

CELL-BASED MONITORING OF CANCER
CIRCULATING TUMOR AND ENDOTHELIAL CELLS

Jaco Kraan

The studies described in this thesis were performed at the Department of Medical Oncology, Erasmus MC Cancer Institute, Rotterdam, Netherlands.

This research project was in part financially supported by Cancer Genomics Netherlands/Netherlands Organization for Scientific Research (NWO), IQ products BV and Janssen, LLC, Raritan, NJ.

Publication of this thesis was financially supported by kind contributions from: Department of Medical Oncology, Erasmus MC Cancer Institute Rotterdam, Erasmus University Rotterdam, Bio-Connect BV Boehringer Ingelheim BV, Chipsoft BV, Distilab BV, GR Instruments and Libra Diagnostica.

ISBN: 978-94-6299-198-9

Cover design by SBG-projects

Lay-out by Ridderprint BV

Printed by Ridderprint BV

Copyright © 2015 Jaco Kraan, Rotterdam, Netherlands

All rights reserved. No part of this thesis may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronically, mechanically, photocopying, recording or otherwise, without the prior written permission of the copyright owner.

CELL-BASED MONITORING OF CANCER CIRCULATING TUMOR AND ENDOTHELIAL CELLS

CEL GEBASEERDE METING VAN KANKER
CIRCULERENDE TUMOR EN ENDOTHEEL CELLEN

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 18 november 2015 om 13.30 uur

door

Jacob Kraan
geboren te Utrecht

PROMOTIECOMMISSIE

Promotor:	Prof.dr. J.A. Foekens
Overige leden:	Prof.dr. L.H.J. Looijenga Prof.dr. L.W.H. Terstappen Prof.dr. C. Verhoef
Copromotor:	Dr. J.W. Gratama

Voor Annemarie

CONTENTS

Chapter 1	General introduction and outline of the thesis	9
	1.1 Cell-based monitoring of cancer	
	1.2 Detection of "rare cellular events"	
	1.3 Circulating Tumor Cells	
	1.4 Circulating Endothelial cells	
	1.5 Aims and outline of the thesis	
Chapter 2	External quality assurance of circulating tumor cell enumeration using the CellSearch® system: A feasibility study. <i>Cytometry Part B (Clinical Cytometry) 2011; 80B:112 - 118</i>	23
Chapter 3	Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. <i>Breast Cancer Research and Treatment 2009; 118:455 - 468</i>	39
Chapter 4	Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. <i>Journal of the National Cancer Institute 2009; 101:61 - 66</i>	65
Chapter 5	Clinical value of circulating endothelial cell detection in oncology. <i>Drug Discovery Today 2012; 17:710 - 717</i>	87
Chapter 6	A new approach for rapid and reliable enumeration of circulating endothelial cells in patients. <i>Journal of Thrombosis and Haemostasis 2012; 10:931 - 939</i>	107
Chapter 7	Endothelial CD276 (B7-H3) expression is increased in human malignancies and distinguishes between normal and tumor derived circulating endothelial cells. <i>British Journal of Cancer. 2014;111:149 - 156</i>	127
Chapter 8	Summary and discussion	145
Appendices	Samenvatting	157
	Dankwoord	
	PhD portfolio	
	Curriculum Vitae	
	List of publications	

Chapter 1

**General introduction
and outline of the thesis**

1.1. Cell-based monitoring of cancer

Cancer is the second leading cause of morbidity and mortality worldwide ¹ and in the Netherlands even the most common cause of death. Greater than 90% of deaths in patients with solid tumors are attributed to metastases². Many patients initially present with late stage, metastatic disease primarily due to the failure of effective clinical detection and treatment of metastatic disease in distant sites ^{3,4}. The metastatic process is comprised of a series of sequential steps⁵, these steps include dissemination of cancer cells from the primary tumor into the bloodstream (intravasation), survival in the circulation, extravasation into secondary sites and initiation and maintenance of growth to form metastases ⁶ (**Figure 1**). In recent years, many new anti-cancer agents have been developed against the different steps of the metastatic process. Although clinical trials have demonstrated a benefit in terms of prolonged survival in many tumor types, for further development of the field there is a compelling need for effective clinical biomarkers for determining the optimal dose, monitoring biologic activity and selecting and stratifying the patients most likely to benefit from a certain treatment.

In this thesis we will focus on the analysis of Circulating Tumor Cells (**CTC**) and Circulating Endothelial cells (**CEC**) that circulate in the blood of cancer patients and on their potential use as cellular biomarkers for monitoring metastatic disease. Both cell types are extremely rare events in the blood circulation, in the range of several cells per mL of blood, compared to leukocytes (millions per mL) and erythrocytes (billions per mL) as shown in **Table 1**

1.2. Detection of "rare cellular events"

Ever since its invention in the 1960s, flow-cytometric techniques of measuring individual cells in a fluid suspension have become increasingly sensitive and specific. These advances have become possible through rapid developments in instrumentation (cytometers), reagents (monoclonal antibodies) and software. In addition, techniques to enrich cells from a suspension have become increasingly refined and accurate. As a result, multiparameter (flow) cytometry now enables the analysis of "rare events", e.g. in the range of a single cell in several mL of blood. This development has extended the range of applications of clinical cell analysis to that of the discipline of oncology focusing on CTC and CEC.

Table 1

Frequency of cell types in peripheral blood and minimal assay volume to obtain a given precision: endothelial and tumor cells are extremely rare.

(adapted and modified from Leon W.M.M. Terstappen)

Peripheral Blood Cells			
Cell Number	Cell Type	Cell Frequency (Leukocytes)	Assay Volume for a CV < 20% *
1,000,000,000 / mL	Erythrocytes	1 in 10^{-2}	0.000025 μ L
	Platelets		
100,000,000 / mL	Reticulocytes	1 in 10^{-1}	0.00025 μ L
10,000,000 / mL	Leukocytes	1 in 10^0	0.0025 μ L
	Neutrophils		
1,000,000 / mL	Lymphocytes	1 in 10^1	0.025 μ L
	Monocytes		
100,000 / mL	Eosinophils	1 in 10^2	0.25 μ L
	Basophils		
10,000 / mL		1 in 10^3	2.5 μ L
	Hematopoietic Progenitors (CD34+)		
1,000 / mL		1 in 10^4	25 μ L
	Nucleated Erythrocytes		
100 / mL		1 in 10^5	250 μ L
	CD34+CD38- Cells		
10 / mL		1 in 10^6	2.5 mL
	Endothelial Cells		
1 / mL	Tumor Cells	1 in 10^7	25 mL
0.1 / mL		1 in 10^8	250 mL

* For cell-based assays in blood, a simple calculation can be used to determine the size of the database/sample that will provide a given precision: $r = (100/CV)^2$; where r is the number of events meeting the required criterion, and CV is the coefficient of variation of a known positive control⁴⁷

1.3. Circulating tumor cells

CTC may be present in the peripheral circulation of patients with solid malignancies and originate from either the primary tumor or their metastases. Although CTC have attracted the interest of cancer researchers for decades, reliable assays to detect such cells have become available only since recently. CTC are only specifically detected using a combination of surface and intracellular markers in assays that typically involve enrichment of a particular cell population.

In clinical settings, the detection of CTC has been established routinely in hematology (i.e., to monitor “minimal residual disease” in leukemia and lymphoma) using multicolor flow cytometry. However, CTC from solid tumors occur at much lower numbers in the peripheral blood compared to malignant cells from hematological cancers. Only in the last decade a sufficiently sensitive and specific technique became available for solid tumors based on the combination of cell enrichment (based on positivity for epithelial cell adhesion molecule [EpCAM]) and image cytometry. This technique (CellSearch) has been implemented in our department and is FDA-cleared for breast cancer⁷⁻¹⁰, colon cancer^{10,11} and prostate cancer^{10,12}.

In addition to enumeration, CTC can be isolated and characterized by techniques such as Immunofluorescence^{13,14}, DNA sequencing¹⁵⁻¹⁸, Fluorescence in situ hybridization (FISH)¹⁹⁻²¹ and real-time reverse transcriptase-quantitative PCR (RT-qPCR)²²⁻²⁴. Apart from enumeration, characterization of CTC has great opportunities for research purposes and patient management. By comparing characteristics of the primary tumor and CTC, novel factors involved in tumor cell dissemination may be identified. Many primary tumor characteristics in a broad range of different tumor types have prognostic and predictive significance. As metastases rather than the primary tumor are the main cause of death from cancer, characteristics of metastasizing cells are likely to yield better prognostic and predictive information than primary tumor cells. Several studies have reported important differences between characteristics of the primary tumor and metastases in terms of genetic alterations and expression of proteins^{25,26}. Another attractive potential application of CTC characterization is the assessment of therapy-induced effects on tumor tissue, for example inhibition of targets for therapeutic drugs, induction of apoptotic pathways and mechanisms conferring resistance against cytotoxic drugs.

1.4. Circulating Endothelial Cells

The endothelium of vessel walls, consisting of approximately 1.2 trillion endothelial cells, is not only a barrier between blood and organs, but is also, and importantly, a highly dynamic structure involved in many aspects of vascular biology. Endothelial cells play an important role in the regulation of vasomotor tonus, hemostasis and

coagulation, fluid and electrolyte transport, inflammation, and angiogenesis. Quantification of specific endothelial features, such as the degree of proliferation (angiogenesis) or the extent of endothelial injury, could be of predictive or prognostic value, or enable to monitor response to therapy.

The assessment of endothelial damage may be of particular interest in oncology. Tumor vasculature, being fragile and leaky, is structurally different from its healthy counterpart. Also, the normal vasculature in cancer patients can be easily damaged, as invasion of the basement membrane is required for the extravasation of malignant cells. Tumor aggressiveness may therefore be reflected by more endothelial damage, of which the extent may be associated with prognosis or disease recurrence.

Appropriate vascularization, is needed for cancer growth and dissemination. Endothelial cells are genetically much more stable when compared to malignant cells, and are therefore unlikely to develop resistance to therapy. This has led to the development of the first anti-vascular drug: bevacizumab, a monoclonal antibody specific for vascular endothelial growth factor (VEGF). In addition to drugs targeting the formation of new vessels, a second group of so-called “vascular disrupting agents” has been developed, which destroy tumor-associated vasculature by inducing hypoxia, starvation, and eventually necrosis. Besides cytoreductive drugs, chemotherapy may also result in endothelial damage. This contention is supported by long-term follow-up of survivors of testicular cancer treated with bleomycin, who have shown a significant increase of cardiovascular complications²⁷.

Thus, the endothelium has an important role in the pathogenesis of cancer and is also a target for treatment. Therefore, markers that provide quantitative information on the extent of tumor vascularization, allow the monitoring of effects of anti-vascular drugs, or have predictive or prognostic value are urgently needed.

While plasma concentrations of endothelial cell-derived proteins such as von Willebrand factor (vWF), thrombomodulin or e-selectin, correlated with response to treatment or survival, their pleiotropic nature could easily compromise specificity. As an alternative, CEC are proposed: these are endothelial cells detached from the vessel wall, and as such act as a promising marker for endothelial damage. Similar to CTC, CEC can be detected by immunomagnetic enrichment followed by image cytometry or flow cytometry. CEC are relatively rare events (up to 20 cells/mL in healthy individuals), but not tumor-specific such as CTC. Increased numbers of CEC have been observed in diseases with vascular involvement such as vasculitis^{28,29}, sickle cell anemia^{30,31}, and cancer³²⁻³⁹.

1.5. Aims and outline of the thesis

The work described in this thesis aimed to improve the predictive and prognostic information that can be obtained from CTC and CEC by improving and standardization their detection and characterization methods.

Since the first anecdotal description of CTC in 1869, only in the last decade different CTC detection methods have been described. The only FDA-cleared CTC detection method is CellSearch, which uses immuno-magnetic and immune-fluorescent labeling to identify and count CTC in blood samples. It is the only system with FDA clearance to predict progression-free survival and overall survival in patients with metastatic breast, colorectal and prostate cancer^{7-9,40-46}. Using threshold levels, CTC enumeration by the CellSearch system can stratify patients with advanced breast, colorectal, and prostate cancer into groups differing in prognosis. In addition, a decrease in CTC count appears to be associated with outcome to systemic therapy in these tumor types. As a result, clinical studies are ongoing to assess whether systemic therapy in certain tumor types can be guided by CTC counts rather than the current standard practice of conventional radiological assessments. For an assay used in daily practice and in multicenter studies, consistency in performance between diverse laboratories is pivotal. In **Chapter 2** we documented the variation in CTC enumeration using the CellSearch system and studied the feasibility of external quality assurance (EQA) of the entire procedure from blood draw to image interpretation.

In addition to enumeration, there is great interest in molecular characterization of these CTC. In **Chapter 3** we aimed to establish a robust method to perform mRNA expression analysis of multiple genes by a RT-qPCR on small numbers of CTC enriched from whole blood by the CellSearch system. We intended the molecular characterization specific for as few as a single CTC, to be used to expand the understanding of the biology of metastasis and, potentially, to improve patient management.

Importantly, the widely used CellSearch system is limited by capturing CTC based on immuno-magnetic enrichment using Ferro fluids coated with anti-EpCam monoclonal antibodies. **Chapter 4** expands on these limitations (i.e. EpCam dependency) when cells belonging to the subgroup of normal like breast tumor cells with epithelial-mesenchymal transition features should be detected and includes recommendations to improve the sensitivity and feasibility of the assay without a loss of specificity.

In the second part of this thesis we address the current state-of-the-art of CEC enumeration as a biomarker in clinical oncology. In view of the central role of the tumor vasculature in the biology of tumors and the various agents that have been developed and are now available or under study for the purpose of targeting the tumor vasculature, **Chapter 5** provides an overview of the technical issues

concerning CEC detection, reviews results from clinical studies and explores future potential applications.

Given the need for a quick, reliable, robust and validated CEC assay that is also affordable, we present in **Chapter 6** a novel and extensively validated approach for enumerating CEC. The agreement with the validated CEC assay on the CellSearch System was tested in healthy subjects and patients with pathologies known to be associated with high CEC levels. Using the multi-parameter flow cytometric approach of our newly developed assay we were able to further characterize CEC in greater detail. As described in **Chapter 7**, this made it possible to discriminate CEC from damaged malignant vasculature from those of damaged normal vasculature to improve the specificity of subsets of CEC in cancer.

Finally, in **Chapter 8**, the results of the studies in this thesis are summarized, conclusions are drawn and future perspectives are presented.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69-90.
2. Wicha MS, Hayes DF. Circulating tumor cells: not all detected cells are bad and not all bad cells are detected. *J Clin Oncol.* 2011;29:1508-1511.
3. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer.* 2002;2:563-572.
4. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer.* 2004;4:448-456.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144:646-674.
6. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer.* 2003;3:453-458.
7. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med.* 2004;351:781-791.
8. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol.* 2005;23:1420-1430.
9. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin Cancer Res.* 2007;13:920-928.
10. Miller MC, Doyle GV, Terstappen LW. Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. *J Oncol.* 2010;2010:617421.
11. Cohen SJ, Punt CJ, Iannotti N, et al. Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. *Ann Oncol.* 2009;20:1223-1229.
12. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res.* 2008;14:6302-6309.
13. Paoletti C, Muniz MC, Thomas DG, et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer. *Clin Cancer Res.* 2015;21:2487-2498.
14. Riethdorf S, Muller V, Zhang L, 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;16:2634-2645.
15. Steinestel J, Luedeke M, Arndt A, et al. Detecting predictive androgen receptor modifications in circulating prostate cancer cells. *Oncotarget.* 2015.
16. Campton DE, Ramirez AB, Nordberg JJ, et al. High-recovery visual identification and single-cell retrieval of circulating tumor cells for genomic analysis using a dual-technology platform integrated with automated immunofluorescence staining. *BMC Cancer.* 2015;15:360.
17. Fernandez SV, Bingham C, Fittipaldi P, et al. TP53 mutations detected in circulating tumor cells present in the blood of metastatic triple negative breast cancer patients. *Breast Cancer Res.* 2014;16:445.
18. Mostert B, Jiang Y, Sieuwerts AM, 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;133:130-141.
19. Leversha MA, Han J, Asgari Z, et al. Fluorescence in situ hybridization analysis of circulating tumor cells in metastatic prostate cancer. *Clin Cancer Res.* 2009;15:2091-2097.

20. Swennenhuis JF, Tibbe AG, Levink R, Sipkema RC, Terstappen LW. Characterization of circulating tumor cells by fluorescence in situ hybridization. *Cytometry A*. 2009;75:520-527.
21. Mayer JA, Pham T, Wong KL, et al. FISH-based determination of HER2 status in circulating tumor cells isolated with the microfluidic CEE platform. *Cancer Genet*. 2011;204:589-595.
22. Guo W, Yang XR, Sun YF, et al. Clinical significance of EpCAM mRNA-positive circulating tumor cells in hepatocellular carcinoma by an optimized negative enrichment and qRT-PCR-based platform. *Clin Cancer Res*. 2014;20:4794-4805.
23. Mostert B, Sieuwerts AM, Bolt-de Vries J, et al. mRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients. *Mol Oncol*. 2015;9:920-932.
24. Smirnov DA, Zweitzig DR, Foulk BW, et al. Global gene expression profiling of circulating tumor cells. *Cancer Res*. 2005;65:4993-4997.
25. Rossi S, Basso M, Strippoli A, et al. Hormone Receptor Status and HER2 Expression in Primary Breast Cancer Compared With Synchronous Axillary Metastases or Recurrent Metastatic Disease. *Clin Breast Cancer*. 2015.
26. Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. *Nature*. 2011;472:90-94.
27. Nuver J, Smit AJ, van der Meer J, et al. Acute chemotherapy-induced cardiovascular changes in patients with testicular cancer. *J Clin Oncol*. 2005;23:9130-9137.
28. Bonello L, Basire A, Sabatier F, Paganelli F, Dignat-George F. Endothelial injury induced by coronary angioplasty triggers mobilization of endothelial progenitor cells in patients with stable coronary artery disease. *J Thromb Haemost*. 2006;4:979-981.
29. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood*. 1999;93:2951-2958.
30. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. *N Engl J Med*. 1997;337:1584-1590.
31. Strijbos MH, Landburg PP, Nur E, et al. Circulating endothelial cells: a potential parameter of organ damage in sickle cell anemia? *Blood Cells Mol Dis*. 2009;43:63-67.
32. Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat Rev Cancer*. 2006;6:835-845.
33. Furstenberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer*. 2006;94:524-531.
34. Goon PK, Boos CJ, Stonelake PS, Blann AD, Lip GY. Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. *Thromb Haemost*. 2006;96:45-52.
35. Goon PK, Lip GY, Boos CJ, Stonelake PS, Blann AD. Circulating endothelial cells, endothelial progenitor cells, and endothelial microparticles in cancer. *Neoplasia*. 2006;8:79-88.
36. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A*. 2007;71:105-113.
37. Strijbos MH, Gratama JW, Schmitz PI, et al. Circulating endothelial cells, circulating tumour cells, tissue factor, endothelin-1 and overall survival in prostate cancer patients treated with docetaxel. *Eur J Cancer*. 2010;46:2027-2035.

38. Beijer N, Kraan J, Taal W, 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.
39. Beijer 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;100:e246-249.
40. Allard WJ, Matera J, Miller MC, 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;10:6897-6904.
41. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging--predicting overall survival in metastatic breast cancer. *Clin Cancer Res*. 2006;12:6403-6409.
42. Cohen SJ, Alpaugh RK, Gross S, et al. Isolation and characterization of circulating tumor cells in patients with metastatic colorectal cancer. *Clin Colorectal Cancer*. 2006;6:125-132.
43. Hayes DF, Cristofanilli M, Budd GT, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin Cancer Res*. 2006;12:4218-4224.
44. Shaffer DR, Leversha MA, Danila DC, et al. Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. *Clin Cancer Res*. 2007;13:2023-2029.
45. Hou JM, Greystoke A, Lancashire L, et al. Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. *Am J Pathol*. 2009;175:808-816.
46. 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;35:463-474.
47. Allan AL, Keeney M. Circulating tumor cell analysis: technical and statistical considerations for application to the clinic. *J Oncol*. 2010;2010:426218.
48. Barnett D, Granger V, Kraan J, et al. Reduction of intra- and interlaboratory variation in CD34+ stem cell enumeration using stable test material, standard protocols and targeted training. DK34 Task Force of the European Working Group of Clinical Cell Analysis (EWGCCA). *Br J Haematol*. 2000;108:784-792.
49. Whitby L, Granger V, Storie I, et al. Quality control of CD4+ T-lymphocyte enumeration: results from the last 9 years of the United Kingdom National External Quality Assessment Scheme for Immune Monitoring (1993-2001). *Cytometry*. 2002;50:102-110.
50. Mostert B, Sieuwerts AM, Kraan J, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. *Ann Oncol*. 2015;26:510-516.
51. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*. 2013;339:580-584.
52. Powell AA, Talasz AH, Zhang H, et al. Single cell profiling of circulating tumor cells: transcriptional heterogeneity and diversity from breast cancer cell lines. *PLoS One*. 2012;7:e33788.
53. Peeters DJ, De Laere B, Van den Eynden GG, et al. Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting. *Br J Cancer*. 2013;108:1358-1367.
54. Choi JH, Ogunniyi AO, Du M, et al. Development and optimization of a process for automated recovery of single cells identified by microengraving. *Biotechnol Prog*. 2010;26:888-895.

55. Swennenhuis JF, Tibbe AG, Stevens M, et al. Self-seeding microwell chip for the isolation and characterization of single cells. *Lab Chip*. 2015;15:3039-3046.
56. Smerage JB, Budd GT, Doyle GV, et al. Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. *Mol Oncol*. 2013;7:680-692.
57. Kao J, Salari K, Bocanegra M, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One*. 2009;4:e6146.
58. Woelfle U, Sauter G, Santjer S, Brakenhoff R, Pantel K. Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer. *Clin Cancer Res*. 2004;10:2670-2674.
59. Mostert B, Kraan J, Bolt-de Vries J, et al. Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146. *Breast Cancer Res Treat*. 2011;127:33-41.
60. Mostert B, Kraan J, Sieuwerts AM, et al. CD49f-based selection of circulating tumor cells (CTCs) improves detection across breast cancer subtypes. *Cancer Lett*. 2012;319:49-55.
61. Paoletti C, Li Y, Muniz MC, et al. Significance of Circulating Tumor Cells in Metastatic Triple-Negative Breast Cancer Patients within a Randomized, Phase II Trial: TBCRC 019. *Clin Cancer Res*. 2015;21:2771-2779.
62. Sieuwerts AM, Kraan J, Bolt J, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J Natl Cancer Inst*. 2009;101:61-66.
63. Lianidou ES, Mavroudis D, Georgoulas V. Clinical challenges in the molecular characterization of circulating tumour cells in breast cancer. *Br J Cancer*. 2013;108:2426-2432.
64. Barradas AM, Terstappen LW. Towards the Biological Understanding of CTC: Capture Technologies, Definitions and Potential to Create Metastasis. *Cancers (Basel)*. 2013;5:1619-1642.
65. Coumans FA, Lighthart ST, Uhr JW, Terstappen LW. Challenges in the enumeration and phenotyping of CTC. *Clin Cancer Res*. 2012;18:5711-5718.
66. Saucedo-Zeni N, Mewes S, Niestroj R, et al. A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. *Int J Oncol*. 2012;41:1241-1250.
67. Goon PK, Boos CJ, Lip GY. Circulating endothelial cells: markers of vascular dysfunction. *Clin Lab*. 2005;51:531-538.
68. Xiong YQ, Sun HC, Zhang W, et al. Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. *Clin Cancer Res*. 2009;15:4838-4846.
69. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J*. 2003;17:984-992.
70. Ouhtit A, Gaur RL, Abd Elmageed ZY, et al. Towards understanding the mode of action of the multifaceted cell adhesion receptor CD146. *Biochim Biophys Acta*. 2009;1795:130-136.
71. Plate KH, Breier G, Millauer B, Ullrich A, Risau W. Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res*. 1993;53:5822-5827.
72. Chapoval AI, Ni J, Lau JS, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol*. 2001;2:269-274.
73. Steinberger P, Majdic O, Derdak SV, et al. Molecular characterization of human 4Ig-B7-H3, a member of the B7 family with four Ig-like domains. *J Immunol*. 2004;172:2352-2359.

74. Lemke D, Pfenning PN, Sahm F, et al. Costimulatory protein 4IgB7H3 drives the malignant phenotype of glioblastoma by mediating immune escape and invasiveness. *Clin Cancer Res.* 2012;18:105-117.
75. Crispen PL, Sheinin Y, Roth TJ, et al. Tumor cell and tumor vasculature expression of B7-H3 predict survival in clear cell renal cell carcinoma. *Clin Cancer Res.* 2008;14:5150-5157.
76. Qin X, Zhang H, Ye D, Dai B, Zhu Y, Shi G. B7-H3 is a new cancer-specific endothelial marker in clear cell renal cell carcinoma. *Onco Targets Ther.* 2013;6:1667-1673.
77. Roth TJ, Sheinin Y, Lohse CM, et al. B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy. *Cancer Res.* 2007;67:7893-7900.
78. Sun Y, Wang Y, Zhao J, et al. B7-H3 and B7-H4 expression in non-small-cell lung cancer. *Lung Cancer.* 2006;53:143-151.
79. Yamato I, Sho M, Nomi T, et al. Clinical importance of B7-H3 expression in human pancreatic cancer. *Br J Cancer.* 2009;101:1709-1716.
80. Zang X, Sullivan PS, Soslow RA, et al. Tumor associated endothelial expression of B7-H3 predicts survival in ovarian carcinomas. *Mod Pathol.* 2010;23:1104-1112.

Chapter 2

External quality assurance of circulating tumor cell enumeration using the CellSearch® system: A feasibility study

Kraan J, Sleijfer S, Strijbos MH, Ignatiadis M, Peeters D,
Pierga JY, Farace F, Riethdorf S, Fehm T, Zorzino L,
Tibbe AG, Maestro M, Gisbert-Criado R, Denton G,
de Bono JS, Dive C, Foekens JA, Gratama JW

Cytometry Part B (Clinical Cytometry) 2011; 80B:112 – 118

Abstract

Background: Circulating tumor cells (CTC) are cells that have detached from solid tumors and entered the blood. CTC can be detected, among others, by semi-automated immunomagnetic enrichment and image cytometry using CellSearch® (Veridex, Raritan, NJ). We studied the feasibility of external quality assurance (EQA) of the entire CellSearch procedure from blood draw to interpretation of results in multiple laboratories.

Methods: Blood samples from 6 cancer patients and controls were distributed to 14 independent laboratories to test between-laboratory, between-assay and between-instrument variation. Additionally, between-operator variability was assessed through the interpretation of blinded images of all blood samples on a website.

Results: Shipment and storage of samples had no influence on CTC values. Between-instrument (coefficient of variation (CV) <12%) and between-assay variation was low (CV ≤20%), indicating high reproducibility. However, between-laboratory CV ranged from 45 - 64%. Although inter-operator agreement on image interpretation (Fleiss' K statistics) ranged from "substantial" to "almost perfect", image interpretation, particularly of samples containing high numbers of apoptotic cells, was the main contributor to between-laboratory variation.

Conclusions: This multicenter study shows the feasibility of an EQA program for CTC detection in patient samples, and the importance of continuation of such a program for the harmonization of CTC enumeration.

Introduction

Detection of CTCs in the peripheral blood holds great promise in oncology for research purposes and patient management. However, CTC enumeration poses a particular challenge due to the very low frequency of cells of interest (i.e., <10 cells/ml) compared to white blood cells. Assays for CTC enumeration have only recently become available. The CellSearch® System (Veridex, Raritan, NJ) uses immunomagnetic and immunofluorescent labeling to identify and count CTCs in blood samples and is the only system with FDA clearance to predict progression-free survival and overall survival in patients with metastatic breast, colorectal or prostate cancer¹⁻¹⁰. Using threshold levels, CTC enumeration by the CellSearch system can stratify patients with advanced breast, colorectal, and prostate cancer into groups differing in prognosis. In addition, a decrease in CTC count appears to be associated with outcome to systemic therapy in these tumor types. As a result, clinical studies are ongoing to assess whether systemic therapy in certain tumor types can be guided on CTC counts rather than the current standard practice of conventional radiological assessments. Given these promising data, the clinical utility of CTC detection and enumeration using this method is likely to increase swiftly.

For an assay used in daily practice and in multi-center studies, consistency in performance between diverse laboratories is pivotal. With respect to the CellSearch System periodic quality assurance of image interpretation is provided by the manufacturer to provide on-going training for CellSearch System certified operators. For this purpose, single-use vials with predefined numbers of fixed, fluorescently labeled SK-BR-3 cells are available. However, it is likely that variability in results for patient samples is greater than for these control samples, as might also be concluded from the varying levels of expression of EpCAM by different breast cancer subtypes¹¹. In addition, we have previously shown for other relatively rare cell subpopulations, such as some lymphocyte subsets and CD34⁺ hematopoietic stem cells, that after establishing the intra- and inter-laboratory variations and their underlying causes, assay performance can be improved by the use of stable test material, standard protocols and targeted training¹²⁻¹⁵.

In this study we aimed to document the extent and reasons of variation in CTC enumerations using the CellSearch® System by 15 European laboratories trained by Veridex. Further, we studied the feasibility of external quality assurance (EQA) of the entire procedure from blood draw to image interpretation.

Design and methods

Patient and sample collection

Six metastatic cancer patients with detectable CTC levels were selected by the coordinating institute (Erasmus University Medical Center, Rotterdam, The Netherlands). Only patients fit enough to donate 120 mL of peripheral blood were enrolled in the study. 120 ml blood was collected by vena puncture in multiple CellSave Preservative tubes (Veridex). To avoid contamination by epithelial skin cells, the first 3 ml was collected in a separate tube and discarded. Blood from all tubes was pooled and re-aliquoted into empty CellSave Preservative tubes (8.5 ml per tube). These tubes were sent to all participating centers at ambient temperature by overnight courier. This study was approved by the Medical Ethical Committee of Erasmus MC and each participating patient provided informed consent.

Variation between laboratories

To evaluate between-laboratory variation in CTC counts including the effects of transport, fresh samples were sent in 2 cohorts consisting of 3 blood samples. Participants were asked to run the sample using the CellSearch Epithelial Cell Kit and CellTracks AutoPrep® System (Veridex) according to the standard procedure¹. In brief, a blood sample of 7.5 ml is mixed with ferrofluid particles coated with anti-EpCAM antibodies. After incubation, unlabeled cells and plasma are removed by magnetic separation. Subsequently, the isolated cells are stained with fluorescent monoclonal antibodies for epithelial cells (cytokeratins 8, 18, 19), contaminating leucocytes (CD45) and a nuclear staining dye (DAPI). During sample processing, 7.5 ml of blood is reduced to ~ 700 μ l containing enriched CTCs and transferred to a cartridge in a MagneSt device. Here, the strong magnetic field moves immunomagnetically labeled cells to the surface of the cartridge caused by the strong magnetic field in order to render them ready for analysis. All steps are performed fully automated by the CellTracks AutoPrep System. Reading of the samples was achieved with the CellTracks Analyzer, a semi-automated fluorescence microscope used to scan the entire surface of the cartridge at four different wavelengths in order to detect objects labeled with all fluorochromes used.

Captured images that contain objects fulfilling predetermined criteria are automatically presented in a gallery format from which classification was performed independently by trained operators in each center. A cell is classified as a CTC on the basis of its morphologic features (i.e., nearly round or oval morphology with a visible nucleus within the cytoplasm) and staining patterns (i.e., DAPI positive, cytokeratin positive and CD45 negative). A CTC must be at least 4 μ m in diameter,

although these cells are highly heterogeneous in terms of size and morphology¹ (Fig 1).

Variation between instruments

To monitor system operation, the manufacturer provides samples containing single-use vials with predefined numbers of fixed, fluorescently labeled SK-BR-3 cells. Each vial contains two different CTC concentrations for high level and low level control. To analyze the inter-instrument variation, two control samples of the same batch were sent to all participants; one batch as part of the first cohort and another batch during the second.

Variation within assays

The intra-assay variation was tested on two blood samples by the coordinating institute. For this purpose 5 extra aliquots were prepared from the same pooled blood sample that was distributed to the participants. All 5 aliquots were tested as a batch on a single instrument.

Between-operator variation of image interpretation

To test the variability on image interpretation between operators, the images obtained by the coordinating institute of all 6 blood samples were made available as a website survey. Participants were asked to analyze all images and to classify for CTCs according to the manufacturer's guidelines.

Statistical analysis

Data processing and statistical analysis were performed using Excel (Microsoft Corporation, Redmond, WA) and Stata (Stata Corporation, College Station, TX) software. Standard statistical methods were used and are specified in Results and legends to the figures.

Results

Variation between laboratories

Aliquots of 6 blood samples from patients with metastatic carcinoma were sent out by courier to 13 participating institutes during the first cohort of 3 samples, and to 14 institutes during the second cohort of 3 samples. One shipment to one center was lost during transport; for all other samples the transport process was successful and the response rate was 100%. Of the 80 reported results, 27 were performed one day after blood collection, 43 after two days, 7 after three days and

only 3 (outside the storage limit of 72 hours) after five days. Pooled CTC data analyzed as a function of time elapsing after blood sampling (Table 1), resulted in similar median CTC counts and ranges up to day 3.

Classification of all 6 samples resulted in median CTC counts ranging from 2 to 36 CTCs per 7.5 ml blood (Table 2). The between-laboratory CV for the 6 samples ranged from 45 to 64% (Table 2). Based on the clinically relevant cut-off levels of ≥ 3 CTC/7.5 ml for metastatic colon carcinoma (7) and ≥ 5 CTC/7.5 ml blood for other advanced carcinomas (1,4,8), samples 1, 2 and 5 were correctly classified by all centers as either below (sample 2) or above the cut-off level (sample 1 and 5). For samples 3, 4 and 6, results were less consistent. Although most participants scored these samples as above the cut-off level, they were classified as below the threshold by 1 out of 14 (7%), 2 out of 13 (15%) and 4 out of 14 (29%) participants, respectively. In total, 6 out of 80 samples (7.5%) were scored discordant in terms of below or above the cut-off levels.

Variation between instruments

In two different high-level control samples, median CTC values were 1105 (inter quartile range [IQR], 1075 to 1126) and 942 (IQR: 911 to 980). For two low-level controls, median CTC values were 51 (IQR: 49 to 56) and 47 (IQR: 45 to 49; Table 3). This low variation between results (CV <12%) fell within the expected values as defined by the manufacturer.

Variation within assays

Samples 1 and 6 were also tested in 5 replicate assays by one instrument in the coordinating laboratory. Results were highly reproducible with a median of 20 CTCs (IQR: 20 to 21; CV, 20%) for sample 1 and a median of 12 CTCs (IQR: 12 to 13; CV, 14%) for sample 6. The cartridge of sample 1 was also scanned again after storage for 24 h in the dark, and yielded a similar result (median 20, IQR: 18 to 21).

Between-operator variation of image interpretation

The majority of the 509 images selected for the website survey, were unanimously classified as not being a CTC (“negative”) by all participants (Table 4). Unanimous classifications of an image as a CTC (“positive”) per sample ranged between 1 (sample 2) to 8 CTCs (sample 1). Discordant classifications ranged between 4% of the images from sample 2, to 31% on images from sample 3 (median 14%). Inter-operator agreement using Fleiss’ kappa statistics (Table 4: 6 surveys; 14 unique readers per image) resulted in the classification “substantial agreement” on 4 samples and “almost perfect agreement” on the remaining 2 samples.

Discussion

The CellSearch System represents the first automated, standardized system with FDA clearance for the detection and quantification of CTCs in peripheral blood^{4,5}. Given the promising data from studies in a wide variety of cancers, it is likely that the role of CTC enumeration by the CellSearch system¹⁻⁹ will gain importance and become more widely used for patient management and translational cancer research.

The aims of this study were to analyze the extent and reasons of between-laboratory variation in results of CTC enumeration in patient samples using the CellSearch System. Previous studies¹²⁻¹⁵ emphasized the importance and confirmed the success of such an approach to reduce the between-laboratory variation in EQA, which is of paramount importance for multicenter studies. The protocol described here could serve as a template for external quality control of this system or any other rare cell detection system.

The CV for between-laboratory variation of CTC counts in this study ranged from 45 to 64% (Table 2). The multicenter survey on variation in rare event counts in whole blood that comes closest, i.e., CD34 hematopoietic stem cell enumeration^{12,14}, showed CVs ranging from 10 to 86% in a standardized assay. Both trials on CTC and CD34 enumeration underscore that there is room for improvement. The approximately 1000-fold lower frequency of CTCs as compared to CD34⁺ stem cells is probably the major contributing factor accounting for the larger between-laboratory variation.

For several tumor types, clinically relevant cut-off values have been established^{3,4,8} with worse outcomes for those patients with CTC counts above these threshold values. Probably, classification of CTC samples either as above or below these cut-off value rather than the absolute number of CTC is more appropriate to determine the variation. Using these cut-off values and on the basis of consensus scores of all laboratories, 5 of the 6 samples were scored as above the cut-off value. Three samples were correctly scored by all laboratories, while 6 misclassifications were made by 1-3 individual participants on the remaining samples. On a total of 80 classifications by 14 laboratories, only 6 (7.5%) were misclassified (Table 2 and Figure 2), which can be deemed acceptable. However, a recent study showed the value of CTC count analyzed as a continuous variable to monitor and classify disease status¹⁶. This particular approach would demand for reproducible assay performance and low between-laboratory variation.

To better address the between-laboratory variation, we studied the factors that contributed to this variability. Although the blood tubes were pooled after collection before being re-aliquoted, the distribution of CTCs will follow a Poisson distribution that will especially influence the variability at low CTC frequencies¹⁷.

Consequently, in the case of patient sample 2 in this study with a median of 2 CTCs, the probability that an aliquot has indeed 2 CTCs is only 27%. In contrast, the probability that an aliquot of this sample has 5 or more CTCs, which is the clinically relevant cut off level for metastatic breast cancer, is only 5% according to the effect of Poisson distribution. Another cause contributing to variation between laboratories is the variation between instruments. This factor was tested using highly stable control samples containing fixed, fluorescently labeled SK-BR-3 cells. As shown in Table 3, this resulted in highly reproducible instrument performance for enrichment and detection of CTCs. Also the variation within assays, as tested on two patient samples by one instrument, was low since results from both samples were within a range that could already be expected by a normal Poisson distribution. Storage time of CTCs after collection might also contribute to large variation between laboratories. However, as already shown ⁷, the use of CellSave Preservative tubes made it possible to ship the blood samples at ambient temperature by courier and stabilized the samples up to 72 hours. Our results were in line with this finding. During this study the manufacturer has adapted the product insert of the CellSave Preservative tubes and increased the time the blood remains stable from 72 hrs to 96 hrs.

Consequently, we conclude that aliquoting blood samples, storage time and instrument have a limited and reproducible role in the variation in CTC enumeration between laboratories and therefore, we established a website survey to address the inter-operator variation on the classification of the images as part of the total assay. Although the inter-operator agreement was statistically interpreted as very good, image interpretation did contribute to between-laboratory variation; specifically, samples containing high numbers of dead or apoptotic CTCs resulted in inconsistency in scoring (e.g., sample 3). As previously described by ¹, dead, apoptotic or enucleated cells could be observed but should not be counted as CTCs (fig 1). So, despite extensive guidance and definitions of which events should be regarded CTCs provided by the manufacturer to the users, inconsistency in image scoring largely contributes to inconsistency in results between laboratories and therefore remains a point of attention. Addition of extra markers specific for CTCs or markers discriminating between vital and dead/apoptotic cells will probably decrease variation and improve the detection of true CTCs. It should be noted however, that enumerating of dead/apoptotic CTCs may also have clinical relevance, as recently addressed by Coumans et al ¹⁸.

In conclusion, our study shows the feasibility of an external quality assurance program for CTC detection in peripheral blood. Obviously, this approach is relevant not only for the CellSearch System, but for other CTC assays as well ¹⁶. Furthermore, this study shows the importance of incorporating 'real life' patient samples to better address between-laboratory variation than when stabilized (blood) samples spiked with cell lines are used. Using clinically relevant cut-off

values, discordance between laboratories is low and can be considered acceptable. In terms of absolute CTC numbers however, the between-laboratory variation is still considerable. Continuation of such an external quality program for the CellSearch instrument and the inclusion of additional markers enabling discrimination of vital versus dead/apoptotic CTCs are important to obtain consistent data from CTC enumeration techniques among different laboratories.

Table 1: Effect of storage time on CTC counts (pooled data of 6 samples)

Time of sample processing (days)	N	Median CTC count	CTC Range
1	27	9	1-65
2	43	9	0-55
3	7	2	1-23
5	3	16	11-19

Table 2: Variation between laboratories on 6 distributed patient samples.

Sample (tumor origin)	Number of participants	CTC count				Classification (discordance*)
		Median (IQR)	Min	Max	CV	
1 (CUP)	13	14 (11-17)	5	32	49%	pos (n=0)
2 (breast)	13	2 (2-3)	1	4	45%	neg (n=0)
3 (prostate)	14	36 (13-45)	5	65	63%	pos (n=0)
4 (prostate)	13	9 (9-13)	1	19	48%	pos (n=2)
5 (colon)	13	10 (7-16)	3	23	52%	pos (n=0)
6 (colon)	14	7 (3-9)	1	16	64%	pos (n=4)

* Using the clinically relevant threshold (≥ 3 CTC/7.5 ml for metastatic colon carcinoma and ≥ 5 CTC/7.5 ml blood for other advanced carcinomas, samples were classified as above (pos) or below these thresholds (neg). Abbreviations: CUP: carcinoma of unknown primary; IQR: inter quartile range; CV: Coefficient of variation.

Table 3: Variation between instruments on 2 distributed control samples.

Sample (control type)	Number of participants	CTC Count				Expected values (samples out of range)
		Median (IQR)	Min	Max	CV	
A478 (Low control)	13	51 (49-56)	43	66	11%	15 - 81 (n=0)
A478 (High control)	13	1105 (1075-1126)	1023	1182	4%	887 – 1387 (n=0)
A767 (Low control)	12	47 (45-49)	40	58	10%	16 - 78 (n=0)
A767 (High control)	12	942 (911-980)	781	1040	7%	725 – 1189 (n=0)

Table 4: Between-investigator variation of image interpretation on one distributed set of images by all participating institutes (n=14).

Sample (tumor origin)	Number of images	CTC Count and image interpretation					Kappa*
		Median (IQR)	CV	Concordant results		Discordant results	
				Positive	Negative		
1 (CUP)	52	14 (12-15)	11%	8	37	7 (13%)	0.89
2 (breast)	50	1 (1-1)	47%	1	47	2 (4%)	0.87
3 (prostate)	62	7 (7-14)	48%	3	40	19 (31%)	0.61
4 (prostate)	152	9 (8-11)	23%	2	131	19 (13%)	0.69
5 (colon)	133	16 (13-19)	19%	5	108	20 (15%)	0.75
6 (colon)	60	7 (6-8)	28%	2	48	10 (17%)	0.75

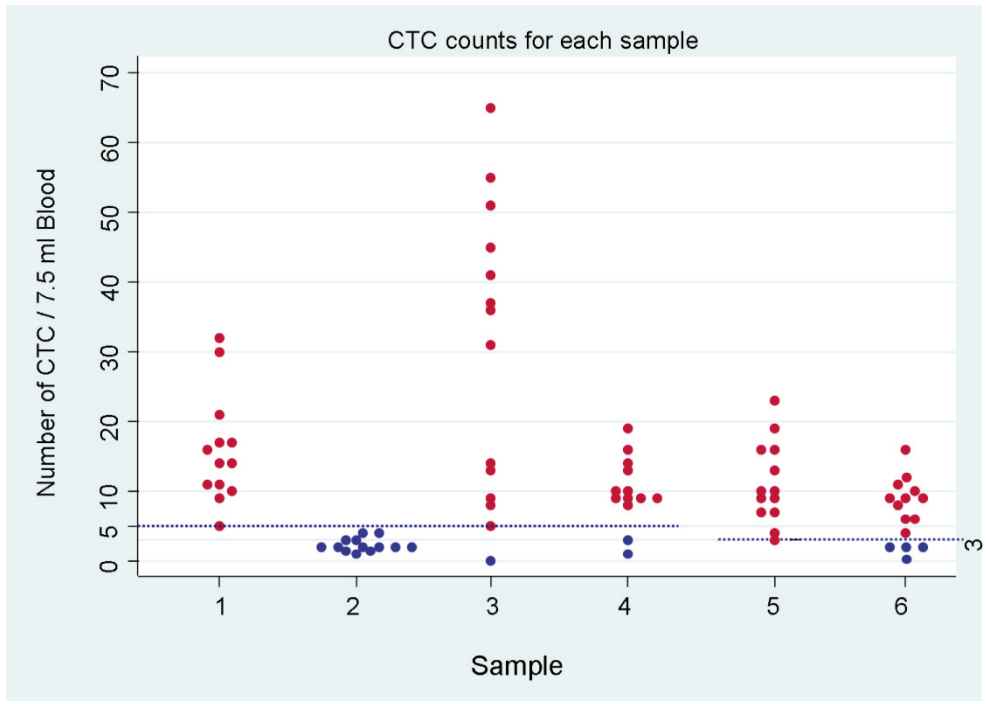
* Interpretation:

<0	Poor agreement
0.0 – 0.20	Slight agreement
0.21 – 0.40	Fair agreement
0.41 – 0.60	Moderate agreement
0.61 – 0.80	Substantial agreement
0.81 – 1.00	Almost perfect agreement

Figure 1: A gallery of images to be screened for CTCs by the operator. The first rows (#1 to #4)) show an image scored as a CTC by all 14 participants. Row #5 and further depict discordant results; the percentage of laboratories scoring the images as a CTC are indicated as the CTC score. Row #8 to #15 provide examples of CTC fragments and apoptotic CTCs, these images are frequently observed in CTC analysis of carcinoma patients but should not be included in the CTC counts according to the manufacturer’s guidelines ^{1,18}.

#	Composite	CD45 APC	FITC	CK PE	DAPI	CTC score
1						100%
2						100%
3						100%
4						100%
5						93%
6						86%
7						86%
8						64%
9						57%
10						57%
11						36%
12						14%
13						14%
14						14%
15						7%

Figure 2: Variation between laboratories on 6 distributed patient samples. Each dot represents the number of CTCs classified by one participant for a sample. Based on the reported clinical cut off levels ≥ 3 CTC/7.5 ml for colon cancer³ and ≥ 5 CTC/7.5 ml for other malignancies^{2,4,8}, red dots represent a positive and blue dots a negative classification.



References

1. Allard WJ, Matera J, Miller MC, 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;10:6897-6904.
2. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging--predicting overall survival in metastatic breast cancer. *Clin Cancer Res*. 2006;12:6403-6409.
3. Cohen SJ, Alpaugh RK, Gross S, et al. Isolation and characterization of circulating tumor cells in patients with metastatic colorectal cancer. *Clin Colorectal Cancer*. 2006;6:125-132.
4. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med*. 2004;351:781-791.
5. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol*. 2005;23:1420-1430.
6. Hayes DF, Cristofanilli M, Budd GT, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin Cancer Res*. 2006;12:4218-4224.
7. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin Cancer Res*. 2007;13:920-928.
8. Shaffer DR, Leversha MA, Danila DC, et al. Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. *Clin Cancer Res*. 2007;13:2023-2029.
9. Hou JM, Greystoke A, Lancashire L, et al. Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. *Am J Pathol*. 2009;175:808-816.
10. 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;35:463-474.
11. Sieuwerts AM, Kraan J, Bolt J, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J Natl Cancer Inst*. 2009;101:61-66.
12. Barnett D, Granger V, Kraan J, et al. Reduction of intra- and interlaboratory variation in CD34+ stem cell enumeration using stable test material, standard protocols and targeted training. DK34 Task Force of the European Working Group of Clinical Cell Analysis (EWGCCA). *Br J Haematol*. 2000;108:784-792.
13. Gratama JW, Kraan J, Keeney M, Granger V, Barnett D. Reduction of variation in T-cell subset enumeration among 55 laboratories using single-platform, three or four-color flow cytometry based on CD45 and SSC-based gating of lymphocytes. *Cytometry*. 2002;50:92-101.
14. Levering WH, Preijers FW, van Wieringen WN, et al. Flow cytometric CD34+ stem cell enumeration: lessons from nine years' external quality assessment within the Benelux countries. *Cytometry B Clin Cytom*. 2007;72:178-188.
15. Levering WH, van Wieringen WN, Kraan J, et al. Flow cytometric lymphocyte subset enumeration: 10 years of external quality assessment in the Benelux countries. *Cytometry B Clin Cytom*. 2008;74:79-90.
16. Scher HI, Jia X, de Bono JS, et al. Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. *Lancet Oncol*. 2009;10:233-239.

17. Tibbe AG, Miller MC, Terstappen LW. Statistical considerations for enumeration of circulating tumor cells. *Cytometry A*. 2007;71:154-162.
18. Coumans FA, Doggen CJ, Attard G, de Bono JS, Terstappen LW. All circulating EpCAM+CK+CD45- objects predict overall survival in castration-resistant prostate cancer. *Ann Oncol*.

Chapter 3

Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR

Jaco Kraan*, Anieta M. Sieuwerts*, Joan Bolt-de Vries,
Petra van der Spoel, Bianca Mostert, John W.M. Martens,
Jan-Willem Gratama, Stefan Sleijfer and John A. Foekens

*Both authors contributed equally to this study.

Abstract

Detection of circulating tumor cells (CTCs) in whole blood from metastatic cancer patients by the CellSearch® CTC Test (Veridex LLC, Warren, NJ) has been shown to have clinical relevance. In addition to enumeration, there is great interest in molecular characterization of these CTCs. We aimed to establish a robust method to perform mRNA expression analysis of multiple genes by a real-time reverse transcriptase (RT)-PCR on small numbers of CTCs enriched from whole blood by the CellSearch system. Despite the 4 log depletion of leukocytes after CellSearch enrichment, the CTC-enriched fractions still contained leukocytes, in particular B-lymphocytes, which severely interfered with our CTC-specific gene expression profiling. After extensive washing and leukocyte-specific depletion by anti-CD45 coated magnetic beads prior to CellSearch enrichment, the number of leukocytes present in the enriched fraction was still high (range 60 – 929). However, by using a set of genes with no or minor expression by leukocytes, we succeeded to perform quantitative gene expression profiling specific for as little as one breast cancer CTC present in a CTC-enriched environment typically containing over 800 contaminating leukocytes. Our method allows molecular characterization specific for as little as one CTC, and can be used to expand the understanding of the biology of metastasis and, potentially, to improve patient management.

Introduction

Circulating cells with the characteristics of tumor cells of epithelial origin have been demonstrated in blood and bone marrow of prostate, melanoma, colon, esophageal, head and neck, lung and breast cancer patients (reviewed in ¹⁻¹¹). These cells have not only been shown in patients with metastatic disease, but also in those whose tumors are apparently localized ^{12,13}. The development and optimization of new technologies to identify and characterize such cells, and the establishment of the association of their presence with potentially clinical significance are highly relevant.

A recently developed technology to quantify the number of CTCs in whole blood of cancer patients is the use of the CellSearch™ CTC Test (Veridex LLC, Warren, NJ), so far the only US Food and Drug Administration (FDA) approved diagnostic test ¹⁴ to automate the detection and enumeration of CTCs for monitoring disease progression and therapy efficacy in metastatic prostate ¹⁵, colorectal ¹⁶ and breast ¹⁷ cancer. After enrichment using magnetic beads coated with EpCAM-specific antibodies, isolated cells are stained with fluorescent monoclonal antibodies specific for epithelial cells (CK-8/18/19), for leukocytes (CD45) and for their nuclei with a nuclear staining dye (DAPI), and subsequently enumerated by a semi-automated fluorescence microscope.

In addition to enumeration, there is great interest in molecular characterization of CTCs to understand fundamental issues such as their genetic composition, and to establish association of these genetic profiles with patient treatment outcomes. However, although this system allows capture of CTCs in blood of cancer patients by selectively isolating EpCAM-positive cells followed by quantification of DAPI- and CK-8/18/19-positive cells, there are still considerable quantities of contaminating leukocytes (DAPI⁺/CD45⁺) present after enrichment. This contamination, together with the fact that CTCs detected by CellSearch™ CTC Test appear to occur in small numbers in humans (frequently fewer than 5 CTCs per 7.5 mL blood in metastatic breast cancer patients¹⁷), forms a pitfall that cannot be ignored when one is interested in a molecular characterization method specific for CTCs.

The purpose of this study was to optimize a robust method to perform mRNA expression analysis of up to 96 genes by real-time RT-PCR, thus conveniently fitting a 96-well plate, on as little as 1 CTC in an environment containing large quantities of contaminating leukocytes.

Materials and Methods

Breast cancer cell lines and healthy blood donors

The MDA-MB-231, CAMA-1 and SK-BR-3 human breast cancer cell lines used in this study were all obtained from American Type Culture Collection (Manassas, VA). Extensive analysis of nearly 150 polymorphic microsatellite markers had shown that all cell lines are unique and monoclonal¹⁸. Clinical blood samples were obtained from breast cancer patients with metastatic disease and blood samples from healthy controls from laboratory volunteers and the Sanquin Blood Bank South-west Region (Rotterdam, The Netherlands). This study was approved by the Erasmus MC Institutional Review Board (METC 2006-248), and all donors gave their written informed consent.

Flow cytometry: absolute number of leukocytes and lymphocyte subsets in whole blood

An aliquot of 100 μ l whole blood was incubated with 10 μ l of the following fluorochrome conjugated antibodies: CD56 PE (DAKO, Glostrup, Denmark), CD45 FITC, CD19 PE-Cy7, CD14 APC, CD3 APC-Cy7 (all BD Biosciences) and 1 μ g/ml 7-amino-actinomycin D (7AAD, Sigma-Aldrich, St. Louis, MO). After a 15 min incubation at room temperature erythrocytes were lysed with 2 ml NH_4Cl containing 100 μ l flow count fluorospheres of known concentration (Beckman Coulter Inc, Miami, FL), which were added to obtain absolute counts. Samples were run on a FACSCanto flow cytometer (BD Biosciences) within 1 h and dead cells (7AAD positive) were excluded from analysis. Cell types could be identified with the following definitions: leukocytes (CD45^+), mature myeloid (CD45^+ , SSC^{high} , CD14^-), monocytes (CD45^+ , CD14^+), basophils (CD45^{dim} , SSC^{low}), lymphocytes (CD45^+ , CD14^- , SSC^{low}), B lymphocytes (CD19^+), T lymphocyte (CD3^+) and NK lymphocytes (CD56^+ , CD3^-).

Flow cytometry: absolute number of leukocytes and lymphocyte subsets in CellSearch-enriched samples

After the CellSearch procedure enriched samples were placed in a magnet (MagCollect, R&D systems, Abingdon, UK) for 10 min and supernatant was discarded. After removing the tube from the magnet the cell pellet was re-suspended in 100 μ l PBS and incubated for 15 min with the same monoclonal antibody cocktail as described for whole blood samples. After washing with 2 ml PBS the tube was placed in a magnet and supernatant discarded. Cells were re-suspended in 500 μ l PBS and 100 μ l Flow-Count fluorospheres (Beckman–Coulter Inc, Miami, FL).

Leukocyte depletion of whole blood with anti-CD45 coated magnetic beads

EDTA blood (5.25 ml) was diluted with CellSearch buffer (3 ml) and mixed with 1 ml anti-CD45 coated magnetic beads (Dynabeads, Invitrogen, Breda, The Netherlands) and after a 30 minute incubation with gently tilting and rotation placed in a magnet for 10 minutes. 7.5 ml blood containing unbound cells was transferred into a CellSearch tube and run on the CellTrack AutoPrep Analyzer (Veridex LLC, San Diego, USA) according to the manufacturer's instructions.

Enumeration of epithelial cells spiked in whole blood

CellSave (Veridex LLC, San Diego, USA) EDTA blood samples (7.5 ml) of various healthy blood donors were quantitatively spiked with 20 μ l cell suspension containing 1-150 cultured human breast cancer cells. To determine the actual viable cell number of epithelial cells to be spiked, a 100 μ l aliquot of the cultured cells was incubated with 10 μ l of 1 μ g/ml 7AAD and 100 μ l of FlowCount fluorospheres. After a 15 min incubation at room temperature, 2 ml PBS was added and samples were run on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). At least 10,000 beads were acquired to estimate the number of 7AAD negative (viable) cells. After serial dilution, spiking efficiency was controlled by counting the exact number of cells in 20 μ l drops in triplicate under a phase contrast light microscope. To establish CTC recovery, samples were processed on the CellTrack AutoPrep Analyzer (Veridex LLC) with the CellSearch CTC profile Kit (Veridex LLC) and CTC counts were determined on the CellSpotter Analyzer (Veridex LLC) according to the manufacturer's instructions.

RNA isolation

For gene expression studies 7.5 ml EDTA blood samples of the same healthy donors were in parallel with the enumeration studies spiked identically as described above and enriched on the CellTrack AutoPrep Analyzer (CellSearch CTC profile kit). After removal of the supernatant using a MagCollect Magnet (R&D Systems, Minneapolis, USA), the CellSearch-enriched cells were lysed by adding 250 μ l of Qiagen RNeasy RLT Lysis buffer (Qiagen BV, Venlo, The Netherlands). The RNA lysate was immediately stored at -80°C. Cultured cells were also directly spiked in Qiagen RNeasy RLT Lysis buffer to serve as positive control. Subsequent RNA isolation was performed with the RNeasy (Micro) Kit (Qiagen) according to the manufacturer's instructions.

cDNA synthesis, pre-amplification and real-time PCR (qRT-PCR)

Two different cDNA synthesis methods (High Capacity cDNA Archive kit from Applied Biosystems (ABI), Nieuwerkerk a/d IJssel, The Netherlands; RevertAid™ H Minus First Strand cDNA Synthesis Kit from Fermentas, St. Leon-Rot, Germany) and three different linear pre-amplification methods (TaqMan™ PreAmp from ABI

suitable for multiplexing up to 100 gene expression targets; Whole Transcriptome Ovation RNA Amplification from NuGEN, Bemmell, The Netherlands; Full spectrum RNA Amplification from System Bioscience, Uden, The Netherlands) were utilized according to the manufacturer's instructions. The resulting pre-amplified cDNA preparations were analyzed by real-time PCR in a 20 μ l reaction volume in a Mx3000P™ Real-Time PCR System (Stratagene, Amsterdam, The Netherlands), using TaqMan® Gene Expression Assays in combination with TaqMan Universal PCR Master Mix No AmpErase UNG (ABI) according to the manufacturer's instructions. PCR efficiency of each gene-specific real-time PCR session was validated with a standard curve constructed from a simultaneously run serially diluted cDNA pool of human breast fibroblasts and cell-lines. Negative controls included samples without reverse transcriptase and samples where total RNA and cDNA was replaced with genomic DNA. Quantitative values were obtained from the threshold cycle (Ct) at which the increase in TaqMan probe fluorescent signal associated with an exponential increase of PCR products reached the fixed threshold value of 0.02, which was in all cases, at least 10-fold the standard deviation of the background signal.

Reference genes and data normalization

Unless stated otherwise, levels of *HMBS*, *HPRT1* and *GUSB* were used to control sample loading and RNA quality, as described previously¹⁹. Bone marrow stromal cell antigen 1 (*BST1*) and protein tyrosine phosphatase receptor type C (*PTPRC* coding for CD45) were the control genes for leukocyte background and keratin 19 (*KRT19*) and tumor-associated calcium signal transducer 1 (*TACSTD1* coding for EpCAM) for CTC quantification. After verification of equal PCR efficiency, the relative expression levels were quantified by using the delta threshold cycle (Ct) method, which is the difference in the target gene Ct minus the average Ct of the appropriate control genes.

Statistical analysis

Statistical analyses were performed with SPSS 15.0, Analyse-it 2.11 and GenEx Pro version 4.3.6 software for real-time PCR expression profiling. Pearson's correlation was used to determine the degree of linear relationship between two variables. The strengths of the associations between continuous variables was tested with the non-parametric Spearman rank correlation test (r_s). Gene expression levels in the various fractions were compared using the non-parametric one-tailed Wilcoxon's test to test the null hypothesis and the Mann-Whitney U test and two-tailed paired t-test to identify genes with significantly different expression levels in between groups. A false discovery rate (FDR) control of 10% was applied to correct for multiple testing²⁰ and principal component analysis (PCA) to reduce

multidimensional data sets to lower dimensions. All statistical tests were two-sided with $P < 0.05$ considered as statistically significant.

Results and discussion

Homogeneous and linear pre-amplification

The purpose of this study was to optimize a sensitive method to perform mRNA expression analysis of up to 96 genes including controls (thus conveniently fitting a 96-well plate) specific for CTCs present in an environment containing large quantities of contaminating leukocytes. Our first challenge was to find a method that would enable us to measure gene transcripts by real-time RT-PCR in total RNA isolated from as little as one cell (~10 pg RNA) in a linear and homogeneous (unbiased) manner. In view of our ultimate goal to molecularly characterize CTCs, any gene expression assay showing as a non-homogeneously amplified outlier in our tests could not be used for further gene expression analysis because the data would not be truly representative for the original sample. Therefore, our criteria to meet were high sensitivity combined with a minimum number of non-homogeneously amplified gene assays. For that, two different cDNA synthesis methods (a: High Capacity cDNA Archive and b: RevertAid H Minus First Strand cDNA Synthesis) and three different linear pre-amplification methods (1: TaqMan PreAmp, 2: Whole Transcriptome (WT) Ovation RNA amplification and 3: Full spectrum RNA amplification) were compared. To validate the linear and homogeneous nature of the various pre-amplification methods, we performed comparative tests between amplified and non-amplified cDNA with the use of total RNA from various sources. Next, the sensitivity (call rate) was calculated from the percentage of real-time PCR assays that required less than 35 cycles (considered as cycle detection threshold) for one or two cells. Thus, a call rate of for example 70% implicates that 70% of the genes can be detected reliably within 35 cycles while 30% of the genes are not detectable within 35 cycles. The homogeneity of amplification was set at a cut-off of 1 Ct, *i.e.*, for an assay to be considered homogeneously amplified, the number of cycles that were required after pre-amplification should be within 1 Ct range of the number of cycles that were required for the non-amplified material.

The results of analyzing our data with the as such defined call rate, using 22 TaqMan Gene Expression Assays in combination with RNA isolated from 2 different cell lines and 32 assays in combination with RNA isolated from 2 different breast tumors, are shown in Table 1. Of the three linear pre-amplification methods (PreAmp, WT Ovation, Full Spectrum), the call rate obtained with total RNA isolated from one or two cells (*i.e.*, ~10-20 pg total RNA) in combination with the

gene specific PreAmp method from ABI with cDNA synthesized with the RevertAid H Minus First Strand cDNA Synthesis Kit from Fermentas was the highest and was significantly higher than the call rate obtained with cDNA generated with the High Archive cDNA synthesis kit (one-tailed Wilcoxon $P = 0.028$, $n=108$). In addition, the homogeneity of amplification was superior for the PreAmp method from ABI (delta Ct amplified versus non-amplified within one Ct range for over 70% of 59 assays analyzed in two different breast tumors, while this was only 9% for the 59 assays analyzed with the WT ovation method and 31% for the 59 assays analyzed with the Full Spectrum method).

Based on these results, we continued with the RevertAid H Minus First Strand cDNA Synthesis Kit to synthesize cDNA and the TaqMan PreAmp method to amplify this cDNA. To further validate the sensitivity plus the linear and homogeneous nature of this cDNA, we performed similar comparative tests between amplified and non-amplified cDNA from various sources (breast tumor specimens, cell lines, healthy blood donors). Representative results are shown in Fig. 1A and Fig. 1B. These experiments showed that, following 14 pre-amplification cycles, all genes were linearly amplified in up to 30 real-time PCR cycles and that most genes could reliably be quantified at the level equivalent to one cell (Fig. 1A, red arrow in left panel). Only quantification of the less abundant transcripts, typically requiring over 30 PCR cycles, such as *MAGEA3*, *TERT*, *KRT20* in this example (Fig. 1A, genes in the top of the right panel) appeared less reliable for assessment of gene transcript levels in low cell numbers such as expected in minimal residual disease. Secondly, the data implied that for 9 out of 144 different genes (6%) quantification might not be representative of the original sample (Fig. 1B; 9 assays outside the ± 1 Ct range depicted by the red and blue dotted lines). Because the latter was not merely associated with transcript abundance (all outliers were found midrange from 23 to 32 Ct and not at the extreme Ct-values that are associated with high and low transcript abundance), these data demonstrated that reliability and reproducibility must be checked beforehand for each individual real-time PCR assay.

Sources of variation

Next, we investigated which procedure in our protocol (CellSearch enrichment, RNA isolation, cDNA synthesis, PreAmp, qPCR), was the most susceptible to variation (see Table 2 for an overview). For this, we randomly selected a panel of 75 genes. Although all steps in the procedure added to the within- and between-run variation (Table 2), the resultant Pearson correlation coefficient between the expression levels of the 75 tested genes in independently performed experiments was never below 0.93 (see Fig. 1C for a graphical presentation of the final resultant with respect to Ct values obtained for two independently performed CellSearch

experiments where 75 different TaqMan Gene Expression Assays were measured in duplicate). Thus, our method to assess transcript levels showed to be reproducible.

qRT-PCR analysis of 94 genes in one breast cancer tumor cell

Having established the feasibility of our approach, *i.e.*, analyzing a large number of genes by pre-amplified RT-PCR in as few as a single cell, we selected 91 TaqMan Gene Expression Assays that were available in our lab at the time of performing the experiment. This set was supplemented with three universal reference genes (*GUSB*, *HPRT1* and *HMBS*) to control sample loading and RNA integrity, one no-assay negative control and one no-template (H₂O only) negative control. Subsequently, we assessed whether or not our method was robust enough to measure these genes in one cell with similar sensitivity and specificity compared with 10 cells. Fig. 1D shows that this was the case: expression profiling obtained with 10 CAMA-1 or 10 MDA-MB-231 cells showed a gene expression profile very similar to the profile obtained with one or two cells (Spearman r_s for one cell vs. 10 cells = 0.94 and 0.92, respectively, $P < 0.0001$). In addition, CAMA-1 and MDA-MB-231 cells clustered together for the 1, 2 and 10 cell samples. Thus, although not all genes were expressed by these two cell types or required for cell type specific clustering, our approach proved to be robust enough to characterize gene expression levels of up to 91 genes and 3 reference genes in RNA isolated from as little as one cultured human breast tumor cell very comparable to what was achieved with RNA obtained from 10 cultured human breast tumor cells.

Selection of pilot genes to study circulating tumor cells

Having established a method that enabled us to measure up to 94 transcripts present in as little as one cell, our next step was to select a pilot panel of genes to be measured in the CTCs after CellSearch enrichment. However, the main challenge in characterizing CTCs is to deal with the contamination with residual white blood cells that is still present after CTC enrichment. That a portion of the white blood cell population is still present in the CellSearch-enriched fraction became evident when we subjected whole blood of 8 healthy individuals to CellSearch enrichment. Not only were we able to measure considerable amounts of gene transcripts in all these EpCAM-based CellSearch-enriched fractions (see for example Fig. 1C), also the variation in the amount of transcripts in-between donor derived white blood cells was enormous, ranging from 13.08 Ct to 19.98 Ct (120-fold difference) for the CD45 marker *PTPRC* and from 18.71 Ct to 28.34 Ct (800-fold difference) for the proliferation marker *MKI67*.

To investigate how these contaminating leukocytes might affect our CTC characterization, gene expression levels measured in CellSearch-enriched HBDs

were compared with gene expression levels measured in three different human breast cancer reference cell lines. Results shown in Fig. 2 show that, depending on the genes included in the panel, material derived from HBD will contribute to the gene expression profile (in this example for the genes at the left side of the graph) thereby potentially interfering with the assessment of genes expressed by CTC.

Reduction of leukocyte contribution

Our first attempt to reduce the confounding contribution of these white blood cells involved extensive washing and resuspension after CellSearch enrichment of the epithelial cells. If the remaining white blood cells were only trapped in the mass of ferrobeads after CellSearch, this procedure should reduce the amount of non-specifically trapped cells. To address this hypothesis of leukocytes a-specifically trapped in the ferrobead mass, 20 CAMA-1 cells were spiked in duplicate in 7.5 ml whole blood from 2 different healthy donors and subjected to CellSearch enrichment. This resulted in both cases in a 75% recovery (15 CAMA-1 cells were counted in the cartridge in both cases). In parallel, enriched fractions were analyzed after 5 cycles of extensive washing with PBS and resuspension in new PBS prior to RNA isolation. Although this rigorous washing did result in an up to 20-fold reduction in contaminating leukocytes as assessed by quantitative RT-PCR, a similar and undesirable reduction was seen in the amount of recovered CAMA-1 tumor cells (only one out of the 20 spiked CAMA-1 cells was recovered). We therefore took another approach and attempted to deplete our samples of leukocytes prior to CellSearch enrichment. For this, ~80 epithelial tumor cells were spiked in 7.5 ml whole blood of 3 donors. The preparations were then subjected to leukocyte-specific depletion with anti-CD45 coated magnetic beads, followed by a standard CellSearch enrichment procedure. Although leukocyte-specific depletion prior to CellSearch did reduce the contribution of leukocytes by up to 92% (n=3, range 63% - 92%), there was still a considerable number of leukocytes (n=3, range 60 – 929) present in the CellSearch-enriched fraction. Thus, it appeared that at least some of the white blood cells were captured by the anti-EpCAM monoclonal antibody and not merely non-specifically trapped in the ferro bead mass.

Origin of concomitantly isolated leukocytes

Because non-specific trapping did not appear the (only) reason for the presence of the leukocytes after CellSearch, we were curious if there was a specific white blood cell population more prone to EpCAM-based CellSearch enrichment and thus the major culprit responsible for the contribution of leukocyte-derived RNA in our CTC-enriched preparations. To address this in more detail, and to get an estimate of the actual number of contaminating leukocytes still present after CellSearch enrichment, we assessed absolute numbers (and percentages) of white blood cell subsets present in 7.5 ml whole blood from two HBDs before and after CellSearch

enrichment. The resulting distributions of specific leukocyte blood cell subsets before and after this enrichment are summarized in Table 3. Although the CellSearch procedure resulted in over 99.9% (4 log) depletion of leukocytes (1381 out of 580×10^5 and 810 out of 600×10^5 leukocytes remained after CellSearch enrichment), there was still a high number of over 800 leukocytes present after EpCam-based enrichment. Apparently, the enrichment particularly favored B lymphocytes (and possibly monocytes) to the detriment of T lymphocytes (Table 3). This specific enrichment of B lymphocytes and depletion of T lymphocytes was confirmed in additional experiments with other HBDs (data not shown).

Profiling whole blood to correct for leukocyte contamination in CellSearch-enriched fractions

Another way that has been used to circumvent this problem of co-assessment of genes derived from contaminating leukocytes is to assess gene expression levels in a fraction of whole blood of the patient that was recovered after the CTCs had been captured. Next, only candidate genes are selected by comparing the corresponding depleted and enriched fractions for minimal expression in the CTC-depleted fraction and significant expression in the CTC-enriched fraction²¹. Also less sophisticated methods such as only selecting markers with high expression in tissue samples from primary tumors and a median 1000-fold lower expression in normal blood⁷ have been used. However, our data in Table 3 show that such approaches may not be accurate for EpCAM-enriched samples because the whole blood cell profile prior to CellSearch is not representative for the blood cell profile after CellSearch. This was confirmed when we analyzed expression of 144 genes by qRT-PCR in 6 different HBDs before and after CellSearch enrichment. Of these 144 genes, most of them selected *in silico*, based on their reported low expression in white blood cells (<http://cgap.nci.nih.gov/SAGE/AnatomicViewer>), 119 genes were still expressed to some extent in these HBDs. After correcting for multiple comparison using a false discovery rate of 10%, expression levels of *TACSTD1* (EpCAM, although higher in the HBDs after CellSearch, not statistically significant with $P_{FDR\ adjusted} = 0.018$), *KRT19*, *PTPRC* (CD45) and *BST1* showed no difference between the two groups. But 14 genes (10.9%) were consistently differentially expressed in all six HBDs after CellSearch enrichment (all lower) ($P_{FDR\ adjusted} < 0.012$).

Selection of pilot genes not expressed by leukocytes to study circulating tumor cells

Obviously, inclusion of genes more dominantly expressed by the surplus of leukocytes will favor characterization of the leukocytes instead of the few, frequently only one, CTC present in the pool of leukocytes that remain after CellSearch enrichment of the patient's blood samples. Having established that

profiling whole blood to correct for leukocyte contribution would be inappropriate, especially for such sensitive methods like real-time RT-PCR with inclusion of a limited number of up to 96 genes, we aimed for another way to circumvent this problem of co-assessment of genes derived from contaminating leukocytes. This was achieved by focusing on genes that are higher expressed in breast cancer cells compared with levels measured in white blood cells after CellSearch enrichment. For this, three different human breast cancer reference cell lines were spiked in 6 different HBDs in a final quantity equivalent to approximately one CTC (~10 pg) per ml whole blood. Gene expression levels using a pilot set of 25 TaqMan Gene Expression Assays were normalized on *KRT19* and compared with PCA with and without inclusion of the 9 genes shown to be predominantly expressed by the leukocytes (genes on the left side of the panel in Fig. 2). Even with this limited and still explorative set of 25 genes, improvement was clearly noticed in our PCA analysis after exclusion of 9 genes that were predominantly expressed by leukocytes (Fig. 3, compare the left panel where all 25 genes were included with the right panel where 9 of the more leukocyte-specific genes were excluded). Although certainly still not optimal, only by excluding the genes more dominantly expressed by the leukocytes did the HBDs spiked with MDA-MB-231 cells cluster separately from the same HBDs spiked with either SKBR-3 cells (Fig. 3, top panel) or CAMA-1 cells (Fig. 3, bottom panel) and together with the respective unspiked cell line preparations (as demonstrated by the closer location of the closed and open symbols in the right panel).

Finally, as proof of principle, we analyzed CellSearch-enriched blood of 10 breast cancer patients with metastatic disease with our multiplex real-time PCR method to molecularly characterize CTCs in large quantities of contaminating leukocytes. For this experiment, 7.5 ml EDTA blood samples were processed in parallel for RNA and CTC count on the CellTrack AutoPrep Analyzer with the CellSearch CTC profile Kit and CTC counts were determined on the CellSpotter Analyzer. From one of the patients we were unable to extract sufficient good quality RNA for a reliable analysis. The results of the remaining 9 patients are described in Figure 4. And indeed, even with this limited and still explorative set of only 16 genes, distinct gene expression patterns were seen in the blood of the 5 patients with CTCs when compared to the 4 patients that were devoid of CTCs. In addition, we noticed a discrepancy between the CTC count in the blood of patient-3, *i.e.*, the third green bar in Figure 4 (no CTCs were detected by the CellSpotter Analyzer), and the gene expression pattern associated with this patient (indicative for the presence of CTC), suggesting that our sensitive PreAmp-RT-PCR method may have detected the presence of a CTC missed by the CellSearch counting method. Alternatively, no CTCs were present in the 7.5 ml of blood used for enumeration while the blood sample used for molecular characterization contained at least 1 CTC.

In summary, our study shows that it is possible to perform mRNA expression analysis of up to 96 genes (thus conveniently fitting a 96-well plate) specific for as little as one cell. If this one cell is a CTC present in an environment containing large quantities of contaminating leukocytes, the only restriction is that the selected genes are not, or below a threshold detection value, *i.e.* much lower, expressed by these leukocytes. With the restriction of not using genes more dominantly expressed by CellSearch-enriched leukocytes, any gene set specific for any cancer type can be implemented in the method we described. These genes may represent markers to identify the tissue origin of the CTCs, for example by implementing the markers described by Xi *et al.* ⁷, as well as more cancer-specific markers such as those useful for drug targeting. The resulting data can be used to further characterize cancer type specific CTCs, thereby potentially improving our insight into biological processes and ultimately patient management.

Table 1: Sensitivity and homogeneity of various pre-amplification methods

Sample	pg total RNA	Method RT procedure Pre-amplification procedure	Call Rate			
			1: Taqman PreAmp		2: WT Ovation	3: Full spectrum
			RevertAid H ⁻ GSP PreAmp [†]	High Archive GSP PreAmp [†]	WT Ovation RNA/2nd strand [‡]	Full spectrum RNA/2nd strand [‡]
CAMA-1	10		68%	50%	<i>nd</i>	<i>nd</i>
MDA-MB-231	10		91%	45%	<i>nd</i>	<i>nd</i>
Breast tumor 1	20		63%	75%	53%	38%
	200		78%	78%	<i>nd</i>	<i>nd</i>
	1000		81%	84%	84%	66%
Breast tumor 2	20		55%	38%	19%	22%
	200		72%	84%	<i>nd</i>	<i>nd</i>
	1000		91%	78%	63%	59%

Following different pre-amplification methods, expression levels of up to 32 different genes were analyzed in 4 samples with real-time PCR (qPCR) using TaqMan Gene Expression Assays to assess sensitivity (call rate or % real-time PCR assays requiring less than 35 cycles for product formation) and homogeneous amplification (delta Ct amplified versus non-amplified within 1 Ct range).

RT = reverse transcriptase; GSP = gene-specific; *nd* = not done.

[†] multiplex gene-specific amplification with cDNA generated from serially diluted total RNA input down to 10 pg (equivalent of 1 cell);

[‡] second strand amplified cDNA from serially diluted total RNA input down to 20 pg (equivalent of 2 cells).

Table 2: Evaluation of sources of variation

Procedure variable	Sample	Input (pg total RNA)	R ²
within-run PreAmp + qPCR	breast tumor	10 vs. 500	0.9622
within-run PreAmp + qPCR	MDA-MB-231	100 vs. 1000	0.9927
in-between runs PreAmp + qPCR	HBD-1	200	0.9620
in-between runs PreAmp + qPCR	HBD-2	20000	0.9836
in between runs cDNA-synthesis + PreAmp + qPCR	HBD-3	20000	0.9889
in-between runs RNA isolation+cDNA-synthesis+PreAmp + qPCR	HBD-1	200	0.9467
in-between runs RNA isolation+cDNA-synthesis+PreAmp + qPCR	HBD-2	20000	0.9841
within-run CellSearch enrichment + RNA isolation + cDNA-synthesis + PreAmp + qPCR	HBD-4	200	0.9414
within-run CellSearch enrichment + RNA isolation + cDNA-synthesis + PreAmp + qPCR	HBD-4	200	0.9310
in-between CellSearch enrichment + RNA isolation + cDNA-synthesis + PreAmp + qPCR	HBD-4	200	0.9377

To evaluate the sources of variation in our procedure, expression levels of a random selection of 75 genes were analyzed in duplicate throughout the entire procedure by step-wise introduction of an additional variable (qPCR, PreAMP, cDNA synthesis, RNA isolation, CellSearch enrichment). Duplicate analyses were performed in RNA isolated from a breast tumor, a cultured cell line or blood cells from healthy donors at the input indicated in the third column (10 pg total RNA ~ one cell). HBD = healthy blood donor; R^2 = Pearson correlation coefficient.

Table 3: Absolute number and distribution of leukocyte subsets before and after EpCAM-based CellSearch enrichment

White blood cell type	HBD-1				HBD-2			
	before enrichment		after enrichment		before enrichment		after enrichment	
	(x 10 ⁵)	%		%	(x 10 ⁵)	%		%
Leukocytes	580 x10⁵	100	1381 ± 215	100	600 x10⁵	100	810 ± 13	100
Mature myeloid	360 x10 ⁵	62	787 ± 73	57	370 x10 ⁵	62	248 ± 14	31
Monocytes	35 x10 ⁵	6	320 ± 112	23	42 x10 ⁵	7	78 ± 14	10
Basophiles	1.5 x10 ⁵	0.2	<10	<1	1.2 x10 ⁵	0.2	<10	<1
Lymphocytes	190 x10⁵	32	274 ± 30	30	180 x10⁵	31	484 ± 33	60
B lymphocytes	19 x10 ⁵	10	117 ± 18	43	26 x10 ⁵	14	223 ± 16	46
T lymphocytes	150 x10 ⁵	79	52 ± 5	19	120 x10 ⁵	66	135 ± 8	28
NK lymphocytes	21 x10 ⁵	11	105 ± 7	38	37 x10 ⁵	20	126 ± 8	26

Absolute numbers and distribution of leukocytes and lymphocyte subsets in 7.5 ml whole blood before enrichment and in the resulting 900 μ l cell suspension after enrichment using the CellTrack AutoPrep Analyzer with the CellSearch CTC profile Kit. Results of two different HBDs, both assessed in duplicate, are expressed as average $\pm$ SD.

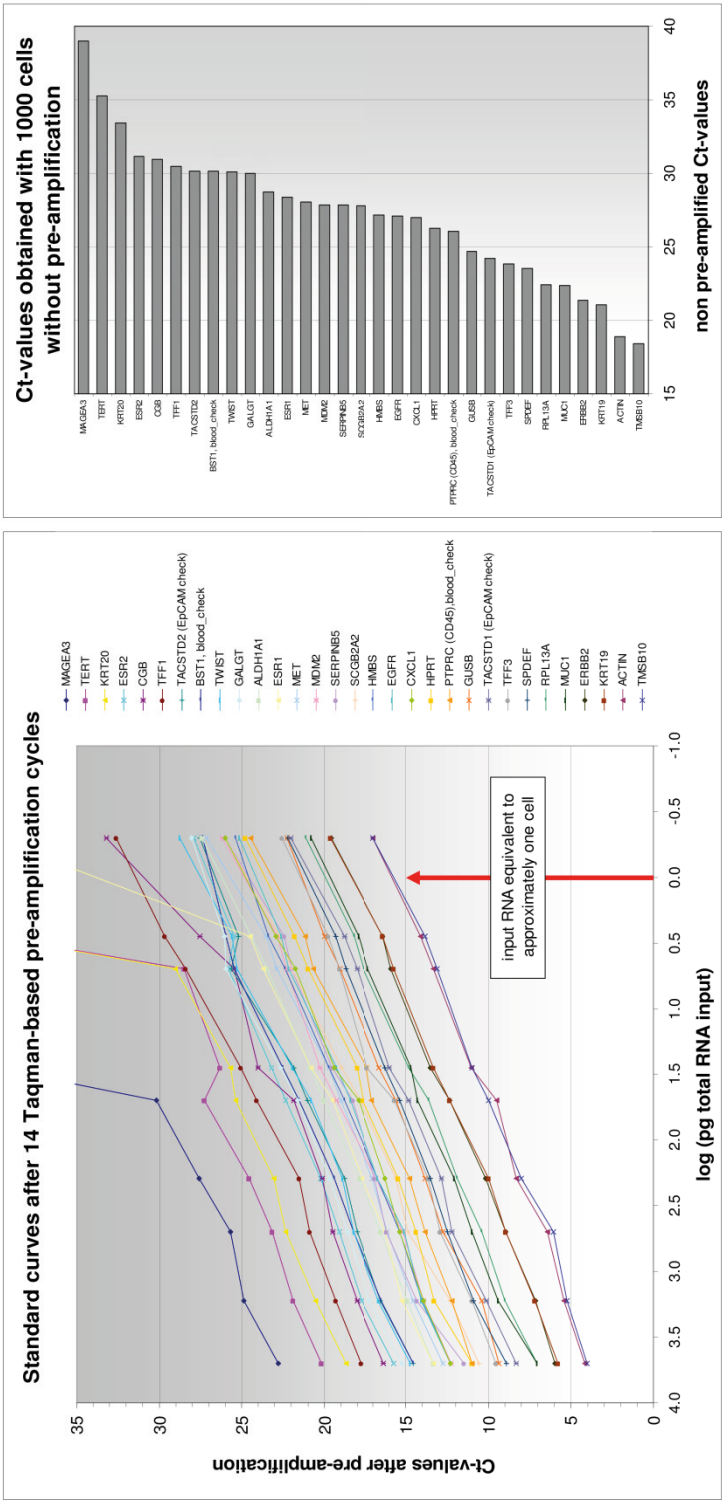


Figure 1A: Linearity and sensitivity

Expression levels of 32 genes were analyzed in cDNA generated from total RNA isolated from a primary human breast tumor. Prior to real-time PCR cDNA was pre-amplified in 14 cycles with the PreAmp method from ABI as described in the Materials and Methods section, using the same TaqMan Gene Expression Assays that were used for the real-time PCR. Linearity and sensitivity of the PreAmp method was assessed by measuring transcript levels in cDNA that was serially diluted down to the equivalent of less than one cell. In the left panel Ct-values obtained after pre-amplification are shown, the right panel shows the Ct-values obtained with RNA equivalent to 1000 cells without pre-amplification.

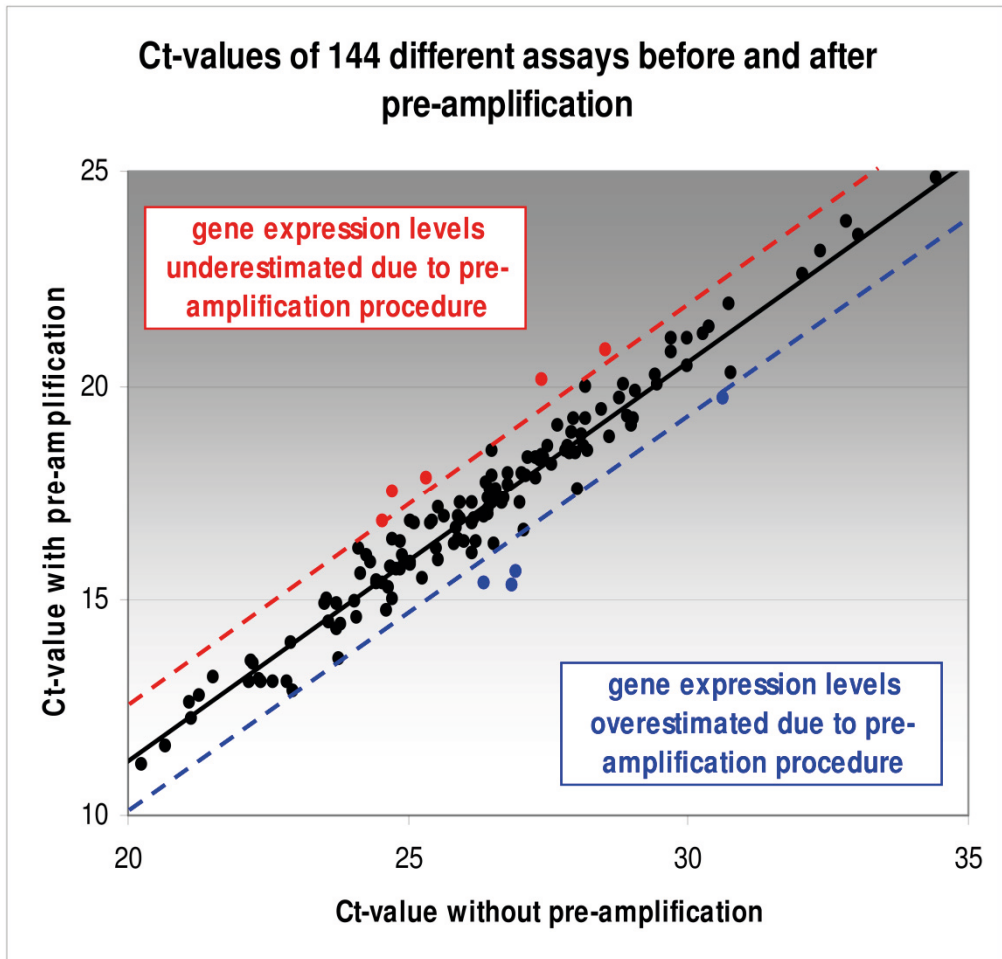


Figure 1B: Homogeneous amplification

Expression levels of 144 genes were analyzed in cDNA generated from total RNA from cultured MDA-MB-231 cells. Prior to real-time PCR cDNA was pre-amplified in 14 cycles with the PreAmp method from ABI as described in the Materials and Methods section, using the same TaqMan Gene Expression Assays that were used for the real-time PCR. Comparative tests between pre-amplified and non pre-amplified cDNA from RNA equivalent to ~2000 cells were performed to establish the degree of homogeneous amplification of the pre-amplified material. Black dotted assays within the boundary set with red and blue dotted lines (delta Ct amplified versus non-amplified within 1 Ct range) are considered to be homogeneously amplified. Red dotted assays indicate an underestimation of gene expression levels after pre-amplification, blue dotted assays an overestimation.

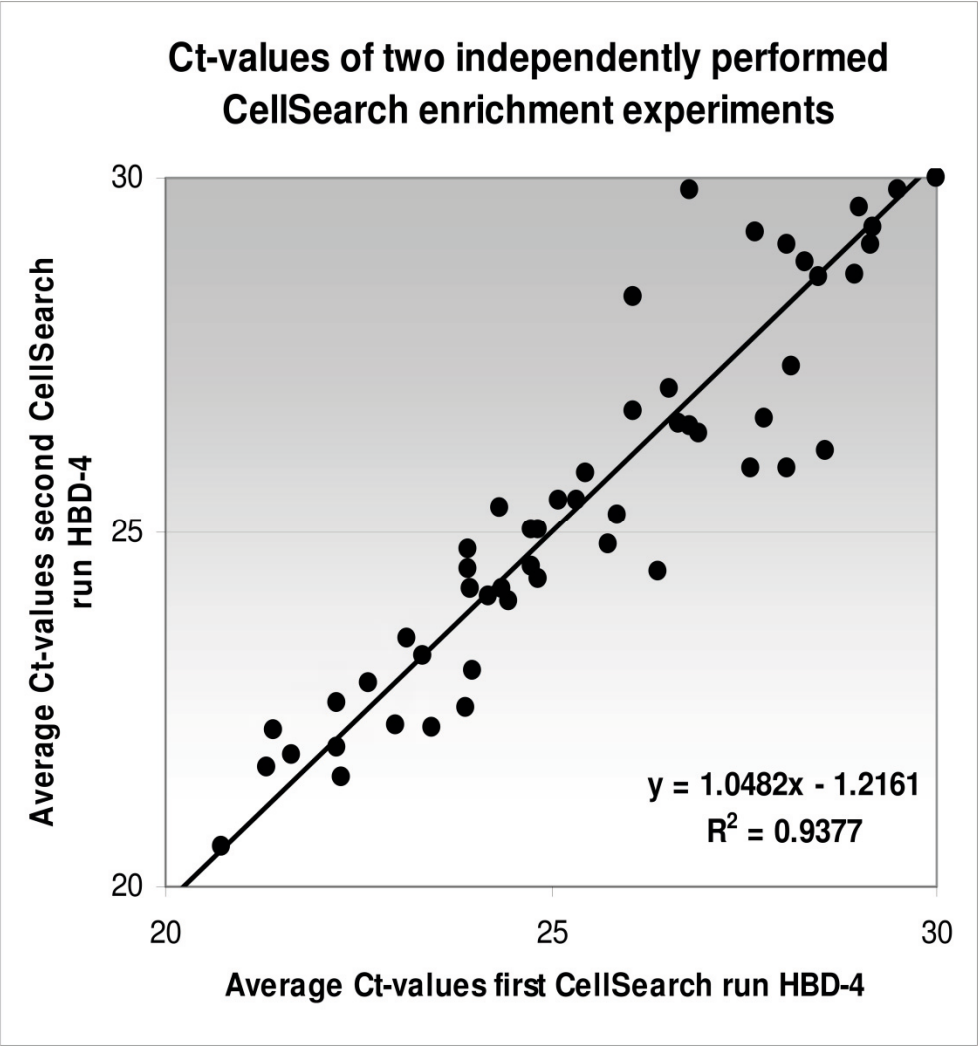


Figure 1C: Overall reproducibility of our method

Reproducibility of the whole procedure was assessed by performing all steps that were needed to establish gene expression levels of 75 genes in two independently performed CellSearch experiments. Each dot represents the Ct levels of a different TaqMan Gene Expression Assay as assessed in two independently performed experiments that involved 7.5 ml whole blood of the same healthy donor (HBD-4). After CellSearch enrichment, but prior to qRT-PCR, the total RNA was diluted to the equivalent of 20 blood cells (~200 pg).



Figure 1D: Comparative gene expression analysis with 91 genes measured in one, two and ten breast cancer tumor cells

Expression levels were analyzed with real-time PCR with 91 TaqMan Gene Expression Assays in cDNA generated from total RNA isolated from 1, 2 and 10 cultured CAMA-1 and MDA-MB-231 (MM231) cells. Sample loading and RNA integrity was controlled with 3 additional universal reference genes (*GUSB*, *HPRT1* and *HMBS*). Prior to real-time PCR cDNA was pre-amplified in 14 cycles with the PreAmp method from ABI as described in the Materials and Methods section, using the same TaqMan Gene Expression Assays that were used for the real-time PCR. Unsupervised two-dimensional hierarchical cluster analysis was performed for comparing mRNA profiles of 1, 2 and 10 human breast cancer cells. Each horizontal row represents a gene, and each vertical column corresponds to a sample. Red color indicates a transcription level above the detection level of the particular assay, black color indicates a transcription level below the detection level of the particular assay. Data shown are the averages of 2 experiments after median normalization of each individual sample across all genes.

← more leukocyte-specific genes — more epithelial-specific genes →

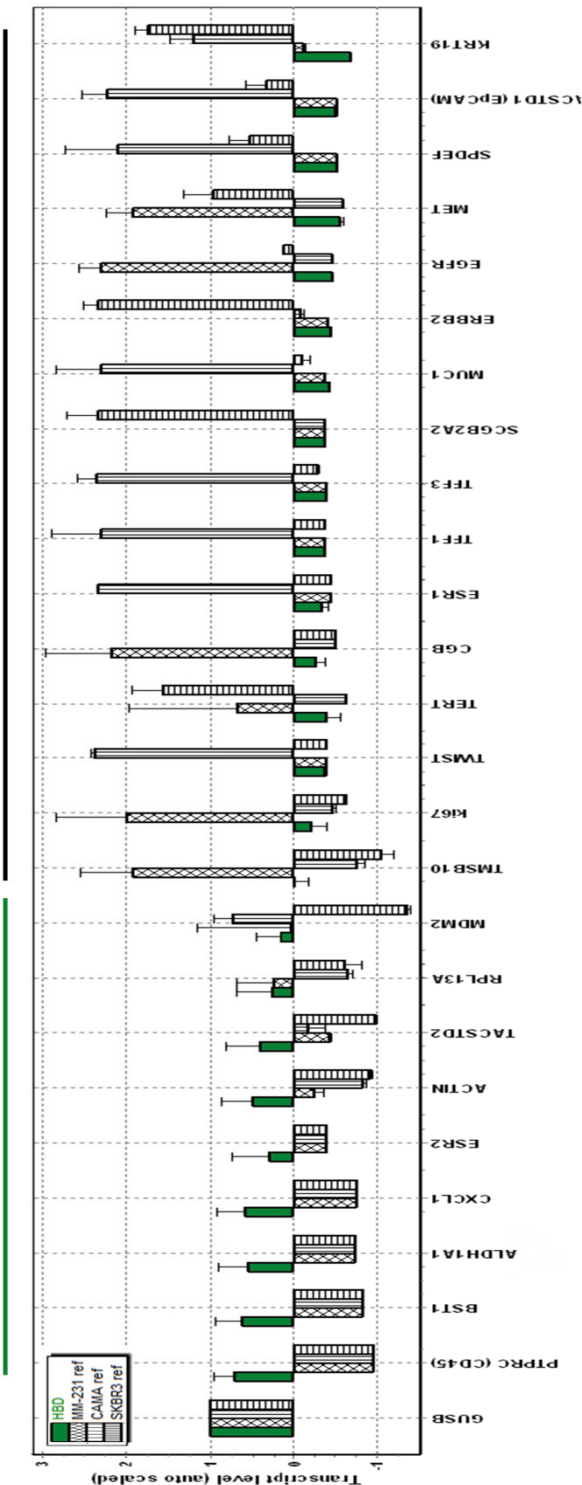


Figure 2: Gene expression in a panel of 3 human breast cancer cell lines and 8 HBDs
(after EpCAM-based CellSearch enrichment)

To evaluate the potential contribution of gene transcript levels derived from 8 different HBDs after CellSearch enrichment, transcript levels of a pilot set of genes were compared with levels measured in 3 human breast cancer reference cell lines (MDA-MB-M231 (MM-231), CAMA-1 (CAMA) and SKBR-3 (SKBR3)). For this, all cDNA preparations were subjected to pre-amplification and qPCR using a pilot set of 26 TaqMan Gene Expression Assays. The average transcript levels $\pm$ SEM for the unspiked HBD (n=8, RNA from 7.5 ml whole blood after CellSearch enrichment) and the reference cell lines (n=2 each, RNA from 10 to 100 cells) are plotted as auto scaled ratios of the universal reference gene *GUSB* to correct for differences in RNA input and quality.

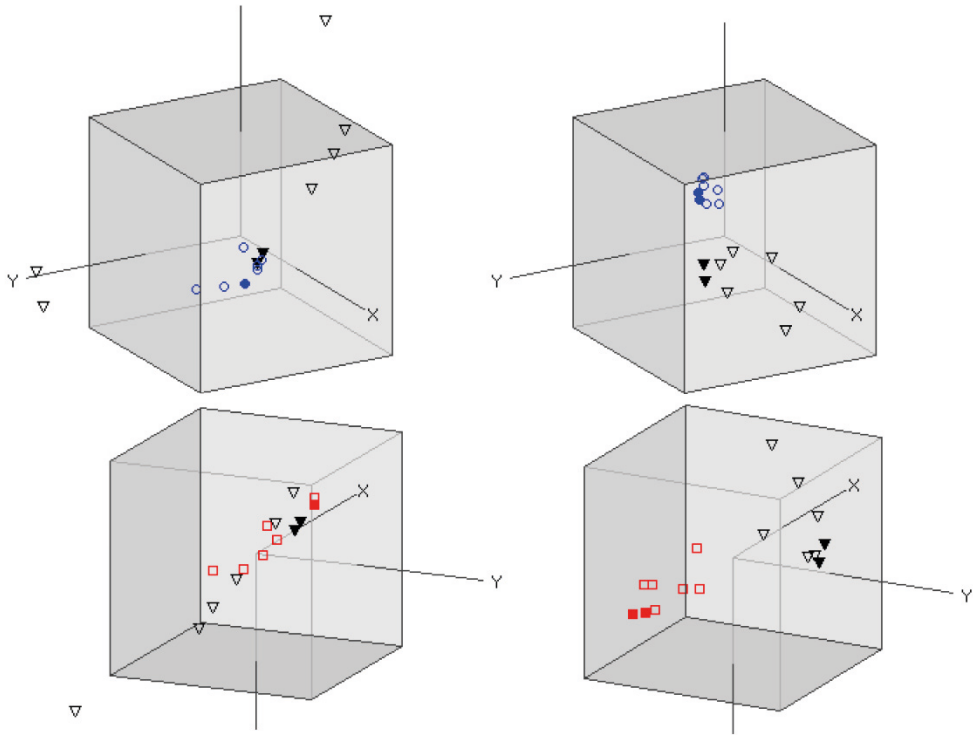


Figure 3: PCA before (left) and after (right) exclusion of genes expressed by leukocytes

To investigate how contaminating leukocytes might affect CTC characterization, gene expression profiles of cells from 3 different human breast cancer reference cell lines (MDA-MB-231 (▼), CAMA-1 (■), and SKBR-3 (●)), were compared before (closed symbols) and after (open symbols) spiking these cells in CellSearch-enriched HBDs. For this, cDNA's of 6 different HBDs were spiked -after RNA isolation and cDNA synthesis, but prior to pre-amplification and real-time PCR- with cDNA from the 3 different human breast cancer reference cell lines in a final quantity equivalent to approximately one CTC (~10 pg) per ml whole blood. Gene expression levels using a pilot set of 25 TaqMan Gene Expression Assays were normalized on *KRT19* and compared by principal component analysis (PCA) with (left panel) and without (right panel) inclusion of the 9 genes shown to be predominantly expressed by the leukocytes (genes on the left side of the panel in Fig. 2).

Reference cells in the absence of HBD: MDA-MB-231, closed triangles; SKBR-3, closed circles; CAMA-1, closed squares.

~1 reference cell spiked in cDNA obtained from 1 ml CellSearch-enriched blood from a HBD: MDA-MB-231, open triangles; SKBR-3, open circles; CAMA-1, open squares.

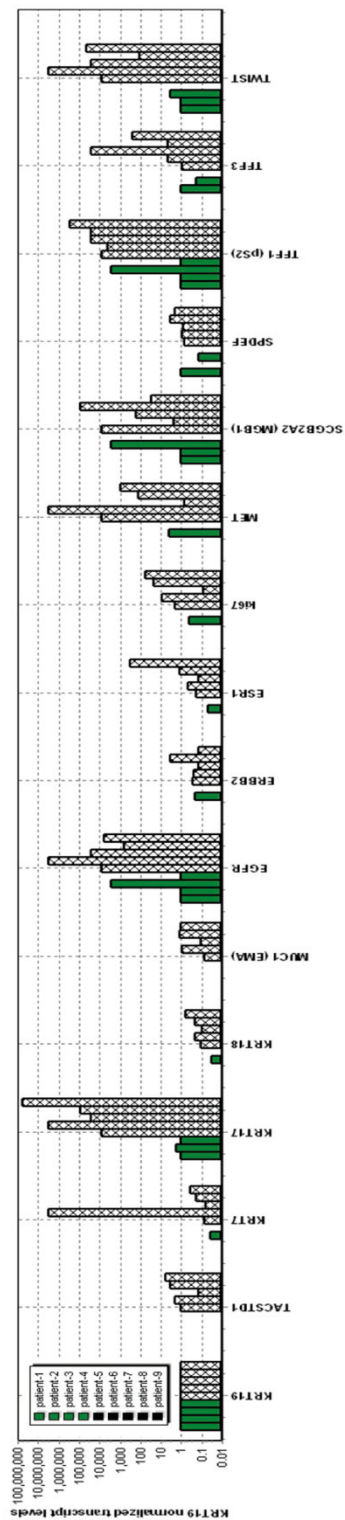


Figure 4: CTC-specific gene expression levels in clinical samples

To evaluate the usefulness of our method for clinical samples, gene expression levels were measured in CellSearch-enriched preparations from 7.5 ml EDTA blood samples of breast cancer patients with metastatic disease. CTC counts were assessed by use of the CellTrack™ AutoPrep Analyzer with the CellSearch™ CTC profile Kit as described in the Materials and Methods section. For the gene expression study 7.5 ml EDTA blood samples of the same breast cancer patients were enriched in parallel. After CellSearch enrichment, RNA was isolated and cDNA was prepared as described in the Materials and Methods section. All cDNA preparations were subjected to pre-amplification and qPCR using a pilot set of 16 TaqMan Gene Expression Assays that had shown not to be expressed in CellSearch enriched preparations of healthy blood donors (see Figure 1), and three universal reference genes. To evaluate the CTC-specific contribution, the transcript levels were first normalized on the average of the universal reference genes *GUSB*, *HMBS*, and *HPRT1* (house keeper set) to correct for differences in RNA input and quality, and next on *KRT19*. Patients are ranked according to the number of CTCs counted in the 7.5 ml blood samples, with no detectable CTCs present in the peripheral blood of 4 patients (closed green bars: patients-1 to -4) and 2 to 342 CTCs in the peripheral blood of the remaining 5 patients (hatched grey bars: patient-5; 2 CTCs, patient-6; 2 CTCs, patient-7; 6 CTCs, patient-8; 12 CTCs, patient-9; 342 CTCs). Note the discrepancy between the CTC count of patient-3 and the gene expression pattern associated with this patient.

References

1. Jiang WG, Martin TA, Mansel RE. Molecular detection of micro-metastasis in breast cancer. *Crit Rev Oncol Hematol*. 2002;43:13-31.
2. Gilbey AM, Burnett D, Coleman RE, Holen I. The detection of circulating breast cancer cells in blood. *J Clin Pathol*. 2004;57:903-911.
3. Ring A, Smith IE, Dowsett M. Circulating tumour cells in breast cancer. *Lancet Oncol*. 2004;5:79-88.
4. Zieglschmid V, Hollmann C, Bocher O. Detection of disseminated tumor cells in peripheral blood. *Crit Rev Clin Lab Sci*. 2005;42:155-196.
5. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging--predicting overall survival in metastatic breast cancer. *Clin Cancer Res*. 2006;12:6403-6409.
6. Morgan TM, Lange PH, Vessella RL. Detection and characterization of circulating and disseminated prostate cancer cells. *Front Biosci*. 2007;12:3000-3009.
7. Xi L, Nicastrì DG, El-Hefnawy T, Hughes SJ, Luketich JD, Godfrey TE. Optimal markers for real-time quantitative reverse transcription PCR detection of circulating tumor cells from melanoma, breast, colon, esophageal, head and neck, and lung cancers. *Clin Chem*. 2007;53:1206-1215.
8. Sleijfer S, Gratama JW, Sieuwerts AM, Kraan J, Martens JW, Foekens JA. Circulating tumour cell detection on its way to routine diagnostic implementation? *Eur J Cancer*. 2007;43:2645-2650.
9. Bertazza L, Mocellin S, Nitti D. Circulating tumor cells in solid cancer: tumor marker of clinical relevance? *Curr Oncol Rep*. 2008;10:137-146.
10. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer*. 2008;8:329-340.
11. Steen S, Nemunaitis J, Fisher T, Kuhn J. Circulating tumor cells in melanoma: a review of the literature and description of a novel technique. *Proc (Bayl Univ Med Cent)*. 2008;21:127-132.
12. Lang JE, Hall CS, Singh B, Lucci A. Significance of micrometastasis in bone marrow and blood of operable breast cancer patients: research tool or clinical application? *Expert Rev Anticancer Ther*. 2007;7:1463-1472.
13. Davis JW, Nakanishi H, Kumar VS, et al. Circulating tumor cells in peripheral blood samples from patients with increased serum prostate specific antigen: initial results in early prostate cancer. *J Urol*. 2008;179:2187-2191; discussion 2191.
14. Medical devices; immunology and microbiology devices; classification of the immunomagnetic circulating cancer cell selection and enumeration system. Final rule. *Fed Regist*. 2004;69:26036-26038.
15. Danila DC, Heller G, Gignac GA, et al. Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. *Clin Cancer Res*. 2007;13:7053-7058.
16. Cohen SJ, Alpaugh RK, Gross S, et al. Isolation and characterization of circulating tumor cells in patients with metastatic colorectal cancer. *Clin Colorectal Cancer*. 2006;6:125-132.
17. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med*. 2004;351:781-791.
18. Harkes IC, Elstrodt F, Dinjens WN, et al. Allelotype of 28 human breast cancer cell lines and xenografts. *Br J Cancer*. 2003;89:2289-2292.
19. Sieuwerts AM, Meijer-van Gelder ME, Timmermans M, et al. How ADAM-9 and ADAM-11 differentially from estrogen receptor predict response to tamoxifen

- treatment in patients with recurrent breast cancer: a retrospective study. Clin Cancer Res. 2005;11:7311-7321.
20. Benjamini Y, Y. H. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc Ser B 1995;57:289-300.
 21. Smirnov DA, Zweitzig DR, Foulk BW, et al. Global gene expression profiling of circulating tumor cells. Cancer Res. 2005;65:4993-4997.

Chapter 4

Anti-Epithelial Cell Adhesion Molecule Antibodies and the Detection of Normal-Like Circulating Breast Tumor Cells

Anieta M. Sieuwerts, Jaco Kraan, Joan Bolt, Petra van der Spoel,
Fons Elstrodt, Mieke Schutte, John W.M. Martens, Jan-Willem
Gratama, Stefan Sleijfer, John A. Foekens

Journal of the National Cancer Institute 2009; 101:61 - 66

Abstract

Identification of specific subtypes of circulating tumor cells in peripheral blood of cancer patients can provide information about the biology of metastasis and improve patient management. However, to be effective, the method used to identify circulating tumor cells must detect all tumor cell types. We investigated whether the five subtypes of human breast cancer cells that have been defined by global gene expression profiling — normal-like, basal, HER2-positive, and luminal A and B — were identified by CellSearch, a US Food and Drug Administration – approved test that uses antibodies against the cell surface – expressed epithelial cell adhesion molecule (EpCAM) to isolate circulating tumor cells. We used global gene expression profiling to determine the subtypes of a well-defined panel of 34 human breast cancer cell lines (15 luminal, nine normal-like, five basal-like, and five Her2-positive). We mixed 50-150 cells from 10 of these cell lines with 7.5 mL of blood from a single healthy human donor, and the mixtures were subjected to the CellSearch test to isolate the breast cancer cells. We found that the CellSearch isolation method, which uses EpCAM on the surface of circulating tumor cells for cell isolation, did not recognize, in particular, normal-like breast cancer cells, which in general have aggressive features. New tests that include antibodies that specifically recognize normal-like breast tumor cells but not cells of hematopoietic origin are needed.

Circulating tumor cells are cells that have detached from a primary tumor or metastatic tumor sites and entered the peripheral circulation. A limited number of markers are used for the isolation (ie, cell surface antigens) or detection (ie, various antigens or mRNAs) of circulating tumor cells. These markers include epithelial cell surface markers such as epithelial cell adhesion molecule (EpCAM; also known as CD326, ESA, HEA125, and TACSTD1); cytokeratins 7, 8, 18, 19, and 20; and more cancer-specific markers, such as HER2-neu and mucin 1 for breast carcinoma ¹⁻³. Commercially available tests for isolation and detection of circulating tumor cells include the CellSearch circulating tumor cell test (Veridex LLC, San Diego, CA) and other tests ¹⁻³. However, it is unclear whether such tests can detect all tumor subtypes. We investigated whether the CellSearch test could recognize all subtypes of breast cancer, including normal-like, basal, HER2-positive, and luminal A and B tumor cells.

The CellSearch™ circulating tumor cell test is the only diagnostic test that is currently approved by the US Food and Drug Administration as an automated test to detect and enumerate circulating tumor cells ⁴. Briefly, blood samples, which contain many leukocytes and few circulating tumor cells, are drawn into 10-mL CellSave Preservative Tubes™ (Veridex LLC), an evacuated blood draw tube containing EDTA as anticoagulant and a cellular preservative, maintained at room temperature, and subsequently processed within 96 hours of collection by use of the CellSearch™ System. The CellSearch System (Veridex LLC) consists of the CellTracks Autoprep (an automated sample preparation system), the CellSearch Epithelial Cell kit to immune-magnetically enrich cells expressing the epithelial cell adhesion molecule EpCAM and fluorescently label all nuclei with 4',6-diamidino-2-phenylindole (DAPI), leukocytes with monoclonal antibodies specific for leukocytes (ie, anti-CD45-Allophycocyan) and epithelial cells with monoclonal antibodies specific for epithelial cells (ie, anti-cytokeratin 8,18,19-Phycoerythrin), and the CellSpotter Analyzer, a semi-automated fluorescence-based microscopy systems that permit computer-generated reconstruction of cellular images. With the CellSearch™ circulating tumor cell test, circulating tumor cells are isolated with anti-EpCAM antibodies coupled to microscopic iron particles, and complexes of circulating tumor cells bound to anti-EpCAM-coupled iron particles are "pulled" out of the blood sample by use of powerful magnets. Unbound cells and remaining plasma is aspirated followed by addition of the staining reagents in conjunction with a permeabilization buffer to fluorescence label the immune-magnetically labeled cells. After incubation on the system, the magnetic separation is repeated, and excess staining reagents is aspirated. In the final processing step, the cells are resuspended in the MagNest Cell Presentation Device (Veridex LLC). This device consists of a chamber and two magnets that orient the immune-magnetically labeled cells for analysis using the CellSpotter Analyzer, a four-color

semiautomated fluorescence microscope. Finally, circulating tumor cells that are defined as nucleated cells with a round to oval morphology that are positive for anticytokeratin antibody binding and negative for anti-CD45 antibody binding⁵ are identified by an operator.

Results of the CellSearch™ test have been used to monitor disease progression and therapy efficacy in metastatic prostate⁶, colorectal⁷, and breast cancer⁸. For patients with advanced breast cancer, the number of circulating tumor cells as determined by the CellSearch™ test has been shown to have prognostic value⁸⁻¹⁴. In addition, a change from the baseline number of circulating tumor cells after the first cycle of therapy appears to distinguish patients who have responded to systemic therapy (who have a decreased number of circulating tumor cells) from those who have not (who have an increased number of circulating tumor cells)¹⁵.

Five subtypes of human breast cancers have been identified by global gene expression profiling, including normal-like, basal, HER2-positive, and luminal A and B¹⁶. The normal- and basal-like subtypes, which represent 7.8% and 25% of all breast cancers, respectively, in our published cohort of 344 breast tumor samples from patients with lymph node-negative disease¹⁷, are in general negative for estrogen receptor (*ESR1*), progesterone receptor, and HER2-neu and so lack targets for endocrine therapy and anti-HER2-neu directed therapy and have therefore poorer treatment options than other subtypes. Because each subtype has distinct prognostic and therapeutic characteristics^{16,18}, it is important to establish whether each breast cancer subtype expresses the cell surface antigens that are used in the circulating tumor cell assays to isolate and detect circulating tumor cells. If certain breast cancer subtypes do not express an antigen, it is important to identify them and to determine which antibodies could be used instead of or in addition to those already in use in present tests for circulating tumor cells.

To investigate whether the CellSearch test recognized all five breast cancer subtypes that are characterized by their intrinsic gene expression profiles as previously described^{16,18}, we first determined the subtypes of our well-defined panel of 34 human breast cancer cell lines¹⁹ by global gene expression profiling, as previously described¹⁷. A previous analysis of nearly 150 polymorphic microsatellite markers in this panel of 34 cell lines has shown that each of these 34 cell lines is unique and monoclonal²⁰. As determined by the expression of genes in the intrinsic gene set^{16,17}, the complete cell line panel contained 15 luminal (ie, no clear-cut discrimination between luminal A and B), nine normal-like, five basal-like, and five HER2-positive cell lines (Table 1). To validate the gene expression profiling data, transcript levels of 14 candidate genes were remeasured with Affymetrix GeneChip Exon 1.0 ST Arrays (Affymetrix UK Ltd., Wickham la Wooburn Grn, UK)

and real-time reverse transcriptase – polymerase chain reaction. The 14 candidate genes that may as such enable discrimination between the breast cancer subgroups included genes that are more specific for cells of hematopoietic origin (*CD44* and *CD45*); epithelial cell – specific genes such as those encoding cytokeratins, EpCAM, and mucin 1; genes encoding markers specific for the breast cancer subgroups (*ESR1*, *ERBB1*, *ERBB2*, *CAV1*, and *CD24*); and genes for two well-known epithelial – mesenchymal transition markers (*TWIST1* and *VIM*). Transcript levels of these 14 genes in all the 34 cell lines were compared with those measured in whole-blood cells from 23 different blood donors before ($n = 6$ samples) and after ($n = 23$ samples) being subjected to EpCAM-based CellSearch enrichment to establish which genes were not expressed by blood cells and could be used to specifically identify the epithelial tumor cells (Table 1). The study was approved by the Erasmus MC Institutional Review Board. Blood samples were collected from healthy volunteers after written informed consent was obtained. Supplementary Table 1 lists the gene expression assays used. We next randomly selected 19 of the 34 cell lines; incubated each cell line with fluorochrome-conjugated antibodies against CD45, CD24, CD44, or EpCAM, which were chosen to enable a distinction between CD45-positive cells of hematopoietic origin, CD45-negative and EpCAM-positive breast cancer cells, and CD45-negative, CD24-negative, and CD44-positive breast cancer stem cells); and used flow cytometry to measure the expression of each marker by each cell line (Table 2). Next, an aliquot (50 – 150 cells per aliquot) of each of these 19 cell lines was added to a separate 7.5-mL sample of peripheral blood from a single healthy volunteer donor, and the mixtures were subjected to the EpCAM-based CellSearch assay to isolate circulating tumor cells. Gene expression data of the isolated cells showed that cells with a luminal or HER2-positive subtype were generally isolated by the assay, whereas cells with a normal-like subtype, which lack EpCAM expression, were not isolated or were only partially isolated (eg, MDA-MB-231 cells, which have marginal EpCAM expression; Figure 1; Supplementary Table 2). We found that the cell lines with the normal-like subtype expressed high mRNA levels of the epithelial – mesenchymal transition markers, TWIST1 (*TWIST1*) and vimentin (*VIM*) (one-way analysis of variance, two-sided $P = .03$ and $P < .001$, respectively) (Table 1); however, MDA-MB-231 cells expressed vimentin but did not appear to express TWIST1 (Supplementary Table 3).

Cells with a normal-like breast cancer subtype express high levels of genes that are characteristic of basal epithelial and adipose cells and low levels of genes that are characteristic of luminal epithelial cells¹⁶. The normal-like breast cancer subtype is the only subtype that displays the putative tumor-initiating stem cell phenotype which includes low expression of CD24 and high expression of CD44^{21,22} (Table 2) and has high expression of both vimentin and TWIST1 (Table 1).

Expression of vimentin and TWIST1 has been used to identify cells that have undergone epithelial–mesenchymal transition, a process that has been linked to the generation of cells with properties of stem cells and to the ability of breast cancer cells to enter the circulation and seed metastases²³⁻²⁵. Thus, the normal-like breast cancer cell subtype is an important target for the development of individualized therapy and should not be overlooked when assessing circulating tumor cells. Intrinsic breast cancer subtypes are easily missed in analyses that use only crude, standard clinical or pathological criteria such as hormone receptor and HER2 status. Therefore, our results, which are based on a distinction in breast cancer subtypes determined by global gene expression profiling, cannot be compared with those of Cristofanilli et al.⁸, who did not find differences in the number of circulating tumor between breast cancer cell subtypes that were defined only by hormone receptor or HER2-neu status.

New tests that include antibodies that specifically recognize normal-like breast tumor cells but not cells of hematopoietic origin are needed. As shown in Table 1, the phenotype of circulating leukocytes in the healthy blood donors before and after EpCAM-based CellSearch enrichment was characterized by the high expression of vimentin and CD44 mRNA and the low expression of CD24 mRNAs. Because antibodies against vimentin and CD44 also bind to cells of hematopoietic origin, these antibodies would not be suitable for the detection of circulating tumor cells that have an epithelial – mesenchymal transition origin or putative breast cancer stem cells. However, current strategies for detecting circulating tumor cells can be improved to allow assessment of these normal-like cells by selecting antigens that are ubiquitously and abundantly present and that can be made accessible for isolation with immunobeads on normal-like cells but that are absent on cells of hematopoietic origin. When selecting antigens to use for isolating circulating normal-like breast cancer cells, it must be emphasized that clinical (breast) cancer samples consist of heterogeneous cell populations and always contain leukocytes and other types of blood cells; we circumvented this problem by using homogeneous cell lines. Examples of membrane antigens that might fulfill the criteria of being ubiquitously and abundantly present on normal-like cells but absent on cells of hematopoietic origin are mucin 1 and caveolin 1. Gene transcripts of caveolin 1, an approximately 22-kDa integral membrane protein, and mucin 1, a 350-kDa glycoprotein that protects the cell surface, were ubiquitously expressed in all normal-like human breast tumor cell lines, with the apparent expression of caveolin 1 being higher (gene transcript expression range = 1.42 – 13.42, relative to the expression of *HMBS*, *HPRT1*, and *GUSB*) than that of mucin 1 (gene transcript expression range = 0.06 – 0.56, relative to the expression of *HMBS*, *HPRT1*, and *GUSB*; Table 1 ; Supplementary Table 3). Furthermore, in our 344 clinical breast cancer¹⁷, decreased expression of EpCAM was associated

with increasing expression of caveolin 1 (Spearman r_s -0.26 , $n = 344$, $P < .001$). Consequently, antibodies against antigens such as caveolin 1 might be able to specifically detect circulating tumor cells from normal-like breast tumors.

A limitation of this study is our use of homogeneous breast cancer cell lines of known subtypes instead of blood samples from patients with breast cancer that had been subtyped. Detection of normal-like breast cancer cells in clinical (breast) cancer samples, which consist of heterogeneous cell populations, will require an assay that uses a mixture of antibodies against different cell surface antigens that are present on circulating tumor cells but absent on cells of hematopoietic origin. Such an assay should be thoroughly validated before clinical use, not only in cultured cell lines but also in clinical samples of known intrinsic breast cancer subtype, as described previously for assays relying only on anti-EpCAM antibodies

⁵.

In conclusion, an EpCAM-dependent assay could not detect normal-like breast tumor cells. In the future, the identification of antibodies that specifically detect normal-like breast cancer cells (which in general have aggressive features) and their inclusion in the CellSearch assay may improve the sensitivity and feasibility of that assay without a loss of specificity.

Table 1. mRNA expression in breast cancer cell lines with different intrinsic subtype characteristics*

Gene symbol (antigen)	mRNA expression (95% CI) relative to <i>HMBS</i> , <i>HPRT1</i> , and <i>GUSB</i>				ANOVA <i>P</i> [†]		mRNA expression (95% CI) relative to <i>HMBS</i> , <i>HPRT1</i> , and <i>GUSB</i>	
	Normal-like (n = 9)	Basal-like (n = 5)	Luminal (n = 15)	HER2-positive (n = 5)			Blood before enrichment (n = 6)	Blood after enrichment (n = 23)
<i>PTPRC</i> (CD45)	0.00 (0.00 to 0.10)	0.00 (0.00 to 0.10)	0.00 (0.00 to 0.10)	0.00 (0.00 to 0.10)	.210		13.39 (10.42 to 16.37)	6.25 (4.99 to 7.50)
<i>CD24</i> (CD24)	0.40 (0.03 to 0.78)	2.31 (0.20 to 4.42)	3.84 (2.14 to 5.54)	2.69 (1.86 to 3.52)	.025		0.03 (0.02 to 0.03)	0.03 (0.01 to 0.06)
<i>CD44</i> (CD44)	0.77 (0.59 to 0.95)	0.75 (0.40 to 1.10)	0.04 (0.02 to 0.06)	0.02 (0.00 to 0.04)	<.001		3.85 (3.13 to 4.57)	3.90 (3.29 to 4.52)
<i>TACSTD1</i> (EpCAM)	0.04 (0.00 to 0.10)	2.04 (0.83 to 3.25)	8.72 (4.02 to 13.43)	4.43 (2.17 to 6.69)	.020		0.00 (0.00 to 0.02)	0.01 (0.00 to 0.02)
<i>MUC1</i> (EMA, mucin 1)	0.32 (0.20 to 0.44)	0.35 (0.15 to 0.55)	1.88 (1.24 to 2.52)	0.53 (0.43 to 0.63)	<.001		0.03 (0.02 to 0.05)	0.01 (0.00 to 0.02)
<i>CAV1</i> (caveolin 1)	5.04 (2.39 to 7.70)	1.70 (0.19 to 3.21)	0.05 (0.02 to 0.08)	0.03 (0.01 to 0.05)	<.001		0.02 (0.01 to 0.04)	0.05 (0.00 to 0.01)
<i>KRT7</i> (cytokeratin 7)	1.53 (0.00 to 3.07)	7.90 (5.63 to 10.17)	6.62 (3.62 to 9.63)	10.46 (6.45 to 14.48)	.009		0.01 (0.00 to 0.02)	0.01 (0.00 to 0.01)
<i>KRT18</i> (cytokeratin 18)	5.58 (0.96 to 10.19)	10.06 (7.88 to 12.24)	16.80 (13.23 to 20.36)	17.17 (12.11 to 22.23)	.001		0.06 (0.03 to 0.09)	0.03 (0.02 to 0.03)
<i>KRT19</i> (cytokeratin 19)	0.09 (0.00 to 0.26)	4.53 (0.00 to 9.11)	28.35 (12.66 to 44.03)	11.99 (1.58 to 22.40)	.022		0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)
<i>ESR1</i> (estrogen receptor)	0.00 (0.00 to 0.30)	0.02 (0.01 to 0.03)	0.76 (0.37 to 1.14)	0.06 (0.00 to 0.16)	.003		0.02 (0.01 to 0.03)	0.01 (0.00 to 0.02)
<i>ERBB1</i> (EGF receptor)	0.20 (0.10 to 0.30)	0.96 (0.00 to 2.01)	0.03 (0.01 to 0.05)	0.07 (0.00 to 0.14)	.003		0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)

<i>ERBB2</i> (HER2)	0.05 (0.03 to 0.06)	0.07 (0.02 to 0.12)	2.53 (0.13 to 4.94)	7.69 (2.28 to 13.09)	.009	0.02 (0.01 to 0.03)	0.01 (0.00 to 0.01)
<i>TWIST1</i> (EMT marker <i>TWIST1</i>)	0.06 (0.01 to 0.11)	0.00	0.01 (0.00 to 0.02)	0.00 (0.00 to 0.00)	.027	0.00	0.00
<i>VIM</i> (EMT marker vimentin)	101.56 (36.90 to 116.23)	0.43 (0.00 to 0.90)	0.30 (0.00 to 0.67)	0.10 (0.00 to 0.27)	<.001	34.47 (22.12 to 46.82)	52.30 (44.10 to 60.50)

*Data in this table are the average and its 95% confidence interval (CI) of one representative experiment performed in duplicate for the number of cell lines and healthy blood donors as indicated. Fourteen candidate genes, which included genes more specific for cells of hematopoietic origin (*CD44* and *CD45*), epithelial specific genes such as those encoding cytokeratins, EpCAM and mucin 1, genes encoding markers specific for the breast cancer subgroups (*ESR1*, *ERBB1*, *ERBB2*, *CAV1*, *CD24*), and 2 well known EMT markers (*TWIST1*, *VIM*), were selected. Data of the individual cell lines are shown in Supplementary Table 3 and the gene expression assays used are shown in Supplementary Table 1. To validate the gene expression profiling data, transcript levels of candidate genes that might enable discrimination among the four breast cancer subtypes, as represented by 19 randomly selected breast cancer cell lines, were analyzed with Affymetrix GeneChip® Exon 1.0 ST Arrays (Affymetrix UK Ltd., Wickham la Woodburn Gm, UK) and real-time reverse transcriptase-PCR (RT-PCR) and compared with levels measured in whole blood of healthy blood donors before (n = 6) or after (n = 23) EpCAM-based CellSearch enrichment for circulating tumor cells. For this, RNA was isolated with the RNeasy (Micro) Kit (Qiagen BV, Venlo, The Netherlands). For the cell lines, cDNA was prepared by use of the Superscript II RNase H- kit from Invitrogen (Breda, the Netherlands). For the healthy blood donor before CellSearch enrichment and the preparations from the healthy blood donor and cell lines after CellSearch enrichment, cDNA was synthesized with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), followed by a linear preamplification for the non-[2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl- (benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium] (SYBR Green I)-based assays (TaqMan™ PreAmp from Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), according to the manufacturer's instructions. The resulting cDNA preparations were analyzed by real-time PCR with TaqMan® gene expression assays and TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems) and Absolute qPCR SYBR Green I mixture (Abgene, Epsom, UK) for the SYBR-based assays. PCRs were performed in a 20-μL reaction volume in an Mx3000P™ Real-Time PCR system (Stratagene, Amsterdam, The Netherlands). Expression of *HMB5*, *HPRT1*, and *GUSB* was used as a reference to control sample loading and RNA quality, as described previously²⁶. To enable comparison between the different gene expression measuring methods (Affymetrix GeneChip® Exon 1.0 ST arrays and real-time RT-PCR), data were normalized by the expression levels as measured with both methods in a set of seven control cell lines (ie, MDA-MB-435s, MDA-MB-231, CAMA-1, MCF-7, T47D, SK-BR-3, and EVSA-T). EGF = epidermal growth factor receptor; EMA = epithelial membrane antigen; EMT = epithelial-mesenchymal transition; EpCAM = epithelial cell adhesion molecule.

† One-way ANOVA was used to test for differences between the four subgroups present in the cell lines with $P < .05$ being considered statistically significant.

Table 2. Immunological assessment of targets in breast cancer cell lines with different intrinsic subtype characteristics and circulating tumor cell recovery*

Intrinsic subtype	No. of cell lines	Flow cytometry, † MFI				CellSearch, ‡ % recovery
		CD45	CD24	CD44	EpCAM	
Normal-like	6	<5	<5	>1000	<5	2 (0 to 6)
Basal-like	5	<5	5–20	200–1000	20–200	48 (36 to 61)
Luminal	5	<5	5–20	5–20	20–200	75 (62 to 89)
HER2-positive	3	<5	5–20	<5	20–200	85 (61 to 108)

*MFI = mean fluorescence intensity; EpCAM = epithelial cell adhesion molecule; CI = confidence interval; 7AAD = 7-amino-actinomycin D.
† Cultured human breast cancer cell lines were incubated with the following fluorochrome-conjugated monoclonal antibodies: EpCAM conjugated with fluorescein isothiocyanate (FITC) (clone EBA-1, BD Biosciences), CD24 conjugated with FITC (clone SN3, eBioscience, Inc., San Diego, CA), CD44 conjugated with R-phycoerythrin coupled to the cyanine dye Cy7 (PE-Cy7) (clone HIB19, eBioscience), and CD45 conjugated with allophycocyanin (APC) (clone 2D1, BD Biosciences). We used (7AAD) (Sigma-Aldrich, St. Louis, MO; 1 µg/mL) to control viability. Cells were then analyzed on a Canto flow cytometer (BD Biosciences). To exclude non-viable cells from analysis, only 7AAD-negative (viable) cells were evaluated for antigen expression. Unstained cells were used as a negative control. Only cells that expressed EpCAM were captured by the anti-EpCAM antibody present in the CellSearch™ circulating tumor cell profile kit. In addition, as shown in Figure 1 and Supplementary Table 2, MDA-MB-231 cells with marginal EpCAM expression were only partially (10 of 82 added tumor cells, or approximately 12%) captured by the CellSearch antibody, indicating that anti-EpCAM antibodies from the EBA-1 clone (BD Biosciences) and from the CellSearch circulating tumor cell kit have similar sensitivities and specificities.

‡ Data are the average (95% CI) of one representative experiment performed in duplicate for the given number of cell lines. Blood samples containing EDTA (7.5-mL aliquots of blood) from a single healthy blood donor were obtained from CellSave Preservative Tubes™ (Veridex LLC). To each sample, 20 µL of a cell suspension containing 50–150 cultured human breast cancer cells was added. To determine the actual viable cell number, a 100-µL aliquot of the cultured cells was incubated with 10 µL of 7AAD (1 µg/mL) and 100 µL of fluorescent beads (Beckman-Coulter Inc., Miami, FL). After a 15-minute incubation at room temperature, 2 mL of phosphate-buffered saline was added and samples were analyzed on a Calibur flow cytometer (BD Biosciences, San Jose, CA). At least 10,000 beads were acquired to estimate the number of 7AAD-negative (viable) cells. The efficiency of retrieving the tumor cells was controlled by counting the exact number of viable cells that were drawn in triplicate by flow cytometry and by light microscopy after serial dilution. To establish the number of circulating tumor cell recovered, samples were processed on the CellTrack™ AutoPrep analyzer (Veridex LLC) with the CellSearch™ circulating tumor cell profile kit (Veridex LLC), which uses microscopic iron particles coupled to anti-EpCAM to enrich for circulating tumor cells. The number of circulating tumor cells (ie, cells that are stained with the nuclear dye, 4',6-diamidino-2-phenylindole, are positive for cytokeratin 8, 18, and 19, and are negative for CD45) were determined on the CellSpotter™ analyzer (Veridex LLC), according to the manufacturer's instructions (for full flow cytometry and CellSearch recovery data on these 19 cell lines, see Supplementary Table 2).

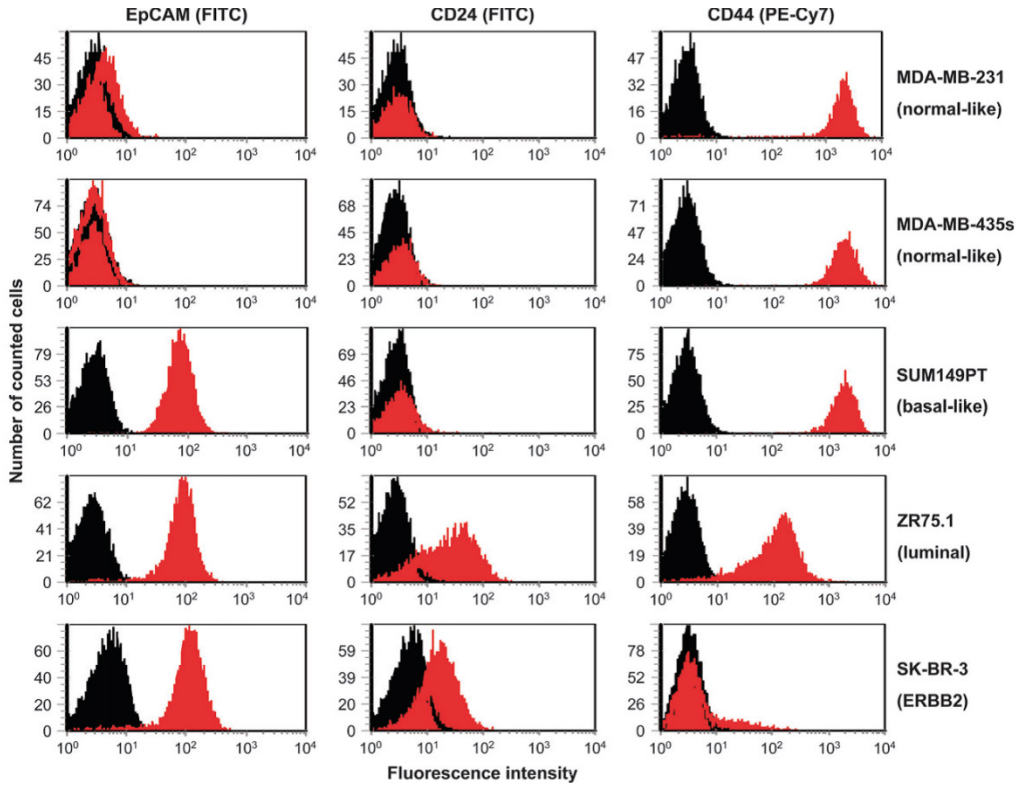


Figure 1. Immunological assessment of targets in breast cancer cell lines representing the four intrinsic breast cancer subtypes. Data from two normal-like breast cancer cell lines, one basal-like cell line, one luminal cell line, and one HER2 (*ERBB2*)-positive cell line are shown. CTC targets were assessed individually by incubating 10^6 cultured human breast cancer cells with the following fluorochrome-conjugated monoclonal antibodies: anti-EpCAM conjugated with fluorescein isothiocyanate (FITC) (clone EBA-1, BD Biosciences), anti-CD24 conjugated with FITC (clone SN3, eBioscience, Inc., San Diego, CA), or anti-CD44 conjugated with R-phycoerythrin coupled to the cyanine dye Cy7 (PE-Cy7; clone HIB19, eBioscience). We used 7-amino-actinomycin D (7AAD; Sigma-Aldrich, St. Louis, MO) to assess viability. Only viable (7AAD-negative) cells were analyzed for antigen expression (red histograms). Unstained cells (black histograms) were used as a negative control. X axis: fluorescence intensity obtained with the specified fluorochrome-conjugated antibody. Y axis: number of counted cells.

References

1. Sleijfer S, Gratama JW, Sieuwerts AM, Kraan J, Martens JW, Foekens JA. Circulating tumour cell detection on its way to routine diagnostic implementation? *Eur J Cancer*. 2007;43:2645-2650.
2. Lacroix M. Significance, detection and markers of disseminated breast cancer cells. *Endocr Relat Cancer*. 2006;13:1033-1067.
3. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer*. 2008;8:329-340.
4. Medical devices; immunology and microbiology devices; classification of the immunomagnetic circulating cancer cell selection and enumeration system. Final rule. *Fed Regist*. 2004;69:26036-26038.
5. Allard WJ, Matera J, Miller MC, 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;10:6897-6904.
6. Danila DC, Heller G, Gignac GA, et al. Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. *Clin Cancer Res*. 2007;13:7053-7058.
7. Cohen SJ, Alpaugh RK, Gross S, et al. Isolation and characterization of circulating tumor cells in patients with metastatic colorectal cancer. *Clin Colorectal Cancer*. 2006;6:125-132.
8. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med*. 2004;351:781-791.
9. Cristofanilli M. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *Semin Oncol*. 2006;33:S9-14.
10. Cristofanilli M, Broglio KR, Guarneri V, et al. Circulating tumor cells in metastatic breast cancer: biologic staging beyond tumor burden. *Clin Breast Cancer*. 2007;7:471-479.
11. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin Cancer Res*. 2007;13:920-928.
12. Stojadinovic A, Mittendorf EA, Holmes JP, et al. Quantification and phenotypic characterization of circulating tumor cells for monitoring response to a preventive HER2/neu vaccine-based immunotherapy for breast cancer: a pilot study. *Ann Surg Oncol*. 2007;14:3359-3368.
13. Ignatiadis M, Georgoulas V, Mavroudis D. Circulating tumor cells in breast cancer. *Curr Opin Obstet Gynecol*. 2008;20:55-60.
14. Nole F, Munzone E, Zorzino L, et al. Variation of circulating tumor cell levels during treatment of metastatic breast cancer: prognostic and therapeutic implications. *Ann Oncol*. 2008;19:891-897.
15. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol*. 2005;23:1420-1430.
16. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406:747-752.
17. Smid M, Wang Y, Zhang Y, et al. Subtypes of breast cancer show preferential site of relapse. *Cancer Res*. 2008;68:3108-3114.
18. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*. 2001;98:10869-10874.

19. Elstrodt F, Hollestelle A, Nagel JH, et al. BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res.* 2006;66:41-45.
20. Harkes IC, Elstrodt F, Dinjens WN, et al. Allelotype of 28 human breast cancer cell lines and xenografts. *Br J Cancer.* 2003;89:2289-2292.
21. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A.* 2003;100:3983-3988.
22. Ponti D, Zaffaroni N, Capelli C, Daidone MG. Breast cancer stem cells: an overview. *Eur J Cancer.* 2006;42:1219-1224.
23. Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell.* 2004;117:927-939.
24. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res.* 2008;68:989-997.
25. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell.* 2008;133:704-715.
26. Sieuwerts AM, Meijer-van Gelder ME, Timmermans M, et al. How ADAM-9 and ADAM-11 differentially from estrogen receptor predict response to tamoxifen treatment in patients with recurrent breast cancer: a retrospective study. *Clin Cancer Res.* 2005;11:7311-7321.

Supplementary data

Supplementary Table 1: Gene expression assay information*

Gene name	Data cell lines	Data HBD	Info Taqman assay	Sense primer	Info SYBR assay	Antisense primer
CAV1	Exon array	SYBR Taqman assay		TCAACGATGACGTGGTCAAG	CTGTGTGTCCCTTCTCTGGTTC	
CD24	Exon array	Taqman assay	Hs00273561_s1			
CD44	Exon array	Taqman assay	Hs00153304_m1			
ERBB1	SYBR	Taqman assay	Hs01076091_m1	CGGGACATAGTCAGCAGTG	GCTGGGCACAGATGATTTTG	
ERBB2	SYBR	Taqman assay	Hs00170433_m1	GTCTACAAGGGCATCTGGAT	GTGGATGTCAGGCAGATGC	
ESR1	SYBR	Taqman assay	Hs00174860_m1	ATCCTACCAGACCCCTTCAGTG	GCCAGACGAGACCAATCATC	
GUSB	Exon array	Taqman assay	Hs999999908_m1			
HMBS	Exon array	Taqman assay	Hs00609297_m1			
HPRT1	Exon array	Taqman assay	Hs01003267_m1			
KRT7	Exon array	Taqman assay	Hs00818825_m1			
KRT18	Exon array	Taqman assay	Hs01920599_gH			
KRT19	Taqman assay	Taqman assay	Hs01051611_gH			
MUC1	Exon array	Taqman assay	Hs00159357_m1			

<i>PTPRC</i> (CD45)	Exon array	Taqman assay	Hs00236304_m1	
<i>TACSTD1</i> (EpCAM)	Taqman assay	Taqman assay	AAGGAGATCACAAACGCGT (MGB)	AGTTTGGCGGACTGCACCTTCA
<i> Twist1</i>	Taqman assay	Taqman assay		AATACTCGTGATAAAATTTTGGATCCA
<i>VIM</i>	SYBR	Taqman assay	Hs00361186_m1	CAGATTTCAGGAACAGCATGTC
				TCAGAGAGGTCAGCAAACTTG

* Gene expression assays that were used to compare mRNA transcript levels among the four breast cancer subtypes in cell lines and the healthy blood donors. To validate the Affymetrix gene expression profiling data, transcript levels of 14 candidate genes that might enable discrimination among the four breast cancer subtypes and cells of hematopoietic origin from healthy blood donors were re-measured with Affymetrix GeneChip® Exon 1.0 ST Array (Affymetrix UK Ltd., Wicksam la Wooburn Grn, UK) taking the average of all core probes (approximately one core probe per exon and roughly 10 core probes per gene), or with real-time reverse transcriptase-PCR with the primer-probe based TaqMan® gene expression assay (Applied Biosystems) or the primers-only based SYBR Green I assay given in the Table. These fourteen candidate genes more specific for cells of hematopoietic origin (*CD44* and *CD45*), epithelial specific genes such as those encoding cytokeratins, EpCAM and mucin 1, genes encoding markers specific for the breast cancer subgroups (*ESR1*, *ERBB1*, *ERBB2*, *CAV1*, *CD24*), and 2 well known EMT markers (*Twist1*, *VIM*). Expression of *HMBS*, *HPRT1*, and *GUSB* was used as a reference to control sample loading and RNA quality.

HBD = healthy blood donor.

Supplementary Table 2: Immunological assessment of targets in 19 randomly selected breast cancer cell lines with different intrinsic subtype characteristics and circulating tumor cell recovery

Intrinsic subtype and cell line	Viability, %	Flow cytometry,† MFI				CellSearch recovery‡		
		CD45	CD24	CD44	EpCAM	No. of viable tumor cells added	No. of cells recovered after enrichment	Viable cells recovered after enrichment, %
Normal-like								
MDA-MB-435s	87	<5	<5	>1000	<5	57	0	0
MDA-MB-436	51	<5	<5	>1000	<5	149	0	0
SK-BR-7	94	<5	<5	>1000	<5	51	0	0
Hs578T	92	<5	<5	>1000	<5	61	0	0
BT549	60	<5	5-20	>1000	<5	85	0	0
MDA-MB-231	94	<5	<5	>1000	5-20	82	10	12
Average ± SD (n = 6)	80 ± 19	<5	<5	>1000	<5	81 ± 36	2 ± 4§	2 ± 5§
Basal-like								
MDA-MB-468	46	<5	5-20	>1000	20-200	161	64	31
BT20	75	<5	<5	200-1000	20-200	91	40	40
SUM149PT	51	<5	5-20	200-1000	20-200	82	50	44
SUM229PE	83	<5	5-20	200-1000	20-200	61	40	65
HCC1937	56	<5	5-20	20-200	20-200	96	30	61
Average ± SD (n=5)	62 ± 16	<5	5-20	200-1000	20-200	98 ± 37	45 ± 13§	48 ± 14§
Luminal								
MPE600	65	<5	20-200	<5	20-200	148	79	53
CAMA-1	66	<5	5-20	5-20	20-200	88	66	75
T47D	74	<5	5-20	<5	20-200	76	57	75
ZR75-1	66	<5	20-200	5-20	20-200	88	85	97
MDA-MB-361	79	<5	5-20	5-20	20-200	86	66	77

Average ± SD (n=5)		70 ± 6	<5	5-20	5-20	20-200	97 ± 29	71 ± 11	76 ± 15
ERBB2									
MDA-MB-453		76	<5	5-20	<5	20-200	89	64	72
EVSA-T		66	<5	<5	<5	20-200	100	109	109
SK-BR-3		93	<5	20-200	5-20	20-200	152	112	74
Average ± SD (n=3)		78 ± 14	<5	5-20	<5	20-200	114 ± 33	95 ± 27	85 ± 21

*MFI = mean fluorescence intensity; EpCAM = epithelial cell adhesion molecule; CI = confidence interval; 7AAD = 7-amino-actinomycin D.

† Cultured human breast cancer cell lines were incubated with the following fluorochrome-conjugated monoclonal antibodies: EpCAM conjugated with fluorescein isothiocyanate (FITC) (clone EBA-1, BD Biosciences), CD24 conjugated with FITC (clone SN3, eBioscience, Inc., San Diego, CA), CD44 conjugated with R-phycoerythrin coupled to the cyanine dye Cy7 (PE-Cy7) (clone HIB19, eBioscience), and CD45 conjugated with allophycocyanin (APC) (clone 2D1, BD Biosciences). We used (7AAD) (Sigma-Aldrich, St. Louis, MO; 1 µg/mL) to control viability. Cells were then analyzed on a Canto flow cytometer (BD Biosciences). To exclude non-viable cells from analysis, only 7AAD-negative (viable) cells were evaluated for antigen expression. Unstained cells were used as a negative control. Only cells that expressed EpCAM were captured by the anti-EpCAM antibody present in the CellSearch™ circulating tumor cell profile kit. In addition, as shown in Figure 1, MDA-MB-231 cells with marginal EpCAM expression were only partially (10 of 82 added tumor cells, or approximately 12%) captured by the CellSearch antibody, indicating that anti-EpCAM antibodies from the EBA-1 clone (BD Biosciences) and from the CellSearch circulating tumor cell kit have similar sensitivities and specificities.

‡ Data are the average (95% CI) of one representative experiment performed in duplicate for the given number of cell lines. Blood samples containing EDTA (7.5-mL blood aliquots) from a single healthy blood donor were obtained from CellSave Preservative Tubes™ (Veridex LLC). To each sample, 20 µL of a cell suspension containing 50-150 cultured human breast cancer cells was added. To determine the actual viable cell number, a 100-µL aliquot of the cultured cells was incubated with 10 µL of 7AAD (1 µg/mL) and 100 µL of fluorescent beads (Beckman-Coulter Inc., Miami, FL). After a 15-minute incubation at room temperature, 2 mL of phosphate-buffered saline was added and samples were analyzed on a Calibur flow cytometer (BD Biosciences, San Jose, CA). At least 10,000 beads were acquired to estimate the number of 7AAD-negative (viable) cells. The efficiency of retrieving the tumor cells was controlled by counting the exact number of viable cells that were added in triplicate drawn samples by flow cytometry and by light microscopy after serial dilution. To establish the number of circulating tumor cell recovered, samples were processed on the CellTrack™ AutoPrep analyzer (Veridex LLC) with the CellSearch™ circulating tumor cell profile kit (Veridex LLC), which uses microscopic iron particles coupled to anti-EpCAM to enrich for circulating tumor cells. The number of circulating tumor cells (ie, cells that are stained with the nuclear dye, 4',6-diamidino-2-phenylindole, are positive for cytokeratin 8, 18, and 19, and are negative for CD45) were determined on the CellSpotter™ analyzer (Veridex LLC), according to the manufacturer's instructions (for full flow cytometry and CellSearch recovery data on these 19 cell lines, see Supplementary Table 2).

§ One-way ANOVA was used to test for differences between the four subgroups present in the cell lines with $P < .05$ being considered statistically significant.

Supplementary Table 3: mRNA expression in individual breast cancer cell lines of known subtype.

Subtype and cell line	mRNA expression relative to HMBS, HPRT1 and GUSB														TWIST1 EMT marker	VIM EMT marker
	PTPRC (CD45)	CD24	CD44	TACSTD1 (EpcAM)	MUC1 (EMA)	CAV1	KRT7	KRT18	KRT19	ESR1	EGFR	ERBB2				
Normal-like																
MDA-MB-435s	0.00	0.01	0.83	0.00	0.14	1.69	0.29	2.28	0.00	0.00	0.01	0.01	0.01	0.01	58.38	
MDA-MB-436	0.00	0.08	0.35	0.01	0.07	1.42	0.41	5.36	0.00	0.00	0.24	0.05	0.04	0.04	26.66	
SK-BR-7	0.00	0.39	0.76	0.01	0.42	2.81	7.19	23.61	0.00	0.00	0.37	0.04	0.01	0.01	14.94	
Hs578T	0.00	0.11	0.93	0.00	0.55	10.00	0.25	3.08	0.00	0.00	0.01	0.05	0.06	0.06	182.03	
BT549	0.00	0.81	0.61	0.00	0.23	3.15	0.24	0.28	0.01	0.00	0.38	0.05	0.04	0.04	69.33	
MDA-MB-231	0.00	0.00	0.57	0.07	0.17	4.61	1.84	4.08	0.77	0.00	0.34	0.05	0.00	0.00	30.54	
MDA-MB-157	0.00	1.76	0.73	0.28	0.41	3.05	0.24	6.33	0.00	0.00	0.18	0.05	0.12	0.12	116.93	
SUM1315MO2	0.00	0.01	1.36	0.01	0.57	13.42	0.17	0.67	0.00	0.00	0.20	0.09	0.24	0.24	326.18	
SUM159PT	0.00	0.46	0.78	0.00	0.30	5.27	3.18	4.48	0.00	0.00	0.07	0.03	0.02	0.02	89.09	
Basal-like																
MDA-MB-468	0.00	6.51	0.96	3.99	0.71	0.22	8.85	11.46	5.52	0.02	3.02	0.07	0.00	0.00	0.16	
BT20	0.00	1.91	0.65	0.78	0.26	0.93	6.93	12.49	2.07	0.01	0.81	0.06	0.00	0.00	0.08	
SUM149PT	0.00	1.15	1.31	2.70	0.27	1.70	10.81	10.07	1.85	0.01	0.12	0.02	0.00	0.00	1.27	
SUM229PE	0.00	0.46	0.59	2.06	0.41	4.64	8.95	10.30	13.18	0.02	0.73	0.16	0.00	0.00	0.65	
HCC1937	0.00	1.53	0.24	0.68	0.10	1.01	3.97	5.96	0.03	0.04	0.12	0.04	0.00	0.00	0.01	
Luminal																
MPE600	0.00	6.56	0.01	11.40	3.18	0.01	11.87	20.66	59.73	1.03	0.05	1.85	0.00	0.00	0.02	
CAMA-1	0.00	1.35	0.01	2.67	1.36	0.01	2.97	7.97	4.25	0.40	0.00	0.13	0.01	0.01	0.01	
T47D	0.00	1.13	0.06	2.07	2.01	0.02	0.79	12.67	11.13	0.97	0.04	0.12	0.00	0.00	0.02	
ZR75-1	0.00	0.24	0.01	3.08	3.28	0.01	0.21	10.55	9.80	1.27	0.00	0.46	0.01	0.01	0.00	
MDA-MB-361	0.00	9.53	0.16	11.75	0.36	0.03	10.49	16.41	11.11	0.54	0.08	3.95	0.00	0.00	0.04	

BT483	0.00	2.27	0.08	4.05	3.54	0.01	5.84	27.54	43.30	1.23	0.01	0.82	0.02	0.02
MDA-MB-134 VI	0.00	5.10	0.02	4.29	0.19	0.05	1.83	7.69	19.41	3.02	0.00	0.04	0.04	2.38
MDA-MB 175 VII	0.00	2.15	0.02	1.82	2.27	0.02	5.39	13.74	25.93	0.35	0.04	1.05	0.05	0.15
MDA-MB-415	0.01	11.56	0.08	10.27	3.79	0.14	16.18	16.32	74.52	0.74	0.00	1.05	0.00	0.09
SUM185PE	0.00	4.15	0.00	5.93	0.87	0.01	7.15	19.89	12.44	0.04	0.00	0.20	0.00	0.03
SUM52PE	0.00	3.11	0.01	4.87	0.93	0.07	5.22	17.66	9.15	0.39	0.00	0.08	0.00	0.01
BT474	0.01	4.90	0.05	35.54	1.14	0.06	19.64	34.74	111.45	0.35	0.14	14.87	0.05	1.72
SK-BR-5	0.00	4.51	0.03	22.77	2.00	0.05	9.85	15.95	13.91	0.04	0.06	0.29	0.01	0.04
OCUB-F	0.01	0.06	0.01	8.81	3.05	0.07	1.17	14.74	15.01	0.00	0.03	12.99	0.01	0.00
MCF-7	0.00	0.98	0.10	1.53	0.18	0.20	0.75	15.40	4.00	1.01	0.00	0.06	0.00	0.01
HER2-positive														
MDA-MB-453	0.01	2.05	0.00	3.05	0.39	0.03	7.24	18.35	3.96	0.00	0.00	2.65	0.00	0.00
EVSA-T	0.00	3.16	0.00	2.09	0.44	0.02	5.03	6.98	1.87	0.00	0.00	0.29	0.00	0.00
SK-BR-3	0.00	2.60	0.02	6.02	0.67	0.06	10.40	19.47	29.84	0.00	0.13	15.49	0.00	0.00
MDA-MB-330	0.00	1.61	0.01	2.83	0.53	0.03	13.10	20.16	5.89	0.26	0.00	9.66	0.00	0.44
UACC893	0.00	4.03	0.06	8.18	0.62	0.01	16.54	20.90	18.40	0.03	0.19	10.36	0.00	0.05
P values†	0.21	0.03	0.03	0.03	0.00	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.03	0.00

*To validate the gene expression profiling data, transcript levels of candidate genes that might enable discrimination among the four breast cancer subgroups were analyzed with Affymetrix GeneChip® Exon 1.0 ST arrays and real-time reverse transcription- polymerase chain reaction. *HMBS*, *HPRT1* and *GUSB* were used as reference genes to control sample loading and RNA quality. To enable comparison between the two gene expression measuring methods data were normalized by the expression levels measured with both methods in a set of seven control cell lines (MDA-MB-435s, MDA-MB-231, CAMA-1, MCF-7, T47D, SK-BR-3 and EVSA-T).

For none of the genes there was a difference in expression level between the 19 cell lines that were tested by CellSearch (printed in italic) and the 15 cell lines that were only analyzed for gene expression (Mann-Whitney's U $P > 0.05$ for all the individual genes within the intrinsic subtype groups). This indicates that the random selection of 19 out of 34 cell lines was representative for the whole cohort.

† One-way ANOVA was used to test for differences between the four subgroups in breast cancer cell lines with $P < .05$ considered as statistically significant. All other statistical tests were two-sided.

Supplementary Table 4: mRNA expression in healthy blood donors before and after CellSearch enrichment*

Subtype and cell line		mRNA expression relative to HMBS, HPRT1 and GUSB												
Healthy blood donor	PTPRC (CD45)	CD24	CD44	TACSTD1 (EpCAM)	MUC1 (EMA)	CAV1	KRT7	KRT18	KRT19	ESR1	EGFR	ERBB2	TWIST1	VIM
													EMT marker	EMT marker
Before CellSearch														
1	14.39	0.03	4.16	0.00	0.05	0.04	0.02	0.08	0.00	0.03	0.00	0.04	0.00	25.81
2	10.46	0.02	4.12	0.00	0.03	0.00	0.01	0.03	0.00	0.02	0.00	0.00	0.00	45.89
3	15.46	0.03	3.41	0.00	0.04	0.05	0.01	0.06	0.00	0.03	0.00	0.01	0.00	60.13
4	10.08	0.04	3.21	0.00	0.02	0.01	0.00	0.08	0.00	0.02	0.00	0.03	0.00	25.99
5	10.56	0.03	2.83	0.00	0.01	0.03	0.00	0.01	0.00	0.01	0.00	0.03	0.00	19.16
6	19.43	0.01	5.35	0.00	0.06	0.02	0.02	0.10	0.00	0.01	0.00	0.02	0.00	29.86
After CellSearch														
1	3.34	0.00	3.95	0.00	0.01	0.02	0.01	0.03	0.00	0.00	0.00	0.00	0.00	44.63
2	8.50	0.04	7.45	0.00	0.01	0.00	0.00	0.03	0.00	0.01	0.00	0.01	0.00	41.64
3	3.86	0.07	1.73	0.01	0.03	0.14	0.00	0.10	0.00	0.02	0.00	0.00	0.00	39.72
4	10.08	0.02	5.29	0.01	0.01	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	80.45
5	11.59	0.00	4.61	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	48.50
6	6.08	0.01	6.47	0.00	0.03	0.00	0.02	0.01	0.00	0.00	0.00	0.00	0.00	54.19
7	3.71	0.01	4.63	0.00	0.01	0.00	0.01	0.02	0.00	0.01	0.00	0.00	0.00	38.32
8	1.88	0.00	6.76	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	60.97
9	4.12	0.00	3.93	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	37.79
10	4.63	0.14	1.84	0.01	0.01	0.00	0.00	0.05	0.00	0.00	0.00	0.02	0.00	43.47
11	8.00	0.00	3.58	0.00	0.01	0.01	0.02	0.01	0.00	0.00	0.00	0.00	0.00	64.45
12	4.77	0.00	4.54	0.00	0.02	0.10	0.01	0.01	0.00	0.00	0.00	0.00	0.00	75.06
13	3.80	0.02	2.58	0.07	0.04	0.00	0.01	0.06	0.00	0.01	0.00	0.02	0.00	27.10
14	5.15	0.00	3.93	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	56.89

15	12.76	0.03	3.74	0.01	0.02	0.09	0.01	0.03	0.00	0.00	0.00	0.00	0.00	63.12
16	2.18	0.03	1.82	0.01	0.00	0.22	0.00	0.03	0.00	0.06	0.00	0.01	0.00	39.45
17	6.23	0.01	3.58	0.00	0.02	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	84.45
18	5.11	0.02	2.82	0.00	0.00	ND	0.00	0.04	0.00	0.00	0.00	0.04	0.00	ND
19	4.80	0.00	2.94	0.00	0.00	0.02	0.01	0.01	0.00	0.01	0.00	0.00	0.00	43.71
20	11.42	0.07	4.01	0.00	0.00	0.57	0.00	0.02	0.00	0.05	0.00	0.00	0.00	14.52
21	9.17	0.01	3.67	0.00	0.03	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	60.13
22	7.24	0.02	3.22	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00	99.04
23	5.29	0.25	2.69	0.05	0.02	0.00	0.01	0.04	0.00	0.04	0.00	0.01	0.00	32.94

*To validate the gene expression profiling data, transcript levels of candidate genes that might enable discrimination among the four breast cancer subgroups were analyzed with Affymetrix GeneChip® Exon 1.0 ST arrays and real-time reverse transcription- polymerase chain reaction. *HMBS*, *HPR1* and *GUSB* were used as reference genes to control sample loading and RNA quality. To enable comparison between the two gene expression measuring methods data were normalized by the expression levels measured with both methods in a set of seven control cell lines (MDA-MB-435s, MDA-MB-231, CAMA-1, MCF-7, T47D, SK-BR-3 and EVSA-T).

Chapter 5

Clinical value of circulating endothelial cell detection in oncology

Jaco Kraan, Stefan Sleijfer, John A. Foekens and Jan W. Gratama

Drug Discovery Today 2012; 17:710 - 717

Abstract

Given the importance of tumor vasculature, both in tumor biology and as target for treatment, there is an increasing need for biomarkers that accurately reflect effects impacting tumor vasculature. Circulating endothelial cells (CEC) are increased in number as a result of vascular damage in cancer and several other diseases. Counting CEC constitutes a promising tool to monitor disease activity with potential to assess prognosis and response to treatment. Here, we address the current state-of-the-art of CEC enumeration as biomarker in clinical oncology. We focus on technical issues concerning their detection, review results from clinical studies and explore future potential applications.

Introduction

The growth of solid tumors is dependent on their capacity to induce the growth of blood vessels to supply them with oxygen and nutrients ^{1,2}. There are several known mechanisms of blood vessel formation. Vessel formation can occur by (i) sprouting of new vessels from existing ones (angiogenesis), (ii) the recruitment of bone-marrow derived and/or vascular-wall-resident endothelial progenitor cells (EPCs) that differentiate into endothelial cells (ECs) (vasculogenesis), or by (iii) vessel splitting (intussusception). In addition, (iv) tumor cells can co-opt preexisting vessels, (v) tumor vessels can be lined by tumor cells (vascular mimicry), and (vi) putative cancer stem-like cells can differentiate into tumor endothelium. Unlike normal tissues, which use sprouting angiogenesis, vasculogenesis and intussusception, tumors can use all six modes of vessel formation ³. Another important difference between normal and tumor vasculature is that normal vasculature is arranged in a hierarchy of evenly spaced, well-differentiated arteries, arterioles, capillaries, venules, and veins, while in contrast, the tumor vasculature is composed of a chaotic mixture of abnormal and hierarchically disorganized vessels ⁴.

In view of the central role of the tumor vasculature in the biology of tumors, various agents have been developed and are now available and under study in order to target tumor vasculature. Examples include vascular endothelial growth factor (VEGF)-pathway inhibiting agents such as monoclonal antibodies against VEGF, and small tyrosine kinase inhibitors targeting VEGF-receptors or targeting Activin Receptor-Like Kinase1 (ALK1) ⁵. Recently a new group of anticancer drugs, vascular disrupting agents (VDA), has been developed to target the endothelial cells and pericytes of the already established tumor vasculature ⁶. Although several clinical trials have demonstrated a benefit in terms of prolonged survival of cancer patients treated with anti-angiogenic therapies, for further development of the field

of anti-angiogenic treatments there is a compelling need for clinically effective biomarkers enabling to determine the optimal dose, to monitor their biologic activity, and to select and stratify the patients who are most likely to benefit from treatment ⁷. There is also a lack of biomarkers identifying escape pathways that underlie resistance against anti-angiogenic agents and that should be targeted after tumors develop resistance to a given antiangiogenic agent ⁸.

Currently, there are several parameters that can serve as surrogate biomarkers to measure angiogenesis and/or anti-angiogenic drug activity. These include, soluble blood factors involved in angiogenesis such as VEGF, s-VEGFR-2, placenta growth factor (PlGF), soluble Tie2, E-Selectine and thrombomodulin, molecular markers of angiogenesis such as von Willebrand factor (vWF) and VE-Cadherin. Blood vessels may be visualized using vascular imaging techniques such as Dynamic-contrast-enhanced MRI and PET scanning. Each of these methods have their own drawbacks regarding specificity, sensitivity or costs and although several studies have shown interesting data, none has been clinically validated ^{7,9}.

CEC is a relative new candidate to serve as a marker to monitor vascular effects. Current opinion states that CEC are mature endothelial cells which have been shed from the vascular cell lining as a result of vascular damage. As numerous diseases, including cancer, are associated with vascular damage, the enumeration of CEC has for long been considered a promising tool to monitor disease activity with a potential to assess prognosis and response to treatment. Accordingly, elevated CEC numbers have been found in multiple diseases, including cardiovascular disorders ^{10,11}, infectious diseases ¹²⁻¹⁴, immune disorders ^{15,16}, pulmonary hypertension ¹⁷, sickle cell anaemia ^{18,19}, status after organ transplantation ^{20,21} and cancer ²²⁻²⁷.

In this review, we will address the current position of CEC enumeration as biomarker in clinical oncology with special attention to technical issues concerning their detection, results from clinical studies obtained so far and future potential applications.

Enumeration of circulating endothelial cells

The first methods to detect CEC were described in the mid-70s and identification methods included separation by density centrifugation, vital light microscopy and May-Grünwald staining. These methods did not isolate and identify CEC reliably, and it took two more decades to establish procedures to quantify CEC. As CEC occur in rather low frequencies in the peripheral blood of healthy donors, there is a consensus in literature for values less than 10 CEC/mL, one of the main challenges is assay sensitivity and accuracy. As a result assays to count CEC are highly prone to errors in sample preparation, and analysis. Given the low frequencies, a volume of more than 1 mL will be needed for a precise enumeration of CEC at a frequency

of less than 1 cell per micro liter blood to obtain a population of at least 100 cells fulfilling the flow cytometric criteria with a maximal coefficient of variation of 10%. Another essential step for proper detection and enumeration step of CEC is the use of specific markers discriminating CEC from other cells present in the peripheral circulation. **Table 1** provides an overview of markers frequently used in various CEC assays. The most common marker to define CEC is CD146 or melanoma cell-adhesion molecule (MCAM). CD146 is present on endothelial cells but also on a subset of activated T-lymphocytes, mesenchymal stem cells and some malignant cells. This implies conformation of the origin of CEC isolated through CD146⁺ enrichment by other CEC markers and the absence of the pan-leukocyte marker CD45. A complication with detection of CEC is that many endothelial surface antigens are secreted, and their interaction with platelet aggregates can result in false-positive signals ²⁸.

In recent years, several methods have been described to enumerate CEC. However, there is no consensus between CEC phenotypes used in these enumeration techniques. Importantly, most methods have not been properly validated ²⁸. In combination with the relative rarity of CEC, this situation has resulted in a wide variation of reported CEC numbers. For example, reported CEC numbers in healthy humans range from 4 to 7,900 cells/mL between the various CEC assays ^{23,25,29-31}. Obviously, this large variation, most likely due to different techniques, hampers comparisons between studies and thereby hinders further progress in the field. As a consequence, there is a need for standardization and validation of the true endothelial origin of cells designated CEC by a particular assay. For the latter, several unique features of endothelial cells can be used. These include uptake of Ulex Europaeus Lectin-1, or UEA-1²⁵. Also immunocytochemistry or immunofluorescence can be used to demonstrate the presence of endothelial markers other than used in the assay to isolate the events thought to be CEC (Table 1). Another means of validation is through gene expression profiling assessing whether or not endothelial genes, such as VE-cadherin are expressed by the population of cells designated as CEC ³².

Manual immunomagnetic isolation of CEC

Currently, most CEC enumeration tests rely on an enrichment step by immunomagnetic bead separation (IMS) using magnetic particles coupled to monoclonal antibody (mAb) targeting an endothelial antigen such as CD146 or CD34, followed by flow cytometry or visual counting with a fluorescent microscope to identify CEC on the basis of morphological or immunophenotypical criteria ^{30,33,34}. A major advantage of this latter approach is that it permits visual identification of CEC, which allows discrimination between CEC and endothelial micro-particles or platelets with a strongly overlapping phenotype. However, any

enrichment step inevitably leads to cell loss and underestimation of the actual CEC number, whilst manual bead-based isolation procedures are labor intensive and difficult to standardize, in terms of purity and recovery. Despite the differences between published IMS methods with respect to the size of the magnetic beads, antibody clones and enumeration techniques most studies are reporting a relatively narrow range of CEC levels, i.e., between 1 and 10 cells/mL in healthy individuals³⁵.

Automated immunomagnetic isolation and staining of CEC

A variant of manual IMS enrichment techniques is the CellSearch® system (Veridex, Raritan, NJ). Initially designed to detect circulating tumor cells, this system provides a fully automated enrichment procedure that is followed by semi-automated image cytometry. The generated images are evaluated for CEC by visual inspection, in which CEC are defined as DAPI⁺, CD105⁺, CD146⁺ and CD45⁻ and morphological criteria such as size and whole intact cells. This assay has a high recovery and good reproducibility, also for low number of CEC as reported in healthy controls (1-20 CEC/mL). Importantly, CEC isolated in this way have been validated by morphology and global gene expression^{26,32}. Drawbacks of the CellSearch® system are the costly equipment and reagents, and the assay cannot be customized. Also, the maximum number of eight samples that can be analyzed in a single run, combined with the relatively long duration of a complete run (approximately 4 h) does not allow high throughput analysis.

Flow Cytometric analysis of CEC

Flow cytometric analysis of CEC has become popular for diagnostics because this approach -in comparison with cellular enrichment- is less time consuming, less costly and easy to standardize once consensus has been reached. Examples of antibodies used in cellular identification includes those against CD31, CD34, CD146, CD309 and DNA stains in combination with CD45 to exclude hematopoietic cells (Table 1). Flow cytometry has significant advantages as it is based on multi-parameter analysis, which allows a highly specific definition of CEC. The first flow cytometry study was published in 2001 by Mancuso et al³¹ identifying CEC as CD146⁺, CD31^{bright} and CD45^{negative} in a commonly applied whole blood assay in a volume of only 100-150 µL. This approach was also applied by other investigators with similar methods. Although this approach appeared promising, the high level of CEC (>7,000/mL in healthy individuals) detected in these studies compared to IMS techniques led to doubts on whether or not the right cell population was designated as CEC. The reason for these discrepancies was

unclear until it was demonstrated that the majority of the cells with the above phenotype were in fact large platelets²⁸. This result underlines the need of including a DNA specific stain such as 4',6-diamidino-2-phenylindole (DAPI) or 1,5-bis[2-(di-methylamino) ethyl]amino-4, 8-dihydroxyanthracene-9,10-dione (DRAQ5) to exclude platelets, aggregates and endothelial micro/macro -particles, which have an overlapping phenotype with CEC. In 2009 Mancuso et al²⁹ presented an improved method by adding the DNA stain syto16 to their earlier described phenotype and proved the endothelial origin of the majority of these cells by the identification of Weibel-Palade bodies using electron microscopy and the presence the endothelial specific gene VE-cadherin in the sorted CEC population. As a result the CEC numbers found in healthy individuals were now much lower than detected by the initial approach (i.e., mean 140 ± 171 cells/mL) and approached the numbers obtained by IMS. Earlier, Goon and colleagues²⁵ compared IMS with a flow cytometry assay using a larger volume of 1 mL blood, which makes it possible to acquire and analyze a high number of cellular events (2 to 5 million), and identified CEC as CD146⁺, CD34^{bright} and CD45^{negative}. They found a reasonable agreement between both methods with a median CEC count between 4 and 8 cells/mL in healthy controls. This result coincides with the values reported by two other groups using flow cytometry methods established in 1 mL blood^{36,37}. Despite these convincing data, most of the recently reported studies using flow cytometry to detect CEC did not include a DNA stain and still determined CEC in a small sample volume of 50-200 μ L^{38,39,40} as a consequence of which incorrectly high CEC levels are found.

Flow cytometric analysis has the possibility to measure CEC viability by adding markers such as Annexin V or 7-AAD. Both, the viable⁴¹ and necrotic/apoptotic CEC populations⁴² as well as changes in the proportion of necrotic/apoptotic CEC⁴³ have been used to study the efficacy of anti-angiogenic therapies. Excluding of necrotic/apoptotic cells from analysis has the advantage that a well-known source of nonspecific binding which can cause artifacts will be deleted, but will underestimate the total counts of CEC. As mature CEC enumeration in particular reflects vascular damage, it is mandatory to study both viable and non-viable populations. In light of these results, CEC enumeration by flow cytometry is not yet a standardized procedure, and there is need for validation studies demonstrating the superiority of one technique over the other regarding sensitivity and reproducibility.

Circulating Endothelial Progenitor Cells

In addition to CEC, several studies also incorporate a strategy to enumerate circulating endothelial progenitor cells (CEPC). In contrast to CEC, CEPC are thought to originate from the bone marrow and contribute actively to vasculogenesis^{44,45}. Mostly CD34, widely used as a marker for hematopoietic

progenitor cells (HPC), is used to identify CEPC with a clonogenic potential. However, CD34 is also expressed on mature endothelial cells, and thus cannot be used to discriminate between CEC and CEPC⁷. At present CD45, CD133 and CD309 (VEGFR-2) in particular are considered appropriate antigens to discriminate between CEC and CEPC⁴⁶. But these antigens are also expressed on HPCs rendering a distinct phenotype between CEPC and HPCs not possible. In fact, the population of CEPC may include a group of cells existing in a variety of stages ranging from immature HPC to differentiated endothelial cells⁴⁷. Accordingly, a robust study by Ingram and colleagues recently showed that endothelial progenitor cells (EPCs) reside in the CD45-CD34+ cell fractions, and do not express CD133. This further complicates the immunophenotypic discrimination between CEPC from CEC, since CD45 and CD133 were thought to discriminate putative CEPC from CEC. Using clonogenic assays it was also shown that CD34+CD45+ cells formed HPCs but not EPCs. Therefore, studies using CD45+, CD34+, CD133+ and/or CD309 as phenotype for CEPC should be reexamined and the search for novel markers and parameters that accurately discriminates CEC from CEPC with flow cytometry is mandatory^{48,49}.

Clinical applications of CEC in oncology

Elevated CEC levels have been described in a range of human malignancies^{22-27,50,51} and mouse models^{43,52}. This result implies that CEC enumeration may be useful to establish prognosis, to predict response to treatment in cancer patients and potentially to determine the most optimal drug dose in early clinical trials.

Baseline CEC levels as prognostic marker in cancer

In colorectal cancer, three studies have reported that a low CEC level at baseline predicts a better outcome compared with a high CEC level⁵³⁻⁵⁵. In contrast, a large study by Simkens et al⁵⁶ using the CellSearch system suggested that CEC numbers at baseline were not prognostic for progression-free or overall survival (Table 2). A similar observation was also made in a CellSearch-based study on prostate cancer by Strijbos et al²⁷. At the other hand, in breast cancer, high CEC levels at baseline were reported by two studies using flow cytometry to indicate a better outcome^{57,58}. However, again these findings could not be confirmed in a study using the CellSearch system⁵⁹ (Table 2). With respect to the potential prognostic value of CEC, there are great differences between the diverse tumor types. This could suggest a difference in vascular turnover according to tumor origin, but may also have resulted from differences in CEC enumeration methods and phenotype. Clearly, more studies are needed to define the value of CEC enumeration as prognostic factor in the diverse tumor types.

Changes in CEC levels and associations with treatment outcome

So far only a limited number of studies have been conducted to explore the value of changes in CEC numbers as a predictor of outcome to treatment. It is important to realize that outcome to treatment in cancer patients can be presented by different parameters including response according to standard criteria such as those from WHO and RECIST, progression free survival, clinical benefit, and overall survival. In 2010, a comprehensive study in prostate cancer patients treated with docetaxel-based regimes reported that an early increase in the number of CEC during treatment was associated with a worse overall survival, in particular when combined with circulating tumor cell numbers and tissue factor levels ²⁷. This data was supported by another study that year⁵⁰ showing that an increase of CEC after 7 days predicts response and survival in cancer patients treated with various chemotherapeutics.

Recently several studies have investigated the association of changes in CEC numbers during treatment with outcome to anti-angiogenic-based therapy (Table 3). Several studies showed that an increase in CEC numbers in patients is associated with clinical benefit in terms of tumor response according to WHO or RECIST criteria or prolonged disease stabilization in diverse tumor types including renal cancer ⁶⁰, GIST ⁶¹ and breast cancer^{43,59}. In contrast, studies in colon cancer³⁸ and glioblastoma⁶² an increase in the number of CEC was associated with worse clinical outcome or was not prognostic in three other studies in colon cancer^{53,56} and renal cancer⁴¹. These discrepancies might be explained by the timing of CEC detection, the inclusion/exclusion of apoptotic CEC within different assays and different anti-angiogenic treatment regimens and should be further explored.

With respect to classic methods to establish anti-tumor effects of systemic agents, which rely on assessing changes in tumor size by radiological assessments, it is increasingly recognized that these not always suffice anymore. This holds true not only when using conventional chemotherapeutic drugs, but in particular when angiogenesis inhibitors are applied. Several studies have monitored CEC levels as a biomarker for response to treatment and showed correlations between changes in CEC levels and response according to standard criteria such as those from WHO and RECIST ^{39,57,63-65}.

Taken together, it is apparent that CEC are increased in patients with different types of malignancies and there is a growing body of evidence that this cell population may evolve into a surrogate biomarker for effectiveness of conventional and targeted therapy.

CEC as biomarker for optimal biological drug dosage

Changes in CEC counts after dose escalation might provide useful insights in establishing the optimal biological drug dose (OBD) when assessing agents targeting vasculature such as VDA or angiogenesis inhibitors. A study by Celik et al. reports a 50% decrease in CEC in tumor bearing mice treated with endostatin. The decrease in CEC showed a clear U-shaped dose relation, suggesting a rationale for using CEC as guideline for optimum dosing. Other pre-clinical studies showed a dose-dependent increase of CEC in tumor bearing mouse treated with VEGFR2 inhibitors⁵² or chemotherapy⁴³. Despite these promising preclinical findings, the use of CEC as a pharmaco-dynamic marker in clinical studies is still limited^{66,67} given the lack of a standardized assay and lack of consensus on the exact phenotype.

Identification of antigens specific for malignant angiogenesis and new targets for anti-angiogenic therapies

It is important to realize that the elevated CEC numbers found in cancer patients are likely a composite of CEC from normal vasculature and from tumor vasculature. It is not clear whether or not the elevated CEC numbers in cancer patients are a result from the accelerated endothelial turnover of tumor vessels, or from a more generalized systemic endothelial damage and/or activation. There is a high need for assays enabling to discriminate CEC originating from normal vasculature from those originating from tumor vessels. Several studies have been conducted to reveal distinctive gene expression profiles of tumor-associated endothelial cells from those of endothelial cells from “normal” tissues, and have attempted to identify markers in this respect. Examination of these tumor-associated endothelial markers (TEM) on CEC can lead to a more appropriate monitoring of follow up and response to treatment and may result in the identification of antigens specific for malignant angiogenesis.

At gene expression level numerous TEM could be detected with either serial analysis of gene expression (SAGE)⁶⁸⁻⁷⁰, selection with in vitro phage display libraries⁷¹ or fluorescence in situ hybridization (FISH)⁷². Several of these TEM were also studied for their surface expression in tumor tissue^{68,69,73} showing increased expression in tumor endothelial cells compared with normal endothelium and with a high specificity. One of these markers, CD276 (B7-H3), has already been reported as predictive for therapeutic outcome when expressed on ovarian tumor endothelium⁷⁴.

In spite of the specificity of several TEM to discriminate between normal and tumor endothelium, cautious evaluation of these TEM for their tissue/cell specificity and biological function should be warranted prior to developing these TEM as therapeutic targets clinically⁷⁵.

Another interesting area of research of TEM will be their detection on CEC to identify the subpopulation originating from tumor vasculature, which will give a more specific marker to establish prognosis and response to treatment, than counting all CEC. In this respect, promising results have recently been reported by Matsusaka and colleagues who showed that the proportion of CXCR4-positive CEC at baseline were correlated with prognosis of breast cancer patients treated with bevacizumab⁷⁶.

Conclusions

The central role of the tumor vasculature in the biology of tumors is unquestionable and new biomarkers that accurately reflect drug effects that impact on tumor vasculature are urgently needed to better design clinical observational studies and trials, and eventually improved individualization of cancer treatment. The serial counting of CEC appears an attractive candidate to meet this demand. Current studies have yielded promising but still inconsistent results; this situation may be caused by differences between patient populations, and differences between enumeration techniques in the various studies. However, recent publications now tend to consensus regarding the phenotype and frequency of CEC in healthy individuals. CEC are increased in patients with different types of malignancies and there is a growing body of evidence that this cell population may evolve as suitable biomarkers for antitumor therapies. The identification of new tumor-associated endothelial markers (TEM) will likely advance monitoring of follow up and response to treatment, and may result in the identification of antigens specific for malignant angiogenesis.

Table 1. Immunophenotype of mature endothelial cells

CD Antigen	Description	EC Subtype Association	Expression Level	Co-Expression	Ref
CD31	PECAM-1	Pan-endothelial	++	Platelets, Leukocytes	[77] [46]
CD34	Stem cell Marker	Pan-endothelial	++	Stem cells	[46] [78] [79] [22] [48]
CD36	CR1	Micro vascular	+	Platelets, Erythrocytes, Monocytes, Dendritic	[80]
CD45	Leukocyte Marker	-	Neg	-	[46] [78] [79] [22] [48]
CD54	ICAM-1	Inflammation	+	Lymphocytes, Monocytes	[46] [81]
CD62-E	E-selectin	Inflammation	++	-	[81]
CD62-P	P-selectin	Inflammation	+	Platelets	[82]
CD90	Thy-1	EPC	Neg	Hematopoietic stem cells	[81]
CD105	Endoglin	Malignant (over expression)	++	Hematopoietic stem cells, Monocyte subset	[46] [26]
CD106	VCAM-1	Inflammation	+	Dendritic cell subset	[81]
CD109	Malignant	Malignant		Hematopoietic stem cells	[68]
CD117	Stem cell Marker	EPC	Neg	Hematopoietic stem cells	[46] [22]
CD133	Stem cell Marker	EPC	Neg	Hematopoietic stem cells	[46] [22] [48]
CD137	ILA/4	Malignant	+	Activated T cells, Monocytes	[68]
CD144	VE-Cadherin	Endothelial specific	++	-	[46] [22] [32]
CD146	MeICAM	Pan-endothelial	++	T cell subset, Pericytes	[46] [78] [79] [22] [26]
CD202b	Tie-2	Angiogenesis	(+)	-	[32]
CD276	B&-H3	Malignant	+	Dendritic cells, Monocytes	[68]
CD309	VEGFR-2	Angiogenesis	(+)	Stem cells	[46] [78] [22] [26] [48]
NA	vWF	Pan-endothelial	++	Platelets	[78] [79] [22] [34]
NA	UEA-1 uptake	Endothelial specific	++	-	[78] [34]
NA	DNA	Pan-endothelial		Nucleated cells	[28] [26] [29]
NA	Ki67	Proliferation		Proliferating cells	[83] [84]

Abbreviations: PECAM-1 = platelet/endothelial cell adhesion molecule 1, CRI = collagen receptor I, ICAM-1 = intracellular adhesion molecule 1, Thy-1 = Thymocyte differentiation antigen 1, VCAM-1 = vascular cellular adhesion molecule 1, ILA/4 = inducible by lymphocyte activation/4, MeICAM = melanoma-associated cellular adhesion molecule, Tie-2 = angiopoietin-1, 2, 4 receptor, B7-H3 = B7 homologue 3, VEGFR-2 = vascular endothelial growth factor receptor 2, vWF = von Willebrand Factor, UEA-1 = Ulex europaeus agglutinin-1, DNA = Deoxyribonucleic acid, Ki67 = antigen encoded by the *MKI67* gene. Expression levels: ++ = strong, + = moderate, (+) = weak.

Table 2. A summary of the current literature reporting CEC levels at baseline in relation to outcome

Tumor Type	Treatment		Patients (n)	Enumeration Method; Phenotype	Blood volume used	Baseline CEC/ml		Ref.
	Anti-angiogenic	Cytotoxic				Healthy controls (median)	Patients (median)	
Colorectal	Bevacizumab	5-FU-based	435	CellSearch	4 mL	4	6.8	Not prognostic for PFS or OS [56]
	Bevacizumab	5-FU-based	33	CellSearch	4 mL	4	NA	CEC <65: increase of PFS and OS [54]
	Bevacizumab	5-FU-based	40	FCM; CD45-, CD146+, CD34+	0.1 ml	20	30	CEC <40: increase of PFS and OS [55]
	Bevacizumab	5-FU-based	97	FCM; CD45-, CD146+, CD31+, 7-AAD-	1 mL	6.5	16	CEC <23: increase of short PFS, not for OS [53]
	Bevacizumab	5-FU-based	46	FCM; CD45-, CD146+, CD34+	0.1 mL	1300	3000 7000	- No Clinical response - Clinical response [58]
Breast	Bevacizumab	5-FU-based	46	FCM; CD45-, CD146+, CD34+	0.1 mL	1300	3500	CEC >2200: Increase of PFS [57]
	Bevacizumab	5-FU-based	67	CellSearch	4 mL	15	17	Not prognostic for PFS [59]
	No	Anthracycline and/or taxane based	35	CellSearch	4 mL	4	NA	Low CEC associated with pCR [85]
Renal	Sunitinib or Sorafenib	No	55	FCM; CD45-, CD146+, CD31+, 7-AAD-	1 mL	6.5	13	Not prognostic for PFS or OS [41]
CRPC	No	Docetaxel	162	CellSearch	4 mL	4	19 (mean)	Not prognostic for OS [27]
NSCLC	No	Carboplatin Pacitaxel	31	CellSearch	4 mL	13 (mean)	595 (mean)	CEC >400: Increase of PFS [63]
Pancreatic	Bevacizumab, Erlotinib	No	36	FCM; CD45-, CD31+, CD34+	NA	NA	11900	Low CEC associated with increase of OS [40]

Abbreviations:

CRPC = Castration resistant prostate cancer, 5-FU = 5-fluorouracil, FCM = flow cytometry, NA = not available, NSCLC = Non-small-cell lung carcinoma, OS = Overall survival, pCR = pathological complete response, PFS = Progression free survival.

Table 3. A summary of the current literature reporting chancing in CEC during treatment in relation to clinical outcome

Tumor Type	Treatment		Patients (n)	Enumeration Method; Phenotype	Blood volume used	Baseline CEC/ml		CEC number Change from baseline and clinical outcome	Ref.
	Anti-angiogenic	Cytotoxic				Healthy controls (median)	Patients (median)		
Colorectal	Bevacizumab	5-FU, EBRT	32	FCM; CD45-, CD31+, CD34+, CD133-	100-150.000 PBMC	0,1-6% (of PBMC)	1,02% (of PBMC)	Increased in patients with residual disease	[38]
	Bevacizumab	5-FU-based	435	CellSearch	4 mL	4	6.8	Not prognostic for PFS or OS	[56]
	Bevacizumab	5-FU-based	97	FCM; CD45-, CD146+, CD31+, 7-AAD-	1 mL	6,5	16	Not prognostic for PFS or OS	[53]
	Bevacizumab	5-FU-based	67	CellSearch	4 mL	15	17	Increase (> 5): associated with improved TtP	[59]
Breast	Thalidomide	Cyclophosphamide, methotrexate	104	FCM; CD45-, CD146+, CD31+	0,1 mL	1300	NA	Increase (>11000) at day 60 is associated with longer PFS and improved OS	[43]
	Sunitinib	No	26	IMS (CD146); UEA-1+	1 mL	8 (mean)	49 (mean)	Early increase is associated with an improved PFS	[60]
Renal	Sunitinib or Sorafenib	No	55	FCM; CD45-, CD146+, CD31+, 7-AAD-	1 mL	6,5	13	Not prognostic for PFS or OS	[41]
	No	Docetaxel	162	CellSearch	4 mL	4	19 (mean)	Increase (>3.8* baseline) is associated with shorter OS	[27]
Glioblastoma	Cediranib	No	16	FCM; CD45-, CD31+, CD34+, CD133-	100-150.000 PBMC	NA	NA	Increased at tumor progression	[62]
GIST	sunitinib	No	73	FCM; CD45-, CD146+, CD31+, CD133-	NA (cyopreserve d PBMC)	540	1090	Increased in patients with clinical benefit	[61]

Abbreviations: 5-FU = 5-fluorouracil, CRPC = Castration resistant prostate cancer, EBRT = external beam radiation therapy, FCM = flow cytometry, IMS = Immunomagnetic isolation, GIST = gastrointestinal stromal tumor, NA = not available, OS = Overall survival, PBMC = peripheral blood mononuclear cells, PFS = Progression free survival, TtP = Time to Progression.

References

1. Naumov GN, Bender E, Zurakowski D, et al. A model of human tumor dormancy: an angiogenic switch from the nonangiogenic phenotype. *J Natl Cancer Inst.* 2006;98:316-325.
2. Srijbos MH, Gratama JW, Kraan J, Lamers CH, den Bakker MA, Sleijfer S. Circulating endothelial cells in oncology: pitfalls and promises. *Br J Cancer.* 2008;98:1731-1735.
3. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature.* 2011;473:298-307.
4. Nagy JA, Chang SH, Shih SC, Dvorak AM, Dvorak HF. Heterogeneity of the tumor vasculature. *Semin Thromb Hemost.* 2010;36:321-331.
5. Hu-Lowe DD, Chen E, Zhang L, et al. Targeting activin receptor-like kinase 1 inhibits angiogenesis and tumorigenesis through a mechanism of action complementary to anti-VEGF therapies. *Cancer Res.* 2011;71:1362-1373.
6. Siemann DW, Bibby MC, Dark GG, et al. Differentiation and definition of vascular-targeted therapies. *Clin Cancer Res.* 2005;11:416-420.
7. Bertolini F, Mancuso P, Shaked Y, Kerbel RS. Molecular and cellular biomarkers for angiogenesis in clinical oncology. *Drug Discov Today.* 2007;12:806-812.
8. Jain RK, Duda DG, Willett CG, et al. Biomarkers of response and resistance to antiangiogenic therapy. *Nat Rev Clin Oncol.* 2009;6:327-338.
9. Longo R, Gasparini G. Anti-VEGF therapy: the search for clinical biomarkers. *Expert Rev Mol Diagn.* 2008;8:301-314.
10. Bonello L, Basire A, Sabatier F, Paganelli F, Dignat-George F. Endothelial injury induced by coronary angioplasty triggers mobilization of endothelial progenitor cells in patients with stable coronary artery disease. *J Thromb Haemost.* 2006;4:979-981.
11. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood.* 1999;93:2951-2958.
12. George F, Brouqui P, Boffa MC, et al. Demonstration of Rickettsia conorii-induced endothelial injury in vivo by measuring circulating endothelial cells, thrombomodulin, and von Willebrand factor in patients with Mediterranean spotted fever. *Blood.* 1993;82:2109-2116.
13. Mutunga M, Fulton B, Bullock R, et al. Circulating endothelial cells in patients with septic shock. *Am J Respir Crit Care Med.* 2001;163:195-200.
14. Percivalle E, Revello MG, Vago L, Morini F, Gerna G. Circulating endothelial giant cells permissive for human cytomegalovirus (HCMV) are detected in disseminated HCMV infections with organ involvement. *J Clin Invest.* 1993;92:663-670.
15. Camoin-Jau L, Kone-Paut I, Chabrol B, Sampol J, Dignat-George F. Circulating endothelial cells in Behcet's disease with cerebral thrombophlebitis. *Thromb Haemost.* 2000;83:631-632.
16. Clancy R, Marder G, Martin V, Belmont HM, Abramson SB, Buyon J. Circulating activated endothelial cells in systemic lupus erythematosus: further evidence for diffuse vasculopathy. *Arthritis Rheum.* 2001;44:1203-1208.
17. Bull TM, Golpon H, Hebbel RP, et al. Circulating endothelial cells in pulmonary hypertension. *Thromb Haemost.* 2003;90:698-703.
18. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. *N Engl J Med.* 1997;337:1584-1590.

19. Strijbos MH, Landburg PP, Nur E, et al. Circulating endothelial cells: a potential parameter of organ damage in sickle cell anemia? *Blood Cells Mol Dis.* 2009;43:63-67.
20. Woywodt A, Scheer J, Hambach L, et al. Circulating endothelial cells as a marker of endothelial damage in allogeneic hematopoietic stem cell transplantation. *Blood.* 2004;103:3603-3605.
21. Woywodt A, Schroeder M, Gwinner W, et al. Elevated numbers of circulating endothelial cells in renal transplant recipients. *Transplantation.* 2003;76:1-4.
22. Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat Rev Cancer.* 2006;6:835-845.
23. Furstenberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer.* 2006;94:524-531.
24. Goon PK, Boos CJ, Lip GY. Circulating endothelial cells: markers of vascular dysfunction. *Clin Lab.* 2005;51:531-538.
25. Goon PK, Boos CJ, Stonelake PS, Blann AD, Lip GY. Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. *Thromb Haemost.* 2006;96:45-52.
26. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A.* 2007;71:105-113.
27. Strijbos MH, Gratama JW, Schmitz PI, et al. Circulating endothelial cells, circulating tumour cells, tissue factor, endothelin-1 and overall survival in prostate cancer patients treated with docetaxel. *Eur J Cancer.* 2010;46:2027-2035.
28. Strijbos MH, Kraan J, den Bakker MA, Lambrecht BN, Sleijfer S, Gratama JW. Cells meeting our immunophenotypic criteria of endothelial cells are large platelets. *Cytometry B Clin Cytom.* 2007;72:86-93.
29. Mancuso P, Antoniotti P, Quarna J, et al. Validation of a standardized method for enumerating circulating endothelial cells and progenitors: flow cytometry and molecular and ultrastructural analyses. *Clin Cancer Res.* 2009;15:267-273.
30. Widemann A, Sabatier F, Arnaud L, et al. CD146-based immunomagnetic enrichment followed by multiparameter flow cytometry: a new approach to counting circulating endothelial cells. *J Thromb Haemost.* 2008;8:869-876.
31. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood.* 2001;97:3658-3661.
32. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LW, O'Hara SM. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. *Cancer Res.* 2006;66:2918-2922.
33. Dignat-George F, Sabatier F, Blann A, Woywodt A. Detection of circulating endothelial cells: CD146-based magnetic separation enrichment or flow cytometric assay? *J Clin Oncol.* 2007;25:e1-2; author reply e3-5.
34. Woywodt A, Blann AD, Kirsch T, et al. Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol. *J Thromb Haemost.* 2006;4:671-677.
35. Goon PK, Boos CJ, Stonelake PS, Lip GY. Circulating endothelial cells in malignant disease. *Future Oncol.* 2005;1:813-820.
36. Vroiling L, Yuana Y, Schuurhuis GJ, et al. VEGFR2 expressing circulating (progenitor) cell populations in volunteers and cancer patients. *Thromb Haemost.* 2007;98:440-450.
37. Jacques N, Vimond N, Conforti R, et al. Quantification of circulating mature endothelial cells using a whole blood four-color flow cytometric assay. *J Immunol Methods.* 2008;337:132-143.

38. Willett CG, Duda DG, di Tomaso E, et al. Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: a multidisciplinary phase II study. *J Clin Oncol*. 2009;27:3020-3026.
39. Torrisi R, Bagnardi V, Cardillo A, et al. Preoperative bevacizumab combined with letrozole and chemotherapy in locally advanced ER- and/or PgR-positive breast cancer: clinical and biological activity. *Br J Cancer*. 2008;99:1564-1571.
40. Ko AH, Venook AP, Bergsland EK, et al. A phase II study of bevacizumab plus erlotinib for gemcitabine-refractory metastatic pancreatic cancer. *Cancer Chemother Pharmacol*. 2010;66:1051-1057.
41. Farace F, Gross-Goupil M, Tournay E, et al. Levels of circulating CD45(dim)CD34(+)VEGFR2(+) progenitor cells correlate with outcome in metastatic renal cell carcinoma patients treated with tyrosine kinase inhibitors. *Br J Cancer*. 2011;104:1144-1150.
42. Rabascio C, Muratori E, Mancuso P, et al. Assessing tumor angiogenesis: increased circulating VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. *Cancer Res*. 2004;64:4373-4377.
43. Mancuso P, Colleoni M, Calleri A, et al. Circulating endothelial-cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. *Blood*. 2006;108:452-459.
44. Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest*. 2000;105:71-77.
45. Peters BA, Diaz LA, Polyak K, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. *Nat Med*. 2005;11:261-262.
46. Timmermans F, Plum J, Yoder MC, Ingram DA, Vandekerckhove B, Case J. Endothelial progenitor cells: identity defined? *J Cell Mol Med*. 2009;13:87-102.
47. Dome B, Timar J, Ladanyi A, et al. Circulating endothelial cells, bone marrow-derived endothelial progenitor cells and proangiogenic hematopoietic cells in cancer: From biology to therapy. *Crit Rev Oncol Hematol*. 2009;69:108-124.
48. Ingram DA, Mead LE, Tanaka H, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood*. 2004;104:2752-2760.
49. Case J, Mead LE, Bessler WK, et al. Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Exp Hematol*. 2007;35:1109-1118.
50. Roodhart JM, Langenberg MH, Vermaat JS, et al. Late release of circulating endothelial cells and endothelial progenitor cells after chemotherapy predicts response and survival in cancer patients. *Neoplasia*. 2010;12:87-94.
51. Bhatt RS, Zurita AJ, O'Neill A, et al. Increased mobilisation of circulating endothelial progenitors in von Hippel-Lindau disease and renal cell carcinoma. *Br J Cancer*. 2011;105:112-117.
52. Beaudry P, Force J, Naumov GN, et al. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. *Clin Cancer Res*. 2005;11:3514-3522.
53. Malka D, Boige V, Jacques N, et al. Clinical value of circulating endothelial cell levels in metastatic colorectal cancer patients treated with first-line chemotherapy and bevacizumab. *Ann Oncol*. 2011.
54. Matsusaka S, Suenaga M, Mishima Y, et al. Circulating endothelial cells predict for response to bevacizumab-based chemotherapy in metastatic colorectal cancer. *Cancer Chemother Pharmacol*. 2010.
55. Ronzoni M, Manzoni M, Mariucci S, et al. Circulating endothelial cells and endothelial progenitors as predictive markers of clinical response to bevacizumab-based first-line treatment in advanced colorectal cancer patients. *Ann Oncol*. 2010;21:2382-2389.

56. 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;21:2447-2448.
57. Calleri A, Bono A, Bagnardi V, et al. Predictive Potential of Angiogenic Growth Factors and Circulating Endothelial Cells in Breast Cancer Patients Receiving Metronomic Chemotherapy Plus Bevacizumab. *Clin Cancer Res.* 2009;15:7652-7657.
58. Dellapasqua S, Bertolini F, Bagnardi V, et al. Metronomic cyclophosphamide and capecitabine combined with bevacizumab in advanced breast cancer. *J Clin Oncol.* 2008;26:4899-4905.
59. Bidard FC, Mathiot C, Degeorges A, 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;21:1765-1771.
60. Gruenewald V, Beutel G, Schuch-Jantsch S, et al. Circulating endothelial cells are an early predictor in renal cell carcinoma for tumor response to sunitinib. *BMC Cancer.* 2010;10:695.
61. Norden-Zfoni A, Desai J, Manola J, et al. Blood-based biomarkers of SU11248 activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor. *Clin Cancer Res.* 2007;13:2643-2650.
62. Batchelor TT, Sorensen AG, di Tomaso E, et al. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell.* 2007;11:83-95.
63. Kawaiishi M, Fujiwara Y, Fukui T, et al. Circulating endothelial cells in non-small cell lung cancer patients treated with carboplatin and paclitaxel. *J Thorac Oncol.* 2009;4:208-213.
64. Ning YM, Gulley JL, Arlen PM, et al. Phase II trial of bevacizumab, thalidomide, docetaxel, and prednisone in patients with metastatic castration-resistant prostate cancer. *J Clin Oncol.* 2010;28:2070-2076.
65. Vroiling L, van der Veldt AA, de Haas RR, et al. Increased numbers of small circulating endothelial cells in renal cell cancer patients treated with sunitinib. *Angiogenesis.* 2009;12:69-79.
66. Yamada K, Yamamoto N, Yamada Y, et al. Phase I dose-escalation study and biomarker analysis of E7080 in patients with advanced solid tumors. *Clin Cancer Res.* 2011;17:2528-2537.
67. Beerepoot LV, Radema SA, Witteveen EO, et al. Phase I clinical evaluation of weekly administration of the novel vascular-targeting agent, ZD6126, in patients with solid tumors. *J Clin Oncol.* 2006;24:1491-1498.
68. Seaman S, Stevens J, Yang MY, Logsdon D, Graff-Cherry C, St Croix B. Genes that distinguish physiological and pathological angiogenesis. *Cancer Cell.* 2007;11:539-554.
69. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. *Science.* 2000;289:1197-1202.
70. Nanda A, Carson-Walter EB, Seaman S, et al. TEM8 interacts with the cleaved C5 domain of collagen alpha 3(VI). *Cancer Res.* 2004;64:817-820.
71. Mutuberria R, Satijn S, Huijbers A, et al. Isolation of human antibodies to tumor-associated endothelial cell markers by in vitro human endothelial cell selection with phage display libraries. *J Immunol Methods.* 2004;287:31-47.
72. Hida K, Hida Y, Amin DN, et al. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res.* 2004;64:8249-8255.
73. Mura M, Swain RK, Zhuang X, et al. Identification and angiogenic role of the novel tumor endothelial marker CLEC14A. *Oncogene.* 2011.
74. Zang X, Sullivan PS, Soslow RA, et al. Tumor associated endothelial expression of B7-H3 predicts survival in ovarian carcinomas. *Mod Pathol.* 2010;23:1104-1112.

75. Li JL, Harris AL. The potential of new tumor endothelium-specific markers for the development of antivascular therapy. *Cancer Cell*. 2007;11:478-481.
76. Matsusaka S, Mishima Y, Suenaga M, et al. Circulating endothelial progenitors and CXCR4-positive circulating endothelial cells are predictive markers for bevacizumab. *Cancer*. 2011;117:4026-4032.

Chapter 6

A new approach for rapid and reliable enumeration of circulating endothelial cells in patients

Jaco Kraan, Michiel H. Strijbos, Petra van der Spoel,
Anieta M. Sieuwerts, John A. Foekens, Michael A. den Bakker,
Cornelis Verhoef, Stefan Sleijfer, Jan W. Gratama

Journal of Thrombosis and Haemostasis 2012; 10:931 - 939

Abstract

Background: Mature circulating endothelial cells (CEC) are surrogate markers of endothelial damage/dysfunction. A lack of standardized assays and consensus on CEC phenotype has resulted in a wide variation of reported CEC numbers (4 to 1,300 per mL).

Objectives: Given the need for a quick, reliable, robust and validated CEC assay at an affordable price, we present a novel approach to enumerate CEC using a multi-parameter flow cytometrical (FCM) method without immunological pre-enrichment.

Methods: CEC were defined as CD34⁺, CD45^{neg}, CD146⁺ and DNA⁺ events based on the immunophenotype of endothelial cells from vein-wall dissections. As CEC express high levels of CD34, we based our assay on absolute CD34 counts after analyzing all CD34 positive events in a total blood volume of 4mL. Endothelial origin of CEC was confirmed by morphology, immunohistochemistry and gene expression. Additionally, our FCM assay was tested in parallel with an already validated assay (i.e., CellSearch®, Veridex [Raritan, NJ]). CEC levels ranged from 4 to 79 CEC/mL in healthy individuals, were significantly higher in carcinoma patients ($P=0.0008$) and in patients with hematological malignancies ($P<0.0001$).

Conclusions: This flow cytometric method should be useful as an economical high-throughput assay to enumerate and characterize CEC.

Introduction

Current opinion states that circulating endothelial cells (CEC) are mature endothelial cells shed from the vessel wall as a result of vascular damage. As numerous diseases are associated with vascular damage, the enumeration of CEC has for long been considered a promising tool to monitor disease activity with a potential to assess prognosis and response to treatment. Elevated CEC numbers have been found in multiple diseases, including cardiovascular disorders¹⁻², infectious diseases³⁻⁵, immune disorders⁶⁻⁷, pulmonary hypertension⁸, sickle cell anaemia⁹⁻¹⁰, status after organ transplantation¹¹⁻¹² and cancer¹³⁻¹⁸.

In recent years, several methods have been described to enumerate CEC. However, there is no consensus between CEC phenotypes used in these enumeration techniques. Importantly, most methods have not been properly validated¹⁹. In combination with the relative rarity of CEC, this situation has resulted in a wide variation of reported CEC numbers, hindering further progress in the field. The great variation in results is exemplified by the reported CEC numbers in healthy humans ranging from 4 to 1,300 cells/mL^{14,16, 20-21}.

Currently, most CEC enumeration tests rely on an enrichment step by immunomagnetic bead isolation (IB) using magnetic particles coupled to monoclonal antibody (mAb) targeting an endothelial antigen such as CD146, followed by flow cytometry or visual counting with a fluorescent microscope to identify CEC on the basis of morphological or immunophenotypical criteria²¹⁻²². A major advantage of this latter approach is that it permits visual identification of CEC, which allows discrimination between CEC and endothelial microparticles or platelets with a strongly overlapping phenotype. However, any enrichment step inevitably leads to cell loss and underestimation of the actual CEC number, whilst manual bead-based isolation procedures are labor intensive and difficult to standardize.

A variant of manual IB enrichment techniques is the CellSearch® system (Veridex, Raritan, NJ). Initially designed to detect circulating tumor cells, the system provides a fully automated enrichment procedure that is followed by semi-automated image cytometry. This assay has a high recovery and good reproducibility. Importantly, CEC isolated in this way have been validated by global gene expression^{17,23}. Drawbacks of the CellSearch® system are the costly equipment and reagents, and the assay cannot be customized. Also, the maximum number of eight samples that can be analyzed in a single run, combined with the relatively long duration of a complete run (approximately 4 h) does not allow high throughput analysis.

Given the need for a quick, reliable, robust and validated CEC assay that is also affordable, we present a novel approach for enumerating CEC using a multi-parameter flow cytometrical method (FCM) *without immunological pre-enrichment*.

The assay is based on absolute CD34 counts after analysis of all CD34 positive events in a total blood volume of 4 mL using a “live gate” on CD34+ events to exclude most of the cells that are not of interest from the analysis, thus allowing the assessment of CEC as CD34⁺, CD45^{neg}, CD146⁺ and DNA⁺ events. The selected markers were tested for CEC specificity on endothelial cells (EC) from vein-wall dissections and the endothelial origin of the cells designated as CEC was confirmed by morphology, immunohistochemistry (IHC) and expression of genes thought to occur specifically in CEC. Additionally, the agreement with another validated assay (CellSearch® system)²⁴ was tested in healthy subjects and patients with pathologies known to be associated with high CEC levels.

Materials and methods

Patients and blood collection

Peripheral blood (PB) was collected using EDTA-containing or CellSave™ tubes (Veridex) from healthy donors (n = 30), carcinoma patients (n = 55) and patients with hematological malignancies in remission (n = 60). All patients provided written informed consent and the study protocols were approved by the local research and ethics committee.

CEC isolation from dissected vein walls

Based on literature²⁴, we defined CEC as CD34⁺, CD45^{neg}, CD146⁺ and DNA⁺ events. To confirm the CEC specificity of the combination of CD34 positivity, CD146 positivity and CD45 negativity, endothelial cells were isolated from dissections of vein walls using collagenase digestion. Vessel dissections (2-3 cm) were opened with sterile scissors, the opened vessel was placed in a petri dish with its endothelial side on a sterile cellulose tissue saturated with collagenase solution (Collagenase Type IV [Sigma C-5138], 2 mg/mL in RPMI). After incubation for 60 min at 37°C, the vessel was placed in a petri dish containing RPMI after which cells from the endothelial side of the vessel were removed using a cell scraper. After the remainder of the vessel had been discarded, the content of the Petri dish was transported to a 50 mL conical tube and the cell suspension centrifuged at 1000 x g for 5 min. The resulting pellet was washed with 50 mL phosphate buffered saline(PBS) and concentrated at 1x10⁶ cells/mL in PBS for flow cytometry.

CD34+ cell-based FCM assay

For CEC analysis, samples were prepared in a lyse-stain wash procedure with minimal sample handling. Four mL of PB were transferred into a 50 mL tube and

45 mL of ammoniumchloride 0.15M was added to induce red-cell lysis. After 20 min of lysis at room temperature (RT), the suspension was centrifuged for 5 min at 1000 x g and the supernatant was removed from the sample tube without disturbing the pellet. To reduce Fc receptor-mediated antibody binding, 50 μ L of CD32 mAb (clone IV3, Stemcell Technologies, Vancouver; 10 μ g/mL in PBS containing 1% bovine serum albumin) was added and the pellet was carefully homogenized using a 100 μ L pipette.

After 10 min of incubation, cells were stained using 50 μ L of a mixture of the following monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC) conjugated CD34 (clone 8G12, BD Biosciences, San Jose, CA); R-phycoerythrin (PE) conjugated CD105 (clone 1G2, IOTest, Marseille France); peridinin chlorophyll protein (PerCP) conjugated CD45 (clone 2D1, BD Biosciences); allophycocyanin (APC) conjugated CD146 (clone 541-10B2, Miltenyi Biotec), and 50 μ L of the DNA dye 5-bis[2-(di-methylamino) ethyl]amino-4, 8-dihydroxyanthracene-9,10-dione (DRAQ5, BioStatus Ltd, Leicestershire, UK). All reagents were diluted in PBS/BSA based on titration, i.e., absence of non-specific staining on negative populations and optimal discriminatory power between negative and positive populations. After 15 min of incubation in darkness at room temperature(RT), 45 mL of PBS were added and the suspension was centrifuged for 5 min at 1000 x g. After removal of supernatant the cells were resuspended in 500 μ L of PBS and transferred to a standard 5 mL flow cytometry tube. The 50 mL tube was rinsed with 500 μ L of PBS, and any remaining cells were also transferred to the cytometry tube to assure optimal recovery of cells. Samples were immediately acquired on a FACSCanto II flow cytometer with FACSDiva v6.1. software (BD Biosciences) or stored on ice for a maximum of 1 h. Data acquisition was started by collecting ungated data of 50,000 nucleated cells (DRAQ5⁺) at a low flow rate (10 μ L/min) (**Fig 1, panel A-F**). Data acquisition was completed by acquiring the remainder of the sample at a high flow rate (120 μ L/min) with a threshold (live-gate) on CD34⁺ events (**Fig 1, panel G-I**).

Absolute cell counting

Absolute counts were obtained by multiplying the CEC percentage within the CD34⁺ population in 4 mL of blood by the absolute CD34 counts obtained on the same blood sample using a single platform FCM assay according to ISHAGE guidelines²⁵⁻²⁶. In brief, 200 μ L of blood was incubated with CD34-FITC (clone 8G12), CD45-PErCP (clone 2D1) and DRAQ5. After 15 min incubation, 2 mL ammonium chloride lysing solution was added, followed by 100 μ L Flow-Count beads. A minimum of 100 CD34⁺ events were acquired on a FASCCanto II flow cytometer (**Fig 1, panel A-F**).

Methods used for assay validation

Cell Sorting

Cells were sorted using a FACSria Cell Sorting System (BD Biosciences) equipped with FACSDiva v6.1. software (BD Biosciences). For morphological and immunohistochemical analysis, cells were sorted on glass slides as spots of 50 cells. For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).studies, cells were sorted in 2 mL Eppendorf tubes, lysed by adding 250 μ L of Qiagen AllPrep DNA/RNA Micro Kit Lysis Buffer (RLT+ lysis buffer) (Qiagen BV, Venlo, The Netherlands) and stored immediately at -80°C until RNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's guidelines.

Morphology

May-Grünwald-Giemsa (MGG) staining was performed using a standard eosin-methylene blue solution and Giemsa solution with an Autostainer XL (Leica Microsystems GmbH, Wetzlar, Germany). All images presented in this study were acquired on a DM2500 microscope equipped with a DC500 digital camera (both from Leica). The acquisition software (Leica IM1000 version 4.0) adjusted focus, brightness and contrast automatically.

Immunohistochemistry

Two-step horseradish peroxidase (HRP)-based immunohistochemistry (IHC) was performed on sorted endothelial cells and lymphocytes using the ChemMate Envision Detection Kit (Dako, Glostrup, DK). Sorted populations were fixed in buffered formalin and stained using mouse-anti-human antibodies against CD31 and von Willebrand Factor (both from Dako). All Immunohistochemical stainings were evaluated by a pathologist.

qRT-PCR

The generation of pre-amplified cDNA from RNA and subsequent Taqman-based qRT-PCR analysis, as well as the validation procedures to ensure homogeneous amplification, were performed as described previously²⁷. RT samples were diluted in nuclease-free ddH₂O and analyzed by real-time PCR in a 20 μ L reaction volume in a Mx3000PTM Real-Time PCR System (Stratagene, Amsterdam, The Netherlands). Negative controls included samples without reverse transcriptase and samples in which RNA and cDNA had been replaced with PCR-grade H₂O or genomic DNA. Quantitative values were obtained from the threshold cycle (Ct) at which the increase in TaqMan probe fluorescent signal associated with an exponential increase of PCR products reached the fixed threshold value of 0.08; In all cases, this was at least ten times the standard deviation of the background signal.

Levels of *HMBS*, *HPRT1* and *GUSB* were the reference genes used to control sample loading and RNA quality with Taqman assays-on-demand from Applied BioSystems (ABI, Nieuwerkerk a/d IJssel, The Netherlands) as described

previously²⁷. Protein tyrosine phosphatase receptor type C (*PTPRC* coding for CD45, ABI: Hs00236304_m1) was the control gene for leukocyte background and *CDH5* (coding for CD144, ABI: Hs00174344_m1), *MCAM* (coding for CD146, ABI: Hs00174838_m1), *VEGFR2* (ABI: Hs00176676_m1) and *VWF* (ABI: Hs00169795_m1) the controls for CEC detection. The delta Ct method was used to evaluate gene transcript levels relative to the reference genes.

CellSearch® Circulating Endothelial Cell assay

CEC were enumerated using the CellSearch® Endothelial Cell Kit and CellTracks® AutoPrep® System (Veridex) according to the standard procedure¹⁷. In brief, a blood sample of 4 mL was mixed with ferrofluid particles coated with anti-CD146 antibodies. After incubation, unlabeled cells and plasma were removed by magnetic separation. Subsequently, the isolated cells were stained with fluorescent monoclonal antibodies for endothelial cells (CD105-PE) and contaminating leucocytes (CD45-APC), and a nuclear staining dye (DAPI). During this sample processing procedure, 4 mL of blood is reduced to ~ 700 μ L containing enriched CEC, which is next transferred to a cartridge in a MagneSt® device. Here, the strong magnetic field moves immunomagnetically labeled cells to the surface of the cartridge caused by the strong magnetic field in order to render them ready for analysis. All steps were performed fully automatically by the CellTracks® AutoPrep® System. The samples were read with the CellTracks Analyzer®, a semi-automated fluorescence microscope used to scan the entire surface of the cartridge at four different wavelengths in order to detect objects labeled with all fluorochromes used.

Captured images that contain objects fulfilling predetermined criteria were automatically presented in a gallery format from which events were classified by a trained operator. A CD146⁺ cell was classified as a CEC on the basis of its morphologic features and staining patterns (i.e., DAPI positive, CD105 positive and CD45 negative).

Statistical analysis

Statistical analysis was performed using Prism™ software (GraphPad Software, La Jolla, CA). To analyze the degree of agreement between the CD34-based FCM assay and the CellSearch reference assay, Bland-Altman plots were generated to relate the inter-assay difference with the mean CEC count of the two methods. The Mann-Whitney U-test was used to compare healthy control with patient samples. $P < 0.05$ was considered to be significant.

Results

Immunophenotypic characteristics of EC isolated from vein dissections.

We validated our phenotypic criteria to analyze CEC by FCM on isolated endothelial cells (EC) from vein wall dissections (n=3). EC were isolated and stained with the same mAb cocktail as used in the assay. As illustrated in **Figure 2, panels A-C**, isolated EC showed a high expression of CD34 and CD146 and were negative for CD45 showing that isolated EC fulfill the phenotypic criteria for CEC (Figure 1) and can be selected by the Boolean gating strategy (DRAQ5+, CD34+, CD45-,CD146+) used in the FCM assay. Furthermore, we demonstrated that isolated EC are positive for several other endothelial markers such as the pan-endothelial markers CD31 and CD144, and the subtype-associated markers CD105, CD309 (VEGFR-2) and CD133 (**Figure 2, panels D-I**).

Validation using morphology, IHC and gene expression

First, CEC were sorted on glass slides and stained for MGG and by IHC the endothelial specific markers CD31 and vWF. More than 95% of all sorted events showed specific EC morphology and were brightly positive for vWF (**Figure 1, panel L**) and CD31 (not shown). In parallel lymphocytes and human hematopoietic stem cells (HSC) were sorted to serve as control cells. Lymphocytes were negative for vWF and partially weakly positive for CD31. HSC were negative for vWF and partly positive for CD31.

Second, sorted CEC were analyzed for mRNA expression by qRT-PCR of both endothelial specific genes and leukocyte specific genes in duplicate assays. This RNA profiling by qRT-PCR showed the presence of endothelial specific markers (*CDH5*, *MCAM*, *VWF*) and absence of an hematopoietic marker (*PTPRC* coding for CD45) in our CEC sorted preparations (**Table 1**). This result indicates an endothelial specific gene expression profile for the cells meeting the phenotypic criteria of the FCM assay.

Reproducibility of CEC measurements between duplicate samples

To measure the variability of CEC counts obtained from two 4 mL aliquots derived from a single blood draw, CEC were enumerated in duplicate using the blood in a single draw from 13 different samples. Regression analysis showed a best-fit line with a slope of 1.006 (95% confidence interval = 0.8702 to 1.141) an Y-intercept of 1.511 (95% confidence interval = -7.854 to 6.206) and an R^2 of 0,96 (**Figure 3**).

Agreement with CellSearch assay

We studied a total of 28 subjects (10 healthy controls and 18 patients with different metastatic carcinomas) for comparison of the two techniques. **Figure 4** shows the correlation between the number of events (red dots) and the obtained absolute number (blue dots) by the FCM method compared with the CellSearch reference assay. Regression analysis for the event count analysis (red dots) showed a best fit line with a slope of 0.66 (95% confidence interval = 0.61 to 0.71), an intercept of 4.16 (95% confidence interval = -1.95 to 10.26) and a R^2 of 0.96. Regression analysis for the CD34-based absolute count analysis (blue dots) showed a best fit line with a slope of 2.66 (95% confidence interval = 0.73 to 2.59), an intercept of 68.97 (95% confidence interval = -41.14 to 179.1) and a R^2 of 0.34. **Figure 5** shows the corresponding Bland-Altman plots. The median number of CEC in the FCM event count analysis was significantly lower compared to the CellSearch reference assay (median 20 versus 26, $P=0.0065$), but significantly higher when expressing CEC as absolute numbers by multiplying the CEC percentage within the CD34⁺ population in 4 mL of blood by the absolute CD34 counts obtained on the same blood sample (median 129 versus 26, $P=0.0002$). This suggests a slightly lower recovery for event counts as a result of limited cell loss during sample preparation and FCM dead volume but a significantly higher recovery when corrected for cell loss using the CD34⁺ cell-based FCM absolute number method.

CEC in healthy individuals, patients with metastatic carcinoma and hematological malignancies in remission.

Although the investigation of CEC levels in different types of cancer other than for validation purposes is beyond the scope of this paper, **Figure 6** shows that CEC levels in healthy individuals ranged from 4 to 79 cells/4 mL ($n = 30$, median 18) and were significantly higher in carcinoma patients (ranging from 5 to 354 ($n = 51$, median 35, $P < 0.005$)) and in patients with hematological malignancies (range: 4 to 653 ($n = 66$, median 37, $P < 0.0005$)).

Discussion

The aims of this study were to (a) develop a quick, reliable, robust and validated FCM method based on CD34 absolute counts without immunological pre-enrichment; (b) confirm the phenotypic criteria for analyzing CEC by FCM on isolated endothelial cells from vein wall dissections; (c) confirm the endothelial origin of all cells designated as CEC by morphology, immunohistochemistry and

gene expression; and (d) test the agreement between the novel FCM method with the validated Cell Search® assay.

We here describe a novel two-step approach using a standardized absolute CD34 count in whole blood²⁸ followed by analyzing all CD34-positive cells in a total of 4 mL blood to reliably count the CEC within this population without immunological pre-enrichment and minimal sample handling. After a mild erythrocyte lysing step, a single wash step and a detection threshold on CD34 positive events to remove most cells not of interest from the analysis, a complete sample could be run within 10 minutes on a flow cytometer without abort counts or impractically large data-files. In contrast to the enrichment procedures reported^{16,17,21} which inevitably leads to cell loss and underestimation of the actual CEC number, one of the advantages of this approach is that only a small degree of random cell loss will occur during the lyse/stain/wash procedure. Additionally, the absolute number of the CEC population can be precisely calculated on the basis of the total absolute CD34 count. Another advantage compared to conventional FCM assays is that we made it possible to overcome the reported obstacle of variability^{29,30} in (extremely) rare events by processing a larger sample volume (i.e. 4 mL instead of 50-200 μ L). A volume of more than 1 mL will be needed for a precise enumeration of CEC at a frequency of less than 1 cell per microliter blood to obtain a population of at least 100 cells fulfilling the flow cytometric criteria with a coefficient of variation of 10%²⁶.

Flow cytometric assays are at risk of overestimating rare events such as CEC by enumerating false positive events caused by non-specific antibody binding. Non-specific binding can be a result of Fc receptor binding, binding to dead cells, or improper use of reagents. In a lyse-stain-wash procedure, the blocking of Fc receptor binding and the use of a 'dump' channel is crucial because these binding sites are no longer saturated by the free plasma immunoglobulins³¹. Next to the interference by non-specific antibody binding, false positivity can also result from specific binding to soluble forms of the antigen. Many endothelial surface antigens are secreted, and their uptake by platelets aggregates can result in false-positive signals. An effective strategy for excluding platelets, aggregates and endothelial micro particles from analysis is by using a DNA specific stain, such as DAPI or DRAQ5. As DRAQ5 is a membrane permeant dye, no additional fixation and permeabilization is needed. Dyes also staining RNA such as PI or LDS751 will fail to exclude platelets by the relatively high expression of RNA by these cells¹⁹.

The difficulties in CEC enumeration is perhaps best illustrated by the enormous range of CEC numbers in healthy controls (4-1,300 cells/mL) in previous studies. This is most certainly a result of using different phenotypic definition of CEC. A phenotypic overlap between CEC and other cell populations may lead to an overestimation due to endothelial microparticles and platelets^{19,32}, whilst the use of markers that are not constantly expressed by all CEC can led to underestimation.

To overcome this problem we confirmed the phenotypic criteria, based on literature and previous experiments, in our assay on endothelial cells isolated from vein-wall dissections. We confirmed the bright expression of CD34³³ and CD146 on these cells as well as the expression of the endothelial associated markers CD31 and CD105 and the endothelial specific marker CD144³⁰. On the basis of these findings, the combination of the brightly expressed pan-endothelial markers CD34 and CD146, the negative selection marker CD45 and the DNA specific stain DRAQ5 to exclude microparticles and platelets was chosen for the immunophenotypic definition of CEC in our assay.

Clinical specimens were used to evaluate the accuracy of the CD34-based FCM for enumerating CECs. Our findings of significantly higher levels in patients with metastatic carcinomas confirm previous publications^{16,17,34}. We also found significantly elevated CEC levels in patients with haematological malignancies in remission and following myeloablative conditioning regimens prior to hematopoietic stem cell transplantation (data not shown). This suggests that these increased CEC counts are merely the result of therapy-induced damage to the vasculature than that they result from tumor growth or bone marrow reconstitution. An advantage of our flow cytometric approach is the possibility to study additional antigens within this assay format. Assessment of viability and activation markers of CEC may provide further insight into their pathophysiology, whilst expression of tumor-associated antigens on CEC may provide clues to their putative tumor origin³⁵⁻³⁶.

To have clinical utility, the method in use must have a low variability and a validation of the true endothelial origin of cells designated as CEC by the assay. Variation in CEC counts taken from the same tube was minimal and more than 95% of designated events could be confirmed as endothelial-specific by morphology, immunohistochemistry and gene expression profile. These findings are important as they may clarify the ongoing debate about over estimation of CEC values in other (conventional) flow cytometry assays lacking this kind of extensive validation.

Clinical specimens were also used to test the agreement with the validated and automated Cell Search assay, showing a high level of agreement. CEC values for healthy subjects for both methods are similar and also line with other recently reported immunomagnetic bead enrichment assays^{16,21}.

In conclusion, our study demonstrates that the new CD34 based FCM assay is able to enumerate a well-defined CEC population in peripheral blood. We envisage that this flow cytometric method can be used as a relatively cheap, high-throughput assay to accurately enumerate and further explore the characteristics of circulating endothelial cells. This will be particularly attractive for large-scale clinical studies to establish the clinical value of CECs in disease with endothelial damage or to monitor angiogenesis-inhibiting therapies.

Table 1: Gene transcripts measured in FCM sorted cells

<i>mRNA transcript</i>	lymphocyte sorted		HSC sorted		CEC sorted	
	<i>Sort 1</i>	<i>Sort 2</i>	<i>Sort 1</i>	<i>Sort 2</i>	<i>Sort 1</i>	<i>Sort 2</i>
<i>PTPRC (CD45)</i>	++	++	+	+	-	-
<i>CDH5</i>	-	-	-	+/-	++	++
<i>MCAM (CD146)</i>	-	-	-	+/-	+	+
<i>vWF</i>	-	-	-	+/-	+++	++
<i>VEGFR2(CD309)</i>	-	-	-	-	-	-

- undetectable
 detectable, less than 1-fold higher expressed relative to the reference genes
 +/-
 + over 1-fold higher expressed relative to the reference genes
 ++ over 10-fold higher expressed relative to the reference genes
 +++ over 100-fold higher expressed relative to the reference genes

Figure 1: Gating strategy for circulating endothelial cell (CEC, purple dots) analysis based on CD34 and CD146 expression. **Panel A-F:** whole blood analysis for absolute counting of CD34 positive cells (red dots). Panel A shows the forward and side scatter light characteristics of the leukocytes. Panel B describes the gating on lymphocytes ($CD45^+$, SCC^{low} , green dots) which serve as an internal (negative) control. Panel C shows gate 3 to include all ($DRAQ5^+$) nucleated cells. Panel D, E and F describes the analysis of $CD34^+$ progenitor cells ($CD34^+$, $CD45^{neg/dim}$, $DRAQ5^+$). **Panel G-K:** Flow cytometric analysis of 4 mL of blood using a CD34 threshold (panel G). CEC were analyzed within the $CD34^+$ gate as a small subset positive for CD146 and negative for CD45 (panel H). CEC are co- expressing the endothelial marker CD105 (panel I). **Panel L** shows the morphology and vWF expression of sorted CECs.

Figure 1:

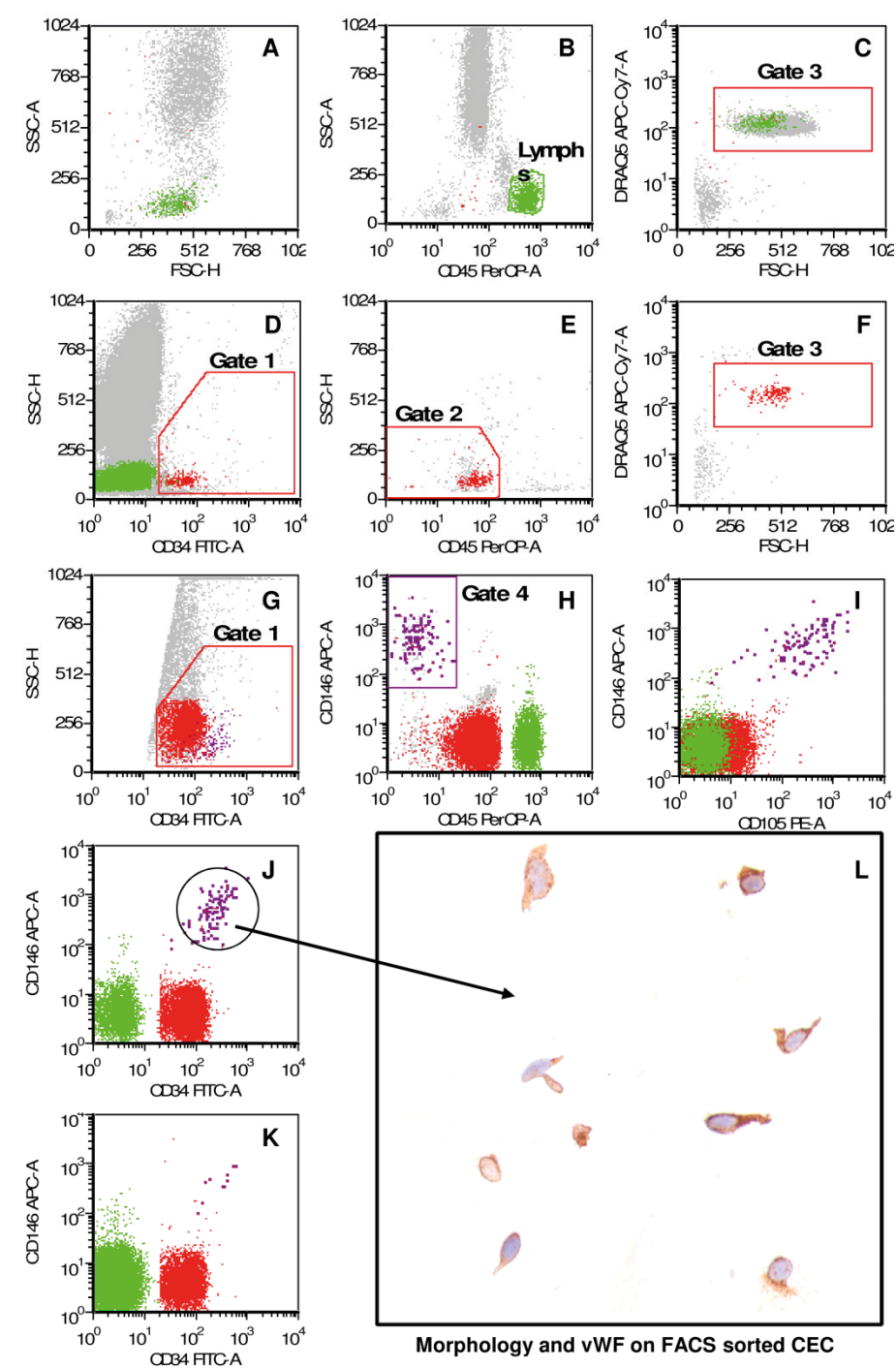


Figure 2: Flow cytometric analysis of endothelial cells isolated from a dissected vein. Firstly, nucleated cells were selected based on DNA content using DRAQ5 expression (**panel A**). Secondly, endothelial cells were identified within the CD34 positive population (**panel B, purple dots**) expressing the pan-endothelial markers CD146, CD144 and CD31 (**panel C, D, H and I respectively**), and subtype associated markers CD105, CD309 (VEGFR-2) and CD133 (**panel E, F, G**). Residual lymphocytes (CD45+, green dots) are serving as an internal (negative) control.

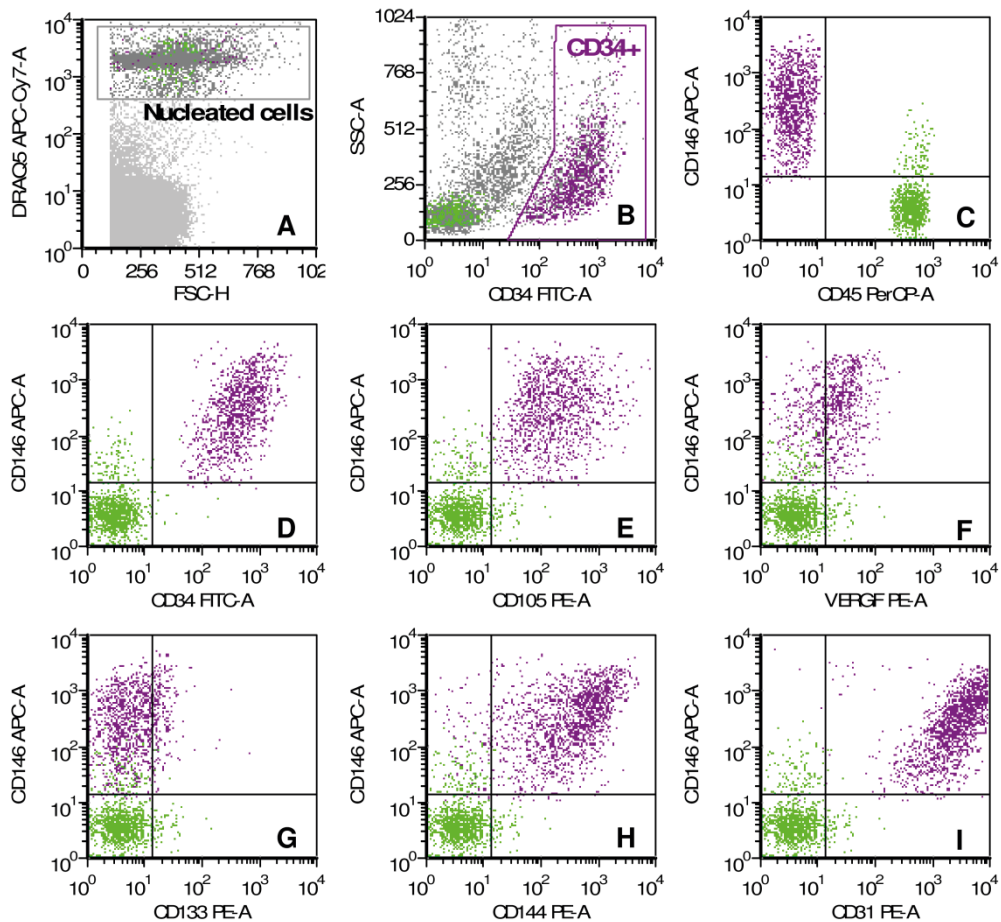
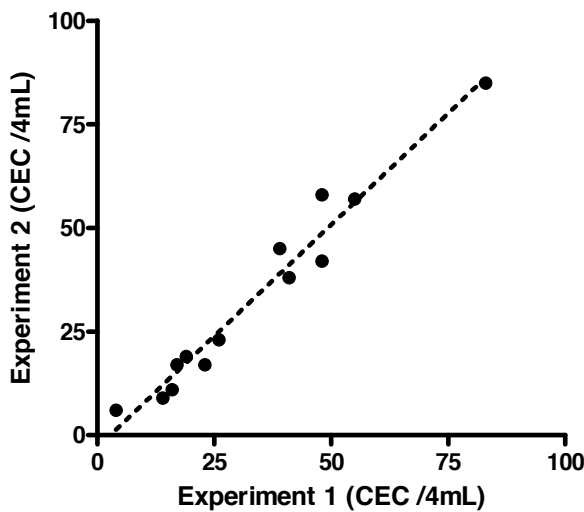


Figure 3: Assay reproducibility. Correlation between duplicate samples assessed in two independently performed experiments. Regression analysis showed a best fit line with R2 of 0.96 and a slope of 1.01 (95% confidence interval (CI); 0.87 to 1.14) an Y-intercept of 1.51 (95% CI; -7.85 to 6.21) .



6

Figure 4: Correlation of CEC values from the two assays between the number of events (red dots; R² of 0.96 with a slope of 0.66 (95% CI; 0.61 to 0.71) and an intercept of 4.16 (95% CI; -1.95 to 10.26)) as well as the obtained absolute number (blue dots; R2 of 0.71 with a slope of 2.60 (95% CI; 1.92 to 3.27) and an intercept of 72.21 (95% CI = -7.88 to 152.3)).

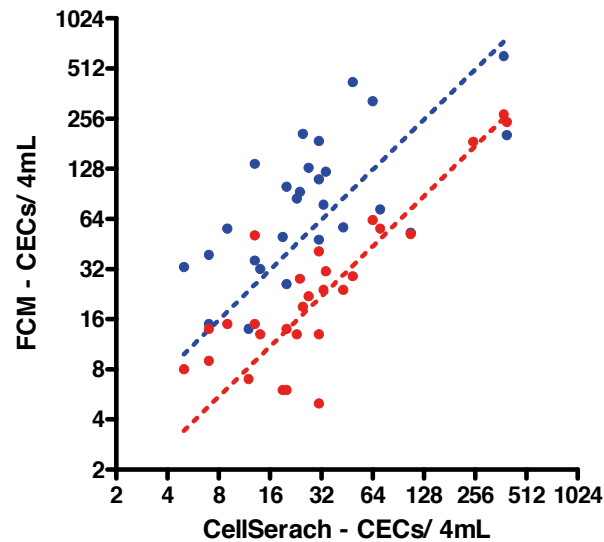


Figure 5: Bland-Altman plots comparing the Cellsearch reference assay with the CD34-based FCM event counts and a absolute count assay. The number of CEC using CD34-based event count analysis was significantly lower compared to the CellSearch reference assay (median; 20 versus 26, $P=0.0065$). Suggesting a slightly lower recovery for event counts as a result of limited cell loss during sample preparation and FCM dead volume but a significantly higher recovery when corrected for cell loss using the CD34+ cell-based FCM absolute number method (median; 129 versus 26, $P<0.0001$).

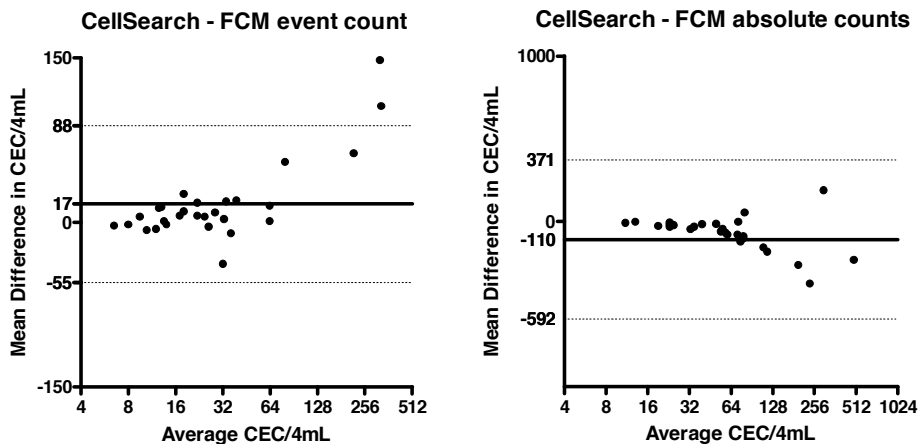
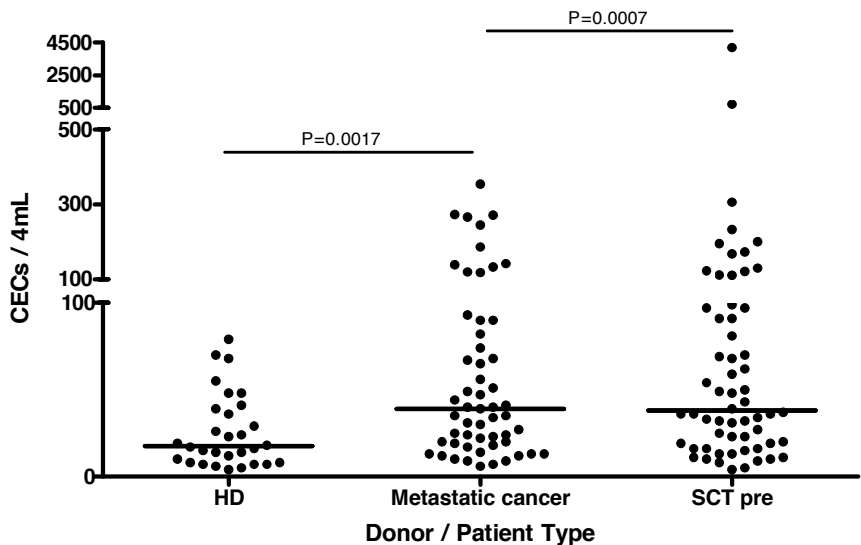


Figure 6: Prevalence of CEC using the CD34-based FCM assay in 4 mL of blood from 30 healthy individuals and significant increase in 55 metastatic carcinoma patients and 39 patients with a haematological malignancy in remission. Solid lines indicate the median value for each group.



References

1. Bonello L, Basire A, Sabatier F, Paganelli F, Dignat-George F. Endothelial injury induced by coronary angioplasty triggers mobilization of endothelial progenitor cells in patients with stable coronary artery disease. *J Thromb Haemost.* 2006;4:979-981.
2. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood.* 1999;93:2951-2958.
3. George F, Brouqui P, Boffa MC, et al. Demonstration of Rickettsia conorii-induced endothelial injury in vivo by measuring circulating endothelial cells, thrombomodulin, and von Willebrand factor in patients with Mediterranean spotted fever. *Blood.* 1993;82:2109-2116.
4. Mutunga M, Fulton B, Bullock R, et al. Circulating endothelial cells in patients with septic shock. *Am J Respir Crit Care Med.* 2001;163:195-200.
5. Percivalle E, Revello MG, Vago L, Morini F, Gerna G. Circulating endothelial giant cells permissive for human cytomegalovirus (HCMV) are detected in disseminated HCMV infections with organ involvement. *J Clin Invest.* 1993;92:663-670.
6. Camoin-Jau L, Kone-Paut I, Chabrol B, Sampol J, Dignat-George F. Circulating endothelial cells in Behcet's disease with cerebral thrombophlebitis. *Thromb Haemost.* 2000;83:631-632.
7. Clancy R, Marder G, Martin V, Belmont HM, Abramson SB, Buyon J. Circulating activated endothelial cells in systemic lupus erythematosus: further evidence for diffuse vasculopathy. *Arthritis Rheum.* 2001;44:1203-1208.
8. Bull TM, Golpon H, Hebbel RP, et al. Circulating endothelial cells in pulmonary hypertension. *Thromb Haemost.* 2003;90:698-703.
9. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. *N Engl J Med.* 1997;337:1584-1590.
10. Strijbos MH, Landburg PP, Nur E, et al. Circulating endothelial cells: a potential parameter of organ damage in sickle cell anemia? *Blood Cells Mol Dis.* 2009;43:63-67.
11. Woywodt A, Scheer J, Hambach L, et al. Circulating endothelial cells as a marker of endothelial damage in allogeneic hematopoietic stem cell transplantation. *Blood.* 2004;103:3603-3605.
12. Woywodt A, Schroeder M, Gwinner W, et al. Elevated numbers of circulating endothelial cells in renal transplant recipients. *Transplantation.* 2003;76:1-4.
13. Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat Rev Cancer.* 2006;6:835-845.
14. Furstenberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer.* 2006;94:524-531.
15. Goon PK, Boos CJ, Lip GY. Circulating endothelial cells: markers of vascular dysfunction. *Clin Lab.* 2005;51:531-538.
16. Goon PK, Boos CJ, Stonelake PS, Blann AD, Lip GY. Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. *Thromb Haemost.* 2006;96:45-52.
17. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A.* 2007;71:105-113.
18. Strijbos MH, Gratama JW, Schmitz PI, et al. Circulating endothelial cells, circulating tumour cells, tissue factor, endothelin-1 and overall survival in prostate cancer patients treated with docetaxel. *Eur J Cancer.* 2010;46:2027-2035.

19. Strijbos MH, Kraan J, den Bakker MA, Lambrecht BN, Sleijfer S, Gratama JW. Cells meeting our immunophenotypic criteria of endothelial cells are large platelets. *Cytometry B Clin Cytom.* 2007;72:86-93.
20. Mancuso P, Antoniotti P, Quarna J, et al. Validation of a standardized method for enumerating circulating endothelial cells and progenitors: flow cytometry and molecular and ultrastructural analyses. *Clin Cancer Res.* 2009;15:267-273.
21. Widemann A, Sabatier F, Arnaud L, et al. CD146-based immunomagnetic enrichment followed by multiparameter flow cytometry: a new approach to counting circulating endothelial cells. *J Thromb Haemost.* 2008;6:869-876.
22. Dignat-George F, Sabatier F, Blann A, Woywodt A. Detection of circulating endothelial cells: CD146-based magnetic separation enrichment or flow cytometric assay? *J Clin Oncol.* 2007;25:e1-2; author reply e3-5.
23. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LW, O'Hara SM. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. *Cancer Res.* 2006;66:2918-2922.
24. 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;98:1731-1735.
25. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *International Society of Hematotherapy and Graft Engineering. J Hematother.* 1996;5:213-226.
26. Sutherland DR, Keeney M, Gratama JW. Enumeration of CD34+ hematopoietic stem and progenitor cells. *Curr Protoc Cytom.* 2003;Chapter 6:Unit 6 4.
27. Sieuwerts AM, Kraan J, Bolt-de Vries J, 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;118:455-468.
28. Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR. Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. *International Society of Hematotherapy and Graft Engineering. Cytometry.* 1998;34:61-70.
29. Gratama JW, Kraan J, Keeney M, Sutherland DR, Granger V, Barnett D. Validation of the single-platform ISHAGE method for CD34(+) hematopoietic stem and progenitor cell enumeration in an international multicenter study. *Cytotherapy.* 2003;5:55-65.
30. Khan SS, Solomon MA, McCoy JP, Jr. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. *Cytometry B Clin Cytom.* 2005;64:1-8.
31. Gratama JW, van der Linden R, van der Holt B, Bolhuis RL, van de Winkel JG. Analysis of factors contributing to the formation of mononuclear cell aggregates ("escapees") in flow cytometric immunophenotyping. *Cytometry.* 1997;29:250-260.
32. Enjeti AK, Lincz LF, Seldon M. Detection and measurement of microparticles: an evolving research tool for vascular biology. *Semin Thromb Hemost.* 2007;33:771-779.
33. Mustafa D, van der Weiden M, Zheng P, Nigg A, Luider TM, Kros JM. Expression sites of colligin 2 in glioma blood vessels. *Brain Pathol.* 2010;20:50-65.
34. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood.* 2001;97:3658-3661.
35. Seaman S, Stevens J, Yang MY, Logsdon D, Graff-Cherry C, St Croix B. Genes that distinguish physiological and pathological angiogenesis. *Cancer Cell.* 2007;11:539-554.
36. Radu A, Pichon C, Camparo P, et al. Expression of follicle-stimulating hormone receptor in tumor blood vessels. *N Engl J Med.* 2010;363:1621-1630.

Chapter 7

**Endothelial CD276 (B7-H3)
expression is increased in
human malignancies
and distinguishes between
normal and tumour derived
circulating endothelial cells**

Jaco Kraan, Patricia van den Broek, Cornelis Verhoef,
Dirk J. Grunhagen, Walter Taal, Jan W. Gratama, Stefan Sleijfer

British Journal of Cancer. 2014;111:149 - 156

Abstract

Background: Mature circulating endothelial cells (CEC) are surrogate markers of endothelial damage. CEC measured in patients with advanced cancer are thought not only to derive from damaged normal vasculature (n-CEC), but also from damaged tumor vasculature (t-CEC). Therefore, assays that allow the discrimination between these two putative types of CEC are thought to improve the specificity of the enumeration of CEC in cancer.

Objectives: Identification of tumour-associated endothelial markers (TEM) to enable distinction between t-CEC and n-CEC and to assess the presence of t-CEC in peripheral blood of cancer patients by incorporating TEM in our novel flow cytometry-based CEC detection assay.

Results: No difference in antigen expression between normal and malignant endothelial cells was found for CD54, CD109, CD137, CD141, CD144 and CXCR7. In contrast, over-expression for CD105, CD146, CD276 and CD309 was observed in tumor endothelial cells compared to normal endothelial cells. CD276 was most differentially expressed and chosen as a marker for further investigation. CD276 expressing CEC were significantly higher in 15 patients with advanced colorectal cancer (median 9 [range 1 to 293 cell/4mL]; $p < 0.005$), in 83 patients with a glioblastoma multiforme (median 10 [range 0 to 804]; $p < 0.0001$) and in 14 patients with advanced breast cancer (median 14 [range 0 to 390] $p < 0.05$) as compared to 24 healthy individuals (median 3 [range 0 to 11]). Of all patients with malignancies, 58% had CD276+CEC counts above the ULN (8 cell/ 4mL).

Conclusions: The present study shows that CD276 can be used to discriminate endothelial cells (ECs) from malignant tissue from ECs from normal tissue. Additionally, CD276+CEC do occur in higher frequencies in patients with advanced cancer.

Introduction

Malignant tumors are critically dependent on the formation of new blood vessels for their growth and dissemination ^{1,2}. Unlike normal tissues, which use sprouting angiogenesis, vasculogenesis and intussusception to form new vessels, tumours also use additional modes of vessel formation. These include the cooptation of pre-existing vessels, the imitation of tumour vasculature by tumour cells (a.k.a. vascular mimicry), and the differentiation of putative cancer stem-like cells into tumor endothelium ³. As a result, tumour vasculature differs morphologically from normal vasculature, as it features an apparently chaotic mixture of abnormal and hierarchically disorganized vessels with an increased permeability ⁴.

Next to morphologic differences, in recent years many tumor-associated endothelial markers (TEM) have been identified by comparing gene and protein expression levels between normal and tumour endothelial cells demonstrating distinctive gene and protein profiles ⁴⁻¹⁰.

Given the central role of vasculature for the growth and development of tumors, various anti-angiogenic agents have been developed and are now under study with the aim to interfere with the vascularization of tumours. Several clinical trials have demonstrated a benefit in terms of prolonged survival of cancer patients treated with such anti-angiogenic therapies. Further development of this field requires the identification of clinically effective biomarkers that assist the determination of the optimal dose of such agents, the monitoring of their biologic activity, and the selection of patients who are most likely to benefit from this treatment ⁵.

Circulating endothelial cells (CEC) are a relative new candidate to monitor vascular effects of anti-angiogenic treatments. CEC are mature endothelial cells which have been shed from the vascular cell lining as a result of vascular damage. Since numerous diseases, including cancer, are associated with vascular damage, enumeration of CEC is being considered a promising tool to monitor disease activity with a potential to assess prognosis and response to treatment¹¹. Importantly, CEC measured in patients with advanced cancer are thought not only to derive from damaged normal vasculature (hereafter termed n-CEC), but also from damaged tumour vasculature (t-CEC). Therefore, assays that allow the discrimination between these two putative types of CEC are thought to improve the specificity of the enumeration of CEC in cancer.

Following dissociation, vascular endothelial cells from tumour and normal tissues can be analyzed by multi-parameter flow cytometry using a combination of exclusion and inclusion markers ^{6,12}. The aim of the current study is to identify tumour-associated endothelial markers (TEM), in order to enable the distinction between t-CEC and n-CEC and to assess the possible detection of t-CEC in

peripheral blood of cancer patients by incorporating TEM enumeration in our novel flow cytometry-based CEC detection assay¹³.

Materials and methods

Blood and tissue collection

Tumor and macroscopically normal tissues were obtained from patients (n=17) undergoing standard surgical procedures for liver metastasis resection of metastatic colorectal cancer (CRC; n=11) or for resection of a retroperitoneal liposarcoma (n=6). Peripheral blood (PB) was collected using EDTA-containing or CellSave™ tubes (Veridex, Raritan, NJ) from healthy donors (n = 18) or patients with advanced CRC (n = 13), advanced breast cancer (n = 9) or patients with a glioblastoma multiforme (GBM; n = 73) who relapsed after prior chemoradiation. Samples were stored at room temperature and examined within 8h (EDTA-containing tubes) or within 96h (CellSave™ tubes) after venipuncture. All patients provided written informed consent and the study protocols were approved by the local research and ethics committee (protocols METC: 2011-260, 2006-248, 2009-405 and P05.182).

Isolation of endothelial cells from tissues

For endothelial cell isolation the resected specimens containing normal or malignant tissue were dissociated using a standardized, semi-automated protocol based on a combination of mechanical tissue disruption and collagenase IV digestion, using a gentleMACS™ dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, tissues were cut in small pieces (2-4 mm) in a petri dish prior to collection in a dedicated gentleMACS tube, to which 10 ml of RPMI 1640 (Lonza, BioWhittaker) with 1% L-Glutamin, 1% Penicillin/Streptomycin, 10% heat-inactivated FBS (Sigma Aldrich, St Louis, MO), as well as collagenase IV (Sigma Aldrich), 1mg/ml, and DNase I (Sigma Aldrich), 10 µg/ml, had been added. The tissues were mechanically minced on the gentleMACS after incubation for 30 min at 37°C in a shaking water-bath. This procedure was repeated once. After the second dissociation step at the gentleMACS, the cell suspension was passed through a 70 µm Falcon cell strainer (BD Biosciences, San Jose, CA). The cell suspension was then collected in a 50 mL conical tube, and centrifuged at 1000 x g for 5 min. The resulting pellet was washed with 50 mL phosphate buffered saline (PBS) and concentrated at 2×10^6 cells/mL in PBS for flow cytometry to detect and characterize the tissue-derived endothelial cells.

Flow cytometry of endothelial cells from tissues

Multiparameter flow cytometry was performed on 100 μ l of cells stained with 10 μ l of each of the following monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC) conjugated CD34 (clone 8G12; BD Biosciences, San Jose, CA); peridinin chlorophyll protein (PerCP) conjugated CD45 (clone 2D1; BD Biosciences); allophycocyanin (APC) conjugated CD146 (clone 541-10B2; Miltenyi Biotec), and 10 μ l of the DNA dye 5-bis[2-(di-methylamino) ethyl]amino-4, 8-dihydroxyanthracene-9,10-dione (DRAQ5; Biostatus, Shepshed, UK) and 5 μ l of the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). Coexpression of endothelial cells was studied using the following phycoerythrin (PE) labeled mAb: CD54 (clone LB2), CD109 (clone TEA 2/16), CD137 (clone 4B4-1) (BD Biosciences), CD105 (clone 1G2), CD144 (clone TEA 1/31) (Beckman Coulter, Miami, FL), CD276 (B7-H3, clone 185504), CD309 (clone 89106) (R&D systems, Minneapolis, MN), CD141 (clone AD5-14H12, Miltenyi Biotec) and CXCR7 (clone 8F11-M16, Biolegend, San Diego, CA). All reagents were diluted in PBS supplemented with 1% bovine serum albumin (BSA) based on titration experiments (i.e. absence of non-specific staining on negative populations and optimal discriminatory power between negative and positive populations). After 15 min of incubation in darkness at room temperature (RT), 2 mL of PBS were added, and the suspension was centrifuged for 5 min at 500 $\times$ g. After removal of supernatant the cells were resuspended in 250 μ l of PBS for acquisition on a BD LSR Fortessa flow cytometer equipped with BD FACSDiva software.

CEC enumeration

CEC enumeration and characterization was performed as described before¹³. The assay is based on absolute CD34 counts after analysis of all CD34 positive events in a total blood volume of 4 mL using a “live gate” on CD34+ events to exclude most of the cells that are not of interest from this analysis. Following this acquisition CEC are defined as CD34++, CD45neg, CD146+ and DNA+ events and combined with PE-labeled markers for their further characterization. The true endothelial origin of this population was previously confirmed by sorting this specific population and characterizing it further using morphology, immunohistochemistry, multi-colour FCM and gene expression profiling¹³. In that study, we demonstrated for this population a specific EC morphology, co-expression of vWF, CD31, CD105 and CD144, which are all considered endothelial cell markers, and additionally found that this population exhibits an EC specific gene profile.

Statistical analysis

Statistical analysis was performed using Prism™ software (GraphPad Software, La Jolla, CA). The Mann-Whitney U-test was used to evaluate differences between

endothelial cells (EC) populations in tissue and peripheral blood. P values < 0.05 are considered significant.

Results

Immunophenotype of tissue-derived endothelial cells.

Based on the previous work of ourselves¹³ and others^{14,15} viable endothelial cells were identified by multiparameter flow cytometry using a combination of exclusion and inclusion markers combined with a permeant DNA-specific dye (DRAQ5) to detect nucleated cells, and a non-permeant dye (DAPI) to exclude dead cells. EC were identified by staining with two pan-endothelial antibodies (CD34 and CD146), whilst CD45 served to exclude leukocytes and nonspecific binding of mAb on tissue cells. Using these criteria we were able to identify a small population of vital EC from tissue specimens containing normal or malignant tissue defined as CD34⁺⁺, CD146⁺, CD45^{neg}, DNA⁺, DAPI^{neg} (fig 1). Additionally, we also identified a population of viable cells with a phenotype reported to be specific for pericytes (Table 1): CD34neg, CD146++, CD45-, DNA+, DAPIneg. In order to verify the nature of endothelial cells and pericytes, we performed additional stainings with a panel of antibodies containing endothelial and pericyte-associated markers that were not used in the flow cytometric marker definition for the detection of these events. These results confirmed that the events assigned as EC and pericytes by this assay indeed represent these cells (table 1 and figure 2).

Comparison of antigen expression on normal versus tumor vasculature

We have studied the expression of endothelial cell-specific and associated antigens on paired cell suspensions prepared from 17 tumors and normal tissues from the same patients.

Macroscopically normal tissues were obtained from (partially) resected liver (N=10), vein (N=4), peritoneum (N=2) and kidney (N=1) tissue. These markers included thrombomodulin (CD141), VE-cadherin (CD144), MCAM (CD146) and VEGFR-2 (CD309). In this study, we also addressed the following putative malignancy-associated antigens, based on published data (Table 2): ICAM-1 (CD54), endoglin (CD105), CD109 (a GPI-linked member of the complement superfamily), CD137 (tumor necrosis superfamily factor 90), CXCR7 (C-X-C chemokine receptor 7) and CD276 (a member of the B7 family of costimulatory molecules), all cell surface expressed proteins. Residual lymphocytes in the cell isolate were used as an internal (negative) control. No difference in antigen expression between normal and malignant endothelial cells was found for CD54, CD109, CD137, CD141, CD144 and CXCR7. In contrast, over-expression for

CD105, CD146, CD276 and CD309 was observed in tumour endothelial cells compared to normal endothelial cells. CD276 was most differentially expressed and chosen as a marker for further investigation. (Figure 3).

CD276 expression on CEC in carcinoma patients

As CD276 was the marker with the largest difference in expression between tumor and normal endothelial cells, it was explored whether or not a subpopulation of CD276+ CEC could be identified in the peripheral blood of solid cancer patients with metastatic disease.

Figure 4 shows the expression of CD276 in two representative patients, one with partial expression and a low number of CD276+ CEC (panel H) and one with a high number of CD276+ CEC (panel I). CD276+ CEC were undetectable or below 12 cells/ 4mL in healthy donors (figure 4 panel G and figure 5).

Although the investigation of CD276+ CEC levels in different types of cancer other than for validation purposes is beyond the scope of this paper, Figure 5 shows that CD276+ CEC levels in healthy individuals ranged from 0 to 11 cells/4 mL (n = 24, median 3) and that the levels of these cells were significantly higher in patients with advanced CRC (ranging from 1 to 293 (n = 15, median 9, p < 0.005)), in patients with a GBM (range: 0 to 804 (n = 83, median 10, p < 0.0001)) and patients with advanced breast cancer (range: 0 to 390 (n = 14, median 13.5, p < 0.05)). Taking all patients with malignancies together, 58% of them had more CD276+ CEC than the upper limit of the normal range (ULN) for healthy individuals, defined as: mean + 1,96x standard deviation (SD), which is 8 CD276+ CEC /4mL. When stratified for the different types of cancer the percentage of patients with CD276+ CEC above ULN were 53%, 58% and 64% for respectively patients with advanced CRC, GBM and advanced breast cancer, respectively.

Discussion

Recent studies suggest that tumor-derived EC possess different characteristics from normal EC including morphologic ¹⁶, cytogenetic ¹⁷ and pathophysiologic ¹⁸ features as well as a different gene expression profile ¹⁹.

In a previous study, we have described a flow-cytometric method to enumerate endothelial cells circulating in the peripheral blood and in the same study, we extensively validated the true endothelial origin of this population assigned as CEC ¹³. This method enabled us to identify markers that can be used to differentiate between CECs from normal vasculature versus CEC derived from damaged tumor vasculature as measured in the peripheral blood of cancer patients. Using this flow-cytometric approach, we were successful to isolate a pure

endothelial cell population from normal and malignant tissues. To the best of our knowledge, this is the first time that such a method is described, which enables the isolation of EC from dissociated tissue by multi-parameter flow cytometry without pre-enrichment and the possibility to distinguish EC from non-EC such as pericytes. We feel that this method can be further used to investigate the characteristics of ECs in more detail.

Subsequently, we studied ECs from normal and malignant tissue for their expression of cell surface markers that have been suggested in the literature as being differentially expressed. Potential TEM such as CD54, CD109, CD137 and CXCR7 did not differ in expression between ECs derived from normal and malignant tissue in a series of colorectal carcinomas and liposarcomas. In contrast, higher expression of CD105, CD146 and CD309 was found on tumor EC compared to EC from normal tissue. These observations are in line with other reports. Over expression of CD105 on tumour-associated EC has been reported for hepatocellular carcinoma²⁰, breast, colon, lung, prostate and brain tumours²¹ while up regulation of CD146 and CD309 has been described in many cancers including breast, prostate, melanoma and glioma^{22,23}. In our series, the largest difference was found for CD276 with higher expression on tumour EC compared to normal tissue EC. CD276 is a member of the B7 family of immunoregulatory molecules that can be induced on T cells, B cells and dendritic cells by a variety of inflammatory cytokines^{24,25}. The ligand for CD276 has not been identified yet as well as its immunoregulatory role. CD276 has been suggested to have a role in the interaction between tumours and the immune system. Lemke and colleagues showed that Natural killer cell-mediated cell lysis was proportionally suppressed as a function of CD276 expression on tumor target cells²⁶. Additionally, several others have shown that expression of CD276 was associated with a relatively aggressive tumour behaviour in a range of various carcinomas²⁷⁻³² and GBM²⁶. In line with our data, also others have shown that CD276 is differentially expressed between normal and malignant ECs. St. Croix and colleagues used SAGE technology in a transcriptomic comparison of gene expression patterns of EC derived from normal resting tissue, from tumours, and from regenerating liver in order to reveal differences between physiological and pathological angiogenesis⁹. Twenty five transcripts were identified to be over-expressed in tumour versus normal endothelium, including 13 that were undetectable in angiogenic endothelium of regenerating liver. Seven of this transcripts encoded known cell surface proteins, of which CD276 (B7-H3) was the most differentially expressed TEM⁹.

As CD276 was the most differentially expressed TEM, we subsequently determined the presence of CD276+CEC in healthy controls and in three groups of patients with advanced tumors (colorectal cancer, GBM and breast cancer).

The levels of CD276+CEC were significantly increased in all patient groups and 58% of all patients had CD276+CEC numbers above the ULN (53%, 58 and 64%,

respectively). Although no clear differences in incidence or numbers of CD276+CECs between these three tumour types could be identified, this study was not powered to detect such differences.

The relatively small series of this study is one of its limitations. Another limitation is that the CD276+CEC numbers were not correlated to clinical outcome as a consequence of which the clinical relevance of this cell population remains to be defined.

In conclusion, the present study shows that CD276 can be used to discriminate ECs from malignant tissue from ECs from normal tissue. Additionally, CD276+CEC do occur in higher frequencies in patients with advanced cancer. In addition to defining potential differences between various tumour types and tumour stages, it is important to further investigate the potential clinical relevance of these cells in oncology.

To do so, further clinical studies are needed to investigate the association of CD276+CEC levels at baseline with outcome, to examine the use of CD276+CEC as a marker for response assessment by serially determining CD276+CEC levels during treatment and to establish whether or not changes are associated with outcome to treatment as determined by conventional means, and to determine whether targets for treatment can be revealed on these cells. This will require large studies in homogenous groups of patients.

Table 1: Reported expression of different endothelial and pericyte associated antigens on isolated endothelial and pericyte populations from healthy donors by flow cytometry

Marker	Endothelial Cells ¹	Pericytes ¹	References
CD9 (<i>TSPAN29</i>)	++	++	7
CD31 (<i>PECAM-1</i>)	++	-	11,33
CD34 (<i>Stem cell marker</i>)	++	-	11,12,33
CD45 (<i>pan-leucocyte</i>)	-	-	11,33
CD54 (<i>ICAM-1</i>)	+	-	11
CD105 (<i>Endoglin</i>)	+	+/-	11,34
CD133 (<i>Stem cell marker</i>)	-	-	11
CD140b (<i>PDGF-Rβ</i>)	-	+	33
CD141 (<i>Thrombomodulin</i>)	+	+	11,34
CD144 (<i>VE-Cadherin</i>)	+	-	11,33
CD146 (<i>MelCAM</i>)	+	++	11,12,33

¹ Expression levels: ++ = strong, + = moderate, - = negative

Table 2: Reported tumour-associated endothelial markers (TEM)

Marker	Cell surface expression on tumor endothelium	References
CD54 (ICAM-1)	Down regulated in renal cell carcinoma	6
CD105 (Endoglin)	Up regulated in breast, colon, cervix, lung, prostate and brain tumors	21,35
CD109	Up regulated in breast, colon and lung carcinoma	9
CD137 (4-1BB)	Up regulated in colon and lung carcinoma	9,36
CXCR7	Up regulated in renal cell carcinoma and meningioma	8,10
CD276 (B7-H3)	Up regulated in breast, colon, lung, renal carcinoma and Glioblastoma	9,26,32

Figure 1: Flow cytometric analysis of endothelial cells and pericytes from dissected normal liver tissue. Viable nucleated cells were selected on DNA content as evidenced by the permeant dye DRAQ5 expression (panel A) and the absence of reactivity of the non-permeant dye DAPI (panel B). Endothelial cells were identified by their expression of CD146 and CD34^{bright}, and absence of CD45 expression. Finally, pericytes were identified by their strong expression of CD146 and dim expression of CD34 (panels C and D). Residual lymphocytes (green dots) in the cell isolate were used as an internal (negative) control. The seven additional markers listed on the right were evaluated using separate PE-labelled antibodies as shown in figure 2.

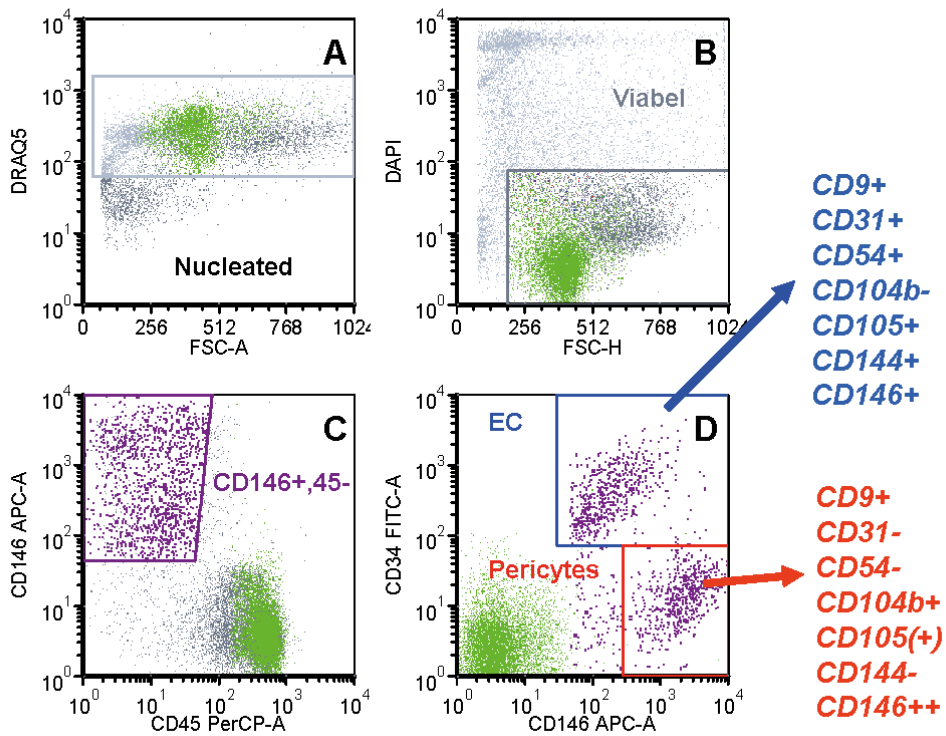


Figure 2: Immunophenotypic characteristics of endothelial cells (blue dots) and pericytes (red dots) isolated from tissue dissections. Lymphocytes (green dots) are serving as an internal (negative) control.

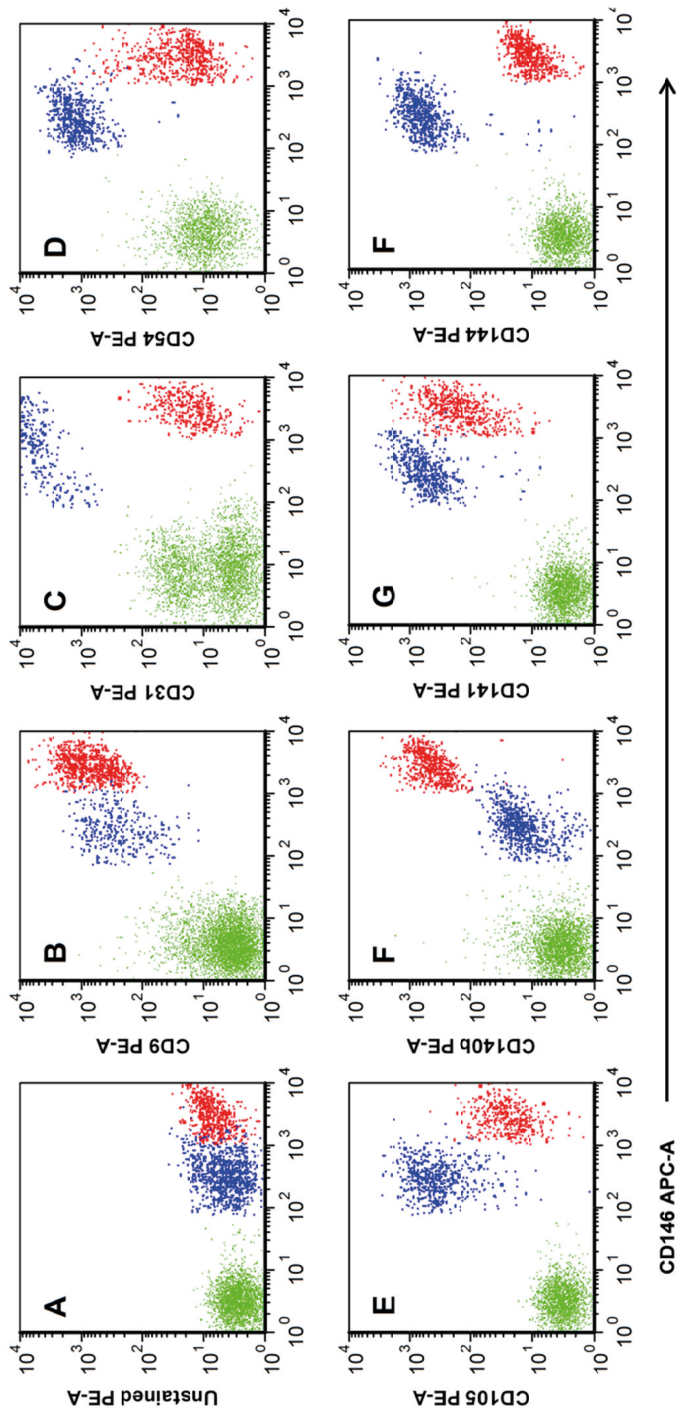


Figure 3: Antigen expression (mean fluorescence intensity) on lymphocytes (left two bars) and endothelial cells (right four bars) isolated from dissected normal and tumour tissue. Sarcoma (N=6), colorectal cancer (N=11) and all tumours (N=17; sarcoma and colorectal taken together) are separately displayed. Solid lines indicate the median value for each group. P-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

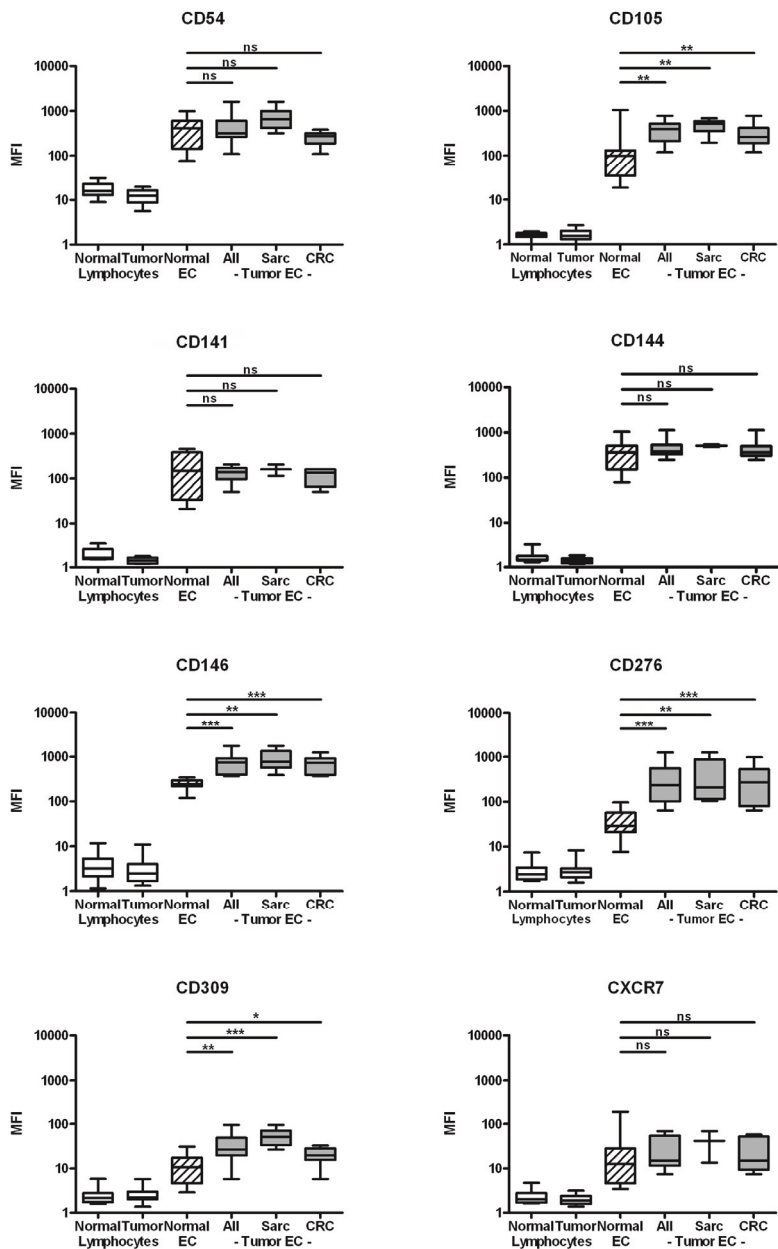


Figure 4: CD276 expression on circulating endothelial cells (CEC, purple dots). CEC are distinguished as a small population within the CD34+ progenitor cells (red dots: CD34+, CD45neg/dim, DNA/DRAQ5+; panels A-C). For optimal detection of the rare CEC population, all CD34+ cells in a total of 4 mL blood were analysed using a threshold on CD34 (panel D). CEC are identified as positive for CD146 and negative for CD45 (panel E). Panels E and F are overlay histograms combining the lymphocytes (internal control, including a small subset of CD146+ T lymphocytes; green dots) from the first 50.000 events acquired without a CD34 threshold, and all CD34 positive events using a threshold on the remaining cells (red and violet dots). Panels G-I shows the absence of CD276 expression on CEC from a healthy individual (panel G) versus partial (panel H) and high (panel I) expression on CEC from two representative carcinoma patients.

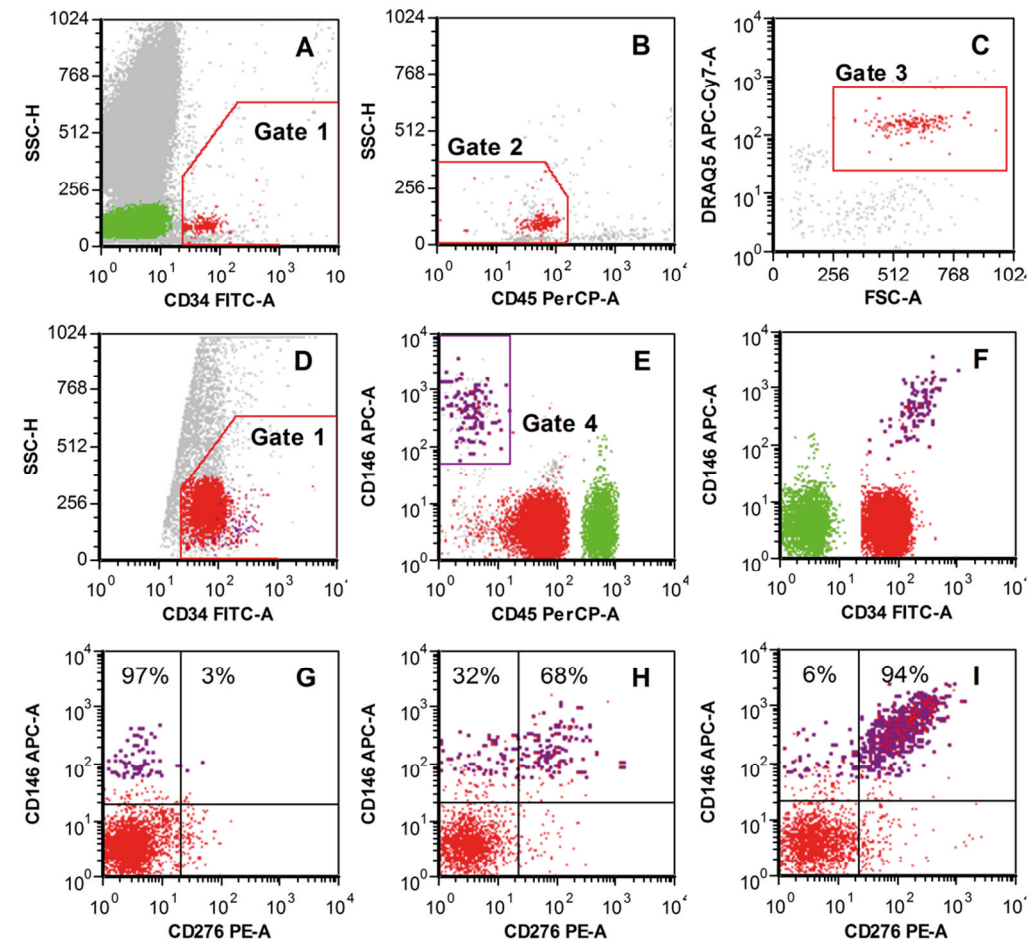
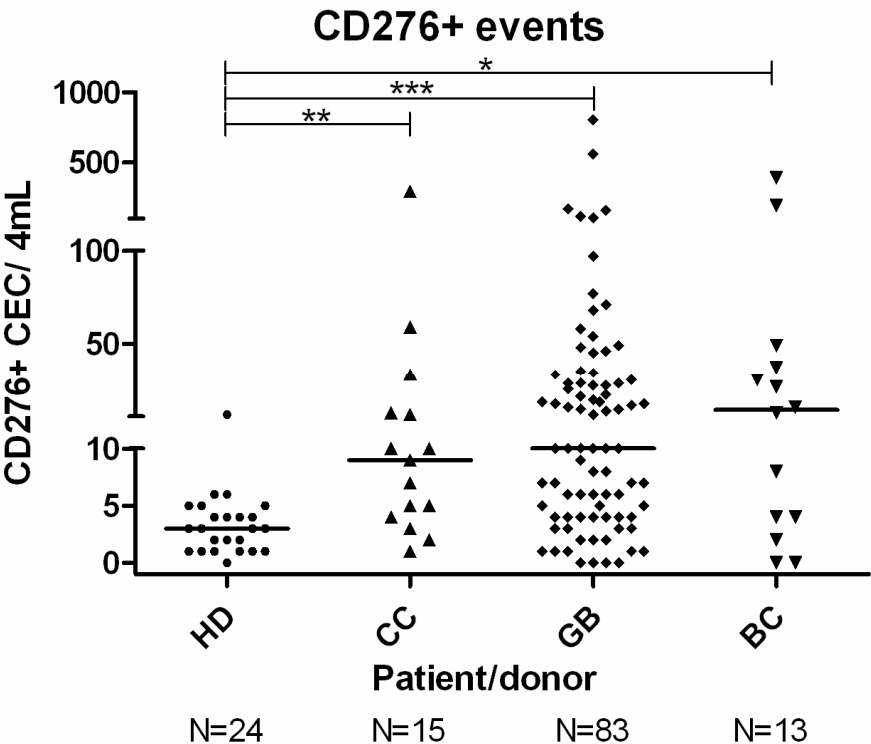


Figure 5: Absolute counts of CD276+ CEC in 4 mL of blood from 24 healthy individuals (HD), 15 patients with advanced CRC (CC), 83 with glioblastoma (GB) and 13 with advanced breast cancer (BC). Solid lines indicate the median value for each group. Differences between HD and cancer patients were significant (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).



REFERENCES

1. Naumov GN, Bender E, Zurakowski D, et al. A model of human tumor dormancy: an angiogenic switch from the nonangiogenic phenotype. *J Natl Cancer Inst.* 2006;98:316-325.
2. 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;98:1731-1735.
3. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature.* 2011;473:298-307.
4. Nagy JA, Chang SH, Shih SC, Dvorak AM, Dvorak HF. Heterogeneity of the tumor vasculature. *Semin Thromb Hemost.* 2010;36:321-331.
5. Bertolini F, Mancuso P, Shaked Y, Kerbel RS. Molecular and cellular biomarkers for angiogenesis in clinical oncology. *Drug Discov Today.* 2007;12:806-812.
6. Griffioen AW, Damen CA, Martinotti S, Blijham GH, Groenewegen G. Endothelial intercellular adhesion molecule-1 expression is suppressed in human malignancies: the role of angiogenic factors. *Cancer Res.* 1996;56:1111-1117.
7. Lijnen HR, Lupu F, Collen D, Le Naour F, Boucheix C. CD9 gene deficiency does not affect smooth muscle cell migration and neointima formation after vascular injury in mice. *Thromb Haemost.* 2000;83:956-961.
8. Maishi N, Ohga N, Hida Y, et al. CXCR7: a novel tumor endothelial marker in renal cell carcinoma. *Pathol Int.* 2012;62:309-317.
9. Seaman S, Stevens J, Yang MY, Logsdon D, Graff-Cherry C, St Croix B. Genes that distinguish physiological and pathological angiogenesis. *Cancer Cell.* 2007;11:539-554.
10. Wurth R, Barbieri F, Bajetto A, et al. Expression of CXCR7 chemokine receptor in human meningioma cells and in intratumoral microvasculature. *J Neuroimmunol.* 2011;234:115-123.
11. Kraan J, Sleijfer S, Foekens JA, Gratama JW. Clinical value of circulating endothelial cell detection in oncology. *Drug Discov Today.* 2012;17:710-717.
12. Zimmerlin L, Donnenberg VS, Pfeifer ME, et al. Stromal vascular progenitors in adult human adipose tissue. *Cytometry A.* 2010;77:22-30.
13. Kraan J, Strijbos MH, Sieuwerts AM, et al. A new approach for rapid and reliable enumeration of circulating endothelial cells in patients. *J Thromb Haemost.* 2012;10:931-939.
14. Goon PK, Boos CJ, Stonelake PS, Blann AD, Lip GY. Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. *Thromb Haemost.* 2006;96:45-52.
15. Dignat-George F, Sabatier F, Blann A, Woywodt A. Detection of circulating endothelial cells: CD146-based magnetic separation enrichment or flow cytometric assay? *J Clin Oncol.* 2007;25:e1-2; author reply e3-5.
16. McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. *Nat Med.* 2003;9:713-725.
17. Hida K, Hida Y, Amin DN, et al. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res.* 2004;64:8249-8255.
18. Hagendoorn J, Tong R, Fukumura D, et al. Onset of abnormal blood and lymphatic vessel function and interstitial hypertension in early stages of carcinogenesis. *Cancer Res.* 2006;66:3360-3364.
19. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. *Science.* 2000;289:1197-1202.
20. Xiong YQ, Sun HC, Zhang W, et al. Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug

- resistance compared with normal endothelial cells. *Clin Cancer Res.* 2009;15:4838-4846.
21. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J.* 2003;17:984-992.
 22. Ouhtit A, Gaur RL, Abd Elmageed ZY, et al. Towards understanding the mode of action of the multifaceted cell adhesion receptor CD146. *Biochim Biophys Acta.* 2009;1795:130-136.
 23. Plate KH, Breier G, Millauer B, Ullrich A, Risau W. Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res.* 1993;53:5822-5827.
 24. Chapoval AI, Ni J, Lau JS, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol.* 2001;2:269-274.
 25. Steinberger P, Majdic O, Derdak SV, et al. Molecular characterization of human 4Ig-B7-H3, a member of the B7 family with four Ig-like domains. *J Immunol.* 2004;172:2352-2359.
 26. Lemke D, Pfenning PN, Sahm F, et al. Costimulatory protein 4IgB7H3 drives the malignant phenotype of glioblastoma by mediating immune escape and invasiveness. *Clin Cancer Res.* 2012;18:105-117.
 27. Zang X, Sullivan PS, Soslow RA, et al. Tumor associated endothelial expression of B7-H3 predicts survival in ovarian carcinomas. *Mod Pathol.* 2010;23:1104-1112.
 28. Crispin PL, Sheinin Y, Roth TJ, et al. Tumor cell and tumor vasculature expression of B7-H3 predict survival in clear cell renal cell carcinoma. *Clin Cancer Res.* 2008;14:5150-5157.
 29. Roth TJ, Sheinin Y, Lohse CM, et al. B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy. *Cancer Res.* 2007;67:7893-7900.
 30. Sun Y, Wang Y, Zhao J, et al. B7-H3 and B7-H4 expression in non-small-cell lung cancer. *Lung Cancer.* 2006;53:143-151.
 31. Yamato I, Sho M, Nomi T, et al. Clinical importance of B7-H3 expression in human pancreatic cancer. *Br J Cancer.* 2009;101:1709-1716.
 32. Qin X, Zhang H, Ye D, Dai B, Zhu Y, Shi G. B7-H3 is a new cancer-specific endothelial marker in clear cell renal cell carcinoma. *Onco Targets Ther.* 2013;6:1667-1673.
 33. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell.* 2008;3:301-313.
 34. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A.* 2007;71:105-113.
 35. Minhajati R, Mori D, Yamasaki F, Sugita Y, Satoh T, Tokunaga O. Organ-specific endoglin (CD105) expression in the angiogenesis of human cancers. *Pathol Int.* 2006;56:717-723.
 36. Broll K, Richter G, Pauly S, Hofstaedter F, Schwarz H. CD137 expression in tumor vessel walls. High correlation with malignant tumors. *Am J Clin Pathol.* 2001;115:543-549.

Chapter 8

Summary and discussion

Summary and discussion

This thesis aimed to optimize the predictive and prognostic information that can be obtained from **Circulating Tumor cells (CTC)** and **Circulating Endothelial Cells (CEC)** in whole blood by improving and standardization of their detection and characterization methods in patients with solid malignancies. Both CTC and CEC are promising biomarkers for research purpose and patient management, that can be easily obtained by a non-invasive blood sampling (i.e. liquid biopsy) on multiple time points.

Standardization of CTC enumeration

CTC can be detected, among others, by semi-automated immuno-magnetic enrichment and image cytometry using CellSearch® system, which is the only system with FDA clearance so far. For an assay used in daily practice and multicenter studies to monitor disease status and therapy efficacy, constituency in performance between tests and between laboratories is pivotal. In **chapter 2** we studied the feasibility of external quality assurance (EQA), i.e. the entire procedure from blood draw to result interpretation by 14 laboratories. We surveyed between-laboratory, between-assay and between-instrument variation. In addition, we assessed between-operator variability by having blinded images of all blood samples interpreted by all participants via a website survey, and participants were asked to analyze the samples for CTC according to the manufacturer's guidelines. Our study showed the feasibility of an external quality assurance program for CTC detection in peripheral blood. Obviously, this approach is relevant not only for the CellSearch System, but for other CTC assays as well. Furthermore, this study showed the importance of incorporating 'real life' patient samples to better address between-laboratory variation than when stabilized (blood) samples spiked with cell lines would be used. Using clinically relevant cut-off values, discordance between laboratories is low and can be considered acceptable. In terms of absolute CTC numbers however, the between-laboratory variation was still considerable. Continuation of such an EQA program for the CellSearch instrument and the inclusion of additional markers enabling characterization of CTC is important to obtain consistent data from CTC enumeration, also for other techniques than the CellSearch system, among different laboratories. However, 'real life' patient samples have an advantage over spiked samples, as this approach is limited by the number of participating laboratories with regards to sample size and availability of patient samples. Alternatively stabilized specimens with minimal matrix effects for all types of reagents and image interpretation, that mimic patient samples as

close as possible, should be developed following the example of running EQA schemes for hematopoietic cells ^{1,2}.

Molecular characterization of CTC

In addition to enumeration, characterization of CTC harbors great opportunities to understand fundamental issues such as their genetic composition, and to establish the association of these genetic profiles with patient treatment outcomes. Given that metastases rather than primary tumors are the main cause of death from cancer and heterogeneity between primary tumor and metastatic sites due to clonal selection and genomic instability ³, characteristics of metastasizing tumor cells are likely to yield better prognostic and predictive information than characteristics of the primary tumor do. In **chapter 3** we demonstrated that it is possible to perform mRNA expression analysis of up to 96 genes (thus conveniently fitting a 96-well plate) specific for as few as a single cell. Even when this single CTC is present in an environment containing large quantities of contaminating leukocytes, the restriction is that the selected genes are not, or below a threshold detection value, i.e. much lower, expressed by these leukocytes. With the restriction of not using genes more dominantly expressed by CellSearch-enriched leukocytes, any gene set specific for any cancer type can be implemented in the method we described. These genes may represent markers to identify the tissue origin of the CTC, as well as more cancer-specific markers such as those useful for drug targeting. The resulting data can be used to further characterize cancer type specific CTC, thereby potentially improving our insight into biological processes and ultimately patient management. Meanwhile, we successfully implemented our developed assay for mRNA expression analysis in published and ongoing clinical studies. In these studies we successfully defined CTC gene expression profiles in metastatic breast ⁴ and colorectal ⁵ cancer patients in order to explore the prognostic value on top of the CTC count. Unfortunately, in the study in patients with metastatic breast cancer, we were not able to validate a prognostic gene expression profile in CTC in an independent validation cohort of patients. One of the underlying reasons might be the current inability to measure all genes of interest in CTC and the possible heterogeneity within the CTC population ^{6,7}. This underscores the need for further development of CTC isolation methods that result in pure CTC populations and/or characterization methods using single CTC to explore CTC heterogeneity with the more powerful next generation sequencing (NGS) technique. To study a single CTC for molecular characterization, the CTC must first be isolated from the (enriched) population. Because of the extremely low frequency of CTC in pre-enriched samples traditional isolation techniques such as flow-sorting are not appropriate and more sophisticated methods using micromanipulation are needed.

At this moment two promising commercial systems are available: (i) the DEP-Array system (Silicon Biosystems) using a microfluidic chip with di-electrophoretic cages to navigate the individual cells by charge after identification with fluorescent markers, recently evaluated by our colleagues in Antwerp⁸; (ii) the CellCollector (Automated Lab Solutions) using a robotic micromanipulator to isolate cells from an immuno-magnetic capture device⁹. Using this system we were able to isolate single CTC from a CellSearch enriched sample for RNA gene profiling and sequencing during a recent demonstration at our laboratory. Another technology that was recently published uses a self-seeding microwell chip to seed single cells in a chip containing 6,400 microwells with a single 5 μm pore in the bottom. After identification by fluorescence microscopy the single cells of interest could be isolated by punching the bottom of the well and characterized by DNA sequencing¹⁰.

Next to isolation of single CTC for molecular characterization, single CTC can also be studied using image techniques combined with *in situ* protein level and DNA level characterizations. This can be performed directly in the CellSearch system using immunofluorescence techniques for protein level characterization, such as for Human Epidermal Growth Factor Receptor 2 (HER2)¹¹, ER estrogen receptor (ER)¹¹, B-cell lymphoma 2 (BCL-2)^{11,12}, proliferation (Ki67)¹¹ and apoptosis (M30)¹², but also for cytogenetics using Fluorescence In Situ Hybridization (FISH)¹³. Recently, we have started a clinical trial within an European consortium to investigate a multiplex *in situ* characterization technology of phosphorylated Her2, ER and mutation in PIK3CA subsequent to CellSearch enumeration and isolation, with the aim to better identify patients responding to trastuzumab treatment (<http://www.caremorectc.eu>).

Limitations of CTC enumeration

Like most CTC detection assays, the CellSearch system relies on EpCam (epithelial cell adhesion molecule, CD326) expression on tumor cells. However, despite the robustness and proven clinical relevance, CellSearch identifies CTC in only 60% of metastatic breast cancer patients. There are three possible explanations for this, (i) the number of CTC are below the detection level (< 1 cell/7.5 mL of blood); (ii) CTC are not captured by the system because of insufficient levels of EpCam expression; (iii) CTC are not identified using the particular cytokeratin staining during the imaging process. In **chapter 4** we described that most “normal-like” breast cancer cell lines, in contrast to other molecular subtypes, lack EpCam expression and were not detected by the EpCam-dependent CellSearch assay. These normal like breast cancer cells have been described to have EMT-characteristics and stem-cell features, and display

aggressive behavior^{14,15}. In recent papers^{16,17} of our group we demonstrated that the majority of EpCam-negative breast cancer cell lines express CD146 and that the detection by the CellSearch system can be improved by using anti-CD146 ferrofluids in combination with pan-cytokeratin and/or CD49f antibodies for imaging. Therefore, we implemented the counting of CD146-positive CTC in a prospective multicenter clinical study in primary breast cancer patients treated with neoadjuvant chemotherapy. In this trial, CD146-positive CTC were enumerated next to EpCAM-positive CTC. We only demonstrated a slightly increased detection rate compared to using the combined enrichment procedure. Currently we are conducting a prospective multicenter clinical study in metastatic breast cancer patients that aims to improve the detection rate of CTC using a dual CD146/EpCAM enrichment approach and to get more insight into the frequency and molecular characteristics of CD146-positive CTC.

Importantly, Hayes et al.¹⁸ reported that using the CellSearch system, CTC were detected in one-third of metastatic triple-negative breast cancer patients (TN MBC), while in particular in these patients, the subtype of CTC with EMT features would be expected, which may lead to low CTC detection rates using an EpCAM-dependent technique. Although these numbers are substantially lower than reported for non-TN MBC, the authors refute our claim that an EpCAM-based assay might not be applicable to patients with TN or 'normal'-like breast cancers due to low expression of this marker¹⁸ as found in our study on our breast cancer cell lines¹⁹.

As the importance of detecting CTC with EMT-characteristics and stem-cell features is widely recognized, several other approaches have been described to measure these events. CTC with mesenchymal features have been detected and thoroughly characterized in the landmark paper by Yu et al.⁶ Using an antibody cocktail against EpCAM, EGFR and HER2, the authors were able to show a CTC subtype with downregulated epithelial RNA transcripts (i.e. encoding for EpCAM, Keratin and Cadherin 1) and upregulated mesenchymal transcripts (i.e. encoding for Fibronectin, Cadherin 2 and Serpin peptidase inhibitor), and suggested a stronger association with disease progression than the epithelial counterpart. Surprisingly, using this assay, which should detect CTC with an epithelial and mesenchymal phenotype, in this study only 41% of MBC patients had detectable CTC. This is lower than using the EpCAM-based CellSearch system²⁰.

Taken together, the question remains whether EpCAM antigen expression is completely absent on CTC with mesenchymal features and are not captured using an EpCAM dependent but sensitive system such as the CellSearch. It is also possible that these cells have a frequency below the detection level of the CellSearch using 7.5 mL of blood and could be only detected by more sensitivity

assays using an larger blood volume such as an apheresis based system^{21,22} or by “fishing” CTC intravenously using a medical wire²³.

Clinical value and standardization of CEC enumeration

Given the importance of tumor vasculature, both in tumor biology and as target for treatment, there is an increasing need for biomarkers accurately reflecting effects affecting tumor vasculature. CEC are increased in number as a result of vascular damage in cancer²⁴⁻²⁹. Counting CEC constitutes a promising tool to monitor disease activity with potential to assess prognosis and response to treatment. In **chapter 5**, we addressed the current state-of-the-art of CEC enumeration as biomarker in clinical oncology. We focussed on technical issues concerning their detection, review results from clinical studies and explore future potential applications. Current studies have yielded promising but still inconsistent results; this situation may be caused by differences between patient populations, and differences between enumeration techniques in the various studies. However, recent publications, including ours, now tend to a consensus regarding the phenotype and frequency of CEC in healthy individuals. A quick, reliable, robust and validated CEC assay that is also affordable was highly needed. In **chapter 6** we presented a novel approach to enumerate CEC using a multi-parameter flow cytometrical (FCM) method without immunological pre-enrichment using a large blood volume and a threshold on a pan epithelial marker. CEC were defined as CD34⁺, CD45^{neg}, CD146⁺ and DNA⁺ events based on the immunophenotype of endothelial cells from vein-wall dissections. As CEC express high levels of CD34, we based our assay on absolute CD34 counts after analyzing all CD34-positive events in a total blood volume of 4 mL. Endothelial origin of CEC was confirmed by morphology, immunohistochemistry and gene expression. Additionally, our FCM assay was tested in parallel with an already validated assay (i.e., CellSearch®, Veridex [Raritan, NJ]). In contrast to other flow cytometric methods, in our method the true endothelial origin of detected events was validated by morphology, immunohistochemistry and gene expression and numbers were significantly higher in carcinoma patients and in patients with hematological malignancies. We envisaged that this flow cytometric method could used as a relatively affordable, high-throughput assay to accurately enumerate and further explore the characteristics of CEC. This will be particularly attractive for large-scale clinical studies to establish the clinical value of CEC in disease with endothelial damage or to monitor angiogenesis-inhibiting therapies.

Our developed assay was used in two subsequent clinical studies to investigate vascular damage. We assessed the kinetics of CEC and explored their prognostic value in patients with recurrent glioblastoma. CEC numbers increased during

treatment with bevacizumab plus lomustine but not during treatment with either agent alone, suggesting that this combination induced the severest vascular damage. Although the absolute number of CEC was not associated with overall survival in patients treated with bevacizumab either alone or in combination, they could serve as a prognostic marker in glioblastoma patients receiving lomustine single agent³⁰. In another study we evaluated the impact of conditioning regimens on CEC as parameter for vascular damage in allogeneic stem cell transplantation. We found that patients receiving myeloablative conditioning have long-term endothelial damage as opposed to patients receiving reduced intensity conditioning. In addition, we observed lower CEC numbers in graft-*versus*-host disease patients, which may possibly be explained by a direct immune response against CEC. Future research should investigate whether such an immune response is indeed present³¹.

Other studies investigating the clinical relevance of such alterations in CEC counts during treatment are warranted.

Improving the specificity of CEC as marker for malignancy

Malignant tumors are critically dependent of the formation of new blood vessels for their growth and dissemination. Unlike normal tissues, which use sprouting angiogenesis, vasculogenesis and intussusception to form new vessels, tumors also use additional modes of vessel formation. These include the cooptation of pre-existing vessels, the imitation of tumor vasculature by tumor cells (also known as vascular mimicry), and the differentiation of putative cancer stem-like cells into tumor endothelium. As a result, tumor vasculature differs morphologically from normal vasculature, as it features an apparently chaotic mixture of abnormal and hierarchically disorganized vessels with an increased permeability. Next to morphologic differences, in recent years many tumor-associated endothelial markers (TEM) have been identified by comparing gene and protein expression levels between normal and tumor endothelial cells demonstrating distinctive gene and protein profiles. CEC measured in patients with advanced cancer are thought not only to derive from damaged normal vasculature (n-CEC), but also from damaged tumor vasculature (t-CEC). Therefore, assays that allow the discrimination between these two putative types of CEC are thought to improve the specificity of the enumeration of CEC in cancer. In **chapter 7** we identified antigens that were overexpressed in endothelial cells isolated from resected tumor tissue compared to corresponding normal tissue. Potential TEM such as CD54, CD109, CD137 and CXCR7 did not differ in expression between EC derived from normal and malignant tissue in a series of colorectal carcinomas and liposarcomas. In contrast, higher expression of CD105, CD146, CD276 and CD309 was found on

tumor EC compared to EC from normal tissue. These observations are in line with other reports. Over-expression of CD105 on tumor-associated EC has been reported for hepatocellular carcinoma³², breast, colon, lung, prostate and brain tumors³³, while up regulation of CD146 and CD309 has been described in many cancers including breast, prostate, melanoma and glioma^{34,35}. Among these antigens CD276 was most differently expressed in our series and most promising as a marker to discriminate t-CEC from n-CEC. CD276 is a member of the B7 family of immune-regulatory molecules that can be induced on T cells, B cells and dendritic cells by a variety of inflammatory cytokines^{36,37}. The ligand for CD276 has not yet been identified and neither has its immunoregulatory role. CD276 has been suggested to have an immunosuppressive role in the interaction between tumors and the immune system³⁸. Additionally, several others have shown that expression of CD276 was associated with a relatively aggressive tumor behavior in a range of various carcinomas³⁹⁻⁴⁴ and glioma³⁸. We subsequently investigated and incorporated CD276 into our CEC detection assay as described before. Indeed, CD276⁺ CEC occurred in higher frequencies in patients with advanced cancer compared to healthy donors. In addition to defining potential differences between various tumor types and tumor stages, it is important to further investigate the potential clinical relevance of these cells in oncology. To do so, further clinical studies are needed to investigate the association of CD276⁺ CEC levels at baseline with outcome, to examine the use of CD276⁺ CEC as a marker for response assessment by serially determining CD276⁺ CEC levels during treatment and to establish whether or not changes are associated with outcome to treatment as determined by conventional means, and to determine whether targets for treatment can be revealed on these cells. Currently the CD276⁺ CEC assay has been implemented in three prospective multicenter clinical studies in patients with recurrent glioblastoma, advanced colon carcinoma and recurrent clear cell carcinoma of the ovary to (partly) answer these research questions.

Altogether, we have described the improvement, standardization and validation of both CTC and CEC assays that have been used in recently published papers, are being used in ongoing clinical trials and can be used in future clinical studies to obtain predictive and prognostic information. This information should ultimately lead to improved individualization of cancer treatment.

References

1. Barnett D, Granger V, Kraan J, et al. Reduction of intra- and interlaboratory variation in CD34+ stem cell enumeration using stable test material, standard protocols and targeted training. DK34 Task Force of the European Working Group of Clinical Cell Analysis (EWGCCA). *Br J Haematol*. 2000;108:784-792.
2. Whitby L, Granger V, Storie I, et al. Quality control of CD4+ T-lymphocyte enumeration: results from the last 9 years of the United Kingdom National External Quality Assessment Scheme for Immune Monitoring (1993-2001). *Cytometry*. 2002;50:102-110.
3. Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. *Nature*. 2011;472:90-94.
4. Mostert B, Sieuwerts AM, Kraan J, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. *Ann Oncol*. 2015;26:510-516.
5. Mostert B, Sieuwerts AM, Bolt-de Vries J, et al. mRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients. *Mol Oncol*. 2015;9:920-932.
6. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*. 2013;339:580-584.
7. Powell AA, Talasz AH, Zhang H, et al. Single cell profiling of circulating tumor cells: transcriptional heterogeneity and diversity from breast cancer cell lines. *PLoS One*. 2012;7:e33788.
8. Peeters DJ, De Laere B, Van den Eynden GG, et al. Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting. *Br J Cancer*. 2013;108:1358-1367.
9. Choi JH, Ogunniyi AO, Du M, et al. Development and optimization of a process for automated recovery of single cells identified by microengraving. *Biotechnol Prog*. 2010;26:888-895.
10. Swennenhuis JF, Tibbe AG, Stevens M, et al. Self-seeding microwell chip for the isolation and characterization of single cells. *Lab Chip*. 2015;15:3039-3046.
11. Paoletti C, Muniz MC, Thomas DG, et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer. *Clin Cancer Res*. 2015;21:2487-2498.
12. Smerage JB, Budd GT, Doyle GV, et al. Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. *Mol Oncol*. 2013;7:680-692.
13. Swennenhuis JF, Tibbe AG, Levink R, Sipkema RC, Terstappen LW. Characterization of circulating tumor cells by fluorescence in situ hybridization. *Cytometry A*. 2009;75:520-527.
14. Kao J, Salari K, Bocanegra M, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One*. 2009;4:e6146.
15. Woelfle U, Sauter G, Santjer S, Brakenhoff R, Pantel K. Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer. *Clin Cancer Res*. 2004;10:2670-2674.
16. Mostert B, Kraan J, Bolt-de Vries J, et al. Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146. *Breast Cancer Res Treat*. 2011;127:33-41.
17. Mostert B, Kraan J, Sieuwerts AM, et al. CD49f-based selection of circulating tumor cells (CTCs) improves detection across breast cancer subtypes. *Cancer Lett*. 2012;319:49-55.

18. Paoletti C, Li Y, Muniz MC, et al. Significance of Circulating Tumor Cells in Metastatic Triple-Negative Breast Cancer Patients within a Randomized, Phase II Trial: TBCRC 019. *Clin Cancer Res.* 2015;21:2771-2779.
19. Sieuwerts AM, Kraan J, Bolt J, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J Natl Cancer Inst.* 2009;101:61-66.
20. Lianidou ES, Mavroudis D, Georgoulas V. Clinical challenges in the molecular characterization of circulating tumour cells in breast cancer. *Br J Cancer.* 2013;108:2426-2432.
21. Barradas AM, Terstappen LW. Towards the Biological Understanding of CTC: Capture Technologies, Definitions and Potential to Create Metastasis. *Cancers (Basel).* 2013;5:1619-1642.
22. Coumans FA, Ligthart ST, Uhr JW, Terstappen LW. Challenges in the enumeration and phenotyping of CTC. *Clin Cancer Res.* 2012;18:5711-5718.
23. Saucedo-Zeni N, Mewes S, Niestroj R, et al. A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. *Int J Oncol.* 2012;41:1241-1250.
24. Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat Rev Cancer.* 2006;6:835-845.
25. Furstenberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer.* 2006;94:524-531.
26. Goon PK, Boos CJ, Stonelake PS, Blann AD, Lip GY. Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. *Thromb Haemost.* 2006;96:45-52.
27. Goon PK, Boos CJ, Lip GY. Circulating endothelial cells: markers of vascular dysfunction. *Clin Lab.* 2005;51:531-538.
28. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A.* 2007;71:105-113.
29. Strijbos MH, Gratama JW, Schmitz PI, et al. Circulating endothelial cells, circulating tumour cells, tissue factor, endothelin-1 and overall survival in prostate cancer patients treated with docetaxel. *Eur J Cancer.* 2010;46:2027-2035.
30. Beijer N, Kraan J, Taal W, 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.
31. Beijer 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;100:e246-249.
32. Xiong YQ, Sun HC, Zhang W, et al. Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. *Clin Cancer Res.* 2009;15:4838-4846.
33. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J.* 2003;17:984-992.
34. Ouhiti A, Gaur RL, Abd Elmageed ZY, et al. Towards understanding the mode of action of the multifaceted cell adhesion receptor CD146. *Biochim Biophys Acta.* 2009;1795:130-136.
35. Plate KH, Breier G, Millauer B, Ullrich A, Risau W. Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res.* 1993;53:5822-5827.

36. Chapoval AI, Ni J, Lau JS, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol.* 2001;2:269-274.
37. Steinberger P, Majdic O, Derdak SV, et al. Molecular characterization of human 4Ig-B7-H3, a member of the B7 family with four Ig-like domains. *J Immunol.* 2004;172:2352-2359.
38. Lemke D, Pfenning PN, Sahm F, et al. Costimulatory protein 4IgB7H3 drives the malignant phenotype of glioblastoma by mediating immune escape and invasiveness. *Clin Cancer Res.* 2012;18:105-117.
39. Crispen PL, Sheinin Y, Roth TJ, et al. Tumor cell and tumor vasculature expression of B7-H3 predict survival in clear cell renal cell carcinoma. *Clin Cancer Res.* 2008;14:5150-5157.
40. Qin X, Zhang H, Ye D, Dai B, Zhu Y, Shi G. B7-H3 is a new cancer-specific endothelial marker in clear cell renal cell carcinoma. *Onco Targets Ther.* 2013;6:1667-1673.
41. Roth TJ, Sheinin Y, Lohse CM, et al. B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy. *Cancer Res.* 2007;67:7893-7900.
42. Sun Y, Wang Y, Zhao J, et al. B7-H3 and B7-H4 expression in non-small-cell lung cancer. *Lung Cancer.* 2006;53:143-151.
43. Yamato I, Sho M, Nomi T, et al. Clinical importance of B7-H3 expression in human pancreatic cancer. *Br J Cancer.* 2009;101:1709-1716.
44. Zang X, Sullivan PS, Soslow RA, et al. Tumor associated endothelial expression of B7-H3 predicts survival in ovarian carcinomas. *Mod Pathol.* 2010;23:1104-1112.

Appendices

SAMENVATTING

Kanker is wereldwijd de op één na voornaamste oorzaak van ziekte en sterfte; in Nederland is kanker zelfs de meest voorkomende doodsoorzaak. Meer dan 90% van de sterfgevallen bij patiënten met solide tumoren wordt veroorzaakt door uitzaaiingen (metastasen) van de primaire tumor. Tijdens het proces van metastasering verspreiden kankercellen van de primaire tumor zich via de bloedbaan naar ander locaties in het lichaam en kunnen daar secundaire tumoren vormen. De laatste jaren zijn veel nieuwe antikankermiddelen ontwikkeld tegen de verschillende stappen in het proces van metastasering. Hoewel de levensverwachting van patiënten met kanker de laatste jaren sterk verbeterd is door de introductie van nieuwe behandelingen is er ook behoefte aan klinisch informatieve bio-markers voor het bepalen van de optimale dosis, het monitoren van de biologische activiteit en het selecteren van de patiënten die hoogstwaarschijnlijk profiteren van een (experimentele) behandeling.

De studies in dit proefschrift hebben als doel om de voorspellende en prognostische informatie die kan worden verkregen uit circulerende tumorcellen (CTC) en circulerende endotheelcellen (CEC) in volbloed te optimaliseren. Zowel CTC als CEC zijn veelbelovende bio-markers voor onderzoeksdoeleinden en behandeling van de oncologische patiënt, die eenvoudig en regelmatig kan worden verkregen door een venapunctie (“vloeibaar bipt”).

Standaardisatie van CTC detectie

Hoewel het tellen van CTC al van grote waarde is gebleken, wordt de implementatie als klinische routinebepaling sterk bemoeilijkt door het zeer lage aantal CTC in bloed. Meestal is er slechts sprake van enkele tumorcellen in een milliliter bloed te midden van miljarden kernhoudende bloed cellen. Pas sinds het begin van deze eeuw is een semi-geautomatiseerde methode beschikbaar (“CellSearch”) methode genaamd, die door de Food and Drug Administration (FDA) is goedgekeurd voor het bepalen van CTC als prognostische factor in verschillende tumortypes van epitheliale origine. Deze methode maakt gebruik van de aanwezigheid van bepaalde eiwitten op CTC. In de eerste stap van de test wordt bloed verrijkt voor cellen die expressie vertonen van epitheliaal celadhesiemolecuul (EpCAM, CD326), een antigeen dat op tumorcellen voorkomt en niet op witte bloedcellen. Deze cellen worden uit het bloed geïsoleerd door gebruik te maken van “ferrofluids”: dit zijn kleine ijzerdeeltjes beladen met een antistof tegen EpCAM, waardoor aantrekking door een sterk magnetisch veld mogelijk wordt. De overige cellen, die niet beladen zijn met anti-EpCAM-ferrofluids, worden weggewassen. De verrijkte celsuspensie kan worden gebruikt voor telling

en verdere (moleculaire)typering. Bij de telling van CTC volgen na de immunomagnetische verrijking een aantal stappen waarin de verrijkte CTC d.m.v. beeldcytometrie worden gedetecteerd op basis van de expressie van (i) cytokeratines, (ii) een kernkleuring en (iii) de afwezigheid van CD45, een merker voor witte bloedcellen.

In **hoofdstuk 2** testen we de haalbaarheid van een extern kwaliteitsborgingsprogramma voor het tellen van CTC op basis van de CellSearch procedure: van bloedafname tot telling van het aantal CTC door 14 laboratoria. We onderzochten niet alleen de variatie binnen de test, de laboratoria en binnen de instrumenten maar ook de variatie in het beoordelen van geblindeerde beeldopnamen van verrijkte celsuspensies uit het CellSearch apparaat door meerdere analisten. Onze studie toont de haalbaarheid van een dergelijk extern kwaliteitsborgingsprogramma voor telling van CTC in het perifere bloed aan en de mogelijke meerwaarde voor standaardisatie van deze en andere CTC bepalingen.

Moleculaire karakterisering van CTC

Naast tellen van CTC is een gedetailleerdere analyse van deze cellen van belang om fundamentele kwesties te begrijpen. Onderzoek naar de genetische opbouw van CTC en onderzoek naar het verband tussen deze genetische profielen en de uitkomst van bepaalde behandelingen zijn van groot klinisch belang. Aangezien metastasen, waarvan CTC worden verondersteld de voorlopers van te zijn, en niet primaire tumoren de voornaamste doodsoorzaak van kanker zijn. En het verder bekend is dat er verschillen zijn tussen de kenmerken van de primaire tumor en metastasen als gevolg van klonale selectie en genomische instabiliteit, zullen de kenmerken van metastaserende tumorcellen waarschijnlijk betere voorspellende informatie opleveren dan de kenmerken van de primaire tumor.

In **hoofdstuk 3** tonen we aan dat het mogelijk is om de mRNA expressie van 96 genen te bepalen in één CTC. Deze detectie is zelfs mogelijk wanneer deze (ene) CTC aanwezig is in een achtergrond van relatief veel witte bloedcellen wanneer de geselecteerde genen niet, of onder een drempelwaarde, tot expressie komen in witte bloedcellen.

Beperkingen van CTC bepalingen

De CellSearch benadering maakt gebruik van de expressie van EpCAM op tumorcellen. Ondanks de robuustheid en bewezen klinische relevantie, identificeert de CellSearch CTC in slechts 60% van de patiënten met gemetastaseerde borstkanker. Er zijn drie mogelijke verklaringen hiervoor, (i) het aantal CTC is lager dan de detectiegrens (<1 cellen / 7,5 ml bloed); (ii) CTC worden niet door het

systeem geïsoleerd vanwege het ontbreken van voldoende EpCAM-expressie op individuele CTC; (iii) CTC worden niet geïdentificeerd met behulp van de cytokeratine antistoffen gebruikt in de CellSearch assay.

In **hoofdstuk 4** onderzoeken we de EpCAM expressie van verschillende borstkanker tumor subtypes op gekweekte cellen(cellijnen). We toonden aan dat EpCAM te laag tot expressie komt op een subgroep van borstkanker cellijnen om gedetecteerd te kunnen worden door het CellSearch systeem. Gezien de karakteristieken van dit subtype zijn deze mogelijk representatief voor een agressieve vorm van borstkanker. Dit hoofdstuk besluit met aanbevelingen gedaan en vervolgstudies gepland om met alternatieve antistoffen te onderzoeken of deze subpopulaties van CTC kunnen worden gedetecteerd in bloed van patiënten.

Klinische waarde en standaardisatie van het bepalen van CEC

De binnenwand van bloedvaten wordt gevormd door ca. 1,2 biljoen endotheelcellen en is niet alleen een barrière tussen bloed en de organen, maar ook een zeer dynamische structuur en betrokken bij vele aspecten van de vaatbiologie. CEC zijn bij gezonde personen slechts in zeer lage aantallen aantoonbaar en een toename van het aantal CEC duidt op vaatschade. Tumoren zijn voor hun groei afhankelijk van de aanleg van nieuwe bloedvaten. Gezien het belang van het bloedvatstelsel voor de tumor, zowel voor de tumorbiologie als ook als doelwit voor behandeling, is er behoefte aan biomarkers die zowel de activiteit als de schade aan het tumor vaatbed door behandeling kunnen meten. In **hoofdstuk 5** beschrijven we de huidige stand van zaken in het CEC onderzoeksveld binnen de oncologie. Recente studies hebben ogenschijnlijk veelbelovende maar inconsistente resultaten opgeleverd; deze variabiliteit kan worden veroorzaakt door verschillen tussen patiëntenpopulaties en de verschillen in de gebruikte technieken voor het bepalen van CEC. Wij concluderen dan ook dat er behoefte is aan een snelle, betrouwbare, robuuste en gevalideerde CEC bepaling.

In **hoofdstuk 6** presenteren we een nieuwe gevalideerde benadering voor het tellen van CEC. Door gebruik te maken van multi-parameter flowcytometrische bepaling en een drempelwaarde op een eiwit dat hoog tot expressie komt op endotheel cellen (CD34) kunnen we, in tegenstelling tot conventionele testen, een veel groter bloedvolume analyseren waardoor de betrouwbaarheid van de test wordt verhoogd.

Verbetering van de specificiteit van CEC als indicator voor maligniteit

De structuur en fysiologie van het tumor vaatbed verschilt sterk van de normale vasculatuur. Tumorvaten zijn chaotisch georganiseerd en fragiel, en hebben een verhoogde doorlaatbaarheid. Naast morfologische verschillen zijn er in de afgelopen jaren veel tumor-geassocieerde endotheliale markers (TEM) geïdentificeerd door vergelijking van gen- en eiwitexpressie niveaus tussen normale en tumor-endotheelcellen.

In **hoofdstuk 7** identificeren we eiwitten die verhoogd tot expressie komen in endotheelcellen geïsoleerd uit tumorweefsel in vergelijking met normaal weefsel van hetzelfde histologische type. Door toevoeging van antistoffen tegen deze eiwitten in de door ons ontwikkelde test wordt het mogelijk onderscheid te maken tussen CEC afkomstig uit de tumoren en CEC afkomstig van normaal weefsel. Hierdoor wordt het mogelijk om vaatschade die specifiek optreedt in tumoren te meten tijdens de behandeling van kanker.

In dit proefschrift hebben we de verbetering, standaardisatie en validatie van zowel CTC als CEC testen die gebruikt werden in recente publicaties beschreven. Deze assays worden toegepast in lopende klinische studies en kunnen worden toegepast in toekomstige klinische studies voor het verkrijgen van voorspellende en prognostische informatie. Deze informatie moet uiteindelijk leiden tot een betere individualisering van de behandeling van kanker.

DANKWOORD

Allen die een bijdrage hebben geleverd aan dit proefschrift wil ik hiervoor hartelijk danken, een aantal van hen wil ik hieronder graag noemen.

Mijn Promotor, prof.dr. J.A. Foekens. Beste John, jij was één van de initiatiefnemers van het CTC programma binnen de afdeling en de aanschaf van het CellSearch instrument, het begin van samenwerking tussen twee verschillende groepen met elk hun eigen expertise. Hierdoor ontstond een succesvol onderzoeksgebied waarin ik met veel plezier werkzaam ben en ik met velen anderen een succesvol promotieonderzoek kon doen. Bedankt voor je ondersteuning gedurende mijn promotietraject, je adviezen en eerlijke standpunten waren van grote waarde.

Mijn Co-promotor, dr. J.W. Gratama. Beste Jan Willem, we begonnen meer dan 25 jaar geleden nagenoeg tegelijkertijd binnen de afdeling Medische en tumor immunologie in de Daniel den Hoed kliniek. Jij als laboratoriumarts en ik in mijn eerste echte baan als analist. Ik kijk met heel veel plezier terug op die periode waarin we succesvol hebben bijgedragen aan de ontwikkeling van de klinische flowcytometrie binnen onze afdeling, maar ook binnen Nederland en ver daarbuiten. Ik ben dankbaar dat je me altijd hebt gestimuleerd om me verder te ontwikkelen. Al eerder adviseerde je mij om promotie onderzoek te gaan doen en hoe mooi dat het CEC/CTC onderzoek deze mogelijkheid gaf. Bedankt voor je onmisbare bijdragen aan dit proefschrift.

De leden van de kleine commissie, prof.dr. L.H.J. Looijenga, prof.dr. L.W.M.M. Terstappen, en prof.dr. C. Verhoef, hartelijk dank voor het plaatsnemen in de kleine commissie en kritisch doorlezen van het manuscript.

De leden van de grote commissie, prof.dr. E.E. Voest, prof.dr. S. Sleijfer, dr. J.W.M. Martens, dank ik voor het plaatsnemen in de commissie.

Prof.dr. S. Sleijfer, afdelingshoofd Interne Oncologie. Beste Stefan, onder jouw bezielende leiding meewerken aan CEC en CTC onderzoek is een feest, door jouw inzet werd er succesvol samengewerkt en konden velen excelleren op hun specifieke expertise. Ik hoop dat er nog vele dinsdagochtend besprekingen met jouw “helden” volgen.

Dr. J.W.M. Martens. Beste John, na de verhuizing van de CTC/CEC groep vanuit de Daniel naar het Josephine Nefkens Instituut zijn we deel uit gaan maken van

jouw werkgroep en dat voelt goed. Nu deze klus is geklaard en we onder één dak zitten kan onze samenwerking alleen maar succesvoller worden.

Patrick Aerts, Petra van der Spoel en Patricia van den Broek, jullie waren achtereenvolgens de analisten die mij bijstonden op het lab voor het CTC/CEC werk, bedankt voor jullie tomeloze inzet. Helaas vertrokken jullie alle drie (hoe zou dat toch komen?) om elders je carrière succesvol voort te zetten.

Mai Van en Zahra Alawi, jullie namen het stokje over en verwerken al enkele jaren op een efficiënte manier het leeuwendeel van de CTC/CEC samples. Mai geweldig hoe je de dingen op het lab regelt en apparatuur met zorg onderhoud.

Anieta Sieuwerts, ook in dit proefschrift was jouw aandeel weer onmisbaar, door jou unieke kennis en vaardigheid op het gebied van PCR hebben we snelle en prachtige resultaten kunnen behalen(en er gaat zeker meer komen) met de karakterisatie van CTC. Ook bedankt voor de gezellige momenten op het lab en tijdens onze congres bezoeken.

Joan Bolt, de vraagbaak van het JNl, dank voor al het werk dat je verzet hebt voor de CTC studies en je hulp en aanwijzingen bij de verhuizing.

Alle mede-promovendi in het CEC/CTC werk door de jaren heen: Michiel Strijbos, Bianca Mostert, Esther Reijm, Wendy Onstenk, Nick Beije en Inge de Kruijff. Dank voor jullie inspirerende inbreng, fijne samenwerking, borrels en ganggesprekken. Veel succes met jullie carrières en promoties. Michiel, bedankt voor je (voor)werk in CEC onderzoek waarmee je een fundamentele bijdrage hebt geleverd aan het CEC gedeelte van dit proefschrift (die CMOIC hebben we maar mooi ontmaskerd). Bianca jij beet het spits af met het CTC onderzoek met een prachtig resultaat en hoewel het een hele heksentoer was om met jou te werken op het lab ben ik toch blij dat je weer terug bent in het EMC en weer samenwerken.

Els Berns, Jozien Helleman en Maurice Janssen, bedankt voor het meedenken en de belangstelling voor dit werk. We werken inmiddels samen in andere onderzoeken en hoop dat succesvol zal worden.

Rik Brooimans, hoewel je niet direct betrokken was bij dit project, hebben we lang samengewerkt en een kamer gedeeld. Bedankt voor de zeer prettige samenwerking, goede gesprekken en gezelligheid.

Cor Lamers, bedankt voor de goede samenwerking en adviezen, we kunnen het gelukkig gewoon voortzetten in het JNl

Marian Reedijk, bedankt voor alle hulp bij administratieve zaken, voor alle verzendingen voor de EQA projecten en voor af toe een gezellig praatje of even stoom afblazen.

Alle collega's van het voormalige lab medische en tumorimmunologie in de Daniel dank ik voor de fijne samenwerking, ondersteuning bij de researchprojecten en gezellige momenten rond de inschrijftafel. Ook de collega's in het JNl dank ik voor hun betrokkenheid bij dit project en het warme onthaal op de 4^e etage.

Mijn paranimfen Wendy Onstenk en Dew Doekharan: ik ben trots dat jullie mij bij willen staan tijdens de verdediging van mijn proefschrift.

Wendy, we zijn alweer bijna 2 jaar kamergenoten en jij bent mijn favoriete roomie (sorry Esther). Bedankt voor het halen van koffie als ik die even nodig had en weer eens geen antwoord gaf, nu ben ik aan de beurt.

Dew, dankzij jouw gouden tip heb ik ooit gesolliciteerd in de Daniel en zijn we lang collega's geweest met verhelderende werkgesprekken en gezellige vrijdag lunches bij van der Steen. Bedankt dat jullie mijn paranimfen wilden zijn.

Jongetje Borsje, heren Lansingh en heren van het Riskgenootschap bedankt voor de ontspanning en goede gesprekken die gelukkig zelden over werk gingen.

Familie en schoonfamilie kan je niet uitzoeken, maar ik heb het bijzonder getroffen met zoveel fijne mensen om me heen, bedankt voor jullie interesse, steun en liefde. Pa en ma, bedankt voor een onbezorgde jeugd en de waarden en normen die jullie mij meegaven.

Karsten en Julia, wat ben ik trots om te zien hoe jullie opgroeien en volwassen worden. Prachtig, hoe jullie al een goede koers gevonden hebben en met jullie scheepsmaatje lekker hoog aan de wind varen, recht zo die gaat.

Allerliefste Anne natuurlijk zijn de laatste worden voor jou, dank voor je steun en liefde, wat ben ik nog steeds ontzettend blij en dankbaar dat jij ervoor mij bent.

PhD PORTFOLIO

PHD TRAINING	Year	Workload (ECTS)
General academic skills		
– Biomedical English writing and communication	2010	4.0
– Photoshop and Illustrator CS5 workshop	2011	0.3
– Biostatistics course on SPSS	2014	1.0
In-depth courses		
– Biomedical Research Techniques	2009	2.0
– Workshop prestatie financiering onderzoek en citatieanalyse	2009	0.2
– NCHA/CGC valorization workshop	2010	0.2
– CPO course Methodologie van patiëntgebonden onderzoek en voorbereiding subsidie aanvragen.	2010	0.3
Seminars and workshops		
– Lorentz Workshop: Circulating tumor cell isolation and diagnostics	2011	2.0
– Cancer Genomics Centre Annual Scientific Meeting	2010 – 2013	1.5
– Quarterly CTC meeting Oncology	2010 – 2014	1.5
– Monthly JNI Oncology lectures	2012 – 2014	1.2
– Weekly Journal Club	2010 – 2014	2.0
Presentations		
– Oral presentation at 7th International symposium on minimal residual cancer, Athens, Greece	2009	1.0
– Oral presentation at the Medical Oncology Science Meetings	2009	1.0
– Oral presentation at NVC conference, Zwolle	2010	1.0
– Oral presentation at annual CYTO conference Seattle, WA	2010	1.0
– Oral presentation at 10 th ESCCA conference Valencia, Spain	2010	1.0
– Oral presentation at 6 th Clinical Course of the ESCCA ‘Cellular biomarkers of cancer’, Valencia, Spain	2010	1.0
– Oral presentation at the Medical Oncology Science Meetings	2012	1.0

–Oral presentations at 8 th Clinical Course of the ESCCA ‘Vascular Biology’, Budapest, Hungary	2012	2.0
–Oral presentations at 13 th ESCCA conference Luxembourg	2012	2.0
–Oral presentation at the Scientific JNI Lab Meeting	2013	1.0
–Poster presentation during 15 th ISLH symposium, Toronto, Canada	2013	1.0
– Oral presentation at 3 rd annual World CTC, Berlin	2013	1.0
–Oral presentations at 1 st Italian CEC workshop, University of Chieti, Italy	2014	2.0
–Oral presentation at 14 th ESCCA conference, Lisbon, Portugal	2014	1.0
–Oral presentation at NVC conference, Zwolle	2014	1.0
–Oral presentations at 1 st Italian CEC workshop, University of Catanzaro, Italy	2015	2.0

National conferences

–Nederlandse vereniging voor Cytometrie	2009-2014	1.0
---	-----------	-----

International conferences

–7th International symposium on Minimal Residual Cancer, Athens, Greece	2009	1.0
–Joint meeting of the Belgian Society for Analytical Cytology and the Dutch Society for Cytometry	2009	1.0
–Annual CYTO conference, Seattle, WA	2010	1.0
–6 th Clinical Course of the ESCCA ‘Cellular biomarker of cancer’, Valencia, Spain	2010	1.0
–10 th ESCCA conference Valencia, Spain	2010	1.0
–11 th ESCCA conference , Dublin, Ireland	2011	1.0
–12 th ESCCA conference, Budapest, Hungary	2012	1.0
–3 rd annual World CTC conference, Berlin, Germany	2013	1.0
–26 th ISLH symposium, Toronto, Canada	2013	1.0
–13 th ESCCA conference, Luxembourg	2013	1.0
–1 st Italian CEC workshop, Chieti, Italy	2014	0.3
–14 th ESCCA conference, Lisbon, Portugal	2014	1.0

TEACHING ACTIVITIES

Supervising

–Junior Med School: 2 high school students	2011	1.0
–21-week research project for 4 th year Medical student	2011-2012	2.5

-Technical support and advise to 8 PhD student	2008-2015	8.0
--	-----------	-----

Lecturing

-MOLMED, Biomedical research techniques, Introduction to Flow cytometry	2009-2014	1.0
-‘Cellular biomarker of cancer’ on 6 TH ESCCA Course, Valencia, Spain	2010	1.0
-Course organizer and lecturer ‘Vascular Biology’ on 8 TH ESCCA Course, Budapest, Hungary	2012	2.0
-‘Clinical Cytometry’ on 1 st Bi-Society ICCCS/ESCCA Course, Lisbon, Portugal	2014	1.0

Reviewing papers for international peer-reviewed journals

- Cytometry part A
- Cytometry part B Clinical Cytometry
- Expert Review of Molecular Diagnostics
- Future Medicine
- Journal of Visualized Experiments

CURRICULUM VITAE

Jaco Kraan werd op 1 augustus 1966 geboren in Utrecht. In 1983 behaalde hij het HAVO diploma aan het Comenius College te Capelle aan den IJssel. In datzelfde jaar begon hij aan de studie Hoger Laboratorium Onderwijs (HLO) aan het van 't Hoff instituut te Rotterdam en behaalde in 1988 de ingenieurs titel in de medisch richting. Aansluitend werd de militaire dienstplicht vervuld waarin hij na de onderofficiersopleiding een jaar lang werkzaam was als analist in het medisch microbiologisch laboratorium van het Militair Hospitaal Dr. A. Mathijssen te Utrecht. In 1989 trad hij in dienst als medisch analist op de afdeling Medische en Tumor Immunologie in de toenmalige Dr. Daniel den Hoed Kliniek (hoofd Dr. R.L.H. Bolhuis). Hier werkte hij achtereenvolgens als analist met specifieke taken, hoofanalist en wetenschappelijk laboratorium medewerker op het gebied van diagnostiek van maligniteiten, kwaliteitsbewaking en onderzoek (hoofd dr. J.W. Gratama). Tijdens dit dienstverband was hij lid van diverse nationale en internationale werkgroepen voor kwaliteitsbewaking, onderzoek en ontwikkeling van diagnostisch bepalingen binnen zijn vakgebied en medeauteur van meerder consensus richtlijnen. In 2010 startte hij een promotie onderzoek op de afdeling Interne Oncologie onder supervisie van Prof. dr. J Foekens en Dr. J.W. Gratama, met dit proefschrift als resultaat. Inmiddels is de Daniel den Hoed Kliniek onderdeel van het Erasmus MC in Rotterdam en werkt hij hier binnen de afdeling Interne Oncologie (hoofd Prof. dr. S. Sleijfer), werkgroep Cancer Genomics and Proteomics (hoofd Dr. J.W.M. Martens), als onderzoeker op het gebied van circulerende tumor cellen en circulerende (tumor)endotheel cellen. Hij is getrouwd met Annemarie Hardam en heeft een zoon en dochter, Karsten en Julia.

LIST OF PUBLICATIONS

Publications related to this thesis

Onstenk W, Sieuwerts AM, **Kraan J**, Van M, Nieuweboer AJ, Mathijssen RH, Hamberg P, Meulenbeld HJ, De Laere B, Dirix LY, van Soest RJ, Lolkema MP, Martens JW, van Weerden WM, Jenster GW, Foekens JA, de Wit R, Sleijfer S. Efficacy of Cabazitaxel in Castration-resistant Prostate Cancer Is Independent of the Presence of AR-V7 in Circulating Tumor Cells. *Eur Urol*. 2015 Jul 15

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.

Onstenk W, Sieuwerts AM, Weekhout M, Mostert B, Reijm EA, van Deurzen CH, Bolt-de Vries JB, Peeters DJ, Hamberg P, Seynaeve C, Jager A, de Jongh FE, Smid M, Dirix LY, Kehler DF, van Galen A, Ramirez-Moreno R, **Kraan J**, Van M, Gratama JW, Martens JW, Foekens JA, Sleijfer S. Gene expression profiles of circulating tumor cells versus primary tumors in metastatic breast cancer. *Cancer Lett*. 2015 Jun 28;362(1):36-44.

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.

Lalmahomed ZS, Mostert B, Onstenk W, **Kraan J**, Ayez N, Gratama JW, Grünhagen D, Verhoef C, Sleijfer S. 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.

Onstenk W, **Kraan J**, Mostert B, Timmermans MM, Charehbili A, Smit VT, Kroep JR, Nortier JW, van de Ven S, Heijns JB, Kessels LW, van Laarhoven HW, Bos MM, van de Velde CJ, Gratama JW, Sieuwerts AM, Martens JW, Foekens JA, Sleijfer S. Improved circulating tumor cell detection by a combined EpCAM and MCAM CellSearch enrichment approach in breast cancer patients undergoing neoadjuvant chemotherapy. *Mol Cancer Ther*. 2015 Mar;14(3):821-7

Mostert B, Sieuwerts AM, **Kraan J**, Bolt-de Vries J, van der Spoel P, van Galen A, Peeters DJ, Dirix LY, Seynaeve CM, Jager A, de Jongh FE, Hamberg P, Stouthard JM, Kehler DF, Look MP, Smid M, Gratama JW, Foekens JA, Martens JW, Sleijfer S. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. *Ann Oncol*. 2015 Mar;26(3):510-6.

Kraan J, van den Broek P, Verhoef C, Grunhagen DJ, Taal W, Gratama JW, Sleijfer S. 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.

Ignatiadis M, Riethdorf S, Bidard FC, Vaucher I, Khazour M, Rothe F, Metallo J, Rouas G, Payne RE, Coombes R, Teufel I, Andergassen U, Apostolaki S, Politaki E, Mavroudis D, Bessi S, Pestrin M, Di Leo A, Campion M, Reinholz M, Perez E, Piccart M, Borgen E, Naume B, Jimenez J, Aura C, Zorzino L, Cassatella M, Sandri M, Mostert B, Sleijfer S, **Kraan J**, Janni W, Fehm T, Rack B, Terstappen L, Repollet M, Pierga JY, Miller C, Sotiriou C, Michiels S, Pantel K. International study on inter-reader variability for circulating tumor cells in breast cancer. *Breast Cancer Res*. 2014 Apr 23;16(2):R43

Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-de Vries J, Biermann K, **Kraan J**, Lalmahomed Z, van Galen A, de Weerd V, van der Spoel P, Ramirez-Moreno R, Verhoef C, Ijzermans JN, Wang Y, Gratama JW, Foekens JA, Sleijfer S, Martens JW. 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.

Mostert B, **Kraan J**, Sieuwerts AM, van der Spoel P, Bolt-de Vries J, Prager-van der Smissen WJ, Smid M, Timmermans AM, Martens JW, Gratama JW, Foekens JA, Sleijfer S. CD49f-based selection of circulating tumor cells (CTC) improves detection across breast cancer subtypes. *Cancer Lett*. 2012 Jun 1;319(1):49-55.

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

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.

Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, Dirix LY, van Dam PA, Van Galen A, de Weerd V, **Kraan J**, van der Spoel P, Ramirez-Moreno R, van Deurzen CH, Smid M, Yu JX, Jiang J, Wang Y, Gratama JW, Sleijfer S, Foekens JA, Martens JW. 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.

Mostert B, **Kraan J**, Bolt-de Vries J, van der Spoel P, Sieuwerts AM, Schutte M, Timmermans AM, Foekens R, Martens JW, Gratama JW, Foekens JA, Sleijfer S. 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.

Kraan J, Sleijfer S, Strijbos MH, Ignatiadis M, Peeters D, Pierga JY, Farace F, Riethdorf S, Fehm T, Zorzino L, Tibbe AG, Maestro M, Gisbert-Criado R, Denton G, de Bono JS, Dive C, Foekens JA, Gratama JW. 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.

Strijbos MH, van Krimpen BA, Debets R, **Kraan J**, Sleijfer S, Gratama JW, Lamers CH. mRNA levels of CD31, CD144, CD146 and von Willebrand factor do not serve as surrogate markers for circulating endothelial cells. *Thromb Haemost.* 2010 Aug;104(2):318-26.

Strijbos MH, Snijder CA, **Kraan J**, Lamers CH, Gratama JW, Duvekot JJ. Levels of circulating endothelial cells in normotensive and severe preeclamptic pregnancies. *Cytometry B Clin Cytom.* 2010 Nov;78(6):382-6.

Lalmahomed ZS, **Kraan J**, Gratama JW, Mostert B, Sleijfer S, Verhoef C. Circulating tumor cells and sample size: the more, the better. *J Clin Oncol.* 2010 Jun 10;28(17):e288-9; author reply e90.

Strijbos MH, **Kraan J**, Lamers CH, Sleijfer S, Gratama JW. Quantification of circulating endothelial cells by flow cytometry. *Clin Cancer Res.* 2009 May 15;15(10):3640; author reply..

Sieuwerts AM, **Kraan J**, Bolt-de Vries J, van der Spoel P, Mostert B, Martens JW, Gratama JW, Sleijfer S, Foekens JA. 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.

Sieuwerts AM, **Kraan J**, Bolt J, van der Spoel P, Elstrodt F, Schutte M, Martens JW, Gratama JW, Sleijfer S, Foekens JA. 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.

Strijbos MH, Rao C, Schmitz PI, **Kraan J**, Lamers CH, Sleijfer S, Terstappen LW, Gratama JW. Correlation between circulating endothelial cell counts and plasma thrombomodulin levels as markers for endothelial damage. *Thromb Haemost.* 2008 Oct;100(4):642-7.

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.

Strijbos MH, **Kraan J**, Sleijfer S, Gratama JW. Potential biomarkers in metastatic gastrointestinal stromal tumors. *Clin Cancer Res.* 2007 Oct 15;13(20):6244.

Strijbos MH, **Kraan J**, den Bakker MA, Lambrecht BN, Sleijfer S, Gratama JW. Cells meeting our immunophenotypic criteria of endothelial cells are large platelets. *Cytometry B Clin Cytom.* 2007 Mar;72(2):86-93.

Sleijfer S, Gratama JW, Sieuwerts AM, **Kraan J**, Martens JW, Foekens JA. Circulating tumour cell detection on its way to routine diagnostic implementation? *Eur J Cancer.* 2007 Dec;43(18):2645-50.

Other publications

de Jongste AH, **Kraan J**, van den Broek PD, Brooimans RA, Bromberg JE, van Montfort KA, Smitt PA, Gratama JW. Use of TransFix cerebrospinal fluid storage tubes prevents cellular loss and enhances flow cytometric detection of malignant hematological cells after 18 hours of storage. *Cytometry B Clin Cytom.* 2014 Jul;86(4):272-9.

de Jongste AH, de Graaf MT, van den Broek PD, **Kraan J**, Smitt PA, Gratama JW. Elevated numbers of regulatory T cells, central memory T cells and class-switched B cells in cerebrospinal fluid of patients with anti-Hu antibody associated paraneoplastic neurological syndromes. *J Neuroimmunol.* 2013 May 15;258(1-2):85-90.

de Jongste AH, de Graaf MT, van den Broek PD, **Kraan J**, Sillevs Smitt PA, Gratama JW. Effector memory and late memory T cells accumulate in the blood of CMV-carrying individuals but not in their cerebrospinal fluid. *Cytometry B Clin Cytom.* 2013 Jul-Aug;84(4):218-21.

de Jongste AH, de Graaf MT, Martinuzzi E, van den Broek PD, **Kraan J**, Lamers CH, Mallone R, Gratama JW, Sillevs Smitt PA. Three sensitive assays do not provide evidence for circulating HuD-specific T cells in the blood of patients with paraneoplastic neurological syndromes with anti-Hu antibodies. *Neuro Oncol.* 2012 Jul;14(7):841-8.

de Graaf MT, van den Broek PD, **Kraan J**, Luitwieler RL, van den Bent MJ, Boonstra JG, Schmitz PI, Gratama JW, Sillevs Smitt PA. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *J Neurol.* 2011 Aug;258(8):1507-12.

de Graaf MT, Smitt PA, Luitwieler RL, van Velzen C, van den Broek PD, **Kraan J**, Gratama JW. Central memory CD4+ T cells dominate the normal cerebrospinal fluid. *Cytometry B Clin Cytom.* 2011 Jan;80(1):43-50.

de Graaf MT, de Jongste AH, **Kraan J**, Boonstra JG, Sillevs Smitt PA, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry B Clin Cytom.* 2011 Sep;80(5):271-81.

Pouw N, Treffers-Westerlaken E, **Kraan J**, Wittink F, ten Hagen T, Verweij J, Debets R. Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. *Cancer Immunol Immunother.* 2010 Jun;59(6):921-31.

Brooimans RA, **Kraan J**, van Putten W, Cornelissen JJ, Lowenberg B, Gratama JW. Flow cytometric differential of leukocyte populations in normal bone marrow: influence of peripheral blood contamination. *Cytometry B Clin Cytom.* 2009 Jan;76(1):18-26.

Levering WH, van Wieringen WN, **Kraan J**, van Beers WA, Sintnicolaas K, van Rhenen DJ, Gratama JW. Flow cytometric lymphocyte subset enumeration: 10 years of external quality assessment in the Benelux countries. *Cytometry B Clin Cytom.* 2008 Mar;74(2):79-90.

Kraan J, Gratama JW, Haioun C, Orfao A, Plonquet A, Porwit A, Quijano S, Stetler-Stevenson M, Subira D, Wilson W. Flow cytometric immunophenotyping of cerebrospinal fluid. *Curr Protoc Cytom.* 2008 Jul;Chapter 6:Unit 6 25.

de Graaf MT, de Beukelaar JW, Burgers PC, Luider TM, **Kraan J**, Smitt PA, Gratama JW. Contamination of synthetic HuD protein spanning peptide pools with a CMV-encoded peptide. *Cytometry A.* 2008 Nov;73(11):1079-85.

de Graaf M, de Beukelaar J, Bergsma J, **Kraan J**, van den Bent M, Klimek M, van Norden Y, Kusuma A, Smitt PS, Gratama JW. B and T cell imbalances in CSF of patients with Hu-antibody associated PNS. *J Neuroimmunol.* 2008 Mar;195(1-2):164-70.

Ratei R, Karawajew L, Lacombe F, Jagoda K, Del Poeta G, **Kraan J**, De Santiago M, Kappelmayer J, Bjorklund E, Ludwig WD, Gratama JW, Orfao A, European Working Group of Clinical Cell A. Discriminant function analysis as decision support system for the diagnosis of acute leukemia with a minimal four color screening panel and multiparameter flow cytometry immunophenotyping. *Leukemia.* 2007 Jun;21(6):1204-11.

Levering WH, Preijers FW, van Wieringen WN, **Kraan J**, van Beers WA, Sintnicolaas K, van Rhenen DJ, Gratama JW. Flow cytometric CD34+ stem cell enumeration: lessons from nine years' external quality assessment within the Benelux countries. *Cytometry B Clin Cytom.* 2007 May;72(3):178-88.

de Beukelaar JW, Verjans GM, van Norden Y, Milikan JC, **Kraan J**, Hooijkaas H, Sintnicolaas K, Gratama JW, Sillevs Smitt PA. No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. *Cancer Immunol Immunother.* 2007 Sep;56(9):1501-6.

de Beukelaar JW, Sillevs Smitt PA, Hop WC, **Kraan J**, Hooijkaas H, Verjans GM, Gratama JW. Imbalances in circulating lymphocyte subsets in Hu antibody associated paraneoplastic neurological syndromes. *Eur J Neurol.* 2007 Dec;14(12):1383-91.

de Beukelaar JW, Gratama JW, Smitt PA, Verjans GM, **Kraan J**, Luider TM, Burgers PC. The impact of impurities in synthetic peptides on the outcome of T-cell stimulation assays. *Rapid Commun Mass Spectrom.* 2007;21(7):1282-8.

Bromberg JE, Breems DA, **Kraan J**, Bikker G, van der Holt B, Smitt PS, van den Bent MJ, van't Veer M, Gratama JW. CSF flow cytometry greatly improves diagnostic accuracy in CNS hematologic malignancies. *Neurology.* 2007 May 15;68(20):1674-9.

Ratei R, Karawajew L, Lacombe F, Jagoda K, Del Poeta G, **Kraan J**, De Santiago M, Kappelmayer J, Bjorklund E, Ludwig WD, Gratama J, Orfao A, European Working Group of

Clinical Cell A. Normal lymphocytes from leukemic samples as an internal quality control for fluorescence intensity in immunophenotyping of acute leukemias. *Cytometry B Clin Cytom.* 2006 Jan;70(1):1-9.

Boonstra JG, van Lom K, Langerak AW, Graveland WJ, Valk PJ, **Kraan J**, van 't Veer MB, Gratama JW. CD38 as a prognostic factor in B cell chronic lymphocytic leukaemia (B-CLL): comparison of three approaches to analyze its expression. *Cytometry B Clin Cytom.* 2006 May;70(3):136-41.

Lamers CH, Gratama JW, Pouw NM, Langeveld SC, Krimpen BA, **Kraan J**, Stoter G, Debets R. Parallel detection of transduced T lymphocytes after immunogene therapy of renal cell cancer by flow cytometry and real-time polymerase chain reaction: implications for loss of transgene expression. *Hum Gene Ther.* 2005 Dec;16(12):1452-62.

van Wering ER, van Lochem EG, Leenders M, van der Sluijs-Gelling AJ, Wind H, Gratama JW, **Kraan J**, Preijers FW. Three-color flowcytometric analysis of mature and immature hematological malignancies. A guideline of the Dutch Foundation for Immunophenotyping of Hematological Malignancies (SIHON). *J Biol Regul Homeost Agents.* 2004 Jul-Dec;18(3-4):313-26.

Perez-Andres M, Santiago M, Almeida J, Mateo G, Porwit-MacDonald A, Bjorklund E, Valet G, **Kraan J**, Gratama JW, D'Hautcourt JL, Merle-Beral H, Lima M, Montalban MA, San Miguel JF, Orfao I, European Working Group on Clinical Cell A, Spanish Network on Multiple M. Immunophenotypic approach to the identification and characterization of clonal plasma cells from patients with monoclonal gammopathies. *J Biol Regul Homeost Agents.* 2004 Jul-Dec;18(3-4):392-8.

Kraan J, Gratama JW, Keeney M, D'Hautcourt JL. Setting up and calibration of a flow cytometer for multicolor immunophenotyping. *J Biol Regul Homeost Agents.* 2003 Jul-Sep;17(3):223-33.

Kraan J, Arroz M, Keeney M, Freire M, Weir K, Heijnen I, Gratama JW, European Working Group on Clinical Cell A. Flow cytometric enumeration of Class I HLA-restricted, peptide-specific CD8+ T lymphocytes using tetramer technology and single-platform absolute T-cell counting. *J Biol Regul Homeost Agents.* 2003 Jul-Sep;17(3):268-78.

Gratama JW, **Kraan J**, Keeney M, Sutherland DR, Granger V, Barnett D. Validation of the single-platform ISHAGE method for CD34(+) hematopoietic stem and progenitor cell enumeration in an international multicenter study. *Cytotherapy.* 2003;5(1):55-65.

Gratama JW, **Kraan J**, Keeney M, Granger V, Barnett D. Reduction of variation in T-cell subset enumeration among 55 laboratories using single-platform, three or four-color flow cytometry based on CD45 and SSC-based gating of lymphocytes. *Cytometry.* 2002 Apr 15;50(2):92-101.

van de Loosdrecht A, Kalk W, Bootsma H, Henselmans JM, **Kraan J**, Kallenberg CG. Simultaneous presentation of sarcoidosis and Sjogren's syndrome. *Rheumatology (Oxford)*. 2001 Jan;40(1):113-5.

Gratama JW, Menendez P, **Kraan J**, Orfao A. Loss of CD34(+) hematopoietic progenitor cells due to washing can be reduced by the use of fixative-free erythrocyte lysing reagents. *J Immunol Methods*. 2000 May 26;239(1-2):13-23.

Barnett D, Granger V, **Kraan J**, Whitby L, Reilly JT, Papa S, Gratama JW. Reduction of intra- and interlaboratory variation in CD34+ stem cell enumeration using stable test material, standard protocols and targeted training. DK34 Task Force of the European Working Group of Clinical Cell Analysis (EWGCCA). *Br J Haematol*. 2000 Mar;108(4):784-92.

Gratama JW, Braakman E, **Kraan J**, Lankheet P, Levering WH, Van Den Beemd MW, Van Der Schoot CE, Wijermans P, Preijers F. Comparison of single and dual-platform assay formats for CD34+ haematopoietic progenitor cell enumeration. *Clin Lab Haematol*. 1999 Oct;21(5):337-46.

Gratama JW, **Kraan J**, Van den Beemd R, Hooibrink B, Van Bockstaele DR, Hooijkaas H. Analysis of variation in results of flow cytometric lymphocyte immunophenotyping in a multicenter study. *Cytometry*. 1997 Aug 15;30(4):166-77.

Gratama JW, **Kraan J**, Levering W, Van Bockstaele DR, Rijkers GT, Van der Schoot CE. Analysis of variation in results of CD34+ hematopoietic progenitor cell enumeration in a multicenter study. *Cytometry*. 1997 Jun 15;30(3):109-17.

Gratama JW, **Kraan J**, Adriaansen H, Hooibrink B, Levering W, Reinders P, Van den Beemd MW, Van der Holt B, Bolhuis RL. Reduction of interlaboratory variability in flow cytometric immunophenotyping by standardization of instrument set-up and calibration, and standard list mode data analysis. *Cytometry*. 1997 Feb 15;30(1):10-22.

Book chapters

Kraan J, Gratama JW, Haioun C, Orfao A, Plonquet A, Porwit A, Quijano S, Stetler-Stevenson M, Subira D, Wilson W. Flow cytometric immunophenotyping of cerebrospinal fluid. International guideline in *Curr Protoc Cytom*. 2008 Jul; Chapter 6:Unit 6 25 [ISBN 0-471-16131-4].

Gratama JW, **Kraan J**, Keeney M, Mandy F, Sutherkand DR, Wood BL. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; *Clinical and Laboratory Standards Institute. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. 2006; CLSI document H42-A2 [ISBN 1-56238-000-0].

