch-enriched fractions of HBDs (22). It should also be kept in mind that, in the current study, only a limited number of samples could be evaluated for presence of splice variants. However, given that the *ESR1* splice variant Δ5 has been linked to endocrine resistance (9, 10), is CTC-specific expressed, and that we found anecdotal evidence of enrichment of this splice variant in paired samples, further research of this splice variant in CTCs is warranted.

In conclusion, *ESR1* mutations and splice variants in CellSearch-enriched CTCs were not enriched in MBC patients progressing on palliative endocrine therapy, but *ESR1* mutations were enriched in those patients when they were assessed in cfDNA. Therefore, cfDNA appears to be a more sensitive and robust source for detecting *ESR1* mutations than DNA from CellSearch-enriched CTCs. However, the use of other CTC enrichment methods might yield better results (29). To improve the sensitivity and specificity of detecting mutations and splice variants, and to really exploit the potential power of CTCs, characterization of pure CTCs with single cell isolation systems is probably required (30). Until that has been proven feasible and superior to analysis of cfDNA, the detection of *ESR1* mutations in cfDNA rather than CTCs is recommended. The increased incidence of *ESR1* mutations in cfDNA at the time of progression on endocrine therapy further adds to the evidence that emergence of *ESR1* mutations is involved in resistance to endocrine therapy in MBC.

05

REFERENCES

1. Pritchard KI. Endocrine therapy: is the first generation of targeted drugs the last? J Intern Med. 2013 Aug;274(2):144-52.

2. De Marchi T, Foekens JA, Umar A, Martens JW. Endocrine therapy resistance in estrogen receptor (ER)-positive breast cancer. Drug Discov Today. 2016 Jul;21(7):1181-8.

3. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet. 2013 Dec;45(12):1439-45.

4. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet. 2013 Dec;45(12):1446-51.

5. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res. 2014 Apr 1;20(7):1757-67.

6. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, et al. Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. J Clin Oncol. 2016 Jun 6.

7. Taylor SE, Martin-Hirsch PL, Martin FL. Oestrogen receptor splice variants in the pathogenesis of disease. Cancer Lett. 2010 Feb 28;288(2):133-48.

8. Poola I, Speirs V. Expression of alternatively spliced estrogen receptor alpha mRNAs is increased in breast cancer tissues. J Steroid Biochem Mol Biol. 2001 Nov;78(5):459-69.

9. Fuqua SA, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, et al. Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res. 1991 Jan 1;51(1):105-9.

10. Bollig A, Miksicek RJ. An estrogen receptor-alpha splicing variant mediates both positive and negative effects on gene transcription. Mol Endocrinol. 2000 May;14(5):634-49.

11. Haber DA, Velculescu VE. Blood-Based Analyses of Cancer: Circulating Tumor Cells and Circulating Tumor DNA. Cancer discovery. 2014;4(6):650-61.

12. Guttery DS, Page K, Hills A, Woodley L, Marchese SD, Rghebi B, et al. Noninvasive detection of activating estrogen receptor 1 (ESR1) mutations in estrogen receptor-positive metastatic breast cancer. Clin Chem. 2015 Jul;61(7):974-82.

13. Chu D, Paoletti C, Gersch C, VanDenBerg D, Zabransky D, Cochran R, et al. ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. Clin Cancer Res. 2015 Aug 10.

14. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med. 2015 Nov 11;7(313):313ra182.

15. Takeshita T, Yamamoto Y, Yamamoto-Ibusuki M, Inao T, Sueta A, Fujiwara S, et al. Droplet digital polymerase chain reaction assay for screening of ESR1 mutations in 325 breast cancer specimens. Transl Res. 2015 Dec;166(6):540-53 e2.

16. Wang P, Bahreini A, Gyanchandani R, Lucas PC, Hartmaier RJ, Watters RJ, et al. Sensitive Detection of Mono- and Polyclonal ESR1 Mutations in Primary Tumors, Metastatic Lesions, and Cell-Free DNA of Breast Cancer Patients. Clin Cancer Res. 2016 Mar 1;22(5):1130-7.

17. Takeshita T, Yamamoto Y, Yamamoto-Ibusuki M, Inao T, Sueta A, Fujiwara S, et al. Clinical significance of monitoring ESR1 mutations in circulating cell-free DNA in estrogen receptor positive breast cancer patients. Oncotarget. 2016 Apr 19.

18. Mostert B, Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, van Galen A, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. Ann Oncol. 2015 Mar;26(3):510-6.

19. Onstenk W, Sieuwerts AM, Weekhout M, Mostert B, Reijm EA, van Deurzen CH, et al. Gene expression profiles of circulating tumor cells versus primary tumors in metastatic breast cancer. Cancer Lett. 2015 Jun 28;362(1):36-44.

20. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res. 2011 Jun 1;17(11):3600-18.

21. Reijm EA, Sieuwerts AM, Smid M, Vries JB, Mostert B, Onstenk W, et al. An 8-gene mRNA expression profile in circulating tumor cells predicts response to aromatase inhibitors in metastatic breast cancer patients. BMC Cancer. 2016;16(1):123.

22. Onstenk W, Sieuwerts AM, Kraan J, Van M, Nieuweboer AJM, Mathijssen RHJ, et al. Efficacy of Cabazitaxel in Castration-resistant Prostate Cancer Is Independent of the Presence of AR-V7 in Circulating Tumor Cells. European urology. 2015 Dec;68(6):939-45.

23. Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, Mostert B, Martens JW, et al. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. Breast Cancer Res Treat. 2009 Dec;118(3):455-68.

24. Spoerke JM, Gendreau S, Walter K, Qiu J, Wilson TR, Savage H, et al. Heterogeneity and clinical significance of ESR1 mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. Nat Commun. 2016;7:11579.

25. Scariano JK, Emery-Cohen AJ, Pickett GG, Morgan M, Simons PC, Alba F. Estrogen receptors alpha (ESR1) and beta (ESR2) are expressed in circulating human lymphocytes. J Recept Signal Transduct Res. 2008;28(3):285-93.

26. Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med. 2014 Sep 11;371(11):1028-38.

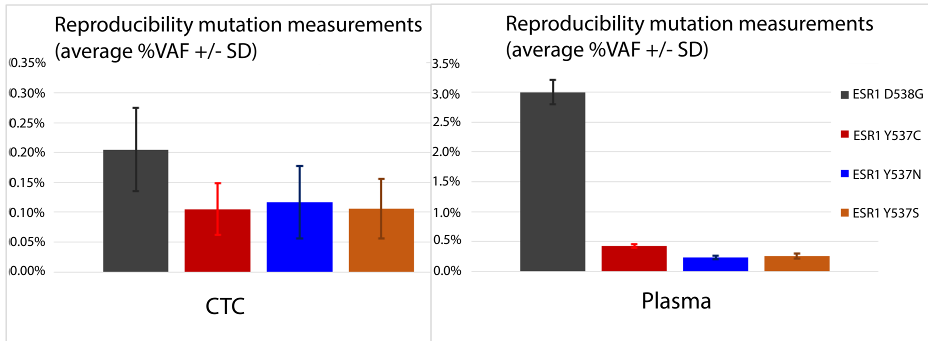
27. Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Nakazawa M, et al. Androgen Receptor Splice Variant 7 and Efficacy of Taxane Chemotherapy in Patients With Metastatic Castration-Resistant Prostate Cancer. JAMA Oncol. 2015 Aug;1(5):582-91.

28. Scher HI, Lu D, Schreiber NA, Louw J, Graf RP, Vargas HA, et al. Association of AR-V7 on Circulating Tumor Cells as a Treatment-Specific Biomarker With Outcomes and Survival in Castration-Resistant Prostate Cancer. JAMA Oncol. 2016 Jun 4.

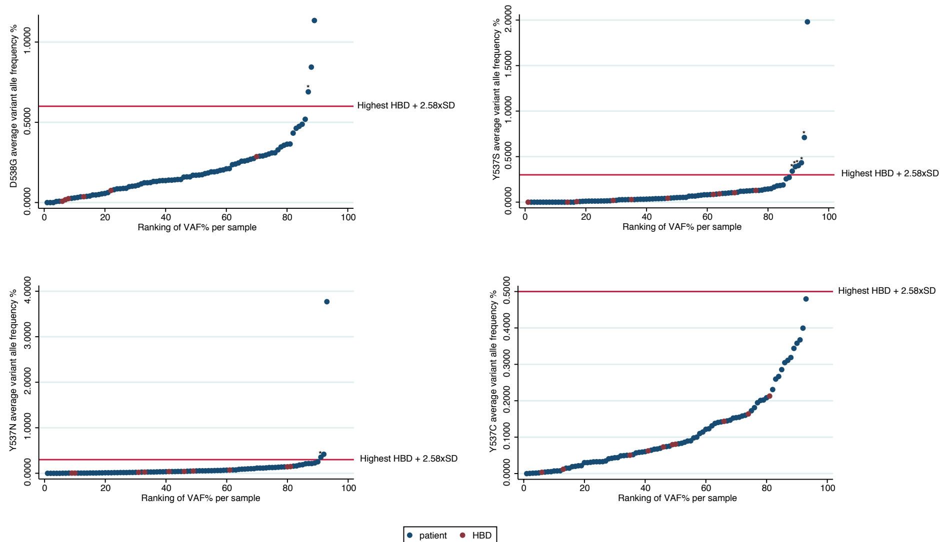
29. Denis JA, Patroni A, Guillerm E, Pepin D, Benali-Furet N, Wechsler J, et al. Droplet digital PCR of circulating tumor cells from colorectal cancer patients can predict KRAS mutations before surgery. Mol Oncol. 2016 Jun 7.

30. Swennenhuis JF, Terstappen L. Sample Preparation Methods Following CellSearch Approach Compatible of Single-Cell Whole-Genome Amplification: An Overview. Methods Mol Biol. 2015;1347:57-67.

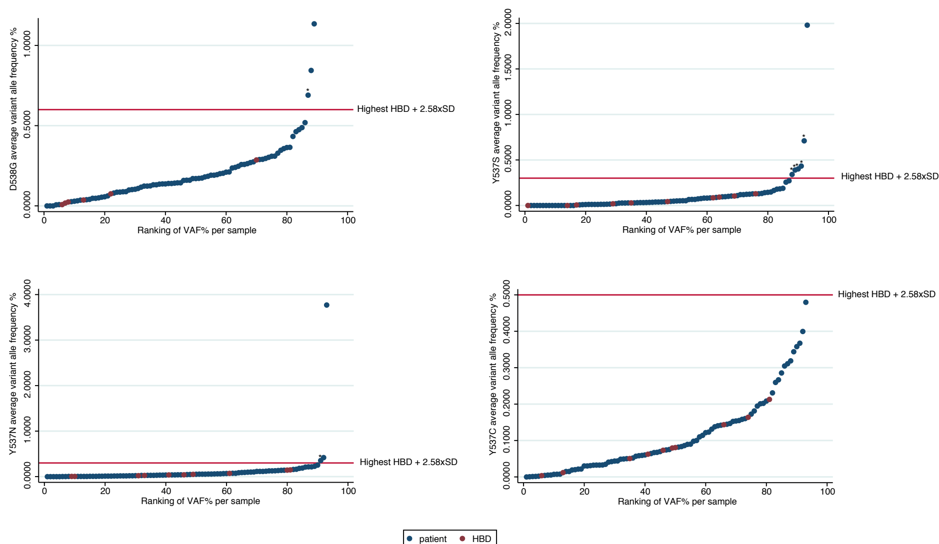
SUPPLEMENTARY DATA



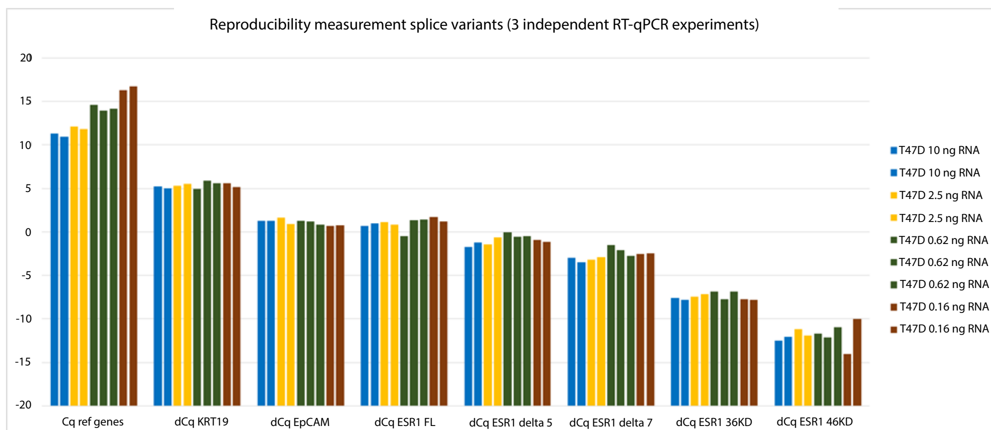
SUPPLEMENTARY FIGURE 1. REPRODUCIBILITY OF *ESR1* MUTATION MEASUREMENTS IN CTCS AND CFDNA. Boxes demonstrate average VAF along with standard deviations (SDs) based on duplicate measurements.



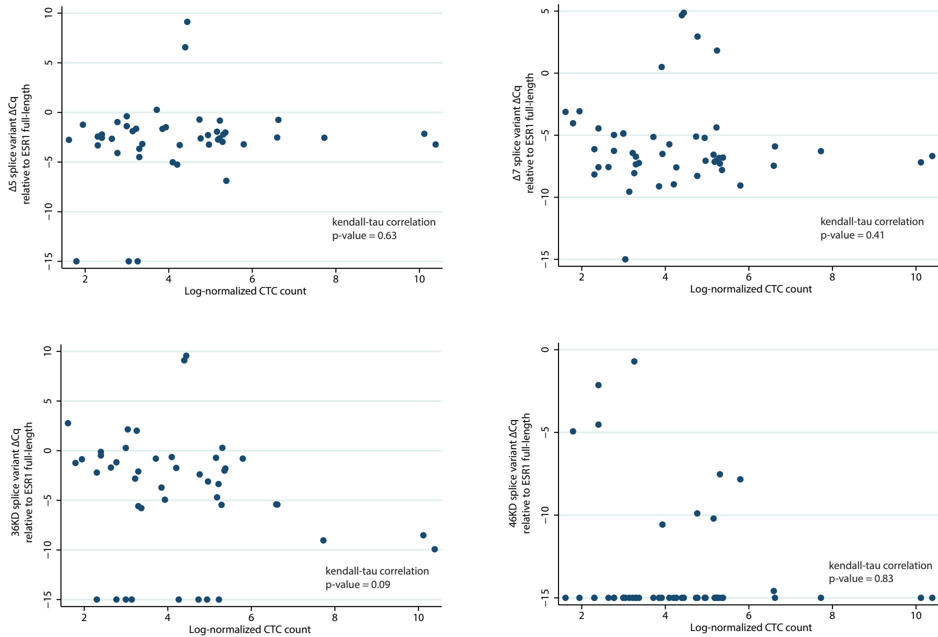
SUPPLEMENTARY FIGURE 2. CUT-OFFS FOR *ESR1* MUTATIONS IN CTCS. Each point represent one sample. Samples were ranked according to the VAF and cut-offs were set at the VAF in the highest HBD (depicted with red dot) + 2.58xSD. Stars indicate that the although the average VAF% was above the cut-off, duplicate experiments were not both above the cut-off.



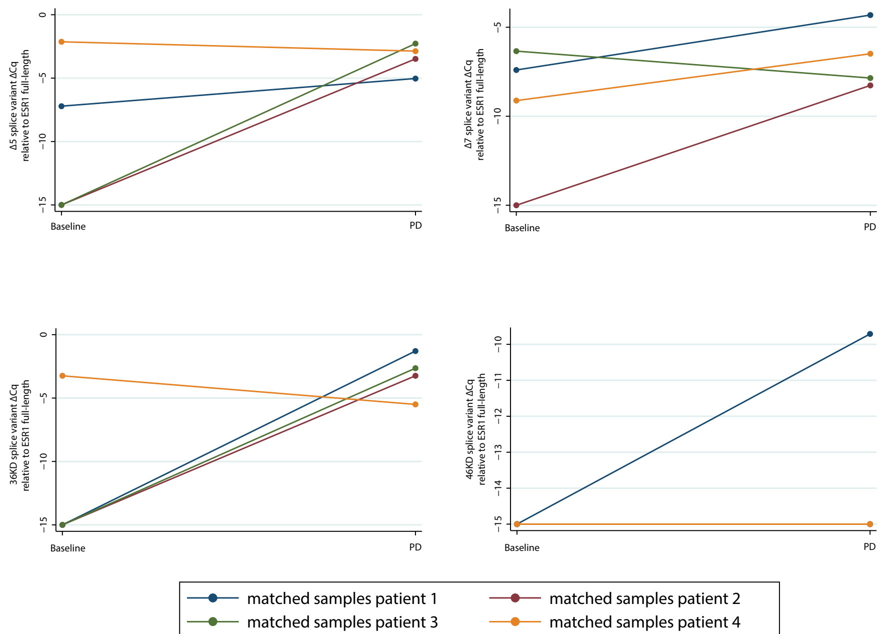
SUPPLEMENTARY FIGURE 3. CUT-OFFS FOR *ESR1* MUTATIONS IN CFDNA. Each point represent one sample. Samples were ranked according to the VAF and cut-offs were set at the VAF in the highest HBD (depicted with red dot) + 2.58xSD.



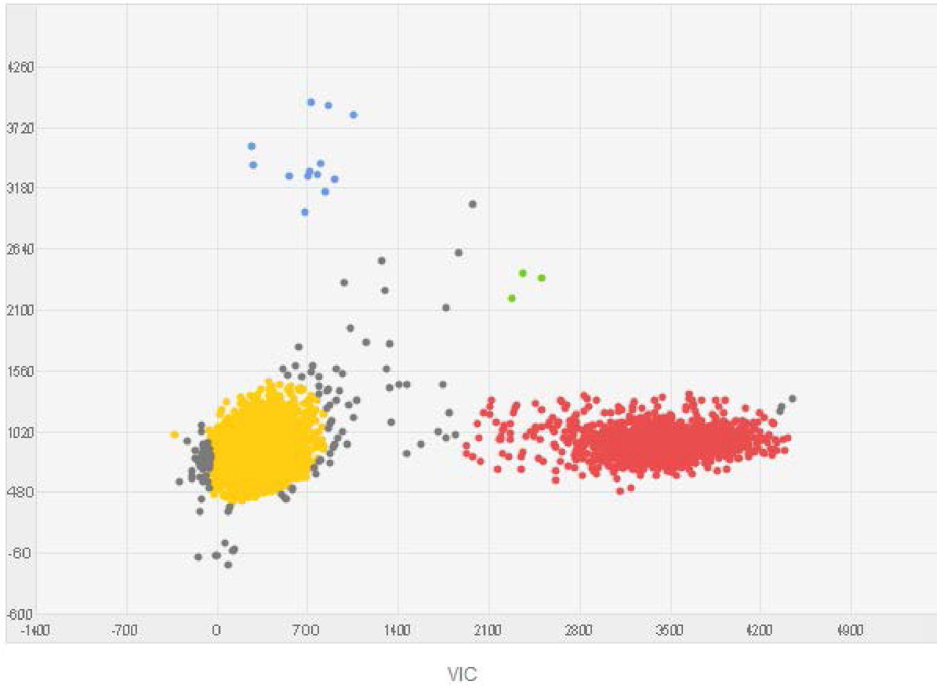
SUPPLEMENTARY FIGURE 4. REPRODUCIBILITY OF SPLICE VARIANT MEASUREMENTS IN T47D CELL LINE WITH VARIOUS INPUTS OF RNA. Various inputs (10 ng, 2.5 ng, 0.62 ng and 0.16 ng) of RNA from cell line T47D were measured in the splice variant panel. Bars represent Cq values of reference genes for all inputs, and ΔCq values (corrected for reference genes) for cytokeratin 19 (KRT19), EpCAM, *ESR1*-wildtype/full length and *ESR1* splice variants.



SUPPLEMENTARY FIGURE 5. CORRELATION BETWEEN *ESR1* SPLICE VARIANT ΔCQ VALUES (RELATIVE TO *ESR1* FULL-LENGTH) AND CTC COUNTS. CTC counts were log-normalized to compress the figure. Samples in which both the *ESR1* splice variant and *ESR1* full-length had no Cq were excluded. Samples in which only *ESR1* full-length but no *ESR1* splice variant was measured got a ΔCq values (relative to *ESR1* full-length) of -15.



SUPPLEMENTARY FIGURE 6. DYNAMICS OF SPLICE VARIANTS IN 4 MATCHED SAMPLES AT BASELINE AND PD. All patients received first-line AI treatment.



SUPPLEMENTARY FIGURE 7. POSITIVE SIGNALS FOR *ESR1* MUTATION Y537N IN CFDNA FROM MALE HBD . Blue dots represent mutant signals, yellow dots represent unamplified signal, red dots represent wildtype signals, green dots represent double positive signals (wildtype+mutant).

SUPPLEMENTARY TABLE 1: Primer and probe sequences used for RT-qPCR and digital PCR

Description	Assay	Symbol	Forward primer	Reverse primer	FAM Taqman probe	VIC Taqman probe	bp product
Reference gene	qPCR	GUSB			Hs99999908_m1		81
Reference gene	qPCR	HMBS			Hs00609297_m1		64
Leukocyte control marker	qPCR	PTPRC			Hs00236304_m1		81
Epithelial control marker	qPCR	KRT19			Hs01051611_gH		66
ESR1 full-length	qPCR	ESR-FL			Hs00174860_m1		62
Epithelial control marker	qPCR	EPCAM	AGTTTGCGGACTGCACTTCA	AATACTCGTGATAAATTTTGGAT	AAGGAGATCACAACGCGT		72
ESR1 splice variant	qPCR	ESR1-delta5	GAGCTGGTTCACATGATC	CATGTCGAAGATCTCCAC	TTTCCCTGGTTCCTGGCAC		225
ESR1 splice variant	qPCR	ESR1-delta7	TTTGTGTGCCTCAAATCTA	TTCATGCTGTACAGATGC	CCATGCCTTTGTTACAGAATTAAGC		254
ESR1 splice variant	qPCR	ESR1-36KD	CCAAGAATGTTCAACCACAACC	GCACGGTTCATTAACATCTTTCTG	TATTTATGTTCCAGTCCCACCTGAGTAGCAAAGTGAACAC		143
ESR1 splice variant	qPCR	ESR1-46KD	CATTCTCCGGGACTGCGGTA	GTACTGGCCAATCTTTCTCTGCC	TGAGGCCAAATTCAGATAATCGACGCCAGGG		140
ESR1 PreAmp	dPCR	ESR1 PreAmp	AGGCATGGAGCATCTGTACA	TTGGTCCGTCTCCTCCA			136
ESR1 mutation	dPCR	ESR1_D538G	CAGCATGAAGTGCAAGAACGT	TGGGCGTCCAGCATCTC	CCCTCTATGGCCTGCT	CCCCTCTATGACCTGCT	63
ESR1 mutation	dPCR	ESR1_Y537S	CAGCATGAAGTGCAAGAACGT	TGGGCGTCCAGCATCTC	CCCTCTCTGACCTGC	CCCCTCTATGACCTGC	63
ESR1 mutation	dPCR	ESR1_Y537C	AGGCATGGAGCATCTGTACA	TTGGTCCGTCTCCTCCA	TGCCCCTCTGTGACCTGCT	TGGTGCCCCTCTATGACCTG	136
ESR1 mutation	dPCR	ESR1_Y537N	AGGCATGGAGCATCTGTACA	TTGGTCCGTCTCCTCCA	TGCCCCTCAATGACCTGC	TGGTGCCCCTCTATGACCTG	136

SUPPLEMENTARY TABLE 2. Characteristics of patients in cfDNA subgroup analysis

Parameter	Baseline cohort (n=18)	Progressing cohort (n=26)
Age at sample draw		
Median age (range)	68 (46 - 83)	64 (35 - 88)
Adjuvant endocrine therapy		
No	11 (61%)	18 (69%)
Yes, tamoxifen only	5 (28%)	4 (15%)
Yes, tamoxifen + AI	2 (11%)	3 (12%)
Yes, AI only		1 (4%)
Adjuvant chemotherapy		
No	15 (83%)	18 (69%)
Yes	3 (17%)	8 (31%)
Number of previous lines endocrine therapy lines for MBC		
0	18 (100%)	
1		16 (62%)
2		8 (31%)
≥3		2 (7%)
Endocrine therapy after start (baseline cohort) or before PD (progressing cohort)		
AI	11 (61%)	16 (62%)
Tamoxifen	7 (39%)	3 (11%)
Fulvestrant		7 (27%)
Previous endocrine therapy lines for MBC (in case of inclusion at PD on ≥2nd-line endocrine therapy)		
Yes, AI only		6 (23%)
Yes, AI + tamoxifen		2 (8%)
Yes, tamoxifen only		2 (8%)
Progression on the current line		
Yes	13 (77%)	26 (100%)

CHAPTER 6

Application of circulating tumor DNA in prospective clinical oncology trials: standardization of pre-analytical conditions

Lisanne F. van Dessel, Nick Beije, Jean C.A. Helmijr, Silvia R. Vitale, Jaco Kraan, Maxime P. Look, Ronald de Wit, Stefan Sleijfer, Maurice P.H.M. Jansen, John W.M. Martens, Martijn P.J.K. Lolkema

Mol Oncol. 2016 in press

ABSTRACT

BACKGROUND Circulating tumor DNA (ctDNA) has emerged as a potential new biomarker with diagnostic, predictive and prognostic applications for various solid tumor types. Before embarking large prospective clinical trials to prove the added value of utilizing ctDNA in clinical practice, it is essential to investigate the effects of various pre-analytical conditions on the quality of cell-free DNA (cfDNA) in general and of ctDNA in particular to optimize and standardize these conditions.

METHODS Whole blood samples were collected from patients with metastatic cancer bearing a known somatic variant. The following pre-analytical conditions were investigated: 1) different time intervals to plasma isolation (1 hour, 24 hours and 96 hours); 2) different preservatives in blood collection tubes (EDTA, CellSave and BCT). Quality of cfDNA/ctDNA was assessed by DNA quantification, digital PCR (dPCR) for somatic variant detection and a β-actin fragmentation assay for DNA contamination from lysed leukocytes.

RESULTS In 11/16 (69%) of our patients we were able to detect the known somatic variant in ctDNA. We observed a time-dependent increase in cfDNA concentrations in EDTA tubes which was positively correlated with an increase in wild type copy numbers and large DNA fragments (> 420bp). Using different preservatives did not affect somatic variant detection ability, but did stabilize cfDNA concentrations over time. Variant allele frequency was affected by fluctuations in cfDNA concentration only in EDTA tubes at 96 hours.

CONCLUSIONS Both CellSave and BCT tubes ensured optimal ctDNA quality in plasma processed within 96 hours after blood collection for downstream somatic variant detection by dPCR.

INTRODUCTION

Circulating tumor DNA (ctDNA) has emerged as a potential new biomarker in the field of oncology. The quantification and characterization of ctDNA in plasma creates numerous potential applications, including detection of minimal residual disease, early evaluation of treatment response and stratification for targeted therapy according to specific genetic changes (1-7).

The application of ctDNA-based diagnostic tests into the clinic still faces several technical difficulties. The biggest hurdle might be the detection limit: ctDNA may comprise less than 1.0% of the total cell-free DNA (cfDNA), making detection of the tumor-specific fraction challenging (1, 8, 9). The majority of cfDNA is derived from apoptotic tissue and hematological cells which release their DNA in the circulation (10, 11). Thus, the absolute quantity of cfDNA ("the background") determines our ability to detect ctDNA, and quantification of the tumor specific variant frequency depends both on the abundance of ctDNA molecules and the total amount of cfDNA. One of the most important factors impacting the total amount of cfDNA is the time to plasma processing after blood collection, which increases the release of wild type DNA from lysed hematological cells present in the blood collection tube (12, 13). To avoid this, plasma needs to be separated from the blood sample within hours after the blood draw, but the maximum time frame to do so, remains to be revealed.

Due to logistical and practical reasons it is often not possible to process and store blood samples immediately after the blood draw to ensure optimal ctDNA quality. Especially in the context of large multi-center prospective clinical trials, which are essential to definitely establish ctDNA as a clinically relevant new biomarker, there is a need for standardization of pre-analytical conditions that allow longer processing time of blood samples. To overcome this problem specialized 'cell-stabilizing' blood collection tubes have been developed. These tubes should not only minimize contamination by wild type DNA from lysed hematological cells in the blood tube, they should also preserve the quality of ctDNA for reliable downstream analyses.

Until today, a number of studies have tested the different available blood collection tubes to optimally preserve cfDNA/ctDNA (12, 14-16). They all demonstrate a time-dependent increase in cfDNA concentrations in EDTA tubes, while cfDNA concentrations remained stable in both BCT and CellSave tubes. Toro et al. (14) included the PAXgene blood DNA tube in their study, but this tube did not improve the results obtained with EDTA tubes. Yet, even though preservation methods have been compared (17), thorough direct comparisons between BCT and CellSave tubes at clinically relevant time frames are missing. We set out to compare the available preservatives for their ability to allow easier implementation of ctDNA-based tests into larger clinical trials where processing of samples within 1 hour presents a major logistical challenge. The purpose of this study was to investigate the effect on the quality of cfDNA in general and of ctDNA in particular in metastatic cancer patients under the following pre-analytical conditions: 1) different time intervals to plasma isolation (1 hour, 24 hours and 96 hours); and 2) different types of preservative in the blood collection tubes (EDTA, CellSave and BCT tubes). To this purpose the amount of cfDNA isolated from plasma was quantitated, its' size determined and the fraction of ctDNA determined.

METHODS

PATIENT CHARACTERISTICS AND SOMATIC VARIANT STATUS OF TUMOR

Between October 2015 and January 2016 cancer patients within the Erasmus MC Cancer Institute in Rotterdam, the Netherlands were invited to contribute blood samples for this study by their treating physician. Patients were included if they had metastatic disease, were not currently receiving systemic treatment and if a validated dPCR assay was available for the known somatic variant in their primary and/or metastatic lesion. Somatic variant status and variant allele frequency (VAF) in tissue had been assessed as part of the standard of care by the molecular diagnostics laboratory of the department of pathology in the Rotterdam region. The calculation of VAF was performed through NGS analysis by calculating the coverage of the variant nucleotide relative to the total coverage on that position. For tissue samples (n=2) analyzed by Sanger sequencing the VAF was calculated by determining the ratio between the variant peak and the wildtype peak. All patients provided written informed consent, and the institutional review board approved the protocols (Erasmus MC ID MEC 15-616).

PRE-ANALYTICAL CONDITIONS

After obtaining written informed consent, 9x10 mL of blood samples were collected within a single blood draw (See Supplemental figure 1). Matched blood samples were collected in sterile 3x 10 mL K_2EDTA vacutainer® (BD, Franklin Lakes, NJ, USA), 3x 10 mL Cell-Free DNA BCT® (Streck, Omaha, NE, USA) and 3x 10 mL CellSave Preservative (Janssen Diagnostics, Raritan, NJ, USA) blood collection tubes according to manufacturer instructions. The blood samples from one of each type of tube (EDTA, BCT and CellSave) were processed for plasma isolation at 3 different time points: within 1 hour after blood draw, at 24 hours and at 96 hours after blood draw (See Supplemental figure 1). Plasma was isolated using 2 sequential centrifugation steps: 1) 1711*g* for 10 minutes at room temperature; 2) 12,000*g* for 10 minutes at room temperature. Plasma was stored at -80°C in 1 ml aliquots immediately after centrifugation until further processing.

CFDNA ISOLATION AND QUANTIFICATION

For cfDNA isolation plasma samples were thawed at 4°C and 3 ml of plasma per sample was used. cfDNA was isolated using the QIAamp® Circulating Nucleic Acid kit (QIAGEN, Venlo, Limburg, The Netherlands) according to manufacturer�s instructions. cfDNA was eluted from the QIAGEN® Mini column using 50 µL buffer AVE which was applied 3 times to the column to obtain the highest cfDNA concentration possible. cfDNA was stored at -20°C. cfDNA concentrations were quantified using the Quant-iT dsDNA high-sensitivity assay (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to manufacturer’s instructions and the Qubit fluorometer (Invitrogen) was used as readout.

DIGITAL PCR TAQMAN® SNP GENOTYPING AND B-ACTIN FRAGMENTATION ASSAY

cfDNA samples were thawed at room temperature. Validated TaqMan® SNP genotyping assays (ThermoFisher Scientific, Waltham, MA USA) were used for somatic variant and wild type detection according to manufacturer’s instructions (**Supplementary table 1**). Accordingly, the limit of detection of this assay is 0.1% (2016). The maximum volume input of 7.8 µl of the final cfDNA eluate was used, unless the amount of cfDNA in this

volume exceeded the maximal input of 30 ng cfDNA, then 30 ng cfDNA was used. Depending on the obtained cfDNA concentration after plasma isolation, at least 2.57 ng cfDNA was analyzed, leading to a detection rate of 0.78% at the most.

The TaqMan® β-actin fragmentation assay was based on the assay developed by Norton et al. (Norton et al., 2013) to detect a small (136 bp) and long (420 bp) β-actin fragments. We adapted the assay so that both fragments were measured within a single experiment using the reported primers, but different probes for each fragment (See Supplemental table 2). For the β-actin fragmentation assay a standardized input of 2 ng cfDNA was used to minimize the change of having multiple DNA fragments in one well. All dPCR reactions were performed with the QuantStudio 3D Digital PCR System (ThermoFisher Scientific) according to the manufacturer's protocol. In short, dPCR reaction mix was prepared containing 8.7 µL QuantStudio 3D Digital PCR Master Mix v2, 0.44 µL Taqman primer/probe mix, up to 7.8 µL of cfDNA and the total volume was completed with PCR grade H_2O to a final volume of 17.4 µL. Using the QuantStudio 3D Digital PCR Chip Loader samples were partitioned on a 20,000 wells QuantStudio 3D Digital PCR Chip v2 followed by a PCR reaction on a ProFlex 2x Flat PCR System with the following program: 10 min at 96°C, 40x cycles of 2 min at 60°C, and followed by 30 sec at 98°C, 2 min at 60°C and pause at 10°C. The dPCR data were then acquired with the QuantStudio 3D Digital PCR Instrument and the data was analyzed with the QuantStudio 3D Analysis Suite by one technician (JH) to account for inter-observer variability.

STATISTICAL ANALYSIS

The Wilcoxon signed rank test was used to compare the difference between matched 1 hour and 24 hour samples relative to the difference between matched 1 hour and 96 hour samples. The Friedman test was used to test the order of the three 1 hour samples. To correct for multiple testing we adjusted the P value for significance using the Bonferroni correction. Significance was thus defined as $P < 0.008$ (0.05/6). Correlations were tested by Spearman's rank correlation coefficient.

cfDNA concentrations determined by the Quant-iT dsDNA high-sensitivity assay were corrected for the plasma input and were converted from ng/ml plasma to copies/ml plasma by taking into consideration that 3.3 pg of human DNA contains 1 copy of a single gene. cfDNA concentrations were then log-transformed.

To correct for differences in plasma input used for cfDNA isolation and for differences in elution volume after cfDNA isolation, we expressed dPCR results as variant/wild type copy numbers per mL plasma. To calculate variant/wild type copy numbers per mL plasma the following equation as described by Lo et al. (18) was used:

$C = Q * (V_{DNA})/V_{PCR}) * (1/V_{ext})$

where C is variant/wild type copy numbers per mL plasma; Q is the total number of variant/wild type copy numbers determined by dPCR; V_{DNA} is the total volume of cfDNA obtained after cfDNA isolation; V_{PCR} is the volume of cfDNA solution used for the dPCR reaction; and V_{ext} is the volume of plasma used for cfDNA isolation.

To calculate VAF we divided the variant copy numbers per mL plasma by the sum of variant and wild type copy numbers per mL plasma. All statistical analyses were performed using STATA version 14.1. All figures were plotted using R version 3.2.3.

RESULTS

SOMATIC VARIANT DETECTION RATE IN CTDNA OF RECRUITED PATIENTS

A total of 16 patients were included who all met the set criteria to investigate the effect of different pre-analytical conditions on the quality of ctDNA. Somatic variant status of the primary and/or metastatic lesion had been previously assessed, either by targeted next generation sequencing (14/16 patients) or by traditional Sanger sequencing (2/16 patients). Table 1 lists the origin of the primary tumor, the site(s) of metastasis and the VAF in the tumor tissue. Using the specific TaqMan SNP genotyping assay (**Supplementary table 1**), we were able to detect in 11/16 (69%) of the patients the known somatic variant in ctDNA isolated within 1 hour from EDTA tubes. This corresponds to the detection of 13/19 (68%) of the total number of somatic variants tested as some patients had multiple known somatic variants.

TABLE 1. Tumor characteristics and somatic variant detection.

Patient ID (#)	Primary tumor	Site and number of metastases (x)	Interval tumor tissue and plasma analysis (months)	Known somatic variant (nucleotide change)	Variant allele frequency in plasma EDTA 1h (%)	Cell-free DNA concentration in plasma EDTA 1h (copies/ml plasma)
01	Cholangio-carcinoma	Li (3), Lu (2), LN (1)	2	KRAS p.G12D (c.35G>A)	0.00	3655
02	Pancreatic cancer	Li (3), Lu (1) LN (6)	9	KRAS p.G12V (c.35G>T)	0.00	4055
	CRC			BRAF p.V600E (c.1799T>A)	0.97	
				PIK3CA p.H1047R (c.3140A>G)	1.86	
03	Breast cancer	LN (>2)	-1*	PIK3CA p.H1047L (c.3140A>T)	0.00	2788
04	Melanoma	Li (2), LN (5)	2	BRAF p.V600E (c.1799T>A)	1.44	1615
05	CRC	Li (6), LN (2)	6	KRAS p.G13D (c.38G>A)	65.46	223130
06	CRC	Li (3), Lu (4)	18	KRAS p.G12D (c.35G>A)	8.61	2215
07	Melanoma	Brain (2), Abd (7)	8	NRAS p.Q61R (c.182A>G)	17.22	4245
08	Melanoma	LN (3), Lu (6), Li (>15), Spleen (1), Bone (4), Peritonitis carcinomatosa, pleuritis carcinomatosa	1	BRAF p.V600E (c.1799T>A)	37.21	22442
09	Melanoma	LN (5)	1	BRAF p.V600E (c.1799T>A)	6.42	2739

TABLE 1. Tumor characteristics and somatic variant detection.

Patient ID (#)	Primary tumor	Site and number of metastases (x)	Interval tumor tissue and plasma analysis (months)	Known somatic variant (nucleotide change)	Variant allele frequency in plasma EDTA 1h (%)	Cell-free DNA concentration in plasma EDTA 1h (copies/ml plasma)
10	CRC	Brain (2), Li (1), Lu (8)	87	KRAS p.G13D (c.38G>A)	0.00	6030
11	CRC	Lu (2)	5	KRAS p.G13D (c.38G>A)	0.84	4670
12	CRC	Li (>20), LN (1)	3	KRAS p.Q61R (c.182A>G)	0.00	16136
13	NSCLC	Brain (8), Adrenal gland (1)	7	EGFR p.T790M (c.2369C>T) EGFR p.L858R (c.2573T>G)	1.18 2.62	5358
14	Melanoma	LN (7), Lu (5), adnexa	22	BRAF p.V600E (c.1799T>A)	5.37	3539
15	NSCLC	Li (unknown)	1	EGFR p.T790M (c.2369C>T)	27.60	14085
16	Melanoma	Brain (1)	38	BRAF p.V600E (c.1799T>A)	0.00	3012

CRC: colorectal cancer; NSCLC: non-small cell lung cancer; Li: liver; Lu: lung; LN: lymph node; Abd: abdomen

TEMPORAL EFFECT OF STORAGE IN EDTA TUBES ON CFDNA QUALITY

To investigate the effect of different time intervals from the blood draw to plasma isolation on cfDNA quality, we measured cfDNA concentration isolated from plasma collected in EDTA tubes. We observed a significant increase in cfDNA concentrations in samples isolated after 96 hours compared to samples isolated within 1 hour (p<0.001; **Figure 1**). This increase in cfDNA concentration was significantly positively correlated to an increase in wild type copy numbers (rho=0.85; p<0.001; **Figure 2A**). If a somatic variant was detected in the 1 hour sample, the somatic variant could also be detected in 24 and 96 hours samples. We also observed a significant positive correlation between variant copy numbers and cfDNA concentration, though this was less strong (rho=0.42; p<0.001; **Figure 2B**).

To investigate whether the increase in cfDNA concentrations and wild type copy numbers was due to the release of intact DNA from lysed leukocytes, we used the β-actin fragmentation assay (**Figure 3A**). In all pre-analytical conditions we detected low amounts of large fragments. We observed significantly more larger fragments in samples from 96 hours than in samples from 1 hour (420bp p<0.001; 2000bp p<0.001; **Figure 3B**). There was also a small but significant increase in fragmented DNA in samples from 96 hours compared to samples from 1 hour (136bp p=0.002; **Figure 3B**).

THE INTERACTION BETWEEN DIFFERENT PRESERVATIVES AND PLASMA ISOLATION TIME INTERVALS AND CFDNA QUALITY

Next, we investigated the effect of different preservatives in blood collection tubes on cfDNA quality. We compared cfDNA concentrations isolated from plasma collected in EDTA, BCT and CellSave tubes processed within 1 hour. cfDNA concentrations were

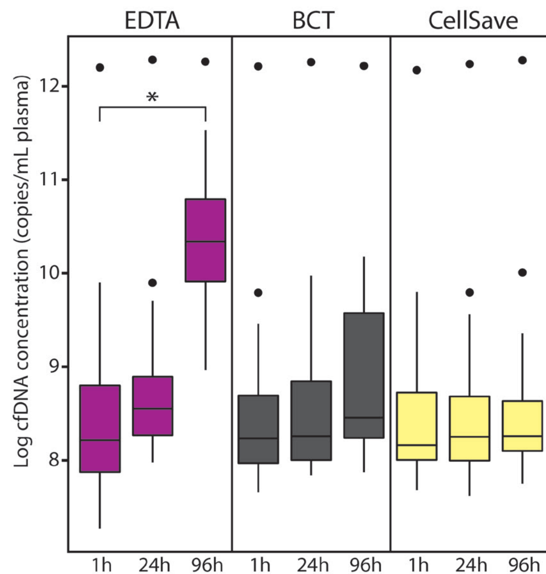


FIGURE 1. CFDNA CONCENTRATIONS FOR DIFFERENT PRE-ANALYTICAL CONDITIONS. Boxes (interquartile ranges (IQR)) and whiskers (1.5x IQR) are shown together with the median (black horizontal line) of the log cfDNA concentrations in ng/mL plasma of 16 patients for the different pre-analytical conditions. Outliers are displayed as black dots. The Wilcoxon signed rank test was used to compare the difference between matched 1 hour and 24 hour samples relative to the difference between matched 1 hour and 96 hour samples. *p<0.001.

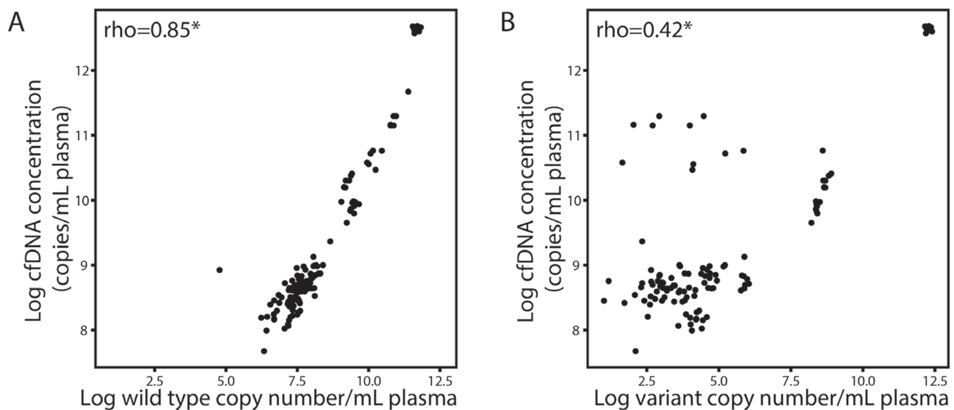


FIGURE 2. CORRELATION BETWEEN WILD TYPE OR VARIANT COPY NUMBERS AND CFDNA CONCENTRATION. The log number of wild type copies (A) or variant copies (B) in copy numbers/mL plasma on the x-as is plotted against the log cfDNA concentrations in ng/mL plasma on the y-as. Data points correspond to single sample measurements from each time interval and each type of preservative. Correlations were tested by Spearman's rank correlation coefficient. *p<0.001. Five patients with undetectable variant copy numbers in ctDNA are removed from plot B.

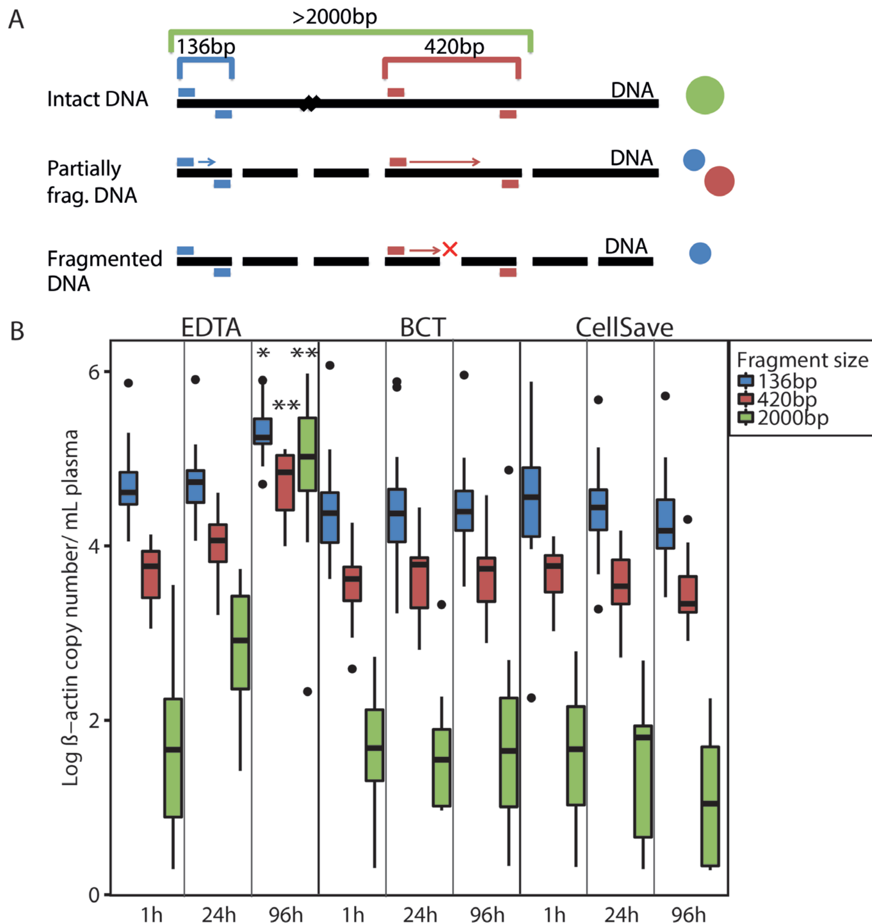


FIGURE 3. Β-ACTIN FRAGMENTATION ASSAY FOR DIFFERENT PRE-ANALYTICAL CONDITIONS. (A) Principles of β-actin fragmentation assay. dPCR wells containing only 136bp signal are indicative of fragmented DNA (fragments <200 bp), whereas the 420 bp set will only binwd to intact DNA (>420bp). When a large intact DNA fragment (>2000 bp) is present in one of the wells both primer sets can bind, resulting in a mixed signal. In theory, this can also occur when a small (<200 bp) and large (>420 bp) DNA fragment is present together in one well. (B) Results of β-actin fragmentation assay. Boxes (interquartile ranges (IQR)) and whiskers (1.5x IQR) are shown together with the median (black horizontal line) of the number of β-actin copies for the different pre-analytical conditions. Outliers are displayed as black points. The Wilcoxon signed rank test was used to compare the difference between matched 1 hour and 24 hour samples relative to the difference between matched 1 hour and 96 hour samples for the different fragment sizes. *p=0.002; **p<0.001.

similar in all blood collection tubes (**Figure 1**). We also did not observe any differences in the DNA fragment size distribution with the β-actin fragmentation assay for the different tubes at 1 hour (**Figure 3B**).

In order to investigate whether the used preservatives in BCT and CellSave tubes could prevent the time-dependent increase in cfDNA concentration observed in EDTA tubes. We observed stable cfDNA concentrations in all 24 and 96 hours samples compared to their matched 1 hour samples (**Figure 1**). Also, we did not observe any differences in the DNA size distribution with the β-actin fragmentation assay for the matched time intervals for both tube types (**Figure 3B**).

THE INTERACTION BETWEEN DIFFERENT PRESERVATIVES AND PLASMA ISOLATION TIME INTERVALS ON SOMATIC VARIANT DETECTION IN CTDNA

To study the effect of time-dependent increase in cfDNA concentrations and wild type copy numbers on somatic variant detection, we analyzed VAF in the different pre-analytical conditions compared to their matched 1 hour sample. If a somatic variant was detected in the EDTA 1 hour sample, the somatic variant could also be detected in BCT and CellSave 1 hour samples. There was no correlation between the VAF in tumor tissue and the VAF in plasma (**Supplementary figure 2**). There was a significant decrease in VAFs in samples from EDTA 96 hours (p=0.003; **Figure 4**), which was not observed for the other pre-analytical conditions. Since all tubes were drawn within a single blood draw we expected, in contrast to VAF, that all tubes within each patient contains similar amounts of variant copy numbers. Indeed, variant copy numbers appeared largely similar between tubes and in all tubes compared to their matched 1 hour sample. (**Figure 5 and Supplementary figure 3**).

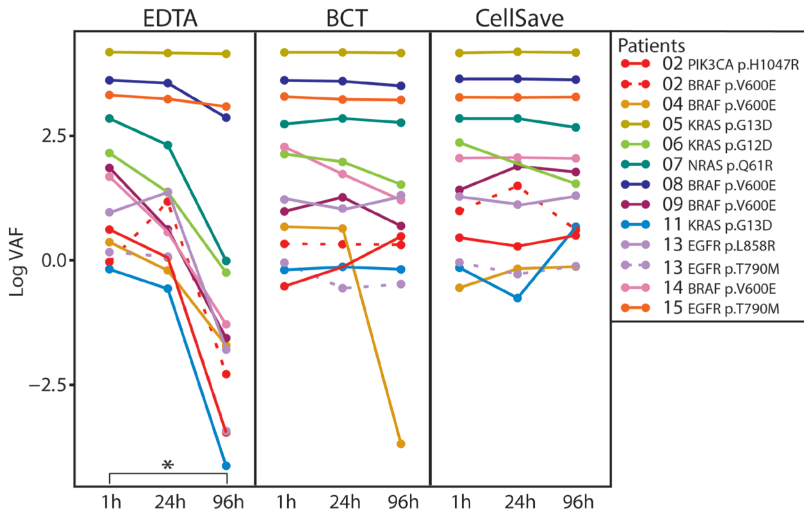


FIGURE 4. VAF FOR DIFFERENT PRE-ANALYTICAL CONDITIONS. The log variant allele frequency (VAF) of 11 patients for the different pre-analytical conditions. Data points correspond to VAF for each individual patient and assay. The Wilcoxon signed rank test was used to compare the difference between matched 1 hour and 24 hour samples relative to the difference between matched 1 hour and 96 hour samples. *p=0.003.

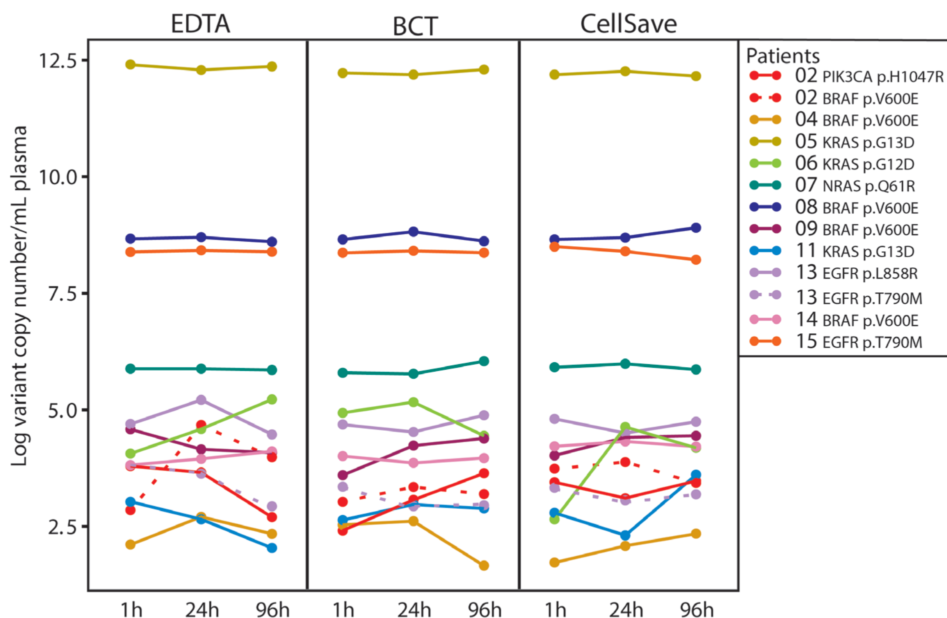


FIGURE 5. Variant copy numbers for different pre-analytical conditions. The log variant copy numbers of 11 patients for the different pre-analytical conditions. Data points correspond to variant copy numbers for each individual patient and assay. The Wilcoxon signed rank test was used to compare the difference between matched 1 hour and 24 hour samples relative to the difference between matched 1 hour and 96 hour samples.

DISCUSSION

The purpose of this study was to investigate the effects of various pre-analytical conditions on the quality of cfDNA in general and of ctDNA in particular. The main aim was to investigate whether BCT and CellSave tubes processed within 96 hours after blood draw into plasma were suitable for downstream analyses of ctDNA.

Patients were recruited with a high prior probability to harbor ctDNA in their plasma, i.e. patients with metastatic disease without current anti-cancer treatment. In 69% of our patients we were able to detect the known somatic variant from tissue in ctDNA and this corresponds to the detection of 68% of all tested somatic variants. In 2/6 missed somatic variants, the somatic variant status in tissue was assessed > 3 years ago. It may be possible that other cancer subclones have emerged, resulting in undetectable somatic variants in ctDNA. Unfortunately, in these cases more recent information on somatic variant status was not available. Detection of somatic variants in plasma may also be influenced by the site and extent of metastases, which is exemplified by patient #05. This patient had a widespread pattern of metastases with corresponding high levels of cfDNA and high levels of variant copy numbers in plasma. However, due to our heterogeneous cohort this relationship could not be tested statistically for the other patients.

The clinical utility and potential importance of our methods is evidenced by our findings in patient #02, who was thought to have metastases from his pancreatic carcinoma (first primary cancer) harboring a *KRAS* mutation. However, we could only detect *BRAF* and *PIK3CA* mutations in his ctDNA, highly suggestive that the metastases were originating from the patients' colorectal cancer (second primary cancer), which can have important implications for his disease management.

The formation of small DNA fragments (180-200 bp lengths) is a biochemical hallmark of apoptosis, whereas during cell lysis or necrosis intact genomic DNA and thus much larger DNA fragments (50-300 kbp) remain (19). Through an increase in wild type copy numbers and mainly intact DNA fragments we were able to demonstrate that the time-dependent increase in cfDNA concentration in EDTA tubes originates from leukocyte lysis. In addition, we observed low levels of intact DNA fragments in all pre-analytical conditions, indicating a background level of leukocyte lysis here. Both Norton et al. (12) and Rothwell et al. (15) observed a similar increase in cfDNA concentrations in samples collected in EDTA tubes. In both BCT and CellSave tubes cfDNA concentrations, wild type copy numbers and β-actin fragment sizes remained stable up to 96 hours, indicating that the preservative in these tubes does not adversely affect cfDNA quality. Interestingly, there was also a significant increase in fragmented DNA in samples from EDTA 96 hours, which might be attributed to nucleases remaining active.

As we only used dPCR for downstream analysis of ctDNA we cannot rule out the possibility that the used preservatives in BCT and CellSave tubes could potentially damage the cfDNA and thus affect other downstream analyses. Rothwell et al. assessed the number of single nucleotide variants through whole genome sequencing of cfDNA isolated from plasma collected in CellSave tubes (15). They did not observe introduction of DNA errors. Thus, the preservative used in CellSave tubes does not seem to influence cfDNA downstream analysis using NGS.

Despite the contamination with intact cfDNA we were still able to detect all somatic variants in ctDNA from EDTA 96 hours samples, in those samples where we were able to detect a somatic variant in the EDTA 1 hour samples. These data suggests that stored samples which have not been processed optimally for ctDNA analysis can still be used to determine the presence of somatic variants in ctDNA. As a consequence of increased cfDNA concentrations and correlated wild type copy numbers, we did observe a significant decrease in VAF in the EDTA 96 hours samples. With respect to ctDNA applications for treatment response evaluation, this could result in serious misinterpretations of VAFs. However, variant copy numbers remained stable in all tubes and might thus be a more accurate outcome measure to evaluate treatment response in cancer patients. However, further investigation is needed to determine the inter-assay variability regarding the range of variant copy numbers and VAFs we observed among the different tubes.

The results in this study indicate that EDTA tubes processed at 96 hours after blood draw are not suitable for blood collection for subsequent cfDNA/ctDNA analysis as the time-dependent increase in cfDNA concentration significantly affects VAF. In patient samples with low variant copy numbers this increase cfDNA concentration, resulting

from leukocyte lysis, may cause variant copies to fall below the limit of detection of the dPCR assay and thus may lead to false-negative results. Both BCT and CellSave tubes preserve cfDNA/ctDNA quality equally well up to 96 hours and the used preservatives did not affect downstream cfDNA/ctDNA analyses by dPCR. Variant copy numbers and VAFs also remained stable in these tubes.

Therefore, we recommend for all future clinical studies, in which flexibility regarding the processing of blood samples is needed, to isolate plasma from blood collected in either BCT or CellSave tubes within 96 hours. This will make large multi-center trials using a central processing facility feasible, and will lead to optimal quality of ctDNA research and diagnostics.

REFERENCES

1. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008 Sep;14(9):985-90.

2. Shinozaki M, O'Day SJ, Kitago M, Amersi F, Kuo C, Kim J, et al. Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. Clin Cancer Res. 2007 Apr 1;13(7):2068-74.

3. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med. 2012 May 30;4(136):136ra68.

4. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013 Mar 28;368(13):1199-209.

5. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 2013 May 2;497(7447):108-12.

6. Bidard FC, Madic J, Mariani P, Piperno-Neumann S, Rampanou A, Servois V, et al. Detection rate and prognostic value of circulating tumor cells and circulating tumor DNA in metastatic uveal melanoma. Int J Cancer. 2014 Mar 1;134(5):1207-13.

7. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol. 2014 Feb 20;32(6):579-86.

8. Diehl F, Li M, Dressman D, He YP, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. P Natl Acad Sci USA. 2005 Nov 8;102(45):16368-73.

9. Holdhoff M, Schmidt K, Donehower R, Diaz LA, Jr. Analysis of circulating tumor DNA to confirm somatic KRAS mutations. J Natl Cancer Inst. 2009 Sep 16;101(18):1284-5.

10. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001 Feb 15;61(4):1659-65.

11. Elshimali YI, Khaddour H, Sarkissyan M, Wu Y, Vadgama JV. The clinical utilization of circulating cell free DNA (CCFDNA) in blood of cancer patients. Int J Mol Sci. 2013;14(9):18925-58.

12. Norton SE, Lechner JM, Williams T, Fernando MR. A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR. Clin Biochem. 2013 Oct;46(15):1561-5.

13. Xue X, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. Clin Chim Acta. 2009 Jun 27;404(2):100-4.

14. Toro PV, Erlanger B, Beaver JA, Cochran RL, VanDenBerg DA, Yakim E, et al. Comparison of cell stabilizing blood collection tubes for circulating plasma tumor DNA. Clin Biochem. 2015 Oct;48(15):993-8.

15. Rothwell DG, Smith N, Morris D, Leong HS, Li Y, Hollebecque A, et al. Genetic profiling of tumours using both circulating free DNA and circulating tumour cells isolated from the same preserved whole blood sample. Mol Oncol. 2015 Nov 19.

16. Sherwood JL, Corcoran C, Brown H, Sharpe AD, Musilova M, Kohlmann A. Optimised Pre-Analytical Methods Improve KRAS Mutation Detection in Circulating Tumour DNA (ctDNA) from Patients with Non-Small Cell Lung Cancer (NSCLC). Plos One. 2016;11(2):e0150197.

17. Kang Q, Henry NL, Paoletti C, Jiang H, Vats P, Chinnaiyan AM, et al. Comparative analysis of circulating tumor DNA stability In KEDTA, Streck, and CellSave blood collection tubes. Clin Biochem. 2016 Apr 27.

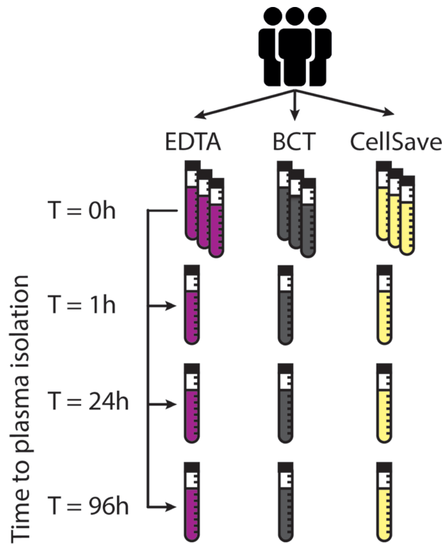
18. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. American Journal of Human Genetics. 1998 Apr;62(4):768-75.

19. Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. Trends Cell Biol. 1995 Jan;5(1):21-6.

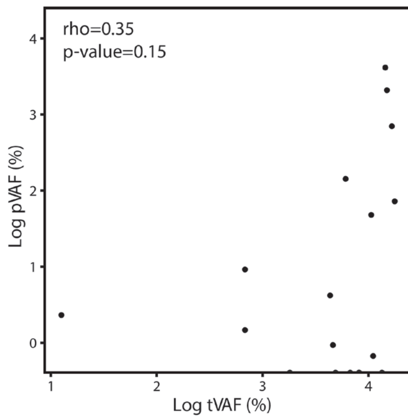
20. Koukourakis MI, Giatromanolaki A, Winter S, Leek R, Sivridis E, Harris AL. Lactate Dehydrogenase 5 Expression in Squamous Cell Head and Neck Cancer Relates to Prognosis following Radical or Postoperative Radiotherapy. Oncology-Basel. 2009;77(5):285-92.

21. Koukourakis MI, Giatromanolaki A, Sivridis E, Bougioukas G, Didilis V, Gatter KC, et al. Lactate dehydrogenase-5 (LDH-5) overexpression in non-small-cell lung cancer tissues is linked to tumour hypoxia, angiogenic factor production and poor prognosis. Brit J Cancer. 2003 Sep 1;89(5):877-85.

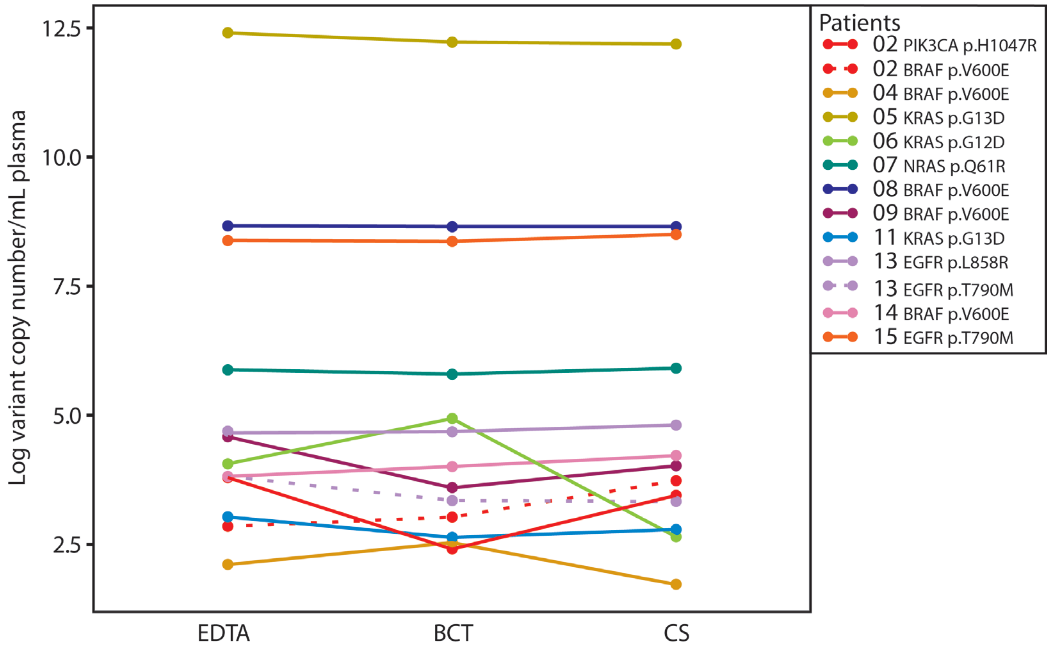
22. Koukourakis MI, Giatromanolaki A, Simopoulos C, Polychronidis A, Sivridis E. Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer. Clin Exp Metastas. 2005 Jan;22(1):25-30.



SUPPLEMENTARY FIGURE 1. Overview of study design. At time point 0h 9x10 mL blood tubes (3x EDTA; 3x BCT; 3x CellSave) were collected within a single blood draw from each recruited patient (N=16). From each type of tube plasma was isolated within 1 hour, after 24 hours and after 96 hours. Plasma was directly stored at -80°C after processing.



SUPPLEMENTAL FIGURE 2. Correlation between variant allele frequency in tumor tissue and in ctDNA in plasma. The log percentage of tumor tissue variant allele frequency (tVAF) on the x-axis is plotted against the log precentage of ctDNA in plasma variant allele frequency (pVAF) on the y-axis. Data points correspond to single somatic variants. Correlation was tested by Spearman's rank correlation coefficient.



SUPPLEMENTARY FIGURE 3. Variant copy numbers for 1 hour samples. The log variant copy numbers from 11 patients for the 1 hour samples. Data points correspond to variant copy numbers for each individual patient and assay.

SUPPLEMENTARY TABLE 1. Used SNP genotyping assays.

Assay ID	Assay Name	Gene	Cosmic ID	Amino acid change	Nucleotide change
AH6R5PH	BRAF_476	*BRAF*	476	p.V600E	c.1799T>A
AHRSROS	EGFR_6240	*EGFR*	6240	p.T790M	c.2369C>T
AHRSRSV	EGFR_6224	*EGFR*	6224	p.L858R	c.2573T>G
AH6R5PI	KRAS_521	*KRAS*	521	p.G12D	c.35G>A
AHX1IHY	KRAS_520	*KRAS*	520	p.G12V	c.35G>T
AHD2BW0	KRAS_532	*KRAS*	532	p.G13D	c.38G>A
AHQJTKH	KRAS_552	*KRAS*	552	p.Q61R	c.182A>G
AHS1P6Q	NRAS_584	*NRAS*	584	p.Q61R	c.182A>G
AHLJ0TP	PIK3CA_776	*PIK3CA*	776	p.H1047L	c.3140 A>T
AHPAVCD	PIK3CA_775	*PIK3CA*	775	p.H1047R	c.3140 A>G

SUPPLEMENTARY TABLE 2. Primer and probe designs for digital PCR.

Gene	Forward primer	Reverse primer	Probe
β-actin 136 bp	5'-GCG CCG TTC CGA AAG TT-3'	5'- CGG CGG ATC GGC AAA -3'	6FAM-ACC GCC GAG ACC GCG TC-MGBNFQ
β-actin 420 bp	5'-CCG CTA CCT CTT CTG GTG-3'	5'-GAT GCA CCA TGT CAC ACT G-3'	VIC-CCT CCC TCC TTC CTG GCC TC-BHQ

06

CHAPTER 7

Somatic mutation detection using various targeted detection assays in paired samples of circulating tumor DNA, primary tumor and metastases from patients undergoing resection of colorectal liver metastases

Nick Beije, Jean C. Helmijr, Marjolein J.A. Weerts, Corine M. Beaufort, Matthew Wiggin, Andre Marziali, Cornelis Verhoef, Stefan Sleijfer, Maurice P.H.M. Jansen, John W.M. Martens

Mol Oncol. 2016 in press

ABSTRACT

Assessing circulating tumor DNA (ctDNA) is a promising method to evaluate somatic mutations from solid tumors in a minimally-invasive way. In a group of twelve metastatic colorectal cancer (mCRC) patients undergoing liver metastasectomy, from each patient DNA from cell-free DNA (cfDNA), the primary tumor, metastatic liver tissue, normal tumor-adjacent colon or liver tissue, and whole blood were obtained. Investigated was the feasibility of a targeted NGS approach to identify somatic mutations in ctDNA. This targeted NGS approach was also compared with NGS preceded by mutant allele enrichment using synchronous coefficient of drag alteration technology embodied in the OnTarget assay, and for selected mutations with digital PCR (dPCR). All tissue and cfDNA samples underwent IonPGM sequencing for a CRC-specific 21-gene panel, which was analyzed using a standard and a modified calling pipeline. In addition, cfDNA, whole blood and normal tissue DNA were analyzed with the OnTarget assay and with dPCR for specific mutations in cfDNA as detected in the corresponding primary and/or metastatic tumor tissue. NGS with modified calling was superior to standard calling and detected ctDNA in the cfDNA of 10 patients harboring mutations in *APC, ATM, CREBBP, FBXW7, KRAS, KMT2D, PIK3CA* and *TP53*. Using this approach, variant allele frequencies in plasma ranged predominantly from 1 to 10%, resulting in limited concordance between ctDNA and the primary tumor (39%) and the metastases (55%). Concordance between ctDNA and tissue markedly improved when ctDNA was evaluated for *KRAS*, *PIK3CA* and *TP53* mutations by the OnTarget assay (80%) and digital PCR (93%). Additionally, using these techniques mutations were observed in tumor-adjacent tissue with normal morphology in the majority of patients, which were not observed in whole blood. In conclusion, in these mCRC patients with oligometastatic disease NGS on cfDNA was feasible, but had limited sensitivity to detect all somatic mutations present in tissue. Digital PCR and mutant allele enrichment before NGS appeared to be more sensitive to detect somatic mutations.

INTRODUCTION

The use of targeted therapies has markedly transformed cancer treatment in the last decade (1). Unfortunately most of the responses to targeted therapies in the advanced setting are transitory at best, because intrinsic or acquired resistance to these agents is present or rapidly develops (2). Tumor heterogeneity is thought to play a pivotal role in the development of acquired resistance (3). Heterogeneity is present in the tumor lesion itself (intra-tumor heterogeneity), while in the advanced setting also heterogeneity between different metastatic lesions (inter-metastatic heterogeneity) can be present (4, 5). Furthermore, during effective treatment the genomic landscape of tumor cells evolves. For example, there are strong indications that the emergence of *KRAS* mutations in metastatic CRC (mCRC) patients who initially harbored a tumor wildtype for *KRAS,* contributes to resistance against anti-EGFR monoclonal antibodies (6, 7). Altogether, this clearly stresses that in the advanced setting, particularly after treatment with agents dependent on a genetic aberration, the analysis of a single biopsy to evaluate the cancer genome and to guide treatment decision making is likely insufficient. The only way to acquire a comprehensive overview of the cancer genome would be to take multiple biopsies from metastases, which is cumbersome and even impossible in some patients due to inaccessibility of lesions.

As an alternative approach to taking biopsies from solid lesions, assessing circulating tumor DNA (ctDNA) in the peripheral blood has been proposed as a minimally-invasive way to evaluate the tumor mutation status. Tumor cells release fragmented DNA into the peripheral blood, and these DNA fragments can be detected as ctDNA in the cell-free compartment (i.e., serum and plasma) of the blood. It is thought that ctDNA can represent the most prevalent tumor clones from primary tumors as well as metastatic lesions. In the last years, various techniques have been introduced to detect and quantify mutations in ctDNA. Generally, for choosing a technique to detect mutant ctDNA one has to take into account the rarity of ctDNA alleles relative to wildtype DNA alleles in the cell-free compartment of the blood. Frequencies of ctDNA vary largely, from roughly <0.1% to >10% (8, 9). Techniques such as digital PCR (dPCR) (10) and BEAMING (11) have the advantage of superior sensitivity, being able to detect ctDNA in frequencies as low as 0.01%. However, using these techniques only one or a limited number of specific somatic mutations can be analyzed simultaneously. Recently, a technique called synchronous coefficient of drag alteration (SCODA) (12, 13) has been used to develop an assay (OnTarget assay) which is able to analyze up to 96 mutant alleles in 9 genes with reported sensitivity similar to dPCR and BEAMING of 0.01 - 0.001% (14). This OnTarget assay firstly enriches for mutant alleles and subsequently genes are targeted next-generation sequenced (NGS). Still, when all somatic variants in numerous genes are of interest, NGS for multiple genes is indicated. A potential drawback of these NGS techniques is however their lack of sensitivity for detecting ctDNA frequencies below 1-2% (15).

The current study set out to explore the feasibility of Ion Torrent PGM (IonPGM) targeted NGS on plasma cfDNA of patients with mCRC undergoing colorectal liver metastasectomy. To this purpose, 12 patients undergoing resection of CRC metastases were investigated. In these patients the primary tumor, the resected liver metastasis, cfDNA and normal tumor-adjacent tissue were sequenced using a 21-gene CRC-specific

panel on the IonPGM platform. In addition, to gain more insight into the advantages and disadvantages of ctDNA detection with different techniques, results generated with the IonPGM platform were compared with the OnTarget assay and with digital PCR for specific variants.

METHODS

PATIENTS AND SAMPLE COLLECTION

Patients with colorectal liver metastases undergoing resection of liver metastases were included as part of a prospective study in the Erasmus MC Cancer Institute evaluating the prognostic value of circulating tumor cells (CTCs) as described before (16, 17). Prior to surgery 30 mL of blood was drawn in EDTA tubes from all patients for DNA isolation from plasma or whole blood as described in the next paragraph. In addition, 30 mL of blood was drawn in CellSave tubes and subsequently processed for CTC enumeration on the CellSearch system (Janssen Diagnostics, Raritan, NJ, USA) as described previously (16). During surgery, the liver metastases and normal tissue of the liver were collected and freshly frozen (FF) for later analyses. In all cases the tissue was also stored as formalin-fixed paraffin-embedded (FFPE). In patients presenting with synchronous CRC metastases, the resection of the colon was combined with the resection of the liver metastases and normal tumor-adjacent colon was also collected.

For this mutation analysis study, only patients with a complete set of available plasma cfDNA, primary tumor tissue, metastatic tumor tissue and normal tumor-adjacent tissue of the liver or the colon were included. Other criteria for inclusion were 1) acquisition of metastatic tissue, normal tissue and plasma on the same day; 2) a minimum percentage of 30% tumor cells in the primary tumor sample and the liver metastasis sample as assessed using hematoxylin and eosin (H&E) slides from macro-dissected tissue by an experienced pathologist; 3) no tumor cells detected in macro-dissected tissue from tumor-adjacent colon and liver as assessed on H&E slides by an experienced pathologist; 4) no adjuvant treatment given in case of metachronous metastases. All patients provided written informed consent and the institutional board approved the protocols (Erasmus MC ID MEC-2006-089).

DNA ISOLATION

DNA was isolated from all tissues using the NucleoSpin DNA tissue kit (Macherey-Nagel, Düren, Germany). For FFPE materials deparaffinization was done prior to isolation of DNA (18). For the isolation of cell-free DNA (cfDNA), 30 mL of peripheral blood was pooled and centrifuged for 10 min at 800g within 24 hours after the blood draw. Subsequently plasma was removed and snap frozen at -80°C. Cell-free DNA was isolated from 3x1 mL plasma and eluted in 20 µL buffer using the QIAamp circulating nucleic acid kit (Qiagen, Venlo, The Netherlands) according to the manufacturer□s instructions. Subsequently the eluate from 3x1 mL of plasma was used for each assay (IonPGM, dPCR and OnTarget) (**Supplementary Table 1**). DNA from whole blood samples was isolated using the QIAamp Blood Mini kit (Qiagen), according to the manufacturer's instructions. Extracted DNA was quantified using the Qubit dsDNA HS Assay kit (Thermo Fisher, Waltham, MA, USA).

TARGETED NGS USING IONPGM

A CRC-specific 21-gene panel was established based on the top 19 most frequently mutated genes for CRC in the Catalogue of somatic mutations in cancer (COSMIC, June 2014). Based on the clinical relevance of *NRAS* in CRC (19) and recent literature suggesting that *KDR* is a marker for hypermutation in CRC (20), these genes were also included in the panel. This resulted in a 21-gene panel consisting of *TP53, APC, KRAS, ATM, PTEN, PIK3CA, BRAF, FBXW7, SMAD4, NF1, RB1, ARID1A, PTCH1, CREBBP, KIT, KMT2D, CDH1, MLH1, EGFR, NRAS & KDR*. These 21 genes were included in a custom amplicon-based sequencing panel (1115 amplicons, ~89kb), where the amplicons covered the whole exome of the gene, except for genes *KRAS, PIK3CA, BRAF, EGFR* and *NRAS* in which only the hotspot regions were sequenced (**Supplementary Table 2**). Libraries for these genes were constructed using the Ion AmpliSeq library kit 2.0 (Thermo Fisher) and subsequently the libraries were sequenced on the Ion Torrent NGS platform (Thermo Fisher), all as described before (21).

IonPGM data was analyzed using our previously described standard calling pipeline (21, 22). In short, raw IonPGM data was first loaded into the TorrentSuite variant caller 4.3, and variants were called with somatic low stringency setting. Then additional filtering was applied: variants were excluded if they were not present in the targeted exonic regions, if they were present in ≥90% of the samples, if they had a Q-score of ≤20, strand bias of ≥90%, read depth ≤100 (≤20 for normal tissue) or mutant allele read depth of ≤10. Then variants detected in normal tissue with a variant allele frequency of ≥35% and/or variants present in the virtual normal database (23) were excluded as somatic variants. Lastly, all detected variants were inspected in all patient-matched samples using raw data without any of the filtering steps (IonPGM hotspot file). The remaining variants were considered to be true somatic variants.

A modified approach to sensitively call known variants in cfDNA was also evaluated. In this modified calling pipeline, all variants observed in the primary tumor or the metastasis as identified with the standard calling pipeline were analyzed in the cfDNA. All of the above criteria to call a variant were used, except for the criterion that variants had to be called by the TorrentSuite variant caller. The somatic low stringency calling was omitted in this approach, as this calling was originally developed for variant calling in tissue, in which variant allele frequencies are usually higher than in cfDNA.

NGS PRECEDED BY ONTARGET ENRICHMENT

DNA isolated from all plasma samples and a selected set of tissue samples and whole blood samples were sent to Boreal Genomics (Vancouver, BC, Canada), and processed with the OnTarget assay (13) followed by targeted sequencing on the MiSeq platform (Illumina, San Diego, CA, USA) as described before (14). Boreal Genomics was blinded from all results obtained with IonPGM and digital PCR.The OnTarget assay targets 96 mutations in 9 genes (*BRAF*, *CTNNB1*, *EGFR*, *KRAS*, *FOXL2*, *GNAS*, *NRAS*, *PIK3CA* and *TP53;* **Supplementary Table 3***)*. The assay detects mutant DNA by electrophoretically removing wild-type DNA from the sample before significant PCR amplification or sequencing. Mutations were called positive if they were detected above a limit of detection (LOD), which was calculated as the mean plus 3 standard deviations of the mutant background (as observed on >100 known wild-type samples), plus two copies of the mutant. In cases where a mutant was called positive on a given PCR amplicon,

the LOD for all other mutants on the same amplicon was raised by 1% of the detected mutant abundance to prevent false positives from PCR errors on the detected mutant. The limit of detection was calculated separately for each mutation in each sample to maximize assay sensitivity and specificity. To evaluate the linearity, accuracy, and precision of the OnTarget assay, a set of samples designed for validation of cfDNA assays (Multiplex I cfDNA Reference Standard, from Horizon DX, Cambridge UK) were tested. These validation experiments demonstrated high sensitivity and specificity of the assay (**Supplementary Table 4**).

DIGITAL PCR

Digital PCR for somatic variants was performed using validated Taqman SNP genotyping assays for *KRAS* p.G12D, *KRAS* p.G12V, *KRAS* p.G13D, *PIK3CA* p.E545K, *TP53* p.R273H and *TP53* p.R248Q (Thermo Fisher) on the Quantstudio 3D digital PCR system (Thermo Fisher), according to the manufacturer's instructions. In case of cfDNA, the maximum input of 7.8 µL DNA was used. The presence of at least 2 mutant signals (FAM-positive, VIC-negative) was considered positive for a certain mutation.

To assess the proportion of cfDNA fragments, a Taqman *β-actin* dPCR assay was used based on the assay described by Norton et al. (24) which is able to detect small (≥136 bp) and long (≥420 bp) *β-actin* fragments. This assay was used to quantify the extent of leukocyte lysis, which is characterized by an increased number of large DNA fragments relative to small DNA fragments (25) . A standardized input of 2 ng was used for the assay, to minimize the chances of double-positive events related to high input. Double-positive events (positive for ≥136 bp & ≥420 bp) were classified as long fragments ≥2000 bp, as the primers for the 136 bp and 420 bp span about 2000 bp. All dPCR experiments were analyzed in the Quantstudio 3D AnalysisSuite (Thermo Fisher) by one experienced technician (JH).

STATISTICAL CONSIDERATIONS

Our primary endpoint was the feasibility of ionPGM sequencing on ctDNA using our standard algorithm. Secondary endpoints included to explore if ctDNA is more resembling of the primary tumor or the metastases, to explore associations between the number of circulating tumor cells and ctDNA detection, to explore associations between cfDNA fragmentation and ctDNA detection, and to explore how ionPGM sequencing relates to other methods detecting mutations in ctDNA. As this study was meant to be exploratory, no formal statistics were performed to compare groups or methods.

RESULTS

PATIENTS AND TISSUES

Twelve patients were identified whom matched the inclusion criteria (**Supplementary Table 5**). Six patients presented with synchronous metastases, and 6 patients had metachronous metastases. Median number of days between the resection of the primary tumor and the resection of the liver metastases for the patients with metachronous metastases was 830 days (range 270-2522 days). None of the patients received adjuvant therapy after surgery of the primary tumor or induction-therapy prior to the surgery for the liver metastases. In the set of 36 tissue samples (primary tumor, metastases and normal liver or colon), 25 tissue samples were FF and 11 tissue samples were FFPE. The median tumor cell percentage was 80% (interquartile range 20%).

PLASMA ISOLATION AND RAW ANALYSIS OF SAMPLES

Median cfDNA concentration after isolation was 564 pg/µL (range 442-1224). A total of 5.75 - 15.91 ng cfDNA was sequenced on the IonPGM platform (**Supplementary Table 6**). Median coverage was 751x (range 582x- 1141x) for tissue samples and 728x (range 527x-812x) for cfDNA samples. Raw data was analyzed and variants not meeting our criteria for somatic mutations were excluded with the exception of one variant (TP53 p.R273H). This variant was observed in one of the 478 virtual normal genomes (0.2%), but since this variant is enriched in TCGA CRC data (3,3%) (26) and linked to enhanced proliferation and invasion (27) it was evaluated as a somatic variant. Following further filtering of variants using the standard variant calling pipeline, a median of 3 variants were called per primary tumor or metastasis. However, in the primary tumors of patients 1, 4 & 5 more than 200 variants remained after filtering. Further investigation of these variants revealed that >97% of the called variants were C > T or G > A variant substitutions, which had previously been linked to sequencings artefacts caused by formalin fixation (28, 29). In an attempt to reduce these artefacts one FFPE-derived DNA was treated with uracil-DNA glycosylase (UDG), which has been described to reduce FFPE-related sequencing artefacts (28, 29). While a great reduction of variants was observed after UDG treatment, still more than 100 variants remained after filtering (data not shown). Since these FFPE-related artefacts severely limited somatic mutation detection, the primary tumor FFPE samples of patients 1, 4 & 5 were omitted from further analysis.

CONCORDANCE BETWEEN PRIMARY TUMOR, METASTASIS AND CFDNA USING IONPGM NGS

Following standard filtering 28 variants were observed in the primary tumor and 33 variants in the metastases, comprising a total of 29 distinct variants (**Table 1**). Of these 29 variants, 10 variants were not previously described in COSMIC. Concordance of all variants between the primary tumor and the metastases was 72%. In the cfDNAs, a total of 11 variants were observed in the blood of 6 patients. Two variants were found exclusively in ctDNA (*KMT2D* p.G794R & *ATM* p.A2301T) which were not previously described in COSMIC. From 28 variants observed in the primary tumor, 5 were retrieved in cfDNA (18%). Of note is that 4 of these 5 retrieved variants were observed in the same patient (patient 9). Out of 33 variants observed in the metastases, 9 variants were retrieved in the cfDNA (27%).

TABLE 1. Variants called in the primary tumor, metastases and plasma.

	Patient	Gene	Position	COSMIC	Primary ionPGM	Meta ionPGM	Plasma ionPGM
metachronous	1	APC	p.Q1388X	yes	X	54	***2.7***
		APC	p.R858X	yes	X	27.4	.
	2	CREBBP	p.P2383L	no	5.4	.	.
		FBXW7	p.D399Y	no	19.8	39.4	.
		KRAS	p.G12D	yes	28.6	38.6	.
		PIK3CA	p.E545K	yes	41.1	74.8	.
		TP53	p.G108S	yes	40.9	78.4	.
		TP53	p.L130I	no	44.1	74.7	.
	3	APC	p.C1369X	no	52.0	78.2	***3.7***
		CREBBP	p.P2383S	no	6.0	.	.
		TP53	p.R273H	yes	50.6	82.0	7.3
	4	APC	p.R223X	yes	X	66.8	25.8
	5	APC	p.E1288X	yes	X	5.4	.
		APC	p.R284X	yes	X	10	***1.6***
		TP53	p.R248Q	yes	X	17.9	***2.2***
	6	APC	p.R481X	yes	.	76.2	15.8
		FBXW7	p.R689W	yes	22.0	.	.
		FBXW7	p.S582L	yes	23.8	.	.
		PIK3CA	p.E545K	yes	5.3	.	.
		KRAS	p.G12V	yes	.	49.7	5.3
		TP53	p.R273H	yes	.	72.3	12.2

TABLE 1. Variants called in the primary tumor, metastases and plasma.

	Patient	Gene	Position	COSMIC	Primary ionPGM	Meta ionPGM	Plasma ionPGM
synchronous	7	KRAS	p.G12D	yes	12.7	8.5	.
		ATM	p.A2301T	no	.	.	3.4
	8	APC	p.E1390X	yes	22.3	50.7	***1.3***
		KRAS	p.G12D	yes	30.9	51.2	***2.3***
		TP53	p.R175H	yes	37.2	69.2	.
		KMT2D	p.G794R	no	.	.	4.2
	9	APC	p.R858X	yes	24.4	41.2	17.2
		CREBBP	p.P937Q	no	4.2	4.3	7.2
		KRAS	p.G12D	yes	39.7	35.0	20.5
		TP53	p.R248Q	yes	37.5	55.7	14.1
	10	TP53	p.R282W	yes	66.8	61.7	.
	11	APC	p.R481X	yes	23.9	16.3	***2.9***
		TP53	p.M237I	yes	55.4	37.3	***1.3***
	12	APC	p.E1390X	yes	26.0	20.9	.
		APC	p.E564X	no	19.1	12.5	.
		CREBBP	p.P975S	no	.	4.2	.
		CREBBP	p.R601Q	no	13.0	9.8	.
		KRAS	p.G13D	yes	37.9	26.6	***1.5***
		PIK3CA	p.E545K	yes	18.0	12.9	.

Variants in plasma only called after modified variant calling are italicized and bold. Excluded samples because of FFPE-related artefacts are indicated with an “X”. Not detected variants are indicated with a dot.

INVESTIGATION OF MODIFIED CALLING PIPELINE FOR CFDNA

To explore whether the sensitivity for calling variants in cfDNA from plasma with the standard filtering strategy could be improved, variants previously observed in the primary tumor or the metastasis were investigated for their presence in cfDNA using a modified calling pipeline. For this pipeline, the variant in cfDNA did not have to be called by the TorrentSuite variant calling program. The modified calling approach led to the identification of 9 additional variants in cfDNA and showed variants in the blood of 10 patients (**Table 1**). In the primary tumor now 11 of 28 variants could be retrieved in the cfDNA samples (39% compared to 18% with standard filtering). In the metastases 18 of 33 variants were retrieved in the cfDNA samples (55% compared to 27% with standard filtering).

After using the modified pipeline, 20 different variants in cfDNA (5 in cfDNA versus primary tumor, 3 in cfDNA versus metastases, 12 in cfDNA versus both primary tumor and metastases) still remained undetected. When the raw data for these variants was explored (**Supplementary Table 7**), for 10 variants no mutant reads were observed at all in cfDNA, while for 5 other variants the number of mutant reads did not exceed the number of mutant reads found in cfDNA of patients without that variant found in their tissue. For 5 additional variants the number of mutant reads did exceed the number of mutant reads observed in cfDNA of patients without that variant in tissue, however, these were generally low-confidence variants with Q-scores below 20.

ONTARGET ENRICHMENT FOLLOWED BY NGS

To explore whether enrichment of mutant alleles with the OnTarget technique followed by NGS would improve sensitivity compared to IonPGM NGS, all cfDNA samples and normal tissues were analyzed with the OnTarget assay (**Supplementary Table 8**). A total of 3.0 - 10.0 ng cfDNA was used for the procedure, which was a similar input as used with IonPGM. The OnTarget assay covers 96 hotspot mutations in 9 genes, and based on our IonPGM sequencing in the primary tumor and metastases, a maximum of 15 mutations could potentially be detected by the OnTarget assay in the samples of 9 patients included in our study (for 3 patients the OnTarget assay did not comprise the observed mutations in the tissues of these patients). These 15 mutations were detected with IonPGM in 5 cfDNA samples with the standard pipeline (33%) and in 8 cfDNA samples with the modified pipeline (58%). Using the OnTarget assay 12 out of 15 variants (80%) could be retrieved in the cfDNA (**Table 2**). No additional variants were detected in cfDNA with the OnTarget assay. Interestingly, the OnTarget assay also detected a total of 13 variants in 7 of 9 normal tumor-adjacent tissues (**Supplementary Table 8**). For 5 patients in which mutations in tumor-adjacent normal tissue were observed there was whole blood available, and all of these variants were absent in whole blood.

DIGITAL PCR

For 14 mutations observed in the primary tumor and/or the metastases, we had validated dPCR assays available (**Supplementary Table 9**). Using dPCR, 13 of 14 mutations (93%) observed in the primary tumor and/or the metastases were detected (**Table 2**). Of note is that the one mutation that was not detected was only observed in the primary tumor of patient 6 at a low frequency (5%), and not the metastases. Digital PCR was however able to detect all mutations that occurred in the liver metastasis of this particular patient.

TABLE 2. Detection of plasma ctDNA mutations using various techniques and strategies.						
Patient	Gene	Position	Plasma IonPGM standard filtering VAF%	Plasma ionPGM modified filtering VAF%	Plasma OnTarget VAF%	Plasma dPCR VAF%
2	KRAS	p.G12D	.	.	.	1.94%
	PIK3CA	p.E545K	.	.	.	2.23%
3	TP53	p.R273H	7.3%	7.3%	3.5%	7.03%
5	TP53	p.R248Q	.	2.2%	1.5%	2.7%
6	KRAS	p.G12V	5.3%	5.3%	3.6%	8.5%
	TP53	p.R273H	12.2%	12.2%	4.9%	7.03%
	PIK3CA	p.E545K	.	.	.	.
7	KRAS	p.G12D	.	.	0.92%	1.57%
8	KRAS	p.G12D	.	2.3%	1.6%	1.66%
	TP53	p.R175H	.	.	2.8%	no assay
9	KRAS	p.G12D	20.5%	20.5%	14.0%	12.87%
	TP53	p.R248Q	14.1%	14.1%	20.0%	15.02%
10	TP53	p.R282W	.	.	0.67%	2.94%
12	KRAS	p.G13D	.	1.5%	3.1%	3.96%
	PIK3CA	p.E545K	.	.	2.7%	2.9%

Not detected variants are indicated with a dot. VAF%= variant allele frequency.

Compared to the OnTarget assay, dPCR detected two additional mutations, both in patient 2. Again, a significant number of mutations (9 out of 14) were detected in tumor-adjacent tissue with normal appearing histology (**Supplementary Table 10**), confirming the findings with the OnTarget assay. None of these mutations were observed in whole blood.

TABLE 3. Associations between the number of CTCs, the percentage of small fragments and detection of somatic mutations with ionPGM using two filtering variants, and with OnTarget and dPCR.

Patient	Number of CTCs/30 mL	% of small DNA fragments	Mutations present in plasma with standard filtering	Mutations present with modified filtering	Mutations present with OnTarget assay	Mutations present with digital PCR
9	0	46%	yes (4 of 4)	yes (4 of 4)	yes (2 of 2)	yes (2 of 2)
10	0	55%	no	no	yes (1 of 1)	yes (1 of 1)
11	0	64%	no	yes (2 of 2)	not applicable	not applicable
1	0	70%	no	yes (1 of 2)	not applicable	not applicable
7	1	61%	yes (2 of 3)	yes (2 of 3)	yes (1 of 1)	yes (1 of 1)
12	1	63%	no	yes (1 of 6)	yes (2 of 2)	yes (2 of 2)
3	1	66%	yes (2 of 3)	yes (2 of 3)	yes (1 of 1)	yes (1 of 1)
4	1	72%	yes (1 of 1)	yes (1 of 1)	not applicable	not applicable
8	2	62%	yes (2 of 5)	yes (4 of 5)	yes (2 of 2)	yes (2 of 2)
2	5	69%	no	no	no	yes (2 of 2)
5	8	73%	no	yes (2 of 3)	yes (1 of 1)	yes (1 of 1)
6	35	71%	yes (3 of 6)	yes (3 of 6)	yes (2 of 3)	yes (2 of 3)

For OnTarget and dPCR, only mutations previously found in the primary tumor or metastases are reported in thi

CTC ENUMERATION & DNA FRAGMENTATION ASSAY

To gain more insight into why ctDNA was or was not detected in some samples, the number of CTCs and fragmentation of cfDNA were assessed. CTC enumeration results of the main study were retrieved (16) for each patient at the time of cfDNA isolation (**Table 3**). In 4 patients, no CTCs were detected in 30 mL of blood, and in 1 of these patients, mutations in ctDNA were detected using IonPGM with our standard calling pipeline. The use of the modified pipeline, the OnTarget assay or dPCR however led to the identification of mutations in most patients, including the patients without CTCs. Because EDTA blood was used that was processed within 24 hours, it was also evaluated whether large DNA fragments from lysed leukocytes diluted out small DNA fragments and decreased sensitivity for ctDNA analyses. A dPCR-based assay was used to detect small fragments (136 bp) and large fragments (>400 bp), the latter indicative of the presence of large DNA fragments from lysed leukocytes. The median percentage of small DNA fragments out of the total number of fragments (small + large) was 65% (range 46% - 73%; **Table 3**). When patients were separated in two groups using the median of small fragments, we found that mutations in ctDNA using IonPGM and modified filtering were detected in 5 of 6 patients (in which 53% of all potentially detectable mutations were detectable) with a number of small fragments above the median, and in 5 of 6 patients (in which 62% of all potentially detectable mutations were detectable) with a number of small fragments below the median.

DISCUSSION

In the era of precision medicine in oncology, there is a high need for accurate biomarkers that can be used before, during and after treatment in a minimally invasive way. Assessing ctDNA has sparked much interest to become such a biomarker. To date, most reports using ctDNA in CRC have reported on strategies using a limited set of genes (most often *KRAS*, *BRAF* & *PIK3CA*) (7, 14, 30-34) or an approach in which personalized assays based on mutations found in the primary tumor or metastases were developed to detect ctDNA (35, 36). Of note is that this latter approach also focuses on a limited set of somatic mutations only. The current study reported on the feasibility of a NGS panel on ctDNA in mCRC patients covering the 21 most prevalent and relevant genes in CRC known to date. It was observed that NGS with IonPGM is feasible on cfDNA, however only a limited number of variants observed in the primary tumor and the metastases could be retrieved in the cfDNA. A number of specific variants not observed in cfDNA with IonPGM could however be detected using alternative methods such as OnTarget enrichment followed by NGS and dPCR.

The sensitivity of IonPGM sequencing with standard filtering to retrieve mutations found in tissue in ctDNA was less than expected. As the plasma was not optimally collected (out of EDTA blood within 24 hours of the blood draw), the percentage of small fragments in cfDNA as a measure of leukocyte lysis was assessed. In addition, the number of CTCs was assessed as the number of CTCs was previously described to be associated with the probability to detect ctDNA in breast cancer (37). However, both the extent of leukocyte lysis as well as the number of CTCs did not provide obvious explanations as to why mutant ctDNA was or was not detected in some patients, although the power of this analysis was limited due to the small sample size of the presented cohort. Interestingly, for example, is patient 9, in whom ctDNA variant allele frequencies of 7 to 20% were observed, but in whom no CTCs were detected and who had the lowest percentage of small DNA fragments. This illustrates that we have limited insight into why some patients have high or low ctDNA frequencies. The ratio of ctDNA versus wildtype cfDNA probably plays an important role, however to date our understanding of this ratio, or a measure how to quantify it, is lacking.

The observation that a modified calling pipeline for ctDNA, instead of a standard calling pipeline based on calls by the TorrentSuite variant caller, resulted in an increased detection of mutations as found in tissue is in accordance with Couraud et al. (38). This group performed IonPGM sequencing on ctDNA in patients with non-small cell lung cancer and observed an increase in concordance rate between matched tissue and ctDNA from 16% to 58% using an in-house calling algorithm instead of using standard IonPGM variant calling. Also similar to our results with the modified calling pipeline is the report by Frenel et al. (39) whom reported that 59% of variants observed in metastases of patients with various tumors could be retrieved with IonPGM sequencing. However, others have also reported higher concordance between tissue and ctDNA using IonPGM of >80-90% (40, 41). A reason for the lower concordance as observed in our study may be that in our study CRC patients with oligometastatic disease were included, while in the studies with higher concordances heavily pretreated patients were included with probably a higher tumor load. This is also reflected by the fact that in these studies, very high ctDNA variant frequencies of >50% were observed in multiple patients.

The presented results of high concordance between tissue and ctDNA when applying dPCR is in accordance with previous reports using similar techniques (30-33, 35, 36). High concordance between tissue and ctDNA with OnTarget followed by NGS was also observed in the current study, which is consistent with a previous report on this assay (14). Interestingly, using dPCR and the OnTarget assay somatic mutations were observed at very low frequencies in the tumor-adjacent control tissue with normal-appearing histology in the majority of patients, which were also observed in the matched tissue samples. As the majority of these mutations were observed by both methods, and the fact that none of these mutations were detected in whole blood of the same patient, we believe these are true somatic mutations present in normal-appearing tumor-adjacent tissue. An explanation for this finding may be that tumor DNA or tumor cells diffused or migrated into the surrounding normal tissue . Further examination of these findings is warranted, for example by investigating whether mutations in normal-appearing tissue are related to an increased chance of local disease relapse.

The study as presented here has some limitations. The small sample size, combined with having to omit 3 primary tumor samples from the analysis due to FFPE-related variant noise, together with the low sensitivity of IonPGM sequencing to detect mutations, makes exploration whether or not ctDNA resembles the metastases rather than the primary tumor impossible. Also for this reason differences between patients with metachronous metastases versus synchronous metastases could not be explored. This data does however provide perspectives on the advantages and disadvantages of current ctDNA methods for detecting somatic mutations, and which one to use for which particular research purpose.

At least in this group of patients with oligometastatic disease, IonPGM sequencing on ctDNA lacks sensitivity to detect mutations, especially if the tissue mutational status is unknown. If the tissue mutational status is known, IonPGM with the modified calling pipeline is a feasible option but still sensitivity remains an issue to evaluate mutations in multiple genes in ctDNA. However, NGS of multiple genes like we performed here is probably the only option if resistance mutations not present in tissue and not previously reported need to be identified. This is also reflected by the fact that two novel mutations in ctDNA were identified that have not been previously reported. However, given the limited sensitivity of IonPGM sequencing to detect mutations this approach is likely to be of restricted value since many variants remain undetected. IonPGM sequencing of ctDNA in patients with higher tumor loads may however yield better results.

If one would like to monitor certain mutations during treatment, an NGS approach using the primary tumor or metastases for discovery and subsequent tracing of mutations with dPCR is likely a successful approach, given the high sensitivity to detect mutations with dPCR as described here. Reinert et al. (35) reported on this approach and developed 2 to 6 individualized dPCR assays per patient for a total of 11 patients. They observed 100% sensitivity and 100% specificity to detect recurrence of CRC using these individualized dPCRs. However, disadvantages of such an approach are labor-intensity because many individualized dPCR assays have to be produced. In addition, novel or known mutations causing resistance not present in the tissue, or developing

during treatment, are missed. Alternatively, the OnTarget assay had high sensitivity to detect many hotspot mutations, tackling the issue of labor-intensity and potentially of mutations not present in the tumor tissue. While dPCR seemed to be slightly more sensitive than the OnTarget assay to detect mutations in ctDNA and tumor-adjacent tissue, the OnTarget assay identified some mutations in tumor-adjacent normal tissue that were not detected in the matched tumor tissue. These mutations would certainly have been missed by an approach using dPCR assays based on previous findings in tumor tissue. Nonetheless, the OnTarget assay is still bound to a limited repertoire of mutations, and currently unknown resistance mutations are potentially missed by the technique. Finally, our sample size is limited to make firm conclusions about the OnTarget assay versus dPCR. At the very least, the OnTarget assay seems like an attractive option for ctDNA detection to screen for multiple mutations if no tumor tissue is available.

In summary, three targeted methods to detect somatic mutations in ctDNA were described and pros and cons were provided for each method. Future efforts using ctDNA as a tool to detect somatic mutations in cancer patients should carefully consider all available methods for ctDNA detection and choose the method most fit to answer the specific research question.

07

REFERENCES

1. Haber DA, Gray NS, Baselga J. The evolving war on cancer. Cell. 2011 Apr 1;145(1):19-24.

2. Leto SM, Trusolino L. Primary and acquired resistance to EGFR-targeted therapies in colorectal cancer: impact on future treatment strategies. J Mol Med (Berl). 2014 Jul;92(7):709-22.

3. Turner NC, Reis-Filho JS. Genetic heterogeneity and cancer drug resistance. Lancet Oncol. 2012 Apr;13(4):e178-85.

4. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW. Cancer genome landscapes. Science. 2013 Mar 29;339(6127):1546-58.

5. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012 Mar 8;366(10):883-92.

6. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012 Jun 28;486(7404):532-6.

7. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med. 2015 Jul;21(7):827.

8. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008 Sep;14(9):985-90.

9. Haber DA, Velculescu VE. Blood-Based Analyses of Cancer: Circulating Tumor Cells and Circulating Tumor DNA. Cancer discovery. 2014;4(6):650-61.

10. Wang J, Ramakrishnan R, Tang Z, Fan W, Kluge A, Dowlati A, et al. Quantifying EGFR alterations in the lung cancer genome with nanofluidic digital PCR arrays. Clin Chem. 2010 Apr;56(4):623-32.

11. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods. 2006 Jul;3(7):551-9.

12. Marziali A, Pel J, Bizzotto D, Whitehead LA. Novel electrophoresis mechanism based on synchronous alternating drag perturbation. Electrophoresis. 2005 Jan;26(1):82-90.

13. Thompson JD, Shibahara G, Rajan S, Pel J, Marziali A. Winnowing DNA for rare sequences: highly specific sequence and methylation based enrichment. PloS one. 2012;7(2):e31597.
14. Kidess E, Heirich K, Wiggin M, Vysotskaia V, Visser BC, Marziali A, et al. Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget. 2015 Feb 10;6(4):2549-61.

15. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol. 2014 Feb 20;32(6):579-86.

16. Lalmahomed ZS, Mostert B, Onstenk W, Kraan J, Ayez N, Gratama JW, et al. Prognostic value of circulating tumour cells for early recurrence after resection of colorectal liver metastases. Br J Cancer. 2015 Feb 3;112(3):556-61.

17. Mostert B, Sieuwerts AM, Bolt-de Vries J, Kraan J, Lalmahomed Z, van Galen A, et al. mRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients. Molecular Oncology. 2015 Apr;9(4):920-32.

18. Banerjee SK, Makdisi WF, Weston AP, Mitchell SM, Campbell DR. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. Biotechniques. 1995 May;18(5):768-70, 72-3.

19. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 2010 Aug;11(8):753-62.

20. Lee SY, Haq F, Kim D, Jun C, Jo HJ, Ahn SM, et al. Comparative genomic analysis of primary and synchronous metastatic colorectal cancers. PloS one. 2014;9(3):e90459.

21. Weerts MJA, van Marion R, Helmijr J, Beaufort CM, Krol NMG, Trapman AMAC, et al. Somatic mutations detected by targeted NGS in minute amounts serum derived cfDNA. Submitted for publication. 2016.

22. Jansen MP, Martens JW, Helmijr J, Beaufort CM, Van Marion R, Krol NMG, et al. Cell-free DNA mutations as biomarkers in breast cancer patients receiving tamoxifen. OncoTarget. 2016 in press.

23. Hiltemann S, Jenster G, Trapman J, van der Spek P, Stubbs A. Discriminating somatic and germline mutations in tumor DNA samples without matching normals. Genome Res. 2015 Sep;25(9):1382-90.

24. Norton SE, Lechner JM, Williams T, Fernando MR. A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR. Clin Biochem. 2013 Oct;46(15):1561-5.

25. Van Dessel LF, Beije N, Helmijr J, Vitale S, Kraan J, Look MP, et al. Application of circulating tumor DNA in prospective clinical oncology trials: standardization of pre-analytical conditions. Mol Oncol. 2016 in press.

26. Cancer Genome Atlas N. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012 Jul 19;487(7407):330-7.

27. Li J, Yang L, Gaur S, Zhang K, Wu X, Yuan YC, et al. Mutants TP53 p.R273H and p.R273C but not p.R273G enhance cancer cell malignancy. Hum Mutat. 2014 May;35(5):575-84.

28. Do H, Dobrovic A. Dramatic reduction of sequence artefacts from DNA isolated from formalin-fixed cancer biopsies by treatment with uracil- DNA glycosylase. Oncotarget. 2012 May;3(5):546-58.

29. Do H, Wong SQ, Li J, Dobrovic A. Reducing sequence artifacts in amplicon-based massively parallel sequencing of formalin-fixed paraffin-embedded DNA by enzymatic depletion of uracil-containing templates. Clin Chem. 2013 Sep;59(9):1376-83.

30. Mouliere F, El Messaoudi S, Gongora C, Guedj AS, Robert B, Del Rio M, et al. Circulating Cell-Free DNA from Colorectal Cancer Patients May Reveal High KRAS or BRAF Mutation Load. Transl Oncol. 2013 Jun;6(3):319-28.

31. Thierry AR, Mouliere F, El Messaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med. 2014 Apr;20(4):430-5.

32. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014 Feb 19;6(224):224ra24.

33. Tabernero J, Lenz HJ, Siena S, Sobrero A, Falcone A, Ychou M, et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: a retrospective, exploratory analysis of the CORRECT trial. Lancet Oncol. 2015 Aug;16(8):937-48.

34. El Messaoudi S, Mouliere F, Du Manoir S, Bascoul-Mollevi C, Gillet B, Nouaille M, et al. Circulating DNA as a strong multi-marker prognostic tool for metastatic colorectal cancer patient management care. Clin Cancer Res. 2016 Feb 4.

35. Reinert T, Scholer LV, Thomsen R, Tobiasen H, Vang S, Nordentoft I, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2015 Feb 4.

36. Tie J, Kinde I, Wang Y, Wong HL, Roebert J, Christie M, et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol. 2015 Aug;26(8):1715-22.

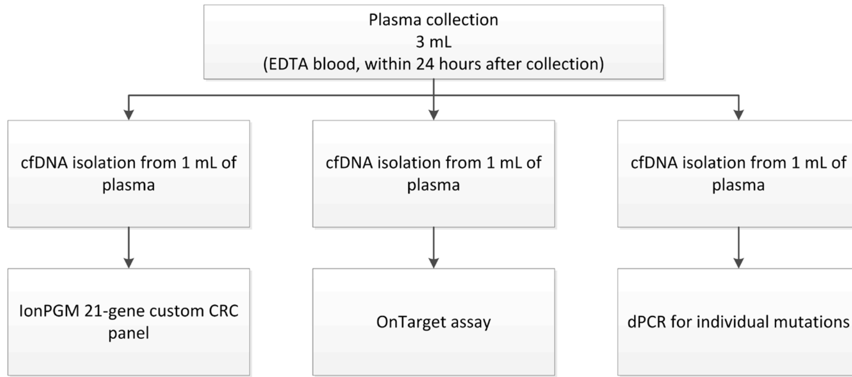
37. Dawson SJ, Rosenfeld N, Caldas C. Circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013 Jul 4;369(1):93-4.

38. Couraud S, Vaca-Paniagua F, Villar S, Oliver J, Schuster T, Blanche H, et al. Noninvasive diagnosis of actionable mutations by deep sequencing of circulating free DNA in lung cancer from never-smokers: a proof-of-concept study from BioCAST/IFCT-1002. Clin Cancer Res. 2014 Sep 1;20(17):4613-24.

39. Frenel JS, Carreira S, Goodall J, Roda D, Perez-Lopez R, Tunariu N, et al. Serial Next-Generation Sequencing of Circulating Cell-Free DNA Evaluating Tumor Clone Response To Molecularly Targeted Drug Administration. Clin Cancer Res. 2015 Oct 15;21(20):4586-96.

40. Rothe F, Laes JF, Lambrechts D, Smeets D, Vincent D, Maetens M, et al. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. Ann Oncol. 2014 Oct;25(10):1959-65.

41. Lebofsky R, Decraene C, Bernard V, Kamal M, Blin A, Leroy Q, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol. 2014 Dec 26.



SUPPLEMENTARY TABLE 1. Study flowchart of cfDNA analyses

SUPPLEMENTARY TABLE 2. Included genes in custom IonPGM CRC 21-gene panel

Gene	Aliases	Number Amplicons Custom IonPGM CRC 21-gene panel	total size design (in bp)
TP53		19	1569
APC		97	8873
KRAS		3	363
ATM		138	9853
NF1		143	9310
PTCH1		57	4962
RB1		43	3084
CREBBP		89	7670
ARID1A		74	7330
SMAD4		27	1780
PIK3CA		3	354
KIT		42	3355
KMT2D	*MLL2*	184	17208
BRAF		2	126
CDH1		35	2825
PTEN		18	1311
FBXW7		35	2675
MLH1		37	2581
EGFR		4	551
KDR	*VEGFR2*	62	4371
NRAS		3	350
Total		**1115**	**90501**

SUPPLEMENTARY TABLE 3. Included genes in OnTarget assay

Gene	Amino Acid Mutation	Coding DNA sequence Mutation
BRAF	*G469A*	*1406G>C*
BRAF	*K601E*	*1801A>G*
BRAF	*L597R*	*1790T>G*
BRAF	*V600E*	*1799T>A*
BRAF	*V600E_cpx*	*1799_1800TG>AA*
BRAF	*V600K*	*1798_1799GT>AA*
CTNNB1	*S33Y*	*98C>A*
CTNNB1	*S45F*	*134C>T*
CTNNB1	*S45P*	*133T>C*
CTNNB1	*T41A*	*121A>G*
EGFR	*G719A*	*2156G>C*
EGFR	*G719C*	*2155G>T*
EGFR	*G719S*	*2155G>A*
EGFR	*E746_A750del*	*2235_2249del15*
EGFR	*E746_A750del*	*2236_2250del15*
EGFR	*E746_A750>IP*	*2235_2248>AATTC*
EGFR	*E746_P753>VS*	*2237_2257>TCT*
EGFR	*E746_S752>A*	*2237_2254del18*
EGFR	*E746_S752>D*	*2238_2255del18*
EGFR	*E746_S752>I*	*2235_2255>AAT*
EGFR	*E746_S752>V*	*2237_2255>T*
EGFR	*E746_T751>A*	*2237_2251del15*
EGFR	*E746_T751>I*	*2235_2252>AAT*
EGFR	*E746_T751>IP*	*2235_2251>AATTC*
EGFR	*E746_T751>V*	*2237_2252>T*
EGFR	*E746_T751>VA*	*2237_2253>TTGCT*
EGFR	*E746_T751del*	*2236_2253del18*
EGFR	*K745_E749del*	*2233_2247del15*
EGFR	*L747_A750>P*	*2238_2248>GC*
EGFR	*L747_A750>P*	*2239_2248>C*
EGFR	*L747_E749del*	*2239_2247del9*
EGFR	*L747_P753>Q*	*2239_2258>CA*

SUPPLEMENTARY TABLE 3. Included genes in OnTarget assay

Gene	Amino Acid Mutation	Coding DNA sequence Mutation
EGFR	*L747_P753>S*	*2240_2257del18*
EGFR	*L747_S752>Q*	*2239_2256>CAA*
EGFR	*L747_S752del*	*2239_2256del18*
EGFR	*L747_T751>P*	*2239_2251>C*
EGFR	*L747_T751>Q*	*2238_2252>GCA*
EGFR	*L747_T751>S*	*2240_2251del12*
EGFR	*L747_T751del*	*2238_2252del15*
EGFR	*T790M*	*2369C>T*
EGFR	*L858R*	*2573T>G*
EGFR	*L861Q*	*2582T>A*
FOXL2	*C134W*	*402C>G*
GNAS	*R201C*	*601C>T*
GNAS	*R201H*	*602G>A*
KRAS	*G12A*	*35G>C*
KRAS	*G12C*	*34G>T*
KRAS	*G12D*	*35G>A*
KRAS	*G12R*	*34G>C*
KRAS	*G12S*	*34G>A*
KRAS	*G12V*	*35G>T*
KRAS	*G13A*	*38G>C*
KRAS	*G13C*	*37G>T*
KRAS	*G13D*	*38G>A*
KRAS	*G13S*	*37G>A*
KRAS	*G13V*	*38G>T*
KRAS	*Q61H*	*183A>C*
KRAS	*Q61H*	*183A>T*
KRAS	*Q61K*	*181C>A*
KRAS	*Q61L*	*182A>T*
KRAS	*Q61R*	*182A>G*
KRAS	*A146T*	*436G>A*
KRAS	*A146V*	*437C>T*

SUPPLEMENTARY TABLE 3. Included genes in OnTarget assay

Gene	Amino Acid Mutation	Coding DNA sequence Mutation
NRAS	*G12C*	*34G>T*
NRAS	*G12D*	*35G>A*
NRAS	*G12S*	*34G>A*
NRAS	*G12V*	*35G>T*
NRAS	*G13D*	*38G>A*
NRAS	*G13R*	*37G>C*
NRAS	*Q61H*	*183A>T*
NRAS	*Q61K*	*181C>A*
NRAS	*Q61L*	*182A>T*
NRAS	*Q61R*	*182A>G*
PIK3CA	*E542K*	*1624G>A*
PIK3CA	*E545K*	*1633G>A*
PIK3CA	*E545Q*	*1633G>C*
PIK3CA	*Q546E*	*1636C>G*
PIK3CA	*H1047L*	*3140A>T*
PIK3CA	*H1047R*	*3140A>G*
PIK3CA	*H1047Y*	*3139C>T*
PIK3CA	*M1043I*	*3129G>A*
PIK3CA	*E81K*	*241G>A*
PIK3CA	*R88Q*	*263G>A*
PIK3CA	*K111E*	*331A>G*
PIK3CA	*R108H*	*323G>A*
PIK3CA	*N345K*	*1035T>A*
PIK3CA	*C420R*	*1258T>C*
TP53	*R175H*	*524G>A*
TP53	*G245D*	*734G>A*
TP53	*G245S*	*733G>A*
TP53	*R248Q*	*743G>A*
TP53	*R248W*	*742C>T*
TP53	*R249S*	*747G>T*
TP53	*R273C*	*817C>T*
TP53	*R273H*	*818G>A*
TP53	*R282W*	*844C>T*

SUPPLEMENTARY TABLE 4. Validation experiment using the OnTarget assay

5% Cell Line			Rep:	1	Rep:	2	Rep:	3
			Input Mass	29.6	Input Mass	32.9	Input Mass	43.2
Gene	**Mutation**	**ddPCR VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**
BRAF	V600E		3498	38.97%	4789	48.00%	5254	40.10%
CTNNB1	S33Y		2492	27.76%	2077	20.82%	2048	15.63%
EGFR	G719S		2382	26.54%	2140	21.45%	2272	17.34%
EGFR	delE746-A750	6.0%	509	5.7%	634	6.4%	590	4.5%
EGFR	T790M	4.5%	826	9.2%	876	8.8%	696	5.3%
EGFR	L858R	4.4%	592	6.6%	535	5.4%	555	4.2%
KRAS	G12D	6.1%	659	7.3%	619	6.2%	601	4.6%
NRAS	Q61K	6.4%	869	9.7%	801	8.0%	808	6.2%
PIK3CA	E545K	6.1%	445	5.0%	666	6.7%	704	5.4%
PIK3CA	H1047R		1915	21.34%	2069	20.74%	2438	18.60%

1% Cell Line			Rep:	1	Rep:	2	Rep:	3
			Input Mass	31.3	Input Mass	31.3	Input Mass	30.9
Gene	**Mutation**	**ddPCR VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**
BRAF	V600E		3304	34.80%	4286	45.12%	4565	48.75%
CTNNB1	S33Y		2024	21.32%	1809	19.05%	1764	18.84%
EGFR	G719S		1866	19.66%	1368	14.41%	1609	17.19%
EGFR	delE746-A750	1.4%	95	1.0%	100	1.0%	107	1.1%
EGFR	T790M	0.9%	131	1.4%	126	1.3%	117	1.2%
EGFR	L858R	0.9%	104	1.1%	63	0.7%	89	1.0%
KRAS	G12D	1.3%	95	1.0%	91	1.0%	87	0.9%
NRAS	Q61K	1.3%	149	1.6%	137	1.4%	126	1.3%
PIK3CA	E545K	1.2%	85	0.9%	120	1.3%	122	1.3%
PIK3CA	H1047R		1702	17.93%	1731	18.22%	2081	22.22%

0.1% Cell Line			Rep:	1	Rep:	2	Rep:	3
			Input Mass	26.1	Input Mass	34.3	Input Mass	28.8
Gene	**Mutation**	**ddPCR VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**
BRAF	V600E		3430	43.44%	4293	41.31%	3857	44.24%
CTNNB1	S33Y		1879	23.79%	1772	17.05%	1511	17.32%
EGFR	G719S		1717	21.74%	1363	13.11%	1500	17.20%
EGFR	delE746-A750	0.16%	16	0.2%	8	0.1%	8	0.1%

SUPPLEMENTARY TABLE 4. Validation experiment using the OnTarget assay

EGFR	T790M	0.09%	14	0.2%	13	0.1%	14	0.2%
EGFR	L858R	0.15%	3	0.0%	6	0.1%	8	0.1%
KRAS	G12D	0.14%	9	0.1%	14	0.1%	11	0.1%
NRAS	Q61K	0.12%	9	0.1%	17	0.2%	14	0.2%
PIK3CA	E545K	0.12%	11	0.1%	8	0.1%	9	0.1%
PIK3CA	H1047R		1497	18.95%	1815	17.46%	1919	22.01%

0.01% Cell Line			**Rep:**	1	**Rep:**	2	**Rep:**	3
			Input Mass	114	**Input Mass**	131	**Input Mass**	111
Gene	**Mutation**	**ddPCR VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**
BRAF	V600E		40	0	55	0	36	0
CTNNB1	S33Y		14	0.04%	16	0.04%	19	0.06%
EGFR	G719S		16	0.05%	18	0.05%	11	0.03%
EGFR	delE746-A750	0.011%	6	0.018%	13	0.032%	6	0.019%
EGFR	T790M	0.015%			9	0.023%	9	0.027%
EGFR	L858R	0.010%	5	0.014%	5	0.011%		
KRAS	G12D	0.025%	8	0.023%	8	0.020%	5	0.014%
NRAS	Q61K	0.008%	6	0.018%	6	0.015%		
PIK3CA	E545K	0.011%	4	0.011%	8	0.021%	3	0.010%
PIK3CA	H1047R		18	0.05%	14	0.03%	20	0.06%

BG WT Control			**Rep:**	1	**Rep:**	2	**Rep:**	3
			Input Mass	93	**Input Mass**	103	**Input Mass**	110
Gene	**Mutation**	**ddPCR VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**	**Copies**	**VAF%**
BRAF	V600E							
CTNNB1	S33Y							
EGFR	G719S							
EGFR	delE746-A750							
EGFR	T790M							
EGFR	L858R							
KRAS	G12D							
NRAS	Q61K							
PIK3CA	E545K							
PIK3CA	H1047R							

SUPPLEMENTARY TABLE 5. Patient characteristics						
Pt	**Metachronous or synchronous metastases**	**Days between resection of primary and resection of metastasis**	**TNM stage at diagnosis**	**Number of liver metastases**	**Primary tumor preservation and % of tumor cells**	**Metastasis preservation and % of tumor cells**
1	Metachronous	281	T2 N0 M0	1	FFPE; 40%	FF; 100%
2	Metachronous	531	T3 N2 M0	1	FFPE; 50%	FF; 100%
3	Metachronous	1841	T3 N2 M0	1	FFPE; 70%	FF; 100%
4	Metachronous	2522	T3 N0 M0	1	FFPE; 90%	FF; 90%
5	Metachronous	1129	T2 N0 M0	1	FFPE; 70%	FFPE; 70%
6	Metachronous	270	T3 N1 M0	1	FFPE; 80%	FF; 100%
7	Synchronous	0	T3 N0 M1	1	FF; 90%	FF; 60%
8	Synchronous	0	T3 N1 M1	2	FF; 80%	FF; 100%
9	Synchronous	0	T4 N1 M1	1	FFPE; 90%	FFPE; 80%
10	Synchronous	0	T3 N0 M1	2	FF; 90%	FF; 60%
11	Synchronous	0	T3 N2 M1	1	FF; 80%	FF; 80%
12	Synchronous	0	T3 N0 M1	1	FFPE; 30%	FFPE; 90%

Supplementary Table 6 is too large to be included in this thesis, can be found online http://www.moloncol.org/article/S1574-7891(16)30110-7/addons

SUPPLEMENTARY TABLE 7. Raw IonPGM sequencing analysis of cfDNA of variants undetected in cfDNA but detected in primary tumor or metastases

Patient and mutation information			cfDNA raw information if not called with standard or modified pipeline						
Patient	Gene	Position	Mutant reads	Depth	VAF%	Q-Score	Strand bias	Highest number of mutant reads in patients without mutation in tissue	Patients in which mutant reads are detected without mutation in tissue
1	APC	p.R858X	7	538.0	1.3	15.36	0.43	8	4
2	CREBBP	p.P2383L	0	31.0	0.0	4.38			
	FBXW7	p.D399Y	0	271.0	0.0	4.33			
	KRAS	p.G12D	8	563.0	1.4	17.14	0.38	1	1
	PIK3CA	p.E545K	3	382.0	0.8	6.45	0.33	0	0
	TP53	p.G108S	0	772.0	0.0	4.32			
	TP53	p.L130I	0	33.0	0.0	4.37			
3	CREBBP	p.P2383S	2	166.0	1.2	6.52	1.0	1	1
5	APC	p.E1288X	4	222.0	1.8	12.26	0.75	0	0
6	FBXW7	p.R689W	2	1195.0	0.2	4.83	0.50	3	4
	FBXW7	p.S582L	0	1456.0	0.0	6.25			
	PIK3CA	p.E545K	0	576.0	0.0	4.32			

SUPPLEMENTARY TABLE 7. Raw IonPGM sequencing analysis of cfDNA of variants undetected in cfDNA but detected in primary tumor or metastases

Patient and mutation information			cfDNA raw information if not called with standard or modified pipeline						
Patient	**Gene**	**Position**	**Mutant reads**	**Depth**	**VAF%**	**Q-Score**	**Strand bias**	**Highest number of mutant reads in patients without mutation in tissue**	**Patients in which mutant reads are detected without mutation in tissue**
7	KRAS	p.G12D	1	2078.0	0.1	8.24	1.00	1	1
8	TP53	p.R175H	96	3001.0	3.2	15.82	0.64	2	6
10	TP53	p.R282W	0	1276.0	0.0	5.47			
12	APC	p.E1390X	0	848.0	0.0	4.33			
	APC	p.E564X	0	542.0	0.0	4.32			
	CREBBP	p.P975S	0	1004.0	0.0	4.25			
	CREBBP	p.R601Q	4	834.0	0.5	5.20	0.50	3	2
	PIK3CA	p.E545K	5	659.0	0.8	7.95	0.40	0	

Supplementary Table 8 and 9 are too large to be included in this thesis, can be found online http://www.moloncol.org/article/S1574-7891(16)30110-7/addons

SUPPLEMENTARY TABLE 10. Occurrence of somatic mutations in normal tissues, compared with ionPGM sequencing findings in primary tumor ("primary"), metastases ("meta"), and whole blood ("WB").

Patient	Gene	Position	Source of normal tissue	Primary ionPGM	Meta ionPGM	Normal ionPGM	Normal OnTarget	WB OnTarget	Normal dPCR	WB dPCR
2	KRAS	p.G12D	liver	28.6	38.6	.	0.31	.	0.26	.
	PIK3CA	p.E545K	liver	41.1	74.8	.	0.62	.	0.76	.
3	TP53	p.R273H	liver	50.6	82	.	0.23	.	0.08	.
	EGFR	p.L861Q	liver	.	.	.	0.07	.	no assay	no assay
	GNAS	p.R201H	liver	.	.	.	.	0.11	no assay	no assay
5	TP53	p.R248Q	liver	X	17.9	.	0.18	not available	0.35	not available
6	PIK3CA	p.E545K	liver	5.3	.	.	.	.	.	.
	KRAS	p.G12V	liver	.	49.7	.	0.27	.	0.5	.
	TP53	p.R273H	liver	.	72.3	.	0.28	.	0.29	.
	TP53	p. R249S	liver	.	.	.	0.16	.	no assay	no assay
7	KRAS	p.G12D	liver	12.7	8.5	.	0.12	.	0.22	.
	CTNNB1	p.S45P	liver	not in panel	not in panel	not in panel	0.02	.	no assay	no assay
8	KRAS	p.G12D	colon	30.9	51.2	.	1.3	not available	1.73	not available
	TP53	p.R175H	colon	37.2	69.2	.	1.8	not available	no assay	no assay
9	KRAS	p.G12D	colon	39.7	35	.	.	.	.	.
	TP53	p.R248Q	colon	37.5	55.7	.	.	.	.	.
	KRAS	p.G12V	colon	.	.	.	0.53	.	.	.
10	TP53	p.R282W	liver	66.8	61.7	.	.	not available	.	not available
12	KRAS	p.G13D	liver	37.9	26.6	.	.	not available	0.27	not available
	PIK3CA	p.E545K	liver	18	12.9	.	.	not available	.	not available

All variants detected by at least one technique are shown. Not detected variants are indicated with a dot. Excluded samples because of FFPE-related artefacts are indicated with an "X".

CHAPTER 8

Exploration of biomarkers for diagnosis and prognostication in pleural effusion and peripheral blood from patients with pleural malignant mesothelioma

Nick Beije, Jaco Kraan, Michael A. den Bakker, Alexander P.W.M. Maat, Cor H. van der Leest, Robin Cornelissen, Ngoc M. Van, John W.M. Martens, Joachim G.J.V. Aerts, Stefan Sleijfer

Submitted

ABSTRACT

BACKGROUND: There is a lack of robust and clinically utilizable markers for the diagnosis and prognostication of malignant pleural mesothelioma (MPM). Here, several novel approaches were optimized and explored for their potential to improve diagnosis and prognostication of MPM in pleural effusion (PE) and peripheral blood (PB).

METHODS: CellSearch-based and flow cytometry (FC)-based assays using melanoma cell adhesion molecule (MCAM) to identify tumor cells in the PE and PB of MPM patients were optimized, validated, explored clinically, and in case of PE compared with cytological analysis. Additionally, tumor-associated circulating endothelial cells (tCECs) were measured in PB. These assay were investigated in a MPM cohort consisting of patients with histology-confirmed MPM (n=27), and in a control cohort of patients with alternative diagnoses (n=22). Exploratory analyses for the prognostic value of all assays were performed.

RESULTS: Malignancy of MCAM-positive cells in PEs from MPM patients was confirmed. Detecting MPM tumor cells in PE with CellSearch had poor specificity. FC had superior sensitivity (48%) to standard cytological analysis (15%) to detect MPM tumor cells (p=0.03). In PB CTCs were observed in 26%, and 42% of the MPM patients had tCECs above the upper limited of normal (ULN). In exploratory analyses the absence of tumor cells in PEs, and tCECs above the ULN appeared to be associated with worse overall survival.

CONCLUSION: MCAM-based FC analysis of PEs was more sensitive than routine cytological analysis. FC analysis of PEs and tCECs in PB are promising markers for the prognostication of MPM patients deserving further study.

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an aggressive and treatment-resistant asbestosis-induced neoplasm, whose incidence is expected to increase in the next years (1). Diagnosing MPM can be a challenging process. Especially the distinction between benign and malignant mesothelial proliferation can be extremely difficult (2). While markers such as mesothelin and osteopontin in plasma and pleural effusions (PEs) have been described, intending to improve diagnosis (3, 4), they are currently not used in clinically. Furthermore, the first encouraging reports of fibulin-3 (5) were shown to be disappointing in clinical practice as a diagnostic marker (6). Therefore, performing a pleural biopsy with histological sampling is still the golden standard for diagnosing MPM (7). A pleural biopsy, either done by video-assisted thoracic surgery (VATS) or with an open procedure, however, is an invasive procedure with associated morbidity, and even with adequate tissue it can be difficult to conclusively diagnose MPM (8). While MPM patients often present with pleural effusion (PE), fluid cytology alone has poor sensitivity of 26% (7).

In addition to the difficulties in diagnosing MPM, another clinical challenge is the current lack of robust prognostic or predictive markers in MPM (9), limiting options to further personalize treatment in MPM patients. Putative interesting tools to improve diagnosis and prognostication of patients with MPM are the assessment of (circulating) tumor cells (CTCs) or circulating endothelial cells (CECs) in PEs and/or in peripheral blood (PB). CTCs are tumor cells detected in the peripheral circulation of patients with solid malignancies, and robust prognostic value of CTCs has been demonstrated for various tumor types (10-12). Of the currently available assays for CTC detection, the CellSearch CTC test is the only one that has been approved by the Food and Drug Administration (FDA). This method isolates tumor cells by immunomagnetic enrichment from bodily fluids, using ferrofluid nanoparticles coated with epithelial cell adhesion molecule (EpCAM) specific antibodies. We previously demonstrated that in breast cancer, melanoma cell adhesion molecule (MCAM or CD146) is an alternative marker that is expressed in EpCAM-negative cell lines (13), and that a modification in the CellSearch CTC enumeration kit can be used to detect MCAM-positive CTCs in breast cancer patients (14). The expression of MCAM in cytological smears of PEs of MPM patients has been suggested as a novel marker to discriminate between malignant and reactive mesothelium (15). However, the malignant nature of MCAM-positive cells was not confirmed, and sensitivity issues are likely to occur in MPM patients with a low occurrence of tumor cells in PE when preparing a cytological smear. Therefore, the use of a CellSearch MCAM-based enrichment approach might specifically detect MPM cells at low occurrences in PE and PB of MPM patients.

CECs are endothelial cells that have been sloughed of the vessel wall, and are increased in the blood of patients with solid malignancies (16, 17). We recently introduced a novel marker, CD276, to distinguish CECs derived from normal endothelium from CECs that are tumor-derived (18). Especially since MPM is a well-vascularized tumor and angiogenesis is thought to be important in MPM (19), CECs may serve as a prognostic marker in MPM (20).

This research aimed to optimize techniques to detect tumor cells in the PE and PB of MPM patients, and to obtain more insight into the potential use of tumor cells detected by these techniques and CECs as biomarkers in MPM. To this end, data are presented here on a cohort of patients with PE due to MPM, and a cohort of patients with PE due to other causes. In all patients, PE was evaluated using MCAM-based methods with CellSearch and flow cytometry (FC). Additionally, the PB of MPM patients was evaluated for the presence of MCAM-CTCs and CECs.

METHODS

MCAM EXPRESSION AND RECOVERY OF MPM TUMOR CELLS

Three primary MPM cell lines were stained with MCAM-APC (clone 541–10B2; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and acquired on the FACS Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Subsequently expression of MCAM was quantified compared to an unstained tube (signal/noise) using FCS Express (De Novo Software, Los Angeles, CA, USA). To determine the recovery of mesothelioma cells by using CellSearch technology (Janssen Diagnostics, Raritan, NJ, USA) with MCAM-based enrichment, 100 cells of MPM cell lines were spiked into 7.5 mL healthy donor blood in duplo. Then MCAM-based CTC enumeration was performed as described before (13, 14). Briefly, to enumerate MCAM-CTCs the anti-EpCAM ferrofluids from the Circulating Epithelial Cell Kit (Janssen Diagnostics) were substituted with anti-MCAM ferrofluids from the Circulating Endothelial Cell Kit (Janssen Diagnostics). Other components of the Circulating Epithelial Cell Kit were left untouched. As an extra marker we used FITC-conjugated CD34 (BD Biosciences, San Jose, CA, USA) to exclude cytokeratin (CK)18-expressing CECs. MCAM-positive tumor cells were defined as CK8/18/19+, DAPI+, CD45- and CD34- after enrichment for MCAM.

SNP ARRAY

PEs were flow cytometrically sorted using a FACS Aria sorter (BD Biosciences). The following populations were sorted: 1) MCAM+, DRAQ5+, CD45- cells; 2) MCAM-, DRAQ5+, CD45- cells and 3) CD45+ cells (leukocyte control), **Supplementary Figure 1**. DNA was isolated from these populations using the Nucleospin DNA kit (Macherey-Nagel, Düren, Germany), and DNA concentrations were quantified using the Qubit dsDNA HS Assay kit (Thermo Fisher, Waltham, MA, USA). DNA from these populations was then subjected to an SNP array using the CytoScan HD Array Kit (Affymetrix, Santa Clara, CA, USA). SNP array data were subsequently analyzed for the presence of copy number variations (CNVs) with the Chromosome Analysis Suite (ChAS) software (Affymetrix).

PATIENTS AND INCLUSION

Two cohorts of patients were included for this prospective study. The first cohort (MPM cohort) consisted of patients with pathology-confirmed MPM or a high suspicion of MPM, presenting with PE who needed to undergo a pleural drainage or video-assisted thoracoscopy as part of standard care. The second cohort (control cohort) consisted of patients who presented with PE with a need to drain the PE as part of standard

care and in whom there was an established diagnosis other than MPM. In all patients the PE was sent to the pathological laboratory and processed as a part of standard care. Additionally, in all patients 20 mL of PE residual material was sent for the MCAM-based CTC enumeration and FC. In the MPM cohort also 2x 10 mL of blood was drawn for MCAM-based CTC enumeration and CEC enumeration. This study ran from March 2014 to January 2016 in two centers in The Netherlands (Erasmus MC Cancer Institute, Rotterdam, The Netherlands and Amphia Hospital, Breda, The Netherlands). All patients provided written informed consent, and the institutional boards of both participating centers approved the protocols (Erasmus MC ID MEC-2014-116; Netherlands Trial Register NTR4575).

PROCESSING OF PE AND PB SAMPLES

PEs were processed for standard cytology analyses as a part of standard care. All PE cytology slides were revised by one pathologist (MdB), who is a member of the Dutch national mesothelioma expert pathology panel. PEs for research purposes were always first filtered on a Falcon Cell Strainer (70 uM; Corning Incorporated, Corning, NY, USA) and processed within 24 hours after the pleural drainage. PB was drawn in CellSave tubes, and processed within 96 hours for MCAM-CTC enumeration and CEC enumeration.

The MCAM-based CTC enumeration in PEs (3 mL) and PB (7.5 mL) was performed as described above and before (13, 14). After centrifugation of the PE a 'dummy' tube with the bottom of the tube marked black (marking the area in which packed red blood cells are expected) was used to process the erythrocyte-poor PE samples on the CellSearch system. All CK+, DAPI+, CD45-, CD34- were considered positive events. In case 5 or more cells were closely connected to each other, the event was counted as a cluster.

For flow cytometry of PE samples, 10 mL filtered PE was washed twice in phosphate buffered saline (PBS), and resuspended in 1 mL of PBS with 1% added bovine serum albumin. Then 100 uL of suspension was stained according to our PE antibody panel containing MCAM, DAPI, pan-cytokeratin (pan-CK), CD45, CD34, two MPM markers thrombomodulin (CD141) & podoplanin (D2-40) and two carcinoma-specific markers CEA (CD66e) and Claudin-4 (**Supplementary Table 1 & 2**). All antibodies were carefully titrated using positive and negative controls. DAPI and pan-CK were stained after fixation and permeabilization of the cells using the FIX&PERM Cell fixation and permeabilization kit (Nordic-MUbio, Susteren, the Netherlands) according to the manufacturers' instructions. Samples were acquired on an FACS Fortessa flow cytometer (BD Biosciences) and were analyzed in FCS Express (De Novo Software). Cells that were MCAM+, DAPI +, pan-CK +, CD45- and CD34- were considered as putative MPM tumor cells. If these cells were also negative for carcinoma markers the cells were considered to be true MPM tumor cells. Positivity for a marker was evaluated against unstained controls for each fluorochrome, and positivity for the MPM and carcinoma markers was defined as ≥20% of the putative MPM tumor cells being positive for that marker.

The enumeration of CECs was performed in 4 mL of PB as described before (17, 18). Cells that were CD34+, DRAQ5+, MCAM+, CD45- were defined as CECs, and CECs expressing CD276 were defined as tumor-associated CECs (tCECs).

STATISTICAL CONSIDERATIONS AND ANALYSIS

Our primary objective was to increase sensitivity of PE evaluation in MPM with the MCAM-based CellSearch CTC enrichment compared to fluid cytology by the pathologist. Based on reports on the expression of MCAM in tissue (21) and PE specimens (15) of MPM patients, which was reported to be >80%, we assumed a sensitivity of at least 80% could be reached by using the CellSearch enrichment technology. According to the literature, fluid cytology by a pathologist has a sensitivity of approximately 30%. We powered the study according to a statistical worst-case scenario of discordant proportions of 0.2 and 0.7 with the McNemar test, and 34 patients with a confirmed diagnosis of MPM would be needed to reach significance at level of α=0.05 and β=0.10. Twenty patients with PE due to another cause than MPM were planned to be included to explore specificity of the test. Secondary objectives of the study were to confirm the malignant nature of MCAM-positive tumor cells in PE, to develop a flow cytometric strategy on PE to diagnose MPM, to investigate the presence of CTCs and tumor-associated CECs in PB, and to perform exploratory analyses on the prognostic value of all measured biomarkers in the context on this study. Numbers of cells between two cohorts were compared using the Mann-Whitney U test, and presence or absence of a biomarker between two groups were compared using the chi-square test. All reported p-values are two-sided, and a significance level α = 0.05 was used. All constructed Kaplan-Meier curves were exploratory as the number of patients and events was low, and no formal statistics were performed on these curves. All data analyses were done using Stata/SE version 12 (StataCorp LP, College Station, TX, USA).

RESULTS

MCAM EXPRESSION AND RECOVERY OF MPM TUMOR CELLS

MCAM expression was evaluated on three primary MPM cell lines. Two cell lines demonstrated high expression of MCAM, while one cell line had moderate to low expression of MCAM. The recovery of mesothelioma cells spiked into PB by using the MCAM-based CellSearch technology (performed in duplicate for each cell line) was 48-63% for two MPM cell lines with high MCAM expression (MESO2 & MESO 4), and 4-8% for MPM cell line MESO 3 with low MCAM expression.

CONFIRMATION OF MALIGNANT NATURE

To confirm the malignant nature of MCAM-positive cells in PE, PEs from two patients with a pathology-confirmed diagnosis of MPM were flow cytometrically sorted for subsequent SNP array analysis. The MCAM-positive populations (MCAM+, DRAQ5+, CD45-) of both patients exhibited a number of CNVs, while the leukocyte population did not have CNVs as expected (**Supplementary Figure 1**). These results demonstrated the malignant nature of MCAM-positive tumor cells in PE of MPM patients and led to the initiation of the clinical study as described below.

TABLE 1. Baseline characteristics

	MPM cohort (n=27)	Control cohort (n=22)
Diagnosis		
Malignant pleural mesothelioma	27	
Epithelial malignancy		9
Non-epithelial malignancy other than mesothelioma		3
Benign		10
Gender		
Female (%)	1 (4%)	8 (36%)
Age		
Years (range)	70 (27 - 90)	67 (30 - 91)
WHO stage		
WHO 0 - 1	14	11
WHO 2	11	8
WHO 3	2	3
Smoking		
Never	9	6
Past	10	11
Current	8	5
Asbestosis exposure		
No	7	3
Yes	20	6
Unknown	0	13
Pathology		
Epithelial MPM	22	
Biphasic MPM	5	
Stage		
MPM stage I-II	14	
MPM stage III-IV	13	
Prior treatments for MPM		
No	19	
Yes	8	
Currently on treatment for MPM		
No	23	
Yes	4	

CLINICAL STUDY - PATIENT CHARACTERISTICS

A total of 49 patients were included. The MPM cohort consisted of 27 patients, and the control cohort of 22 patients (**Table 1**). Patients in the control cohort were a mixed population with regard to malignant PEs (52%) and benign PEs (48%). In the MPM cohort, the majority of the patients had an epithelial type MPM (81%). The MPM patients were evenly divided between stage I-II (52%) and stage III-IV (48%), and most of them (70%) did not receive any prior treatments for MPM.

PE ANALYSIS USING MCAM-BASED ENRICHMENT

PEs of 41 patients could be evaluated with the CellSearch MCAM-based enrichment. Cells meeting our criteria for tumor cells were observed in 21 of 23 patients (91%) in the MPM cohort, and in 17 of 18 patients (94%) in the control cohort. Median number of cells was 416/3 mL in the MPM cohort, and 440/3 mL in the control cohort (p=0.86). Cell clusters were present in 15 patients (65%) in the MPM cohort and 11 patients (61%) in the control cohort (p=0.79).

PE ANALYSIS USING FC ASSAY

Using the FC assay, tumor cells meeting our criteria for MPM tumor cells (MCAM+, CK+, DAPI+, CD45-, CD34-, CEA-, Claudin-4-) were observed in the PE of 12 of 25 patients in the MPM cohort (48%). One patient (6%) in the control cohort had these cells, leading to a specificity of 94%. The median number of MPM tumor cell events in the MPM patients was 337 (range 23 – 10017 events). No MPM tumor cells were observed in 4 patients with biphasic MPM histology. In the majority of MPM patients the tumor cells expressed thrombomodulin (92%); podoplanin expression on these cells was observed in only one patients' PE (8%). MPM tumor cells expressed EpCAM in 33% of the cases, and GLUT1 in 58% of the cases. Of note was that in the control group two patients had MCAM-positive cells, not meeting the criteria for MPM tumor cells given the strong expression of epithelial markers CEA and Claudin-4, suggesting carcinoma cells. These patient indeed had metastatic epithelial cancers (breast cancer and thyroid cancer, respectively).

Observations with the FC assay were compared to analysis by the pathologist, which is the current clinical standard. Paired observation data were present for 20 patients in the MPM cohort, and 18 patients in the control cohort. Pathology review correctly identified 3 MPM patients as having MPM, and none of the patients in the control cohort were scored as having MPM, yielding a sensitivity of 15% and specificity of 100%. The FC assay identified 6 more MPM patients than pathology review of PE (**Table 2,** McNemar p= 0.03). The MPM patients in whom MPM tumor cells were observed using FC may have a better overall survival than patients in whom these cells were not observed (**Figure 1**).

TABLE 2. Pathology review versus FC assay for patients with MPM in whom matched cytology and FC results from PE obtained on the same day were available			
	FC positive for MPM	**FC negative for MPM**	*Total*
PA positive for MPM	3	0	3
PA negative for MPM	6	11	17
Total	9	11	20

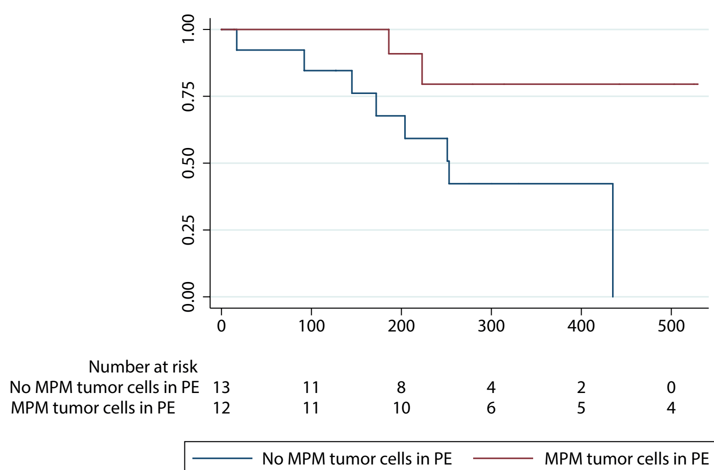


FIGURE 1. Overall survival according to the detection of tumor cells in pleural effusion using flow cytometry in the MPM cohort

CIRCULATING TUMOR CELLS AND CIRCULATING ENDOTHELIAL CELLS

CTCs were observed in 6 of 23 MPM patients (26%). In five patients one CTC per 7.5 mL of blood was observed, while one patient had 3 CTCs/7.5 mL. Two patients with CTCs had stage I-II disease, the other 4 patients with CTCs had stage III-IV disease. Four of the patients with CTCs had received prior chemotherapy. CEC enumeration results were available for 24 MPM patients. The median number of CECs was 37/4 mL (range 3 – 179). The tumor-associated marker CD276 was expressed in a median of 24% (range 7% - 78%) of the CECs, resulting in 10 patients (42%) having tCECs higher than the upper limit of normal (≥8 tCECs/4 mL (18)). Four of these patients had stage I-II disease, and 6 patients had stage III-IV disease.

In exploratory survival analyses, the presence of CTCs or a CEC number above the median did not appear to be associated with OS. However, the presence of tCECs higher than the upper limit of normal may be associated with OS (**Figure 2**).

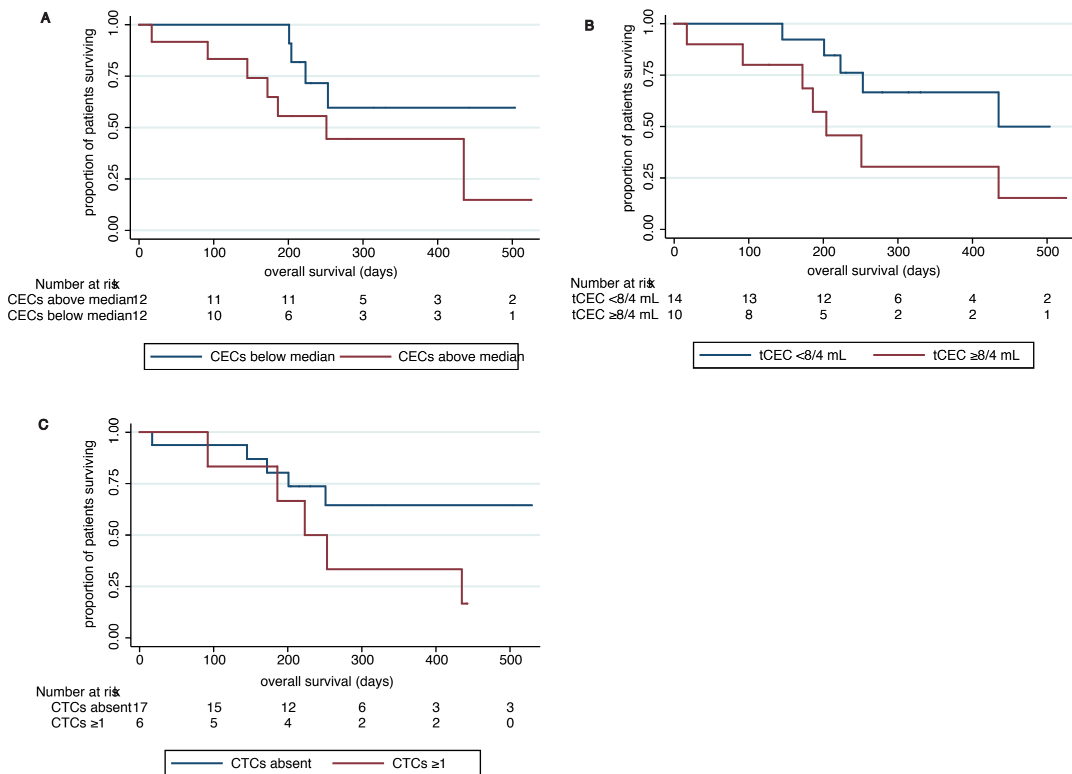


FIGURE 2. Overall survival in the MPM cohort according to biomarkers in peripheral blood. Panel A demonstrates the patients separated by the median number of CECs. Panel B demonstrates patients above and below the upper limit of normal for tCECs. Panel C demonstrates overall survival according to the presence of CTCs.

DISCUSSION

The diagnosis and treatment of MPM are hampered by limited availability of biomarkers that are clinically utilizable. The current manuscript presented and validated techniques to identify tumor cells in PE and PB of MPM patients and subsequently explored whether detecting these tumor cells in PEs might improve the diagnosis of MPM, and whether enumerating CTCs and CECs improves prognostication of MPM patients. It was observed that the enumeration of tumor cells in PE using the FDA-cleared CellSearch machine had limited specificity. Using FC higher sensitivity than with standard pathological assessment was observed and accompanied by acceptable specificity. Additionally, flow cytometrically enumerating tumor cells in PE and enumerating tCECs in PB seemed to have prognostic value.

It was hypothesized that sensitivity and specificity for diagnosing MPM could be improved by enriching for the presumed MPM-specific marker MCAM. The malignant nature of MCAM-positive cells was confirmed using flow cytometric sorting combined with genomic analyses, and the CellSearch system was chosen to enrich and

enumerate these cells, as an MCAM-specific enrichment was already up-and-running on this machine (13, 14). Using this latter technique, however, it was observed that a large number of non-mesothelioma PEs had high numbers of cells meeting the criteria for tumor cells. This observation of limited specificity with the CellSearch machine is in accordance with the report by Schwed Lustgarten and colleagues (22). This group intended to improve diagnosis of malignant PEs by using the CellSearch system using EpCAM to enrich for CTCs. They observed up to 2556 EpCAM-positive cells/3 mL in patients with benign effusions and non-epithelial effusions. Since in the present study high number of cells with CellSearch were observed in patients who had no detectable MCAM-positive cells using FC, we hypothesize that reactive mesothelium is aspecifically enriched if it is excessively present in PE. When one of the PE samples was treated with immunoglobulins prior to CellSearch enrichment to block aspecific binding of MCAM antibodies to Fc receptors, 60% less cells were observed than without treatment with immunoglobulins. These findings suggest that aspecific binding of reactive mesothelium through Fc receptor binding partly underlies the limited specificity using CellSearch. The poor specificity as observed with CellSearch led to early discontinuation of the study, because the primary study endpoint could not be reached anymore. Using FC, better specificity than with the CellSearch system was observed. In addition, the FC assay had improved sensitivity over standard cytological analysis by an experienced pathologist. However, where the cytological review had 100% specificity, the FC assay had one false-positive finding leading to 94% specificity. This finding should however be interpreted with caution, because beyond the diagnosis of a benign mesothelial proliferation in this patient, the patient is currently still clinically suspect for MPM. However, during a follow-up period of 1.5 years, MPM has not been pathologically confirmed.

In the past years a lot of research has been done on novel markers to diagnose MPM in PEs, but none of these markers have been widely incorporated in clinical practice. Most recently, others have reported that p16 FISH and BRCA1-associated protein 1 (BAP1) immunohistochemistry may improve sensitivity of PE cytology, and have reported sensitivity of 45% to 84% for p16 and of 33% to 74% for BAP1 (23). The sensitivity of the flow cytometric assay in the current study falls into the range of these assays. Unfortunately, there was no availability of cytological slides to compare the FC assay with p16, BAP1 or MCAM staining on cytological slides. Importantly, however, the numbers of observed MPM tumor cells in PE using FC were often low, suggesting that there is a fairly low chance of them being detected with standard cytological analysis.

Surprisingly, we observed that patients who had MPM tumor cells in their PE using FC, had improved OS compared to patients who did not have MPM tumor cells in the PE. If true, there are two possible explanations for this observation: 1) MCAM is a marker for good prognosis; 2) the presence of tumor cells in PE is related to an increased chance of response to therapy. Obviously, validation of this finding and if confirmed, exploration of the underlying reason in a larger cohort is necessary.

In a quarter of the patients CTCs in the PB were observed using our MCAM-based enrichment. This CTC-positivity rate using MCAM-based CTC enrichment as reported here was lower than previously reported by other groups using the standard EpCAM-

based CTC enrichment. The group of Yoneda and colleagues (24) reported 33% of their MPM patients to have EpCAM-positive CTCs, while Raphael et al. (25) detected EpCAM-positive CTCs in 44% of their MPM patients. As MCAM is more widely expressed than EpCAM in MPM tissues, the lower CTC-positivity rate as observed in the current study was surprising. This finding may be explained by the inclusion of more patients with stage IV disease in both studies evaluating EpCAM-positive CTCs than in the current study. Our observations along with the observations regarding EpCAM-positive CTCs suggest that the presence of CTCs is limited in patients with MPM, as is also reflected by the fact that clinically detectable metastasis outside the thorax is a relatively rare and late event in MPM (26). The presence of MCAM-positive CTCs did not appear to be associated in exploratory overall survival analysis in our cohort. Also the presence of CECs above the median did not appear to be related to OS, however when only tCECs were analyzed they seemed to be of prognostic value for OS. Yoneda et al. (20) previously reported a modest relation between poor OS and high numbers of CECs in a cohort of 79 MPM patients. Our findings in a much smaller cohort of patients suggest that if a marker specific for tumor-associated CECs is added, thereby omitting CECs derived from normal vasculature (27), the prognostic power of CECs possibly improves.

In conclusion, this exploratory study demonstrated the malignant nature of MCAM-positive cells in PEs of patients with MPM, and evaluated several markers to improve diagnosis and prognostication of patients with MPM. Enumerating tumor cells using FC and enumerating tCECs may lead to improvement of both and warrant further research in a larger cohort of MPM patients.

REFERENCES

1. Robinson BW, Musk AW, Lake RA. Malignant mesothelioma. Lancet. 2005 Jul 30-Aug 5;366(9483):397-408.

2. Churg A, Galateau-Salle F. The separation of benign and malignant mesothelial proliferations. Archives of pathology & laboratory medicine. 2012;136(10):1217-26.

3. Creaney J, Yeoman D, Demelker Y, Segal A, Musk AW, Skates SJ, et al. Comparison of osteopontin, megakaryocyte potentiating factor, and mesothelin proteins as markers in the serum of patients with malignant mesothelioma. J Thorac Oncol. 2008 Aug;3(8):851-7.

4. Grigoriu BD, Scherpereel A, Devos P, Chahine B, Letourneux M, Lebailly P, et al. Utility of osteopontin and serum mesothelin in malignant pleural mesothelioma diagnosis and prognosis assessment. Clin Cancer Res. 2007 May 15;13(10):2928-35.

5. Pass HI, Levin SM, Harbut MR, Melamed J, Chiriboga L, Donington J, et al. Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. N Engl J Med. 2012 Oct 11;367(15):1417-27.

6. Creaney J, Dick IM, Meniawy TM, Leong SL, Leon JS, Demelker Y, et al. Comparison of fibulin-3 and mesothelin as markers in malignant mesothelioma. Thorax. 2014 Oct;69(10):895-902.

7. Boutin C, Rey F. Thoracoscopy in pleural malignant mesothelioma: a prospective study of 188 consecutive patients. Part 1: Diagnosis. Cancer. 1993 Jul 15;72(2):389-93.
8. Campbell N, Kindler H. Update on malignant pleural mesothelioma. Seminars in respiratory and critical care medicine. 2011;32(1):102-10.

9. Liu H, Wu L, Ji K, Wang W. Prognostic value of several biomarkers for the patients with malignant pleural mesothelioma. Tumour Biol. 2015 Sep;36(10):7375-84.

10. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014 Apr;15(4):406-14.

11. de Bono J, Scher H, Montgomery R, Parker C, Miller M, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(19):6302-9.

12. Cohen S, Punt C, Iannotti N, Saidman B, Sabbath K, Gabrail N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26(19):3213-21.

13. Mostert B, Kraan J, Bolt-de Vries J, van der Spoel P, Sieuwerts AM, Schutte M, et al. Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146. Breast Cancer Res Treat. 2011 May;127(1):33-41.

14. Onstenk W, Kraan J, Mostert B, Timmermans MM, Charehbili A, Smit VT, et al. Improved Circulating Tumor Cell Detection by a Combined EpCAM and MCAM CellSearch Enrichment Approach in Patients with Breast Cancer Undergoing Neoadjuvant Chemotherapy. Mol Cancer Ther. 2015 Mar;14(3):821-7.

15. Sato A, Torii I, Okamura Y, Yamamoto T, Nishigami T, Kataoka T, et al. Immunocytochemistry of CD146 is useful to discriminate between malignant pleural mesothelioma and reactive mesothelium. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2010;23(11):1458-66.

16. Kraan J, Sleijfer S, Foekens JA, Gratama JW. Clinical value of circulating endothelial cell detection in oncology. Drug Discov Today. 2012 Jul;17(13-14):710-7.

17. Kraan J, Strijbos MH, Sieuwerts AM, Foekens JA, den Bakker MA, Verhoef C, et al. A new approach for rapid and reliable enumeration of circulating endothelial cells in patients. J Thromb Haemost. 2012 May;10(5):931-9.

18. Kraan J, van den Broek P, Verhoef C, Grunhagen DJ, Taal W, Gratama JW, et al. Endothelial CD276 (B7-H3) expression is increased in human malignancies and distinguishes between normal and tumour-derived circulating endothelial cells. Br J Cancer. 2014 Jul 8;111(1):149-56.

19. Edwards JG, Cox G, Andi A, Jones JL, Walker RA, Waller DA, et al. Angiogenesis is an independent prognostic factor in malignant mesothelioma. Br J Cancer. 2001 Sep 14;85(6):863-8.

20. Yoneda K, Tanaka F, Kondo N, Orui H, Hashimoto M, Takuwa T, et al. Circulating endothelial cell (CEC) as a diagnostic and prognostic marker in malignant pleural mesothelioma (MPM). Ann Surg Oncol. 2012 Dec;19(13):4229-37.

21. Bidlingmaier S, He J, Wang Y, An F, Feng J, Barbone D, et al. Identification of MCAM/CD146 as the Target Antigen of a Human Monoclonal Antibody that Recognizes Both Epithelioid and Sarcomatoid Types of Mesothelioma. Cancer research. 2009;69.

22. Schwed Lustgarten DE, Thompson J, Yu G, Vachani A, Vaidya B, Rao C, et al. Use of Circulating Tumor Cell Technology (CELLSEARCH(R)) for the Diagnosis of Malignant Pleural Effusions. Ann Am Thorac Soc. 2013 Nov 15.

23. Churg A, Sheffield BS, Galateau-Salle F. New Markers for Separating Benign From Malignant Mesothelial Proliferations: Are We There Yet? Arch Pathol Lab Med. 2016 Apr;140(4):318-21.

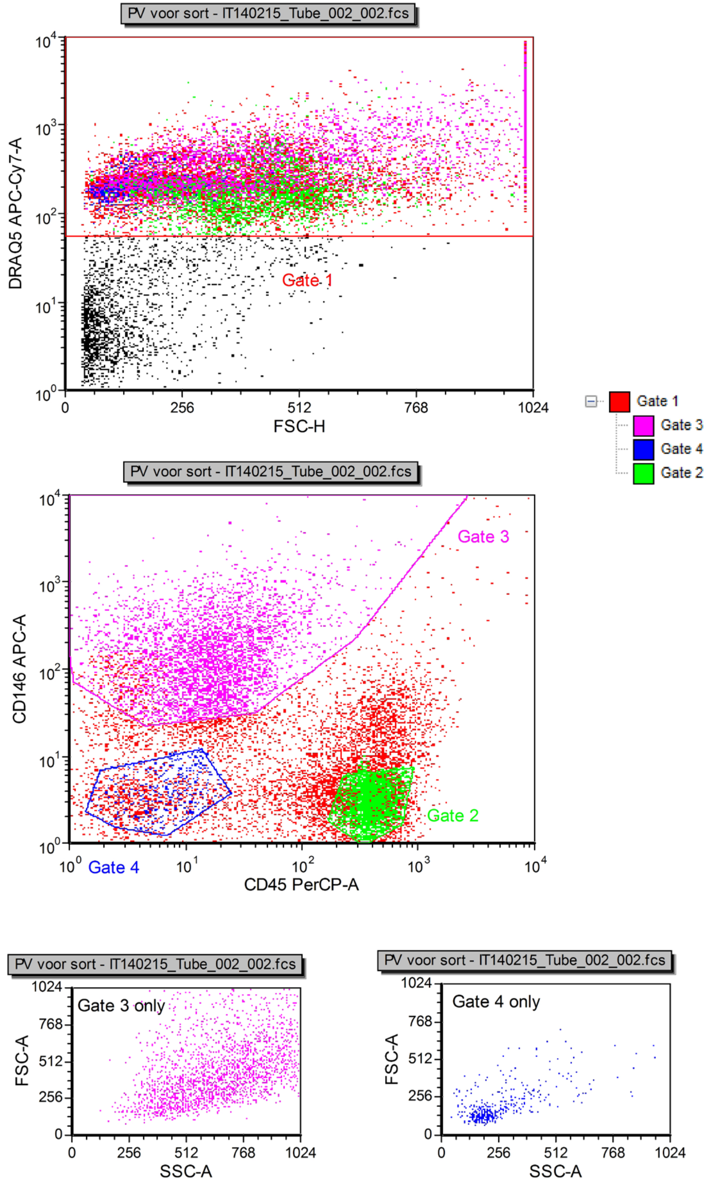
24. Yoneda K, Tanaka F, Kondo N, Hashimoto M, Takuwa T, Matsumoto S, et al. Circulating tumor cells (CTCs) in malignant pleural mesothelioma (MPM). Ann Surg Oncol. 2014 Dec;21 Suppl 4:S472-80.

25. Raphael J, Massard C, Gong IY, Farace F, Margery J, Billiot F, et al. Detection of circulating tumour cells in peripheral blood of patients with malignant pleural mesothelioma. Cancer Biomark. 2015;15(2):151-6.

26. Finn RS, Brims FJ, Gandhi A, Olsen N, Musk AW, Maskell NA, et al. Postmortem findings of malignant pleural mesothelioma: a two-center study of 318 patients. Chest. 2012 Nov;142(5):1267-73.

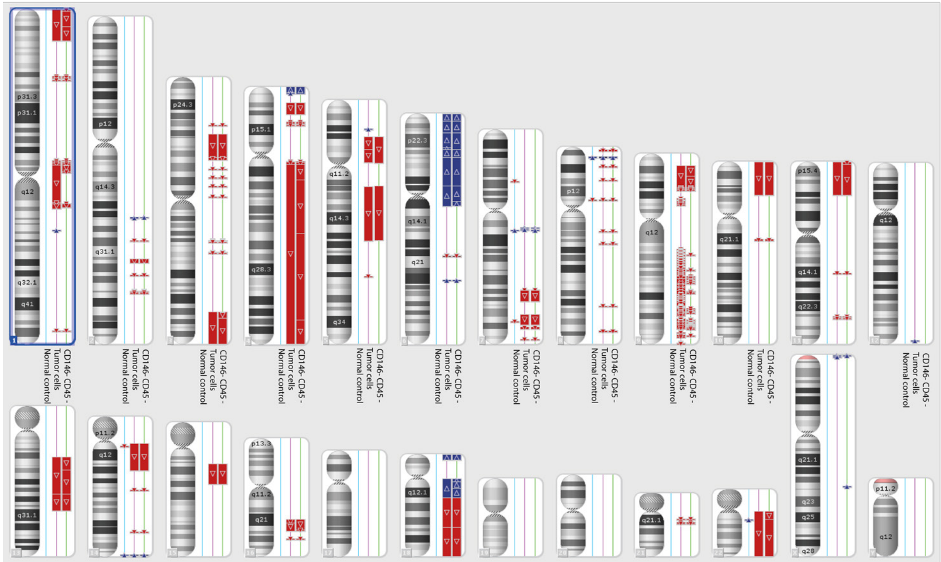
27. Beije N, Kraan J, Taal W, van der Holt B, Oosterkamp HM, Walenkamp AM, et al. Prognostic value and kinetics of circulating endothelial cells in patients with recurrent glioblastoma randomised to bevacizumab plus lomustine, bevacizumab single agent or lomustine single agent. A report from the Dutch Neuro-Oncology Group BELOB trial. Br J Cancer. 2015 Jul 14;113(2):226-31.

08



SUPPLEMENTARY FIGURE 1. Gating strategy for flow cytometric sorting

Affymetrix CytoScan HD SNP-array
PT 15



Blue lines = normal control lymphocytes (CD146- CD45+ DNA+) - Purple lines = tumor cells (CD146+ CD45- DNA+) - Green lines= CD146- CD45- DNA+

SUPPLEMENTARY FIGURE 2. SNP arrays of gated populations presented in Supplementary Figure 1. Blue lines represent the normal control lymphocytes (CD146-neg, CD45-pos, DNA+; gate 2), Purple lines represent putative tumor cells (CD146-pos, CD45-neg, DNA+; gate 3), and green lines represent a CD146-neg, CD45-neg, DNA+ population (gate 4).

SUPPLEMENTARY TABLE 1. Flow cytometric panel for pleural effusion

Tube	Pacific blue	Brilliant Violet 711	FITC	PE	PerCP	PE-Cy7	APC
1	DAPI*	CD146	unstained	unstained	CD45	CD34	unstained
2	DAPI*	unstained	unstained	unstained	CD45	CD34	pan CK*
3	DAPI*	CD146	unstained	unstained	CD45	CD34	pan CK*
4	DAPI*	CD146	CD66e	CD141	CD45	CD34	pan CK*
5	DAPI*	CD146	EpCam	D2-40	CD45	CD34	pan CK*
6	DAPI*	CD146	Claudin-4	GLUT1	CD45	CD34	pan CK*

*: intracellular staining after fixation and permeabilization

SUPPLEMENTARY TABLE 2. Details of flow cytometric panel for pleural effusions

Antibody	Alternative name	Fluorochrome	Company	Clone	Used dilution	Comments
DAPI		Pacific Blue	Janssen Diagnostics		1:10	from CellSearch CTC kit
MCAM	CD146	Brilliant Violet 711	BD Biosciences	P1H12	1:10	
CEA	CD66e	FITC	BioConnect	MCA1744F	1:1	
EpCAM	CD326/Ber-EP4	FITC	BD Biosciences	EBA-1	1:1	
Claudin-4		FITC	R&D systems	38231	1:2	
Thrombomodulin	CD141	PE	Miltenyi Biotec	AD5-14H12	1:10	
Podoplanin	D2-40	PE	eBioscience	8.1.1	1:20	
GLUT1		PE	R&D systems	202915	1:1	
CD45		PerCP-Cy5	BD Biosciences	2D1	1:1	
CD34		PE-Cy7	Beckman Coulter	581	1:2	
Pan-cytokeratin		APC (eFluor 660)	eBioscience	AE1/AE3	1:10	

09

CHAPTER 9

Prognostic value and kinetics of circulating endothelial cells in patients with recurrent glioblastoma randomised to bevacizumab plus lomustine, bevacizumab single-agent or lomustine single-agent. A report from the Dutch Neuro-Oncology Group BELOB trial

Nick Beije, Jaco Kraan, Walter Taal, Bronno van der Holt,
Hendrika M. Oosterkamp, Annemiek M. Walenkamp,
Laurens Beerepoot, Monique Hanse, Myra E. van Linde,
Aja Otten, Rene M. Vernhout, Filip Y.F de Vos,
Jan W. Gratama, Stefan Sleijfer, Martin J. van den Bent

Br J Cancer. 2015 Jul 14;113(2):226-31

ABSTRACT

BACKGROUND: Angiogenesis is crucial for glioblastoma growth, and anti-vascular endothelial growth-factor (VEGF) agents are widely used in recurrent glioblastoma patients. The number of circulating endothelial cells (CECs) is a surrogate marker for endothelial damage. We assessed their kinetics and explored their prognostic value in patients with recurrent glioblastoma.

METHODS: In this side-study of the BELOB trial, 141 patients with recurrent glioblastoma were randomised to receive single-agent bevacizumab or lomustine, or bevacizumab plus lomustine. Before treatment, after 4 weeks and after 6 weeks of treatment, CECs were enumerated.

RESULTS: The number of CECs increased during treatment with bevacizumab plus lomustine, but not during treatment in the single-agent arms. In patients treated with lomustine single-agent, higher absolute CEC numbers after 4 weeks ($\log_{10}$CEC HR 0.41, 95% CI 0.18-0.91) and 6 weeks ($\log_{10}$CEC HR 0.16, 95% CI 0.05-0.56) of treatment were associated with improved OS. Absolute CEC numbers in patients receiving bevacizumab plus lomustine or bevacizumab single-agent were not associated with OS.

CONCLUSIONS: CEC numbers increased during treatment with bevacizumab plus lomustine but not during treatment with either agent alone, suggesting that this combination induced the greatest vascular damage. Although the absolute number of CECs was not associated with OS in patients treated with bevacizumab either alone or in combination, they could serve as a marker in glioblastoma patients receiving lomustine single-agent.

INTRODUCTION

Glioblastoma is the most common and most aggressive malignant primary brain tumor in adults. Angiogenesis is crucial for glioblastoma growth, and the presence of endothelial proliferation is a key WHO-criterion for diagnosing glioblastoma (1). Tumor vessels in glioblastoma are morphologically and functionally different from normal blood vessels and are characterised by their high complexity, disorganisation and leakiness (2). Hypoxic glioblastoma tumor cells are able to interact with endothelial cells and promote angiogenesis by producing high numbers of vascular endothelial growth factor (VEGF) (3, 4). Therefore, a strong rationale exists for using anti-VEGF agents such as bevacizumab, a humanized monoclonal antibody against circulating VEGF, in the treatment of glioblastoma. Accordingly, numerous clinical trials have explored the value of bevacizumab in glioblastoma patients (5-7), but none of them showed clear survival benefit of single-agent bevacizumab in newly diagnosed or recurrent glioblastoma.

Treatment response is critical in these studies and in daily clinical practice and is generally assessed by radiographic response on MRI. However, besides inter-observer (8) and intra-observer (9) variability in radiographic assessments, clear limitations are encountered with current treatment response evaluation in glioblastoma, especially in patients treated with anti-VEGF monoclonal antibodies. Most importantly, the administration of anti-VEGF monoclonal antibodies may result in radiographic pseudoresponse caused by the rapid normalisation of abnormally permeable blood vessels (10). While these issues were taken into account in the revised response assessment criteria proposed by the Response Assessment in Neuro-Oncology (RANO) working group (11), alternative methods of response assessment focusing on the extent of angiogenesis could be helpful to guide prognosis and treatment success in glioblastoma.

Circulating endothelial cells (CECs) are mature endothelial cells that are present in the peripheral circulation and are presumed to be a marker of vascular injury (12) and angiogenesis. CECs were shown to have a clinically relevant prognostic value in various solid tumors (13). Given the high rate of angiogenesis in glioblastomas and the lack of prognostic markers for anti-VEGF treatments in general, we prospectively assessed the kinetics and prognostic relevance of CECs in the BELOB trial (14). In this randomised phase II trial, patients were treated for recurrent glioblastoma with bevacizumab plus lomustine, or with bevacizumab or lomustine single-agent.

METHODS

PATIENTS AND TREATMENT

This prospective study was a side-study of the randomised multi-centre phase II trial from the Dutch Neuro-Oncology Group (LWNO) “BELOB” (Netherlands Trial Register ID NTR1929). In-depth information regarding eligibility criteria, treatment and outcome assessments were described in the paper regarding the primary clinical endpoints of the study (14). Briefly, patients with recurrent glioblastoma were stratified according to centre, ECOG performance status and age, to be subsequently randomised between bevacizumab in combination with lomustine, bevacizumab single-agent or lomustine single-agent.

Lomustine was given orally every 6 weeks, for a maximum of 6 cycles. Bevacizumab was given intravenously every two weeks until disease progression. One treatment cycle was defined as 6 weeks. Overall survival was measured from the day of randomisation until death from any cause.

The central and local institutional review boards approved the protocol and all patients provided written informed consent. Peripheral blood (PB) samples for CEC analyses were acquired in CellSave™ tubes (Janssen Diagnostics, Raritan, NJ, USA) before the start of treatment (baseline) and after 4 and 6 weeks of treatment. Samples were maintained at room temperature and processed within 96 hours of blood collection.

ENUMERATION OF CIRCULATING ENDOTHELIAL CELLS

CECs were enumerated according to our previously reported flow cytometric approach (15), in which we demonstrated excellent reproducibility of the assay between duplicate CEC samples. The following directly conjugated monoclonal antibodies were used to identify CEC: CD34-FITC (clone 8G12; BD Biosciences, San Jose, CA, USA), CD146-APC (clone 541-10B2; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and CD45-PerCP (clone 2D1; BD Biosciences). DRAQ5 (Biostatus Ltd, Shepshed, UK) was used as a cell permeable nuclear dye to exclude platelets and microparticles. The definition of a CEC was CD34+, CD146+, CD45- and DRAQ5+.

Samples were acquired on a FACS Canto II or Fortessa flow cytometer (BD Biosciences) and were later analysed using FCS Express (De Novo Software, Los Angeles, CA, USA). One experienced technician (JK) evaluated and checked all analyses before the result of the CEC enumeration were considered final to minimise inter-rater variability.

STATISTICAL ANALYSIS

Several time intervals were constructed to minimise inter-patient variability regarding the time of CEC measurement. For a sample to be eligible as a baseline sample, the blood should have been drawn at least within two weeks before the start of treatment. A sample was eligible as a 4 weeks sample if the sample was drawn between day +25 and +31 after treatment start, while a sample was eligible as a 6 weeks sample if the sample was drawn before the administration of the second cycle and between day +39 and +45 after treatment start.

For comparisons between CEC numbers at baseline versus during treatment in the same treatment arm, the Wilcoxon signed-rank test was used. Correlation between corticosteroid use and CEC numbers was assessed using point biserial correlation. Correlation between maximum tumor diameter and CEC numbers was assessed using Spearman correlation. CEC values were $\log_{10}$-normalised before inclusion in Cox regression analyses. For the increase or decrease of CECs relative to baseline, the logarithm was calculated of the quotient between baseline (t0) and the appropriate time point (tx), formula: $\log_{10}(tx/t0)$.

In case a statistically significant result was observed in the univariate Cox regression crude hazard ratio (HR) calculation, an adjusted HR was calculated adjusting for maximum tumor diameter and corticosteroid use at baseline or after 6 weeks of treatment. These

TABLE 1. Baseline patient characteristics (n=141)	
Parameter	Value
Age, median (range)	57 (24-77)
Sex female (%)	55 (39%)
WHO status (%)	
WHO 0	40 (28%)
WHO 1	86 (61%)
WHO 2	15 (11%)
Patients using corticosteroids (%)	68 (48%)
Maximum enhancing tumor diameter in mm, median (range)	35 (11-93)
Treatment arm (%)	
Bevacizumab + Lomustine	51 (36%)
Bevacizumab single-agent	48 (34%)
Lomustine single-agent	42 (30%)

parameters were not available for the 4 weeks after start of treatment sample, thus no adjusted HRs were calculated for this sample. Maximum enhancing tumor diameter was assessed by RANO criteria (11). For a maximum diameter to be eligible for adjustment in the multivariate model after 6 weeks of treatment, the corresponding MRI should have been performed before the administration of the second cycle of lomustine (only if applicable). All reported *p* values are two-sided, and a significance level $\alpha = 0.05$ was used. All data analyses were done using Stata/SE version 12 (StataCorp LP, College Station, TX, USA).

RESULTS

PATIENT AND TREATMENT CHARACTERISTICS

A total of 153 patients were enrolled in the BELOB study between December 2009 and November 2011. From the 148 eligible patients as reported in the paper regarding the primary endpoint (14), 141 patients were included in this side-study. Patient and treatment characteristics are presented in **Table 1**. 51 patients were randomised in the bevacizumab plus lomustine arm, 48 patients received bevacizumab single-agent therapy, while 42 patients received lomustine single-agent therapy. Only four patients were still alive at the end of follow-up, and these patients had a median follow-up of 35.3 months (range 28.9 - 41.5 months).

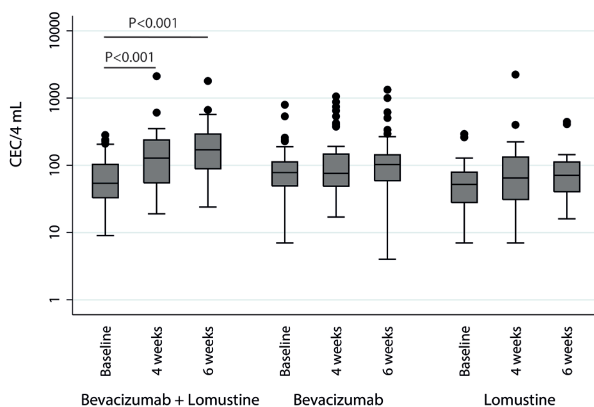


FIGURE 1. Box-whisker plots of CEC kinetics during treatment per treatment arm. (boxes show 25th percentile, median and 75th percentile, whiskers show the lower and upper adjacent values, according to Tukey. Dots represent outliers).

CEC MEASUREMENTS AND KINETICS

A total of 382 samples were evaluated for the presence of CECs. 30 samples were excluded from the analysis because they were not drawn in the appropriate time interval (n=28) or because they were drawn after the administration of the second cycle (n=2). Consequently, out of 423 expected samples, 352 samples (83% of expected samples) were included in the final analysis, of which 129 were eligible as baseline samples, 119 were eligible as 4 weeks samples and 104 were eligible as 6 weeks samples **(Table 2)**.

Baseline CEC numbers were not significantly correlated with corticosteroid use (r=0.06, P=0.54) or the maximum diameter of the tumor lesion (r=-0.11, P=0.22). Since we expected that CEC kinetics would differ between the three treatment arms, we analysed CEC kinetics separately in each treatment arm **(Figure 1)**. In patients receiving bevacizumab plus lomustine combination therapy, patients had higher CECs after 4 weeks (P<0.001) and 6 weeks (P<0.001) of treatment compared to the baseline value. In contrast, in patients receiving bevacizumab single-agent and in patients receiving lomustine single-agent, CECs at baseline were similar to CECs after 4 weeks and 6 weeks of treatment.

TABLE 2. Characteristics of CEC measurements per treatment arm

	Bevacizumab + Lomustine			Bevacizumab single-agent			Lomustine single-agent		
Month of CEC count	**n**	**median (CEC/4 mL)**	**range (CEC/4 mL)**	**n**	**median (CEC/4 mL)**	**range (CEC/4 mL)**	**n**	**median (CEC/4 mL)**	**range (CEC/4 mL)**
Baseline	47	54	9 - 282	40	78	7 - 793	37	52	7 - 292
4 weeks	43	128	19 - 2122	41	76	17 - 1056	35	65	7 - 2232
6 weeks	39	170	24 - 1796	38	103	4 - 1330	32	71	16 - 442

ASSOCIATION OF CEC NUMBERS WITH OVERALL SURVIVAL

The prognostic relevance of CECs with respect to overall survival in this study was explored using a Cox regression model. No association was found between the absolute number of CECs at baseline and overall survival. Since we observed that CEC kinetics differed between the three treatment arms, Cox regression for absolute CEC counts after 4 weeks and 6 weeks of treatment with respect to OS was performed separately for each treatment arm (**Table 3**). In patients receiving bevacizumab plus lomustine and bevacizumab single-agent, no significant associations between absolute CEC counts and OS were observed. However, in patients receiving lomustine single-agent, an association between OS and higher absolute CEC counts at 4 weeks (log_{10}CEC 4 weeks HR 0.41, 95% CI 0.18-0.91) and 6 weeks (log_{10}CEC 6 weeks HR 0.16, 95% CI 0.05-0.56) was observed. After addition of data regarding corticosteroid use and the maximum tumor diameter after 6 weeks of treatment to the model, the CEC count after 6 weeks was still significantly associated with OS (log_{10}CEC 6 weeks HR 0.18, 95% CI 0.05-0.74). Besides the absolute threshold model as described above, we also analysed whether changes in CECs from baseline to 4 weeks and baseline to 6 weeks were associated with OS in the Cox regression model. In contrast to our findings using the absolute CEC numbers after 4 weeks and 6 weeks of treatment, we did not observe any association between CEC changes and OS.

TABLE 3. Cox regression absolute CEC numbers

CEC time point & treatment arm	log_{10}CEC crude HR (95% CI)	P-value log_{10}CEC
Baseline all treatment arms	1.10 (0.68 - 1.80)	0.69
4 weeks bevacizumab + lomustine	1.21 (0.59 – 2.50)	0.59
4 weeks bevacizumab single-agent	1.32 (0.66 – 2.65)	0.44
4 weeks lomustine single-agent	0.41 (0.18 - 0.91)	0.03
6 weeks bevacizumab + lomustine	0.71 (0.27 - 1.86)	0.49
6 weeks bevacizumab single-agent	1.09 (0.49 - 2.46)	0.83
6 weeks lomustine single-agent	0.16 (0.05 - 0.56)	0.004 *

* log_{10}CEC adjusted HR 0.18 (95% CI 0.05-0.74), P=0.02. Adjusted HR was only calculated if crude HR was significant and adjusted for corticosteroid use and maximum tumor diameter after 6 weeks of treatment.
No data regarding corticosteroid use and tumor diameter after 4 weeks of treatment were available.

DISCUSSION

There is a clear clinical unmet need for alternative response evaluation during treatment of glioblastoma. As glioblastomas are highly angiogenic tumors, we proposed CEC enumeration as a surrogate marker for endothelial damage and assessed CEC kinetics and explored their possible prognostic relevance during the randomised BELOB trial. We observed that CECs increased only during treatment in patients receiving combination treatment with bevacizumab plus lomustine. We also found an association between higher absolute CEC numbers and improved overall survival during treatment with lomustine single-agent, but not in bevacizumab treated patients.

Our observation of increased CECs during treatment with bevacizumab plus lomustine in contrast to the single-agent treatment arms suggests that there is a synergistic effect of bevacizumab and lomustine in triggering endothelial damage. The results from the BELOB study suggested survival benefit from the combination of bevacizumab and lomustine, as the primary endpoint (OS at 9 months) was reached to justify the exploration of this combination treatment in a phase III trial (14). Our results indicate that this treatment combination may have had a positive effect in triggering endothelial damage in the glioblastoma tumor itself. Our observation of increased CECs during treatment with bevacizumab and lomustine cytotoxic chemotherapy is in accordance with previous reports in metastatic breast cancer and advanced colorectal cancer (16, 17), in which CEC numbers were increased after combination treatment with cytotoxic chemotherapy and bevacizumab.

During treatment with the single-agent lomustine cytotoxic chemotherapy, CEC numbers remained stable. Reynes and colleagues observed a similar pattern in glioblastoma patients before and after treatment with cytotoxic chemotherapy and radiotherapy (18). To our knowledge, no other studies have reported on CEC changes during bevacizumab single-agent therapy, therefore our finding of stable CECs during bevacizumab single-agent therapy remains to be confirmed by other studies.

We did not observe an association between baseline CEC counts and OS. In addition, we explored whether or not absolute CEC numbers during treatment or the relative changes during treatment were associated with outcome. During single-agent therapy with lomustine, an association was revealed between improved OS and higher absolute CEC numbers after 4 weeks and 6 weeks of treatment. Since we observed the association between CECs and OS in the patients receiving single-agent lomustine after both 4 weeks and 6 weeks of treatment, and this association remained statistically significant in multivariable Cox regression analysis, it is unlikely that these findings are false positives. Our findings that CEC changes relative to baseline did not correlate with OS, suggests that the absolute CEC number, which reflects the extent of endothelial damage during treatment at a specific point in time, is more important than the actual pattern of endothelial damage over time.

The lack of association between baseline CECs and OS is in contrast to two other glioblastoma studies (18, 19). It should however be noted that in these studies other CEC enumeration techniques were used. It is known that the CEC compartment in patients with solid tumors consists of both tumor-derived endothelial cells (tumor CECs; tCECs)

and normal tissue-derived endothelial cells (normal CECs; nCECs). There are a few potential pitfalls associated with measuring the nCEC compartment. Since the nCEC compartment is relatively large, smaller changes taking place in the tCEC compartment may be masked. In addition, changes in the nCEC compartment because of non-tumor related causes might lead to incorrect interpretations regarding the tCEC compartment. The use of tumor-endothelial markers therefore allows more precise tCEC measurement. Cuppini and colleagues used a putative tumor-endothelial specific marker (CD109) to detect CECs in their study (19).

The study by Cuppini et al. reported decreased CD109-positive CECs in patients who responded to bevacizumab plus irinotecan and bevacizumab single-agent after 2 months of treatment, while we could not find such associations for the bevacizumab-containing regimens in our study. Interestingly, while the study by Cuppini et al. did not observe such an association between CD109-CECs and response in patients receiving cytotoxic chemotherapy, we observed that higher CEC numbers were associated with improved OS only in the single-agent lomustine cytotoxic chemotherapy group. It should be realised however that there are important differences between the used CEC enumeration method by Cuppini and colleagues and our CEC enumeration method, which may explain differences in prognostic value between our studies. Cuppini et al. investigated an entirely different CEC population than we did, with no CD146 expression (20). While they used CD31 to identify a CD109-positive, CD146-negative CEC population and a separate CD146-positive, CD109-negative CEC population with no overlap of both markers, we used CD34 to identify CD146-positive CECs. Apart from these differences in CEC enumeration techniques, differences in the chosen fixed time points and differences in treatment regimens may also explain the differences between our studies. Another explanation for the differences between our studies may be that single-agent irinotecan has no proven efficacy in glioblastoma (5), in contrast to lomustine.

The most likely explanation that can be thought of as to why we observed prognostic value of circulating endothelial cells in the patients receiving lomustine single-agent but not in patients receiving bevacizumab-based therapy is our sample size. Although the number of patients per treatment arm was relatively large for any study evaluating recurrent glioblastoma patients, only a limited number of patients were evaluated per treatment arm. This may have provided insufficient statistical power to associate CEC numbers with OS in the bevacizumab-based treatment arms. However, we can speculate on biological mechanisms that may have accounted for the lack of prognostic value in the bevacizumab-based treatment arms as well. Since CEC numbers remained stable in the whole group of lomustine single-agent treated patients, high CEC numbers during treatment in the lomustine single-agent arm may represent only those patients in whom a larger extent of endothelial damage was present as a consequence of more lomustine-induced damage of the tumor. In contrast to the lomustine single-agent arm, CECs increased in the whole group of patients receiving bevacizumab plus lomustine, suggesting that endothelial damage occurred in the majority of these patients. The origin of CECs responsible for the increased CEC counts (from the tumor vasculature or the normal vasculature) is however unknown. Endothelial damage of tumor vessels might have occurred in the majority of patients because of an improved penetration of lomustine into the tumor, which has been demonstrated in preclinical experiments using neuroblastoma xenografts treated with bevacizumab and systemic chemotherapy

(21). Alternatively, combination treatment with lomustine and bevacizumab might have triggered significant endothelial damage in normal tissues in addition to tumor tissue, consequently masking changes in the tCEC compartment. Both these mechanisms may have made it more challenging to associate CECs with outcome in the bevacizumab plus lomustine combination arm, which further stresses the high need for robust assays to discriminate tCECs from nCECs. The lack of association between CEC numbers and outcome in the bevacizumab single-agent arm is probably due to the fact that bevacizumab as a single-agent has low activity in glioblastoma (14).

There are several strong points of our study including the randomised set-up of our study, the high number of collected samples and the use of OS as the endpoint for associations with CECs. However, our study also had some limitations. The number of patients per treatment was relatively limited. In addition to limiting the statistical power of associating CEC numbers with OS in the bevacizumab-based treatment arms as mentioned before,, the limited number of patients prevented a definition of CEC cut-off points associated with prognosis and did not allow intergroup comparisons.

Another limitation is that the CEC assay used in our study does not specifically detect tumor-derived CECs. Our observation that CECs increased during treatment with bevacizumab plus lomustine but were not associated with prognosis, may have been caused by changes occurring in the nCEC compartment, therefore masking changes in the tCEC compartment. We recently reported that CD276 is a putative tCEC marker that was expressed more highly in CECs from patients with glioblastoma than in CECs from healthy donors (22). We are currently participating in an EORTC phase III study that compares outcomes following bevacizumab plus lomustine versus lomustine single-agent in recurrent glioblastoma (trial registry number NCT01290939); it includes a side-study to evaluate the clinical value of CD276+ tCECs. This will enable us to validate our findings regarding the prognostic value of CECs and possibly CD276+ tCECs in glioblastoma patients receiving lomustine single-agent. In addition, as we believe CD276+ tCECs are more sensitive for measuring the tCEC compartment, we hope to identify the true clinical value in patients receiving bevacizumab plus lomustine combination therapy.

Altogether, the results from our study indicate that it may be worthwhile to further explore CEC enumeration as a marker in recurrent glioblastoma, as was also suggested by Cuppini and colleagues. Given the heterogeneity of tumor types and patient populations, different anti-tumor agents administered and different CEC enumeration techniques used, one should nonetheless be careful in interpreting CEC data between studies (13, 23). Consensus is needed on the optimal CEC enumeration technique, as this would enable researchers to compare findings between studies and ultimately take the application of CECs to the next level. In addition, the initiation of studies using promising tCEC markers will be essential for CECs to eventually make it as a reliable and robust biomarker in clinical oncology.

REFERENCES

1. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. 2007 Aug;114(2):97-109.

2. Jain RK, di Tomaso E, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT. Angiogenesis in brain tumours. Nat Rev Neurosci. 2007 Aug;8(8):610-22.

3. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature. 1992 Oct 29;359(6398):845-8.

4. Salmaggi A, Eoli M, Frigerio S, Silvani A, Gelati M, Corsini E, et al. Intracavitary VEGF, bFGF, IL-8, IL-12 levels in primary and recurrent malignant glioma. J Neurooncol. 2003 May;62(3):297-303.

5. Friedman HS, Prados MD, Wen PY, Mikkelsen T, Schiff D, Abrey LE, et al. Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. J Clin Oncol. 2009 Oct 1;27(28):4733-40.

6. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. N Engl J Med. 2014 Feb 20;370(8):699-708.

7. Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. N Engl J Med. 2014 Feb 20;370(8):709-22.

8. Hayward RM, Patronas N, Baker EH, Vezina G, Albert PS, Warren KE. Inter-observer variability in the measurement of diffuse intrinsic pontine gliomas. J Neurooncol. 2008 Oct;90(1):57-61.

9. Provenzale JM, Mancini MC. Assessment of intra-observer variability in measurement of high-grade brain tumors. J Neurooncol. 2012 Jul;108(3):477-83.
10. van den Bent MJ, Vogelbaum MA, Wen PY, Macdonald DR, Chang SM. End point assessment in gliomas: novel treatments limit usefulness of classical Macdonald's Criteria. J Clin Oncol. 2009 Jun 20;27(18):2905-8.

11. Wen PY, Macdonald DR, Reardon DA, Cloughesy TF, Sorensen AG, Galanis E, et al. Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. J Clin Oncol. 2010 Apr 10;28(11):1963-72.

12. Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, Clancy RM, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost. 2005 Feb;93(2):228-35.

13. Kraan J, Sleijfer S, Foekens JA, Gratama JW. Clinical value of circulating endothelial cell detection in oncology. Drug Discov Today. 2012 Jul;17(13-14):710-7.

14. Taal W, Oosterkamp HM, Walenkamp AM, Dubbink HJ, Beerepoot LV, Hanse MC, et al. Single-agent bevacizumab or lomustine versus a combination of bevacizumab plus lomustine in patients with recurrent glioblastoma (BELOB trial): a randomised controlled phase 2 trial. Lancet Oncol. 2014 Aug;15(9):943-53.

15. Kraan J, Strijbos MH, Sieuwerts AM, Foekens JA, den Bakker MA, Verhoef C, et al. A new approach for rapid and reliable enumeration of circulating endothelial cells in patients. J Thromb Haemost. 2012 May;10(5):931-9.

16. Bidard FC, Mathiot C, Degeorges A, Etienne-Grimaldi MC, Delva R, Pivot X, et al. Clinical value of circulating endothelial cells and circulating tumor cells in metastatic breast cancer patients treated first line with bevacizumab and chemotherapy. Ann Oncol. 2010 Sep;21(9):1765-71.

17. Simkens LH, Tol J, Terstappen LW, Teerenstra S, Punt CJ, Nagtegaal ID. The predictive and prognostic value of circulating endothelial cells in advanced colorectal cancer patients receiving first-line chemotherapy and bevacizumab. Ann Oncol. 2010 Dec;21(12):2447-8.

18. Reynes G, Vila V, Fleitas T, Reganon E, Font de Mora J, Jorda M, et al. Circulating endothelial cells and procoagulant microparticles in patients with glioblastoma: prognostic value. PloS one. 2013;8(7):e69034.

19. Cuppini L, Calleri A, Bruzzone MG, Prodi E, Anghileri E, Pellegatta S, et al. Prognostic value of CD109+ circulating endothelial cells in recurrent glioblastomas treated with bevacizumab and irinotecan. PloS one. 2013;8(9):e74345.

20. Mancuso P, Calleri A, Gregato G, Labanca V, Quarna J, Antoniotti P, et al. A Subpopulation of Circulating Endothelial Cells Express CD109 and is Enriched in the Blood of Cancer Patients. PloS one. 2014;9(12):e114713.

21. Dickson PV, Hamner JB, Sims TL, Fraga CH, Ng CY, Rajasekeran S, et al. Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. Clin Cancer Res. 2007 Jul 1;13(13):3942-50.

22. Kraan J, van den Broek P, Verhoef C, Grunhagen DJ, Taal W, Gratama JW, et al. Endothelial CD276 (B7-H3) expression is increased in human malignancies and distinguishes between normal and tumour-derived circulating endothelial cells. Br J Cancer. 2014 Jul 8;111(1):149-56.

23. Strijbos MH, Gratama JW, Kraan J, Lamers CH, den Bakker MA, Sleijfer S. Circulating endothelial cells in oncology: pitfalls and promises. Br J Cancer. 2008 Jun 3;98(11):1731-5.

CHAPTER 10

Circulating endothelial cell enumeration demonstrates prolonged endothelial damage in recipients of myeloablative allogeneic stem cell transplantation

Nick Beije, Jurjen Versluis, Jaco Kraan, Jan W. Gratama, Stefan Sleijfer and Jan J. Cornelissen

Haematologica. 2015 Jun;100(6):e246-9

ABSTRACT

Although outcomes following allogeneic stem cell transplantation (allo-SCT) have improved, graft-versus-host disease (GVHD), infections and conditioning related toxicity still cause significant morbidity and mortality. Circulating endothelial cells (CECs) are mature endothelial cells found in the peripheral blood and are presumed to be a marker of vascular damage. We here used CEC enumeration to assess the extent of conditioning-related endothelial damage, enabling to compare the extent and duration of vascular damage between myeloablative (MAB) and reduced intensity conditioning (RIC) regimens. In addition, we explored the use of CECs as a marker for GVHD or post-transplant infections. CECs were enumerated by a flow-cytometric approach in 112 adult patients undergoing allo-SCT at fixed time points up to 24 months post-transplant. Recipients of MAB conditioning had significantly more CECs than patients receiving RIC in the first year post-transplant. Strikingly, we observed lower CEC numbers in patients with either acute or chronic GVHD, evoking the hypothesis whether alloreactivity towards endothelial cells might play a role. CECs appeared to express both class I and class II HLA antigens. Collectively, the lower CEC counts during RIC regimens suggest less vascular damage compared to MAB, which may translate into less cardiovascular morbidity in the long term. Further studies are warranted to investigate the clinical relevance of the increased CEC numbers in MAB conditioned allo-SCT patients, as well as to investigate alloreactivy towards endothelial cells during GVHD.

INTRODUCTION

Allogeneic stem cell transplantation (allo-SCT) has the potential to cure patients with various hematological malignancies. Significant morbidity and mortality however occurs following allo-SCT due to complications such as graft-versus-host disease (GVHD), infections and conditioning related toxicity. Additionally to early morbidity, it is increasingly appreciated that long-term allo-SCT survivors have an increased incidence of cardiovascular risk factors and have a greater burden of cardiovascular morbidity (1, 2) with odds ratios ranging from 2.3 to 3.0 in recipients of allo-SCT compared to a matched general population (2).

There is mounting evidence that many of the complications of allo-SCT are at least partially related to endothelial damage. Consequently, there is a high need for parameters to accurately assess allo-SCT conditioning regimen-related effects on the endothelium as well as the potential role of the endothelium in the untoward events accompanying allo-SCT. Circulating endothelial cells (CECs) are mature endothelial cells present in the peripheral circulation and are a surrogate marker for endothelial damage. In a previous study to investigate the impact of conditioning regimen-related endothelial damage following allo-SCT, it was demonstrated that patients who received reduced-intensity conditioning (RIC) had significantly lower CEC numbers than patients who underwent myeloablative (MAB) conditioning (3). However, patients were only followed for 21 days post-transplant and consequently the extent of long-term endothelial damage was not established.

Given the current trend in allo-SCT towards the use of more RIC regimens (4), we investigated the impact of RIC versus MAB conditioning on endothelial damage in greater detail. CECs were enumerated at fixed time points in a large group of adults undergoing allo-SCT for up to 2 years post-transplant. We also explored the use of CECs as a putative marker for GVHD and infections.

METHODS

PATIENTS AND BLOOD COLLECTION

This retrospective, single-center study included adult patients receiving allo-SCT in the Erasmus MC Cancer Institute in Rotterdam between August 2009 and November 2011 in two prospective trials.

One of these trials involved sibling donor patients and matched unrelated donor patients (Netherlands Trial Registry -NTR- number NTR2252, HOVON 96), while the other trial involved double umbilical cord blood patients (NTR1573, HOVON 106) (5, 6). The institutional review board approved the protocols, and all patients and donors provided written informed consent. Peripheral blood (PB) samples were acquired in EDTA tubes at baseline (one month before transplantation) and 3, 6, 12 and 24 months post-transplant to determine post-transplant kinetics of CECs. In patients undergoing a double umbilical cord blood transplantation (dUCBT), additional PB samples for the same purpose were acquired at 1 and 2 months post-transplant. Samples were maintained at room temperature and processed within 24 hours of blood collection.

CONDITIONING REGIMEN AND SUPPORTIVE CARE

All patients received either a RIC, RIC-umbilical cord blood (RIC-UCB) or a MAB regimen. TBI in the RIC regimen consisted of 2 Gy or 4 Gy, while TBI in the RIC-UCB regimen consisted of 4 Gy. MAB TBI dose was 10 Gy or 12 Gy in all patients.

All MUD and sib donors received granulocyte colony-stimulating factor (G-CSF; 2x 5 μg/kg s.c.) to mobilize peripheral blood stem cells, starting at day -5 and ending at the last day of apheresis. Stem cells were infused at day 0 in all cohorts. In the dUCBT cohort, grafts were routinely infused at two consecutive days (day 0 and day +1). Hematopoietic growth factors (G-CSF) were not routinely given to allo-SCT recipients in any of the cohorts.

All patients received cyclosporine A (CsA; trough level 250-350 μg/l) and mycophenolate mofetil (MMF; 2 x 16 mg/kg) as additional post-transplant GVHD prophylaxis for at least three months and one month, respectively, with gradual tapering of the drug thereafter. Acute GVHD (aGVHD) was graded according to the Glucksberg criteria updated according to Przepiorka et al. (7, 8). All patients who suffered from aGVHD grade II-IV received prednisone (2 mg/kg/day). Chronic GVHD (cGVHD) was scored according to the Seattle classification for limited and extensive chronic GVHD (9). Chronic GVHD for which local therapy was not applicable, was treated with a combination of prednisone and cyclosporine according to clinical response.

All patients received prophylactic cotrimoxazol (1 x 480 mg) to prevent infections with pneumocystis carinii and valaciclovir (3 x 500 mg) to prevent CMV-reactivations for at least one year following allo-SCT. In the case of chronic GVHD or delayed immunosuppressive tapering, infectious prophylaxis was prolonged.

INFECTIONS

All infections were scored according to the NCI common toxicity criteria (CTC) version 3.0 (10) between day 1 and day 365 post-transplant, as described before (11, 12). All CTC grade 3-4 infections were scored and, if applicable, the location and causative microorganism of the infection were documented. In addition, CTC grade 2 CMV reactivations were scored, because CMV is known to infect endothelial cells and promote angiogenesis.

ENUMERATION OF CIRCULATING ENDOTHELIAL CELLS

Enumeration of circulating endothelial cells (CECs) was performed according to our previously reported flow cytometric approach (13). We used the following directly conjugated monoclonal antibodies for the identification of CEC: CD34-FITC (clone 8G12; BD Biosciences, San Jose, CA, USA), CD146-APC (clone 541-10B2; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and CD45-PerCP (clone 2D1; BD Biosciences). DRAQ5 (Biostatus Ltd, Shepshed, UK) was used as a cell permeable nuclear dye to exclude platelets and microparticles. CECs were defined as CD34+, CD146+, CD45- and DRAQ5+.

Samples were acquired on a FACS Fortessa flow cytometer (BD Biosciences) and were subsequently analyzed using FCS Express (De Novo Software, Los Angeles, CA, USA). Analyses were always checked by one experienced technician to minimize inter-rater variability.

EXPRESSION OF HLA CLASS I AND II ANTIGENS ON CECS

To study expression of HLA-DR on CECs, HLA-DR-PE (clone L243, BD Biosciences) was used. For the HLA-class I and HLA-mismatch analyses, HLA-A2, HLA-A9, HLA-B12, HLA-B27 & HLA-Bw6 biotinylated monoclonal antibodies (IgG2b; One Lambda, Canoga Park, CA, USA) were used and subsequently coupled to Streptavidin-PE (BD Biosciences).

STATISTICAL CONSIDERATIONS

Several time intervals were constructed to define which CEC measurements were eligible for a given time point. These time intervals were also used to define the presence of absence of GVHD at that given time point. CEC samples that were drawn after disease relapse were excluded from the analysis, as the presence of very high numbers of disease-related CD34+ stem cells in these patients may interfere with the CEC analysis. CEC numbers between conditioning types or the presence of GVHD were compared using the Mann-Whitney U test. For the comparison of CEC numbers within the same patients on different time points, the Wilcoxon signed-rank test was used. Multivariable linear regression was performed using log-normalized CEC numbers to assure normality of the CEC data. Parameters used as variables included age, gender, HCT-CI score, donor source, conditioning intensity, occurrence of GVHD and occurrence of infections. A backward stepwise approach was used with a significance level of ≥0.2 to omit a given variable from the model. Age and gender were then subsequently added to the model, even if they did not have a significant contribution to the model, to assure that the most clinically relevant model was used. All reported *p* values are two-sided, and a significance level $\alpha = 0.05$ was used. All data analyses were done using Stata/SE 12 (StataCorp LP, College Station, TX, USA).

RESULTS

Our retrospective study included 112 adult patients receiving allo-SCT in the Erasmus MC Cancer Institute. One patient was excluded because of the application of a unique, alternatively intensified conditioning regimen prior to double umbilical cord blood transplantation (dUCBT), which differed from other dUCBT recipients and also differed from RIC and MAB conditioned patients. Patient characteristics from the 111 remaining patients are presented in **Supplementary Table 1**. All patients were transplanted between August 2009 and November 2011 in the context of two prospective trials. Sibling donor patients (n=37) and matched unrelated donor patients (n=56) were included in the context of the HOVON 96 study (Netherlands Trial Registry - NTR2252), while dUCBT (n=18) were included in the context of the HOVON 106 study (NTR1573) (5, 6). MAB conditioning was received by 24 patients, consisting mainly of myeloablative TBI (12 Gy) and cyclophosphamide. RIC was received by 69 patients, consisting mainly of 2 Gy TBI combined with fludarabin (14). Lastly, 18 patients received a RIC-UCB consisting of 2x2 Gy TBI combined with fludarabin and cyclophosphamide prior to UCBT. None of the

patients received in vivo T cell depletion. The minimal follow-up time was one year, and the median follow-up time for living patients was 34 months.

A total of 357 peripheral blood samples were evaluated for the presence of CECs at baseline, 1 (dUCBT recipients only), 2 (dUCBT recipients only), 3, 6, 12 and 24 months post-transplant. Based on the number of follow-up days, we expected 473 samples, while 357 were analyzed, indicating that 75% of the expected samples were analyzed. CECs were defined as CD34+, CD146+, DRAQ5+, CD45- events and enumerated according to our previously described flow cytometric approach (13). Absolute CD34 counts did not correlate with CEC counts (r=0.09, **Supplementary Methods Figure 1**). The influence of RIC and MAB conditioning on CEC kinetics is presented in **Figure 1 (left panel)**. While CEC numbers did not differ between RIC and the MAB conditioned patients pre-transplant (P=0.71) , patients who received MAB conditioning had higher CEC numbers than RIC recipients for up to 12 months following allo-SCT (P=0.000, P=0.000 and P=0.002 at 3, 6 and 12 months post-allo-SCT, respectively). At 24 months following allo-SCT, CEC numbers were similar in RIC and MAB conditioned patients (P=0.64). In the MAB group, CEC numbers were higher 12 months post-transplant than pre-transplant (one-sided Wilcoxon signed-rank test P=0.04).

In patients receiving an RIC-UCB conditioning additional CEC numbers at 1 and 2 months post-allo-SCT were available. A significant rise in CEC numbers was observed at one month following dUCBT (P=0.006), to decrease significantly towards baseline values from 2 months post-allo-SCT onwards (P=0.009) (**Figure 1, right panel**).

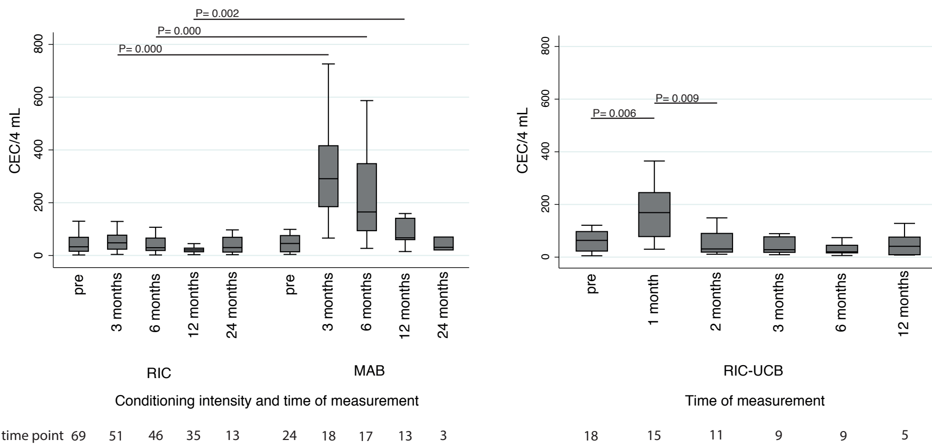


FIGURE 1. Box-and-whisker plots showing the influence of conditioning intensity on CECs. Left panel shows CEC kinetics in MAB and RIC conditioned patients. Right panel shows CEC kinetics in dUCBT patients receiving RIC-UCB conditioning. (Boxes show 25th percentile, median and 75th percentile, whiskers show the lower and upper adjacent values, according to Tukey).

We observed a CTC grade III-IV infection in 54%, 17% and 18% of the patients in the first 3 months, month 3 to 6 and month 6 to 12 post-transplant, respectively. No significant differences in CEC numbers were observed at 3 months (P=0.12), 6 months (P= 0.51) and 12 months (P=0.99) post-transplant between those patients with versus those without a grade III-IV infection. Apart from CTC grade III-IV infections, CMV reactivations including those meeting CTC grade II criteria were separately scored in all patients. In 37 patients (33%), a CMV reactivation was observed. No significant differences in CEC numbers and the occurrence of CMV reactivation at 3, 6 and 12 months post-allo-SCT were observed.

In multivariable analysis at 3, 6 and 12 months post-transplant taking age, gender, HCT-CI score, donor source, conditioning intensity (MAB, RIC and UCB conditioning), occurrence of GVHD and occurrence of infections into account, MAB conditioning was associated with higher CEC numbers (P=0.000, P=0.000 and P=0.008, respectively) (**Table 1**). At 3 months following allo-SCT, the occurrence of aGVHD grade II-IV appeared associated with lower CEC numbers (P=0.003). The occurrence of cGVHD, limited and/or extensive was also associated with lower CEC numbers at 6 (P=0.019) and 12 (P=0.012) months post-transplant.

TABLE 1. Multivariable linear regression analysis on variables associated with the number of CECs.

Month of CEC count	Variable	Beta	*P*-value
Pre	No variables associated with CEC numbers		
3	aGVHD grade 2-4	-0.26	0.003
	MAB conditioning	0.70	0.000
6	cGVHD present	-0.22	0.019
	MAB conditioning	0.57	0.000
12	cGVHD present	-0.33	0.012
	MAB conditioning	0.47	0.008

Negative standardized betas represent a correlation with lower CECs, while positive standardized betas represent a correlation with higher CECs.

We further explored the reasons underlying the differences in CEC numbers between patients experiencing GVHD versus those who had not. To exclude that our findings were due to the occurrence of donor-derived CECs in our assay, we evaluated CEC-chimerism 1 month after transplantation by using HLA class II mismatch-specific monoclonal antibodies in two dUCBT recipients with a class II mismatch with their donor graft (**Figure 2A-B**). We did not observe CEC-chimerism: all CECs appeared of recipient origin. We then hypothesized that the unexpected lower number of CEC in patients with overt GVHD could be due to a direct immune response of alloreactive donor lymphocytes towards recipient CEC. Unfortunately it appeared technically impossible to visualize an immune response towards the small number of CECs that were detected by flow cytometry. Since an immune response of alloreactive donor T-cells to CECs would require HLA-expression on CECs, we evaluated in peripheral blood mononuclear cell (PMBC) samples the percentage of CECs expressing a HLA class I antigen (8 samples) and HLA-class II antigens (13 samples). A large subset of CECs was found to express a HLA class I antigen (median CEC HLA class I positive 94%, range 81-100%) as well as HLA class II antigens (median CEC HLA-DR positive 86%, range 80-99%) **(Figure 2C-D)**.

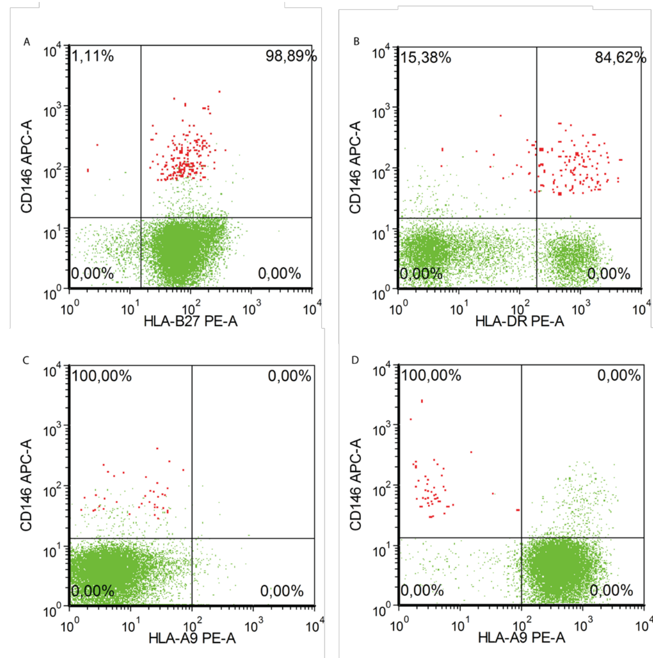


FIGURE 2. Panel A & B show the presence of donor-specific HLA-A9 on lymphocytes (green) and CECs (red). Panel A shows that 100% of all CECs did not express HLA-A9 prior to SCT. Panel B shows that all CECs at 1 month following allo-SCT are of recipient origin, while virtually all lymphocytes are of donor origin and express HLA-A9. Panel C & D show representative images of HLA class I and HLA class II expression on lymphocytes (green) and CECs (red). Panel C shows that HLA-B27 is expressed in 98,89% of all CECs at 3 months post-transplant (both donor and recipient harbored the HLA phenotype HLA-B27). Panel D shows HLA-DR expression in 84,62% of all CECs at 3 months post-transplant.

DISCUSSION

This study confirmed the previous observation that MAB induces more endothelial damage than RIC in the first month following allo-SCT (3, 15). We now showed for the first time that in MAB conditioned patients, endothelial damage is present for at least 12 months following transplantation. In contrast, in dUCBT patients receiving a 4 Gy TBI conditioning, a significant rise in CEC numbers as opposed to baseline was observed only at one month following allo-SCT. This suggests that endothelial damage following a relatively modest dose of TBI is only present for a short period of time. At 24 months post-transplant, CEC numbers of RIC and MAB conditioned patients were similar. The prolonged endothelial damage in patients receiving MAB conditioning may possibly be associated with more long-term cardiovascular conditions, as compared to RIC. This may be an important observation, especially since MAB conditioning is predominantly applied in younger patients, who will have more time to actually develop cardiovascular conditions. Thereby, our data may support the suggestion to further examine the use of RIC regimens in subsets of younger patients (16), especially in younger patients who already have relevant cardiovascular risk factors or comorbidity.

In contrast with some reports that suggested that GVHD is associated with increased endothelial damage (17-19), we observed significant lower CEC numbers in patients who experienced GVHD. It should however be noted that these previous studies were not all performed in humans, and different methods to assess endothelial damage were used. Following our observations that CECs strongly express HLA class I and class II antigens, we formulated the hypothesis that an alloreactive immune response may be exerted against CECs.

Because we did not observe CEC-chimerism in 2 patients at 1 month following SCT, it is unlikely that the lower CEC numbers in patients with overt GVHD were due to the occurrence of donor-derived CECs. Unfortunately no suitable PBMC samples were available to test the occurrence of CEC-chimerism at later time points following transplantation. Prospective studies investigating whether or not CEC-chimerism occurs in the post-transplant period, and if so from what time point onwards, are needed.
We also hypothesized that GVHD-associated treatments, such as steroids and calcineurin inhibitors (CNI), which were routinely given to all patients might account for the occurrence of less CECs in GVHD patients. However, since increased endothelial dysfunction has been linked to prednisone use (20) and cortisol excess (21) and therefore likely leads to higher CEC numbers, it is unlikely that the lower CEC numbers in GVHD patients are due to steroid treatment. Additionally, patients with and without GVHD in our study were fairly balanced regarding CNI treatment and had proper ciclosporin or tacrolimus trough levels at the time of CEC measurement, further rendering it unlikely that our findings were due to differences in CNI treatment or CNI toxicity.

Another explanation for the lower CEC numbers in GVHD patients could be that vascular damage occurs to such an large extent that only endothelial fragments remain, which do not meet our criteria for intact endothelial cells and are therefore missed by the current flow cytometric approach.

There are several potential limitations of this study. Fixed time points were chosen to evaluate long-term changes related to the conditioning intensity, but are less suitable for the analysis of allo-SCT related complications such as GVHD. Clearly, these complications do not necessarily coincide with these fixed time points and therefore rapid CEC kinetic changes might be missed by this approach. Other limitations include the relatively small number of patients for subgroup analyses, especially at 24 months post-allo-SCT, and the relatively short follow-up, which made it impossible to explore whether those patients with highest CEC numbers are indeed at increased risk to develop cardiovascular diseases.

In summary, we present the largest study to date evaluating the impact of conditioning regimens on CECs as parameter for vascular damage in allo-SCT. We found that patients receiving MAB conditioning have long-term endothelial damage as opposed to patients receiving RIC. Further studies are warranted to investigate the clinical relevance of the increased CEC numbers in MAB patients, especially regarding the possible association with long-term cardiovascular outcomes. In addition, we observed lower CEC numbers in GVHD patients, which may possibly be explained by a direct immune response against CECs. Future research should investigate whether such an immune response is indeed present.

10

REFERENCES

1. Armenian SH, Sun CL, Vase T, Ness KK, Blum E, Francisco L, et al. Cardiovascular risk factors in hematopoietic cell transplantation survivors: role in development of subsequent cardiovascular disease. Blood. 2012 Nov 29;120(23):4505-12.

2. Chow EJ, Baker KS, Lee SJ, Flowers ME, Cushing-Haugen KL, Inamoto Y, et al. Influence of conventional cardiovascular risk factors and lifestyle characteristics on cardiovascular disease after hematopoietic cell transplantation. J Clin Oncol. 2014 Jan 20;32(3):191-8.

3. Woywodt A, Scheer J, Hambach L, Buchholz S, Ganser A, Haller H, et al. Circulating endothelial cells as a marker of endothelial damage in allogeneic hematopoietic stem cell transplantation. Blood. 2004 May 1;103(9):3603-5.

4. Passweg JR, Baldomero H, Bregni M, Cesaro S, Dreger P, Duarte RF, et al. Hematopoietic SCT in Europe: data and trends in 2011. Bone Marrow Transplant. 2013 Sep;48(9):1161-7.

5. Somers JA, Braakman E, van der Holt B, Petersen EJ, Marijt EW, Huisman C, et al. Rapid induction of single donor chimerism after double umbilical cord blood transplantation preceded by reduced intensity conditioning: results of the HOVON 106 phase II study. Haematologica. 2014 Aug 8.

6. Somers J, Brand A, van Hensbergen Y, Mulder A, Oudshoorn M, Sintnicolaas K, et al. Double Umbilical Cord Blood Transplantation: A Study of Early Engraftment Kinetics in Leukocyte Subsets using HLA-Specific Monoclonal Antibodies. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2013;19(2):266-73.

7. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation. 1974 Oct;18(4):295-304.

8. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant. 1995 Jun;15(6):825-8.

9. Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. Am J Med. 1980 Aug;69(2):204-17.

10. CancerTherapyEvaluationProgram. Common Terminology Criteria for Adverse Events v3.0 (CTCAE)2006: Available from: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf.

11. Wils EJ, van der Holt B, Broers AE, Posthumus-van Sluijs SJ, Gratama JW, Braakman E, et al. Insufficient recovery of thymopoiesis predicts for opportunistic infection in allogeneic hematopoietic stem cell transplantation recipients. Haematologica. 2011 Aug 22.

12. Broers AE, van der Holt B, Haze S, Braakman E, Gratama JW, Lowenberg B, et al. A comparison of postengraftment infectious morbidity and mortality after allogeneic partially T cell-depleted peripheral blood progenitor cell transplantation versus T cell-depleted bone marrow transplantation. Exp Hematol. 2005 Aug;33(8):912-9.

13. Kraan J, Strijbos MH, Sieuwerts AM, Foekens JA, den Bakker MA, Verhoef C, et al. A new approach for rapid and reliable enumeration of circulating endothelial cells in patients. J Thromb Haemost. 2012 May;10(5):931-9.

14. Niederwieser D, Maris M, Shizuru JA, Petersdorf E, Hegenbart U, Sandmaier BM, et al. Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological diseases. Blood. 2003 Feb 15;101(4):1620-9.

15. Zeng L, Yan Z, Wang L, Du B, Pan X, Xu K. Irradiation is an early determinant of endothelial injury during hematopoietic stem cell transplantation. Transplant Proc. 2008 Oct;40(8):2661-4.

16. Estey EH. Intensity of conditioning for allogeneic haemopoetic cell transplantation. Lancet Oncol. 2012 Oct;13(10):966-8.

17. Salat C, Holler E, Kolb HJ, Pihusch R, Reinhardt B, Hiller E. Endothelial cell markers in bone marrow transplant recipients with and without acute graft-versus-host disease. Bone Marrow Transplant. 1997 May;19(9):909-14.

18. Yan Z, Zeng L, Jia L, Xu S, Ding S. Increased numbers of circulating ECs are associated with systemic GVHD. Int J Lab Hematol. 2011 Oct;33(5):507-15.

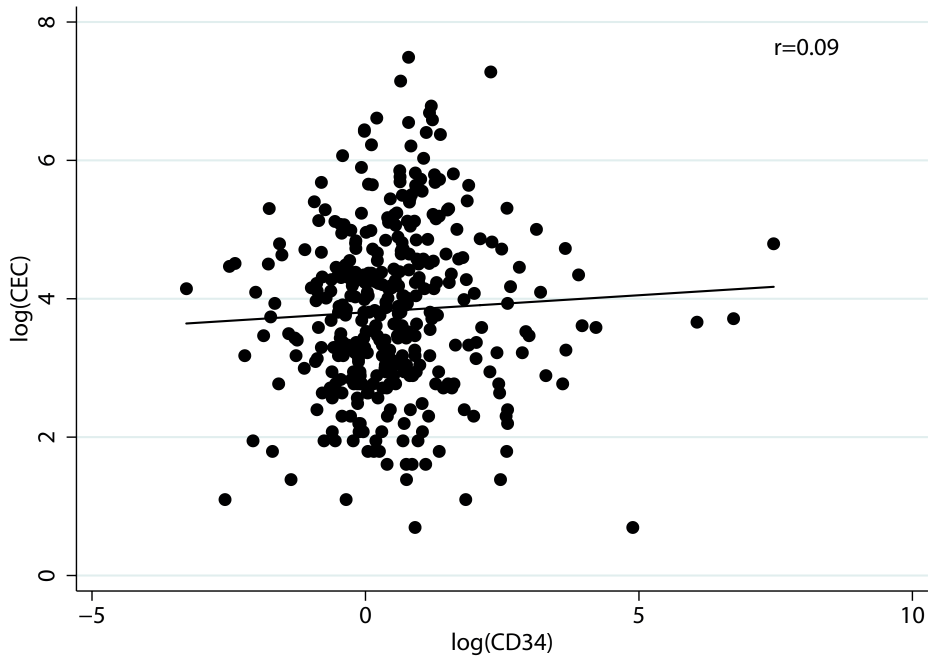
19. Almici C, Skert C, Verardi R, Di Palma A, Bianchetti A, Neva A, et al. Changes in circulating endothelial cells count could become a valuable tool in the diagnostic definition of acute graft-versus-host disease. Transplantation. 2014 Oct 15;98(7):706-12.

20. Turner E, Dishy V, Chung CP, Harris P, Pierces R, Asanuma Y, et al. Endothelial function in systemic lupus erythematosus: relationship to disease activity, cardiovascular risk factors, corticosteroid therapy, and coronary calcification. Vasc Health Risk Manag. 2005;1(4):357-60.

21. Whitworth JA, Williamson PM, Mangos G, Kelly JJ. Cardiovascular consequences of cortisol excess. Vasc Health Risk Manag. 2005;1(4):291-9.

SUPPLEMENTARY TABLE 1. Patient and graft characteristics (n=111).

Parameter	RIC (n=69)	RIC-UCB (n=18)	MAB (n=24)
Age, median(range)	55 (26-66)	53 (34-64)	32 (19-52)
Sex female (%)	31 (45)	6 (33)	10 (42)
Diagnosis			
ALL	4	0	8
AML	28	9	13
CLL	6	1	0
CML	2	2	2
MDS	6	2	1
MM	3	0	0
NHL	9	1	0
Other	11	3	0
Graft source			
Sib	27	0	10
MUD	42	0	14
dUCBT	0	18	0
Conditioning regimen			
Cyclo+TBI 12 Gy	0	0	21
Cyclo+Busu	0	0	2
Flu+TBI 12 Gy	0	0	1
Flu+Cyclo+TBI 2x2 Gy	9	18	0
Flu+TBI 2 Gy	57	0	0
TBI 2 Gy	3	0	0



Supplementary Figure 1. Correlation plot between CEC numbers and CD34 numbers. All values were log transformed in order to compress the figure. Spearman correlation coefficient was calculated with non-normalized values.

CHAPTER 11

Summary and general discussion

INTRODUCTION

The capacity to optimally treat cancer patients is nowadays challenged by several factors. The introduction of this thesis already addressed several of these challenges, in particular tumor heterogeneity and plasticity, causing tumor characteristics to change over time and under treatment pressure. Besides these challenges, which are related to the biology of cancer, we also have to deal with important exterior factors that may challenge our ability to deliver the best possible cancer care. Demographics such as an increasing age of the population, in combination with an increasing availability of new diagnostics, surgical techniques and especially the continuous stream of new and expensive cancer medicine have driven cancer-related costs to be sky-high (1, 2). Hospitals in the Netherlands have reported an annual increase of about 10% in costs spent on expensive cancer medicine, and this trend is expected to continue in the next years (3).

While the problem of increasing costs related to cancer care is a complex issue, in which efforts on multiple levels are necessary (which are outside the scope of this thesis), the ability to personalize treatment for cancer patients is likely to decrease costs. This was for example demonstrated for metastatic colorectal cancer (mCRC) patients, in which screening for KRAS and BRAF mutations in the primary tumor prior to anti-EGFR therapy has been proven to be cost-effective (4, 5). The availability of robust biomarkers which can tailor the right therapeutic strategy, for the right person, at the right time, is where we can use knowledge on the biology of cancer to treat cancer in the most optimal fashion, combined with a decrease in cancer-related costs in the long run. Liquid biopsies are of great interest to further personalize cancer treatment. However, their clinical utility is still rather limited. The next paragraphs will further discuss the current status of liquid biopsies on their way to personalize cancer treatment, and will set the observations as done in this thesis into a broader perspective.

CIRCULATING TUMOR CELLS

CLINICAL APPLICATIONS OF ENUMERATING CTCS USING THE CELLSEARCH MACHINE

The CellSearch assay to count CTCs is FDA-approved for diagnostic purposes in patients with metastatic colorectal, prostate and breast cancer, but not recommended in the ASCO or ESMO guidelines for clinical use in any of those tumor types. In **Chapter 2** of this thesis, an effort was made to come to recommendations for the use of CTC enumeration by the CellSearch machine in breast cancer patients. We observed a lack of sufficient data to recommend the use of CTC enumeration in patients with primary breast cancer. New data from a meta-analysis in primary breast cancer patients (n=3,173), not published yet at the time that the review article was written, demonstrated that the presence of CTCs was associated with shorter overall survival in multivariate analysis (HR 1.97, 95%CI 1.5-2.6) (6). However, the clinical relevance of these results and how it should impact treatment decision-making is still unclear. In patients without CTCs, disease-free survival (DFS) after 5 years was ±85%, while assays as currently used in the clinic such as Oncotype DX or Mammaprint are able to identify patients with a DFS of >94% after 5 years (7, 8), meaning that CTC enumeration is unlikely to be superior to such assays in primary breast cancer.

As further narrated in **Chapter 2**, in metastatic breast cancer (MBC) patients the use of CTC enumeration can be recommended in two clinical situations. First, given that CTCs are superior over serum biomarkers such as CEA and CA15.3 (9), CTC counting should be done in MBC patients with poorly evaluable disease such as those with bone metastases only. Second, CTC enumeration can be advocated to be used in clinical trials as a marker for response, as enumerating CTCs is an excellent early response marker. This could lead to a quicker examination of the efficacy of new drugs, already 3-4 weeks after treatment start.

While we recommend CTC enumeration in certain clinical situations in MBC patients, its true clinical utility is still only applicable to a small proportion of patients. Especially disappointing were the results from the SWOG S0500 interventional study. In this study, early switches in chemotherapeutic regimens, based on changes in CTC counts during treatment, did not results in an overall survival (OS) benefit (10). These results were somewhat discouraging for the future of CTC enumeration as a clinical tool, as these are the kind of strategies that everyone had hoped would bring benefits for large groups of patients.

New studies are now on the way to further examine whether counting CTCs may still have clinical utility. Concerning MBC patients, some French studies are now investigating other strategies to use CTC enumeration to guide treatment decision-making (11). An example is the CirCe01 trial, in which early switches are performed based on CTC response after one cycle of chemotherapy in MBC patients receiving third-line chemotherapy, as this allows to more easily search for the regimen that is most worthy to be pursued for more than one cycle.

In tumor types other than breast cancer opportunities are also present for the use of CTC enumeration. For instance, in patients with muscle-invasive bladder cancer (MIBC) we are currently performing the CirGuidance study (12). Because the OS benefit of neoadjuvant chemotherapy (NAC) in MIBC patients is small (5% 10-year OS benefit), we hypothesize that MIBC patients without CTCs have such a good prognosis that these patients do not need NAC. This is a good example of a study in which CTCs are being investigated as a tool to guide treatment-decision making.

ALTERNATIVE CTC ENUMERATION ASSAYS

Given that the EpCAM-dependancy of the CellSearch assay has disadvantages, a lot of other assays to enumerate CTCs have been described, for example other EpCAM-dependent assays (e.g., IsoFlux), RNA-based assays (e.g., AdnaTest), size-based assays (e.g., Screencell, ISET, VyCAP Microsieve), immunofluorescence-based assays (e.g., Epic Sciences, CytoTrack), vimentin-based assays (13), and assays based on the depletion of leukocytes (e.g., CTC-iChip (14, 15)). Especially of interest are techniques that are able to capture more mesenchymal CTCs. Previously, Yu et al. (16) reported interesting results that CTCs (microfluidically enriched using an antibody cocktail of EpCAM, EGFR and HER2) may exhibit either epithelial characteristics, mesenchymal characteristics or a combination of both. In addition, they reported that the presence of mesenchymal-type CTCs appeared to be associated with disease progression in a series of 11 MBC patients. However, these results, published in 2013, have to date not

been validated in a larger cohort of patients. Also of particular interest are results by Satelli and colleagues, who have used an assay that is able to detect mesenchymal CTCs using cell-surface vimentin (CSV) in patients with epithelial cancers (17, 18). Lastly, our group has suggested the use of MCAM as a marker for EpCAM-negative CTCs (19), and have shown that a combined EpCAM/MCAM capturing approach increases the capture rate of CTCs in primary breast cancer patients (20).

These assays, which are EpCAM-independent, and for the CSV assay also cytokeratin-independent, are nice additions to the arsenal of CTC assays currently available. While the true clinical relevance of mesenchymal-like CTCs in patients with epithelial tumors still seems unclear, the use of the assays for EpCAM-negative or more mesenchymal-type cancers is promising, for instance as shown with the CSV assay in sarcoma patients (13). Another example is the detection of MCAM-positive tumor cells in malignant pleural mesothelioma (MPM) patients. In contrast to EpCAM, MCAM is widely expressed in mesothelioma tissues (21). In **Chapter 8**, we investigated whether MCAM-positive CTCs are present in the peripheral blood (PB) and pleural effusion (PE) of patients with MPM, and explored their relevance in diagnosing MPM and in improving prognostication in MPM. MCAM-CTCs were detected in the peripheral blood of 26% of the patients, but did not appear to be of prognostic significance in MPM patients. MPM-CTCs measured in PEs using flow cytometry were detected in half of the patients, and this technique was superior to the current clinical standard, cytology review by the pathologist, for diagnosing MPM. In addition, the presence of MPM tumor cells in PE appeared to be associated with good prognosis. These results provide new options to improve the diagnosis and prognostication of MPM patients, and show the potential of the available assays able to detect EpCAM-negative CTCs.

CHARACTERIZATION OF CTCS

Counting CTCs is merely a one-dimensional use of CTCs. Because CTCs are intact tumor cells, they contain DNA and express RNA and proteins, and their characterization thus provides interesting opportunities to get multidimensional insight into tumor characteristics. Recently, a lot of research has been done on both the technical side of CTC characterization, as well as on the biological relevance and potential clinical relevance of CTC characterization.

PROTEIN LEVEL CHARACTERIZATION

Characterization of CTCs on the protein level has generally been limited to immunofluorescence directly on CTCs enumerated with CellSearch. Several protein markers have been described, some of them related to targets for therapy (i.e., ER, HER2, AR), apoptosis (M30), proliferation (Ki-67) or other markers associated with particular subtypes of CTCs (e.g., EGFR, CD44, Bcl-2). The characterization of HER2 and ER in MBC patients have received the most attention till date. Discrepancies between the ER-status and the HER2-status of the primary tumor and the CTCs in MBC have been demonstrated by numerous groups (22-36). However, reports on the clinical relevance of these discrepancies have been scant (35, 36). In **Chapter 3**, the clinical relevance of HER2-positive CTCs in MBC patients with a HER2-negative primary tumor was investigated in the context of endocrine resistance, as HER2-positivity in primary tumors had previously been linked to endocrine resistance. In addition, the prognostic impact of HER2-positive CTCs was assessed in a control cohort of patients receiving

chemotherapy. Notably, HER2-positive CTCs occurred in 29% of all patients (n=154) in this study, demonstrating that HER2-positive CTCs are frequently present in patients with a HER2-negative primary tumor. However, in the cohort of MBC patients receiving endocrine therapy, the occurrence of HER2-positive CTCs was not associated with impaired response to endocrine therapy. Also in the control cohort of patients receiving chemotherapy, the HER2 status of the CTCs did not have any prognostic impact.
The relatively high frequency of HER2-positive CTCs as demonstrated in our study provides opportunities to target patients with HER2-positve-CTCs with anti-HER2 targeting therapies Therefore, we recently initiated a study in which chemotherapy-naive MBC patients with HER2-negative primary tumors but HER2-positive CTCs are treated with the combination of trastuzumab and docetaxel (CareMore-Trastuzumab study; Netherlands Trial Register number NTR5115 (37)). Another interesting approach may be to treat HER2-positive CTC patients with trastuzumab emtansine (T-DM1), which targets HER2 overexpressing cells with a cytotoxic agent (DM1) (38, 39). The delivery of the cytotoxic agent might yield additional benefit, given that HER2 overexpression alone on CTCs does not necessarily mean that the HER2-signaling pathway is truly activated. Studies evaluating the success of treating patients with HER2-positive CTCs with these sorts of anti-HER2 treatments are of utmost importance to answer the clinically important question whether HER2-positive CTCs are predictive for outcome to anti-HER2 targeted therapies.

Besides the immunofluorescent staining of HER2 on CellSearch-enumerated CTCs, other markers have only been assessed anecdotally. Some other interesting results were obtained with a marker panel containing HER2, ER, Bcl-2 and Ki-67 on CellSearch-enumerated CTCs, in which a score derived from the combination of these markers was dubbed the CTC-endocrine therapy index (CTC-ETI) (40). In patients with MBC, CTC-ETI risk classifications were constructed (low, intermediate, high) and the outcome of patients for each risk classification is now investigated in a prospective trial.

RNA LEVEL CHARACTERIZATION

Our group previously demonstrated that molecular characterization of RNA from CTCs in a background of leukocytes is feasible in CTCs from MBC patients. We developed a 96-gene mRNA profile, of which 65 genes were considered to be CTC-specific. Using a similar approach as in MBC, a gene expression profile consisting of 34 CTC-specific genes was generated for use in CTCs from mCRC patients (41). Since then, using these mRNA profiles, we have carried out multiple studies to further evaluate whether CTCs are resembling of the primary tumor or the metastases, and to assess the prognostic relevance of these mRNA profiles. We demonstrated that discrepancies between the primary tumor and CTCs from MBC patients regarding gene expression are frequently present (48% discrepant) (35), however, without that having prognostic consequences. In line with the results in MBC patients, we found that CTCs from mCRC patients undergoing resection of liver metastases were often discrepant with the primary tumor (43% discrepant), while the CTCs better resembled the liver metastases (42). Regarding the prognostic value of the mRNA profile in MBC patients, we identified a 16-gene profile in CTCs that was associated with poor prognosis, but this profile could not be validated in an independent cohort (43). An 8-gene profile for resistance to endocrine therapy was also identified, but similarly to the 16-gene profile this profile could not be validated.

In addition to these mRNA gene expression profiles, also single genes of interest can be analyzed. In **Chapter 3**, besides characterization of HER2 on the protein level as discussed above, the expression levels of the gene coding for the ER, *ESR1*, were assessed in CTCs. We observed a discordance in ER status between the primary tumor and the CTCs in 25% of the patients, which did not seem to have prognostic relevance in exploratory analysis. Interestingly, frequently switches in ER status were observed from ER-negative in the primary tumor to ER-positive in the CTCs. As these patients might benefit from endocrine therapy, these results warrant further investigation.

Recently it has also become of huge interest to determine aberrant splice variants in mRNA from CTCs. In a landmark study by Antonarakis and colleagues, the presence of the AR-V7 splice variants in CTCs (enriched with AdnaTest) from patients with mCRPC predicted resistance to endocrine agents, demonstrating a 0% response rate in patients harboring an AR-V7 splice variant (44). Following these results, our group showed that the response to chemotherapy with cabazitaxel is not impaired in patients with the AR-V7 splice variants (45). Both the resistance to endocrine therapy (46) and the intact response to taxanes (46, 47) have now been independently validated using various assays (AdnaTest, CellSearch, Epic AR-V7 Test) to detect AR-V7 in CTCs. These data suggest that mCRPC patients with an AR-V7 splice variant in CTCs should receive chemotherapy, and patients without an AR-V7 splice variant should receive either endocrine therapy or chemotherapy.

Given the strong predictive value of AR splice variants in mCRPC, in **Chapter 5** we evaluated whether splice variants of the ER were present in CTCs from MBC patients and whether they were enriched during endocrine therapy. We found the Δ5 *ESR1* splice variant to be more highly expressed in CellSearch-enriched CTC fractions of MBC patients than in healthy blood donors. Since this splice variant has been linked to constitutive activity of the ER, this finding may be relevant, however, the Δ5 splice variant was not enriched during endocrine therapy.

DNA LEVEL CHARACTERIZATION

Characterization of DNA from CTCs has generally been hampered by the lack of purity of CTCs after isolation. In 2013, our group used several PCR methods to analyze CellSearch-enriched DNA from CTCs, but these PCR techniques lacked sensitivity to detect low-frequency mutations (48). More recently highly sensitive digital PCR (dPCR)-based assays have become available and may be used to detect mutations in DNA from CTCs. For example, *ESR1* mutations may be assessed using dPCR-based techniques. As reviewed in **Chapter 4**, these mutations are rare in primary breast cancer tumors, but enriched in patients with metastatic disease who have had prior treatment with aromatase inhibitors (AIs). As these mutations can only be assessed in metastatic tumor cells, the use of liquid biopsies to assess these mutations is of interest. We described in **Chapter 5** that dPCR is able to detect *ESR1* mutations in DNA from CellSearch-enriched CTCs, however, cell-free DNA was observed to be a superior substrate for *ESR1* mutation detection. We hypothesize that the background of nonspecifically CellSearch-enriched, leukocyte-derived, wildtype DNA hampers the detection of mutations in CTCs.

ANALYSIS OF CTCS AT THE SINGLE CELL LEVEL

The described results on the DNA and RNA level, limited by leukocyte contamination, have called for analysis of single or pure CTCs. A potential avenue could be to perform fluorescence in situ hybridization (FISH) or use padlock probes (PLP), both in-situ assays, to characterize single cells for single markers (49). However, when we want to quantify multiple genes or mutations, for example by sequencing, what is really needed are pure CTCs, without contaminating leukocytes. This has recently become feasible by using devices that are able to isolate single CTCs using for example micro-manipulation (e.g., CellCelector), electric fields (DEP-Array) or a punching needle (VyCAP puncher). The promise of these techniques has been demonstrated in DNA from single CTCs from patients with mCRC (50), mCRPC (51) and MBC (52). DNA from these single CTCs is subjected to whole-genome amplification and followed by targeted NGS or even whole-exome sequencing. The analysis of single CTCs in these studies generally demonstrated mutational heterogeneity between CTCs, and characteristics of CTCs seem to be representative of metastatic tumor lesions (51).

INCREASING THE NUMBER OF CTCS SUITABLE FOR CHARACTERIZATION

CTC characterization is not only complex because CTC enrichment methods yield CTC-containing fraction that is not pure. CTCs are also rare cells, and if an increased number of CTCs could be obtained, more cells would be available for characterization. Some have reported on the culture of CTCs to obtain more cells for molecular analysis and to perform direct drug sensitivity analyses on CTC (53-55). While long-term cultures have been achieved by multiple groups, the chance of success is very low, and seems to be limited to patients with very high CTC counts >300 CTCs/7.5 mL (55).

Another method to obtain more cells for analysis is to obtain higher volumes of blood for CTC enrichment. A mathematical extrapolation of increasing blood volume for CTC enrichment to 5 liters predicted that in about 99% of metastatic cancer patients at least one CTC would be detectable (56). Therefore, efforts have been undertaken to perform diagnostic leukapheresis to increase the blood volume (57), which in non-metastatic cancer patients was demonstrated to markedly increase the number of available CTCs for analysis (58). We recently initiated the CIRCLE study, in which the two concepts mentioned in this paragraph will be combined. In this study, diagnostic leukapheresis will be performed to obtain CTCs for long-term culture and drug sensitivity analyses in mCRPC patients.

CIRCULATING TUMOR DNA

As summarized above, CTCs have certain disadvantages, mainly their rarity and complexity for characterization. When CTCs or tumor cells in the tumor themselves go into apoptosis, DNA is released which can be found as cfDNA in the circulation. In patients with primary cancer and metastatic cancer, ctDNA (the tumorous part of cfDNA as a whole) is more frequently detected than CTCs (59, 60). The introduction of several high-throughput technologies has ignited research on cfDNA in the past years, with high potential for clinical utility. This had recently led to FDA-approval of a companion diagnostic EGFR mutation test to identify metastatic lung cancer patients eligible

for treatment with erlotinib (61). While the exact prognostic and predictive relevance of cfDNA is not clear yet, and procedures to process blood for cfDNA analysis and downstream techniques are still being optimized, non-FDA-approved assays like the Guardant360 (62), able to detect mutations in the cfDNA, are currently already offered as commercial assays to patients. The availability of these kind of commercial tests illustrates the enthusiasm in the community to incorporate these techniques into clinical practice.

STANDARDIZATION OF PRE-ANALYTICAL CONDITIONS

The enthusiasm for cfDNA analysis is understandable. Plasma from whole blood can be easily obtained, and downstream techniques to detect ctDNA are relatively user-friendly. The processing of plasma and the use of downstream techniques, however, vary widely at this point. In **Chapter 6**, we demonstrated that plasma DNA isolated from CellSave tubes and BCT tubes 96 hours after the blood draw, have similar cfDNA concentrations and mutation frequencies as plasma isolated from blood one hour after the blood draw. Since blood for CTC enumerations is also collected in CellSave tubes, this means that plasma isolated from CellSave tubes collected for CTC enumeration have optimal cfDNA quality and can be used in the context of multicenter trials.

CHALLENGES OF SEVERAL TECHNIQUES FOR DOWNSTREAM CFDNA ANALYSES

While most groups have used dPCR to detect mutations in cfDNA, the downside of using dPCR-based techniques is that you have to know beforehand what mutations to look for, and that only a limited number of mutations can be assessed. Therefore, assays in which more mutations or whole genes can be assessed are of interest. In **Chapter 7**, we compared several techniques for the detection of ctDNA in mCRC patients undergoing liver metastasectomy. We used a CRC-specific 21-gene NGS panel, which performed well on DNA from tissues of these patients, but had limited sensitivity to detect mutations in the cfDNA of patients. In contrast, dPCR was very sensitive for the detection of mutations in cfDNA. The OnTarget assay, able to assess 96 hotspot mutations, also appeared to be very sensitive for the detection of mutations in cfDNA.

This work further highlighted the pros and cons of current assays for cfDNA analysis. The assay of choice should be based on the research question. When one wants to identify novel mutations, for example related to resistance, NGS remains the only available option. While we observed poor sensitivity using NGS, its sensitivity may be improved by using optimally collected plasma, by the use of other NGS-based techniques using pre-enrichment (e.g., SafeSeqS (63), CAPP-Seq (64)), or by assessing patients with a higher tumor load. Panels with hotspot mutations like the OnTarget assay are especially of interest if the mutation status of the primary tumor or the metastases are unknown. Digital PCR can also be of interest if the mutation status of the tissue is unknown, however, it can only be used if a few specific mutations need to be assessed. For example, in **Chapter 5** we demonstrated that dPCR is able to readily detect 4 *ESR1* hotspot mutations in the cfDNA of MBC patients.

CLINICAL RELEVANCE OF CFDNA ANALYSIS

The efforts as performed by our group provided insight into the optimal pre-analytical conditions, and into the different available choices of assays and their relative strengths and weaknesses. The next step is to unravel the true clinical relevance of cfDNA analysis. As mentioned before, in **Chapter 4** we reviewed current evidence on the clinical relevance of *ESR1* mutations in MBC patients, where we made a case for the use of cfDNA to assess these mutations. In **Chapter 5**, we demonstrated that CellSearch-enriched CTCs were inferior to cfDNA regarding the detection of *ESR1* mutations using dPCR. In addition, we demonstrated that *ESR1* mutations are rarely present in patients starting first-line endocrine therapy, but are enriched in patients progressing on endocrine therapies, further emphasizing the role of *ESR1* mutations in endocrine resistance.

These results have been amongst the first to further substantiate the potential clinical relevance of cfDNA. Clinical utility of cfDNA analyses could be present through various avenues.

First, like *ESR1* mutations, there may be clinical utility by detecting resistance mutations in cfDNA. For instance, a mutation in the *EGFR*, T790M, confers resistance against tyrosine kinase inhibitors (TKIs) in lung cancer patients (65), and patients with an *EGFR* T790M mutation benefit from new third-generation TKIs such as osimertinib (66). Another example is the occurrence of *KRAS* mutations that cause resistance to monoclonal antibodies targeting the *EGFR* (67). Recent evidence has also suggested that upon discontinuation of EGFR-specific antibodies *KRAS* mutations decay and drug sensitivity is regained, meaning that re-challenges with *EGFR*-specific antibodies may be effective (67).

Second, there may be clinical relevance by using cell-free DNA to identify patients suitable for a certain treatment. The above mentioned EGFR mutations for treatment with erlotinib (in case of EGFR exon 19 deletions or L858R mutation) and osimertinib (in case of EGFR T790M mutation) are examples of this. An analysis of 171 patients who were analyzed by the Guardant360 cfDNA assay revealed that in about 40% of the screened patients with various types of cancer, a potential target for therapy was found and suggested that patients could be treated accordingly (62). While it is currently still debated whether these kind of strategies indeed provide true PFS-benefit for patients (68), it is likely that this could offer new treatment possibilities for at least a small subset of these patients.

Third, relapse after surgery may be detected earlier using cfDNA. Several groups have reported on an approach in which the primary tumor is sequenced to identify mutations, and then subsequently “personalized” dPCR assays are made for one or more mutations found in the primary tumor. These personalized dPCR assays are then used to monitor the plasma for ctDNA. This approach was found to be associated with relapse-free survival in relatively small studies of patients after surgery for primary breast cancer (69), colorectal cancer (70, 71) and bladder cancer (72). While this certainly is very exciting data, disadvantages are that these sorts of approaches are labour-intensive and that novel clones or resistant mutations that were not present in the primary tumor are still missed.

Fourth, cfDNA may be used as a surrogate of drug response. When a specific mutation, or multiple mutations, are identified in a patient, these mutations can be sampled longitudinally. To date, most of the studies have been performing longitudinal sampling merely anecdotal, for example as reviewed in **Chapter 4** for *ESR1* mutations, or as done for other personalized mutations in MBC patients (59), in mCRC patients receiving EGFR monoclonal antibodies (67, 73) or in lung cancer patients receiving erlotinib (74).

GOING BEYOND SOMATIC MUTATIONS

Most reports thus far have focused on determining somatic mutations in cfDNA. While it may also be interesting to assess structural variants (75) in cfDNA, most of these options are limited by the fact that you need to know beforehand what abberations to look for. As we demonstrated in **Chapter 7**, NGS targeting multiple genes currently still lacks sensitivity, and hotspot panels (e.g., OnTarget assay) are often tumor-type dependent and limited to hotspot mutations. Another promising alternative to detect ctDNA is to identify tumor-specific copy number alterations (CNAs) using whole-genome sequencing (WGS) techniques rather than to focus on specific point mutations. Two groups have suggested that WGS techniques may be used to detect CNAs in ctDNA (76, 77). However, these techniques were optimized to detect CNAs in the metastatic setting (76) or only when CNAs were as large as whole chromosome arms (77), meaning that more research on the use of CNAs to detect cfDNA will be necessary.

CIRCULATING ENDOTHELIAL CELLS

CTCs and cfDNA are both able to provide important insights into the biology of tumor cells. However, besides the broad spectrum of morphologies, gene expression profiles, and functional roles of tumor cells, also infiltrating lymphocytes, endothelial cells and other stromal cell types from the microenvironment form an integral part of the tumor (78). While the characterization of immune cells and stromal cells are outside of the scope of this thesis, we did focus on endothelial cells that circulate in the blood called CECs. Reports on these CECs have been scarce in the past years, which can be explained by several reasons. First, there is a lack of consensus in the field on how to optimally enumerate CECs. Second, as CEC assays also detect CECs from normal, non-tumor-derived, endothelium, assessing tumor-specific effects is more challenging. Third, the community is treating circulating endothelial progenitor cells (EPC) as a separate entity, while the reported phenotypes of CECs and EPCs often overlap (79). All these factors, combined with the almost sole availability of small and heterogeneous studies, have led to contrasting findings with CECs, limiting the evaluation of CECs as a bona fide biomarker in oncology and thereby its clinical validity.

TOWARDS CLINICAL VALIDITY: VALIDATION OF THE CEC ASSAY

Our group has presented a CEC enumeration assay in which CECs are defined as DRAQ5/DNA+, CD34+, MCAM+ & CD45-, and confirmed the endothelial nature of these cells using morphology, immunohistochemistry and gene expression (80). Using this definition for CECs, in **Chapter 9** we evaluated the prognostic relevance of CECs in patients with recurrent glioblastoma multiforme (GBM) from the BELOB study, who

were randomized to the combination of lomustine/bevacizumab, lomustine alone or bevacizumab alone. We observed that the number of CECs significantly increased in patients receiving lomustine/bevacizumab, but not in patients receiving either of these agents alone, suggesting that lomustine and bevacizumab are synergistic in their ability to induce endothelial damage. In the patients treated with lomustine alone, which is still the agent with the most activity in recurrent glioblastoma (81), an increase in CEC counts was associated with improved OS. These findings have underlined the prognostic potential of CECs. However, in the subgroups of patients randomized to bevacizumab-containing regimens, CEC changes did not have any prognostic relevance, while an endothelial marker would be expected to provide information on the efficacy of anti-angiogenic treatments.

DETECTING TUMOR-SPECIFIC CECS

The fact that CECs represent both the compartment of CECs from normal endothelium as the compartment of CECs from tumor endothelium, may have played a role in the limited prognostic power of CECs in patients receiving bevacizumab and lomustine. Therefore, our group identified a marker, CD276, which can be reliably determined on CECs and which we showed to be tumor-associated (82). The use of CD276 to identify tumor-associated CECs (tCECs) is likely to improve the specificity of the CEC assay to measure CECs from tumor vasculature. We are currently investigating the prognostic relevance of tCECs in the follow-up study of the BELOB study, the EORTC 26101 study, in which patients are randomized to lomustine alone or bevacizumab/lomustine. While the main study did find a longer PFS in patients receiving bevacizumab/lomustine, this PFS benefit did not translate in an OS benefit (81). We hope that the measurement of tCECs will give more insight into which patients are experiencing benefit from bevacizumab/lomustine.

In our mesothelioma study described in **Chapter 8**, we already demonstrated the potential for tCECs as a prognostic marker. In this study 42% of the patients had tCEC levels above the upper limit of normal as observed in healthy donors. In exploratory analysis, a higher number of tCECs appeared to be associated with poorer OS, in contrast to the number of CECs, which was not associated with OS. While these results should be validated in a larger cohort, they might emphasize the added value of measuring tCECs over CECs.

APPLICATION OF CECS OUTSIDE THE FIELD OF ONCOLOGY

While the obtained results with tCECs look promising, it should be noted that CECs can also be used outside of the field of oncology, without the need to specifically identify tumor-associated CECs. CECs have for example been described to play a role in myocardial infarction (83) and in short-term endothelial damage in allogeneic stem cell transplantation (allo-SCT) recipients (84). In **Chapter 10**, we investigated whether the number of CECs during allo-SCT was associated with conditioning regimen-related endothelial damage, and allo-SCT-related complications. We observed that a myeloablative conditioning regimen, which is often given to younger patients, resulted in higher CEC counts for at least 12 months post-transplantation compared to CEC counts in patients receiving a reduced-intensity conditioning regimen. This suggests that patients receiving myeloablative conditioning have prolonged endothelial damage,

which might be related to long-term cardiovascular outcome, meaning that further studies on this association are warranted. In addition, we observed that CEC count were lower in patients who experienced graft-versus-host disease (GVHD), and that CECs express HLA class I and HLA class II antigens, leading to the hypothesis that the lower CEC numbers in patients who had GVHD may be caused by an alloreactive immune response against CECs.

FUTURE PERSPECTIVES

This thesis provided more insight into the technical aspects and possible clinical relevance of the liquid biopsies CTCs, cfDNA and CECs in cancer patients. Regarding CTC enumeration, we should keep in mind that although CTC enumeration by the CellSearch system has been thoroughly validated in almost every epithelial-type cancer and is already on the market for quite some time, its use is still limited to small groups of patients. If CTC enumeration is to reach its full potential, it is crucial that new studies evaluating CTC enumeration in PB of patients with epithelial tumors should be focused on the clinical applicability of CTC enumeration, preferably in the context of an interventional trial. An exception may be the enumeration of CTCs in bodily fluids other than PB, for example in PE as described above, or in cerebrospinal fluid (85), in which CTC enumeration is not validated as well as in PB and seems promising because CTC enumeration may be more sensitive as currently used methods by the pathologist. In addition, the enumeration of CTCs in patients with mesenchymal tumors is worth further investigation.

The characterization of CTCs, over just counting CTCs, is likely to significantly broaden the clinical utility of CTCs. The AR-V7 results, for example, are likely to be practice-changing in due time, and underline the potential of CTC characterization to have real consequences for clinical decision-making. A recent cost-savings analysis of AR-V7 testing revealed that upfront testing of the AR-V7 status in mCRPC patients could potentially save $150 Million in the USA per year (86), also emphasizing the prospect of optimizing cost-effectiveness in cancer medicine by using liquid biopsies to personalize treatment. Unfortunately, the results obtained in this thesis regarding CTC characterization in MBC patients on the protein level (HER2), RNA level (*ESR1* expression and splice variants) and DNA level (*ESR1* mutations) did not point towards direct clinical utility of CTC characterization in MBC patients; what is more, cfDNA analysis was superior to CTC characterization for detecting *ESR1* mutations. For the *ESR1* mutations, this certainly means that cfDNA should be used over CTCs. Does this mean that CTC characterization is futile? Certainly not. Still, CTCs remain the only substrate in which characterization can be performed at multiple dimensions. An extremely important marker for endocrine resistance such as the AR-V7 splice variant can never be measured at the (cf)DNA level (unless there is an associated mutational pattern), and the same goes for important targets for therapy that should be measured at the protein level, such as ER. In addition, at a more fundamental level, one could argue that a fragment from an apoptotic tumor cell, does not tell you anything about the biology of the living tumor (87).

Obtaining single and/or pure CTCs will, however, be necessary to conquer a lot of the problems now encountered with CTC characterization. When pure CTCs are obtained, without leukocyte characterization, this allows for more reliable RNA and DNA analysis. Furthermore, heterogeneity between single CTCs could be analyzed, as techniques sequencing DNA and mRNA from the same single cell have recently become feasible (88). These sorts of strategies will provide import insights into the biology and subclonal diversity of CTCs, and will especially be interesting to track during treatment with anti-cancer agents.

The characterization of cfDNA is likely to be complementary to single CTC analyses. First, not in every patient CTCs are detected, and while a correlation between higher CTC counts and cfDNA presence has been reported, cfDNA is still generally more often detected than CTCs (59). This will especially play a role in applications for early detection of relapse, a setting in which CTCs are rare. In this setting of minimal residual detection, cfDNA analyses are likely to offer more than CTCs, especially when techniques such as the described CNA assay to detect ctDNA are further optimized. In addition, these sorts of strategies provide opportunities in patients with mesenchymal or EpCAM-negative tumors.

Second, it is now unknown if a single CTC resolution is always necessary, and whether that really adds to its clinical utility. If measuring a single mutation or mutational panel on cfDNA provides the clinician with enough information to direct a patient towards a certain treatment, or to evaluate drug response, this may just be enough. Single CTCs could then be used to analyze certain subclones in more detail.

Third, while early studies on single CTCs suggested that most mutations from metastases are represented in the exome of CTCs (51), the question remains whether characterization of only a few CTCs, in particular in patients with low CTC counts, will represent the whole genomic landscape of the tumor.

Lastly, we should keep in mind that the characterization of single CTCs will probably be more labor-intensive and in need of more specialized personnel and instruments than the characterization of cfDNA. For cfDNA characterization, blood collected in a CellSave tube can be sent to a diagnostics lab for processing of plasma within 96 hours, and subsequently downstream analyses such as digital PCR or NGS can be performed swiftly, and these techniques are available in most research laboratories. For characterization of single CTCs, blood can similarly be collected, but then first an enrichment step will be needed, followed by staining for CTC markers, followed by the collection of the single CTCs, and if multiple single CTCs are then subsequently analyzed, lots of data will become available. For RNA analyses on single CTCs, the blood will have to shipped to the lab within 24 hours, as quick isolation of single CTCs will be necessary to obtain the highest quality of RNA. In addition, it should be realized that an apparatus especially for the isolation of these single CTCs is necessary, which is not readily available in most research labs. While all the work to obtain single CTCs will be feasible in a dedicated CTC research lab (such as the lab where the work for this thesis was performed), it can be imagined that the majority of other labs will go with cfDNA characterization, given the above mentioned advantages of its analysis.

The overflow of data on CTCs and cfDNA raises the question if there is a place for CEC enumeration in clinical oncology. At this point, we do not know what its place will be. The small study on tCECs in mesothelioma patients as presented in this thesis is one of the first studies in which we were able to measure CECs coming from the tumor vasculature, and this is a big step forward. If there is a place for CECs, it will probably be in patients receiving anti-cancer agents targeting the vasculature or in patients with highly vascularized tumor types (such as mesothelioma), however, studies on the clinical relevance of tumor-associated CECs in these patients are urgently needed.

In conclusion, the characterization of single CTCs along with complementary cfDNA analyses is likely to play an important role in clinical oncology in due time. For single CTC research, it will be vital to establish pipelines for optimal DNA and RNA characterization of single CTCs. Future research on cfDNA should focus on optimizing cfDNA detection methods to be able to overcome the current problem of being limited by gene panels or the detection of single mutations. This could be done by improving NGS techniques so that more genes (or even whole genomes) can be sequenced for detecting somatic mutations, or by measuring CNAs to detect ctDNA, depending on the research question. Eventually, it will be of utmost importance to establish the true clinical relevance of both single CTCs and cfDNA, to see in which cases one of these substrates will be superior to the other, and to see where these substrates can complement each other. Liquid biopsies will help us to an improved understanding of heterogeneity and plasticity of cancer, and ultimately this information along with its clinical use is going to strike a major blow in the war on cancer.

REFERENCES

1. Sullivan R, Peppercorn J, Sikora K, Zalcberg J, Meropol NJ, Amir E, et al. Delivering affordable cancer care in high-income countries. Lancet Oncol. 2011 Sep;12(10):933-80.

2. Luengo-Fernandez R, Leal J, Gray A, Sullivan R. Economic burden of cancer across the European Union: a population-based cost analysis. Lancet Oncol. 2013 Nov;14(12):1165-74.

3. KWF Kankerbestrijding. Effective new anti-cancer drugs, But the funding system is creaking at the seams. 2015.

4. Frank M, Mittendorf T. Influence of pharmacogenomic profiling prior to pharmaceutical treatment in metastatic colorectal cancer on cost effectiveness : a systematic review. Pharmacoeconomics. 2013 Mar;31(3):215-28.

5. Behl AS, Goddard KA, Flottemesch TJ, Veenstra D, Meenan RT, Lin JS, et al. Cost-effectiveness analysis of screening for KRAS and BRAF mutations in metastatic colorectal cancer. J Natl Cancer Inst. 2012 Dec 5;104(23):1785-95.

6. Janni WJ, Rack B, Terstappen LW, Pierga JY, Taran FA, Fehm T, et al. Pooled Analysis of the Prognostic Relevance of Circulating Tumor Cells in Primary Breast Cancer. Clin Cancer Res. 2016 May 15;22(10):2583-93.

7. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Prospective Validation of a 21-Gene Expression Assay in Breast Cancer. N Engl J Med. 2015 Nov 19;373(21):2005-14.

8. Cardoso F, van't Veer LJ, Bogaerts J, Slaets L, Viale G, Delaloge S, et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. N Engl J Med. 2016 Aug 25;375(8):717-29.

9. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014 Apr;15(4):406-14.

10. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol. 2014 Nov 1;32(31):3483-9.

11. Bidard F-CC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabières C, Janni W, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer metastasis reviews. 2013;32(1-2):179-88.

12. Beije N, Sleijfer S, Boormans JL. De rol van circulerende tumorcellen bij het urotheelcarcinoom van de blaas. Tijdschrift voor Urologie. 2015(Nov).

13. Satelli A, Mitra A, Cutrera JJ, Devarie M, Xia X, Ingram DR, et al. Universal marker and detection tool for human sarcoma circulating tumor cells. Cancer Res. 2014 Mar 15;74(6):1645-50.

14. Karabacak NM, Spuhler PS, Fachin F, Lim EJ, Pai V, Ozkumur E, et al. Microfluidic, marker-free isolation of circulating tumor cells from blood samples. Nat Protoc. 2014 Mar;9(3):694-710.

15. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, Miyamoto DT, Brachtel E, et al. Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. Sci Transl Med. 2013 Apr 3;5(179):179ra47.

16. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating Breast Tumor Cells Exhibit Dynamic Changes in Epithelial and Mesenchymal Composition. Science. 2013 Feb 1;339(6119):580-4.

17. Satelli A, Brownlee Z, Mitra A, Meng QH, Li S. Circulating tumor cell enumeration with a combination of epithelial cell adhesion molecule- and cell-surface vimentin-based methods for monitoring breast cancer therapeutic response. Clin Chem. 2015 Jan;61(1):259-66.

18. Satelli A, Mitra A, Brownlee Z, Xia X, Bellister S, Overman MJ, et al. Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression. Clin Cancer Res. 2015 Feb 15;21(4):899-906.

19. Mostert B, Kraan J, Bolt-de Vries J, van der Spoel P, Sieuwerts AM, Schutte M, et al. Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146. Breast Cancer Res Treat. 2011 May;127(1):33-41.

20. Onstenk W, Kraan J, Mostert B, Timmermans MM, Charehbili A, Smit VT, et al. Improved Circulating Tumor Cell Detection by a Combined EpCAM and MCAM CellSearch Enrichment Approach in Patients with Breast Cancer Undergoing Neoadjuvant Chemotherapy. Mol Cancer Ther. 2015 Mar;14(3):821-7.

21. Bidlingmaier S, He J, Wang Y, An F, Feng J, Barbone D, et al. Identification of MCAM/CD146 as the Target Antigen of a Human Monoclonal Antibody that Recognizes Both Epithelioid and Sarcomatoid Types of Mesothelioma. Cancer research. 2009;69.

22. Meng S, Tripathy D, Shete S, Ashfaq R, Haley B, Perkins S, et al. HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci U S A. 2004 Jun 22;101(25):9393-8.

23. Fehm T, Becker S, Duerr-Stoerzer S, Sotlar K, Mueller V, Wallwiener D, et al. Determination of HER2 status using both serum HER2 levels and circulating tumor cells in patients with recurrent breast cancer whose primary tumor was HER2 negative or of unknown HER2 status. Breast Cancer Res. 2007;9(5):R74.

24. Tewes M, Aktas B, Welt A, Mueller S, Hauch S, Kimmig R, et al. Molecular profiling and predictive value of circulating tumor cells in patients with metastatic breast cancer: an option for monitoring response to breast cancer related therapies. Breast Cancer Res Treat. 2009 Jun;115(3):581-90.

25. Pestrin M, Bessi S, Galardi F, Truglia M, Biggeri A, Biagioni C, et al. Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients. Breast Cancer Res Treat. 2009 Dec;118(3):523-30.

26. Munzone E, Nole F, Goldhirsch A, Botteri E, Esposito A, Zorzino L, et al. Changes of HER2 status in circulating tumor cells compared with the primary tumor during treatment for advanced breast cancer. Clin Breast Cancer. 2010 Oct 1;10(5):392-7.

27. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res. 2011 Jun 1;17(11):3600-18.

28. Ignatiadis M, Rothe F, Chaboteaux C, Durbecq V, Rouas G, Criscitiello C, et al. HER2-positive circulating tumor cells in breast cancer. PloS one. 2011;6(1):e15624.

29. Pestrin M, Bessi S, Puglisi F, Minisini AM, Masci G, Battelli N, et al. Final results of a multicenter phase II clinical trial evaluating the activity of single-agent lapatinib in patients with HER2-negative metastatic breast cancer and HER2-positive circulating tumor cells. A proof-of-concept study. Breast Cancer Res Treat. 2012 Jul;134(1):283-9.

30. Hayashi N, Nakamura S, Tokuda Y, Shimoda Y, Yagata H, Yoshida A, et al. Prognostic value of HER2-positive circulating tumor cells in patients with metastatic breast cancer. Int J Clin Oncol. 2012 Apr;17(2):96-104.

31. Nadal R, Fernandez A, Sanchez-Rovira P, Salido M, Rodriguez M, Garcia-Puche JL, et al. Biomarkers characterization of circulating tumour cells in breast cancer patients. Breast Cancer Res. 2012;14(3):R71.

32. Onstenk W, Gratama J, Foekens J, Sleijfer S. Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. Cancer Treat Rev. 2013;39(7):691-700.

33. Liu Y, Liu Q, Wang T, Bian L, Zhang S, Hu H, et al. Circulating tumor cells in HER2-positive metastatic breast cancer patients: a valuable prognostic and predictive biomarker. BMC Cancer. 2013;13:202.

34. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, et al. Development of Circulating Tumor Cell-Endocrine Therapy Index in Patients with Hormone Receptor Positive Breast Cancer. Clin Cancer Res. 2014 Nov 7.

35. Onstenk W, Sieuwerts AM, Weekhout M, Mostert B, Reijm EA, van Deurzen CH, et al. Gene expression profiles of circulating tumor cells versus primary tumors in metastatic breast cancer. Cancer Lett. 2015 Jun 28;362(1):36-44.

36. Wallwiener M, Hartkopf AD, Riethdorf S, Nees J, Sprick MR, Schonfisch B, et al. The impact of HER2 phenotype of circulating tumor cells in metastatic breast cancer: a retrospective study in 107 patients. BMC Cancer. 2015;15(1):403.

37. CareMoreCTC studies. http://www.caremorectc.eu/.

38. Krop IE, Kim SB, Gonzalez-Martin A, LoRusso PM, Ferrero JM, Smitt M, et al. Trastuzumab emtansine versus treatment of physician's choice for pretreated HER2-positive advanced breast cancer (TH3RESA): a randomised, open-label, phase 3 trial. Lancet Oncol. 2014 Jun;15(7):689-99.

39. Krop IE, Lin NU, Blackwell K, Guardino E, Huober J, Lu M, et al. Trastuzumab emtansine (T-DM1) versus lapatinib plus capecitabine in patients with HER2-positive metastatic breast cancer and central nervous system metastases: a retrospective, exploratory analysis in EMILIA. Ann Oncol. 2015 Jan;26(1):113-9.

40. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer. Clin Cancer Res. 2015 Jun 1;21(11):2487-98.

41. Mostert B, Sieuwerts AM, Bolt-de Vries J, Kraan J, Lalmahomed Z, van Galen A, et al. mRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients. Molecular Oncology. 2015 Apr;9(4):920-32.

42. Onstenk W, Sieuwerts AM, Mostert B, Lalmahomed Z, Bolt-de Vries JB, van Galen A, et al. Molecular characteristics of circulating tumor cells resemble the liver metastasis more closely than the primary tumor in metastatic colorectal cancer. Oncotarget. 2016 Jun 20.

43. Mostert B, Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, van Galen A, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. Ann Oncol. 2015 Mar;26(3):510-6.

44. Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med. 2014 Sep 11;371(11):1028-38.

45. Onstenk W, Sieuwerts AM, Kraan J, Van M, Nieuweboer AJM, Mathijssen RHJ, et al. Efficacy of Cabazitaxel in Castration-resistant Prostate Cancer Is Independent of the Presence of AR-V7 in Circulating Tumor Cells. European urology. 2015 Dec;68(6):939-45.

46. Scher HI, Lu D, Schreiber NA, Louw J, Graf RP, Vargas HA, et al. Association of AR-V7 on Circulating Tumor Cells as a Treatment-Specific Biomarker With Outcomes and Survival in Castration-Resistant Prostate Cancer. JAMA Oncol. 2016 Jun 4.

47. Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Nakazawa M, et al. Androgen Receptor Splice Variant 7 and Efficacy of Taxane Chemotherapy in Patients With Metastatic Castration-Resistant Prostate Cancer. JAMA Oncol. 2015 Aug;1(5):582-91.

48. Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-de Vries J, Biermann K, et al. KRAS and BRAF mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue. Int J Cancer. 2013 Jul;133(1):130-41.

49. Swennenhuis JF, Tibbe AG, Levink R, Sipkema RC, Terstappen LW. Characterization of circulating tumor cells by fluorescence in situ hybridization. Cytometry A. 2009 Jun;75(6):520-7.

50. Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, et al. Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. Cancer Res. 2013 May 15;73(10):2965-75.

51. Lohr JG, Adalsteinsson VA, Cibulskis K, Choudhury AD, Rosenberg M, Cruz-Gordillo P, et al. Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. Nat Biotechnol. 2014 May;32(5):479-84.

52. Shaw JA, Guttery DS, Hills A, Fernandez-Garcia D, Page K, Rosales BM, et al. Mutation analysis of cell-free DNA and single circulating tumor cells in metastatic breast cancer patients with high CTC counts. Clin Cancer Res. 2016 Jun 22.

53. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science. 2014 Jul 11;345(6193):216-20.

54. Zhang L, Ridgway L, Wetzel M, Ngo J, Yin W, Kumar D, et al. The identification and characterization of breast cancer CTCs competent for brain metastasis. Science translational medicine. 2013;5(180).

55. Cayrefourcq L, Mazard T, Joosse S, Solassol J, Ramos J, Assenat E, et al. Establishment and characterization of a cell line from human circulating colon cancer cells. Cancer Res. 2015 Mar 1;75(5):892-901.

56. Coumans FA, Ligthart ST, Uhr JW, Terstappen LW. Challenges in the enumeration and phenotyping of CTC. Clin Cancer Res. 2012 Oct 15;18(20):5711-8.

57. Stoecklein NH, Fischer JC, Niederacher D, Terstappen LW. Challenges for CTC-based liquid biopsies: low CTC frequency and diagnostic leukapheresis as a potential solution. Expert Rev Mol Diagn. 2016;16(2):147-64.

58. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. Proc Natl Acad Sci U S A. 2013 Oct 8;110(41):16580-5.

59. Dawson SJ, Rosenfeld N, Caldas C. Circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013 Jul 4;369(1):93-4.

60. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014 Feb 19;6(224):224ra24.

61. FDA approves first blood test to detect gene mutation associated with non-small cell lung cancer. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm504488.htm.

62. Schwaederle M, Husain H, Fanta PT, Piccioni DE, Kesari S, Schwab RB, et al. Detection rate of actionable mutations in diverse cancers using a biopsy-free (blood) circulating tumor cell DNA assay. Oncotarget. 2016 Mar 1;7(9):9707-17.

63. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011 Jun 7;108(23):9530-5.

64. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014 May;20(5):548-54.

65. Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, et al. Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. Nat Med. 2015 Jun;21(6):560-2.

66. Janne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. N Engl J Med. 2015 Apr 30;372(18):1689-99.

67. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med. 2015 Jul;21(7):827.

68. Le Tourneau C, Delord JP, Goncalves A, Gavoille C, Dubot C, Isambert N, et al. Molecularly targeted therapy based on tumour molecular profiling versus conventional therapy for advanced cancer (SHIVA): a multicentre, open-label, proof-of-concept, randomised, controlled phase 2 trial. Lancet Oncol. 2015 Oct;16(13):1324-34.

69. Garcia-Murillas I, Schiavon G, Weigelt B, Ng C, Hrebien S, Cutts RJ, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med. 2015 Aug 26;7(302):302ra133.

70. Reinert T, Scholer LV, Thomsen R, Tobiasen H, Vang S, Nordentoft I, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2015 Feb 4.

71. Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med. 2016 Jul 6;8(346):346ra92.

72. Birkenkamp-Demtroder K, Nordentoft I, Christensen E, Hoyer S, Reinert T, Vang S, et al. Genomic Alterations in Liquid Biopsies from Patients with Bladder Cancer. Eur Urol. 2016 Jul;70(1):75-82.

73. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012 Jun 28;486(7404):532-6.

74. Mok T, Wu YL, Lee JS, Yu CJ, Sriuranpong V, Sandoval-Tan J, et al. Detection and Dynamic Changes of EGFR Mutations from Circulating Tumor DNA as a Predictor of Survival Outcomes in NSCLC Patients Treated with First-line Intercalated Erlotinib and Chemotherapy. Clin Cancer Res. 2015 Jul 15;21(14):3196-203.

75. McBride DJ, Orpana AK, Sotiriou C, Joensuu H, Stephens PJ, Mudie LJ, et al. Use of cancer-specific genomic rearrangements to quantify disease burden in plasma from patients with solid tumors. Genes Chromosomes Cancer. 2010 Nov;49(11):1062-9.

76. Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med. 2012 Nov 28;4(162):162ra54.

77. Jiang P, Chan CW, Chan KC, Cheng SH, Wong J, Wong VW, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci U S A. 2015 Mar 17;112(11):E1317-25.

78. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

79. Danova M, Comolli G, Manzoni M, Torchio M, Mazzini G. Flow cytometric analysis of circulating endothelial cells and endothelial progenitors for clinical purposes in oncology: A critical evaluation. Mol Clin Oncol. 2016 Jun;4(6):909-17.

80. Kraan J, Strijbos MH, Sieuwerts AM, Foekens JA, den Bakker MA, Verhoef C, et al. A new approach for rapid and reliable enumeration of circulating endothelial cells in patients. J Thromb Haemost. 2012 May;10(5):931-9.

81. Wolfgang W, Brandes AA, Gorlia T, Bendszus M, Sahm F, Taal W, et al. EORTC 26101 phase III trial exploring the combination of bevacizumab and lomustine in patients with first progression of a glioblastoma. J Clin Oncol. 2016;34:(suppl; abstr 2001).

82. Kraan J, van den Broek P, Verhoef C, Grunhagen DJ, Taal W, Gratama JW, et al. Endothelial CD276 (B7-H3) expression is increased in human malignancies and distinguishes between normal and tumour-derived circulating endothelial cells. Br J Cancer. 2014 Jul 8;111(1):149-56.

83. Damani S, Bacconi A, Libiger O, Chourasia AH, Serry R, Gollapudi R, et al. Characterization of circulating endothelial cells in acute myocardial infarction. Sci Transl Med. 2012 Mar 21;4(126):126ra33.

84. Woywodt A, Scheer J, Hambach L, Buchholz S, Ganser A, Haller H, et al. Circulating endothelial cells as a marker of endothelial damage in allogeneic hematopoietic stem cell transplantation. Blood. 2004 May 1;103(9):3603-5.

85. Lee JS, Melisko ME, Magbanua MJ, Kablanian AT, Scott JH, Rugo HS, et al. Detection of cerebrospinal fluid tumor cells and its clinical relevance in leptomeningeal metastasis of breast cancer. Breast Cancer Res Treat. 2015 Nov;154(2):339-49.

86. Markowski MC, Frick KD, Eshleman JR, Luo J, Antonarakis ES. Cost-savings analysis of AR-V7 testing in patients with metastatic castration-resistant prostate cancer eligible for treatment with abiraterone or enzalutamide. Prostate. 2016 Jul 12.

87. Inside the race to diagnose cancer from a simple blood draw. Stat News https://wwwstatnewscom/2016/03/21/cancer-liquid-biopsy/.

88. Dey SS, Kester L, Spanjaard B, Bienko M, van Oudenaarden A. Integrated genome and transcriptome sequencing of the same cell. Nat Biotechnol. 2015 Mar;33(3):285-9.

APPENDICES

NEDERLANDSE

SAMENVATTING

INTRODUCTIE

Kanker is de belangrijkste doodsoorzaak in Nederland. In de laatste decennia zijn belangrijke succesverhalen te vertellen wat betreft de behandeling van kanker, bijvoorbeeld de genezing van allerlei soorten kankers die voorkomen in kinderen, lymfeklierkanker en zaadbalkanker. Daarnaast is er ook sprake geweest van een verbeterde vroege detectie van kanker en behandeling na de operatie (adjuvante therapie), welke hebben geleid tot een verbetering in de 5-jaars overleving van patiënten met kanker. Echter, ondanks al deze verbeteringen, blijft kanker de belangrijkste doodsoorzaak in de westerse samenleving, en blijft verdere voortgang dus hard nodig.
In de afgelopen decennia hebben we veel geleerd over de achterliggende oorzaken die het zo moeilijk maken om kanker te behandelen. Allereerst moet men zich realiseren dat patiënten met kanker niet zo zeer overlijden aan de tumor die het eerst aanwezig is (primaire tumor), maar aan het optreden van uitzaaiingen op afstand vanuit deze primaire tumor. De huidige behandeling van patiënten met kanker is over het algemeen gebaseerd op de karakteristieken van de primaire tumor. Jaren aan onderzoek heeft ons echter geleerd dat tumorcellen plastisch zijn: hun eigenschappen veranderen naar mate de tijd vordert en onder druk van anti-tumor behandelingen. Daarnaast hebben we geleerd dat tumorcellen niet allemaal hetzelfde zijn: als een tumor cel voor cel wordt bekeken, blijken er zelfs belangrijke verschillen te zijn (heterogeniteit) tussen elke individuele cel in een tumor. Dit betekent dat het erg onwaarschijnlijk is dat een enkel anti-kankermiddel een effect zal sorteren op álle kankercellen in een tumor, hetgeen genezing dus ook onwaarschijnlijker maakt, vooral in patiënten met uitgebreidere, uitgezaaide ziekte.

Al deze inzichten laten zien dat er behoefte is aan manieren om de diagnosestelling, prognosebepaling en predictie van behandelsucces te verbeteren in patiënten met kanker, en dan het liefst voor, tijdens en na de therapie. Het biopteren van uitzaaiingen zou een optie kunnen zijn om naar de plasticiteit en heterogeniteit van kankercellen te kijken. Echter is dit een pijnlijke en soms moeilijke procedure, die realistisch gezien niet regelmatig uitgevoerd kan worden. Daarnaast krijgt men hiermee maar het beeld van één plek in één uitzaaiing. Daarom is er grote behoefte aan manieren om toch inzicht te krijgen in de eigenschappen van de kankercellen, die minder belastend zijn voor de patiënt. Zo'n manier is het nemen en karakteriseren van zogenaamde "vloeibare biopsies".

VLOEIBARE BIOPSIES

Met een 'vloeibare biopsie' wordt bedoeld dat door middel van een relatief simpele ingreep zoals een bloedafname, er een bron wordt verkregen waaruit we meer te weten kunnen komen over de eigenschappen van de tumor van een patiënt. Dit proefschrift heeft zich gericht op drie soorten vloeibare biopsies: circulerende tumorcellen (CTCs), circulerend tumor DNA (ctDNA) en circulerende endotheelcellen (CECs). De volgende paragrafen zullen deze vloeibare biopsies verder toelichten, en het werk zoals gedaan in dit proefschrift verder in perspectief zetten.

CIRCULERENDE TUMORCELLEN

CTCs zijn kankercellen van een tumor die in het bloed terecht komen en vervolgens uitzaaiingen in andere organen kunnen vormen. Er bestaan allerlei technieken om deze tumorcellen uit het bloed of uit andere lichaamsvochten te isoleren, waarvan de meest gebruikte techniek de 'CellSearch' machine is. Deze machine maakt gebruikt van magnetische bolletjes die gekoppeld zijn aan een EpCAM antilichaam. EpCAM komt tot expressie op tumorcellen, hetgeen betekent dat die tumorcellen in bloed aan het EpCAM antilichaam gekoppeld aan een magnetisch bolletje binden, en zo met een magneet uit het bloed gehaald kunnen worden. Met behulp van deze techniek worden in ongeveer 60% van de patiënten met uitgezaaide kanker CTCs aangetroffen. Vervolgens zijn verschillende opties: 1) het aantal CTCs kan simpelweg geteld worden, waarvan we weten dat dit sterk gecorreleerd is met de prognose van patiënten met epitheliale uitgezaaide kanker zoals borstkanker, prostaatkanker en dikke darmkanker; of 2) de erfelijke (genetische) eigenschappen (eiwit, DNA en RNA) van de CTCs kunnen verder geanalyseerd worden.

TELLEN VAN CTCS

Het tellen van CTCs is goedgekeurd door de Amerikaanse Food and Drug Administration voor diagnostisch gebruik in patiënten met uitgezaaide borstkanker, prostaatkanker en dikke darmkanker. Echter wordt het gebruik hiervan in de kliniek momenteel niet aangeraden in de ESMO (Europese Oncologie Organisatie) of ASCO (Amerikaanse Oncologie Organisatie) richtlijnen voor deze tumortypes. In **hoofdstuk 2** van dit proefschrift werden aanbevelingen gedaan voor het gebruik van CTC tellingen in patiënten met borstkanker. In patiënten met borstkanker die niet is uitgezaaid, vonden we onvoldoende bewijs voor het gebruik van CTC tellingen. In patiënten met uitgezaaide borstkanker kunnen CTC tellingen echter toegepast worden in twee klinische situaties: 1) in patiënten met slecht evalueerbare ziekte, zoals patiënten waarbij de uitzaaiingen en het effect van de behandeling op de uitzaaiingen slecht in kaart te brengen zijn met gebruikelijke technieken zoals CT-scans, en 2) als marker voor vroeg succes op anti-kankertherapie, hetgeen kan leiden tot een snellere beoordeling van de efficiëntie van nieuwe anti-kankermedicijnen, al 3-4 weken na start van de behandeling.

Ondanks dat er dus toepassingen zijn van CTC tellingen, gelden deze toepassingen helaas maar voor kleine groepen patiënten. Daarnaast is een probleem dat de CellSearch techniek om CTCs te tellen niet werkt voor alle soorten tumoren. Dit is bijvoorbeeld het geval bij patiënten met asbestkanker (maligne pleuraal mesothelioom), waarbij de tumor nauwelijks EpCAM tot expressie brengt, waardoor deze tumorcellen niet gevangen worden met de CellSearch machine. Daarom heeft onze groep een andere techniek ontwikkelt, waarbij tumorcellen niet met EpCAM worden gevangen, maar met MCAM. In **hoofdstuk 8** werd onderzocht of met deze techniek CTCs konden worden gedetecteerd in het bloed en het pleuravocht ('vocht achter de longen') van patiënten met asbestkanker. In 26% van de patiënten werden CTCs in het bloed gevonden, echter bleek het aanwezig zijn van deze CTCs niet van prognostische waarde. De detectie van CTCs in het pleuravocht was superieur aan de huidige klinische standaard (beoordeling door de patholoog van het pleuravocht). Dit betekent dat in de toekomst de diagnosestelling van asbestkanker mogelijk verbeterd kan worden met behulp van onze techniek.

ANALYSE VAN GENETISCHE EIGENSCHAPPEN VAN CTCS

Het tellen van CTCs is een erg beperkt gebruik van CTCs. CTCs worden slechts geteld, terwijl zij ook allerlei informatie bezitten over de genetische eigenschappen van de tumorcellen. Daarom is in dit proefschrift tevens uitgebreid aandacht besteed aan het bepalen van de genetische eigenschappen van de CTCs.

In **hoofdstuk 3** werd gekeken naar de HER2-status van de CTCs in patiënten met uitgezaaide borstkanker. HER2 is, indien uitgebreid aanwezig op de tumorcel, een receptor die betrokken is bij de groei van een tumorcel, en is een belangrijk doelwit voor therapie met de bestaande anti-HER2 therapieën. Zoals eerder gezegd, zijn tumoren plastisch, en kunnen eigenschappen zoals de HER2 status over de tijd of onder invloed van anti-kanker behandelingen veranderen. Wij onderzochten of HER2-positieve CTCs voorkwamen in patiënten met een HER2-negatieve primaire tumor, en in hoeverre dit van invloed was resistentie op hormonale therapie, aangezien patiënten met een HER2-positieve primaire tumor een slechtere uitkomst hebben op hormonale therapie. We vonden dat HER2-positieve CTCs voorkwamen in 29% van alle patiënten (n=154) in deze studie. Het voorkomen van deze HER2-positieve CTCs bleek echter geen relatie te hebben met de uitkomst op hormonale therapie, en ook niet op de uitkomst op chemotherapie zoals geanalyseerd in een controlegroep.

Het feit dat HER2-positieve CTCs voorkomen in zo'n grote groep patiënten met een HER2-negatieve primaire tumor, biedt opties om deze patiënten te behandelen met anti-HER2 therapie. Daarom zijn we recent een studie gestart waarin patiënten met HER2-positieve CTCs, maar een HER2-negatieve primaire tumor, behandeld worden met anti-HER2 therapie. Het doen van dit soort studies is van het grootste belang om uiteindelijk de vraag te beantwoorden of patiënten met HER2-positieve CTCs inderdaad baat hebben bij therapie met anti-HER2 middelen.

Naast HER2 is een ander belangrijk doelwit voor therapie in patiënten met uitgezaaide borstkanker de oestrogeen receptor. Patiënten met een oestrogeen receptor-positieve primaire tumor, krijgen hormonale therapie voorgeschreven. Dit is een behandeling waar een hoop patiënten baat bij hebben, met relatief milde bijwerkingen. In 40% van de patiënten met uitgezaaide borstkanker treedt echter direct resistentie tegen de behandeling op, en in de rest van de patiënten zal resistentie uiteindelijk ook altijd optreden. In dit proefschrift werd daarom onderzoek gedaan naar meerdere mechanismen die te maken hebben met resistentie op hormonale therapie.

Eén van de reden voor resistentie op hormonale therapie zou kunnen zijn het verlies van expressie van de oestrogeen receptor in de uitgezaaide tumorcellen. In **hoofdstuk 3** werd gekeken naar discrepanties tussen de oestrogeen receptor-status van de primaire tumor, en die van de CTCs. In 25% van de patiënten bleek de oestrogeen receptor-status van de primaire tumor anders dan die van de CTCs, hetgeen overigens geen invloed had op de prognose van deze patiënten in exploratieve analyses. Interessant was de bevinding dat veel patiënten met een ER-negatieve primaire tumor, ER-positieve CTCs bleken te hebben. Dit zou kunnen betekenen dat deze patiënten baat zouden kunnen hebben bij hormonale therapie, hetgeen in nieuw onderzoek bewezen zal moeten worden.

Een andere reden voor resistentie op hormonale therapie zou het optreden van mutaties in de oestrogeen receptor kunnen zijn. In **hoofdstuk 4** werd verder uiteengezet wat we op dit moment weten van deze zogenaamde "*ESR1* mutaties". Deze *ESR1* mutaties zijn nauwelijks aanwezig in de primaire tumor van patiënten met uitgezaaide borstkanker, maar treden vaak op in patiënten met uitgezaaide borstkanker die eerder hormonale therapie hebben gekregen. Dit betekent dat het voorkomen van deze mutaties onderzocht zal moeten worden in uitgezaaide tumorcellen, waarbij vloeibare biopsies natuurlijk interessant zijn. In **hoofdstuk 5** onderzochten we of deze *ESR1* mutaties te detecteren waren in CTCs. Deze mutaties bleken te detecteren in CTCs, echter bleek detectie in een andere vloeibaar biopsie, namelijk circulerend tumor DNA, veel gevoeliger dan in de CTCs (daarover meer in de paragraaf over circulerend tumor DNA). We denken dat dit komt doordat bij de manier van isoleren van CTCs ook witte bloedcellen worden geïsoleerd. Deze witte bloedcellen bevatten ook DNA en komen terecht in de CTC-verrijkte fractie die geanalyseerd wordt. Omdat dit DNA geen tumor-afwijkingen bevat, wordt het tumor-specifieke DNA uitgedund door dit normale DNA, waardoor de detectie van tumormutaties bemoeilijkt wordt.

In **hoofdstuk 5** werd ook een ander mechanisme van resistentie op hormonale therapie onderzocht. We keken of bepaalde splice varianten van de oestrogeen receptor, dit zijn RNA varianten die er mogelijk voor zorgen dat er een veranderde oestrogeen receptor tot expressie komt dan normaal, voorkomen in CTCs van patiënten met uitgezaaide borstkanker. We vonden dat één splice variant, de Δ5 splice variant, die eerder al geassocieerd werd met resistentie op hormonale therapie, vaker voorkomt in CTCs van patiënten dan in gezonde donoren. Er werd echter geen verrijking van deze splice variant gezien in patiënten die hormonale therapie hadden gekregen. Opvallend was dat alle gemeten splice varianten in onze studie ook tot expressie kwamen in de CTC-verrijkte fracties van gezonde donoren (terwijl zij geen CTCs hebben), hetgeen de complexiteit van het bepalen van genetische eigenschappen in CTCs nog eens weergeeft.

CIRCULEREND TUMOR DNA

Eén van de problemen met CTCs, zoals ook hierboven toegelicht, is dat zij vrij zeldzaam zijn en dat het bepalen van de genetische eigenschappen van de CTCs erg complex is. Als CTCs of tumorcellen in de tumor zelf dood gaan (in apoptose gaan), kan dit in het bloed gevonden worden als circulerend tumor DNA (ctDNA). Als een buis bloed afgenomen wordt en wordt afgedraaid, wordt de bovenste laag gevormd door het plasma. Uit dit plasma kan DNA geïsoleerd worden, hetgeen we celvrij DNA (cfDNA) noemen. Iedereen heeft cfDNA, ook gezonde personen. Binnen dit cfDNA, is er in kankerpatiënten een fractie van DNA die afkomstig is van tumorcellen, het ctDNA. Vergeleken met CTCs, is de verwerking en analyse van cfDNA relatief eenvoudiger en lijkt ctDNA vaker voor te komen. Echter kan alleen het DNA geanalyseerd worden van het cfDNA en niet andere genetische eigenschappen (zoals eiwitten en RNA), hetgeen weer wel kan bij CTCs.

Door de introductie van allerlei nieuwe technieken is het onderzoek naar cfDNA de laatste jaren snel toegenomen. Dit heeft te maken met het feit dat plasma makkelijk te verkrijgen is, en vervolgens te analyseren is met technieken die al veel laboratoria

in huis hebben. Echter is nog weinig inzicht in de optimale condities om het plasma te verzamelen en te verwerken. In **hoofdstuk 6** werd daarom het effect van de tijd tot isolatie van het plasma bepaald en verschillende bloedbuizen om het plasma uit te isoleren vergeleken. Plasma uit CellSave of BCT buizen, dat binnen 96 uur geïsoleerd werd, bleek van even goede kwaliteit voor cfDNA analyses als plasma dat binnen 1 uur werd verwerkt. Dit betekent dat CellSave buizen, die ook gebruikt worden om bloed te verzamalen om CTCs te kunnen tellen, gebruikt kunnen worden om CTCs en cfDNA met optimale kwaliteit te bepalen tot 96 uur na afname.

Op het moment dat het plasma optimaal is verzameld, kunnen vervolgens mutaties in het cfDNA worden bepaald. Hoe de verschillende technieken om mutaties te detecteren zich verhouden tot elkaar, is echter nog maar weinig onderzocht. In **hoofdstuk 7** werden verschillende technieken om mutaties in cfDNA te detecteren naast elkaar gezet in patiënten met leveruitzaaiingen van dikke darmkanker, waarbij de uitzaaiing in de lever operatief werd verwijderd. Het gebruik van next-generation sequencing (NGS) bleek zeer goed in staat om mutaties te detecteren in tumorweefsel van de dikke darm en de leveruitzaaiing. Het voordeel van deze techniek is dat meerdere mutaties van meerdere genen bekeken kunnen worden. NGS was echter minder goed in staat om mutaties te detecteren in het cfDNA, wegens een te lage sensitiviteit. De OnTarget techniek, die in staat is om 96 van tevoren gedefinieerde mutaties te meten, bleek wel erg gevoelig om mutaties te detecteren in cfDNA. Daarnaast bleek een digitale PCR techniek de meest gevoelige techniek om mutaties te detecteren in cfDNA. Dit onderzoek bood meer inzicht in de verschillende technieken voor detectie van mutaties in cfDNA, en hoe deze technieken zich verhouden tot elkaar.

Uiteindelijk is de belangrijkste vraag in hoeverre deze technieken ingezet kunnen worden om de behandeling van patiënten met kanker te verbeteren. In **hoofdstuk 4** zetten wij, zoals al eerder genoemd, uiteen in hoeverre *ESR1* mutaties gebruikt zou kunnen worden in de kliniek. Daarnaast raadden wij aan om *ESR1* mutaties in cfDNA te meten en niet in biopsies van uitzaaiingen. In **hoofdstuk 5** werd de digitale PCR techniek gebruikt om onderzoek te doen naar *ESR1* mutaties in cfDNA. Hierin lieten we zien dat *ESR1* mutaties nauwelijks voorkomen in patiënten die voor het eerst begonnen aan hormonale therapie voor uitgezaaide borstkanker, maar dat deze mutaties frequent voorkomen in patiënten die hormonale therapie hebben gekregen. Dit laat zien dat *ESR1* mutaties een belangrijke rol spelen in resistentie op hormonale therapie, en dit zal in de toekomst hoogstwaarschijnlijk invloed gaan hebben op de behandeling van patiënten met uitgezaaide borstkanker.

De bevindingen in hoofdstuk 4 en 5 zijn gerelateerd aan het gebruik van cfDNA voor het bepalen van resistentie op therapie. Vervolgens kan een patiënt op basis hiervan een andere therapie krijgen. Er zijn meer toepassingen van cfDNA die verder niet in dit proefschrift behandeld worden, namelijk 1) om patiënten te identificeren voor een bepaalde behandeling (bij een bepaalde mutatie of andere afwijking in het cfDNA hoort dan een bepaalde behandeling), 2) voor de vroege detectie van nieuwe tumoren of voor de detectie van terugkomende ziekte na een operatie, en 3) als een marker voor respons op therapie. Toekomstig onderzoek, mede door onze groep, zal belangrijke antwoorden gaan bieden over de potentiële toepasbaarheid van cfDNA in de kliniek.

CIRCULERENDE ENDOTHEELCELLEN

CTCs en cfDNA geven beiden belangrijke inzichten in de biologie van de tumorcel. Het is echter ook belangrijk om te kijken naar andere factoren die ook van invloed zijn op de tumor: bijvoorbeeld de aanwezigheid van bepaalde immuuncellen die belangrijk zijn in de eigen afweer tegen een tumor, of bloedvatcellen die de tumor zelf maakt en nodig zijn om de tumor van voedingsstoffen te voorzien. Deze bloedvatcellen, ook endotheelcellen genoemd, kunnen als ze loskomen van het vaatbed gemeten worden in het bloed. Ook gezonde mensen hebben deze circulerende endotheelcellen (CEC), ten gevolge van een normale vervanging van bloedvatcellen. In kankerpatiënten is het aantal CECs echter verhoogd, en er wordt gedacht dat het aantal CECs iets zegt over in hoeverre de tumorcel bloedvaten aanmaakt en afbreekt. Onze groep heeft eerder een techniek gepresenteerd waarin CECs betrouwbaar in het bloed van patiënten met kanker gemeten kunnen worden.

In **hoofdstuk 9** hebben we onderzocht of CECs, gemeten met deze techniek vooraf en tijdens de behandeling, iets zeggen over de prognose en reactie op behandeling van patiënten met de hersentumor glioblastoom. Dit werd gedaan in patiënten uit de BELOB studie, waarin patiënten werden gerandomiseerd tussen 1) chemotherapie, 2) een medicijn dat nieuwe vaatgroei van de tumor remt (bevacizumab), of 3) chemotherapie en bevacizumab. Patiënten die een combinatie van beide middelen kregen, hadden tijdens de behandeling meer CECs, terwijl dit niet werd gezien in patiënten die maar één van beide middelen kregen. Dit suggereert dat de combinatie van middelen op een synergistische manier meer schade aan de bloedvaten geeft. Het stijgende aantal CECs hield echter geen verband met de overleving van deze patiënten. In patiënten behandeld met chemotherapie alleen, nog steeds de belangrijkste behandeling in patiënten met glioblastoom, hield een stijging van het aantal CECs wel verband met een betere prognose. Alhoewel dit mooie data was, relevant voor een grote groep glioblastoom patiënten die chemotherapie krijgen, werd juist verwacht dat CECs specifiek iets zouden zeggen over patiënten die therapie krijgen gericht tegen de bloedvatvorming.

Het probleem is echter dat CECs van het gezonde vaatbed niet onderscheiden kunnen worden van CECs van het tumorvaatbed. Onze groep heeft recent een marker ontdekt, CD276, welke specifieker lijkt te zijn voor CECs afkomstig van tumorvaten, ook wel tumor-CECs (tCECs) genoemd. In **hoofdstuk 8** werd deze marker ingezet in patiënten met asbestkanker, en we zagen dat het aantal CECs geen verband hield met de overleving van patiënten, maar het aantal tCECs wel. Aangezien deze bevindingen werden gedaan in een vrij kleine patiëntengroep, is verder onderzoek hiernaar aangewezen, maar het laat wel zien dan dit een interessante marker voor de detectie van tCEC is.

CECs kunnen ook gebruikt worden buiten het vakgebied van de interne oncologie. In dat geval is er ook geen noodzaak om tumor-specifieke CECs aan te tonen. Als toepassingen buiten de interne oncologie kan gedacht worden aan de cardiologie en de transplantatiegeneeskunde. In **hoofdstuk 10** onderzochten we in hoeverre het aantal CECs veranderde tijdens een allogene stamceltransplantatie (allo-SCT). Bij een allo-SCT krijgt een patiënt met een bloedziekte of bloedkanker (b.v. leukemie) een transplantatie met stamcellen van een familiedonor of een geschikte ongerelateerde donor. Hiertoe

krijgen de patiënten een zogenaamd ‘conditioneringsschema’, waarbij door middel van chemotherapie en bestralingen het eigen immuunsysteem kapot wordt gemaakt, om zo de stamcellen van de donor vrij spel te geven om de bloedziekte of bloedkanker te genezen. Wij lieten zien dat bij patiënten die een intensief conditioneringsschema kregen, het aantal CECs tot wel 12 maanden na transplantatie sterk verhoogd bleef, wijzend op langdurige vaatschade na de allo-SCT. Aangezien dit soort schema’s met name worden gegeven aan jonge patiënten, waarvan we weten dat die een verhoogde kans hebben op hart- en vaatziekten op de lange termijn, zou deze bevinding van lange vaatschade daar mogelijk meer te maken hebben, en dit verdient verder onderzoek. Daarnaast vonden we een verband tussen een daling van het aantal CECs en een belangrijke complicatie van allo-SCT, namelijk graft-versus-host ziekte. Dit zou kunnen betekenen dat T-cellen van de donor de endotheelcellen van de patiënt aanvallen, en dit wordt nu experimenteel onderzocht.

DE TOEKOMST VAN VLOEIBARE BIOPSIES

Dit proefschrift heeft meer inzicht geboden in de technische aspecten en mogelijke klinische relevantie van de vloeibare biopsies CTCs, cfDNA en CECs in patiënten met kanker. Wat betreft het tellen van CTCs, moeten we ons realiseren dat na vele jaren van onderzoek het gebruik in de kliniek nog vrij beperkt is. Daarom moeten nieuwe studies zich ook richten op de klinische toepassing van CTC tellingen, het liefst in de vorm van een studie waarbij een bepaalde interventie wordt gedaan op basis van CTC tellingen. Zo’n studie wordt bijvoorbeeld momenteel door onze groep gedaan in patiënten met blaaskanker die door de spierlaag is heen gegroeid (CirGuidance studie).

Het bepalen van de genetische eigenschappen van CTCs zal echter op de lange termijn de echte toekomst hebben. Een eerste voorbeeld van een klinische toepassing hiervan is bijvoorbeeld het bepalen van een splice variant V7 van de androgeen receptor (AR) in CTCs van patiënten met uitgezaaide prostaatkanker. Patiënten met deze AR-V7 splice variant in CTCs reageren nauwelijks op hormonale therapie, maar wel op chemotherapie. In de toekomst zullen patiënten dan ook zeer waarschijnlijk therapie krijgen op basis van hun AR-V7 status in CTCs.

Voor patiënten met uitgezaaide borstkanker heeft dit proefschrift onderzoek gedaan naar allerlei klinische toepassingen van het bepalen van de genetische eigenschappen CTCs. Alhoewel dit voor enkele bevindingen aanleidingen heeft gegeven tot nieuw onderzoek, hebben de meeste bevindingen wat betreft CTCs in dit proefschrift nog geen klinische toepassing. In het onderzoek naar *ESR1* mutaties, bleek dat deze mutaties veel beter te detecteren waren in het cfDNA dan in de CTCs. Dit betekent echter niet dat het onderzoek naar de genetische eigenschappen van CTCs niet zinvol is. Een zeer belangrijke marker als AR-V7 zal nooit in cfDNA gemeten kunnen worden, en hetzelfde geldt bijvoorbeeld voor eigenschappen op eiwitniveau zoals de oestrogeen receptor.
De karakterisatie van CTCs zal echter sterk verbeterd moeten worden om uiteindelijk het optimale resultaat eruit te halen. Wanneer pure CTCs verkregen worden, zonder witte bloedcellen die de bepaling van de genetische eigenschappen van CTCs vertroebelen, zijn we een flinke stap verder. Dit is sinds kort mogelijk, en het lab waar het onderzoek voor dit proefschrift werd verricht krijgt binnenkort de beschikking over een machine

om deze zuivere en ‘single’/individuele CTCs in handen te krijgen. Het bepalen van genetische eigenschappen in cfDNA zal in de toekomst waarschijnlijk complementair zijn aan dit soort analyses op zuivere CTCs. Het is op dit moment de vraag hoe cfDNA en zuivere CTCs zich tot elkaar verhouden, en in welke situaties welke van de twee het beste ingezet kan gaan worden.

De grote hoeveelheid data over CTCs en cfDNA doet de vraag opkomen of er nog wel een plaats is voor CEC tellingen. Op dit moment weten we niet wat de plaats van CECs gaat zijn. Onze groep heeft belangrijk werk verricht met het opzetten van een techniek om betrouwbaar CECs te meten, om tumor-specifieke CECs te meten, en in dit proefschrift lieten we zien dat het meten van CECs en tCECs mogelijk ook klinisch interessant is. Dit laatste is een grote stap voorwaarts, maar nieuwe studies om de klinische relevantie van (t)CECs te onderzoeken zijn van cruciaal belang om vooruit te blijven gaan. Deze studies zullen zich dan vooral moeten richten op patiënten met tumoren die heel sterk doorbloed zijn en veel nieuwe bloedvaten aanmaken (zoals asbestkanker) of in patiënten die anti-kanker medicijnen krijgen die gericht zijn tegen de bloedvaten van de tumor.

Concluderend, zal het bepalen van de genetische eigenschappen van CTCs, gecombineerd met complementaire cfDNA analyses, te zijner tijd een belangrijke rol gaan spelen in de oncologie. Het is nu van het grootste belang om de technische aspecten van het onderzoek naar zuivere CTCs te optimaliseren, om vervolgens de klinische relevantie te testen. Voor cfDNA analyses zullen de technieken ook geoptimaliseerd worden, en de vraag is vervolgens welke techniek (cfDNA of CTCs of allebei) het meest bruikbaar is in de kliniek. Dit zal waarschijnlijk afhankelijk zijn van de klinische vraagstelling en het tumortype van de patiënten. Vloeibare biopten zullen ons informatie over kanker gaan verschaffen van onschatbare waarde, en uiteindelijk zal deze informatie, en het gebruik hiervan in de behandeling van kanker, een geweldige stap vooruit gaan betekenen voor patiënten met kanker.

CURRICULUM

VITAE

CURRICULUM VITAE

Nick Beije werd op 21 augustus 1987 geboren in Rotterdam. Hij groeide op in Spijkenisse, en haalde in 2005 zijn VWO diploma op het Penta Collega CSG Blaise Pascal in Spijkenisse. Vervolgens volgde hij één jaar de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden, hetgeen resulteerde in een propedeuse. In 2006 begon hij aan de opleiding Geneeskunde aan de Erasmus Universiteit. Tijdens zijn opleiding werkte hij als afdelingsassistent op de afdeling maag-, darm- en leverziekten en de afdeling kindergeneeskunde van het Sint Franciscus Gasthuis. Daarnaast werkte hij als coördinator van het studententeam dat meehielp aan een gerandomiseerde studie betreffende het bevolkingsonderzoek naar dikke darmkanker in het Erasmus MC. Van 2010 tot 2012 liep hij coschappen, en nadat een groot deel van het coschap Interne Geneeskunde in het Vlietland Ziekenhuis werd gevolgd op de afdeling oncologie, werd zijn interesse in dit vakgebied gewekt. Zijn oudste coschap volgde hij bij de afdeling Interne Geneeskunde van het Ikazia Ziekenhuis (opleider: dr. Zandbergen). In 2012 verrichte hij zijn wetenschappelijke stage op het laboratorium Medische Tumor Immunologie in de Daniël den Hoed Kliniek, onder supervisie van dr. Gratama, dr. Kraan en prof. dr. Cornelissen. De daaruit volgende thesis leidde vervolgens tot een promotieplek bij de Interne Oncologie in het Erasmus MC op het onderzoek naar “*liquid biopsies”,* onder supervisie van prof. dr. Sleijfer en dr. Martens, zoals in dit proefschrift verder beschreven. Tijdens zijn promotietraject gaf hij veelvuldig onderwijs aan allerlei groepen studenten, hetgeen in 2016 leidde tot het behalen van de Basiskwalificatie Onderwijs. Daarnaast presenteerde hij op meerdere (inter)nationale congressen, en was hij hoofdauteur van gehonoreerde subsidieaanvragen door de Stichting Coolsingel, de Daniel den Hoed Stichting, de Stichting Mitialto en Pink Ribbon. Vanaf september 2016 was hij werkzaam als ANIOS in het Havenziekenhuis (opleider: dr. Wismans), en vanaf januari 2017 is hij aan de slag gegaan als AIOS Interne Geneeskunde in het Havenziekenhuis, met het uiteindelijke doel om internist-oncoloog te worden.

PHD

PORTFOLIO

Name PhD student: Nick Beije
Erasmus MC Department: Medical Oncology
Research School: Erasmus postgraduate school Molecular Medicine (MolMed)
PhD period: November 2012 – September 2016
Promotor: prof. dr. S. Sleijfer
Copromotor: dr. J.W.M. Martens

	Year	Workload (ECTS)
1. PHD TRAINING		
General academic and research skills		
BROK course (GCP course)	2013	0.9
Research Integrity	2013	2
Biostatistical Methods I: Basic Principles (CC02)	2013	5.7
Photoshop and Illustrator CS6 workshop	2014	0.3
InDesign CS6 workshop	2014	0.15
Biomedical English Writing and Communication	2014	3
In-depth courses		
Basic and Translational Oncology	2012	1.8
Medical Oncology Journal Club	2012-2016	1.5
Course on Molecular Diagnostics VIII	2013	1
NGS in DNA Diagnostics Course	2015	1
Teach The Teacher Course I	2015	0.7
Training on coaching medical students	2015-2016	0.4
Training on formulating exam questions	2016	0.2

	Year	Workload (ECTS)
Oral presentations		
Scientific JNI Lab Meeting, Erasmus MC, Rotterdam	2013-2016	1
Translational Cancer Genomics and Proteomics Meeting, Erasmus MC, Rotterdam	2014-2015	1
Ikazia Ziekenhuis Scientific Meeting, Ikazia, Rotterdam	2015	0.2
Pharmacokinetics Scientific Meeting, Erasmus MC, Rotterdam	2015	0.2
International Congress of Breast Disease Centers, ESMO Young Oncologist Meeting, Antwerp, Belgium	2015	0.2
Scientific Meeting Medical Oncology, Erasmus MC, Rotterdam	2015	0.2
European Cancer Congress, Vienna, Austria	2015	0.2
Bladder Cancer Research Day, Erasmus MC, Rotterdam	2015	0.2
Internal Medicine Clinical Demonstration, Erasmus MC, Rotterdam	2015	0.2
Medical Oncology Research Meeting, Erasmus MC, Rotterdam	2015	0.2
Dutch Uro-Oncology Studygroup Symposium, Utrecht	2015	0.2
Cancer Genomics Netherlands Annual Scientific Meeting, Utrecht	2016	0.2
Breast Cancer Research Meeting, Erasmus MC, Rotterdam	2016	0.2
EORTC Pathobioloy Group Meeting, Rotterdam	2016	0.2
Borstkanker Behandeling Beter Symposium, Rotterdam	2016	0.2

	Year	Workload (ECTS)
Poster presentations		
European Cancer Congress, Vienna, Austria	2015	1
Cancer Genomics Netherlands Meeting: "Molecular and Cellular Aspects of Cancer"	2015	1

(Inter)national conferences		
Center for Personalized Cancer Treatment (CPCT) Symposium, Utrecht	2014	0.2
Daniel 100 years Symposium, Rotterdam	2014	0.3
Cancer Genomics Netherlands Annual Meeting, Amsterdam	2014-2015	0.5
International Congress of Breast Disease Centers, Antwerp, Belgium	2015	0.2
European Cancer Congress, Vienna, Austria	2015	1
San Antonio Breast Cancer Meeting, San Antonio, USA	2015	1

2. TEACHING		
Lecturing		
Junior Med School Oncology Course, Erasmus MC, Rotterdam	2014-2016	1
Dell Inc. (on invitation from Pink Ribbon), Amsterdam	2014	0.3
Gezondheidsbeurs (on invitation from Pink Ribbon), Utrecht	2015	0.3

Supervising student theses		
Co-supervising 21-week research project for 6th year medical school student (D. van Rappard)	2013	1
Supervising 21-week research project for 3th year higher laboratory education student (E. Bont)	2014-2015	2.5
Supervising 12-week research project for bachelor thesis Medical Natural Sciences student (C. Bersee)	2015	1.4

	Year	Workload (ECTS)
Medical school training-associated teaching and counselling		
Tutorial class first-year medical students	2013-2015	4.5
Supervisor "clinical orientation on the medical profession" for first-year medical students	2013-2015	1.5
Supervisor/coordinator 4-week Junior Med School Medical Oncology Research Program	2015	1
Medical School Bachelor Phase Coaching Program	2015-2017	1.5

Teaching certificates		
University Teaching Qualification ("Basiskwalificatie onderwijs"/ BKO)	2016	2

3. Other

Grant allocation		
Stichting Coolsingel (together with prof. Aerts): € 27,351	2014	
Daniel den Hoed Foundation (principal author. PIs: dr. Martens, prof. Fodde, prof. Hendriks): € 267,140	2016	
Mitialto Foundation (together with prof. Sleijfer): € 13,280	2016	
Pink Ribbon (together with prof. Sleijfer, dr. Martens, dr. Onstenk and dr. Sieuwerts): € 59,491	2016	

Peer review of manuscripts for international peer-reviewed journals

Current Cancer Drug Targets (IF 3.7)

Critical Reviews in Oncology/Hematology (IF 5.0)

Clinical Chemistry (IF 7.5)

LIST OF PUBLICATIONS

Beije N, Angus L, Jager A, Martens JWM, Sleijfer S
"*ESR1* mutations: moving towards guiding treatment decision-making in metastatic breast cancer patients"
Cancer Treat Rev. 2016 in press

Beije N, Helmijr JC, Weerts MJA, Beaufort CM, Wiggin M, Mai L, Verhoef C, Sleijfer S, Jansen MPHM, Martens JWM
"Somatic mutation detection using various targeted detection assays in paired samples of circulating tumor DNA, primary tumor and metastases from patients undergoing resection of colorectal liver metastases"
Mol Oncol. 2016 in press

van Dessel LF, **Beije N**, Helmijr JCA, Vitale SR, Kraan J, Look MP, de Wit R, Sleijfer S, Jansen MPHM, Martens JWM, Lolkema MPJK
"Application of circulating tumor DNA in prospective clinical oncology trials: standardization of pre-analytical conditions"
Mol Oncol. 2016 in press

Beije N, Onstenk W, Kraan J, Sieuwerts AM, Hamberg P, Dirix LY, Peeters DJ, de Jongh FE, Jager A, Seynaeve CM, Van NM, Foekens JA, Martens JW, Sleijfer S
"Prevalence and prognostic impact of HER2 and ER status of circulating tumor cells in metastatic breast cancer patients with a HER2-negative primary tumor"
Neoplasia. 2016 Oct 17;18(11):647-653

Beije N, Sleijfer S, Boormans JL
"De rol van circulerende tumorcellen bij het urotheelcelcarcinoom van de blaas"
Tijdschr Urol. 2015 Nov; 5:223-228

Beije N, Kraan J, Taal W, van der Holt B, Oosterkamp HM, Walenkamp AM, Beerepoot L, Hanse M, van Linde ME, Otten A, Vernhout RM, de Vos FY, Gratama JW, Sleijfer S, van den Bent MJ
"Prognostic value and kinetics of circulating endothelial cells in patients with recurrent glioblastoma randomised to bevacizumab plus lomustine, bevacizumab single agent or lomustine single agent. A report from the Dutch Neuro-Oncology Group BELOB trial."
Br J Cancer. 2015 Jul 14;113(2):226-31.

Beije N, Versluis J, Kraan J, Gratama JW, Sleijfer S, Cornelissen JJ
"Circulating endothelial cell enumeration demonstrates prolonged endothelial damage in recipients of myeloablative allogeneic stem cell transplantation"
Haematologica. 2015 Jun;100(6):e246-9.

Beije N, Jager A, Sleijfer S
"Circulating tumor cell enumeration by the CellSearch system: the clinician's guide to breast cancer treatment?"
Cancer Treat Rev. 2015 Feb;41(2):144-50.

DANKWOORD

DANKWOORD

Uiteraard is dit proefschrift tot stand gekomen door de samenwerking met en de steun van vele andere mensen. Een aantal daarvan zou ik hier graag nog eruit willen lichten.

Allereerst natuurlijk veel dank aan mijn promotor, prof. dr. Sleijfer. Beste Stefan, ik kwam ooit binnen als student die bij Jan-Willem en Jaco zijn keuzeonderzoek deed op een alternatief project, en heb toen de kans gekregen om bij de CTC groep aan de slag te gaan. In de vier daarop volgende jaren heb ik verschrikkelijk veel opgestoken, niet in de laatste plaats door jouw begeleiding. Met name de besprekingen op dinsdagochtend waren van grote waarde, waarbij jij duidelijk het overzicht had over alle projecten die liepen, en altijd op duidelijke wijze de lijnen wist uit te zetten en de rode draad wist te bewaken. Ook je commentaren op manuscripten en andere stukken waren altijd nuttig, en ik blijf me verbazen over het feit dat commentaren ondanks je drukke bestaan altijd zo snel terugkwamen. Daarnaast ben ik dankbaar voor het feit dat ik van jou echt de kans heb gekregen om een belangrijk deel van mijn promotie zelf in te vullen. Ik kreeg veel vrijheid om behoorlijk wat projecten te exploreren en zelf op te starten. Ook de mogelijkheid die ik kreeg om een aantal grants (mee) te schrijven heb ik verschrikkelijk veel van geleerd, en gelukkig hebben we nog wat binnengehaald ook! Ik hoop dat ik ook na dit promotieonderzoek nog vele samenwerkingen met jou en de afdeling aan kan gaan.

Mijn copromotor, beste dr. Martens, beste John, na de verhuizing van onze groep naar het JNI zijn wij heel goed opgenomen in jouw club aldaar. Ik denk dat de verhuizing één van de beste dingen was die de CTC groep kon overkomen. Er was natuurlijk al sprake van veel samenwerking, maar de verhuizing zorgde ervoor dat jij en de andere mensen van de 4e verdieping nog veel dichter bij het CTC onderzoek betrokken konden raken, met inmiddels heel wat mooie resultaten tot gevolg. Ook zorgde het voor een verbreding van mijn blikveld buiten de kliniek. Jouw kennis en input vanuit een moleculair biologische hoek hebben veel manuscripten beter gemaakt. Daarnaast ook aan jou veel dank voor alle dingen die ik naast het promoveren heb mogen doen, met het krijgen van de Daniel den Hoed Stichting grant voor het VyCAP systeem als hoogtepunt. Als laatste ook bedankt voor de gezelligheid, niet alleen op de afdeling, maar ook vaak als vaste gast op één van de vele borrels die we over de jaren hebben gehad.

Prof. dr. van den Bent,en prof. dr. Aerts, beste Martin en Joachim, hartelijk dank voor het plaatsnemen in mijn kleine commissie, en aan jullie bijdragen aan respectievelijk het hoofdstuk over CECs in de BELOB studie en de MESOPA studie. Deze hoofdstukken tonen aan dat uit multidisciplinaire samenwerking mooie resultaten kunnen komen, en ik kijk uit naar nieuwe samenwerkingen.

Prof. dr. Verheul wil ik graag danken voor het participeren in mijn kleine commissie, en prof. dr. van Laere en dr. Dinjens voor het plaatsnemen in mijn grote commissie. De tijd die jullie hebben geïnvesteerd in het lezen van het proefschrift wordt zeer gewaardeerd, en ik kijk uit naar de discussie.

Beste dr. Jager, beste Agnes, als co-auteur op vier van de manuscripten zoals in dit proefschrift beschreven vond ik het niet meer dan logisch om jou in mijn grote commissie plaats te laten nemen. Ik vind dat al deze manuscripten, alsmede ook meerdere klinische protocollen, door jouw scherpe input allemaal beter zijn geworden. Met name jouw pragmatische klinische blik ("wat is de échte klinische relevantie") heb ik erg nuttig gevonden. Ik zie er naar uit om verder van gedachten te wisselen over het proefschrift.

Beste prof. dr. Cornelissen, beste Jan, als geneeskundestudent deed ik onderzoek naar allogene stamceltransplantaties, en ik vind het nog steeds een fascinerend gebied om me mee bezig te houden. Het stuk over CEC's na allo-SCT hebben wij uiteindelijk voornamelijk door jouw input heel mooi weg kunnen zetten in Haematologica. Dank voor het plaatsnemen in mijn grote commissie.

Graag zou ik ook prof. dr. Foekens willen bedanken. Beste John, toen ik kwam had je de tent praktisch gezien al overgedragen aan de andere John, maar je bent toch bij veel projectplannen en manuscripten betrokken geweest. Jouw commentaar is altijd met verschrikkelijk veel oog voor detail, en heeft ervoor gezorgd dat manuscripten tot in de puntjes verzorgd zijn, tot aan de referenties aan toe. Ik vind het een goede zaak dat je met al jouw ervaring betrokken blijft bij al het onderzoek wat we doen, en hopelijk ga je dat nog lang volhouden.

Beste prof. dr. Berns, beste Els, jouw bijdrage aan dit proefschrift is relatief beperkt. Op het gebied van het onderwijs wat ik heb gegeven ben je echter wel degelijk belangrijk geweest. Ik heb zeer gewaardeerd dat je mij en ook andere PhD studenten de kans hebt gegeven om veel onderwijs te kunnen geven, bijvoorbeeld voor de Junior Med School. Daarnaast heeft jouw enthousiasme en passie voor het onderwijs er ook zeker aan bijgedragen dat ik uiteindelijk zelfs mijn BKO certificaat heb behaald, waarvoor dank.

Beste dr. Gratama, beste Jan-Willem, ik kwam binnen om onder jouw leiding mijn keuzeonderzoek als medisch student te doen. Nog voordat ik klaar was met mijn keuzeonderzoek, had ik al de toezegging om een PhD te mogen doen, waar jij je mede sterk voor hebt gemaakt. Ik ben erg dankbaar voor de kans die jij mij mede hebt gegeven om toentertijd van start te gaan met het promotieonderzoek.

Dr. Sieuwerts, beste Anieta, jouw bijdrage aan een aantal hoofdstukken in dit proefschrift is heel belangrijk geweest. Jouw expertise omtrent (digital) PCR is ongekend, en heeft het moleculaire CTC werk naar een hoog niveau gedreven. De grotendeels door jou ontwikkelde karakterisatie van CellSearch CTCs op RNA niveau is in de wereld vrij uniek, en heeft onze groep en mij enorm veel mooie data en publicaties opgeleverd. Ik ben met name trots op het stuk over de *ESR1* mutaties en splice varianten, waar van ons allebei heel veel uren inzitten, en wat uiteindelijk een prachtig stuk is geworden. Daarnaast ook zeker dank voor de gezelligheid, de trip naar San Antonio met jou, John en Marjolein was één van de hoogtepunten van mijn PhD tijd. Ik kijk uit naar de toekomst, waar we op single CTC niveau met het VyCAP systeem en jouw expertise nog veel mooie dingen kunnen gaan doen.

Uiteraard wil ik hier ook dr. Kraan bedanken. Jaco, jij hebt de meeste begeleiding gedaan toen ik als geneeskundestudent mijn keuzeonderzoek kwam doen. We begonnen toen met een CEC project wat niet goed van de grond kon komen, en uiteindelijk eindigde ik met een mooi afstudeerproject over immuunreconstitutie na stamceltransplantatie. Jouw inbreng op het technische vlak is voor veel stukken in dit proefschrift van groot belang geweest. De expertise die je hebt o.a. wat betreft het optimaal verwerken van bloed, het correct verrijken van CTCs en flow cytometrie is voor de afdeling onmisbaar. Daarnaast lever je natuurlijk een belangrijke bijdrage aan de sfeer. Het koffie drinken na de lunch is een mooie traditie en af en toe vonden daar nog nuttige discussies plaats ook!

Dr. Jansen, beste Maurice, vooral de laatste anderhalf jaar hebben we veel samengewerkt op allerlei projecten omtrent cell-free DNA. Voor mij was dit een heel nieuw gebied om in te duiken, en mede met jouw hulp heb ik daar veel kennis in opgedaan. Door jouw ideeën omtrent het RMD paper en gelegde contacten met Boreal is dat bijna afgeschreven manuscript uiteindelijk nog een succes geworden en kan het nu in dit proefschrift opgenomen worden. Daarnaast heb ik ook onze discussies over de data, maar ook over het grote geheel (“waar gaan we uiteindelijk heen met de liquid biopsies”) gewaardeerd.

Dr. Onstenk, beste Wendy, het was natuurlijk zeer pijnlijk om mij als kamergenoot te laten gaan na onze periode in de Daniël, en mij in te moeten ruilen voor Jaco. Gelukkig heb je dat goed opgepakt daarna, en kwam je af en toe nog eens in Be-414a buurten. Bedankt voor al je hulp als senior AIO bij het schrijven van de manuscripten, het omgaan met het promovendus zijn, en niet in de laatste plaats met de totstandkoming van dit boekje met o.a. alle offertes, adreslijsten en de totaal niet neurotische tijdsplanning!

Ook de huidige bezetting dames in Be-414, die helaas niet als 4 paranimfen konden fungeren (in verband met een restrictie tot 2), ben ik natuurlijk dank verschuldigd. Er was altijd een goede sfeer op onze kamer, en alle borrels, etentjes en andere gezellige dingen waren echt top. Ook ben ik er heel erg trots op dat dit proefschrift maar liefst 3 hoofdstukken bevat waarbij wij als Be-414 promovendi met elkaar hebben samengewerkt, hetgeen maar aangeeft dat buiten alle gezelligheid er ook zeker hard werd gewerkt.

Marjolein, jij bent niet alleen heel belangrijk geweest voor de sfeer op de kamer, maar zeker ook met al jouw technische en biologische kennis. Je hebt deze cliniclown steeds, en soms meerdere keren opnieuw, moeten uitleggen hoe nu precies zat met al die NGS technieken en nog veel meer andere technische zaken, en daardoor snapte ik er uiteindelijk oprecht een stuk meer van. Op een PhD kamer zijn juist mensen zoals jij met een andere invalshoek dan de arts-onderzoekers van belang om weer nieuwe ideeën te ontwikkelen en met sommige projecten verder te komen. Ik hoop echte van harte voor je dat de *onderwerp gecensureerd door Philips* het helemaal gaan worden. Ook natuurlijk dank voor de gezelligheid buiten het werk om: de verschillende etentjes, borrels, de trips naar verschillende binnen- en buitenlandse congressen en als kers op de taart de trip naar NYC.

Inge, als mijn “raamgenoot” heb ik ook jouw positieve inbreng en humor op de kamer enorm gewaardeerd. Je hebt enorm veel projecten van mij mogen overnemen, waarvan ik weet dat ze in goede handen bij jou zijn. Jouw winst van de Pieter de Mulder award vond ik echt heel tof, de trip om jou op te zoeken in Stockholm was fantastisch, en ik hoop heel erg dat de PLP op CTC’s van de grond gaat komen. Ons gezamenlijke paper gaat er nog komen.

Lisanne, onze (zelfbenoemde) favoriete collega. Ook jij heel erg bedankt voor alle gezelligheid. Ik vond het echt heel tof dat wij toen te gast mochten zijn op jouw bruiloft, dat was echt een prachtige dag. Ik ben heel erg benieuwd wat jouw onderzoek gaat opleveren, met hele veelbelovende projecten zoals cfDNA in prostaatkanker, cfDNA in de urine en de gekweekte CTC’s na leukoferese.

Lindsay, ondanks dat je nog relatief nieuw bent heb je je wel snel thuis gemaakt in onze kamer (nadat we je van de kamer van Jaco en Wendy moesten kapen!) en de CTC groep. Je bent net een jaar bezig en hebt toch al mooie dingen bereikt, met alle nucleaire studies die nu uiteindelijk dankzij jou open zijn, het schrijven van de recente KWF aanvraag, en ook met de acceptatie van ons review. Ik ben echt heel erg benieuwd wat je in de komende jaren nog meer in petto hebt.

Daarnaast zou ik ook alle andere promovendi en oud-kamergenoten willen bedanken voor alle gezelligheid: Arjen, Diana, JingJing, Rene, Tommaso, Esther, en ook de mensen van de PK-groep Annemieke, Jacqueline, Lisette, Roelof, Bodien en Femke. Special shout-out voor Sander, jij bent vlak na mij begonnen, en was altijd in voor een goede kop koffie en een mooi gesprek over voetbal, of juist over de toekomst van de oncologie en het onderzoek. Ik kijk er naar uit om over een paar jaar als internist-oncologen i.o. de afdeling te gaan runnen.

Ook heel veel dank aan onze vaste analisten Mai, Zahra en Joan. Zonder al jullie werk op het lab hadden deze stukken er nooit kunnen komen. Ook bedankt voor alle hulp van de andere mensen op en rondom het lab: Jean, Corine, Silvia, Wendy, Vanja, Kirsten, Michelle, Mieke, Anita en Renee, de post-docs van de groep Jozien en Antoinette, en secretaresse Rosita.

Graag zou ik ook alle mensen willen bedanken waarmee ik heb samengewerkt door de jaren heen. Allereerst alle nog niet genoemde oncologen in het Erasmus MC. Dr. Seynaeve, beste Caroline, dank voor alle nuttige en kritische input die je hebt geleverd op mijn stukken. Dr. Homs, Marjolein, bedankt voor je hulp bij het indienen en van de grond laten komen van de CareMore-Trastuzumab studie. Prof. dr. Mathijssen, beste Ron, bedankt voor alle adviezen door de jaren heen over het vak en professionele ontwikkeling. Dr. Lolkema, beste Martijn, bedankt voor de discussies die we hebben gehad over cell-free DNA en nieuwe studievoorstellen. Ook alle andere oncologen uit het Erasmus MC die ik niet heb genoemd bedankt voor alle hulp die ik van jullie heb gekregen bij alle studies die we hadden lopen, en de inclusies in de verschillende studies.

Een belangrijke samenwerking in huis was met dr. Boormans van de afdeling urologie. Beste Joost, mede door jouw enorme inzet voor de studie is de CirGuidance studie een succes geworden, en mogen we zeer binnenkort de laatste patiënt, nummer 300, gaan begroeten. Ook al komt het betreffende stuk uiteindelijk niet in mijn boekje, wel hebben we een mooi Nederlands review geschreven, ben ik dankbaar voor de kans om op een aantal symposia zoals de DUOS meeting te hebben mogen presenteren op voorspraak van jou, en gaan we hopelijk in de toekomst nog verder samenwerken op andere blaaskanker-gerelateerde projecten.

Ook belangrijke was de samenwerking met de ziekenhuizen in de regio Rotterdam.
Dr. Hamberg, beste Paul, het Franciscus Gasthuis heeft enorm bijgedragen aan de inclusie in verschillende studies die inmiddels zijn gepubliceerd of nog lopen. Bijna in elke studie is het Franciscus Gasthuis één van de top includerende centra, en dat is niet in de laatste plaats door jouw inzet voor al deze studies. Daar wil ik je graag voor bedanken, en ook zeker voor jouw altijd kritische input op nieuwe studievoorstellen en manuscripten.

Dr. de Jongh, beste Felix, ook in het Ikazia kunnen wij altijd terecht om nieuwe studies op te starten. Ik weet vanuit mijn oudste coschap wat ik ooit in het Ikazia heb gelopen hoe bezeten je bent van het vak, en dat enthousiasme zag ik altijd terug bij onze besprekingen over nieuw te starten studies, en je commentaren op stukken. Hopelijk kunnen we samenwerking met het Ikazia nog lang voortzetten.
Ook zou ik graag de researchverpleegkundigen uit deze centra willen bedanken, met name Karin Wensing en Corry Leunis, die altijd scherp zijn geweest op het verzamelen van materialen voor onze studies, wat van enorm belang is voor de kwaliteit en volledigheid van de data van onze studies.

Buiten Nederland hebben we ook samengewerkt. Met name wil ik de mensen van de Translational Cancer Research Unit in Antwerpen bedanken. Onder leiding van dr. Dirix en prof. dr. van Laere hebben we vele mooie onderzoeksvragen mogen beantwoorden, en de interlab-meetings waren nuttig en ook gezellig. Ook dank aan Dieter en Anja voor de samenwerking omtrent verschillende studies en het uitwisselen van data.

Also thanks to Matthew, David and Andre from Boreal Genomics, who were kind enough to collaborate on our manuscript on cfDNA in colorectal cancer with their OnTarget assay. The addition of the OnTarget data really strengthened our manuscript and resulted in a nice publication in Molecular Oncology.

Dank ook aan al mijn collega's in het Havenziekenhuis. Ik ben daar hartstikke goed opgevangen. In vrije korte tijd ben ik me mede door jullie in het team thuis gaan voelen, en ook in mijn (nieuwe) rol als clinicus. Ik zie er naar uit om me in het Havenziekenhuis verder te ontwikkelen als dokter.

Graag wil ik ook al mijn Ardennengroep buddies Weeda, Mariska, Polder, Do, JP, Nicole, VDV, Ellen, Lost, Fab, Leonie, Vos, Lé, Melda, Marjolein, Niels, Suus en Joe bedanken. Niet zozeer omdat jullie nu een weergaloze bijdrage hebben geleverd aan dit boekje, maar wel vanwege alle mooie dingen die we afgelopen jaren hebben gedaan en zeker vol gaan houden.

Ook natuurlijk dank voor alle gezellige etentjes met mijn oude studiegroep-genoten Mariëtte, Line en Aimee. Aimee, je bent niet voor niets mijn paranimf, we kennen elkaar nu al echt weer lang (alweer 10+ jaar), en ik word er altijd blij van om met je af te spreken, wat te gaan eten (/koken te vermijden), en het dan te hebben over serieuze en (vaak ook) minder serieuze zaken. Je merkt het, ook in dit proefschrift stoppen de complimenten aan jouw adres niet. Bedankt dat je mijn paranimf wil zijn!

Graag wil ik ook al mijn familie bedanken. Opa en oma van der Schee voor alle lieve berichten en belangstelling. Opa en oma Beije voor alle support door de jaren heen. Ilona en Suus voor alle positiviteit en aanmoedigingen.

Britt, ik vind het leuk dat jij mijn paranimf wil zijn. Ben trots op wat jij allemaal in korte tijd al hebt bereikt met je studie en werk, en wie weet sta jij ooit ook nog eens op mijn positie als promovendus. De Giuliano lunches gaan we zeker volhouden!

Mijn ouders wil ik ook graag bedanken. Pap, mam, van kleins af aan ben ik nooit iets tekort gekomen, en hebben jullie eigenlijk elke beslissing gesteund die ik ooit gemaakt heb. Ik ben enorm trots dat ik dit boekje heb kunnen maken, en jullie hebben daar zeker in geholpen.

Als laatste wil ik graag alle patiënten bedanken die hebben deelgenomen aan de verschillende onderzoeken die in dit proefschrift zijn beschreven. Ondanks dat zij vaak niets hadden aan het afstaan van bloed en/of weefsels, wilden zij graag met hun deelname voor de patiënten na hen iets betekenen. Vele van de patiënten die hebben geparticipeerd in onze onderzoeken zijn inmiddels overleden, maar ik weet zeker dat we mede door hun deelname ervoor kunnen zorgen dat de zorg en kwaliteit van leven van toekomstige kankerpatiënten verder zal verbeteren.