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# Quantification of Circulating Endothelial Cells as Surrogate Marker for Vascular Damage

Kwantificering van circulerende endotheelcellen als surrogaatmerker voor vaatschade

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Voor Els

In herinnering aan Guy Malherbe

# CHAPTER 1

General Introduction and Scope of This Thesis

## The vascular endothelium

The inner lining of our vascular system consists of approximately 1.2 trillion endothelial cells [1]. Rather than being a mere wall between the blood and organs, the endothelium is a highly dynamic structure involved in many aspects of vascular biology. Endothelial cells play an important role in regulation of vasomotor tonus, hemostasis and coagulation, fluid and electrolyte transport, inflammation, and angiogenesis [2]. Understanding the biology of the endothelium in specific diseases that can be associated with vascular damage could be of great interest for physicians. 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 could enable physicians to monitor the response to treatment.

## Vascular biomarkers in oncology

In particular in cancer, determination of endothelial damage might be of interest. Tumor vasculature is structurally different from its healthy counterpart, as it is fragile and leaky. Also, the normal vasculature in cancer patients can easily be damaged; as invasion of the vessels' basement membrane is required for the extravasation of malignant cells. Tumor aggressiveness may therefore be reflected by more endothelial damage. Consequently, extent of endothelial damage may be associated with prognosis, or could be a biomarker for detecting disease recurrence of progression.

In addition to being a prognostic biomarker, markers informative on the extent of endothelial injury might predict outcome to therapy at an early stage. In 1971, Judah Folkman was the first to postulate the therapeutic potential of drugs targeting the tumors' vasculature [3,4]. Nowadays, it has been unambiguously shown that proper vascularisation is needed for growth and they are a necessity for cancer growth and dissemination [5]. Also, studies demonstrated that endothelial cells are genetically much more stable when compared to malignant cells, and are therefore unlikely to develop resistance to therapy [6]. These findings have led to the development of the first anti-vascular drug, three decades after Folkmans' initial publication: bevacizumab (Avastin®, Roche), a monoclonal antibody (mAb) against VEGF [7]. Subsequently, the multi target tyrosine kinase inhibitors such as sunitinib (Sutent®, Pfizer) and sorafenib (Nexavar®, Bayer) became available that also target the VEGF pathway [8-10], and of which efficacy has been established in randomized trials [8,9] .

Next to targeting the formation of new vessels, a second class of anti-vascular drugs is currently being investigated: the vascular disrupting agents (VDA), such as combretastatin A4 (Oxigene). Rather than impeding the formation of new vessels, these drugs act by destroying established vessels by tumors, causing hypoxia, starvation and eventually necrosis [11]. Clinical trials on VDAs are currently ongoing [12,13].

In addition to such agents, also effects of conventional chemotherapy are likely to be translated into changes in parameters of endothelial damage. For example, long term follow up of patients treated with chemotherapy for testicular cancer has shown a significant increase in cardiovascular complications such as hypertension, hypercholesterolemia, insulin resistance, as well as an increase in plasma and clinical biomarkers of endothelial dysfunction and damage in these patients [14-16]. These findings, combined with data obtained in the setting of *in vitro* studies, suggest vascular toxicity of a large number of cytotoxic agents.

Given the important role of the endothelium in the pathogenesis of cancer, as well as being a target for treatment, markers that provides quantitative information on the extent of tumor vascularization, allow the monitoring the effect of anti-vascular drugs, or have predictive or prognostic value in oncology, are urgently needed [17].

Over the last decades, a large number of techniques have been deployed to obtain information on the vasculature. However their usability as predictive of prognostic marker, or as aid in monitoring response to treatment, is often compromised by either requiring invasive procedures or by lacking sensitivity and specificity. For example, increased intratumoral microvessel density (MVD) is associated with poor survival in several forms of cancer [18-20], but the need for tumor tissue make this technique unsuitable as monitoring tool. By the same token, plasma concentrations of endothelial cell derived proteins, such as von Willebrand factor (vWF) [21-23], thrombomodulin [24-26], and E-selectin [27-29], have been found to correlate with response to treatment or survival. However, the pleiotropic effects of these proteins could easily compromise specificity. In contrast to endothelial derived proteins, evaluation of plasma or tumor levels of angiogenesis related proteins such as VEGF, failed to demonstrate a predictive of prognostic value for survival in various tumor types [30-32].

## Circulating endothelial cells

A novel endothelial biomarker currently under investigation, is the number of circulating endothelial cells (CEC). CEC are endothelial cells detached from the vessel wall, and as such are considered a promising marker for endothelial damage [33,34].. Increased numbers of CEC have been observed in diseases with documented vascular involvement such as vasculitis, sickle cell anemia, and cancer [35-38]. Also, their number correlates to putative plasma markers of endothelial injury [39]. Nevertheless, the clinical implementation of CEC as biomarker for vascular damage has been hindered by several factors.

CEC occur in very low numbers in blood, typical counts being 0-20 cells per mL of blood in healthy donors [36,40]. In comparison, the number of hematopoietic stem cells in peripheral blood, generally considered to be rare cells, typically range from 500-6500 cells per mL in healthy, non-mobilized donors. This low number, but more importantly, the lack of consensus on the immunophenotype of CEC hinders their reliable and reproducible detection. Nowadays, there is good agreement that some form of sample enrichment is mandatory [40] to increase assay sensitivity and specificity.

Frequently used enrichment techniques include density gradient centrifugation and immunomagnetic isolation, but enrichment inevitably leads to cell loss. Enrichment based techniques are therefore likely to give an underestimation of the actual CEC count. Moreover, positive immunomagnetic isolation requires the presence of an antigen highly specific for the cells of interest, whereas negative immunomagnetic isolation (depletion) can easily lead to entrapment of other rare cells which are not CEC.

Next, as a result of their functional heterogeneity, endothelial cell characteristics are highly variable which is reflected in the changes in morphology and immunophenotype in response to disease or treatment. The archetypal endothelial cell is characterized by having a nucleus, expressing the antigens CD31, CD34, CD144, and CD146 [35,41], absence of the pan-leukocyte antigen CD45, and the presence of the intracellular von Willebrand Factor protein [42] (Table 1). In pathological conditions, inflammatory mediators, cytokines and growth factors can induce the expression of specific antigens onto the endothelial cell surface, such as growth factor receptors, adhesion molecules, and selectins. This heterogeneity has led to a large number of different definitions of CEC in literature, which makes it difficult to extrapolate conclusions from one study to another.

**Table 1 – Markers used in CEC assays**

| Marker | Description | Subtype Association | Co expression | Ref |
|---|---|---|---|---|
| CD31 | PECAM-1 | Pan-endothelial | P | 35 |
| CD34 | Stem cell Marker | Pan-endothelial | S | 43 |
| CD36 | Collagen receptor I | Micro vascular | P, E, M, D | 44 |
| CD45 | Pan-leukocyte marker | None | - | 36 |
| CD54 | ICAM-1 | Inflammation | L, M | 45 |
| CD62-E | E-selectin | Inflammation | - | 45 |
| CD62-P | P-selectin | Inflammation | P | 46 |
| CD105 | Endoglin | Angiogenesis, malignant | S, M[1] | 36 |
| CD106 | VCAM-1 | Inflammation, malignant | - | 45 |
| CD137 | ILA/4 | Malignant | L[1], D | 47 |
| CD144 | VE-Cadherin | Pan-endothelial | - | 48 |
| CD146 | MelCAM | Pan-endothelial | L[1] | 49 |
| CD202b | Tie-2 | Angiogenesis | - | 48 |
| CD276 | B7-H3 | Malignant | D, M[1] | 47 |
| CD309 | VEGFR-2 | Angiogenesis | S | 35 |
| VWF | Von Willebrand Factor | Pan-endothelial | P | 40 |
| UEA-1 | Ulex Europaeus Lectin-1 | Pan-endothelial | - | 40 |
| DiL-AcLDL | Acetylated LDL | Pan-endothelial | - | 50 |

## Scope of the thesis

The objectives of this thesis are: 1) to investigate different techniques of quantification of CEC in view of their use as surrogate marker for vascular damage, 2) to study the relation of CEC to alternative blood based markers of endothelial injury and function, and 3) to assess the clinical relevance of CEC in cancer and non-malignant diseases. To this end, we review the possible clinical applications of CEC enumeration in oncology, and the problems that are frequently encountered in this process in **Chapter 2.** In **Chapter 3**, we examine the origin of cells designated CEC as detected by a widely applied single platform flow cytometer assay. Based on the results of this study, we decided to perform all additional studies using the CellSearch™ assay, developed by Immunicon (now Veridex, Raritan, NJ). This assay uses CD146 immunomagnetic isolation, with additional immunophenotyping of these $CD146^+$ cells by assessing the expression of the endothelial marker CD105, the presence of a nucleus (determined by positive staining for the DNA specific dye 4',6-diamidino-2-phenylindole [DAPI]), and lack of the pan-leukocyte antigen CD45. In a study of 40 healthy controls using this assay, we found CEC counts of 0-20 /mL, which is comparable to counts found by other validated CEC detections assays. Also, the elevated CEC numbers in patients with cancer and sickle cells disease as reported by others, could be reproduced using the CellSearch ™ assay. In **Chapter 4**, we explore the effect of biological parameters such as age and gender on CEC numbers, as well as the relation between CEC and plasma markers of endothelial function and damage in healthy individuals and patients with metastatic carcinoma. **Chapter 5** describes the use of a model of vascular damage to confirm the origin and phenotype of CEC with a validated endothelial phenotype, detected with the CellSearch™ assay. **Chapter 6** focuses on a possible alternative CEC detection strategy. We examined whether blood levels of mRNA of the endothelial proteins vWF, CD31, CD144, and CD146, detected by real-time polymerase chain reaction (RT-PCR), could serve as a sensitive and less expensive alternative to the CellSearch™ assay..In Chapters 7, 8 and 9, the clinical relevance of CEC, as well as their relation to clinical, cellular, and soluble markers of disease activity is studied. In **Chapter 7**, these parameters are studied in sickle cell anemia, in **Chapter 8** in preeclampsia, and **Chapter 9** in hormone refractory metastatic prostate cancer. **Chapter 10** provides a general discussion of the results obtained by studies in this thesis, and gives an overview of the future perspectives of CEC as surrogate marker. **Chapter 11** provides a simplified overview of this thesis in Dutch.

## References


1 Aird WC. Endothelium as an organ system. Crit Care Med. 2004;32:S271-279.
2. Pries AR, Kuebler WM. Normal endothelium. Handb Exp Pharmacol. 2006:1-40.
3. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285:1182-1186.
4. Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. Ann Surg. 1972;175:409-416.
5. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature. 1993;362:841-844.
6. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature. 1997;390:404-407.
7. Kabbinavar F, Hurwitz HI, Fehrenbacher L, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol. 2003;21:60-65.
8. Escudier B, Eisen T, Stadler WM, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med. 2007;356:125-134.
9. Motzer RJ, Michaelson MD, Redman BG, et al. Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol. 2006;24:16-24.
10. Rini BI, Sosman JA, Motzer RJ. Therapy targeted at vascular endothelial growth factor in metastatic renal cell carcinoma: biology, clinical results and future development. BJU Int. 2005;96:286-290.
11. Hinnen P, Eskens FA. Vascular disrupting agents in clinical development. Br J Cancer. 2007;96:1159-1165.
12. Oh Y, Herbst RS, Burris H, et al. Enzastaurin, an oral serine/threonine kinase inhibitor, as second- or third-line therapy of non-small-cell lung cancer. J Clin Oncol. 2008;26:1135-1141.
13. Rehman F, Rustin G. ASA404: update on drug development. Expert Opin Investig Drugs. 2008;17:1547-1551.
14. Nuver J, Smit AJ, Sleijfer DT, et al. Microalbuminuria, decreased fibrinolysis, and inflammation as early signs of atherosclerosis in long-term survivors of disseminated testicular cancer. Eur J Cancer. 2004;40:701-706.
15. 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.
16. Nuver J, Smit AJ, Wolffenbuttel BH, et al. The metabolic syndrome and disturbances in hormone levels in long-term survivors of disseminated testicular cancer. J Clin Oncol. 2005;23:3718-3725.
17. 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.
18. Li C, Gardy R, Seon BK, et al. Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis. Br J Cancer. 2003;88:1424-1431.

19. Martone T, Rosso P, Albera R, et al. Prognostic relevance of CD105+ microvessel density in HNSCC patient outcome. Oral Oncol. 2005;41:147-155.
20. Rasheed S, Harris AL, Tekkis PP, et al. Assessment of microvessel density and carbonic anhydrase-9 (CA-9) expression in rectal cancer. Pathol Res Pract. 2008.
21. Damin DC, Rosito MA, Gus P, Roisemberg I, Bandinelli E, Schwartsmann G. Von Willebrand factor in colorectal cancer. Int J Colorectal Dis. 2002;17:42-45.
22. Wang WS, Lin JK, Lin TC, et al. Plasma von Willebrand factor level as a prognostic indicator of patients with metastatic colorectal carcinoma. World J Gastroenterol. 2005;11:2166-2170.
23. Rohsig LM, Damin DC, Stefani SD, Castro CG, Jr., Roisenberg I, Schwartsmann G. von Willebrand factor antigen levels in plasma of patients with malignant breast disease. Braz J Med Biol Res. 2001;34:1125-1129.
24. Hanly AM, Redmond M, Winter DC, et al. Thrombomodulin expression in colorectal carcinoma is protective and correlates with survival. Br J Cancer. 2006;94:1320-1325.
25. Kim SJ, Shiba E, Ishii H, et al. Thrombomodulin is a new biological and prognostic marker for breast cancer: an immunohistochemical study. Anticancer Res. 1997;17:2319-2323.
26. Tamura A, Hebisawa A, Hayashi K, et al. Prognostic significance of thrombomodulin expression and vascular invasion in stage I squamous cell carcinoma of the lung. Lung Cancer. 2001;34:375-382.
27. Bewick M, Conlon M, Lee H, et al. Evaluation of sICAM-1, sVCAM-1, and sE-Selectin levels in patients with metastatic breast cancer receiving high-dose chemotherapy. Stem Cells Dev. 2004;13:281-294.
28. Guney N, Soydinc HO, Derin D, et al. Serum levels of intercellular adhesion molecule ICAM-1 and E-selectin in advanced stage non-small cell lung cancer. Med Oncol. 2008;25:194-200.
29. Silva HC, Garcao F, Coutinho EC, De Oliveira CF, Regateiro FJ. Soluble VCAM-1 and E-selectin in breast cancer: relationship with staging and with the detection of circulating cancer cells. Neoplasma. 2006;53:538-543.
30. Deprimo SE, Bello CL, Smeraglia J, et al. Circulating protein biomarkers of pharmacodynamic activity of sunitinib in patients with metastatic renal cell carcinoma: modulation of VEGF and VEGF-related proteins. J Transl Med. 2007;5:32.
31. Jubb AM, Hurwitz HI, Bai W, et al. Impact of vascular endothelial growth factor-A expression, thrombospondin-2 expression, and microvessel density on the treatment effect of bevacizumab in metastatic colorectal cancer. J Clin Oncol. 2006;24:217-227.
32. Kindler HL, Friberg G, Singh DA, et al. Phase II trial of bevacizumab plus gemcitabine in patients with advanced pancreatic cancer. J Clin Oncol. 2005;23:8033-8040.
33. Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost. 2005;93:228-235.
34. Shantsila E, Blann AD, Lip GY. Circulating endothelial cells: from bench to clinical practice. J Thromb Haemost. 2008;6:865-868.
35. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol. 2004;15:139-145.

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. 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.
38. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis. Lancet. 2003;361:206-210.
39. Strijbos MH, Rao C, Schmitz PI, et al. Correlation between circulating endothelial cell counts and plasma thrombomodulin levels as markers for endothelial damage. Thromb Haemost. 2008;100:642-647.
40. 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.
41. Duda DG, Cohen KS, di Tomaso E, et al. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J Clin Oncol. 2006;24:1449-1453.
42. Babich V, Meli A, Knipe L, et al. Selective release of molecules from Weibel-Palade bodies during a lingering kiss. Blood. 2008;111:5282-5290.
43. 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.
44. Moroni G, Del Papa N, Moronetti LM, et al. Increased levels of circulating endothelial cells in chronic periaortitis as a marker of active disease. Kidney Int. 2005;68:562-568.
45. Dixon GL, Heyderman RS, van der Ley P, Klein NJ. High-level endothelial E-selectin (CD62E) cell adhesion molecule expression by a lipopolysaccharide-deficient strain of Neisseria meningitidis despite poor activation of NF-kappaB transcription factor. Clin Exp Immunol. 2004;135:85-93.
46. Corcoran TB, O'Shea A, Engel A, Shorten GD. The influence of propofol on P-selectin expression and nitric oxide production in re-oxygenated human umbilical vein endothelial cells. Acta Anaesthesiol Scand. 2006;50:348-354.
47. 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.
48. 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.
49. 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.
50. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J Cell Biol. 1984;99:2034-2040.

# CHAPTER 2

## CIRCULATING ENDOTHELIAL CELLS IN ONCOLOGY: PITFALLS AND PROMISES

*Br J Cancer. 2008 Jun 3;98(11):1731-5.*

## SUMMARY

Adequate blood supply is a prerequisite in the pathogenesis of solid malignancies. As a result, depriving a tumour from its oxygen and nutrients, either by preventing the formation of new vessels, or by disrupting vessels already present in the tumour, appears to be an effective treatment modality in oncology. Given the mechanism by which these agents exert their anti-tumour activity together with the crucial role of tumour vasculature in the pathogenesis of tumours, there is a great need for markers properly reflecting its impact. Circulating endothelial cells (CEC), which are thought to derive from damaged vasculature, may be such a marker. Appropriate enumeration of these cells appears to be a technical challenge. Nevertheless, first studies using validated CEC assays have shown that CEC numbers in patients with advanced malignancies are elevated compared to healthy controls making CEC a potential tool for amongst other establishing prognosis and therapy-induced effects. In this review, we will address the possible clinical applications of CEC detection in oncology, as well as the pitfalls encountered in this process.

## INTRODUCTION

Angiogenesis is thought to be an absolute prerequisite for the growth and dissemination of malignant tumours. When a tumour reaches a size of 1-2 $mm^3$, its microenvironment can no longer provide the required amount of nutrients and oxygen by diffusion. The resulting cellular hypoxia initiates a response in malignant cells: the so-called “angiogenic switch” [1]. By upregulating the expression of proangiogenic proteins, the tumour induces sprouting of pre-existing capillaries, which result in the formation of new vessels.

The pivotal role of angiogenesis in tumour growth prompted the development of several agents targeting receptors or signal transduction pathways involved in angiogenesis. Based on data from randomized clinical trials, the benefit of compounds that inhibit angiogenesis has already been demonstrated in several types of cancer, including metastatic colon carcinoma [2], renal cell carcinoma [3], and non-small lung cell carcinoma [4]. In addition to these drugs inhibiting angiogenesis, another class of anticancer drugs, vascular disrupting agents (VDAs), has recently been developed and is designed to target already established tumour vasculature. Clinical studies exploring the latter drugs are currently ongoing. Given the important role of angiogenesis in oncology in terms of pathogenesis as well as being a target for treatment, there is an increasing need for markers that accurately reflect effects impacting tumour vasculature.

A promising candidate to serve as such a marker is the enumeration of circulating endothelial cells (CEC). CEC are mature endothelial cells, sloughed off the vessel wall as a result of vascular insults. Their number in peripheral blood is considered to reflect the extent of vascular damage in patients with vasculopathies. Recently, several assays for their detection and quantification have been developed. Although consensus on the phenotypic definition of CEC as well as on the optimal

enumeration technique is still lacking, the number of clinical studies assessing CEC in cancer patients is rapidly expanding. Several studies on CEC also incorporate a strategy to enumerate endothelial progenitor cells (EPC), which, in contrast to CEC, are thought to originate from the bone marrow and to contribute actively to angiogenesis. A study by Lyden showed in a murine model that, all tumor vessel endothelial cells were bone marrow derived[5], and similar observations were done in humans [6], stressing the importance of EPC in angiogenesis. However, as the frequency of EPC in blood is suggested to be several times lower than those of CEC, while additionally, their exact phenotype has not been elucidated, it is very difficult to reliably detect and enumerate EPC by currently available assays. EPC are therefore not included in this manuscript. This review provides an overview of the uses of CEC as surrogate marker for vascular damage in oncology, in particular technical issues concerning their detection, its potential applications, and results from clinical studies obtained so far.

## TECHNICAL ISSUES IN CEC ENUMERATION

Currently, there are several assays described for the detection and enumeration of CEC. All these assays have to deal with the low number of CEC in peripheral blood (PB), (typically 0-20 CEC/mL in healthy donors) rendering such assays highly susceptible to errors in sampling, preparation, and analysis. For instance, several groups have demonstrated the negative impact of venipuncture, as traumatically detached CEC contribute significantly to CEC counts[7,8]. Because of this low number, enrichment steps are applied in several approaches, which inevitably leads to cell loss and underestimation of the actual CEC number. Next to proper sampling, thorough analysis of enrichment efficacy, reported in terms of purity and recovery is therefore mandatory before using such assays based on enrichment in the clinic. The EUROCEC network has provided useful suggestions on how such a validation might be performed in practice (www.eurocec.com)[9].

Of key importance for proper detection and enumeration of CEC is the use of specific markers. However, the endothelium is a highly dynamic structure, closely involved in hemostasis, inflammation, regulation of vascular tonus, and angiogenesis. This dynamic process is accompanied by changing immunophenotype of endothelial cells that may largely account for the large number of different CEC phenotypes reported in literature. For enumeration of the total CEC number, and not a particular subpopulation, it is necessary to identify markers that are specific for and are constantly expressed by all CEC. Currently, no marker is considered to meet these criteria. Consequently, assays rely on multiple characteristics to define CEC. The majority of current assays define CEC as being positive for the CD146 antigen, present on endothelial cells, but also on a subset of activated T-cells, and melanomas [10]. Table 1 provides an overview of markers frequently used in CEC assays. As the different reported phenotypes render it impossible to compare results between various studies, consensus on a common endothelial cell phenotype is a key issue.

Another mandatory step in the development of CEC assays is the validation of the true endothelial origin of cells designated CEC by that assay. For this purpose, several unique features of endothelial cells can be used. These include uptake of Ulex Europaeus Lectin-1, or UEA-1 [7] or the ability of CEC to scavenge acetylated low-density lipoproteins, which can be visualized when labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil Ac-LDL) [11]. In addition, immunohistochemistry or immunofluorescence can be used to demonstrate the presence of von Willebrand factor (VWF), or other endothelial surface markers (Table 1) [12]. Another means of validation is through gene expression profiling assessing whether or not expression of endothelial genes such as vascular endothelial cadherin (VE-cadherin), is present in the population of cells designated as CEC [13].

**Table 1 – Markers used in CEC assays**

| Marker | Description | Subtype Association | Expression Level | Co expression | Ref |
|---|---|---|---|---|---|
| CD31 | PECAM-1 | Pan-endothelial | ++* | P,Pan-leukocyte | 12 |
| CD34 | Stemcell Marker | Pan-endothelial | ++* | S | 26 |
| CD36 | CR-I | Micro vascular | +* | P, E, M, D | 27 |
| CD54 | ICAM-1 | Inflammation | +* | L, M | 28 |
| CD62-E | E-selectin | Inflammation | ++ | - | 28 |
| CD62-P | P-selectin | Inflammation | + | P | 29 |
| CD105 | Endoglin | Malignant | ++* | S, M[1] | 8 |
| CD106 | VCAM-1 | Inflammation | +* | - | 28 |
| CD137 | ILA/4 | Malignant | + | L[1], D | 23 |
| CD144 | VE-Cadherin | Pan-endothelial | +* | - | 13 |
| CD146 | MelCAM | Pan-endothelial | ++* | L[1] | 30 |
| CD202b | Tie-2 | Angiogenesis | (+)* | - | 13 |
| CD276 | B7-H3 | Malignant | + | D, M[1] | 23 |
| CD309 | VEGFR-2 | Angiogenesis | (+)* | S | 12 |
| VWF | | Pan-endothelial | ++* | P | 9 |
| UEA-1 | | Pan-endothelial | ++ | - | 9 |
| DiL-AcLDL | | Pan-endothelial | ++ | - | 11 |

*Abbreviations: PECAM-1=platelet/endothelial cell adhesion molecule 1, CR-I=collagen receptor I, ICAM-1=intracellular adhesion molecule 1, VCAM-1 =vascular cellular adhesion molecule 1, ILA/4 =inducible by lymphocyte activation /4, MelCAM= 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. S =hematopoietic stem cells, L =lymphocytes, P =platelets, M =monocytes, E =erythrocytes, D =dendritic cells. [1] indicates presence on activated cells. Expression levels: ++ =strong, + =moderate, (+) =weak. *indicates data based on flow cytometric results from the authors.*

## AVAILABLE ASSAYS

*Manual immunomagnetic isolation*
The most widely used method to isolate CEC, is by the use of magnetic beads coupled to a monoclonal antibody (mAb) targeting CD146, as first described by Dignat-George [14]. After isolation, additional techniques, such as flow cytometry or microscopic analysis, are used to identify CEC based on morphological or immunophenotypical criteria. A major advantage of this technique is that it permits visual identification of CEC, which allows them to be discriminated from endothelial micro particles, anuclear cells, and cellular conglomerates. However, manual bead based isolation has several shortcomings, especially with regard to large monitoring studies. It is labour-intensive, requires a high level of operator skills, and requires additional steps to positively identify CEC, which make them unsuitable for high throughput monitoring. Furthermore, magnetic enrichment may give rise to an underestimation of the actual number of CEC. As CD146 expression on CEC is lower than on the HUVEC used to test assay recovery, its expression by CEC might be insufficient to bind to magnetic beads.

*Automated isolation and staining*
A variant on techniques applying a manual immunomagnetic enrichment step, is the CellTracks AutoPrep and CellTracks Analyzer II System (Immunicon Corp, Huntington Valley, PA), initially designed to detect circulating tumour cells. As with manual immunomagnetic isolation, cells are isolated by CD146-coupled ferrofluids, with the main difference being that the CellTracks System is fully automated, and is therefore not operator dependent. After isolation, the suspension of $CD146^+$ cells is stained with (i) DAPI to identify nucleated cells, (ii) CD105, fairly unique on endothelial cells, and (iii) CD45, to exclude CD146-expressing T-cells. The enriched and stained sample is dispensed in a magnetic cartridge to form a monolayer of cells, which is scanned by the CellTracks Analyzer II System. The generated images are evaluated for CEC by visual inspection, in which CEC are defined as $DAPI^+$, $CD105^+$, $CD146^+$, $CD45^-$ [8]. The endothelial feature of the cells meeting these phenotypic criteria was further demonstrated by global gene expression profiling, which clearly demonstrated the presence of endothelial markers [13].

Drawbacks of this method are the costly equipment and reagents, while assay customization is not possible. Also, the maximum number of only 8 samples than can be analysed in a single run, combined with the relative long duration of a complete run (approximately 4 hours), limits high throughput analysis.

*Flow cytometry*
Flow cytometry is a widely accepted tool for immunophenotyping of cells. A cocktail of fluorochrome-labelled mAb is used to identify CEC, allowing a highly specific definition of CEC, and possibly, the identification of endothelial cell subsets such as EPC. Another benefit of flow cytometry is, that by multi-colour staining cells with an immunophenotypical overlap with CEC, such as endothelial micro particles and platelets, can be excluded from evaluation, which increases specificity. However, flow cytometry assays in whole blood are at risk to overestimate CEC by enumerating false

positive cells. A well-known cause of false positivity is non-specific antibody binding. Non-specific binding can be the result of Fc receptor binding, binding to dead cells, or by improper use of reagents. A recent study clearly showed that the exceptionally high CEC counts found with a commonly applied flow cytometry based assay [15], were due to the fact that the majority of the cells designated CEC were in fact large platelets [16]. As a result of improper titration of the CD146 mAb, cells with a $CD45^{dim}$, $CD31^{+}$ phenotype stained positively for this antigen, suggesting that CEC were enumerated. Such problems can be prevented through staining whole blood rather than a cell suspension, or through the use of a blocking reagent, by which Fc receptors are kept saturated and non-specific binding is prevented. Furthermore, addition of a real-time viability stain, such as 7-aminoactinomycin D or propidium iodide, allows the exclusion of dead cells. Next to interference by non-specific antibody binding, false positivity can also result from specific binding to soluble forms of the antigen. Many endothelial surface antigens such as sCD144, and sCD146 are secreted, and their uptake by platelet aggregates can result in positivity. An effective strategy for excluding platelets, aggregates and endothelial micro particles from analysis is by using 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).

Recently, absolute CEC counts obtained by both magnetic isolation and flow cytometry assays were compared [7]. The reasonable agreement in CEC counts between both methods, in combination with an established CEC phenotype (i.e. $CD34^{+}$, $CD146^{+}$, $CD45^{-}$), is suggestive that actual CEC counts are being approached by this flow cytometry assay. However, isolation and validation as done on the magnetically isolated cells (Rowand et al, 2007), was not performed on the CEC as defined by Goon et al (2006).

## CLINICAL RESULTS

Although a large number of studies on CEC in cancer patients have been published, many of these studies rely on a flow cytometric approach, which defines CEC as being $CD45^{-}$, $CD31^{+}$, and $CD146^{+}$. Although this is a generally accepted CEC immunophenotype, no reports are currently available in which the endothelial origin of cells enumerated by those assays has been demonstrated unambiguously[17,18].
As a result, studies on CEC that are in our opinion reliable, are scarce: only three studies are currently available, all based on CD146 driven magnetic isolation of CEC [8,9,12].

In the first study, CEC were enumerated by magnetic isolation, followed by visual analysis of CD31, CD309 and VWF expression, determined by immunofluorescence. CEC numbers in cancer patients (n=146, mean CEC 399±36/mL) were found to be increased 3.3 fold when compared to healthy controls (n=46, mean CEC 121±16/mL). Further analysis of CEC numbers in cancer patients, demonstrated significantly higher CEC in patients with progressive disease vs. those with stable disease (95 patients, 438±65 CEC/ml and 17 patients, 179±61 CEC/ml, respectively), whereas no difference was seen between patients with stable disease and healthy

donors [12]. The increased CEC count in cancer patients was confirmed in a study of Rowand using the CellTracks System. Cells were isolated by automated CD146 driven magnetic isolation, and assay accuracy, sensitivity, linearity and precision were assessed, Hereafter, nucleated ($DAPI^+$) cells, expressing CD105 but lacking CD45 were enumerated in 249 healthy donors and 206 patients with metastatic cancer [8]. The observed mean CEC count of 21 in healthy controls is comparable by those found by the EUROCEC assay. Strikingly, the number of CEC reported in healthy donors was tenfold lower when compared to those reported by Beerepoot, namely 0-20/mL [8]. Both groups isolate CEC using beads targeting CD146, but Beerepoot uses CD31, CD309 and VWF, rather than CD105 to confirm the endothelial origin of cells. Where CD31 and VWF are considered to be pan-endothelial cell markers, CD105 is expressed predominantly on proliferative and/or malignant CEC. Given its high recovery and reproducibility and extensive validation, we favour the CellTracks assay for isolating CEC.

## POTENTIAL APPLICATIONS IN ONCOLOGY

Provided that an adequately validated, sensitive and specific assay is used, the detection and enumeration of CEC in patients with solid malignancies offer a wide spectrum of potential applications.

*CEC: Marker for establishing prognosis and for follow-up?*
The observation that advanced cancer patients have higher CEC counts than healthy controls [8,12], whereas patients with stable disease and healthy individuals have similar numbers [12], may imply that CEC enumeration may be used for prognosis and during follow-up in cancer patients. Obviously, verification of this statement would require studies in which large numbers of patients are included with enumeration of CEC at baseline and during follow-up.

*CEC: Marker for response to treatment?*
Classic methods to establish anti-tumour effects of systemic agents, which rely on assessing changes in tumour size by radiological assessments, are not longer sufficient. This holds true not only when using conventional chemotherapeutic drugs, but in particular when angiogenesis inhibitors are applied.
In several randomized trials exploring such drugs, enhanced progression-free periods were observed, yet the response rates as defined by RECIST, were minimal [19]. Such findings emphasize the limitations of conventional imaging, as well as the need to incorporate novel, sensitive indicators of response.

The increase in CEC numbers in mice stimulated with VEGF, and the consecutive decrease of these numbers after treatment with the angiogenesis inhibitor endostatin [20], are suggestive for a possible role for CEC in monitoring response to treatment when using agents targeting the VEGF-VEGFR pathway. Recently, a xenograft prostate cancer model showed that an increase in apoptotic CEC after treatment with thalidomide and docetaxel, was predictive for response to treatment [21].

However, as these results were based on a non-validated assay, it is important to confirm these findings using different, validated techniques.

*CEC: Guideline for optimal drug dosing?*
Nowadays it is increasingly recognised that the recommended dose for further exploration of a drug should be the optimal biological drug dose (OBD) rather than the MTD. The OBD is defined as the dose that is feasible to be applied in humans and yields biological effects. Changes in CEC counts after dose escalation might provide useful insights in establishing the OBD when assessing agents targeting vasculature such as VDA or angiogenesis inhibitors. A recent study by Celik et al. reports a 50% decrease in CEC in tumour bearing mice treated with endostatin. The decrease in CEC showed a clear U-shaped dose relation. The optimum dose, determined by assessment of tumour micro vessel density and analysis of tumour blood flow, resulted in the largest decrease in CEC numbers, whereas under- or over treatment resulted in a diminished decrease or even increase in CECs [22], suggesting a rationale for using CEC as guideline for optimum drug dosing. However, whether this holds true for humans, and for anti-angiogenic drugs other than endostatin, remains to be established.

*CEC: Towards identification of new targets in oncology?*
One of the major advances in the management of cancer in the last decades is the introduction of targeted therapy. By identifying tumour factors that play a crucial role in the pathogenesis of a disease, an avalanche of new targets for therapy has been identified. Examination of endothelial cells from tumour vasculature, may result in the identification of antigens specific for malignant angiogenesis, such as the recently identified H3 homologue of the co- stimulatory molecule B7 (CD276) [23], and allow the development of agents selectively targeting tumour vasculature.

*CEC: Marker for vascular toxicity?*
With better treatment outcomes for some patient populations with advanced malignancies, there is growing interest for long-term side effects of anti-tumour therapies. An increased incidence of cardiovascular events has emerged as one of the most important long-term untoward sequelae. For example, patients treated with chemotherapy for advanced germ-cell cancer have a 2-7 fold increased risk for cardiovascular events when compared to the general population [24]. Although the exact mechanism of this increased risk is unknown, in vitro data suggest that cytotoxic agents directly cause endothelial damage. Frequently used agents as cisplatin, bleomycin and etoposide, can cause thickening of the carotid artery intima, Raynaud's phenomenon, and an increase in plasma C-reactive protein (CRP), VWF and PAI-1, all associated with endothelial dysfunction [25]. Monitoring biomarkers of endothelial dysfunction or damage such as CEC levels, both during and after treatment might provide more insight into the vascular toxicity profile of chemotherapeutic agents. Based on such data, less vasotoxic treatments with equivalent anti-tumour activity would be warranted, especially for patients for whom treatment is likely to result in long-term survival, such as in germ cell cancers, childhood lymphoblastic leukaemia and lymphoma subtypes. A recent phase I study with the protein kinase ß C inhibitor enzastaurin (Rademaker-Lakhai et al, 2007), in which CEC were enumerated by immunomagnetic isolation, did not find an effect of enzastaurin on CEC numbers. In

contrast, a study on the VDA ZD6126 demonstrated a significant increase in immunomagnetically isolated CEC 2-8 hours after infusion (Beerepoot et al, 2006). Given the presumed mechanism of action of VDAs, this finding strongly suggests that CEC serves as a marker to assess vascular toxicity of drugs.

## CONCLUSIONS

In view of the growing recognized role of angiogenesis in oncology, and the integration of drugs targeting tumour vasculature, biomarkers that enable monitoring effects on vasculature are urgently needed. Many soluble markers, including VWF and thrombomodulin have been proposed as such markers. However, most of these are acute phase products and therefore susceptible to interference by other events frequently encountered in cancer patients such as infection. By contrast, monitoring CEC appears an attractive candidate. Several techniques have been developed to detect and enumerate CEC, but the lack of consensus on the phenotype of CEC, as well as their low numbers in blood, have resulted in conflicting results and have severely hindered progress in this important field. Providing a clear definition of CEC together with careful confirmation and validation that cell population designated "circulating endothelial cells" are indeed CEC, are the most important issues that need to be addressed. In order to overcome these issues, suggestions on phenotype and detection strategy have been provided by the EUROCEC network [9]. Despite these problems, validated assays enabling the proper enumeration of CEC are since recently available and the first results have revealed that patients with advanced cancer have indeed higher levels than healthy controls. In theory, there are many potential applications for the assessment of CEC counts in patients with solid malignancies including establishing prognosis and therapy-induced effects, but whether or not CEC can actually be used for these purposes is currently the subject of clinical studies. So only time will tell whether CEC can fulfil its promise as biomarker in clinical oncology.

## 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. Kabbinavar F, Hurwitz HI, Fehrenbacher L, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol. 2003;21:60-65.
3. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med. 2007;356:115-124.
4. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006;355:2542-2550.
5. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med. 2001;7:1194-1201.
6. 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.
7. 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.
8. 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.
9. 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.
10. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 2005;106:2923-2924.
11. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J Cell Biol. 1984;99:2034-2040.
12. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol. 2004;15:139-145.
13. 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.
14. George F, Brisson C, Poncelet P, et al. Rapid isolation of human endothelial cells from whole blood using S-Endo1 monoclonal antibody coupled to immuno-magnetic beads: demonstration of endothelial injury after angioplasty. Thromb Haemost. 1992;67:147-153.
15. 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.

16. 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.
17. Duda DG, Cohen KS, di Tomaso E, et al. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J Clin Oncol. 2006;24:1449-1453.
18. 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.
19. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med. 2003;349:427-434.
20. Schuch G, Heymach JV, Nomi M, et al. Endostatin inhibits the vascular endothelial growth factor-induced mobilization of endothelial progenitor cells. Cancer Res. 2003;63:8345-8350.
21. Li H, Raia V, Bertolini F, Price DK, Figg WD. Circulating endothelial cells as a therapeutic marker for thalidomide in combined therapy with chemotherapy drugs in a human prostate cancer model. BJU Int. 2007.
22. Celik I, Surucu O, Dietz C, et al. Therapeutic efficacy of endostatin exhibits a biphasic dose-response curve. Cancer Res. 2005;65:11044-11050.
23. 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.
24. Meinardi MT, Gietema JA, van der Graaf WT, et al. Cardiovascular morbidity in long-term survivors of metastatic testicular cancer. J Clin Oncol. 2000;18:1725-1732.
25. 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.
26. 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.
27. Moroni G, Del Papa N, Moronetti LM, et al. Increased levels of circulating endothelial cells in chronic periaortitis as a marker of active disease. Kidney Int. 2005;68:562-568.
28. Dixon GL, Heyderman RS, van der Ley P, Klein NJ. High-level endothelial E-selectin (CD62E) cell adhesion molecule expression by a lipopolysaccharide-deficient strain of Neisseria meningitidis despite poor activation of NF-kappaB transcription factor. Clin Exp Immunol. 2004;135:85-93.
29. Corcoran TB, O'Shea A, Engel A, Shorten GD. The influence of propofol on P-selectin expression and nitric oxide production in re-oxygenated human umbilical vein endothelial cells. Acta Anaesthesiol Scand. 2006;50:348-354.
30. 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.

# CHAPTER 3

## CELLS MEETING OUR IMMUNOPHENOTYPIC CRITERIA OF ENDOTHELIAL CELLS (CMOIC) ARE LARGE PLATELETS

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***Background***
Circulating endothelial cells (CEC) are shed from damaged vasculature, making them a rational choice to serve as surrogate marker for vascular damage. Currently, various techniques and CEC definitions are in use, and their standardisation and validation is needed. A flow cytometric single platform assay defining CEC as forward light scatter $(FSC)^{low\ to\ intermedate}$, sideward light scatter $(SSC)^{low}$, $CD45^{-}$, $CD31^{++}$ and $CD146^{+}$ is a promising approach to enumerate CEC because of its simplicity (Mancuso *et al.,* Blood 2001: 97, 3658-3661). Here, we set out to confirm the endothelial nature of these cells.

***Methods***
We isolated cells with a $FSC^{low\ to\ intermediate}$, $SSC^{low}$, $CD31^{++}$, $CD45^{dim}$ immunophenotype (termed "cells meeting our immunophenotypic criteria for endothelial cells" [CMOIC]) from healthy donors to study the expression of endothelium-associated markers using several techniques. Special attention was paid to reagents identifying the endothelial cell-specific marker CD146. We compared antigen expression patterns of CMOIC with those of the HUVEC endothelial cell line and lymphocytes. Electron microscopy was used to detect the presence of endothelial cell-specific Weibel-Palade bodies in the sorted cells.

***Results***
CD146 expression was negative on CMOIC for all tested CD146 mAbs, but positive on HUVEC cells and a minor subset of T lymphocytes. Using flow cytometry, we found no expression of any endothelium-associated marker except for CD31 and CD34. HUVEC cells were positive for all endothelial markers except for CD34. Evaluation of CMOIC morphology showed a homogenous population of cells with a highly irregular nucleus-like structure and positive endothelial immunohistochemistry. CMOIC contained neither nuclei nor DNA. Electron microscopy revealed the absence of a nucleus, the absence of endothelial specific Weibel-Palade bodies, and revealed CMOIC to be large platelets.

***Conclusion***
The vast majority of cells with the immunophenotype $FSC^{low\ to\ intermediate}$, $SSC^{low}$, $CD45^{-}$, $CD31^{++}$ do not express CD146 and are large platelets rather than endothelial cells.

## INTRODUCTION

Circulating endothelial cells (CEC) are mature endothelial cells shed from injured vasculature [1,2]. The concentrations of CEC are reportedly increased in disease conditions associated with vascular damage such as sickle cell anaemia [3], pulmonary hypertension [4], rickettsial infection [5], myocardial infarction [6], ANCA-associated vasculitis [7] and several types of cancer [8]. Furthermore, it appeared that the concentrations of CEC frequently correlated with disease severity. Enumeration of CEC may be especially relevant for clinical oncology. Since several new anti-tumor drugs target tumor angiogenesis or tumor vasculature, CEC counts are a potential surrogate marker for assessing drug efficiency and therefore hold promise to avoid over- or under-treatment of individual patients. In addition, reductions in CEC concentrations may reflect tumor growth and thereby serve as a parameter to reveal disease progression at an early stage [9].

Various enumeration methods for CEC have been reviewed by Blann *et al.* [1] and Khan *et al.* [10]. Approaches based on cell enrichment (i.e. immunomagnetic sorting, density gradient isolation, affinity separation), as well as approaches based on flow cytometry only are used. Currently, there is no consensus about the immunophenotype of CEC and numerous combinations of cell surface and intracellular markers are in use. Commonly used markers include CD31, CD34, CD105, CD144, CD146, CD202b and CD309, with CD146 being the most widely used as endothelial cell marker. As a consequence of these differences in terms of techniques and definitions, the reported concentrations of CEC in healthy donors and cancer patients vary widely, i.e., ranging between 0.7 and 7,900 cells per mL of peripheral blood (PB) [10]. Therefore, and in view of the increasingly recognized importance of CEC, standardization and validation of methods to enumerate CEC are needed.

Given its minimal sample manipulation, the approach developed by Mancuso *et al.* [8] appears to be very attractive. This method is based on a single platform whole blood assay without prior cell enrichment, in which CEC are defined as events having low to intermediate forward light scatter (FSC), low sideward light scatter (SSC), and being $CD31^{++}$, $CD45^{-}$ and $CD146^{+}$. Mean $CD146^{+}$ CEC counts in healthy donors reported were 7,900 cells per mL using this approach (8), which is in sharp contrast to numbers observed using enrichment-based methods (typically <100 CEC per mL)[1,10]. In view of the large discrepancy between results of single platform methodology and cell enrichment-based methods, we set out to confirm the endothelial nature of $FSC^{low\ to\ intermedate}$, $SSC^{low}$, $CD31^{++}$, $CD45^{-}$ cells in the first approach, which we refer to as 'cells meeting our immunophenotypic criteria of circulating endothelial cells' (CMOIC).

## MATERIALS AND METHODS

### *Subjects and sample collection*

PB was obtained from 43 healthy donors. Blood was drawn form the antecubital vein into 7 mL tubes containing ethylenediaminetetraacetic acid (EDTA). All subjects were volunteers and had signed informed consent. The study protocol was reviewed and approved by the institutional Medical Ethical Review Board.

### *Flow cytometry*

*Monoclonal antibodies (mAb) and dyes.*

Various conjugated mAb, viability stains and nuclear dyes were used to assess the immunological phenotype of CMOIC in greater detail (Table 1). Antibodies were conjugated to fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). All reagents were diluted based on titration, i.e., absence of non-specific staining on negative populations and optimal discriminatory power between negative and positive populations (Figure 1). To assess the presence of nuclei and DNA, we used four nuclear dyes, i.e., DRAQ5 (BioStatus, Leicester, UK), 7-aminoactinomycin D (7-AAD; Sigma-Aldrich, St. Louis, MO), propidium iodide (PI; Sigma-Aldrich) and Laser Dye Styryl-751 (LDS 751; Exciton, Dayton, OH). All dyes were used in concentrations as recommended by their manufacturers. In addition, samples stained for 7-AAD and PI were permeabilized using Fix & Perm (An der Grub, Kaumberg, Austria) in order to stain DNA.

*Data acquisition and analysis.*

Acquisition was done using a FACSCalibur flow cytometer equipped with a second red-diode laser (BD Biosciences, San Jose, CA). Flow cytometer setup and calibration was performed as described previously [11]. Data analysis was performed using CellQuest Pro v5.2 (BD Biosciences). At least 100,000 leukocytes per sample were acquired, and analysis was considered informative if >100 events had been collected that met the CMOIC criteria, i.e., $FSC^{low\ to\ intermedate}$, $SSC^{low}$, $CD31^{++}$, $CD45^{-}$ (see Results).

### *HUVEC*

The HUVEC-C CRL-1730 cell line was purchased from ATTC (Manassas, VA) and was cultured and harvested as recommended by the supplier. The viability of HUVEC cells was established by staining with trypan blue before each use.

### *Cell Sorting*

For cell sorting, blood samples were prepared using a stain-lyse-wash procedure in order to increase the nucleated cell concentration in the sample. Staining and lysing of whole blood was performed as for absolute cell count enumeration. After lysis, the cell suspension was centrifuged for 5 min. at 500 x *g* at room temperature (RT). The supernatant was removed from the sample tube.

The cell pellet was resuspended in 2 ml of phosphate-buffered saline (PBS), and centrifuged for 5 min. at 500 x *g* at RT. Finally, the supernatant was removed and the pellet was resuspended in 250 µl PBS. For EM analysis, pre-enrichment of mononuclear cells was done by means of density gradient centrifugation using

Lymphoprep (Axis Shield PoC, Norway, density: 1.077). Individual cellular subsets were sorted using a FACSAria Cell Sorting System (BD Biosciences) equipped with FACSDiva v4.1.2 software (BD Biosciences) and a 70µm nozzle; sheet fluid pressure was 70 PSI (pounds per square inch). For studies based on morphology, immunohistochemistry and fluorescence in situ hybridization (FISH), we sorted 4 spots of 200 CMOIC and 4 spots of 200 lymphocytes on standard glass slides. For QRT-PCR and EM, the required amount of cells was sorted in 15ml tubes containing 750µl of fetal calf serum.

***Morphology***

May-Grünewald-Giemsa (MGG) staining was done using a Autostainer XL (Leica Microsystems GmbH, Wetzlar, DE). Images were acquired on a DM2500 microscope equipped with a DC500 digital camera (both from Leica). The acquisition software (Leica IM1000 version 4.0) set focus, brightness and contrast automatically.

***Immunohistochemistry***

Two-step horseradish peroxidase (HRP)-based immunohistochemistry (IHC) was performed 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, von Willebrand Factor (both from Dako) and Fli-1 (Santa Cruz Biotechnologies, Santa Cruz, CA). Images were acquired on an Axioplan 2 Imaging microscope (Carl Zeiss, Göttingen, Germany).

***FISH***

Freshly prepared cytospin preparations were used made from 200 sorted CMOIC or 200 lymphocytes. Pre-treatment of slides and hybridisation of 0.5-1 µl of probes for centromere 7 and 8 (CEP 7 and CEP 8, Vysis, Abbott Laboratories, Des Plaines, IL) was performed as described elsewhere [12]. Slides were counterstained with DAPI and embedded in Vectashield/DABCO (Vector Laboratories, Burlingame, VT). For each sample as much as interphase cells as possible were scored. Images were captured with an epifluorescence microscope (Axioplan 2, Carl Zeiss) using MacProbe version 4.3 software (Applied Imaging, Newcastle upon Tyne, UK).

***Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR)***

QRT-PCR for the endothelial cell-specific gene vascular endothelial cadherin (VE-Cadherin, CD144) was performed on sorted CMOIC, HUVEC and lymphocytes using the protocol and primers described by Rabascio *et al.* [13]. RNA was isolated using the ChargeSwitch Total RNA Cell Kit (Invitrogen, Paisley, UK). Complementary DNA synthesis was done using Reverse Transcriptase Superscript III (Invitrogen). Standard curves were obtained using plasmid kindly donated by Dr. Rabascio (Milan, Italy). RT-PCR was performed on a Taqman RT-PCR instrument with the probe 6-FAM-TGT-GAG-AAC-GCT-GTC-CAT-GGC-CAG-TAMRA (Applied Biosciences, Foster City, CA). For quantification, the 18S ribosomal RNA component was used using the Taqman Ribosomal RNA Control Reagent (Applied Biosciences) as endogenous control. HUVEC cells were spiked in 5,000 lymphocytes counted by cell sorting. Fifty, 500 and 5,000 HUVEC cells per sample was done to define assay sensitivity. Specificity of the assay was confirmed by comparing a positive control (5,000

HUVEC) with a negative control (5,000 lymphocytes per sample). For detection of VE-Cadherin in CMOIC, 5,000 sorted cells per test sample were used.

***Electron microscopy (EM)***

Cells were fixed in EM fixative 4cF-1G (4% of commercial formalin, 1% of glutaraldehyde) for 24 hours at RT. Secondary fixation was done by osmium tetroxide 1% for 24 hours. The sample was dehydrated by centrifugation in 50%, 70%, 90%, 95% and 100% aqueous acetone and in equimolar parts of 100% acetone/epoxy resin consecutively (each step: 5 minutes at 1000 x g at RT). The dehydrated pellet was embedded in pure epoxy resin (Embed 812, EMS, Hatfield, PA). Polymerisation took place at 60 degrees Celsius for 18 hours, followed by appropriate sectioning of the embedded cell pellet. Electron micrographs were acquired using a Morgagni 268(D) transmission electron microscope (FEI Electron Optics, NL) at an excitation voltage of 80 kV.

**Table 1 - Markers used for immunophenotyping of CMOIC**

| Marker | Description | Clone | Manufacturer |
|---|---|---|---|
| CD31 | PECAM-1 | WM59 | BD Biosciences |
| CD34 | Hematopoietic stem cell marker | 8G12 | BD Biosciences |
| CD45 | Pan-leukocyte marker | 2D1 | BD Biosciences |
| CD45 | Pan-leukocyte marker | J33 | Immunotech |
| CD105 | TGF-β1 receptor | 1G2 | Immunotech |
| CD106 | VCAM-1 | 51-10C9 | BD Biosciences |
| CD133 | Progenitor cell marker | AC133/1 | Miltenyi Biotec |
| CD146 | MelCAM | P1H12 | BD Biosciences |
| | | P1H12 | Chemicon |
| | | S-Endo 1 | BioCytex |
| CD202b | Angiopoietin receptor | 83751 | R&D Systems |
| CD309 | VEGFR-2 | 89106 | R&D Systems |
| 7-AAD | DNA specific viability stain. | - | Sigma-Aldrich |
| DRAQ5 | DNA specific dye | - | BioStatus |
| LDS 751 | Nuclear and mitochondrial dye | - | Exciton |
| PI | DNA and ds RNA binding dye | - | Sigma-Aldrich |

*Abbreviations: PECAM-1 platelet/endothelial cell adhesion molecule; TGF-β1 transforming growth factor β1; VCAM-1 vascular cellular adhesion molecule 1; MelCAM melanoma-associated cellular adhesion molecule; VEGFR-2 vascular endothelial growth factor receptor 2. Locations of manufacturers: BD Biosciences, San Jose (CA); Immunotech, Miami (FL); Miltenyi Biotec, Bergisch Gladbach (Germany); Chemicon, Temecula (CA); BioCytex, Marseille (France); R&D Systems, Minneapolis, (MN); Sigma-Aldrich (St. Louis, MO); BioStatus (Leicester, UK); Exciton (Dayton, OH).*

## RESULTS

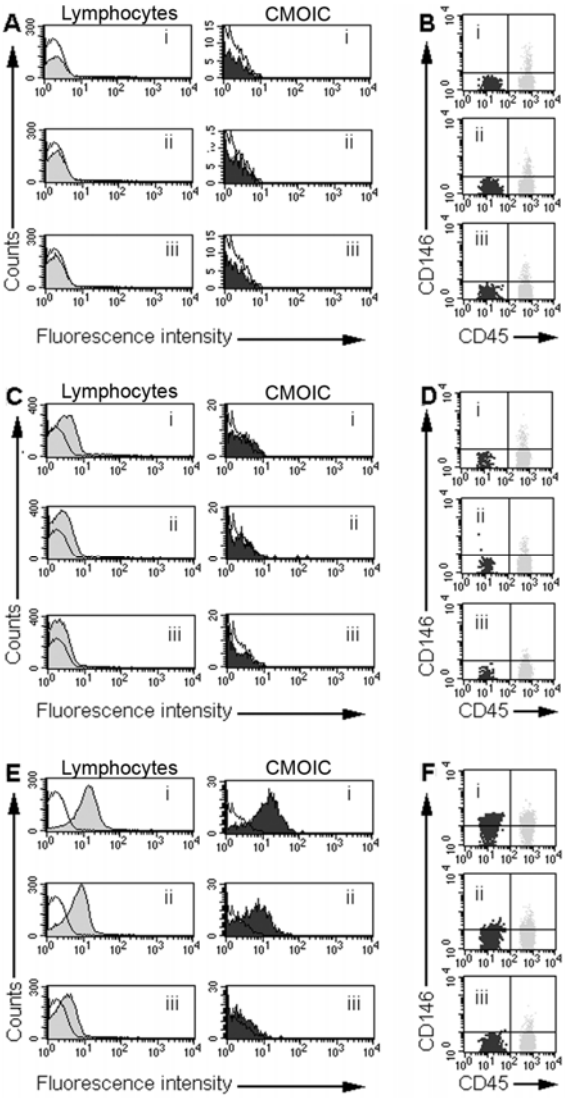
### *Flow cytometric enumeration of CMOIC*

Our protocol is based on that of Mancuso *et al.* [8] with two modifications. Briefly, 100 µL of PB is incubated with 10µL each of CD31 FITC mAb, CD45 PE and 10µL of 7-AAD for 15 minutes in darkness at room temperature (RT). After 15 minutes of lysis in $NH_4Cl$ (0.154mM in purified water), 100µL of counting beads (Flow-Count fluorospheres, Beckman-Coulter, Miami, FL) were added for calculating absolute cell numbers. The first protocol modification was implementation of back-gating on viable lymphocytes, similar to that used in the ISHAGE guidelines for flow cytometrical $CD34^+$ enumeration of hematopoietic stem cells [14]. This approach allows a more reproducible positioning of the lower FSC margin of the analysis gate. The second modification was the exclusion of CD146 from the staining panel as we found it to be negative on CMOIC (see below). We compared our protocol with that of Mancuso *et al.* [8] by testing 43 samples side-by-side. There was a strong correlation between the counts of CEC [8] and CMOIC (Pearson's correlation of r = 0.93 ). Specifically, the median CEC count was 31,000/mL (range, 12,000 to 64,000) and the median CMOIC count was 33,000/mL (range 9,500 to 65,000). The two-tailed t-test for paired samples revealed no significant difference between both methods ($P = 0.21$).

### *CMOIC do not express CD146*

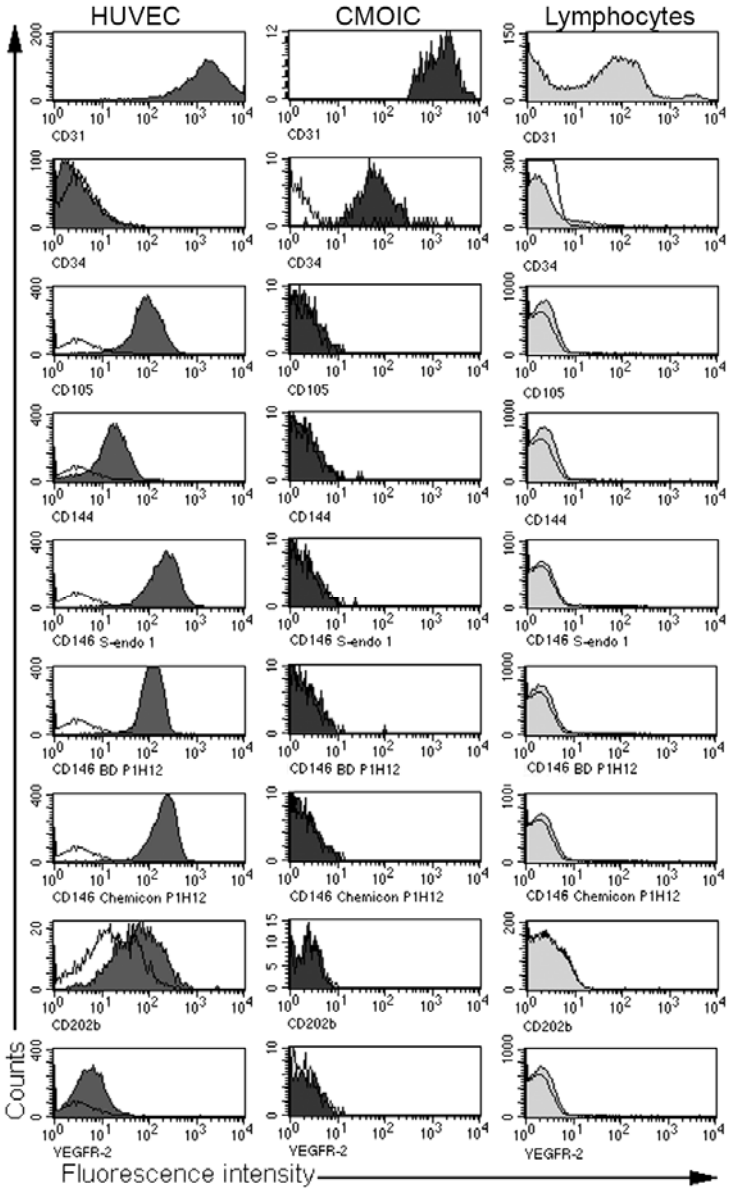
We studied the expression of CD146 by CMOIC (i.e., $FSC^{low\ to\ intermediate}$, $SSC^{low}$, $CD31^{++}$, $CD34^+$, $CD45^-$, $7\text{-}AAD^-$ events) using 3 PE-conjugated mAb. Expression of CD146 is known to be limited to endothelial cells [1,15,16] and a subset of activated T-cells [17,18]. Two mAb derived from the P1H12 clone (one from BD Biosciences [San Jose, CA] and one from Chemicon [Temecula, CA]), and a third mAb derived from the S-Endo 1 clone (BioCytex [Marseille, France]) were titrated on HUVEC, CMOIC and lymphocytes ($CD45^{bright}$, $SSC^{low}$). No expression of CD146 was detected within CMOIC and the majority of lymphocytes using the BD Biosciences and BioCytex mAb (Figure 1, panels A and C). The small $CD146^+$ subset of lymphocytes consisted of activated $CD3^+$ T cells (Ref. 22 and data not shown). As expected, all CD146 mAb showed bright and homogenous staining on HUVEC (Figure 2, left panels). In contrast to the other two CD146 mAb, the Chemicon reagent stained both CMOIC and lymphocytes when applied in the same concentration as originally described by Mancuso *et al.* [8], i.e., 0.2 µg per test (Figure 1, panels Ei). A negative signal with this mAb on the majority of lymphocytes was only obtained at a much lower concentration, i.e., 0.025µg/test (Figure 1, panel Eiii). At that concentration, no reactivity was observed against CMOIC (Figure 1, panel Eiii), whilst HUVEC (Figure 2) and a small subset of lymphocytes (Figure 1, panel Fiii) remained positive. Based on visual comparison of the BD Biosciences and Chemicon CD146 PE conjugates, we suggest that the reactivity of CMOIC with the latter conjugate is due to large amounts of free fluorochrome in this reagent (Figure 3).

**Figure 1 - Titration of three CD146 PE mAbs on CMOIC and lymphocytes**




*A and B: BBiosciences (clone P1H12); C and D: BioCytex (clone S-Endo-1); E and F: Chemicon (clone P1H12). Blood samples were stained using a stain, lyse, no-wash protocol using 10µl of antibody per test. The amounts of mAb (µg per test) used were: panels Ai-iii, Bi-iii: 0.015-0.008-0.003, panels Ci-iii, Di-iii 0.075-0.038-0.015:, panels Ei-iii, Fi-iii: 0.200-0.100-0.025. In each of Panels A, C and E, open (unstained) histograms show the fluorescence patterns of unstained cells. The filled histograms show CD146 fluorescence patterns on lymphocytes (gray) and CMOIC (black). The BD Biosciences P1H12 clone and BioCytex S-Endo 1 clone do not stain CMOIC and lymphocytes in any dilution (Panels Ai-iii, Ci,iii). Plotting both CMOIC and lymphocytes using CD45 vs. CD146 bivariate histograms reveals the presence of a minor CD146+ lymphocyte subset as expected [17], whilst CMOIC are CD146 negative with these mAb (Panels Bi-iii, Di-iii). In contrast, the Chemicon P1H12 reacts positively with CMOIC at 0.2 µg/test, but this is due to high background fluorescence, as the total lymphocyte population shows a similar staining pattern (Panels Ei-iii). Dim lymphocyte background fluorescence is still visible at a dilution of 1:400 (0.025µg/test; Panel Eiii); at this concentration, CMOIC are negative.*

**Figure 2 - Immunological characterization of HUVEC, CMOIC and lymphocytes**




*The open histograms show the fluorescence patterns of unstained cells. The filled histograms show the fluorescence patterns after staining with the mAb as indicated at the bottom of each panel. HUVEC show homogenous and positive staining patterns for all endothelium-associated markers except for CD34. Lymphocytes show heterogeneous staining for CD31, but are negative for the other markers. CMOIC are only positive for CD31 and CD34, but are negative for all other endothelial specific markers.*

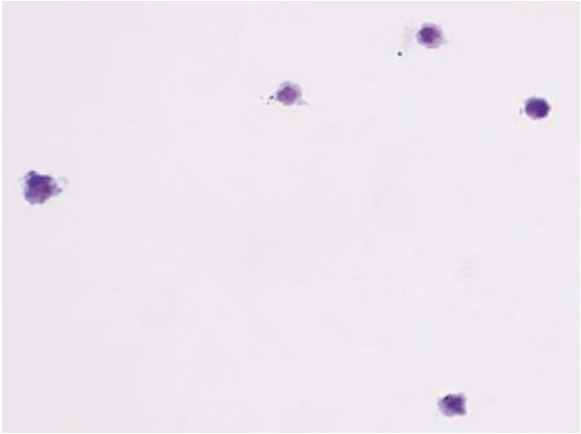
**Figure 3 - Vials containing various dilutions of PE-conjugated CD146 mAb**

*On the left, CD146 PE (clone P1H12, BD Biosciences) is shown as comparison. The BD Biosciences reagent is colorless, whilst the Chemicon (P1H12 clone) reagent is pink in all dilutions up to 1:400 (0.025 µg/test).*

***Cell morphology***

Evaluation of cell morphology of approximately 150 sorted CMOIC using light microscopy and May-Grünewald-Giemsa staining revealed a homogenous cell population. These cells were typically around 5µm in diameter, and contained an irregularly shaped nucleus-like structure and variable amounts of cytoplasm (Figure 4).

**Figure 4 - May-Grünewald-Giemsa staining of sorted CMOIC**



*This representative sample shows cells that contain an irregularly shaped structure and variable amounts of cytoplasm. Cell size ranges between 4 and 6 µM.*

### *Immunological phenotyping and comparison to HUVEC*

Because of the absence of CD146 on CMOIC, we tested various mAb commonly used in CEC enumeration: CD31, CD34 (class III), CD105, CD144, CD146, CD202b and CD309 (VEGFR-2) [8,10,19,20] on PB from 6 healthy donors for their expression on CMOIC. These expression patterns were compared to those of HUVEC, a cultured cell line used as model for endothelial cells, and to those of lymphocytes, which were used as a negative control (Figure 2). All these mAb except CD34 were reactive with HUVEC. Lymphocytes showed a very heterogeneous expression pattern of CD31, and were negative for all other endothelial markers. CMOIC were only dimly positive for CD34 (Figure 2). The expression of several other lymphocytic, myeloid and platelet markers, i.e., CD3, CD4, CD8, CD14, CD19, CD33, CD41, CD56, CD57 and CD61, was compared between CMOIC and leukocyte subsets. The expected staining patterns were observed for each leukocyte subset, whilst all markers except for the platelet markers CD41 and CD61 were negative on CMOIC (data not shown).

In addition to immunophenotyping by flow cytometry, we also addressed the expression of endothelial specific antigens by means of immunohistochemistry. We sorted CMOIC based on our definition of $FSC^{low\ to\ intermediate}$, $SSC^{low}$, $CD31^{++}$ and $CD45^{dim}$. Lymphocytes were sorted as a reference. Unstained cells were used as negative control. CMOIC showed an intense staining for CD31, vWF and Fli-1, which is consistent with the phenotype of endothelial cells and platelets [21]. Expectedly, lymphocytes did not stain for CD31 and vWF, and only dimly for Fli-1 (Figure 5).

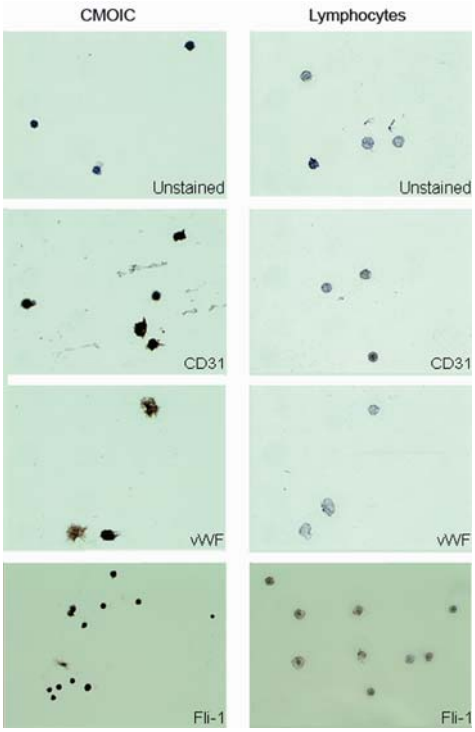
### *QRT-PCR for VE-Cadherin*

Because of the apparent lack of endothelial marker expression by CMOIC, we studied the presence of VE-Cadherin mRNA in these cells. We obtained a positive signal for VE-Cadherin mRNA from a minimum of 50 HUVEC spiked in 5,000 leukocytes. However, we did not detect a positive signal for VE-Cadherin mRNA when 5,000 CMOIC or 5,000 lymphocytes were tested (data not shown).

### *Assessment of DNA and/or RNA content of CMOIC*

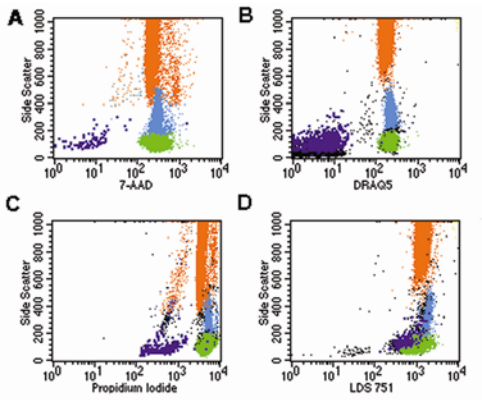
The lack of expression of endothelial specific surface antigens, the positive expression of thrombocyte markers CD41 and CD61, the immunohistochemical positivity of CD31, vWF and Fli-1 and the absence of endothelial specific VE-Cadherin mRNA prompted us to address whether or not CMOIC contained DNA and/or RNA. The DNA specific dyes 7-AAD and DRAQ5 stained brightly positive on all leukocytes but not on CMOIC (Figure 6, panels A and B). Both PI, which stains double-stranded DNA and RNA, and LDS 751, which stains RNA in nuclei and mitochondria, showed bright reactivity with leukocytes, but relatively dim reactivity with CMOIC (Figure 6, panels C and D). We then performed FISH using probes against the centromeres of chromosomes 7 and 8 in combination with counterstaining by DAPI. This approach revealed no signal in CMOIC but showed two copies of each centromere and a positive DAPI staining in lymphocytes (data not shown). These combined results indicate that CMOIC do not contain DNA.

**Figure 5 - Analysis by immunohistochemistry.**




*Expression of the endothelium-associated antigens vWF, CD31 and Fli-1 of sorted cells (left panels) were compared to lymphocytes (right panels). Unstained samples were used to assess positivity. Magnification: 400x. CMOIC show an intense but highly irregular staining for all tested markers. Lymphocytes do not stain for any of these markers.*

**Figure 6 - Assessment of presence of DNA, RNA and mitochondria in CMOIC and leukocyte subsets**

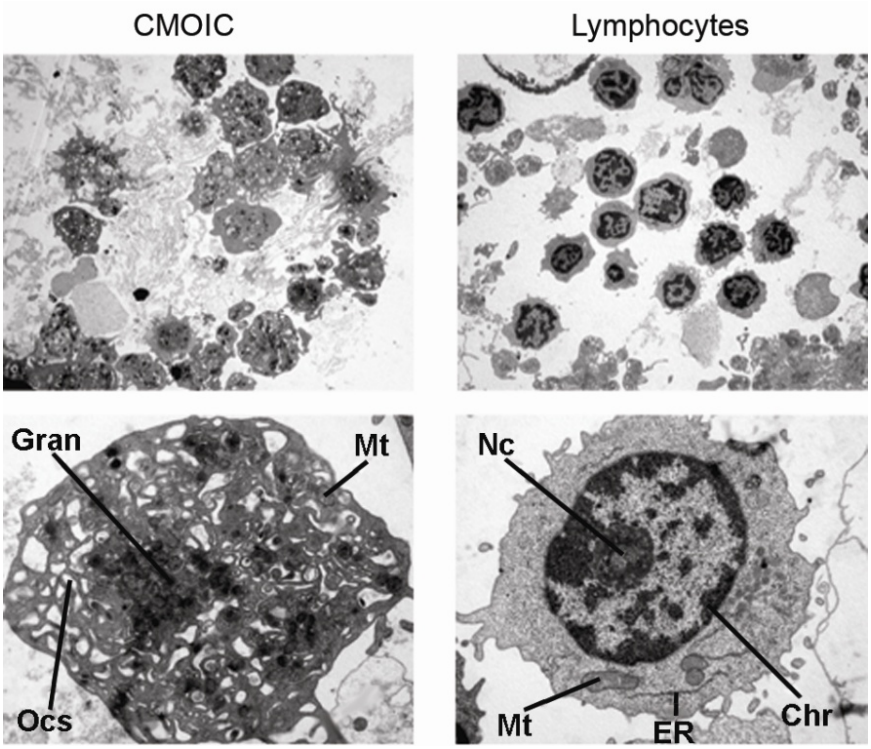



*One hundred µl of blood were stained with the stain, lyse, no-wash protocol using 10µl of CD31 FITC, 10 µl of CD45 PE and 10µl of the nuclear dyes in appropriate concentrations. Granulocytes are shown in orange, monocytes in blue, lymphocytes in green, CMOIC in purple and cellular debris in black. 7-AAD and DRAQ5 are positive on all leukocytes, demonstrating the presence of DNA in these cells. The negative staining of CMOIC implicates that these cells do not contain DNA (Panels A and B). PI and LDS751 are brightly positive on leucocytes. A less intense staining is observed on CMOIC, which is the result of staining double strand RNA in the case of PI (panel C) and the staining of mitochondria in the case of LDS751 (Panel D).*

***Electron microscopic analysis***

Weibel-Palade bodies are rod-shaped organelles that are specific for vascular endothelium in vivo [22] and in vitro [23]. Presence of these organelles would provide definite proof of the endothelial origin of our sorted CMOIC. For this purpose, 600,000 CMOIC were isolated by flow sorting, and a similar number of lymphocytes were sorted as control. A homogenous population of both CMOIC and lymphocytes was observed (Figure 7, upper 2 panels). At higher magnification (Figure 7, lower 2 panels), CMOIC appeared to be 4 µm in size not nucleated and to lack Weibel-Palade bodies. Instead, CMOIC were found to contain mitochondria, an open canalicular system and centrally located electron dense granulae: these morphological characteristics are consistent with large platelets [24-26].

**Figure 7 - Electron micrographs of CMOIC and lymphocytes**




*The upper panels show a representative overview of each sample at 2,200x magnification. The corresponding lower panels show a single CMOIC and a single lymphocyte at 11,000x magnification. Here, the lymphocyte shows a clearly distinguishable nucleus with chromatin (Chr) and a nucleolus (Nc). In the cytoplasm, mitochondria (Mt) and endoplasmatic reticulum (ER) are present. The CMOIC lacks a nucleus. A wide open canalicular system is present (Ocs), as well as some mitochondria (Mt). In the middle, mainly electron dense granulae (Gran) are present.*

## DISCUSSION

As the number of new molecules inhibiting angiogenesis increases, a monitoring tool for their effectiveness is needed in order to avoid over- or undertreatment with these compounds [1,10]. For this purpose the enumeration of CEC, which are thought to be shed from mature normal and tumor vasculature, is a promising [3-7,27] option. The single-platform flow cytometric approach as described by Mancuso *et al.* [8,28-30] appeared to be highly attractive for this purpose, as it would obviate the need for prior cell enrichment and, at the same time, generate absolute CEC counts. However, review of the results obtained with various methods of CEC enumeration revealed that the single-platform flow cytometric method yielded 100 to 1,000-fold higher CEC counts than methods using CD146-based enrichment of CEC [3-7,27] Therefore, we considered it necessary to confirm the endothelial nature of the cells identified by the single-platform flow cytometric approach. To this end, we investigated "cells meeting our immunophenotypic criteria of endothelial cells" (CMOIC), i.e., $FSC^{low\ to\ intermediate}$, $SSC^{low}$, $CD45^{dim}$ and $CD31^{++}$ .

Here, we showed that CMOIC are actually not CEC but large platelets. First, their reactivity with CD146 mAb was only demonstrated using the Chemicon CD146 PE conjugate and not with 2 other CD146 PE conjugates (i.e., the P1H12 from BD Biosciences and S-Endo 1 from BioCytex). This result, combined with our observation that the Chemicon CD146 PE conjugate reacted with most lymphocytes in the same concentration as which they stained CMOIC (i.e., 0.2 μg/test), but were negative with CMOIC at a much lower concentration where lymphocytes showed only dim reactivity, led us to conclude that CMOIC do not express CD146. Using immunophenotyping and sorting of CMOIC followed by morphology, IHC, and QRT-PCR, we were not able to detect cells with endothelial characteristics among these cells. The positive staining of CMOIC for vWF and Fli-1 was not conclusive with respect to their endothelial origin, as cells from the megakaryocytic lineage express these antigens as well [21,31]. Furthermore, nuclear staining and FISH failed to detect cells with a nucleus in this population and finally, electron microscopy revealed CMOIC to be large platelets. The absence of CMOIC (and platelets) in patients with severe aplastic anemia and chemoradiotherapy-induced bone marrow aplasia, and their reappearance in the circulation of engrafting recipients of an allogeneic stem cell transplant (data not shown) further suggests that CMOIC are derived from hematopoietic stem cells and not from endothelial cells lining the vasculature.

Our findings provide an explanation for the significantly higher $CD146^{+}$ CEC counts as reported using the single platform flow cytometric assay [8,28-30] and those determined by CD146-based enrichment methods [3-7,27] .We suggest that the CD146 positivity of CEC as defined by Mancuso *et al.*[8,28-30], which largely overlap with CMOIC, is due to non-specific reactivity of the Chemicon CD146 PE conjugate used in the published concentration [8]. These results emphasize the importance of appropriate mAb titration using well-defined positive and negative controls, and the use of internal negative controls (such as lymphocytes) in addition to unstained cells.

Recent approaches to CEC enumeration use immunomagnetic enrichment using paramagnetic particles coated with CD146 mAb in combination with markers to exclude hematopoietic cells (i.e., CD45) [32], or in combination with markers that confirm endothelial origin (Ulex Europaeus Lectin-1 and CD105) [32,33], as enrichment for CD146 also yields $CD146^+$ T cells. With these approaches, CEC counts in healthy individuals generally do not exceed 20 cells per mL [32,33].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## REFERENCES


1. Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost. 2005;93:228-235.
2. Delorme B, Basire A, Gentile C, et al. Presence of endothelial progenitor cells, distinct from mature endothelial cells, within human CD146+ blood cells. Thromb Haemost. 2005;94:1270-1279.
3. 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.
4. Bull TM, Golpon H, Hebbel RP, et al. Circulating endothelial cells in pulmonary hypertension. Thromb Haemost. 2003;90:698-703.
5. 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.
6. 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.
7. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis. Lancet. 2003;361:206-210.
8. 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.
9. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol. 2004;15:139-145.
10. 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.

11. 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;17:223-233.
12. van Zutven LJ, Velthuizen SC, Wolvers-Tettero IL, et al. Two dual-color split signal fluorescence in situ hybridization assays to detect t(5;14) involving HOX11L2 or CSX in T-cell acute lymphoblastic leukemia. Haematologica. 2004;89:671-678.
13. 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.
14. 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.
15. Bardin N, Frances V, Lesaule G, Horschowski N, George F, Sampol J. Identification of the S-Endo 1 endothelial-associated antigen. Biochem Biophys Res Commun. 1996;218:210-216.
16. Bardin N, George F, Mutin M, et al. S-Endo 1, a pan-endothelial monoclonal antibody recognizing a novel human endothelial antigen. Tissue Antigens. 1996;48:531-539.
17. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 2005;106:2923-2924.
18. Pickl WF, Majdic O, Fischer GF, et al. MUC18/MCAM (CD146), an activation antigen of human T lymphocytes. J Immunol. 1997;158:2107-2115.
19. Young MR. Tumor skewing of CD34+ progenitor cell differentiation into endothelial cells. Int J Cancer. 2004;109:516-524.
20. 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.
21. Jackers P, Szalai G, Moussa O, Watson DK. Ets-dependent regulation of target gene expression during megakaryopoiesis. J Biol Chem. 2004;279:52183-52190.
22. Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. Arterioscler Thromb Vasc Biol. 2006;26:1002-1007.
23. Friis T, Hansen AB, Houen G, Engel AM. Influence of angiogenesis inhibitors on endothelial cell morphology in vitro. Apmis. 2006;114:211-224.
24. Flaumenhaft R, Dilks JR, Rozenvayn N, Monahan-Earley RA, Feng D, Dvorak AM. The actin cytoskeleton differentially regulates platelet alpha-granule and dense-granule secretion. Blood. 2005;105:3879-3887.
25. Escolar G, Leistikow E, White JG. The fate of the open canalicular system in surface and suspension-activated platelets. Blood. 1989;74:1983-1988.
26. Weis L. Cell and Tissue Biology - A Textbook of Histology. Cell and tissue biology (ed 6). Baltimore: Urban & Schwartzenberg; 1983:434-435.
27. Nakatani K, Takeshita S, Tsujimoto H, Kawamura Y, Tokutomi T, Sekine I. Circulating endothelial cells in Kawasaki disease. Clin Exp Immunol. 2003;131:536-540.

28. Mancuso P, Calleri A, Cassi C, et al. Circulating endothelial cells as a novel marker of angiogenesis. Adv Exp Med Biol. 2003;522:83-97.
29. 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.
30. Mancuso P, Rabascio C, Bertolini F. Strategies to investigate circulating endothelial cells in cancer. Pathophysiol Haemost Thromb. 2003;33:503-506.
31. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998;67:395-424.
32. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LW, O'Hara S M. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. Cancer Res. 2006;66:2918-2922.
33. 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.

# CHAPTER 4

## CORRELATION BETWEEN CIRCULATING ENDOTHELIAL CELL COUNTS AND PLASMA THROMBOMODULIN LEVELS AS MARKERS FOR ENDOTHELIAL DAMAGE

*Thromb Haemost. 2008 Oct;100(4):642-7*

## SUMMMARY

***Background***
Increased numbers of circulating endothelial cells (CEC) in peripheral blood have been observed in diseases with vascular involvement, and are considered a promising surrogate marker for vascular damage.

***Objectives***
To evaluate the correlation between putative soluble markers of endothelial injury, activation, and endothelial proliferation, and absolute numbers of CEC.

***Patients/Methods***
CEC were evaluated in 125 healthy donors and 40 patients with metastatic carcinoma by automated CD146 driven immunomagnetic isolation. Plasma concentrations of E-selectin, endoglin, and thrombomodulin were assessed by ELISA in plasma obtained from 40 healthy donors and 40 patients.

***Results***
CEC numbers in blood were positively correlated with plasma thrombomodulin levels, but not with levels of E-selectin and endoglin. Multivariate analysis demonstrated a significant increase in CEC numbers with age. The levels of plasma biomarkers were not influenced by age. Higher levels of thrombomodulin and E-selectin were observed in males when compared to females.

***Conclusions***
CEC numbers correlate positively with plasma levels of thrombomodulin.

## INTRODUCTION

Angiogenesis plays a crucial role in the growth and dissemination of cancer [1]. Accordingly, inhibition of angiogenesis has recently been found to improve treatment efficacy in various types of cancer, such as renal cell carcinoma [2,3] and colorectal carcinoma [4]. Since angiogenesis inhibitors are considered cytostatic rather than cytotoxic drugs [5], response evaluation based on changes in tumor size, such as the widely applied Response Evaluation Criteria in Solid Tumors (RECIST), may not be appropriate [6]. Therefore, novel sensitive and specific surrogate markers are needed for accurate monitoring of clinical efficacy using these drugs. A specific marker for vascular damage would serve this purpose. Next to monitoring of anti-tumor activity, such a marker would ideally reveal tumor growth and disease progression at an earlier stage than conventional imaging in view of the known fragility of tumor vasculature compared to healthy vasculature [7,8]. Blood levels of circulating endothelial cells (CEC) are considered a rational choice to serve as a surrogate marker for endothelial damage since these cells are thought to have detached directly from the vessel wall [9].

Until recently, interpretation of CEC in clinical studies has been hampered by lack on consensus on the exact immunophenotype, as well as on the optimal detection strategy. As a result, studies yielded conflicting results. For example, CEC numbers in healthy donors varied from 0-7,900/mL, depending on the used assay. Enrichment based approaches, such as the assay proposed by the EUROCEC network (www.eurocec.com) [10], and the assay used in this study, typically report 0-20 CEC/mL in healthy controls, whereas single platform flow cytometry based assays result in CEC counts 100-1,000 fold higher [11,12]. This discrepancy in CEC counts has been addressed in a recent study, in which it was clearly demonstrated that these assays actually report the number of large platelets rather than CEC [13].

Recently, a novel approach using an automated rare cell detection system has proven to be highly accurate and reliable in enumeration and isolation of CEC from peripheral blood (PB) using CD146 automated immunomagnetic enrichment [14]. Gene expression profiling of these isolated CEC clearly demonstrated their endothelial origin [15]. Using this automated method, higher CEC numbers were revealed in blood of cancer patients with metastatic disease, when compared to healthy controls [14].

Nowadays, there is a good degree of agreement on the CEC immunophenotype, namely being CD146 positive, followed by a confirmatory step: Ulex-Europaeus-Lectin-1 staining [10] or image cytometry [14].

In addition to CEC numbers in blood, other markers are thought to reflect the presence and extent of vascular damage and endothelial cell dysfunction or activation, such as blood concentrations of soluble factors including soluble endothelial cell surface proteins, adhesion molecules secreted from dysfunctional endothelium, and pro-angiogenic cytokines. Elevated levels of these soluble markers have shown to be of prognostic value in diseases with vascular involvement.

In this respect, the following markers have been frequently discussed: soluble E-selectin (CD62E), which is an adhesion molecule expressed on activated endothelial cells [16-19]; endoglin (CD105), a component of the TGF-beta receptor complex and involved in regulating endothelial cell proliferation and differentiation, and the endothelial cell thrombin receptor thrombomodulin (CD141), which is directly associated with endothelial damage [20-22].

The main object of this study was to assess the correlation between CEC numbers in blood as a novel biomarker for endothelial injury, and the plasma levels of soluble markers known to be informative of the endothelial status. Additionally, we studied how these markers were influenced by age and gender. We included in this study a heterogeneous population of healthy controls and a randomly selected group of patients with metastatic cancer, as our major aim was to assess the correlation between CEC numbers and plasma concentrations endothelial related soluble markers, rather than to assess the effect of disease or treatment.

## PATIENTS AND METHODS

### *Donors and blood sampling*

Peripheral blood (PB) was obtained from 125 apparently healthy donors (56 male, 69 female; mean age 52 years, range 21-86) and 40 patients (13 male, 27 female; mean age 65 years, range 23-87). None of participants had severe renal dysfunction. Patients suffered from metastatic cancer originating from the colon (n=7), breast (n=7), colon/breast (n=1), ovary (n=8), lung (n=4), prostate (n=2), stomach (n=2), kidney (n=3), thyroid (n=2), bladder (n=2), mandible (n=1) and tongue (n=1). PB was drawn into CellSave preservative tubes (Immunicon Corp., Huntington Valley, PA, USA), which contain a proprietary preservative, and ethylenediaminetetraacetic acid (EDTA) as anticoagulant. To avoid contamination with traumatically detached endothelial cells, the first tube was discarded. Samples were kept at room temperature and were processed within 24 hours. The protocol and informed consent forms were approved by the Independent Investigational Review Board (Plantation, FL) and all donors (all US citizens) provided written consent prior to participation in the trial.

### *CEC enumeration*

The CellTracks® AutoPrep® System and the CellSpotter® Analyzer II System (Immunicon Corp) were used to enumerate CEC, and have been described elsewhere (10). Briefly, four milliliters (mL) of blood were used for immunomagnetic enrichment using ferrofluids coupled to an anti-CD146 antibody. This marker is present on endothelial cells, a subset of activated T-lymphocytes, and melanoma cells [23-25]. After enrichment, the following reagents were added: the nuclear dye 4,6-diamidino-2-phenylindole (DAPI), and fluorochrome-conjugated monoclonal antibodies (mAb): phycoerythrin (PE)-conjugated CD105, which is present on endothelial cells, activated monocytes, and pre-B-lymphocytes [26], and allophycocyanin (APC)-conjugated CD45, a pan-leukocyte antigen included in order to exclude hematopoietic cells from analysis. Analysis was done using image cytometry, where CEC were defined as being $CD146^+$, $DAPI^+$, $CD105^+$ and CD45 negative [14].

### *Assessment of soluble endothelial markers by ELISA*

Plasma was obtained from 40 asymptomatic donors (18 male, 22 female; mean age 53 years, range 21-86), and 40 patients (13 male, 27 female; mean age 64 years, range 23-87) with metastatic cancer, from the same CellSave preserved whole blood sample in which CEC numbers were enumerated. Plasma concentrations of thrombomodulin, E-selectin, and endoglin were determined using ELISA according to the manufacturers' instructions. The thrombomodulin-specific ELISA was purchased from American Diagnostica Inc. (Stanford, CT, USA), the other ELISA were from R& D Systems (Minneapolis, MN, USA). Absorbance was read at 450 nm using a Titertek 212 MS microplate reader (Titertek, Huntsville, AL, USA). Samples were tested in duplicate and related to the standard curve. The lower detection limits of the assays were 2.0 ng/mL for E-selectin; 1.2 ng/mL for thrombomodulin, and 0.07 ng/mL for endoglin.

### *Validation of CellSave preservative tube -derived plasma for ELISA*

Currently, no reference values are available for the levels of the markers under study in plasma derived from CellSave preservative tubes. Therefore, we assessed the levels of these markers in CellSave and EDTA derived plasma obtained from 6 healthy donors (5 male, 1 female; mean age 34 years, range 29-41). All samples were aliquotted after collection and frozen at -80 °C. As the experimental samples for this study had been subjected to one previous freezing and thawing cycle, we additionally determined the effects of repeated freezing and thawing (i.e., 1, 2 or 3 times) on the levels of E-selectin, endoglin, and thrombomodulin.

### *Statistical analysis*

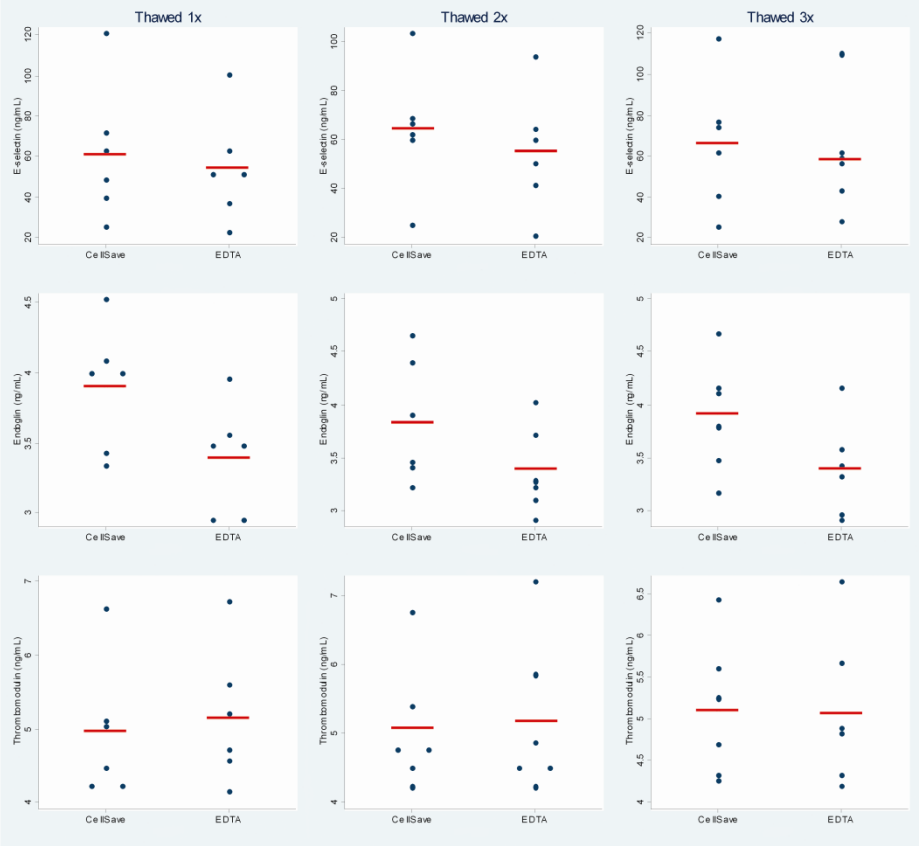
Assessment of skewness and kurtosis demonstrated a non-normal distribution of CEC numbers and plasma levels of endoglin, E-selectin and thrombomodulin in healthy donors. To approximate normality, a natural logarithmic transformation was performed on CEC, endoglin and E-selectin values. An inverse transformation was performed on levels of thrombomodulin. Differences in mean CEC counts between healthy controls and patients were assessed by the Mann-Whitney U test on untransformed data. Equality of paired data was done using the Wilcoxon signed-rank test. Correlations between parameters were calculated using Spearman's correlation coefficient. Relationships between variables were assessed using both uni- and multivariate linear regression analysis. Statistical differences were considered significant if $P < 0.05$. Values are displayed as mean ± standard deviation (SD) unless stated otherwise. Analyses were performed using the statistical software package (STATA 9.2, StataCorp College Station, TX, USA).

## RESULTS

### *Suitability of CellSave plasma for ELISA*

To assure appropriate quantification of soluble endothelial biomarker concentrations in plasma stored in CellSave preservative tubes, we compared the levels of these markers in CellSave plasma with those in EDTA plasma derived from 6 healthy controls. Samples were tested in ELISA before and after repeated freezing. This evaluation revealed no differences between CellSave and EDTA plasma levels of E-selectin, thrombomodulin and endoglin, regardless of the number of freeze-thaw cycles (Figure 1).

**Figure 1 - CellSave plasma is suitable for assessment of soluble endothelial biomarkers by ELISA.**




*Soluble marker concentrations were determined in CellSave and EDTA derive plasma obtained from 6 healthy controls following 1, 2 or 3 freezing/thawing cycles by ELISA and displayed as nanograms /mL. Presented are individual and median (line) concentrations.*

***Effect of prolonged storage at room temperature on plasma protein concentrations***

To exclude possible interference of prolonged storage on plasma levels of E-selectin, endoglin, thrombomodulin, we assessed concentrations of these proteins in plasma obtained within 30 minutes after sampling, and in plasma isolated after 72 hours of storage at room temperature. Subsequently, concentrations were compared using the Wilcoxon signed-rank test, which revealed a significant increase in plasma thrombomodulin levels (0.68 ± 0.4 ng/mL vs. 1.34 ± 0.2 ng/mL; P=0.03), but not those of endoglin and E-selectin (Table 1).

**Table 1 - Increased levels of thrombomodulin after 72 hours of storage at room temperature**

| | 30 minutes | 72 hours | Difference | P-value |
|---|---|---|---|---|
| Endoglin (ng/mL) | 3.7 ± 1.0 | 3.6 ± 1.2 | 3% decrease | NS |
| E-selectin (ng/mL) | 20.2 ± 9.9 | 15.2 ± 7.9 | 25% decrease | NS |
| Thrombomodulin (ng/mL) | 0.68 ± 0.4 | 1.34 ± 0.2 | 97% increase | P=0.03 |

*Soluble marker concentrations were determined in CellSave derived plasma obtained from 3 healthy donors and 3 cancer patients within 30 minutes after sampling and after 72 hours of storage at room temperature. Data is shown as mean ± standard deviation.*

***Correlation of CEC and plasma endothelial biomarkers***

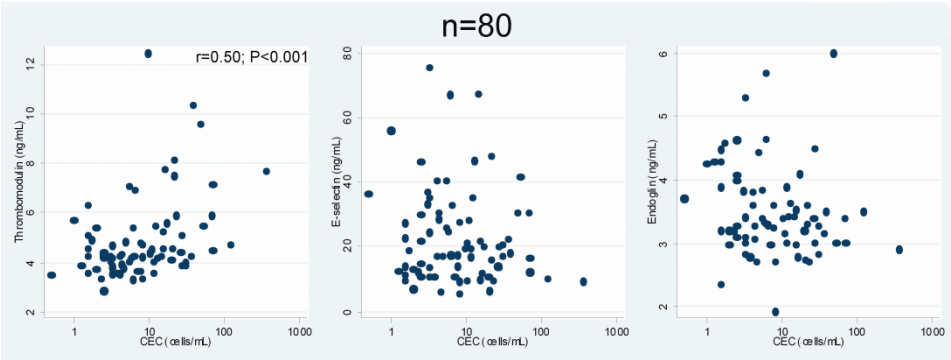
Details on the enumeration of CEC in the current cohorts of patient and healthy controls has been published elsewhere [14], and showed higher numbers of CEC in blood of patients (median 15 CEC/ml, range 0-360, n=40) than in healthy controls (median 5 CEC/ml, range 0-97, n=125; P=0.0001). The correlation between CEC numbers and plasma endothelial markers was analyzed in the 40 patients samples, extended with 40 healthy control samples that had been randomly selected from the total group of 125 controls. Analysis of these 80 samples, with CEC numbers ranging from 0-360 CEC/mL, demonstrated a positive correlation between CEC numbers and plasma levels of thrombomodulin (r=0.50; P<0.0001), but not with endoglin or E-selectin (Figure 2). Analysis of healthy controls alone revealed a similar correlation between CEC numbers and plasma thrombomodulin (r=0.51; P<0.001). In patients suffering from metastatic cancer, this correlation was weaker (r=0.38; P=0.02), possibly the result of the large biological heterogeneity of this group.

***Effect of age and gender on CEC and plasma biomarker levels***

Both uni- and multivariate analyses were performed to assess the effects of age, gender, and the interaction thereof on CEC numbers and plasma protein concentrations. In univariate analyses, we found a modest but significant increase of CEC ($R^2$=0.08; P=0.001) and thrombomodulin ($R^2$=0.13; P=0.024) with age. Age did not influence E-selectin and endoglin levels (data not shown). In addition, a significant effect of gender on thrombomodulin levels was observed, for which concentrations were higher in males when compared to females ($R^2$=0.22; P=0.03).

The effect of gender on E-selectin did not reach statistical significance in univariate analysis ($R^2$=0.08; P=0.07), despite the somewhat higher levels detected in males (27.0 ± 14.3 ng/mL vs. 20.5 ±15.3 ng/mL in females, respectively; P=0.05). No effects of gender were observed on CEC numbers, and plasma endoglin concentrations. After multivariate analysis, CEC numbers were only influenced by age ($R^2$=0.09; P=0.001), and the concentrations of thrombomodulin and E-selectin only by gender ($R^2$=0.28; P=0.009, and $R^2$=0.13; P=0.04, respectively). Endoglin levels were unaffected by age and gender.

**Figure 2 - Correlation between CEC numbers and plasma levels of soluble markers of endothelial injury or activation.**




*CEC numbers correlate significantly to plasma concentrations of thrombomodulin, but not those of E-selectin or endoglin*

## DISCUSSION

Enumeration of CEC numbers in blood is a promising candidate tool for monitoring of diseases that are known with vascular damage. Lack of consensus on the exact phenotype of CEC, as well as different assays used to enumerate them, resulted in inconsistent outcomes severely hindering the suitability of CEC as a marker for endothelial damage. Reviews on CEC enumeration by Blann *et al* [9] and Khan *et al* [27] revealed a large discrepancy in CEC numbers between CD146 driven enrichment based assays versus flow cytometry based assays, the latter yielding 100-1,000× more CEC in healthy controls [11,12].

Nowadays, enrichment based approaches are generally favored as the technique of choice for CEC detection. Substantial technical differences between assays are encountered mainly when the endothelial origin of the CD146 enriched cells is studied. Nevertheless, Ulex Europaeus Lectin-1 uptake [10], CD105/CD45/DAPI staining [14], or flow cytometrical analysis [28] produce similar results in this respect. In the current study, we used the combination of automated immunomagnetic enrichment and image cytometry, as this technique is fully automated, thoroughly validated, and detects CEC with high recovery and reproducibility (14) . However, a limitation of this approach is that the inclusion of CD105 in the CEC definition might result in an actual underestimation of the number of CEC, as several reports suggest absence of this marker on parts of the endothelium [8]. Also, the definition used here (14) does not exclude the enumeration of circulating endothelial progenitor cells, whichare also $CD45^-$ and $CD146^+$, [29].

To explore the correlation of this potentially novel marker for vascular injury to putative soluble markers of endothelial injury, activation, and proliferation, we have studied CEC counts and plasma levels of thrombomodulin, E-selectin, and endoglin, in 40 healthy controls and 40 patients with metastatic cancer. Specifically, We found a significant correlation between CEC numbers and thrombomodulin levels, but not between CEC and endoglin or E-selectin. Of note, none of the study subjects had renal dysfunction, which in itself is associated with increased serum thrombomodulin levels [30]. In this context it is relevant that increased concentrations of soluble thrombomodulin have been reported in lung, gastric and pancreatic [31], glioblastoma multiforme [32] and hepatocellular carcinoma [33]. Here, the increment in soluble thrombomodulin concentrations is considered to reflect vascular damage in relation to tumor invasiveness and metastasis. However, part of such increments in thrombomodulin concentrations may have been artifactual, as we have observed upon storage of such samples for 72h at room temperature.

Prior to the widespread use of CEC enumeration as monitoring tool for prognosis and in cancer treatment, it is important to assess any interaction between CEC counts and age and gender. Multivariate regression analysis showed that CEC numbers were not affected by gender, in contrast to plasma concentrations of thrombomodulin and E-selectin. For both plasma markers, higher concentrations were noticed in males when compared to females. In addition, age was found to increase CEC numbers in blood.

Age is a well-known risk factor for the occurrence of vascular events such as myocardial infarction and stroke [34], and several studies have demonstrated a decrease in endothelial function [35-37] with age. Our finding is consistent with the hypothesis that vascular integrity diminishes with age. On the contrary, none of the plasma endothelial biomarker levels was affected by age.

Collectively, we have shown that CEC numbers in blood increase with age, and that these CEC levels correlate with plasma levels of thrombomodulin, but not E-selectin or endoglin. This observation suggests that CEC enumeration offers a specific monitoring tool for vascular damage. Whether CEC numbers are predictive of clinical outcome in the treatment of cancers with angiogenesis inhibitors, needs to be elucidated in future prospective studies.

**ACKNOWLEDGEMENTS**

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## REFERENCES


1. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285:1182-1186.
2. Escudier B, Eisen T, Stadler WM, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med. 2007;356:125-134.
3. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med. 2007;356:115-124.
4. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med. 2004;350:2335-2342.
5. Ellis LM. Mechanisms of action of bevacizumab as a component of therapy for metastatic colorectal cancer. Semin Oncol. 2006;33:S1-7.
6. Curran SD, Muellner AU, Schwartz LH. Imaging response assessment in oncology. Cancer Imaging. 2006;6:S126-130.
7. Cao Y, Liu Q. Therapeutic targets of multiple angiogenic factors for the treatment of cancer and metastasis. Adv Cancer Res. 2007;97:203-224.
8. di Tomaso E, Capen D, Haskell A, et al. Mosaic tumor vessels: cellular basis and ultrastructure of focal regions lacking endothelial cell markers. Cancer Res. 2005;65:5740-5749.
9. Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost. 2005;93:228-235.
10. 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.
11. 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.
12. Duda DG, Cohen KS, Scadden DT, Jain RK. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc. 2007;2:805-810.
13. 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.
14. 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.
15. 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.
16. Hrachovinova I, Cambien B, Hafezi-Moghadam A, et al. Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. Nat Med. 2003;9:1020-1025.
17. McEver RP. Adhesive interactions of leukocytes, platelets, and the vessel wall during hemostasis and inflammation. Thromb Haemost. 2001;86:746-756.
18. Rini BI, Rathmell WK. Biological aspects and binding strategies of vascular endothelial growth factor in renal cell carcinoma. Clin Cancer Res. 2007;13:741s-746s.

19. Zoppini G, Targher G, Zamboni C, et al. Effects of moderate-intensity exercise training on plasma biomarkers of inflammation and endothelial dysfunction in older patients with type 2 diabetes. Nutr Metab Cardiovasc Dis. 2006;16:543-549.
20. Boffa MC, Karmochkine M. Thrombomodulin: an overview and potential implications in vascular disorders. Lupus. 1998;7 Suppl 2:S120-125.
21. Ishii H, Uchiyama H, Kazama M. Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. Thromb Haemost. 1991;65:618-623.
22. Kozuka K, Kohriyama T, Nomura E, Ikeda J, Kajikawa H, Nakamura S. Endothelial markers and adhesion molecules in acute ischemic stroke--sequential change and differences in stroke subtype. Atherosclerosis. 2002;161:161-168.
23. Bardin N, George F, Mutin M, et al. S-Endo 1, a pan-endothelial monoclonal antibody recognizing a novel human endothelial antigen. Tissue Antigens. 1996;48:531-539.
24. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 2005;106:2923-2924.
25. George F, Poncelet P, Laurent JC, et al. Cytofluorometric detection of human endothelial cells in whole blood using S-Endo 1 monoclonal antibody. J Immunol Methods. 1991;139:65-75.
26. Gougos A, St Jacques S, Greaves A, et al. Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukemic cells, and syncytiotrophoblasts. Int Immunol. 1992;4:83-92.
27. 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.
28. Widemann A, Sabatier F, Arnaud L, et al. CD146-based immunomagnetic enrichment followed by multi-parameter flow cytometry: a new approach to count circulating endothelial cells. J Thromb Haemost. 2008.
29. Alvarez DF, Huang L, King JA, ElZarrad MK, Yoder MC, Stevens T. Lung microvascular endothelium is enriched with progenitor cells that exhibit vasculogenic capacity. Am J Physiol Lung Cell Mol Physiol. 2008;294:L419-430.
30. Rustom R, Leggat H, Tomura HR, Hay CR, Bone JM. Plasma thrombomodulin in renal disease: effects of renal function and proteinuria. Clin Nephrol. 1998;50:337-341.
31. Xu GB, Luo LH, Lu XG. [Detection of thrombomodulin in both plasma and tissue extracts of cancer patients and its clinical significance]. Zhejiang Da Xue Xue Bao Yi Xue Ban. 2003;32:141-144.
32. Salmaggi A, Eoli M, Frigerio S, Ciusani E, Silvani A, Boiardi A. Circulating intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and plasma thrombomodulin levels in glioblastoma patients. Cancer Lett. 1999;146:169-172.
33. Biguzzi E, Franchi F, Bucciarelli P, Colombo M, Romeo R. Endothelial protein C receptor plasma levels increase in chronic liver disease, while thrombomodulin plasma levels increase only in hepatocellular carcinoma. Thromb Res. 2007;120:289-293.

34. Goessens BM, van der Graaf Y, Olijhoek JK, Visseren FL. The course of vascular risk factors and the occurrence of vascular events in patients with symptomatic peripheral arterial disease. J Vasc Surg. 2007;45:47-54.
35. Tschudi MR, Barton M, Bersinger NA, et al. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. J Clin Invest. 1996;98:899-905.
36. Brown KA, Didion SP, Andresen JJ, Faraci FM. Effect of Aging, MnSOD Deficiency, and Genetic Background on Endothelial Function. Evidence for MnSOD Haploinsufficiency. Arterioscler Thromb Vasc Biol. 2007.
37. Schrage WG, Eisenach JH, Joyner MJ. Ageing reduces nitric-oxide- and prostaglandin-mediated vasodilatation in exercising humans. J Physiol. 2007;579:227-236.

# CHAPTER 5

ON THE ORIGIN OF

(CD105+) CIRCULATING ENDOTHELIAL CELLS

*Thromb Haemost. 2009 Aug;102(2):347-5*1

## ABSTRACT

### *Background*
Cells designated by the CellSearch™ assay as circulating endothelial cells (CEC), defined as $CD146^+$, $CD105^+$, and $CD45^-$ nuclear cells, are thought to derive from damaged vasculature. As CD105 has been suggested to be expressed by endothelial cells from malignant vasculature in particular, it is currently unknown whether or not this assay is suitable to determine CECs in non-malignant diseases. Also, more insight is needed whether the CECs as detected by this assay predominantly measures CECs or also endothelial progenitor cells (EPCs), which originate from the bone marrow and reflect angiogenesis rather than vascular damage.

### *Methods*
CEC counts were determined in 9 patients treated with isolated limb perfusion with tumor necrosis factor α and melphalan, and in 10 healthy donors. Given the severe vascular damage caused by venesection and cannulation of the main vessels, we expected a significant increase in CEC counts in case CEC were of vascular rather than of bone marrow origin. Additionally, this finding, as well as the presence of $CD105^+$ CEC in the blood of healthy controls, would confirm that healthy endothelial cells do express CD105.

### *Results*
Numbers of $CD146^+/CD105^+/CD45^-$ nuclear CEC increased significantly after venesection and cannulation. After administration of TNF, a large fraction of non-intact, possibly apoptotic CEC appeared.

### *Conclusions*
This study shows that the CellSearch™ assay detects CECs originating from damaged vasculature. Furthermore, CD105 expression is found on CEC from damaged normal vasculature rendering further exploration of the value of CEC determined by this assay worthwhile not only in malignant diseases but also in non malignant disorders characterized by vascular damage.

## INTRODUCTION

In the last few years, phase III trials have clearly demonstrated the efficacy of drugs targeting the tumor vasculature in a wide variety of solid tumors, including breast cancer, non-small cell lung cancer, and renal cancer (1-3). However, the unique mechanisms by which these drugs exert their anti-tumor effect frequently does not cause tumor shrinkage as typically observed with classical cytotoxic therapy. As a result, response evaluation based on changes in tumor size, such as the Response Evaluation Criteria in Solid Tumors (RECIST), are unsuitable to monitor their efficacy (4). Surrogate markers that are informative on the extent of vascular damage due to tumor growth and inflicted by these novel compounds will therefore form a valuable addition to the currently available means to establish tumor aggressiveness and to monitor response to treatment.

Circulating endothelial cells (CEC) are endothelial cells present in the peripheral circulation. Their number is thought to reflect vascular damage, and increased numbers of CEC have been reported in diseases with overt vascular involvement such as cancer (5, 6). It is hypothesized that the increased number of CEC observed in patients suffering from metastatic cancer is caused by shedding of endothelial cells from the structurally inferior tumor vessels (7). Therefore, CEC might be informative on the vascular capacity of tumors, and their number could serve as an alternative for RECIST in cancer patients.

Through technical advances, several assays enabling the detection and enumeration of CECs have been described in recent years. Unfortunately, only a few of these have been properly validated (reviewed in ref. 7). One assay that has extensively validated the endothelial origin of enumerated cells (8) is the CellSearch™ assay, developed by Veridex (Veridex, Raritan, NJ). This assay relies on CD146 driven immunomagnetic isolation, followed by staining with CD45, to exclude $CD146^+$ T-cells (9), DAPI to show the presence of a nucleus, and CD105 to positively identify CEC (6). Using this technique, elevated CEC counts have been reported in patients with metastatic cancer (6), and their number in blood correlates to plasma concentrations of thrombomodulin, a marker of endothelial damage, in both cancer patients and healthy donors (10). However, two uncertainties still remain concerning CEC detected by the CellSearch™ assay. First, little is known about the precise origin of $CD146^+$, $CD105^+$, and $CD45^-$ CEC. Do they originate from damaged vasculature, or are a substantial part of these cells actually bone marrow derived endothelial progenitor cells (EPC), which are thought to reflect angiogenesis rather than vascular injury (11)? A second issue is the use of CD105 as pan endothelial marker. This assumption is made by the CellSearch assay (6). In contrast to von Willebrand factor or CD31, which are present on all endothelial cells (12, 13), CD105 (or endoglin), is thought to be present only on angiogenic, malignant endothelial cells in particular (14, 15). As a result, the number of CEC reported by the CellSearch™ assay might be an underestimation of the actual number of CEC in cancer patients, as CD105 negative CEC are excluded from analysis, and renders the application of this method in non-malignant diseases questionable.

To test the hypothesis that the endothelial cells as detected by the CellSearch™ assay are vascular derived CEC rather than bone marrow derived EPC, we determined CEC in the blood and perfusate of patients suffering from hemangioma, soft tissue sarcoma or squamous cell carcinoma of the skin located in the extremities before, during and after treatment with isolated limb perfusion with melphalan and tumor necrosis factor α (TNF). As this procedure inflicts severe local vascular damage by the opening of the major artery and vein for cannulation, followed by perfusion of high doses of TNF and melphalan, which cause rapid and severe endothelial cell damage (16, 17), a significant increase in CEC count should be observed if CECs are derived from damaged vasculature indeed. Alternatively, the absence of such an increase would provide evidence that CEC are not of vascular origin, and likely originate from the bone-marrow.

Furthermore, by inflicting damage to healthy vasculature by incision and cannulation, we tested the hypothesis that non malignant CEC do express CD105 as well and can also be detected with the CellSearch™ assay, thereby rendering its application in non-malignant diseases worthwhile to study as well. For the same purpose; we examined the presence of CD105 expressing CEC in the blood from 10 healthy volunteers.

## MATERIALS AND METHODS

### *Patients and sampling*

Blood was collected from 9 patients (3 male, 6 female; mean age 66 years, range 33-80) in 10 mL CellSave tubes (Veridex) at 5 time points: sample 1 was drawn prior to any incision, sample 2 after cannulation of the main vessels of the perfused limb, sample 3 after 15 minutes of perfusion with TNF, sample 4 after 1.5 perfusion with melphalan, and sample 5 after ending of the procedure. Patients were suffering from hemangioma (n=1), soft tissue sarcoma (n=4) or squamous cell skin carcinoma (n=4) of the extremities and were treated with hyperthermia and ILP as described earlier (18). Briefly, the main vessels of the involved limb were clamped, cannulated and subsequently connected to an extracorporeal oxygenator. Systemic leakage of melphalan and TNF was prevented by ligation of collateral vessels and application of a tourniquet. After mild limb hyperthermia was established, TNF was injected into the arterial cannula (1mg for the arm and 2mg for the leg). After 15 minutes, melphalan was added (13mg/L for the arm and 10mg/L for the leg). Finally, after 1.5 hours of perfusion, the extremity was rinsed with an electrolyte solution for 15 minutes and the vessels were closed. Patients suffering from melanoma were excluded from this study, as circulating melanoma cells also express the CD146 antigen used for the definition of endothelial cells. To assess the expression of CD105 on CEC in healthy donors, we examined the blood obtained from 10 apparently healthy controls (3 males, 7 females; mean age 60.1 years, range 30-84). All patients signed informed consent prior to participation. This study was approved by the local Institutional Medical Ethical Review Boards, and is in agreement with the Helsinki declaration of 2000.

### *CEC enumeration*

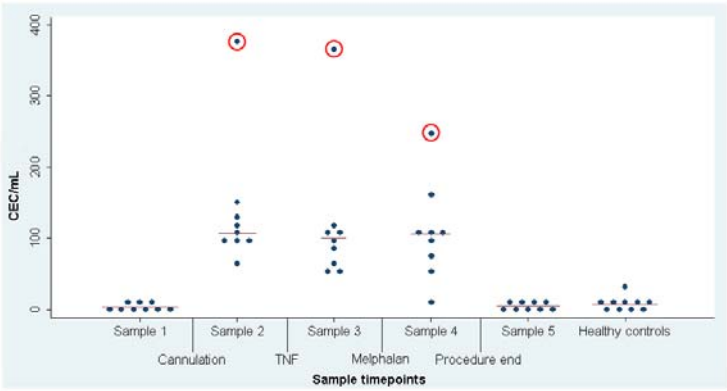
The CellTracks® AutoPrep® System (Veridex) was used to immunomagnetically isolate CEC using ferrofluids coupled to an anti-CD146 monoclonal antibody (mAb) from 4 mL of blood collected in CellSave tubes. Subsequently, the following staining reagents were added: the nuclear dye 4,6-diamidino-2-phenylindole (DAPI), and fluorochrome-conjugated mAb: phycoerythrin-conjugated CD105, expressed on endothelial cells, and allophycocyanin -conjugated CD45, a pan-leukocyte marker included in order to exclude hematopoietic cells from analysis. Finally, analysis of the stained cell isolate is done on the CellSpotter® Analyzer II System (Veridex) fluorescence microscope, where CEC were visually examined and identified as intact cells meeting the $DAPI^+$, $CD146^+$, $CD105^+$ and $CD45^-$ criteria, with a minimum size of 4 μm.

***Statistical analysis***
CEC data was logarithmically transformed to correct skewness where appropriate. Comparison of 2 groups was done using the Mann-Whitney u test. Multiple group comparisons were done using the Kruskal-Wallis test. Data is reported as mean ± standard deviation (SD). Differences were considered significant if P<0.05. Analysis was performed using Stata 10.1 software (StataCorp, college Station, TX).

## RESULTS

Baseline CEC counts in patients were 5±3 cells/mL and did not differ significantly from numbers found in 10 healthy controls (9±10 cell/mL; P=0.25). When compared to the baseline, we found a significant increase in CEC counts in the perfusate after cannulation of the major vessels (sample 2: 136±92 vs. sample 1: 5±3 CEC vs. CEC; P=0.0008). No additional increase in CEC was detected after administration of TNF, 15 minutes of perfusion with TNF, or after 90 minutes of perfusion after the addition of melphalan (sample 2 136±92 CEC vs. sample 3: 117±98 CEC vs. sample 4: 109±67; P=0.54). CEC counts returned to baseline values within 20 minutes after surgery (Sample 5: 5±3 CEC vs. sample 1: 5±3 CEC; P=0.60). Data is shown in figure 1.

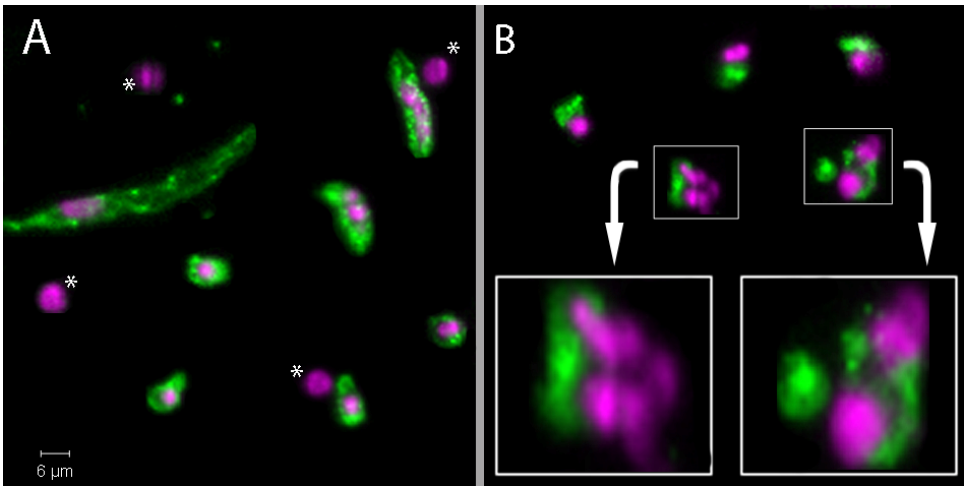
**Figure 1 – CEC numbers during ILP and in healthy controls**




*Sample 1 is served as reference CEC number and was drawn prior to any incision. CEC numbers were within the normal range for all patients (0-20 CEC/mL). Sample 2 was drawn after attachment of the extracorporeal circuit to assess possible contamination of the perfusate with CEC detached by cannulation. Sample 3 was drawn prior to melphalan administration to assess the effect of TNF on CEC counts. Sample 4 was drawn after 1.5 hours of perfusion with both TNF and melphalan. Sample 5 was drawn within 20 after finishing the surgical procedure. Sample 1 and 5 were drawn from an arterial line inserted into the radial artery. Samples 2-4 were drawn from the cannula inserted into the major draining vein from the perfused limb. A significant increase in CEC counts was observed after insertion of the cannulae. No effect of melphalan or TNF chemotherapy on CEC counts could be detected. After treatment, CEC numbers returned to baseline values. The outliers observed at sample 2, 3 and 4, (encircled in red) were obtained from the same patient. Horizontal lines indicate median CEC counts.*

Interestingly, visual analysis of all isolated cells revealed the presence of a large number of non-intact DAPI$^{+}$ CD146$^{+}$ CD45$^{-}$ CD105$^{+}$ cells in specimens 2, 3 and 4 (Figure 2). Although exact quantification of these abnormal cells is difficult, as they do not meet specific, well defined criteria, we attempted to enumerate CD146$^{+}$/CD105$^{+}$/CD45$^{-}$ cells whose nucleus fell outside the cytoplasm for more than 50% or whose cell membrane was not intact in 1 patient suffering from synovial sarcoma. In this sample, we found none of these cells at baseline (sample 1) and after cannulation (sample 2), 53 (14% of CEC) after administration of TNF (sample 3), 300 (81% of CEC) after administration of melphalan (sample 4), and 79 (32% of CEC) after ending the procedure (sample 5).

**Figure 2 – Occurrence of structurally abnormal CEC after perfusion with TNF**




*Panel A: Typical morphology of CEC as evaluated by florescence microscopy prior to the administration of TNF. Cells are intact, with nuclei (stained with DAPI; violet) found within the cytoplasm (stained with CD105-PE; green). Asterisks show CD146$^{+}$ leukocytes which lack CD105 staining.*

*Panel B: After TNF treatment, a large number of atypical CEC occur. These cells, which have CEC associated phenotype (i.e. DAPI$^{+}$, CD146$^{+}$, CD105$^{+}$, CD45$^{-}$) appear to have lost their structural integrity and the cytoplasm disintegrates around the nuclei.*

## DISCUSSION

Studies on the vascularization of solid tumors have led to the development of a new class of drugs in oncology, the angiogenesis inhibitors. By targeting pro-angiogenic ligands, such as VEGF, or their receptors, clinical outcome has markedly improved in many cancer patients. However, the lack of direct cytotoxicity of these new drugs makes traditional response evaluation, which relies on changes in tumor size, unreliable. Given their direct relation to the vessel wall, CEC are considered a promising marker for vascular damage, and although their number is increased in patients with manifest vasculopathy, proof that CEC originate from damaged vasculature is still lacking. In this study, we determined CEC by CD146 based immunomagnetic isolation followed by image cytometry in patients treated with ILP with melphalan and TNF. We hypothesized that if CEC, as measured by the CellSearch™ assay, are derived from the damaged vasculature instead of having been mobilized from the bone marrow. Based on this assumption, their number would increase in the perfusate after venesection and cannulation of the main vessels of the limb, which we indeed found. Furthermore, as TNF exerts its anti-tumor effect by the rapid and selective targeting of the tumor vasculature (18), we expected an additional increase in CEC numbers during treatment, which we did not encounter.

We found a significant increase in CEC numbers in the perfusate after incision and cannulation, when compared to CEC numbers determined before surgery. This shows that CEC determined with the CellSearch™ assay are cells of vascular rather than bone marrow origin. The rapid normalization of CEC numbers after restoration of the circulation is likely to be the result of dilution by the blood and added infusion fluids during the procedure, and ongoing apoptosis of endothelial cells, which could cause them to fail to fulfill all criteria required designating cells as CEC (i.e. cells not intact, nucleus not ≥ 50% inside the cytoplasm).

Next, we questioned the suitability of CD105 as confirmatory staining of CEC. CD105, or endoglin, is a component of the receptor complex of transforming growth factor-β(19) and is closely involved proliferation and differentiation of endothelial cells (20). Immunohistochemical staining for CD105 revealed high levels of CD105 on intratumoral endothelial cells in a large number of solid and hematopoietic tumors (14), whereas expression on healthy endothelium was weak or absent (21). As a result, the use of CD105 might lead to an underestimation of CEC, as non-angiogenic and/or non-malignant CEC are excluded from analysis. The increase in $CD105^+$ CEC occurred not only in perfusions in patients with malignancies also occurred in a patients with a non malignant disease, hemangioma (22, 23). The observation that the rise is CECs was seen directly after cannulation, which is unlikely to affect the vasculature of the tumor, and prior to the administration of TNF, which selectively targets tumor endothelial cells (18), as well as the presence of $CD105^+$ CEC in healthy donors, strongly suggests that CD105 is also present on endothelial cells from normal vasculature.

In contrast to our hypothesis, administration of TNF did not induce an additional increase in CEC counts after cannulation, but lead to the appearance of non-intact cells with an endothelial phenotype ($CD146^+$, $CD45^-$, $CD105^+$) not meeting the definitions of CECs according to this technique. As TNF causes detachment and apoptosis of tumor endothelium (17), we hypothesize that these cells are apoptotic or necrotic CEC, detached from the tumor vasculature. However, studies using apoptosis markers such as Annexin V on enriched CEC should confirm this hypothesis, as the number of apoptotic CEC could provide additional information on the effect of treatment with vascular targeting drugs. Assays that allow the quantification of apoptotic CEC should be developed for this purpose. There was insufficient material available to perform apoptosis tests in our study.

The vascular origin of CEC as determined by the CellTracks™ assay supports the rationale of using their number as surrogate marker for vascular damage rather than of angiogenesis.

## REFERENCES


1. Miller KD. E2100: a phase III trial of paclitaxel versus paclitaxel/bevacizumab for metastatic breast cancer. Clin Breast Cancer 2003 Feb;3(6):421-2.
2. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 2007 Jan 11;356(2):115-24.
3. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med 2006 Dec 14;355(24):2542-50.
4. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000 Feb 2;92(3):205-16.
5. Khan SS, Solomon MA, McCoy JP, Jr. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. Cytometry B Clin Cytom 2005 Mar;64(1):1-8.
6. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. Cytometry A 2007 Feb;71(2):105-13.
7. Strijbos MH, Gratama JW, Kraan J, et al. Circulating endothelial cells in oncology: pitfalls and promises. Br J Cancer 2008 Jun 3;98(11):1731-5.
8. Smirnov DA, Foulk BW, Doyle GV, et al. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. Cancer Res 2006 Mar 15;66(6):2918-22.
9. Elshal MF, Khan SS, Raghavachari N, et al. A unique population of effector memory lymphocytes identified by CD146 having a distinct immunophenotypic and genomic profile. BMC Immunol 2007;8:29.
10. Strijbos MH, Rao C, Schmitz PI, et al. Correlation between circulating endothelial cell counts and plasma thrombomodulin levels as markers for endothelial damage. Thromb Haemost 2008 Oct;100(4):642-7.

11. Ding YT, Kumar S, Yu DC. The role of endothelial progenitor cells in tumour vasculogenesis. Pathobiology 2008;75(5):265-73.
12. Beerepoot LV, Mehra N, Vermaat JS, et al. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol 2004 Jan;15(1):139-45.
13. 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 Mar;4(3):671-7.
14. Fonsatti E, Altomonte M, Nicotra MR, et al. Endoglin (CD105): a powerful therapeutic target on tumor-associated angiogenetic blood vessels. Oncogene 2003 Sep 29;22(42):6557-63.
15. Fonsatti E, Altomonte M, Arslan P, et al. Endoglin (CD105): a target for anti-angiogenetic cancer therapy. Curr Drug Targets 2003 May;4(4):291-6.
16. ten Hagen TL, Eggermont AM. Solid tumor therapy: manipulation of the vasculature with TNF. Technol Cancer Res Treat 2003 Jun;2(3):195-203.
17. ten Hagen TL, Seynhaeve AL, Eggermont AM. Tumor necrosis factor-mediated interactions between inflammatory response and tumor vascular bed. Immunol Rev 2008 Apr;222:299-315.
18. Verhoef C, de Wilt JH, Grunhagen DJ, et al. Isolated limb perfusion with melphalan and TNF-alpha in the treatment of extremity sarcoma. Curr Treat Options Oncol 2007 Dec;8(6):417-27.
19. Cheifetz S, Bellon T, Cales C, et al. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem 1992 Sep 25;267(27):19027-30.
20. Zhu HJ, Burgess AW. Regulation of transforming growth factor-beta signaling. Mol Cell Biol Res Commun 2001 Nov;4(6):321-30.
21. Wang JM, Kumar S, Pye D, et al. A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissues. Int J Cancer 1993 May 28;54(3):363-70.
22. Goh SG, Calonje E. Cutaneous vascular tumours: an update. Histopathology 2008 May;52(6):661-73.
23. Requena L, Sangueza OP. Cutaneous vascular proliferation. Part II. Hyperplasias and benign neoplasms. Journal of the American Academy of Dermatology 1997 Dec;37(6):887-919; quiz 20-2.

# CHAPTER 6

## PERIPHERAL BLOOD MRNA LEVELS OF THE ENDOTHELIUM-ASSOCIATED MARKERS CD31, CD144, CD146 AND VON WILLEBRAND FACTOR DO NOT SERVE AS SURROGATE MARKERS FOR CIRCULATING ENDOTHELIAL CELLS

*Submitted for publication*

## ABSTRACT

***Purpose***
Circulating endothelial cells (CEC) are considered a promising marker to determine the extent of vascular damage. However, currently available and validated CEC enumeration assays are laborious, time consuming and costly, which limits their clinical utility. Here, we evaluated the feasibility of quantifying mRNA levels of the endothelium-associated markers CD31, CD144, CD146 and von Willebrand factor (vWf) in peripheral blood (PB) of healthy donors, patients, and human umbilical veins by real time reverse transcriptase polymerase chain reaction (RT-PCR) and their use as surrogate markers for CEC.

***Experimental design***
We determined sensitivity and specificity of RT-PCR for endothelial markers using human umbilical vein endothelial cells (HUVEC). Next, CEC were isolated from peripheral blood of apparently healthy donors, patients as well as umbilical cord blood specimens by automated CD146-based immunomagnetic isolation. Whole blood samples and the $CD146^+$ cell-enriched fractions were assessed for mRNA and protein expression of CD31, CD144, CD146 and vWf by RT-PCR and flow cytometry, respectively.

***Results***
We showed the feasibility to detect endothelial mRNA isolated from HUVEC numbers as low as 10. However, no endothelial mRNA could be measure in whole blood samples, and only low levels of CD31 and CD146 mRNA were detected in suspensions of isolated CEC with numbers up to 4,450 CEC per sample.

***Conclusions***
We conclude that mRNA levels of CD31, CD144, CD146 and vWf in whole blood as detected by real time RT-PCR cannot be used as biomarkers for end-stage endothelial cells such as CEC.

## INTRODUCTION

Neovascularisation plays a pivotal role in tumour growth and metastasis, making it a promising target in cancer treatment (1, 2). Drugs targeting angiogenesis-related receptors or pathways have been developed, and randomized clinical trials have demonstrated their efficacy in several types of cancer, including metastatic colon cancer (3), metastatic renal cell carcinoma (4) and non-small-cell lung carcinoma (5). In addition to angiogenesis inhibitors, another class of drugs targeting the tumour vasculature, i.e. the vascular disrupting agents such as combretastatin and ZD6126 are currently under investigation (6). As these new agents are considered to be cytostatic rather than cytotoxic, response evaluation based on changes in tumour size, such as by the widely applied RECIST criteria, is not considered relevant (7).

With a growing number of anti-angiogenic and vascular disrupting compounds entering clinical trials, there is a need for surrogate markers that allow accurate monitoring of treatment response, and, ideally, predict response to therapy.

Circulating endothelial cells (CEC) in peripheral blood (PB) are mature, end-stage endothelial cells that have detached from vessel walls as a result of vascular insults. Consequently, the number of CEC in blood is considered to reflect the extent of vascular damage and accordingly, increased CEC counts have been observed in disorders with vascular involvement, such as vasculitis, myocardial infarction, and several forms of cancer (8). Currently, several methods to enumerate CEC have been developed and validated. Typically, such assays combine CD146-based immunomagnetic isolation followed by staining with Ulex Europaeus Lectin-1 (UEA-1)(9), uptake of fluorescent low density lipoproteins (Dil-Ac-LDL) (10) or immunohistochemistry combined with image cytometry for endothelial specific markers (11, 12) to confirm the endothelial lineage of the isolated cells. In addition to enrichment-based assays, reports on flow cytometric CEC detection have been published, although thorough validation of these assays is still lacking (reviewed in (13)). Generally, these assays are labour intensive, time consuming and costly, and therefore considered unsuitable for high-throughput monitoring studies.

In search for alternative methods to quantify CEC, we examined whether quantification of RNA levels of endothelial cell markers in blood could serve as a straightforward, rapid and sensitive method to detect CEC. To this end, we have set up real time reverse transcriptase polymerase chain reaction (RT-PCR) assays to estimate the concentrations of the endothelial markers CD31 (platelet/endothelial cell adhesion molecule 1 or PECAM-1), CD144 (vascular endothelial cadherin or VE-cadherin), CD146 (melanoma associated cellular adhesion molecule or MelCAM), and von Willebrand factor (vWf). After validating these assays using human umbilical vein endothelial cells (HUVEC), their feasibility as biomarker for CEC was assessed on blood samples obtained from healthy donors and from patients with elevated CEC numbers, as well as on samples enriched for CEC by automated CD146-based immunomagnetic isolation (12).

## MATERIALS AND METHODS

### *Patients and blood collection*

Peripheral blood (PB) was obtained from three healthy donors and three patients suffering from chronic graft versus host disease (GvHD), metastatic lung cancer and sarcoma, respectively, following written informed consent. Blood was collected in 10 millilitres (mL) CellSave tubes containing ethylenediaminetetraacetic acid (EDTA) and a proprietary preservative (Veridex, Raritan, NJ); the first 4 ml of blood were discarded to prevent contamination with endothelial cells detached as a result of venipuncture. From patient 3, who was treated with an isolated limb perfusion using high-dose chemotherapy and hyperthermia, two blood samples were obtained. The first sample was taken prior to treatment and served as a reference; the second was taken from a cannula introduced in the femoral vein prior to hyperthermia and chemotherapy. Here, we expected a large number of CEC that were detached by trauma. In addition to the six samples mentioned, two umbilical cord blood (UCB) specimens were collected by aspiration from the umbilical vein immediately post partum, and were preserved in a phosphate-buffered saline solution containing heparin (final concentration 10-20 IU/mL). All samples were analyzed within 24 h after collection. The study protocol was reviewed and approved by the Erasmus MC Medical Ethical Review Board.

**Table 1– Donor characteristics and CEC counts**

| | Age / Gender | Morbidity | Total isolated CEC[1] | CEC per mL |
|---|---|---|---|---|
| Donor 1 | 31 / Male | Healthy | 220 | 5.5 |
| Donor 2 | 30/ Male | Healthy | 400 | 10 |
| Donor 3 | 51 / Male | Healthy | 330 | 8.25 |
| Patient 1 | 54 / Male | Lung cancer | 290 | 7.25 |
| Patient 2 | 51 / Female | Chronic mild GvHD | 1810 | 45.3 |
| Patient 3 | 40 / Male | Sarcoma | 3200 | 800[2] |
| UCB 1 | Female | Healthy | 4450 | 1112 |
| UCB 2 | Female | Healthy | 2890 | 723 |

*[1] CEC numbers are enumerated in image cytometry using CellTracks® Analyser II in CD146-enriched cell fractions generated from 40 mL blood samples (donors 1 and 2; patient 1 and 2) or from 4 mL blood samples (donor 3 and UCB 1 and 2) .*
*[2] CEC numbers immediately after introduction of a femoral cannula for hyperthermic isolated limb perfusion; prior to surgery were 5 CEC/mL.*

***Cell culture***
HUVEC cell line CRL-1730 (passage no. 16, ATCC, Manassas, VA) were grown in tissue culture flasks coated with 0.2% gelatine, in endothelial cell basal medium supplemented with bovine brain extract, hydrocortisone, human epidermal growth factor, gentamicin, amphotericin B and 10% of fetal bovine serum (all from Lonza, Verviers, Belgium). Cells were harvested at 80% confluency with trypsin/EDTA, and subcultured up to maximum passage no. 26.

***Immunomagnetic endothelial cell enrichment and enumeration.***
Endothelial cell enrichment was done using the Endothelial Cell Reagent Kit in combination with the CellTracks® AutoPrep® System, both from Veridex), which allows for a fully automated immunomagnetic isolation of CEC as recently described (12). In brief, a ferrofluid coated with a mAb recognizing CD146, which is expressed on endothelial cells as well as on a subset of activated T-cells (14), is used to magnetically isolate $CD146^+$ cells from peripheral blood. Subsequently, the isolated cell fraction was either used for CEC immunophenotyping by flow cytometry (detailed below), endothelial marker-based RT-PCR or for image cytometry using a CellTracks® Analyzer II (Veridex). CD146-enriched cell fractions were stained with phycoerythrin (PE)-conjugated CD105 mAb, allophycocyanin (APC)-conjugated CD45 mAb and the nuclear dye 4',6-diamidino-2-phenylindole (DAPI) using the Endothelial Cell Reagent Kit (Veridex). Subsequently, CEC were immunophenotypically defined as: $CD45^-$, $CD105^+$, $CD146^+$, $DAPI^+$.

***Flow cytometric detection of CEC and assessment of apoptosis***
The CD146-enriched cell fractions were analysed by flow cytometry for expression of the endothelium-associated markers CD31, CD34, CD144 and CD146 using 2 four-color stainings. We previously demonstrated expression of these antigens on HUVEC (19). No fluorochrome-conjugated mAb to vWf is currently available.
The following mAbs were used: CD31 mAb conjugated with fluorescein isothiocyanate (FITC; clone WM59, BD Biosciences); CD105 FITC (clone MEM-226, Affinity BioReagents, Golden, CO); CD144 PE; clone TEA 1/31, Beckman Coulter Inc, Fullerton, CA); CD146 PE (Clone P1H12, BD Biosciences); CD45 conjugated with peridinin-chlorophyll-protein complex (PerCP; clone 2D1, BD Biosciences); and CD34 conjugated with allophycocyanin (APC; clone 8G12, BD Biosciences). In addition, the DNA-specific dye 1,5-bis[2-(di-methylamino) ethyl]amino-4, 8-dihydroxyanthracene-9,10-dione (DRAQ5, Biostatus Ltd, Shepshed, UK) was used to include only nucleated cells. Data acquisition was performed using a FACSCanto flow cytometer equipped with a second red-diode laser (BD Biosciences), and instrument setup and calibration was done as described previously (Kraan et al, 2003). Data were analysed by using FCS Express v3 (De Novo software, Los Angeles, CA), applying a sequential gating strategy as detailed in Figure 1 to identify CEC in the CD146-enriched cell fraction. CEC were assessed by flow cytometry as $CD146^+$ cells that also were $DRAQ5^+$, $CD45^-$ and $CD31^+$ or $CD146^+$, $DAPI^+$, $CD105^+$, $CD34^+$, $CD144^+$.

Flow cytometric assessment of apoptosis and cell death were done by the simultaneous staining of cells with Annexin V-FITC mAb (BD Biosciences) and 7-aminoactinomycin D (7-AAD, Sigma-Aldrich, St. Louis, MO) in Annexin binding buffer (BD Biosciences). Apoptotic cells were defined as Annexin $V^+$ and 7-$AAD^-$, whereas necrotic cells were positive for both markers.

***Real time reverse transcriptase polymerase chain reaction***

Real time RT-PCR for the endothelial markers CD31, CD144, CD146 and vWf was performed on whole blood samples, on predetermined numbers of sorted HUVEC, lymphocytes, and on CD146-enriched cell fractions prepared from PB obtained from healthy donors and patients, and from UCB. For blood samples, 40 mL samples were processed in 8 parallel isolations on 5 mL aliquots each; the CD146-enriched fractions were pooled prior to processing. In addition, we examined the presence of CD3 and CD146 RNA in T-cells sorted or immunomagnetically enriched for CD146. RNA was isolated from blood samples and cell suspensions using the QIAamp® RNA Blood Minikit (Qiagen, Valencia, CA) and ChargeSwitch Total RNA Cell Kit (Invitrogen, Carlsbad, CA), respectively, according to the manufacturer's guidelines. Complementary DNA (cDNA) synthesis was done using Reverse Transcriptase Superscript III (Invitrogen) under standard conditions.

RT-PCR and real time measurements of amplicons were performed essentially as described elsewhere (Rabascio et al, 2004). Real time PCR was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Bioscience, Foster City, CA), with an initial heating profile of 50°C for 2 min and 95°C for 10 min, followed by 40-50 cycles of alternating cycles of 95°C for 15 s and 60°C for 1 min each. For CD144, primer and probe sequences were as follows. CD144 TQM forward primer: 5'-AAG-CCC-TAC-CAG-CCC-AAA-GT; CD144 TQM reverse primer: 5'-TTG-CGG-AGA-TCT-GCA-GGA-C; and CD144 6-carboxyfluorescein (FAM)-probe: TGT-GAG-AAC-GCT-GTC-CAT-GGC-CAG- 6-carboxy-tetramethyl-rhodamine (TAMRA) (Eurogentec Ltd, Southampton, UK).

A 10-fold serially diluted CD144-containing plasmid (kindly provided by Dr. C. Rabascio, Milan, Italy), ranging from 100 pg to 0.1 fg, was used to generate a standard curve and to allow quantification of CD144 in fg-scale levels. RT-PCR for CD3, CD31, CD146 and vWf were performed with TaqMan® Gene Expression Assays (MGB probes, FAM™ dye-labelled, no. 4331182; Applied Biosciences, Foster City, CA) for CD3 (no: HS00167894_m1.), CD31 (no: Hs00169777_m1), CD146 (no: Hs00174838_m1) and vWf (no: Hs00169795_m1). A no-template water control was used as negative control for every PCR.

Relative mRNA levels were calculated as $2^{-\Delta CT}$ (15), with CT being the cycle number at which the amplified fluorescence signal crosses a threshold pre-set under non-amplified conditions, and ΔCT being the difference between the CT value of the marker of interest (FAM label) and that of the endogenous ribosomal 18S RNA, with the latter determined by the TaqMan 18S Ribosomal RNA Control Reagent (VIC-labelled probe; Applied Biosciences)(16).

Presented CT values are the mean of at least two subsequent sample dilutions. CT values were considered valid when they fulfilled the following criteria: (1) they showed the expected decrease in at least two subsequent sample dilutions, (2) they were at least 2 cycles lower than the water control (and thus lower than or equal to a CT of 38), and (3) duplicate measurements were within a 1-cycle range. The Student's t-test was used to compare data from two experimental conditions. All P-values were two-sided, and P-values $< 0.05$ were considered statistically significant

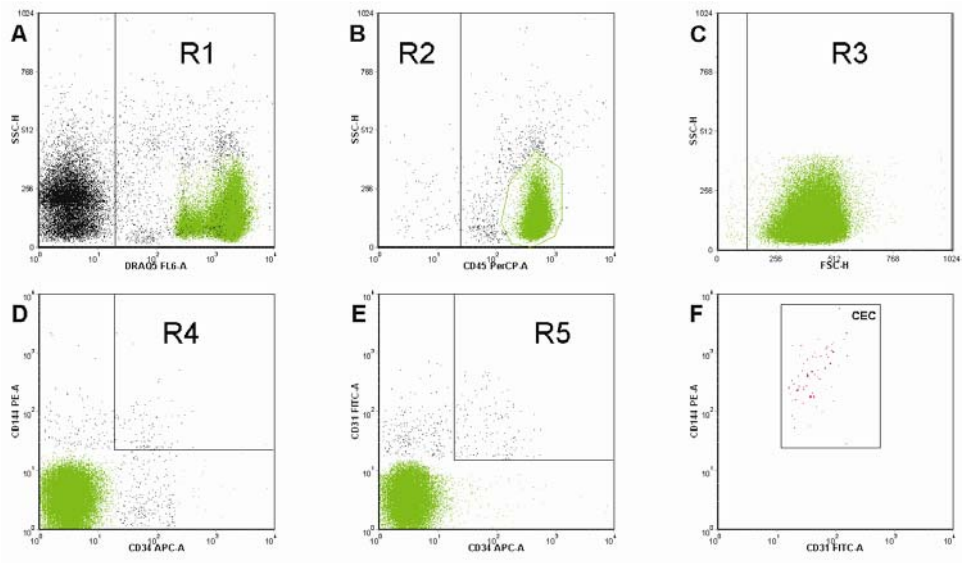
***Cellular control samples to validate RT-PCR of endothelial markers***

Aliquots with predetermined cell numbers were prepared using a FACSAria Cell Sorting System (BD Biosciences, San Jose, CA) equipped with FACSDiva v4.1.2 software (BD Biosciences). HUVEC cells, used to determine assay sensitivity, were spiked into whole blood samples following recovery by cell sorting based on their relatively high forward and sideward light scatter properties. Lymphocytes, used to determine assay specificity, were sorted from peripheral blood mononuclear cells (PBMC) stained with CD45 PerCP based on their intermediate forward scatter, low sideward light scatter properties and high expression of CD45. $CD146^+$ and $CD146^-$ T-cells, used to determine mRNA of T cell markers as a measure of contaminating lymphocytes, were sorted using CD3 FITC (clone SK7, BD Biosciences, San Jose, CA) and CD146 PE. The accuracy of cell enumeration by FACSAria was confirmed by counting predefined numbers of sorted cells on slides, followed by microscopic enumeration.

## RESULTS

CEC show surface membrane expression of CD31, CD34, CD105, and CD144. To verify isolation of CEC, we first assessed surface expression of these markers by cells obtained using CD146-based automated immunomagnetic isolation and flow cytometry. Using sequential gating, we confirmed the presence of CD31, CD34, CD105 and CD144, and absence of the pan-leukocyte marker CD45 on CEC in CD146-enriched cell fractions (Figure 1). Notably, Figure 1 shows the presence of a large number of $CD45^+$ positive lymphocytes in the CD146-based immunomagnetically isolated suspension (Figure 1 [panels B and C], lymphocytes shown in green). Earlier, we have shown similar surface expression of these endothelial markers on HUVEC (Strijbos et al, 2007).

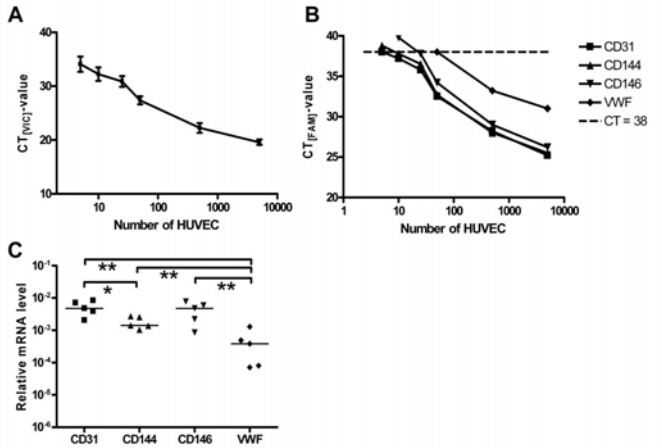
**Figure 1 - Flow cytometric assessment of CD31, CD34 and CD144 on CD146$^{+}$ CEC**




*A CD146-enriched cell fraction was prepared from 40 ml of blood using automated immunomagnetic isolation and subsequent staining by a mAb cocktail prior to flow cytometry (see Materials and Methods). The following analytical strategy was followed, a.k.a. "sequential gating". Plot A: Identification of nucleated cells in the CD146$^{+}$ enriched cell fraction by the DNA specific nuclear dye DRAQ5 (gate R1). Plot B: Identification of the CD45$^{-}$ fraction within the nucleated cells (gate R2 in a CD45 vs. SSC plot). Lymphocytes (DRAQ5$^{+}$,CD45$^{+}$,FSC$^{intermediate}$,SSC$^{low}$) are shown in green and taken along in plots D and E. Plot C: Inclusion of cells with at least the size of lymphocytes (gate R3) in a FSC plot, indicative of cell size). Plot D: Inclusion of CD34$^{+}$, CD144$^{+}$ cells using lymphocytes as internal negative control (gate R4). Plot E: Inclusion of CD31$^{+}$, CD34$^{+}$ cells (gate R5). Plot F: Identification of CEC (i.e., 97 of 25,000 nucleated cells [0.39%]) shown in a CD31 vs. CD144 plot (highlighted in red). In addition, CD105 FITC was used instead of CD31 FITC, which confirmed the surface expression of CD105 on isolated CEC (data not shown).*

***Levels of CD31, CD144, CD146 and vWf mRNA can be detected in 5-50 HUVEC***
Real time RT-PCR for endothelial markers was set up using HUVEC. For assessment of assay sensitivity, RNA was isolated from 5,000, 500, 50, 25, 10 and 5 flow-sorted HUVEC. As few as 5 HUVEC could be detected using 18S rRNA specific VIC reagents (Figure 2A). Endothelial specific marker RNA levels, expressed as $CT_{[FAM]}$-values, of the different numbers of HUVEC are plotted in Figure 2B. ΔCT-values ($CT_{[FAM]}$ for marker of interest - $CT_{[VIC]}$ 18S RNA) were constant for the tested range of HUVEC cell numbers down to as low as 10 HUVEC. Based on analysis criteria (see M&M section) and having a constant ΔCT-value for the different numbers of HUVEC, the minimum numbers of HUVEC that was considered to reliably detect endothelial markers were 10 for CD31 and CD144, 25 for CD146 and 50 for vWf. Looking at actual expression levels, we observed that endothelial markers were differentially expressed in HUVEC (see Figure 2C), with highest levels for CD31 and CD146 and lowest for vWF. The relative mRNA levels (expressed as fraction of 18S rRNA) were for CD31: $4.8x10^{-3}$ (median, range $2.1x10^{-3}$ – $8.4x10^{-3}$), for CD144: $1.4x10^{-3}$ ($9.8x10^{-4}$ – $2.8x10^{-3}$), for CD146: $4.8x10^{-3}$ ($8.5x10^{-4}$ – $7.8x10^{-3}$), and for vWF: $3.9x10^{-4}$ ($7.1x10^{-5}$ – $1.3x10^{-3}$; Figure 2D). In addition, CD144 mRNA levels in HUVEC were quantified using the CD144-plasmid standard curve, and equalled 0.95 fg per cell (median; range 0.49 – 1.65 fg per cell; n=5).

**Figure 2 – RT-PCR shows differential expression of the endothelial specific markers and allows detection of at least 10 HUVEC**




*A-C) RNA was isolated from 6 cell suspensions containing 5 to 5,000 HUVEC. Following cDNA synthesis, the amount of 18 S RNA and endothelial specific maker RNA (CD31, CD144, CD146 and vWF) were assessed (see Materials and Methods). A) Significant 18 S rRNA levels were isolated from as few as 5 HUVEC. $CT_{[VIC]}$-values (mean +/- 1 SD from 4 independent measurements) for 1:90 diluted cDNA samples were shown. B) Endothelial specific RNA levels, expressed as $CT_{[FAM]}$-values from a representative experiment for 1:10 diluted cDNA samples. The dashed line (CT = 38) indicates the upper limit for a valid assay. C) RNA was isolated from 5000 HUVEC in 5 independent experiments. Individual and median values of the relative endothelial marker mRNA levels are expressed as $2^{-\Delta CT}$. Significant differences are shown: 2-sided t-test P-values: *, 0.01< P < 0.05; **,0.001< P < 0.01; ***, P <* 0.001.

***Endothelial specific mRNA can be detected after immunomagnetic enrichment but not in blood from healthy donors and patients nor UCB***

As CEC occur in PB in very low numbers, typically up to 20 cells per mL in healthy controls (9, 12), we determined the sensitivity of the RT-PCR to detect CEC markers in blood. A priori, we noted a lack of endothelium-derived mRNA in any of the tested whole blood samples with normal (i.e. ≤20 CEC/mL) and increased concentrations of CEC (i.e. 45.3-1112 CEC/mL)(data not shown).

Then, we tested whether endothelial specific mRNA can be detected in CD146-enriched PB fractions. First, we investigated whether the immunomagnetic isolation procedure would affect endothelium-derived RNA levels. To that end, we assessed the endothelial specific RNA levels in 500 and 5,000 sorted HUVEC that had or had not been spiked into 4 mL of PB prior to immunomagnetic isolation, and observed similar levels of endothelial specific marker mRNA in both types of samples (data not shown). Subsequently, we tested whether or not endothelial specific mRNA could be detected in CD146-enriched cell fractions from blood, containing CEC numbers equivalent to those for which HUVEC samples showed a positive result. To this end, we isolated CEC from 40 mL of blood obtained from 3 healthy donors, 3 patients, and 2 UCB samples (Table 1). The CD146-enriched cell fractions, typically containing 20,000 – 30,000 nucleated cells, yielded between 220 and 4,450 CEC (i.e., $CD146^+$, $CD45^-$, $CD105^+$, $DAPI^+$; Table 1). The blood sample containing the highest CEC count (Patient 3) was from a patient undergoing hyperthermic isolated limb perfusion, a procedure that was shown to yield transiently very high CEC numbers (Strijbos et al, unpublished). The total RNA level of CD146-enriched PB cell fractions on a per cell basis was significantly lower (i.e., over a 100-fold) than that of HUVEC (Figure 3A). Also the relative mRNA levels of the endothelial markers CD31 and CD146 were lower in CD146-enriched PB cell fractions (Figure 3B) when compared to HUVEC (Figure 2C), i.e., for CD31: $1.4x10^{-4}$ (median, range $8.8x10^{-6}$ – $2.7x10^{-3}$) vs. $4.8x10^{-3}$ (median, range $2.1x10^{-3}$ – $8.4x10^{-3}$) [P = 0.001], and for CD146: $4.1x10^{-5}$ (median, range $1.6x10^{-5}$ – $2.1x10^{-4}$) vs $4.8x10^{-3}$ (median, range $8.5x10^{-4}$ – $7.8x10^{-3}$) [P = 0.005]. Negligible CD144 and vWf mRNA was detected in the CD146-enriched PB cell fractions with CEC counts up to 3,200 (Figure 3B). Combining the results of the total RNA and relative mRNA levels, the absolute levels of endothelial specific mRNA in CEC are up to 10,000-fold lower than in HUVEC, e.g., for CD31: 100 x ($4.8x10^{-3}/1.4x10^{-4}$) = 3,400-fold lower, and for CD146: 100 x ($4.8x10^{-3}/4.1x10^{-5}$) = 11,700-fold lower.

***CD146-expressing lymphocytes are devoid of detectable levels of CD146 mRNA***

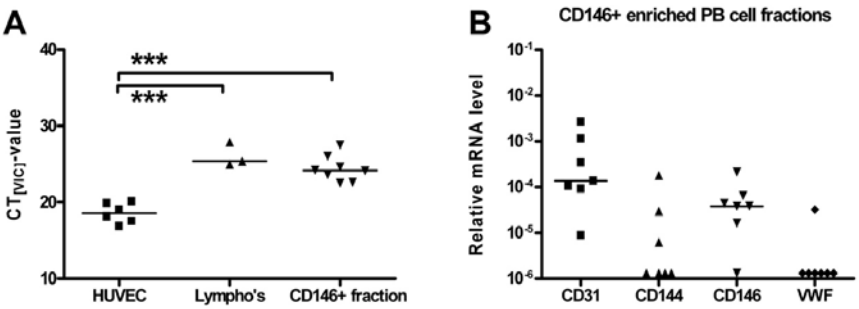
Finally, we assessed the role of 'contaminating $CD146^+$ lymphocytes' present in the CD146-immunomagnetically purified PB samples. Therefore, we examined 18S RNA levels as a measure of total RNA levels and CD3 and CD146 mRNA levels in CD146-immunomagnetically enriched PB samples and compared these values with those of 20,000 flow-sorted $CD45^+$, $CD3^+$, $CD146^-$ and 6,000 flow-sorted $CD45^+$, $CD3^+$, $CD146^+$ lymphocytes.

As reported earlier, the CD146 membrane expression on $CD45^+/CD3^+/CD146^+$ lymphocytes is relative low when compared to the CD146 membrane expression on CEC (17). The total RNA levels of the CD146-enriched PB cell fraction and the sorted lymphocyte fractions were comparable with previous (not shown) experimentations (Figure 4A).

It is noteworthy, that we were able to detect a CD3 specific signal in all three samples, demonstrating the presence of (NK)T cells in samples immunomagnetically enriched for CD146 (Figure 4B). Remarkably, the CD3 mRNA level was lower in the CD146-enriched PB cell fraction when compared to levels in sorted T-cell fractions. In contrast, CD146 mRNA could only be detected in the CD146-immunomagnetically purified sample, but not in both the $CD146^-$ (n=20,000) and $CD146^+$ (n=6000) sorted T lymphocyte fractions (Figure 4B).

To assure that the lack of CD146 mRNA in the sorted $CD146^+$ T lymphocyte suspension was not the result of apoptosis due to sample preparation, we stained all samples with Annexin V and 7-AAD. Flow cytometric analysis demonstrated that only 3% of $CD146^+$ T-cells were apoptotic (defined as Annexin $V^+/7\text{-}AAD^-$), whereas less than 2% of these cells were necrotic (Annexin $V^+/7\text{-}AAD^+$; data not shown).

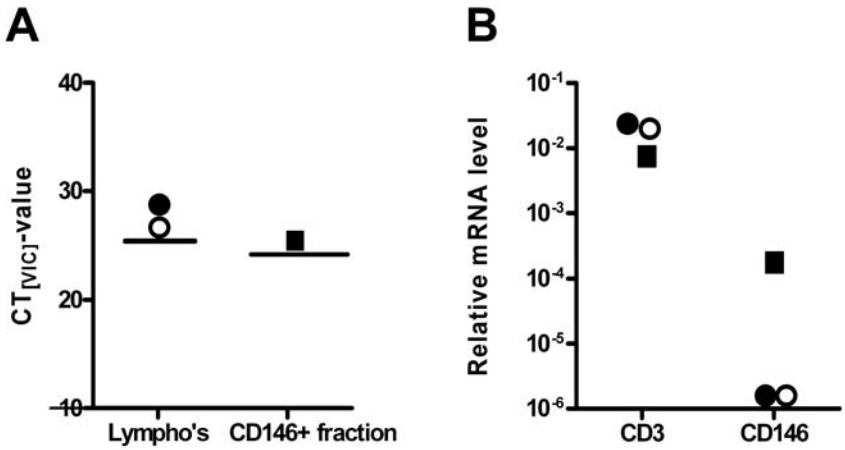
## Figure 3 – Lower total and endothelial specific RNA yield from $CD146^+$ enriched PB fractions than from HUVEC




*A) RNA was isolated from suspensions of 5.000 HUVEC (n=6), 5.000 lymphocytes (n= 3) and CD146-enriched PB fractions (n=8), each containing approximately 19,000 to 31,000 nucleated cells. Following cDNA synthesis the amount of 18 S rRNA was assessed (see Materials and Methods). Individual and median CT-values for 1:90 diluted cDNA samples are shown.*

*B) Endothelial marker RNA levels in the CD146-enriched PB cell fractions (n=7; due to technical failure one sample was eliminated). Individual and median (CD31, CD146) values of the relative endothelial marker mRNA levels, expressed as $2^{-\Delta CT}$ are shown. Median values were not shown for CD144 and vWF, as no RNA levels were detectable in 4 and 6 samples, respectively. Non detectable RNA levels were put at $1x10^{-6}$ (base line). See legend to Figure 2 for technical details.*

**Figure 4 – Low CD146 specific mRNA levels in the CD146$^+$ enriched PB fractions but not in sorted CD146$^+$ and CD146$^-$ T lymphocytes**




*A) RNA was isolated from suspensions of 6,000 sorted CD3$^+$CD146$^+$ T lymphocytes (filled circle) and 20,000 CD3$^+$CD146$^-$ T lymphocytes (open circle) and the CD146$^+$ enriched PB fraction of a healthy donor (filled square). Individual (18 sRNA) CT$_{[VIC]}$-values for 1:90 diluted cDNA samples are shown. For reference, the median CT$_{[VIC]}$-values taken from Fig. 3A are shown (horizontal lines).*

*B) Relative CD3 and CD146 RNA levels in sorted CD3$^+$CD146$^+$ T lymphocytes (filled circles), 20,000 CD3$^+$CD146$^-$ T lymphocytes (open circles) and the CD146$^+$ enriched PB fraction of a healty donor (filled squares). Non detectable RNA levels were arbitrarily put at 1x10$^{-6}$ (base line). See legend to Figure 2 for technical details.*

## DISCUSSION

Drugs that target the tumours' vasculature are becoming a well-established treatment modality for cancer. As a result, there is a growing need for biomarkers that allow monitoring of the efficacy and toxicity of these agents. Results from several studies have already demonstrated the feasibility of monitoring CEC as a novel marker for vascular damage in oncology, but these assays are laborious and costly. In search for an alternative and sensitive assay that is capable to process samples with a semi-high throughput, we have evaluated the feasibility of RT-PCR to quantify endothelial marker-specific RNA levels in blood. The high sensitivity, relative ease of inter-laboratory standardization, and possibility to analyse a large number of both fresh and frozen samples, make PCR an attractive alternative for cell-based assays.

We studied RNA levels of 4 endothelial markers, namely CD31, CD144, CD146 and vWf, and showed that these endothelial marker mRNAs can be reliably detected in as few as 10 HUVEC (using strict criteria for analysis as indicated in M&M). Also, we were able to detect CD31 and CD146 mRNA but not vWf and CD144 in CD146-enriched blood samples with CEC numbers ranging from 220-4450 per sample, although absolute mRNA levels were up to 10,000-fold lower than those observed in similar numbers of HUVEC. In addition, we showed that the detected CD146 mRNA levels in the CD146-enriched blood samples could not be attributed to contaminating $CD146^+$ T lymphocytes, and thus - highly probably - are truly CEC related. Our results are in agreement with those of Smirnov et al (18), showing endothelial specific mRNA in CD146-immunomagnetically enriched PB fractions, although they used preamplification of total RNA prior to their RT-PCR.

In the contrary, we failed to detect significant levels of endothelial marker mRNA in whole blood samples. These results were unexpected as others have described their ability to detect CD146 mRNA in peripheral whole blood of cancer patients and healthy controls (19). Several factors could explain these differences.

First, Furstenberger *et al.* state that CD146 mRNA can be detected from a minimum of 500 CEC/mL, whereas there is a good consensus that in healthy donors, CEC counts do not exceed 20 cells/mL (9, 12). In their study, 0.5 mL is used for RNA isolation, containing a theoretical average of only 10 cells, which is 50 times below their given detection threshold. We believe that their results could be caused by insufficient specificity of their assay. In addition, the correlation that Furstenberger and colleagues report between CEC numbers and levels of CD146 mRNA should be interpreted with care, as we have shown that the CEC enumeration method used in their study (19) may detect platelets rather than CEC (20).

Second, circulating endothelial cells are highly quiescent with an estimated turnover rate of approximately 1,000 days and a limited ability to proliferate (21). It is likely that CEC, which are end-stage endothelial cells, have a low metabolic and consequently transcriptional activity, whilst still expressing the endothelial surface markers at protein level. In contrast, our control cells, HUVEC, are cultured cells which actively proliferate and have a high metabolic and transcriptional activity and are therefore not a representative model of CEC in the setting of this study. In addition, our negative CD146 mRNA results in sorted $CD146^+$ T-lymphocytes might be related to the fact that CD146 membrane expression in T cells is even considerably lower than in CEC (17).

In conclusion, we suggest that our inability to detect endothelial specific mRNA in whole blood enriched blood samples is the combined result of a low number of CEC in peripheral blood and the low transcriptional activity in these cells. In conclusion, the RT-PCR for endothelial specific markers by the approach described in this study does not provide a technical alternative to detect CEC as biomarker for vascular damage by either CD146-based enrichment or flow cytometry.

## REFERENCES


1. Bicknell R, Harris AL. Mechanisms and therapeutic implications of angiogenesis. Current opinion in oncology 1996;8: 60-5.
2. Folkman J. Angiogenesis--retrospect and outlook. Exs 1992;61: 4-13.
3. Kabbinavar F, Hurwitz HI, Fehrenbacher L*, et al.* Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 2003;21: 60-5.
4. Motzer RJ, Hutson TE, Tomczak P*, et al.* Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. The New England journal of medicine 2007;356: 115-24.
5. Sandler A, Gray R, Perry MC*, et al.* Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. The New England journal of medicine 2006;355: 2542-50.
6. Patterson DM, Rustin GJ. Vascular damaging agents. Clinical oncology (Royal College of Radiologists (Great Britain)) 2007;19: 443-56.
7. Van De Wiele C, Oltenfreiter R, De Winter O, Signore A, Slegers G, Dierckx RA. Tumour angiogenesis pathways: related clinical issues and implications for nuclear medicine imaging. European journal of nuclear medicine and molecular imaging 2002;29: 699-709.
8. Khan SS, Solomon MA, McCoy JP, Jr. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. Cytometry 2005;64: 1-8.
9. 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.
10. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J Cell Biol 1984;99: 2034-40.
11. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol 2004;15: 139-45.
12. 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: 105-13.
13. 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: 1731-5.
14. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood 2005;106: 2923-4.
15. Chan EC, Fraser S, Yin S*, et al.* Human myometrial genes are differentially expressed in labor: a suppression subtractive hybridization study. The Journal of clinical endocrinology and metabolism 2002;87: 2435-41.
16. Gabert J, Beillard E, van der Velden VH*, et al.* Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain

reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 2003;17: 2318-57.
17. 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.
18. 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 research 2006;66: 2918-22.
19. Furstenberger G, von Moos R, Senn HJ, Boneberg EM. Real-time PCR of CD146 mRNA in peripheral blood enables the relative quantification of circulating endothelial cells and is an indicator of angiogenesis. Br J Cancer 2005;93: 793-8.
20. 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 2007;72: 86-93.
21. Foreman KE, Tang J. Molecular mechanisms of replicative senescence in endothelial cells. Experimental gerontology 2003;38: 1251-7.

# CHAPTER 7

## CIRCULATING ENDOTHELIAL CELLS: A POTENTIAL PARAMETER OF ORGAN DAMAGE IN SICKLE CELL ANEMIA?

*Blood Cells Mol Dis. 2009 Jul-Aug;43(1):63-7*

**ABSTRACT**

Objective laboratory tools are needed to monitor developing organ damage in sickle cell disease (SCD). Circulating endothelial cells (CEC) are indicative of vascular injury. We determined whether elevated CEC can be detected in asymptomatic SCD with the CellSearch system and whether the CEC count is related to clinical and blood-based biomarkers of disease severity. Fifteen consecutive clinically asymptomatic HbSS patients and 15 matched HbAA controls were analysed for CEC counts, laboratory parameters of disease severity (Hb, leukocyte counts, HbF%), plasma levels of markers for endothelial activation (sVCAM-1, VWF:Ag) and of endogenous inhibitors of nitric oxide synthase (asymmetrical dimethylarginine [ADMA]). CEC counts were significantly higher in patients (12 cells/mL, IQR 8-29) as compared to controls (4 cells/mL, 3-10) (p=0.007). CEC counts were significantly higher in patients with pulmonary hypertension (PHT) (p=0.015), and increased with increasing number of affected organs (0-4 involved organs, p=0.002). No significant correlations between CEC and any other laboratory parameter were detected. In conclusion, CEC's could prove to be an important new tool for assessing developing vasculopathy and organ damage in SCD.

## INTRODUCTION

Sickle cell disease (SCD) is a heterogeneous disorder characterized by chronic hemolysis, increased susceptibility to infections and acute and chronic vaso-occlusive complications culminating in significant morbidity and early death. Both improved supportive care (timely vaccination, penicillin prophylaxis, blood transfusions) and rational drug therapy (hydroxyurea) have contributed to an increasing life expectancy of patients with SCD, with most patients in Western countries surviving into the fourth and fifth decades of life [1]. With this, the impact of accumulating organ damage on patient outcome has become increasingly clear. Both the ongoing vaso-occlusive process with its acute exacerbations, as well as the sequelae of chronic intra-vascular hemolysis, result in continuous endothelial insults, ultimately leading to widespread organ damage. Evidence of endothelial perturbation in SCD comes from animal models [2] and studies in humans demonstrating elevated blood levels of endothelial activation markers [3] , endothelial dysfunction [4] and increased numbers of circulating endothelial cells (CEC's) [5].

Large patient cohort studies have identified laboratory markers associated with the clinical course of SCD, such as hemoglobin concentrations, leukocyte counts, lactate dehydrogenase (LDH) levels and the fetal hemoglobin percentage (HbF%) [6]. Such markers are, however, neither sufficiently reliable in predicting developing organ damage in individual patients, nor are they suitable for monitoring the effect of therapeutics in daily clinical practice [7]. Accurate objective laboratory parameters that reflect the rate of developing organ damage in SCD are therefore needed. Attempts to assess the value of monitoring endothelial activation markers in relation to SCD severity have been made [3]. However, most endothelial activation markers are neither specific for endothelial cells (EC's) (e.g., also expressed on or originating from non-EC's), nor specific for endothelial damage or dysfunction [8]. Therefore, measuring CEC's may be more accurate for studying *in vivo* endothelial damage as is demonstrated in other disease states characterized by endothelial injury [8]. CEC's are elevated and activated in SCD [5], but their relationship to organ damage in SCD has not been reported.

We performed a pilot study to determine circulating CEC numbers in SCD with a validated automated rare cell analysis system [9] and to assess their relationship to SCD related organ damage, laboratory markers of disease severity, markers of endothelial activation and plasma levels of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA).

## MATERIALS

### *Patients*

Consecutive patients attending the out-patient hematology clinics of the Erasmus Medical Center (Rotterdam) and the Academic Medical Center (Amsterdam) were screened. Inclusion criteria were high performance liquid chromatography confirmed diagnosis of sickle cell anemia (HbSS) and age ≥ 18 years. Exclusion criteria were blood transfusions in the preceding three months, any acute SCD related complication in the preceding 2 weeks, pregnancy, cancer, infection, and connective tissue diseases. Race, age, and sex matched blood donors served as controls (HbAA). Written informed consent was obtained from all subjects. This study was approved by the local Institutional Medical Ethical Review Boards, and is in agreement with the Helsinki declaration of 2000.

### *Clinical data*

In the participating centers, sickle cell patients are screened for the presence of organ damage as recently defined and described [10]. From chart review, the following SCD related complications were scored if assessed within one year of sample collection: *Pulmonary hypertension (PHT):* tricuspid regurgitation jet flow velocity (TRV) ≥2.5 m/s in rest detected by echocardiography. PHT was considered absent with no or trace TRV. *Micro-albuminuria (MA):* urinary creatinine (mmol/l) to urinary albumin (mg/l) ratio >3.5 (males)/ >2.5 (females) confirmed with 24 hour urine collection (micro-albuminuria >30 mg/24 hours). *Retinopathy (RET)*: presence of at least mild non-proliferative retinopathy. Leg ulcers (ULC): chronic ulcers of the ankle not otherwise explained. The number of admissions for treatment of a *painful crisis (PC) and/or acute chest syndrome (ACS)* in the year before sample collection were recorded.

### *Sample collection*

Whole blood, ethylenediaminetetraacetic acid (EDTA) blood, citrated blood, and CellSave preserved blood (contains EDTA and a proprietary preservative, Immunicon Corp, Huntingdon Valley, PA, USA) were collected by veni-puncture. To avoid contamination with traumatically detached EC's, CellSave tubes were drawn last.

### *CEC enumeration*

The CellTracks AutoPrep and CellSpotter Analyzer II System (both Immunicon) were used to count CEC [9]. Four milliliters (mL) of CellSave preserved blood were incubated with ferrofluids coupled to a monoclonal antibody (mAb) against CD146 (clone S-Endo 1), present on EC's, a subset of activated T-cells and melanoma cells. After enrichment, cells were stained with fluorochrome-conjugated mAb: phycoerythrin (PE)-conjugated CD105, present on EC's and certain leukocytes, allophycocyanin (APC)-conjugated CD45, a pan leukocyte marker, and the nuclear dye 4, 6-diamidino-2-phenylindole (DAPI). CEC were immuno-phenotypically defined as $DAPI^+$, $CD146^+$, $CD105^+$ and $CD45^-$.

***Sample analysis***
Standard laboratory tests were performed according to local protocols. Serum levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) were determined using ELISA (R&D Systems, Minneapolis, MN). Von Willebrand Antigen (VWF:Ag) and VWF ristocetin cofactor activity (VWF:RCo) were measured in citrated plasma [11] and EDTA plasma concentrations of arginine and ADMA were also determined [12].

***Statistical analysis***
No published data on CEC distribution as determined with the methods above in SCD were available. Furthermore, no reports on CEC's in relation to organ damage in SCD were available and thus the number of CEC that are clinically relevant in relation to organ damage in SCD is presently unknown. Therefore, in this pilot study, a power calculation was not performed. Between group differences were assessed with the Mann-Whitney U test. Trends within ordered groups of non-parametric data were assessed using Cuzick's test for trends [13]. For correlation studies Spearman's rank correlation coefficient ($r_s$) was determined. Statistical significance was considered with $P < 0.05$ (STATA statistical software package 10, StataCorp College Station, TX, USA).

**Table 1 – Patient characteristics and laboratory data**

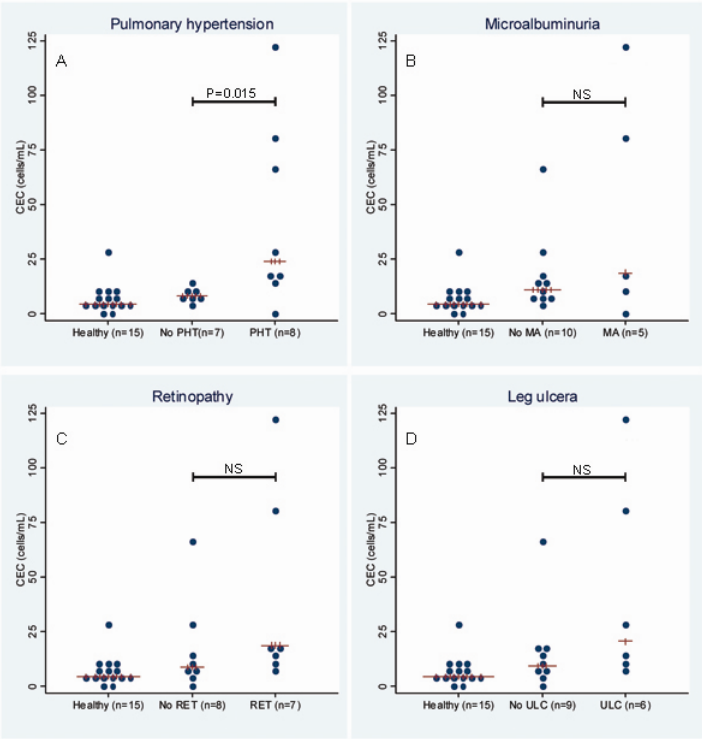
| | **HbAA (n=15)** | **HbSS (n=15)** | **P-Value** |
|---|---|---|---|
| Age | 24 (21-28) | 24 (21-28) | - |
| Gender (M:F) | 4:14 | 4:14 | - |
| Hemoglobin (g/L) | 14.1 (13.1-15.9) | 8.7 (7.4 - 10.0) | <0.0001 |
| Leukocytes (*$10^9$/L) | 6.7 (5.8-7.3) | 8.7 (7.2–11.1) | 0.003 |
| LDH (IU/L) | 144 (139-151) | 487 (392-630) | <0.0001 |
| Creatinin (µmo/L) | 75 (55.6-95.6) | 52 (49-57) | <0.0001 |
| HbF (%) | <1.0% | 7.7 (4.4-10) | <0.0001 |
| Hydrea use (yes:no) | NA | 5:10 | |
| | | | |
| CEC (cells/mL) | 4 (3-10) | 12 (8-29) | 0.007 |
| | | | |
| vWF:Ag (IU/mL) | 0.95 (0.81-1.44) | 2.01 (1.23-2.51) | 0.002 |
| vWF:RCo (IU/mL) | 0.96 (0.74-1.25) | 1.54 (1.20-1.95) | 0.007 |
| VCAM-1 (ng/mL) | 921( 765-1050) | 1879 (1398-2568) | 0.0001 |
| | | | |
| ADMA (µmol/l) | 0.42 (0.39-0.45) | 0.70 (0.59-0.80) | <0.0001 |
| Arginine (µmol/l) | 80.8 (72.7-90.9) | 79.4 (53.0- 88.7) | NS |

*Data are shown as medians and corresponding inter quartile ranges. NS=not significant***.**

## RESULTS

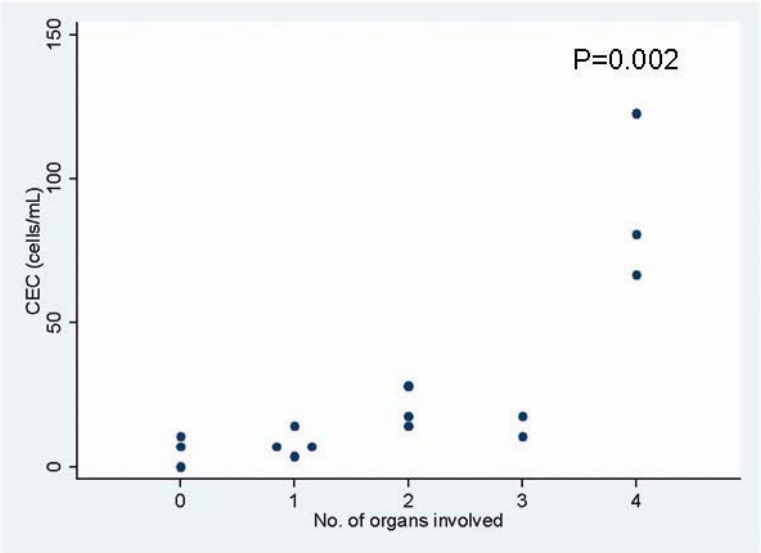
Results of all measured parameters are shown in table 1. CEC counts were significantly higher in sickle cell patients. Also, CEC counts were higher in sickle cell patients with PHT as opposed to patients without PHT, with no significant differences obsered in relation to other forms of organ damage (Figure 1). CEC counts increased significantly with increasing numbers of affected organs (Figure 2). CEC counts were not related to painful crises (data not shown) and no acute chest syndromes or strokes had occured in the year prior to CEC determination. CEC counts did not differ between patients on hydroxyurea and those who did not use hydroxyurea (data not shown). Additionally, no effect of hydroxyurea was observed on any of the other measured parameters (data not shown) in this cross sectional study. No statistically significant correlations were detected between CEC counts and hemoglobin concentrations, leukocyte counts, LDH concentrations or HbF% (data not shown) or any of the tested markers of endothelial actvation (Figure 3). Additionally; no significant correlations were found between CEC numbers and plasma arginine or ADMA concentrations in either heatlhy controls ($r_s$=-0.13, P=0.64) or sickle cell patients ($r_s$=0.05, P=0.88).

**Figure 1 – CEC numbers and relation to specific organ complications**



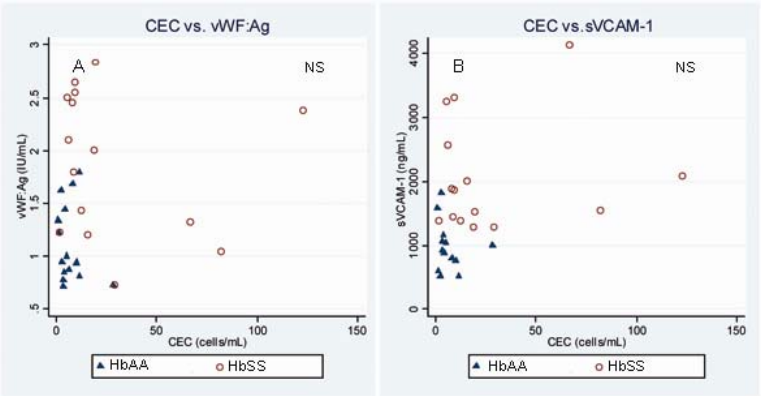

*CEC counts were compared using the Mann-Whitney U test in SCD patients with and without specific organ complications. Bars indicate median values. NS=not significant.*

**Figure 2 – Relation of CEC and the extent of organ involvement**




*The significance of trends in CEC counts with increasing organ involvement was assessed using the Cuzick's test for trends.*

**Figure 3- Correlation of CEC and vWF:Ag and VCAM-1 levels in patients and**




*Healthy controls: CEC vs. vWF:Ag: $r_s$=-0.14, P=0.61. CEC vs. sVCAM-1: : $r_s$=-0.28, P=0.34. SCD patients: CEC vs. vWF:Ag: $r_s$=-0.27, P=0.33. CEC vs. sVCAM-1: : $r_s$=-0.01, P=0.82*

## DISCUSSION

Continuous endothelial perturbation culminates in significant organ damage in SCD, and accurate monitoring of this process could be of great value in the study and management of SCD. We detected elevated CEC counts in the same range as previously reported in clinically asymptomatic sickle cell anemia patients [5] using a validated automated CEC detection assay developed to overcome shortcomings of assays based on manual enrichment know to be susceptible to cell loss and to have low reproducibility [14]. CEC counts appeared to be highest in those patients with most extensive organ damage. Importantly, patients with minimal organ involvement were characterized by CEC counts comparable to control values. No significant relation was observed between CEC counts and laboratory markers of disease severity, nor to crisis frequency.

Direct EC injury by rigid sickled erythrocytes, high arterial vascular wall shear stress [8], neutrophil degranulation, present even in the clinically asymptomatic state of SCD [15], all likely contribute to the detachment of EC from the vessel wall. EC apoptosis can also lead to elevated CEC's [8], however, previous work on sickle cell CEC's and plasma VEGF levels [16] were suggestive of an anti-apoptotic tone.

Elevated blood levels of sVCAM-1 [3] and VWF:Ag [17] indicate EC activation in SCD, with further increments occurring during acute vaso-occlusive events [17; 18]. Levels of sVCAM-1 are related to hemolytic rate [3] and either directly or indirectly to SCD related complications associated with a high hemolytic rate, such as PHT and leg ulcers [19]. Also, sVCAM-1 levels have been associated with endothelial dysfunction in SCD as well [20]. Neither sVCAM-1 nor VWF:Ag correlated significantly to CEC counts. EC activation was manifest even in patients with minimal organ damage, whereas CEC counts were comparable to controls in these patients. Importantly, these data suggest that the extent of EC activation/dysfunction is not necessarily related to the extent of EC damage and detachment in SCD.

Recently ADMA has been recognized as an important contributor to the characteristically reduced nitric oxide bioavailability of SCD and its plasma level may be an important biomarker of disease severity, with associations reported to PHT [21] and mortality [22] in adult sickle cell patients. Plasma levels are elevated in SCD and, likely via NOS inhibition, contribute to endothelial activation [21]. However, ADMA levels were not significantly associated to CEC counts in either patients or controls, perhaps suggesting that elevated ADMA levels contribute primarily to endothelial activation but not to endothelial damage.

Several shortcomings should be addressed when interpreting these data. Firstly, our sample size was small, precluding multivariate analysis. Evidently, these findings are preliminary, but the differences in CEC counts between patients without organ damage and those at the other end of the spectrum with all organs affected can be clearly appreciated. Secondly, several clinical complications did not occur in our patients (e.g. acute chest syndromes) and some forms of organ damage were not screened for (e.g. silent ischemic brain injury). Also, we did not monitor patients after blood sampling, so we cannot exclude that acute events were developing at the time of sampling, possibly affecting CEC numbers. Lastly, as we did not characterize the phenotype of CEC's, their vascular origin is unknown.

In conclusion, these preliminary data show the feasibility of the employed technique for measuring CEC in SCD. Our findings suggest a possible relation of CEC counts to SCD related organ damage, and CEC's do not seem to be associated with either traditional or novel biomarkers markers of disease severity. Prospective studies in large patient cohorts addressing chronic organ damage, acute complications and the effect of established therapeutics (e.g. red cell transfusion and hydroxyurea) with longitudinal CEC determinations are warranted to analyze whether CEC can be used as a reliable parameter to monitor developing organ damage in SCD.

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## REFERENCES

[1] W. Hagar, and E. Vichinsky, Advances in clinical research in sickle cell disease. Br J Haematol 141 (2008) 346-56.
[2] J.D. Belcher, C.J. Bryant, J. Nguyen, P.R. Bowlin, M.C. Kielbik, J.C. Bischof, R.P. Hebbel, and G.M. Vercellotti, Transgenic sickle mice have vascular inflammation. Blood 101 (2003) 3953-9.
[3] J.B. Schnog, R.A. Rojer, M.R. Mac Gillavry, H. Ten Cate, D.P. Brandjes, and A.J. Duits, Steady-state sVCAM-1 serum levels in adults with sickle cell disease. Ann Hematol 82 (2003) 109-13.
[4] L. Belhassen, G. Pelle, S. Sediame, D. Bachir, C. Carville, C. Bucherer, C. Lacombe, F. Galacteros, and S. Adnot, Endothelial dysfunction in patients with sickle cell disease is related to selective impairment of shear stress-mediated vasodilation. Blood 97 (2001) 1584-9.
[5] A. Solovey, Y. Lin, P. Browne, S. Choong, E. Wayner, and R.P. Hebbel, Circulating activated endothelial cells in sickle cell anemia. N Engl J Med 337 (1997) 1584-90.
[6] M.H. Steinberg, Predicting clinical severity in sickle cell anaemia. Br J Haematol 129 (2005) 465-81.

[7] C.T. Quinn, N.J. Lee, E.P. Shull, N. Ahmad, Z.R. Rogers, and G.R. Buchanan, Prediction of adverse outcomes in children with sickle cell anemia: a study of the Dallas Newborn Cohort. Blood 111 (2008) 544-8.
[8] E. Shantsila, A.D. Blann, and G.Y. Lip, Circulating endothelial cells: from bench to clinical practice. J Thromb Haemost 6 (2008) 865-8.
[9] J.L. Rowand, G. Martin, G.V. Doyle, M.C. Miller, M.S. Pierce, M.C. Connelly, C. Rao, and L.W. Terstappen, Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. Cytometry A 71 (2007) 105-13.
[10] E.J. van Beers, C.F. van Tuijn, M.R. Mac Gillavry, A. van der Giessen, J.J. Schnog, and B.J. Biemond, Sickle cell disease-related organ damage occurs irrespective of pain rate: implications for clinical practice. Haematologica 93 (2008) 757-60.
[11] T.N. Bongers, M.P. de Maat, M.L. van Goor, V. Bhagwanbali, H.H. van Vliet, E.B. Gomez Garcia, D.W. Dippel, and F.W. Leebeek, High von Willebrand factor levels increase the risk of first ischemic stroke: influence of ADAMTS13, inflammation, and genetic variability. Stroke 37 (2006) 2672-7.
[12] J.B. Schnog, T. Teerlink, F.P. van der Dijs, A.J. Duits, and F.A. Muskiet, Plasma levels of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, are elevated in sickle cell disease. Ann Hematol 84 (2005) 282-6.
[13] J. Cuzick, A Wilcoxon-type test for trend. Stat Med 4 (1985) 87-90.
[14] M.H. Strijbos, J.W. Gratama, J. Kraan, C.H. Lamers, M.A. den Bakker, and S. Sleijfer, Circulating endothelial cells in oncology: pitfalls and promises. Br J Cancer 98 (2008) 1731-5.
[15] L.R. Lard, F.P. Mul, M. de Haas, D. Roos, and A.J. Duits, Neutrophil activation in sickle cell disease. J Leukoc Biol 66 (1999) 411-5.
[16] A. Solovey, L. Gui, S. Ramakrishnan, M.H. Steinberg, and R.P. Hebbel, Sickle cell anemia as a possible state of enhanced anti-apoptotic tone: survival effect of vascular endothelial growth factor on circulating and unanchored endothelial cells. Blood 93 (1999) 3824-30.
[17] J.J. Schnog, J.A. Hovinga, S. Krieg, S. Akin, B. Lammle, D.P. Brandjes, M.R. Mac Gillavry, F.D. Muskiet, and A.J. Duits, ADAMTS13 activity in sickle cell disease. Am J Hematol 81 (2006) 492-8.
[18] A.J. Duits, R.C. Pieters, A.W. Saleh, E. van Rosmalen, H. Katerberg, K. Berend, and R.A. Rojer, Enhanced levels of soluble VCAM-1 in sickle cell patients and their specific increment during vasoocclusive crisis. Clin Immunol Immunopathol 81 (1996) 96-8.
[19] G.J. Kato, S. Martyr, W.C. Blackwelder, J.S. Nichols, W.A. Coles, L.A. Hunter, M.L. Brennan, S.L. Hazen, and M.T. Gladwin, Levels of soluble endothelium-derived adhesion molecules in patients with sickle cell disease are associated with pulmonary hypertension, organ dysfunction, and mortality. Br J Haematol 130 (2005) 943-53.
[20] M.T. Gladwin, A.N. Schechter, F.P. Ognibene, W.A. Coles, C.D. Reiter, W.H. Schenke, G. Csako, M.A. Waclawiw, J.A. Panza, and R.O. Cannon, 3rd, Divergent nitric oxide bioavailability in men and women with sickle cell disease. Circulation 107 (2003) 271-8.

[21] P.P. Landburg, T. Teerlink, E.J. van Beers, F.A. Muskiet, M.C. Kappers-Klunne, J.W. van Esser, M.R. Mac Gillavry, B.J. Biemond, D.P. Brandjes, A.J. Duits, and J.J. Schnog, Association of asymmetric dimethylarginine with sickle cell disease-related pulmonary hypertension. Haematologica 93 (2008) 1410-2.
[22] C.R. Morris, Mechanisms of vasculopathy in sickle cell disease and thalassemia. Hematology Am Soc Hematol Educ Program 2008 (2008) 177-85.

# CHAPTER 8

## LEVELS OF CIRCULATING ENDOTHELIAL CELLS IN NORMOTENSIVE AND PREECLAMPTIC PREGNANCIES

*Submitted for publication*

**ABSTRACT**

**Background:** Preeclampsia is a disease hypothesized to originate from widespread endothelial dysfunction or damage. The present study investigated whether CEC can serve as a surrogate marker for disease severity in patients with preeclampsia, and whether or not their number correlated to serum endothelial biomarkers for activation, dysfunction or damage of those cells.

**Methods:** Blood was drawn from 15 patients consecutively admitted with the diagnosis severe preeclampsia. Fifteen healthy, normotensive, patients, matched for age, body mass index and gestational age, served as a control group. We determined the number of CEC and serum concentrations of biomarkers indicative of endothelial damage (thrombomodulin) and activation (E-selectin), and the antiangiogenic protein (endoglin), which reflects endothelial dysfunction.

**Results:** CEC counts did not differ significantly between these patients and the control group (median 5.0 vs. 3.1 CEC/mL, respectively), and were within the normal range (<20 CEC/ml). However, serum concentrations of thrombomodulin (median 2.3 vs. 4.1 ng/mL; P=0.002), E-selectin (median 19.8 vs. 29.0 ng/mL; P=0.02), and especially endoglin (median 6.4 vs. 62.7 ng/mL; P<0.0001), were significantly increased in severely pre-eclamptic patients. CEC counts were negatively correlated to systolic blood pressure in preeclamptic patients ($R_s$=-0.54; P=0.04). Also, CEC correlated to serum concentrations of thrombomodulin in healthy controls, ($R_s$=0.54; P=0.05), but not to any of the clinical parameters or investigated laboratory indices.

**Conclusion:** Preeclampsia is characterized by endothelial dysfunction and activation rather than actual endothelial damage as characterized by aberrant CEC counts.

## INTRODUCTION

Preeclampsia, defined as the occurrence of hypertension and proteinuria after 20 weeks of gestation, affects up to five percent of all pregnancies worldwide, and is one of the leading causes of maternal and fetal morbidity and mortality (1,2). Although the exact etiology of preeclampsia is still unknown, there is sufficient clinical and biochemical evidence that the endothelium plays an important role in its pathogenesis (3).

Endothelial dysfunction in preeclampsia is thought to be the result of several mechanisms triggered in response to the placental hypoxia caused by abnormal placentation. These mechanisms include the release of soluble antiangiogenic factors, such as soluble fms-like tyrosine kinase 1 receptor (Flt-1, a soluble receptor for the vascular endothelial growth factor [VEGF]), and soluble endoglin, (a co-receptor for transforming growth factor β-1[TGF]). Also, release of proinflammatory mediators such as tumor necrosis factor alpha, interferon gamma, and interleukin 1 and 6 has been reported (3). Prolonged exposure to these mediators is known to causes endothelial activation and dysfunction in pregnant women (4). Indeed, increased serum concentrations of E-selectin and von Willebrand factor, putative markers of endothelial dysfunction, have been observed in preeclamptic women (5).

In addition to endothelial dysfunction, actual damage to the maternal vascular endothelium contributes to the clinical syndrome. For example, it has been shown that Flt-1 leads to the production of endothelin-1, which subsequently causes glomerular endothelial injury and proteinuria (6). Blood based evidence for endothelial damage in preeclampsia has recently been demonstrated by Canbakan and Grundmann. Both authors reported increased numbers of circulating endothelial cells (CEC) in preeclamptic women (7,8). Increments in CEC counts are considered to reflect the extent of endothelial injury (9,10). However, these studies rely on manual enumeration of CEC, which is known to be susceptible to cell loss and therefore to underestimation of counts (11).

In this cross-sectional study, we hypothesize that CEC numbers, determined by a well-validated semi-automatic CEC detection assay with excellent recovery of these cells, are elevated in preeclamptic pregnancies, and would reflect endothelial injury. Furthermore, we expected that such increment would not correlate to increments in serum markers of endothelial dysfunction (soluble E-selectin and soluble endoglin) and endothelial injury (soluble thrombomodulin).

## MATERIALS AND METHODS

### *Patients and blood sampling*

Peripheral blood (PB) was obtained from 15 women consecutively admitted to the Department of Obstetrics of the Erasmus Medical Centre with a diagnosis of severe preeclampsia. As a control group served 15 healthy, normotensive, pregnant controls, visiting the outpatient clinic. Both groups were matched for age, gestational age and body mass index (BMI). In preeclamptic patients blood sampling was done immediately on admission, to exclude any interference of treatment on CEC counts. Preeclampsia was diagnosed according to the definition of the International Society for the Study of Hypertension (ISSHP). Severe preeclampsia was diagnosed if one or more of the following criteria was present: a blood pressure of 160 mmHg systolic (SBP) or higher or 110 mmHg diastolic (DBP) or higher on two occasions at least 6 hours apart; proteinuria of 5 grams or more in a 24 hour urine specimen or 3+ or greater on two random urine samples collected at least 4 hours apart; oliguria of less than 500 mL in 24 hours; cerebral or visual disturbances; pulmonary edema or cyanosis; epigastric or right upper-quadrant pain; impaired liver function; thrombocytopenia; fetal growth restriction (12). Patients with previous pregnancies complicated by preeclampsia, gestational diabetes or growth retardation were excluded from this study. The protocol and informed consent forms were approved by the institutional Medical Ethical Review Board, and all patients provided written consent prior to participation. The study is in agreement with the Helsinki declaration of 2000.

### *Collected clinical and laboratory parameters*

For each patient, the following demographic data and clinical parameters were noted: maternal age, length, weight, body mass index (BMI), systolic and diastolic blood pressure at admission and the laboratory parameters: hemoglobin concentration, hematocrit, reticulocytes, thrombocytes, ureum, creatinin, uric acid, total bilirubin, sodium, potassium, calcium, magnesium, total protein, albumin, haptoglobin, aspartate transaminase (ASAT), alanine transaminase (ALAT), lactate dehydrogenase (LDH) and gamma glutamyl transpeptidase (GGT).

### *Sample collection*

In total, 20 mL of blood was collected: 10 mL was drawn into a serum separator tube and 10 mL in CellSave preservative tubes, which contain EDTA as anticoagulant and a proprietary preservative (Veridex, Raritan, NJ). To avoid contamination with traumatically detached CEC, the CellSave tube was drawn last. All samples were processed within 24 hours. Serum was prepared within 1 hour after sampling by centrifugation at 1700 x *g* for 10 minutes. Aliquots were immediately frozen at -80°C until analysis.

### *CEC enumeration*

The CellTracks® AutoPrep® System and the CellSpotter® Analyzer II System (Veridex) were used to enumerate CEC, and have been described elsewhere (13) . Briefly, four milliliters (mL) of blood were used for immunomagnetic enrichment using ferrofluids coupled to an anti-CD146 antibody.

This marker is present on endothelial cells, a subset of activated T-lymphocytes, and melanoma cells. Subsequently, the following staining reagents were added: the nuclear dye 4,6-diamidino-2-phenylindole (DAPI), and fluorochrome-conjugated monoclonal antibodies (mAb): phycoerythrin-conjugated CD105, which is present on endothelial cells, and allophycocyanin-conjugated CD45, which is a pan-leukocyte antigen included in order to exclude hematopoietic cells during analysis. Analysis was done using image cytometry, where CEC were defined as being at least 4 μm in diameter, intact (with the nucleus ≥50% of the surface of the cytoplasm), $DAPI^+$, $CD146^+$, $CD105^+$ and CD45 negative..

***Assessment of soluble endothelial markers by ELISA***

Serum concentrations of thrombomodulin, E-selectin, and endoglin were determined using ELISA according to the manufacturers' instructions. The thrombomodulin-specific ELISA was purchased from American Diagnostica Inc. (Stanford, CT, USA), the other ELISAs were from R& D Systems (Minneapolis, MN, USA). Absorbance was read at 450 nm using a Titertek 212 MS microplate reader (Titertek, Huntsville, AL, USA). Samples were tested in duplicate and related to the relevant standard curves. The lower detection limits of the assays were 2.0 ng/mL for E-selectin; 1.2 ng/mL for thrombomodulin, and 0.07 ng/mL for endoglin.

***Statistical analysis***

Comparison between groups was done using the Mann-Whitney U test. Correlations between parameters were calculated using Spearman's correlation coefficient ($r_s$). Statistical differences were considered significant if $P < 0.05$. Analyses were performed using STATA 10 (StataCorp College Station, TX, USA).

## RESULTS

The demographical, obstetrical and clinical characteristics of patients and controls are shown in Table 1, whilst a summary of relevant laboratory parameters is presented in Table 2. Expectedly, healthy, normotensive controls and severe preeclamptic patients differed with respect to blood pressure (Table 1), liver function tests, serum creatinin, uric acid, haptoglobin and number of reticulocytes and thrombocytes (Table 2). Also, all severe preeclamptic patients had proteinuria (Table 2). Four patients showed the characteristic combination of hemolysis, elevated liver tests, and low platelet counts, known as the HELLP syndrome. There were significantly more primigravidae and nulliparae among the pre-eclamptic patients than among the control patients (Table 1), but we did not expect these differences to have an effect on the parameters of interest in this study. None of the control patients developed signs of hypertension or preeclampsia later in their pregnancies.

**Table 1 – Demographical, obstetrical, and clinical data of patients and healthy controls**

| | Control group (n=15) | Patients with severe preeclampsia (n=15) | P-value |
|---|---|---|---|
| Demographics | | | |
| Age (years) | 31 (28-34) | 31 (28-34) | NS |
| BMI (kg/m$^2$) | 27.4 (22.5-32.1) | 29.6 (23.4-38.7) | NS |
| SBP (mm Hg) | 120 (100-125) | 160 (145-180) | P<0.0001 |
| DBP (mm Hg) | 75 (70-80) | 100 (90-110) | P<0.0001 |
| Smoking | | | |
| Yes | 1 | 0 | |
| No | 14 | 15 | |
| Obstetrics | | | |
| Gestational age (days) | 203 (191-214) | 208 (202-216) | NS |
| Gravidity | | | |
| 1 | 0 | 11 | |
| 2 | 8 | 2 | |
| ≥3 | 7 | 2 | |
| Parity | | | |
| 0 | 1 | 11 | |
| 1 | 8 | 3 | |
| 2 | 5 | 1 | |
| ≥3 | 1 | 0 | |
| Medication | | | |
| Methyldopa | 0 | 11 | |
| $Ca^{2+}$ reentry blocker | 0 | 7 | |
| Magnesium sulphate | 0 | 4 | |

*Data are shown as medians and interquartile ranges. NS=not significant*

Upon analysis of CEC numbers, no significant difference could be detected between the normotensive control group and the patients with severe preeclampsia (median 3.1 CEC [IQR 2-4.5] vs. 5.0 CEC [IQR 3.3-8.3], respectively, P=0.10) (Table 2). We did not find any correlation between CEC numbers and any other demographic data and clinical parameters (data not shown)

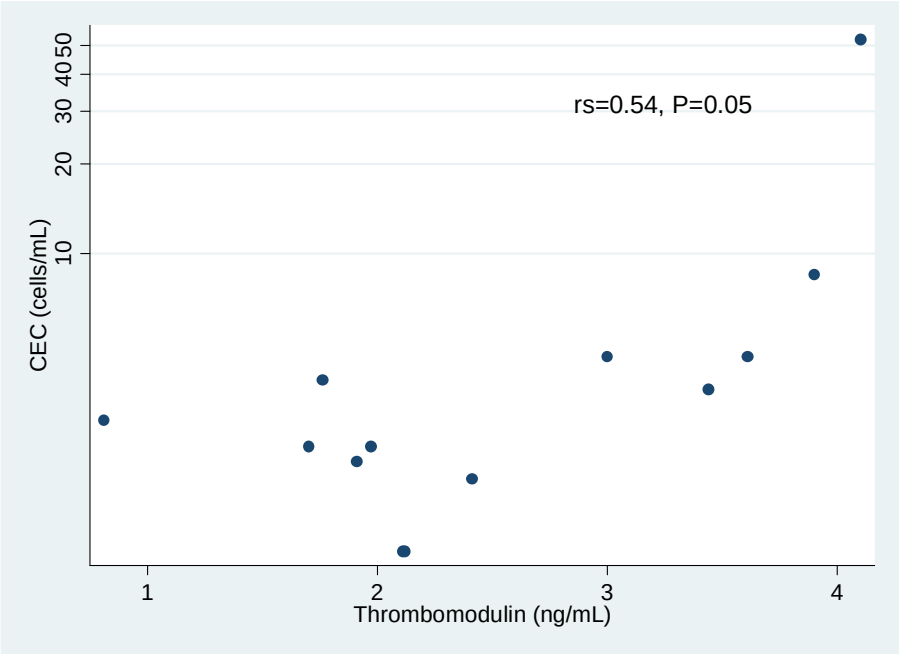
Serum concentrations of thrombomodulin, E-selectin and endoglin were all markedly elevated in preeclamptic patients as compared to controls (Table 2). Whilst median thrombomodulin and E-selectin levels doubled in preeclampsia (2.0 vs. 4.2 ng/mL and 14.1 vs. 28.2 ng/mL, respectively), endoglin levels were even increased 16-fold from 4.2 to 66.2 ng/mL. CEC numbers showed a weak correlation to serum concentrations of thrombomodulin in normotensives, which is in accordance to our earlier report ($r_s$=0.54; P=0.05; Figure 1) (10).

**Table 2 – Laboratory parameters.**

| | Controls | Patients | P-value |
|---|---|---|---|
| Hematology | | | |
| Hematocrit (%) | 36 (35-37) | 36 (35-39) | NS |
| Thrombocytes (*$10^9$/L) | 277 (223-291) | 150 (81-181) | P=0.0001 |
| Hemolysis | | | |
| LDH (IU/L) | 312 (278-366) | 551 (494-802) | P<0.0001 |
| Haptoglobin (g/L) | 0.97 (0.77-1.49) | 0 (0-0.32) | P=0.0001 |
| Reticulocytes (%) | 2.2 (2-3) | 16 (14-20) | P<0.0001 |
| Total bilirubin (mg/dL) | 7 (6-10) | 7.5 (6-13) | NS |
| Liver function | | | |
| ASAT | 18 (16-21) | 45 (33-127) | P<0.0001 |
| ALAT | 13 (9-20) | 49 (31-117) | P<0.0001 |
| GGT | 11 (8-15) | 20 (14-27) | P=0.001 |
| Renal function | | | |
| Creatinin (μmo/L) | 49 (43-51) | 68 (57-82) | P=0.0007 |
| Proteinuria (mg/dL) | 0 * | 1.1 (0.3-4.5) | P<0.0001 |
| Uric acid (mmol/L) | 0.23 (0.18-0.27) | 0.41 (0.37-0.45) | P<0.0001 |
| Endothelial cell biology | | | |
| CEC (cells/mL) | 3.1 (2-4.5) | 5 (3.3-6.5) | NS |
| E-selectin (ng/mL) | 14.1 (7.2-19.7) | 28.2 (21.7-42.9) | P=0.02 |
| Endoglin (ng/mL) | 4.2 (6.8-7.8) | 66.2 (46.6-106.0) | P=0.001 |
| Thrombomodulin (ng/mL) | 2.0 (1.8-2.4) | 4.2 (3.3-5.2) | P=0.005 |

*Data are shown as medians and interquartile ranges. NS=not significant. *=negative urine dipstick test.*

**Figure 1 – Correlation between CEC and thrombomodulin**




*CEC numbers correlated significantly with serum concentrations of thrombomodulin in normotensive pregnancies but not in preeclamptic patients.*

## DISCUSSION

In this study, we compared serum biomarkers of endothelial dysfunction (E-selectin and endoglin) and endothelial injury (thrombomodulin) to numbers of CEC in 2 groups of pregnant women: one with severe preeclampsia, and one of normotensive women. The groups were matched for gestational age and BMI. CEC counts in both groups of women were within normal ranges (i.e., <20 CEC/ml; ref. (13)]; these counts were somewhat higher in the pre-eclamptic patients than in the normotensives, but this difference did not reach significance. However, soluble endothelial markers E-selectin, endoglin, and thrombomodulin were significantly increased in patients with severe preeclampsia.

The absence of increased CEC counts in preeclampsia was unexpected, as previous studies have reported increased CEC numbers in these patients (7,8). We believe that this discrepancy can be attributed to several factors. First, the power of our study was insufficient to be able to detect significant differences between 2 small groups that both still fall within the reference ranges of normal.

The standard deviation (SD) of CEC counts in healthy, non-pregnant women in our study is substantially larger (9.3) than the reported SD's in the study by Canbakan ($SD_{healthy}$=1.3; no power calculation was provided by Grundmann), possibly the result of a different immunophenotypical definition of CEC. Based on this SD, we calculated that we would need at least 174 patients to be able to detect a difference of 2.8 CEC with 80% power.

Second, methodological issues seem to play a role. CEC counts were significantly higher in healthy non-pregnant and pregnant women in the study by Grundmann. Theoretically, the inclusion of CD105 in the immunophenotypic definition of CEC in our study leads to an underestimation of CEC, as CD105 negative CEC (if they exist) are excluded from analysis. However, the CEC numbers in non-pregnant and normotensive pregnant women reported by Canbakan are similar to those in our study, despite the fact that they use a similar technique as Grunmann, namely manual CD146-driven immunomagnetic isolation, followed by a confirmatory staining (Canbakan: acridine orange, Grundmann: Ulex Europaeus Lectin -1). Within-assay differences (i.e. Canbakan vs. Grundmann) appear to contribute to the reported differences in CEC numbers in preeclampsia.

Various hypotheses are considered to elucidate the pathogenesis of vascular damage in women with preeclampsia. The key event in the pathogenesis of preeclampsia is thought to be abnormal placentation and failing vascularization of the placenta (14). The resulting placental hypoperfusion and hypoxia trigger the subsequent release of soluble factors. Three soluble factors related to endothelial damage (thrombomodulin), activation (E-selectin), and especially the antiangiogenic protein endoglin, were increased in our study but CEC levels were not. These findings suggest that endothelial dysfunction and activation rather than actual damage occurs in preeclamptic patients. However, additional prospective studies - which should be sufficiently powered - will be needed to elucidate the exact role of the endothelium in preeclampsia and the role of endoglin as a potential early surrogate marker. Importantly, a generally accepted consensus on the optimal CEC detection technique will be of critical importance when such studies will be carried out (15).

## REFERENCES


1. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. Lancet 2006;367(9516):1066-74.
2. Gilbert JS, Ryan MJ, LaMarca BB, Sedeek M, Murphy SR, Granger JP. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. Am J Physiol Heart Circ Physiol 2008;294(2):H541-50.
3. Myatt L, Webster RP. Vascular biology of preeclampsia. J Thromb Haemost 2009;7(3):375-84.
4. Haider S, Knofler M. Human tumour necrosis factor: physiological and pathological roles in placenta and endometrium. Placenta 2009;30(2):111-23.
5. Nadar SK, Al Yemeni E, Blann AD, Lip GY. Thrombomodulin, von Willebrand factor and E-selectin as plasma markers of endothelial damage/dysfunction and activation in pregnancy induced hypertension. Thromb Res 2004;113(2):123-8.
6. Hauser PV, Collino F, Bussolati B, Camussi G. Nephrin and endothelial injury. Curr Opin Nephrol Hypertens 2009;18(1):3-8.
7. Grundmann M, Woywodt A, Kirsch T, Hollwitz B, Oehler K, Erdbruegger U, Haller H, Haubitz M. Circulating endothelial cells: a marker of vascular damage in patients with preeclampsia. Am J Obstet Gynecol 2008;198(3):317 e1-5.
8. Canbakan B, Keven K, Tutkak H, Danisman N, Ergun I, Nergizoglu G. Circulating endothelial cells in preeclampsia. J Hum Hypertens 2007;21(7):558-63.
9. Lee KW, Lip GY, Tayebjee M, Foster W, Blann AD. Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. Blood 2005;105(2):526-32.
10. 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;100(4):642-7.
11. 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(11):1731-5.
12. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. Obstet Gynecol 2002;99(1):159-67.
13. Rowand JL, Martin G, Doyle GV, Miller MC, Pierce MS, Connelly MC, Rao C, Terstappen LW. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. Cytometry A 2007;71(2):105-13.
14. McMaster MT, Zhou Y, Fisher SJ. Abnormal placentation and the syndrome of preeclampsia. Semin Nephrol 2004;24(6):540-7.
15. Strijbos MH, Kraan J, Lamers CHJ, Sleijfer S, Gratama JW. Quantification of circulating endothelial cells by flow cytometry. Clin Cancer Res 2009;In press.

# CHAPTER 9

EARLY CHANGES IN CIRCULATING ENDOTHELIAL CELLS, CIRCULATING TUMOUR CELLS AND SERUM LEVELS OF TISSUE FACTOR PREDICT OVERALL SURVIVAL IN CASTRATION RESISTANT PROSTATE CANCER TREATED WITH DOCETAXEL.

*Submitted for publication*

## ABSTRACT

### Purpose
In this study, we determined whether baseline levels of endothelin-1 (ET-1), tissue factor (TF), and circulating endothelial cells (CEC), or their relative changes during treatment, were prognostic for overall survival (OS) in patients with castration resistant prostate cancer (CRPC) treated with docetaxel and prednisone. In addition, we examined whether the combined use of these markers with the number of circulating tumour cell (CTC) could aid in the construction of a predictive nomogram for outcome to treatment.

### Patients and Methods
Numbers of CEC and CTC, and serum concentrations of ET-1 and TF were studied in 162 patients treated with a docetaxel containing regimen before treatment, and after 2-5 weeks and 6-8 weeks of treatment.

### Results
In contrast to CTC, baseline levels of CEC, TF, and ET-1 were not associated with OS. A relative increase in CEC counts greater than 3.8* baseline CEC counts at 2-5 weeks was associated with decreased OS (median 10.9 vs. 16.8 months; P=0.015), as was a decrease in TF levels at this time (median 11.9 vs. 21.5 months; P=0.0005). CTC counts ≥5/7.5mL at 2-5 weeks was also predictive of worse OS (median 10.4 vs. 17.4 months; P=0.0002). Combining changes in TF, CEC, and CTC at 2-5 weeks after treatment initiation, yielded four groups differing in OS (median OS 24.2 vs. 16.0 vs. 11.4 vs. 6.1 months, respectively; P<0.0001).

### Conclusion
CEC, and CTC numbers and TF levels, in particular when combined, allows the discrimination of CRPC patients with respect to outcome to docetaxel-based therapy already at an early stage during treatment. If prospectively confirmed, the combined use of these markers is useful tool for clinical trial design and to tailor patient management.

## INTRODUCTION

Prostate cancer caused approximately 26,000 deaths worldwide in 2007, and is the second most frequent cause of cancer related deaths in males [1]. After failure of local treatment by surgery and / or radiation therapy, androgen deprivation is the treatment of choice, but hormone resistance eventually occurs. Standard treatment for castration resistant prostate cancer (CRPC) consists of docetaxel plus prednisone, resulting in a median overall survival (OS) of 18 months [2]. Given the palliative setting of this treatment and its side-effects, over treatment should be avoided, which underscores the need for markers enabling the clinician to discriminate patients with respect to outcome to therapy.

In addition to the widely used prostate specific antigen (PSA), both tumour- and vascular derived markers have been investigated for this purpose in the last few years. It has been shown that circulating tumour cell (CTC) counts, determined at base-line but also during therapy, predict OS in several forms of cancer, including metastatic breast, colorectal and prostate cancer [3-5]. A recent study by Scher *et al* in CRPC revealed that CTC counts clearly outperformed serum concentrations of PSA as predictor for OS [6].

Several studies suggest that markers indicative of angiogenesis and vascular damage could also be of prognostic value in prostate cancer [7-9]. For example, quantization of angiogenesis by histological assessment of micro vessel density, has shown to be independently associated with survival in prostate cancer [10,11], but this approach requires an invasive procedure, is time consuming, and therefore impractical in clinical use. Alternative parameters reflecting angiogenesis are soluble serum proteins such as endothelin 1 (ET-1) and tissue factor (TF), both not widely explored in prostate cancer yet. ET-1 acts synergistically with vascular endothelial growth factor (VEGF) and is secreted primarily by vascular endothelial cells [12,13]. Following binding to the endothelin A receptor, it induces endothelial cell proliferation, invasion, and tubule production. Levels of ET-1 are associated with microvessel density in several forms of cancer, including breast, colorectal, and ovarian cancer [14-17]. TF is a transmembrane glycoprotein derived form platelets, endothelial cells and leukocytes, which is involved in coagulation and tumor neovascularization [18]. After splicing, it can be measured in the peripheral blood. Both ET-1 and TF receptor antagonists are currently being investigated as anti-angiogenic treatment .[14,15,19,20]

Also the number of circulating endothelial cells (CEC) is considered a promising parameter with prognostic value in cancer patients [9]. CEC are mature endothelial cells detached from the vessel wall, and are considered a marker of vascular damage. The latter is supported by the correlation of CEC numbers and plasma concentrations of thrombomodulin, a putative plasma marker of endothelial injury [21]. In this study, we examined whether or not base-line levels of CEC, ET-1, and TF and changes thereof early during therapy are associated with OS in patients with CRPC treated with docetaxel. In addition, as parameters reflecting different processes involved in malignant behaviour may improve the prognostic value of each separate parameter, we examined whether or not the combined use of these markers with CTC counts results in a better model associated with outcome in these patients.

## PATIENTS AND METHODS

### *Patients*

Blood samples were collected from 231 patients with CRPC by venesection into 2 x 10 mL CellSave tubes (Veridex, Raritan NJ) and 1 x 6 mL serum tube in a multi-center prospective study. One CellSave tube was used to determine circulating tumour cells (CTC) for OS. Results on CTC levels measured in this group have been previously published [5]. The second CellSave tube was used to analyze CEC counts. In order to assess a homogenous group of patients with respect to systemic therapy, we selected from these 231 patients, all patients who were scheduled to receive docetaxel or a docetaxel containing regimen (n=162). Treatment was continued until progression or unacceptable toxicity. Demographics of the patients included in the present study are shown in table 1. Main eligibility criteria included age ≥18 years, pathological diagnosis of adenocarcinoma of the prostate, first or later line of chemotherapy, serum testosterone < 1.7 nmol/L (50 ng/mL), Eastern Cooperative Oncology Group (ECOG) performance status 0-2, pretreatment serum PSA ≥ 5 ng/mL, PSA progression (2 rises above a reference value), and ability to sign informed consent. CEC and CTC counts were determined prior to chemotherapy, after 2-5 weeks of treatment (first follow-up draw), and after 6-8 weeks (second follow-up draw). In addition, serum concentrations of ET-1 and TF levels were determined at the same time points. Prior to treatment, all patients had a complete blood count analysis and assessment of serum concentrations of lactate dehydrogenase (LDH), alkaline phosphatase (Alk. Phos), haemoglobin,and albumin. This study was approved by the local Institutional Medical Ethical Review Boards, and is in agreement with the Helsinki declaration of 2000. Written consent was obtained prior to participation.

### *Enumeration of CTC and CEC*

The CellTracks AutoPrep and CellSpotter Analyzer II System (both Veridex) were used to count CTC and CEC as previously described [4,22]. CTC were defined as intact cells, positive for 4' 6-diamidinophenylindole (DAPI), epithelial cell adhesion molecule (EpCAM) and for cytokeratins 8, 18 and 19, and that lack expression of the panleukocyte marker CD45. CEC were identified as intact cells, positive for DAPI, CD146 and CD105, also lacking expression of CD45. The technical data of both assays have been published earlier [4,22]. CTC counts are reported as cells/7.5mL whole blood, CEC counts as cells/4mL.

### *Assessment of serum levels of TF and ET-1*

Serum concentrations of ET-1 and TF-1 were determined by ELISA. The ET-1 specific ELISA was purchased from Assay Designs (Ann Arbor, MI, USA), and used according to the manufacturers' instructions. The TF specific ELISA was purchased from AssayPro (St. Charles, MO, USA). For this assay, samples were diluted twofold rather than the fourfold suggest by the manufacturer, to improve sensitivity. Absorbance was read at 450 nm using a Titertek 212 MS microplate reader (Titertek, Huntsville, AL). Samples were tested in duplicate and related to the standard curve. The lower detection limits of the assays were 1.3 pg/mL for ET-1 and 20.0 pg/mL for TF.

### *Statistical analysis*

CEC and CTC numbers, as well as serum concentrations of LDH, alkaline phosphatase, PSA, ET-1 and TF were skewed. Therefore a logarithmic transformation was applied. Longitudinal data were analysed using a random effects linear regression model using the maximum likelihood random effects estimator (the "xtreg, mle" command in STATA), in order to incorporate both between- and within-subject effects as well as random effects. OS was defined as the time between the first blood draw to the date of death or last contact. Kaplan-Meier survival plots were made using stratified data. For CTC numbers, a cutoff value of 5 CTC/7.5mL was used. In order to obtain a cutoff value for survival analysis for CEC numbers and serum concentrations of ET-1 and TF, patient data were stratified according the quantile (p0-p25; p25-p50, p50-p75, and p75-p100) in which either their baseline value or the change between baseline values and those determined at 2-5 weeks would fall. In case of a clear outlier, determined by inspection of the four strata in the Kaplan-Meier plots, this quantile was used to dichotomize data. In case no clear outlier could be observed, the median value was used. The logrank test was used to compare survival between strata. We used Cox proportional-hazards regression to identify baseline parameters associated with survival. Significant baseline parameters i.e. p-values $< 0.05$ were subsequently used in multivariate analysis. The predictive accuracy of the multivariate Cox models was assessed by concordance analysis and is reported as Harrel's C index. Data are reported as mean ± standard deviation (SD), unless stated otherwise. All analyses were performed using STATA 10 software (StataCorp. College Station, TX, USA).

## RESULTS

### *Alterations in CEC, CTC, ET-1 and TF levels during treatment*

Base-line levels of CEC, CTC, ET-1, and TF are shown in table 1. During therapy, a significant increase in CEC numbers was observed at the first follow-up blood draw, 2-5 weeks after initiation of docetaxel, when compared to baseline. No further increase was found at the second follow-up draw, 6-8 weeks after treatment initiation. Repeated linear regression for longitudinal data was performed to correct for inter-individual variation in CEC counts, which confirmed the significant general increase in CEC counts after 2-5 weeks of treatment (Figure 1, panel A and E). No additional change was observed after 6-8 weeks of treatment. The CTC numbers from the subgroup of patients included in this study are depicted in Figure 1, panels B and F. CTC decreased after 2-5 weeks of treatment, but remained stable hereafter.

In contrast to serum concentrations of ET-1, which remained constant during docetaxel treatment (Figure 1, panels C and G), a significant decrease of TF was observed after 6-8 weeks of therapy compared to baseline and first follow up draw (Figure 1, panels D and H).

**Table 1 – Patient demographics and laboratory parameters**

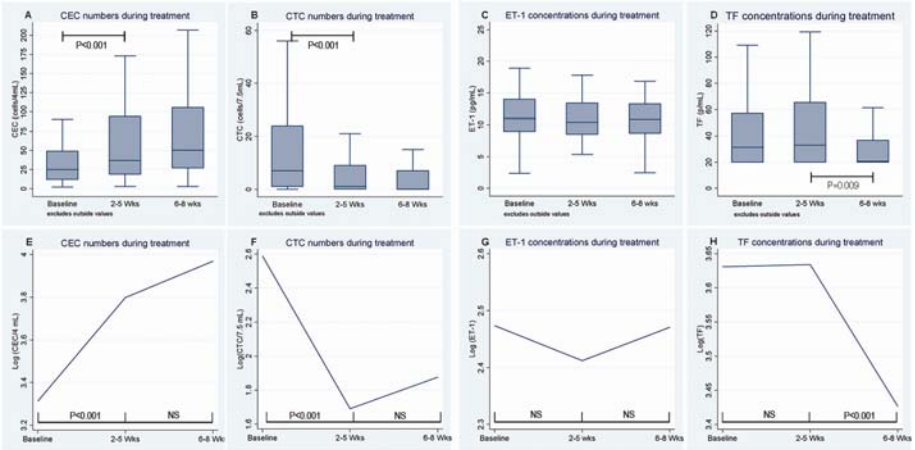
| Parameter | Baseline | Wks 2-5 | Wks 6-8 |
|---|---|---|---|
| N | 162 | 134 | 89 |
| Time from baseline (days) | | | |
| Mean ± SD | 0 | 26.6 ± 6.7 | 47.7 ± 6.6 |
| Range | 0 | 19 - 41 | 42 -61 |
| Age (years) | | | |
| Mean ± SD | 69.8 ± 9.6 | | |
| Range | 45 – 92 | | |
| Race | | | |
| White | 148 | | |
| Black | 11 | | |
| Hispanic | 1 | | |
| Asian | 2 | | |
| ECOG status | | | |
| 0 | 74 | | |
| 1 | 71 | | |
| 2 | 12 | | |
| Stage at diagnosis | | | |
| 1 | 9 | | |
| 2 | 24 | | |
| 3 | 39 | | |
| 4 | 13 | | |
| Gleason score | 7.1 ± 1.5 | | |
| Mean ± SD | 7.1 ± 1.5 | | |
| Range | 2 -10 | | |
| Prior local radiation | | | |
| Yes | 66 | | |
| No | 96 | | |
| Prior surgery | | | |
| Yes | 103 | | |
| No | 59 | | |
| Prior chemotherapy | | | |
| Mitoxantrone | 24 | | |
| Estramustine | 5 | | |
| Gemcitabine | 3 | | |
| Carboplatin | 3 | | |
| None | 127 | | |
| Line of chemotherapy | | | |
| 1 | 126 | | |
| 2 | 20 | | |
| 3 | 12 | | |
| 4 | 2 | | |

| | | | |
|---|---|---|---|
| 5 | 1 | | |
| 6 | 1 | | |
| Site of metastasis | | | |
| Bone | 147 | | |
| Visceral | 65 | | |
| Baseline hemoglobin (g/dL) | 12.4 ± 1.5 | | |
| Baseline LDH (IU/mL) | 287 ± 212 | | |
| Baseline Alk. Phos. (IU/mL) | 238.7 ± 280.1 | | |
| Baseline albumin (g/dL) | 4.0 ± 3.0 | | |
| Baseline testosterone (ng/mL) | 0.26 ± 0.2 | | |
| PSA (ng/mL) | | | |
| Mean ± SD | 566.5 ± 1832.9 | 428.4 ± 1526.4 | 347.3 ± 1307.4 |
| Range | 1.9 – 17.800 | 0.3 – 17420 | 0.3 – 12940 |
| CEC (cells/4mL) | | | |
| Mean ± SD | 75 ± 200 | 102 ± 176 | 94 ± 119 |
| Range | 2 - 1939 | 3 - 1102 | 0 – 701 |
| CTC (cells/7.5mL) | | | |
| Mean ± SD | 107.2 ± 534.8 | 22.7 ± 72.6 | 37.0 ± 160.8 |
| Range | 0 – 5925 | 0 – 525 | 0 - 1367 |
| ET-1 (pg/mL) | | | |
| Mean ± SD | 19.3 ± 52.5 | 14.1 ± 21.9 | 14.9 ± 17.4 |
| Range | 2.3 – 489.7 | 1.3 – 200.0 | 2.5 – 110.9 |
| TF ( pg/mL) | | | |
| Mean ± SD | 46.6 ± 39.3 | 56.0 ± 102.3 | 45.6 ± 98.0 |
| Range | 20.0 – 243.4 | 20.0 - 945.9 | 20.0 - 877.6 |

***Early changes in CEC, CTC and TF levels are prognostic for poor overall survival***
After dichotomizing data around their median baseline values, no prognostic value for OS could be determined for baseline levels of CEC, ET-1 or TF (Figure 2, panels A, C and D, respectively). As already reported for the whole group [5], also in the subgroup included in the present study, baseline CTC numbers ≥5 cells/7.5mL were associated with significantly decreased OS when compared to patients with baseline CTC numbers<5 cells/7.5/mL (10.9 vs. 17.4 months, respectively; P=0.0004, Figure 2, panel B ). With respect to CEC, analyses with various quantiles of the ratio between CEC at 2-5 weeks and CEC at baseline, revealed that patients in the p75-p100 quantile (i.e. an increase in CEC counts greater than 3.8* baseline CEC numbers), showed significantly worse outcome compared to those with a more limited CEC increase (Figure 2, panel E). Median survival for patients with an increase greater than 3.8 * baseline was 10.9 months, while those with an increase smaller than 3.8 * baseline had an OS of 16.8 months (P=0.015).

Although no significant overall difference could be observed between baseline TF levels and levels at 2-5 weeks of treatment (Figure 1, panels D and H), we found a significantly worse OS in those patients in whom a decrease in TF concentrations was observed (Figure 2, panel H). Median OS was 11.9 months vs. 21.5 months; P<0.001).

**Figure 1 – Course of tumor and vascular markers during treatment.**

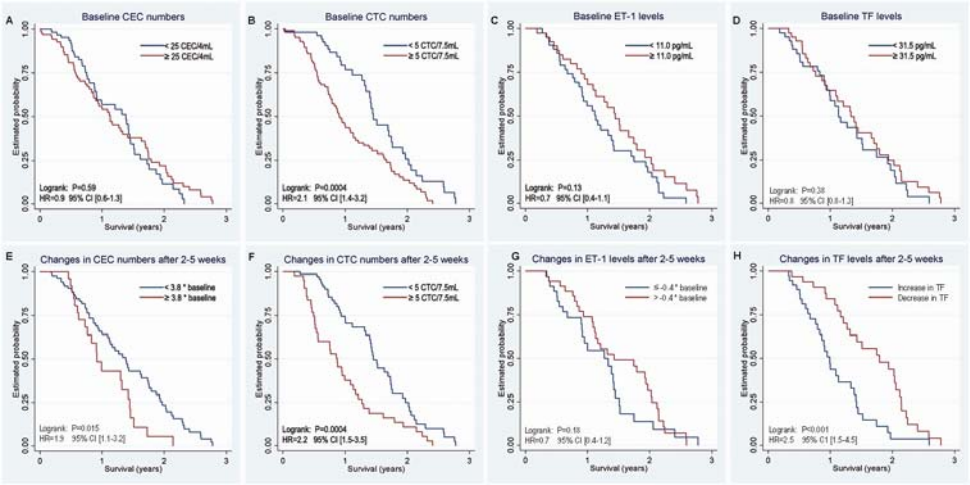



*Panels A-D: CEC numbers increased significantly within 2-5 weeks of treatment, whereas CTC numbers decreased. Both remained stable hereafter. No effect of treatment on ET-1 levels was observed. Serum TF declined after 6-8 weeks of treatment. NS=not significant.*

*Panels E-H: To assure that the observed alterations in biomarkers was not the result of inter individual differences; repeated linear regression analyses were performed. This confirmed the reported alterations in biomarker levels.*

Additionally, we assessed whether or not the combined use of all markers found to be prognostic for worse OS assessed in this study, i.e. CEC and CTC numbers and TF levels, could provide additional information on survival at 2-5 weeks of treatment. Sixty-nine patients were eligible for this combined analysis, meaning that all clinical and laboratory was available. First, we assessed any possible relation between each prognostic marker by both uni- and multivariate regression analysis. No significant associations were found, implying the independent prognostic value of each factor (data not show). Next, we stratified patients based on the number of risk factors for poor survival present at 2-5 weeks, namely an increase in CEC numbers greater than 3.8 times baseline counts, a decrease in TF levels when compared to baseline counts, and CTC counts equal or greater than 5 per 7.5 mL blood after 2-5 weeks of treatment. Here, we found a significant decrease in OS with increasing risk factors present (Logrank test for trend P<0.0001) All data is shown in Figure 3.

**Figure 2 – Associations of baseline levels and changes in CEC, ET-1, and TF levels with OS**




*Panels A–D: No prognostic value for OS could be determined for baseline values of the tested markers but CTC numbers. HR=hazard ratio, HR=hazard ratio determined in univariate Cox proportional-hazards regression. CI= confidence interval.*

*Panel E-H: Patients with an increase in CEC of ≥ 3.8 * baseline CEC counts weeks, with CTC counts ≥5 cells/7.5mL or with a decrease in TF levels during treatment were characterized by a markedly worse OS*

***CEC and TF levels increase the prognostic accuracy of CTC at 2-5 weeks***

To determine which parameters would provide the most accurate survival model, we performed the following procedure. First, we identified all parameters that were prognostic at baseline in a univariate Cox regression analysis (shown in Table 2), as these might affect the survival model at 2-5 weeks. Parameters found to be significant were subsequently used in a multivariate analysis, which also included changes in CEC, CTC and TF (Table 3). Four variables were found to be independently significant in multivariate analysis, namely CTC counts≥5 cells/7.5mL at 2-5 weeks, an increase in CEC≥3.8 * baseline CEC counts at 2-5 weeks, and a decrease in TF levels at 2-5 weeks, and baseline LDH concentration. To assess whether patient stratification yielded a more accurate survival model than the combined use of each individual prognostic parameter, we performed a concordance analysis, which results in a C index. Briefly, the C index describes how accurate the Cox regression model predicts survival, where a C index near 0.5 means that the model does not predict survival, whereas values approaching 1.0 indicate that the model nearly always predicts if a patient has a better prognosis [23]. Here, we found that the stratification based on the number of risk factors present, resulted in an increased predictive power fit of the Cox proportional-hazards regression model (Table 4).

**Table 2 - Univariate Cox proportional-hazards regression analysis of baseline characteristics**

| Parameter | Categories | | OS Risk from baseline | |
|---|---|---|---|---|
| | Positive | Negative | HR [CI] | p-value |
| Age at baseline | ≥70 | <70 | 1.4 [1.0-2.1] | 0.078 |
| Gleason score | 10-2 | | 1.1 [0.9-1.2] | 0.34 |
| Stage at diagnosis | 4 vs. 3 vs. 2 vs. 1 | | 0.9 [0.7-1.3] | 0.64 |
| ECOG status | 2 vs. 1 vs. 0 | | 2.1 [1.5-2.9] | <0.001 |
| Line of chemotherapy | Continuous | | 1.0 [0.8-1.3] | 0.97 |
| Hemoglobin (g/L) | Continuous | | 0.9 [0.8-1.0] | 0.011 |
| Testosterone (ng/mL) | Continuous | | 1.1 [1.0-1.0] | 0.62 |
| Albumin (g/dL) | Continuous | | 1.0 [1.0-1.1] | 0.20 |
| LDH (IU/mL) | Continuous | | 5.0 [3.2-7.9] | <0.001 |
| Alk. Phos. (IU/mL) | Continuous | | 1.4 [1.1-1.8] | 0.011 |
| PSA (ng/mL) | Continuous | | 1.1 [1.0-1.3] | 0.053 |
| Bone metastasis | Yes | No | 1.0 [1.0-1.0] | 0.51 |
| Visceral metastasis | Yes | No | 1.2 [0.8-1.8] | 0.39 |
| CEC (cells/4mL)[1] | ≥25 | <25 | 0.9 [0.6-1.3] | 0.59 |
| CTC (cells/7.5mL) | ≥5 | <5 | 2.1 [1.4-3.2] | 0.001 |
| ET-1 (pg/mL)[1] | ≥11.0 | <11.0 | 0.7 [0.4-1.1] | 0.14 |
| TF (pg/mL)[1] | ≥31.5 | <31.5 | 0.8 [0.5-1.3] | 0.39 |

[1] *Median value used as cutoff*

***PSA declines after 2-5 weeks of treatment are not prognostic for poor overall survival***

Additionally, we analysed baseline levels of prostate specific antigen (PSA) and for a decrease in PSA of both 30% and 50% from baseline values using identical patient stratification. We observed a decreased OS for patients with baseline PSA levels equal or higher than 493 ng/mL (median 16.6 months vs. 9.5 months; P=0.002). Neither a 30% nor 50% decrease in PSA levels at 2-5 weeks after start of docetaxel was associated with OS in this cohort (data not shown).

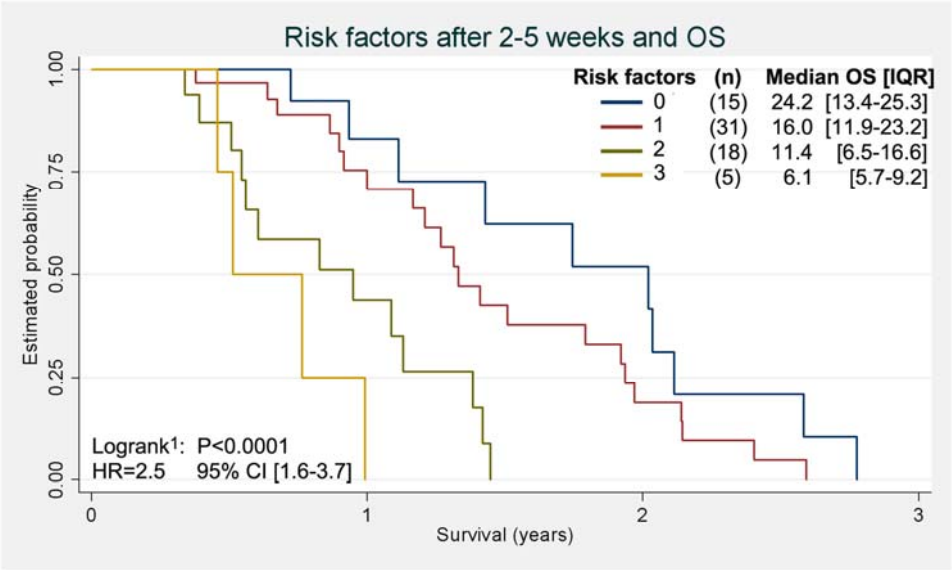
**Table 3 - Multivariate Cox proportional-hazards regression analysis of parameters prognostic for OS after 2-5 weeks of treatment**

| | OS Risk from baseline | |
|---|---|---|
| Parameter | HR [CI] | p-value |
| CTC (≥5 vs. 5 cell/7.5mL) | 2.0 [1.0-4.1] | 0.047 |
| CEC (≥3.8 vs. <3.8 * baseline CEC/4mL) | 2.3 [1.1-4.6] | 0.022 |
| TF (decrease vs. increase from baseline) | 2.4 [1.1-5.0] | 0.026 |
| ECOG status (2 vs. 1 vs. 0) | 2.0 [0.9-3.9] | 0.06 |
| Baseline hemoglobin (g/L) | 1.2 [0.9-1.5] | 0.13 |
| Baseline LDH (IU/mL) | 3.4 [1.5-7.7] | 0.003 |
| Baseline AP (IU/mL) | 1.1 [0.7-1.7] | 0.78 |

**Table 4 – Combined use markers adds to the predictive strength**

| Individual use | HR [CI] | p-value | C index |
|---|---|---|---|
| CTC (≥5 vs. 5 cell/7.5mL) | 2.0 [1.0-3.8] | 0.049 | 0.74 |
| CEC (≥3.8 vs. <3.8 * baseline CEC/4mL) | 2.5 [.2-4.9] | 0.011 | |
| TF (decrease vs. increase from baseline) | 2.9 [1.5-5.6] | 0.002 | |
| Baseline LDH (IU/mL) | 3.7 [1.7-8.0] | 0.001 | |
| Combined use | | | |
| Risk factors (3 vs.2 vs. 1 vs.0) | 2.5 [1.6-3.8] | <0.001 | 0.76 |
| Baseline LDH (IU/mL) | 3.2 [1.5-6.6] | 0.002 | |

**Figure 3 –Risk factors and overall survival**




[1] *Test for trend of survivor functions.*
*Association of risk factors present at 2-5 weeks (increase greater than 3.8 * baseline CEC, an decrease of TF, and a CTC number of ≥5 cells/7.5mL) with OS. HR=hazard ratio determined in univariate Cox proportional-hazards regression. CI= confidence interval.*

## DISCUSSION

In CRPC patients, docetaxel combined with prednisone is currently the only treatment that has been shown to yield an OS benefit in randomized studies [2]. However, the gain in OS is relatively modest given a median prolongation of 2 months compared to mitoxantrone and prednisone, which comes at the expense of sometimes severe side-effects. With this in mind, many attempts have been made to identity CRPC patients at risk for rapid progression and poor survival.

For this purpose, PSA is the most widely explored marker. Multivariate analysis on baseline PSA levels obtained from the TAX327 study in CRPC in which docetaxel with prednisone was randomly compared to mitoxantrone with prednisone, demonstrated increased OS for patients with a baseline PSA <114 ng/mL when compared to those with higher levels [24]. Furthermore, data from the same study allowed the development of a nomogram predictive for survival. Using baseline parameters such as PSA, lactate dehydrogenase, alkaline phosphatase and haemoglobin concentrations, patients likely to have rapid disease progression can be identified[25]. In this study, we confirmed the prognostic value of baseline PSA levels. But in contrast to our hypotheses, we found no prognostic value of baseline CEC, ET-1, or TF levels for survival, three markers that have not previously been assessed in CRPC. In addition to a marker that provides information on OS prior to initiation of chemotherapy, there is also a great need for markers that discriminate at an early stage during therapy those patients who clearly benefit from chemotherapy from those who do not. Previously, it was demonstrated that a PSA decrease of 30% after 3 months of treatment was a good surrogate marker for survival in patients treated with docetaxel / estramustine or mitoxantrone / prednisone [26]. Similar results have been observed in the TAX 327 study [24].

In the current study, we found that an increase greater than 3.8 * baseline CEC counts already after 1 or 2 cycles of docetaxel was prognostic for worse OS. CEC numbers are thought to reflect the extent of vascular damage. Several studies have shown an anti-vascular effect for both paclitaxel and docetaxel *in vitro* and in murine models [27,28]. If this holds true for humans as well, then the rise in CEC numbers, which was seen in all patients, is likely the result of vascular damage inflicted by docetaxel. The additional CEC increase in patients with the worst OS may be attributed to the continued endothelial cell shedding from vessels in tumours progressing during treatment. Furthermore, we found that an increase in TF concentration during the first 2-5 weeks of treatment was associated with a better survival. We hypothesize that this observed association is the result of massive shedding from apoptotic tumour cells, which, similar to platelets, endothelial cells and leukocytes, have also been reported to express TF [29]. Additionally, and as previously reported for the whole group from which the group was selected for the present study [5], CTC counts ≥5/7.5mL during the first 2-5 weeks of treatment were associated with a worse survival.

As all the explored markers are considered to represent different processes involved in tumour progression, we explored whether or not their combined use could aid clinicians in classifying docetaxel-treated CRPC patients into groups differing in OS. Concordance analysis demonstrated that the use of CEC, CTC and TF levels are independent risk factors for OS. Importantly, their combined use after 2-5 weeks yielded four groups with statistically different and clinically relevant differences in OS (median OS: 24.2 vs. 16.0 vs 11.4 vs 6.1 months). At this time point, PSA values were not informative for survival. This strongly suggests that the evaluated risk stratification examined in this study outperform PSA levels as early markers for ultimate outcome during docetaxel-based chemotherapy in CRPC.

To the best of our knowledge, this is the first study that demonstrates a prognostic value for CEC and TF changes during cytotoxic therapy in a well defined study population. Although the number of patients with a decreased OS survival in our study was relatively small, these results are encouraging. Furthermore, the combined use of CTC number and relative changes of CEC and TF allows the identification of CRPC patients not responding to docetaxel-based therapy already at an early stage during therapy. Whether or not this holds also true for other therapies and other tumour types deserves further study. Moreover, prospective studies should confirm our findings, and compare them to conventional biomarkers such as PSA. If confirmed, the model established here may serve as a useful tool for clinical trial design and to tailor patient management.

## REFERENCES


1. ACS: American Cancer Society: Cancer Statistics 2007 Presentation. Atlanta, 2008
2. Tannock IF, de Wit R, Berry WR, et al: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 351:1502-12, 2004
3. Cohen SJ, Punt CJ, Iannotti N, et al: Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol 26:3213-21, 2008
4. Cristofanilli M, Budd GT, Ellis MJ, et al: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 351:781-91, 2004
5. 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 14:6302-9, 2008
6. 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 10:233-9, 2009
7. Beerepoot LV, Mehra N, Vermaat JS, et al: Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol 15:139-45, 2004

8. 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 24:1491-8, 2006
9. Strijbos MH, Gratama JW, Kraan J, et al: Circulating endothelial cells in oncology: pitfalls and promises. Br J Cancer 98:1731-5, 2008
10. Offersen BV, Borre M, Overgaard J: Quantification of angiogenesis as a prognostic marker in human carcinomas: a critical evaluation of histopathological methods for estimation of vascular density. Eur J Cancer 39:881-90, 2003
11. Weidner N, Carroll PR, Flax J, et al: Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 143:401-9, 1993
12. Bagnato A, Spinella F, Rosano L: The endothelin axis in cancer: the promise and the challenges of molecularly targeted therapy. Can J Physiol Pharmacol 86:473-84, 2008
13. Spinella F, Rosano L, Di Castro V, et al: Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. J Biol Chem 277:27850-5, 2002
14. Asham E, Shankar A, Loizidou M, et al: Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. Br J Cancer 85:1759-63, 2001
15. Salani D, Rosano L, Di Castro V, et al: ABT-627, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma growth in vitro. Clin Sci (Lond) 103 Suppl 48:318S-321S, 2002
16. Shankar A, Loizidou M, Aliev G, et al: Raised endothelin 1 levels in patients with colorectal liver metastases. Br J Surg 85:502-6, 1998
17. Wulfing P, Kersting C, Tio J, et al: Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. Clin Cancer Res 10:2393-400, 2004
18. Yu J, May L, Milsom C, et al: Contribution of host-derived tissue factor to tumor neovascularization. Arterioscler Thromb Vasc Biol 28:1975-81, 2008
19. Lalich M, McNeel DG, Wilding G, et al: Endothelin receptor antagonists in cancer therapy. Cancer Invest 25:785-94, 2007
20. Wang HY, Yang YM, Zhuang Y, et al: The effect of celecoxib on tissue factor expression in pancreatic cancer cells. Chin Med J (Engl) 120:1753-6, 2007
21. Strijbos MH, Rao C, Schmitz PI, et al: Correlation between circulating endothelial cell counts and plasma thrombomodulin levels as markers for endothelial damage. Thromb Haemost 100:642-7, 2008
22. Rowand JL, Martin G, Doyle GV, et al: Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. Cytometry A 71:105-13, 2007
23. Azen S, Xiang A, Lapuerta P, et al: Comparison of Predictive Accuracy of Neural Network Methods and
Cox Regression for Censored Survival Data.
24. Berthold DR, Pond GR, de Wit R, et al: Survival and PSA response of patients in the TAX 327 study who crossed over to receive docetaxel after mitoxantrone or vice versa. Ann Oncol 19:1749-53, 2008

25. Armstrong AJ, Garrett-Mayer ES, Yang YC, et al: A contemporary prognostic nomogram for men with hormone-refractory metastatic prostate cancer: a TAX327 study analysis. Clin Cancer Res 13:6396-403, 2007
26. Petrylak DP, Ankerst DP, Jiang CS, et al: Evaluation of prostate-specific antigen declines for surrogacy in patients treated on SWOG 99-16. J Natl Cancer Inst 98:516-21, 2006
27. Grant DS, Williams TL, Zahaczewsky M, et al: Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). Int J Cancer 104:121-9, 2003
28. Hotchkiss KA, Ashton AW, Mahmood R, et al: Inhibition of endothelial cell function in vitro and angiogenesis in vivo by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol Cancer Ther 1:1191-200, 2002
29. Gouault-Helimann M, Josso F: [Initiation in vivo of blood coagulation. The role of white blood cells and tissue factor (author's transl)]. Nouv Presse Med 8:3249-53, 1979

# CHAPTER 10

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In view of the prominent role of the endothelium in disease, enumeration of circulating endothelial cells (CEC) could provide valuable information on disease severity, on prognosis, or could serve as a tool for monitoring the response to treatment. Therefore, validated and robust detection assays are needed. The thesis deals with the detection and clinical usability of circulating endothelial cells as surrogate marker for vascular injury. The low number of CEC in blood makes their detection highly susceptible to errors in sampling, preparation, and analysis. Also, no endothelial specific markers are available, so false positives can easily lead to overestimation of the actual CEC count [1]. Therefore, prior to its implementation as clinical monitoring tool, a CEC enumeration assay has to be extensively validated. First, assay sensitivity and specificity should be determined by confirming the endothelial origin of cells of cells labelled CEC. Second, assay performance has to be examined as inter- and intra-test variability should be below certain limits. In **Chapter 2**, we reviewed the currently available CEC detection assays, and discussed the possible applications of CEC in oncology. We found that only a very limited number of assays met the requirements concerning validation. Furthermore, despite having great potential as biomarker for vascular damage [1,2], the clinical use of CEC has only been evaluated in small studies, and most often in studies featuring heterogeneous patient cohorts. Table 1 provides an overview of frequently used CEC enumeration assays.

**Table 1 - Overview of currently available CEC enumeration assays**

| Group | Technique | CEC phenotype | Ref |
|---|---|---|---|
| Mancuso *et al.* | FCM | Syto16$^{+}$/CD45$^{-}$/CD31$^{+}$/CD146$^{+}$ | 49 |
| Widemann *et al.* | IMS + FCM | CD45$^{-}$/CD146$^{+}$ | 50 |
| Beerepoot *et a*l. | IMS + IC | CD31$^{+}$/CD146$^{+}$/CD309$^{+}$/vWF$^{+}$ | 14 |
| Rowand *et al.* | IMS + IC | DAPI$^{+}$/CD45$^{-}$/CD105$^{+}$CD146$^{+}$ | 5 |
| Solovey *et al.* | IMS + IC | CD146$^{+}$/vWF$^{+}$ | 16 |
| Bull *et al.* | IMS + IC | CD146$^{+}$/CD309$^{+}$/vWF$^{+}$ | 51 |
| Woywodt *et al.* | IMS + IC | CD146$^{+}$/UEA-1$^{+}$ | 6 |
| Shaffer *et al.* | FCM | CD31$^{+}$/CD34$^{+}$/CD146$^{+}$/CD133$^{-}$ | 52 |

*Abbreviations: FCM=flow cytometry; IMS=immunomagnetic isolation; IC=immunocytochemistry; UEA-1=Ulex Europaeus Lectin 1; vWF=von Willebrand Factor*

**The origin of endothelial cells**

In our initial attempts to detect CEC, we applied a widely applied flow cytometer assay first published by Mancuso *et al* [3]. This method is based on a single platform whole blood assay without prior cell enrichment, in which CEC are defined as events having $FSC^{low\ to\ intermediate}$ and $SSC^{low}$, and being $CD31^{++}$, $CD45^{-}$ and $CD146^{+}$. Despite the use of an immunophenotype consistent with endothelial cells, as well as the reported clinical relevance of these CEC in cancer patients, we questioned the endothelial origin of cells detected by this assay. Proper validation had not been reported for this assay, while CEC counts in healthy donors reported by this assay (i.e., up to 7,900 CEC /mL) greatly exceed those reported by enrichment based assays, which typically report less than 20 CEC/mL [4-6]. In **Chapter 3**, we described our experiments aimed to identify the nature of cells meeting our immunophenotypic criteria of endothelial cells (CMOIC), i.e. according to the definition of CEC by Mancuso *et al.* Using extensive flow cytometrical immunophenotyping, and cell sorting followed by assessment of morphology, immunohistochemistry, FISH, RT-PCR, and electron microscopy, we demonstrated that CMOIC are actually large platelets, rather than endothelial cells [7]. However, despite our reports on this finding on international meetings and in relevant medical journals [8], Mancuso's method, or minor modifications thereof, still remain among the most frequently used flow cytometrical CEC assays [9-12].

During our search for a validated assay, it appeared that most enrichment based studies rely on manual isolation of CEC [13-17]. The extensive sample manipulation makes these assays highly dependent on operator skills. In contrast, the CellSearch™ assay is semi automated and yields very low inter- and intra-laboratory variations [5]. Therefore, we decided to perform additional studies on CEC using the CellSearch™ system. This system was initially developed by Immunicon (currently Veridex LLC, Raritan, NJ) to detect circulating tumor cells, but was modified to allow the detection of CEC. Cells positive for CD146 are isolated by a monoclonal antibody (mAb) coupled to ferrofluids, magnetically isolated and stained with CD45, CD105 and DAPI. The endothelial nature of these $DAPI^{+}$, $CD146^{+}$, $CD105^{+}$, and $CD45^{-}$ cells was clearly shown by gene expression profiling, which demonstrated the presence of endothelial specific genes such as vWF and VE-Cadherin [18]. However, the exact site of origin of these endothelial cells, being either the bone marrow or the vascular wall, was still unanswered. Based on our findings reported in **Chapter 3**, showing that interference of cells with an overlapping immunophenotype, such as platelets, can easily be considered to be endothelial cells, we considered it paramount to demonstrate prior to the implementation of this assay in our studies, that the endothelial cells detected with the CellSearch™, are actually derived from the vessel wall, but not from the bone marrow, which would justify its use to determine vascular damage.

To this end, we used a model, described in **Chapter 4**, in which vascular damage was inflicted by incision and cannulation of the main artery and vein of a limb for the purpose of isolated limb perfusion. Subsequently, high doses of the vasotoxic agent tumor necrosis factor-α (TNF), and the alkylating agent melphalan were administered to the extracorporeal circulation. We hypothesized that a significant increase in CEC count would only be observed if CECs were derived from damaged

vasculature. Alternatively, the absence of such an increase would provide evidence that CEC as determined by the CellSearch™ technique are not of vascular origin, and likely originate from the bone-marrow. In addition and as previously mentioned, CD105 is used in the definition of CEC as determined by the CellSearch™ technique. However, CD105 may be mainly expressed by endothelial cells from damaged tumor vasculature rather than from normal vasculature [19]. By inflicting damage to healthy vasculature by incision and cannulation during isolated limb perfusion, we additionally tested the hypothesis that non-malignant CEC do express CD105 and can also be detected with the CellSearch™ assay, thereby rendering its application in non-malignant diseases worthwhile to study.

We observed a large increase in CEC in patients with both malignant and non-malignant diseases after isolated limb perfusion, which confirmed our hypothesis that these cells are actually of vascular origin, from non-malignant as well as malignant vasculature. In addition, we examined the correlation between CEC numbers and related plasma biomarkers of endothelial activation (E-selectin or CD62-E), endothelial proliferation (endoglin or CD105), and endothelial injury (thrombomodulin or CD141) in **Chapter 5**.

We found a significant correlation between CEC and plasma concentrations of thrombomodulin in healthy controls and in patients with metastatic carcinoma, which provided us with additional evidence for the use of CEC as biomarker for vascular damage in clinical studies on patients with malignant as well as non-malignant diseases.

**The clinical relevance of CEC**

Ever since the first report on the presence of circulating endothelial cells in 1976, then described as *"endothelaemia"* by Hladovek [20], researchers have studied the possible clinical applications of CEC, many of which are reviewed in **Chapter 2** [20]. A possible role for CEC as predictive or prognostic biomarker has been investigated in diseases including sickle cell anemia, infection, myocardial infarction, and cancer. With respect to latter, however, studies have typically been performed on heterogeneous groups. Also, most of the studies on CEC have included only a small number of patients, whereas others use assays that rely on non validated assays.[5,14]. Large scale studies that rely on well validated assays are scarce. [1] Therefore, the large majority of studies performed on CEC should be considered *"hypothesis generating"*.

Based on the correlation between increased tumor vasculature and tumor aggressiveness in metastatic prostate cancer, we investigated in **Chapter 7** whether or not CEC counts at baseline, at 2-5 weeks, and after 6-8 weeks of treatment, could provide prognostic information on survival in a relatively large and homogenous group. In addition to CEC, we examined serum concentrations of two proteins considered to be novel prognostic biomarkers of tumor vascularization and aggressiveness in prostate cancer, namely endothelin 1 (ET-1), a protein secreted primarily by vascular endothelial cells [21], and tissue factor (TF), a trans membrane glycoprotein derived from platelets, endothelial cells and leukocytes.

Both ET-1 and TF play an important role in tumor angiogenesis and metastasis [22-24], for ET-1 and TF are currently being investigated as target in cancer treatment [25-27]. In this study, we found that in patients suffering from hormone refractory metastatic prostate cancer (HRMPC) treated with the standard chemotherapeuticum docetaxel, CEC counts increased during the first 5 weeks of treatment in all patients. Furthermore, we demonstrated that the largest increase in CEC counts during 2-5 weeks of treatment occurred in patients with poorer outcome. In addition, we found no prognostic relevance for either baseline ET-1 levels or levels at 2-5 weeks. Surprisingly, patients with a decrease in TF levels after 2-5 weeks of docetaxel treatment also had significantly reduced survival when compared to patients in which an increase was observed during treatment. We speculate that the surge observed in TF levels in patients responding to treatment is the result of apoptosis and necrosis of tumor cells that, like endothelial cells, are known to express TF, or possibly, these patients had larger initial tumor volumes.

Based on these findings, we hypothesize that the additional increase in CEC observed in those patients with a poor outcome to docetaxel is the result of ongoing tumor growth and vascularization, despite no significant difference in serum levels of prostate specifi antigen (PSA) could be detected between both groups. Additionally, CEC might serve as an early marker that can discriminate between those patients benefiting from therapy, and those who do not and may therefore be withheld unnecessary toxicity. However, this needs to be established in prospective studies.

In addition to its possible prognostic value and early marker for response to chemotherapy in malignant diseases, we evaluated the clinical usefulness of CEC in two benign diseases, namely sickle cell disease (SCD) and preeclampsia. For both diseases, there is sufficient evidence that suggests a prominent role of the endothelium in its pathogenesis [16,28-33]. In the case-control cohort study performed on 15 homozygous, clinically asymptomatic SCD patients and 15 controls matched for age, race, and gender, we found increased CEC numbers as more organs were affected by the disease (**Chapter 8**). Interestingly, we found no correlation to serum concentrations of markers typically associated with endothelial damage and activation in SCD, such as von Willebrand Factor antigen (vWF:Ag) and vascular cell adhesion molecule 1 (VCAM-1). Also, no correlation was found between CEC numbers and any clinical or laboratory marker of disease severity. These findings suggest a possible role for CEC limited to the identification of patients at risk for severe organ damage, but a larger, prospective study should confirm this hypothesis.

A similar pilot study performed on 15 women with severe preeclampsia and 15 normotensive controls is described in **Chapter 9**. Here, we determined CEC numbers and serum concentrations of three endothelial proteins informative of the endothelial cell status, more specifically E-selectin, endoglin, and thrombomodulin. We found similar numbers of CEC in both cohorts (both within the normal range of 0-20 CEC/mL), whereas levels of all three endothelial biomarkers were significantly increased in preeclamptic pregnancies. These findings suggest that endothelial activation and/or dysfunction rather than actual vascular injury occur in this disease.

## FUTURE PERSPECTIVES

The studies reported in this thesis strongly support the hypothesis that CEC are a surrogate marker for vascular damage in malignant, and possibly, in non-malignant diseases. Obviously, large scale prospective studies on CEC are definitely warranted. In addition, uncertainty remains on the most appropriate means to determine CEC, while there is a great need for novel assays enabling the detection of CEC subpopulations (see below).

### Development of novel CEC detection assays

The flow cytometrical enumeration of CEC requires a multi-parameter approach. In addition to confirming the endothelial origin of CEC, which in our opinion requires at least examination of expression of the markers CD34, CD45, CD146 and a nuclear dye, robust assay performance should be demonstrated. The studies reported in this thesis were performed using the CellSearch™ assay.

Notwithstanding the prognostic value of CEC detected by this assay, as shown in **Chapter 9**, this assay has several disadvantages. First, although the CellSearch™ assay does allow the addition of only one extra marker, further characterization, as would be required for the detection of CEC subsets or the identification of novel targets for therapy, is almost impossible within this setting. Using the CellSearch technique it is not possible to get a pure fraction of isolated CEC as isolation of CEC through immunomagnetic beads loaded with anti-CD146 antibodies always results in cell suspensions mainly consisting of CD146 expressing T-cells, whilst a minority is CEC [34]. Also, the assay is costly (approx. €150 per test), and time consuming (min. 1.5 hours for one test). In view of these limitations of the CellSearch™ assay, we examined an alternative strategy that could allow the fast and reliable quantification of CEC. In **Chapter 6**, we described an alternative approach by real time polymerase chain reaction (RT-PCR) for the endothelial proteins vWF, CD31, CD144, and CD146. This approach allowed the detection of as few as 10 spiked human umbilical vein endothelial cells (HUVEC), but failed to detect similar numbers of CEC.

Currently, we are investigating the feasibility of a multicolor flow cytometer assay that uses the endothelial markers CD34, CD105 and CD146, the pan-leukocyte marker CD45, and the DNA specific nuclear dye DRAQ5 to identify CEC. Immunohistochemical staining of CEC (defined as $CD34^{+}/CD45^{-}/CD105^{+}/CD146^{+}/DRAQ5^{+}$) with von Willebrand Factor and CD31 clearly demonstrated the endothelial nature of these cells. Further validation and studies on assay performance are currently ongoing.

**Completing the picture**

We strongly believe that in order to get a complete view of the role of the endothelium in disease, assays should be developed that give detailed information on the equilibrium between vascular formation and vascular damage. Therefore, the clinical relevance of each endothelial cell (subset) or related particles, of which Figure 1 provides an overview, should be investigated.

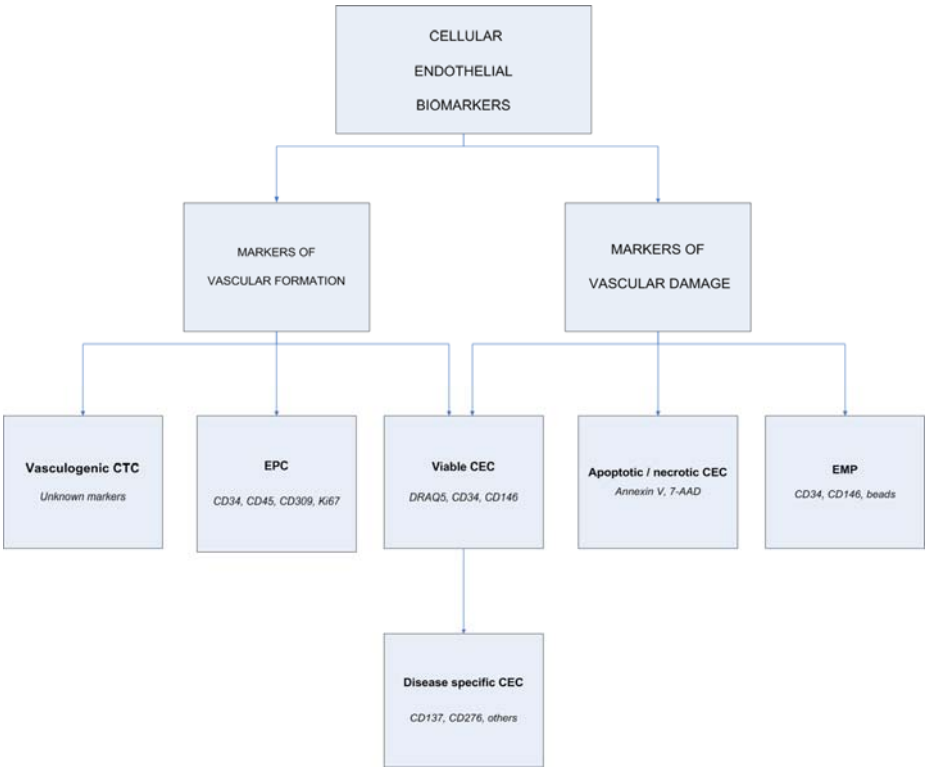
*Detection of viable, apoptotic and necrotic CEC*

Viable and proliferating endothelial cells could possibly aid in neovascularization and have been observed in the circulation [35] . Their number in the blood might therefore be considered indicative of angiogenesis rather than vascular damage. In contrast, apoptotic and necrotic CEC would be considered markers for vascular damage. *In vitro* studies have shown that detachment from the vascular matrix induces apoptosis or endothelial cells by activating caspase 8 [36], and several clinical studies have indeed shown that a significant proportion of CEC are apoptotic or necrotic [17,37]. Also, results from our study in chapter 4 clearly show an increase of non-intact, possibly apoptotic CEC after administration of the apoptosis inducing agent TNF. We hypothesize that each different CEC viability subset (i.e. viable, apoptotic and necrotic CEC) might therefore yield prognostic information. Studies using apoptosis markers such as Annexin V and caspases, and viability stains such as propidium iodide and 7-aminoactinomycin D should confirm this hypothesis.

*Identification of disease specific CEC*

Based on the fact that endothelial cells forming tumor vessels are immunophenotypically different from their normal counterpart [38], the existence of malignant CEC was hypothesized. Already, global gene expression profiling of CEC has demonstrated a difference in gene expression for several endothelial specific genes such as vWF and VE-Cadherin [18]. Also, tumor specific endothelial markers have been identified, such as CD137 (ILA/4, part of TNF receptor family) and CD273 (B7 homolog 3) [38]. Quantification of these tumor vessel-derived CEC could possibly provide additional prognostic information. By the same token, activated CEC could be present in the circulation of patients suffering from SCD or other diseases with overt vascular damage. Isolation by using a customized version of the previously described flow cytometer assay. For example, the addition of the activation markers CD54 (ICAM-1 or intercellular adhesion molecule 1) or CD106 (VCAM-1 or vascular cell adhesion molecule 1), and subsequent isolation and molecular characterization of these cells, could result in new insights into pathophysiological mechanisms, and ideally, lead to the identification of new vascular targets for treatment.

**Figure 1 – Schematic overview of potential cellular endothelial biomarkers**




*Overview of endothelial cells and particles that could provide specific information on the vascular endothelium. Abbreviations: CTC=circulating tumor cell; EPC=endothelial progenitor cell, CEC=circulating endothelial cell; EMP=endothelial microparticle.*

*Description of used markers:*
*7-AAD: DNA binding viability stain; Annexin V: phosphatidylserine binding apoptosis marker; CD34: Hematopoietic stem cell marker; CD45: Pan-leukocyte antigen; CD137: ILA/4, part of TNF receptor family; CD144: Vascular endothelial cadherin 5; CD146: Melanoma associated cellular adhesion molecule; CD276: B7 homolog 3; CD309: Vascular endothelial growth factor receptor 2.*

*Detection of endothelial progenitor cells*
Although not within the scope of our past and present studies on endothelial cells, endothelial progenitor cell (EPC) detection is very likely to yield valuable additional information on our vasculature, more specifically on angiogenesis. EPC are defined as progenitor cells derived from the bone marrow that circulate in the peripheral blood, home to a site where new vasculature is formed to become integrated into the vasculature. Currently, four mechanisms are known to contribute to neovascularization in cancer, namely 1) EPC derived angiogenesis, 2) local endothelial cell proliferation and differentiation, 3) co-opting of already established healthy vessels, and 4) vascular mimicry, the incorporation of epithelial tumor cells in the endothelial lining, functioning as endothelial cells [39]. Given the important role of EPC, a properly validated assay that allows the detection of EPC would definitely broaden our insights on the vascular equilibrium between vascular formation (EPC) and vascular decay (CEC), although we believe that EPC counts do not reflect all ongoing angiogenesis.

Unfortunately, as the frequency of EPC in blood is suggested to be several times lower than those of CEC, and as their exact phenotype has not been elucidated, it is very difficult to reliably detect and enumerate EPC by currently available assays. Also, results obtained by studies focusing on EPC have yielded conflicting results regarding the vascular tube forming capabilities of cells designated EPC. As functional assays, such as tube formation, are important in validating the progenitor capabilities of these cell [40,41], lack of such outgrowth makes the use of these cells as angiogenesis marker in cancer patients questionable. Possibly, more insight in EPC phenotype, combined with novel, highly sensitive detection techniques can overcome the limitations currently encountered in EPC enumeration.

*Enumeration of endothelial microparticles*
Another parameter that might reflect endothelial damage or angiogenesis are the enumeration of endothelial microparticles (EMP),. EMP are non-nucleated vesicles, approximately and are released from the membrane surface during cell activation or apoptosis [42]. They originate from various cell types, displaying the typical surface cell proteins and cytoplasmic components of their cell origin, such as CD31, CD105, CD144, and CD146 for endothelial microparticles (EMPs), and are approximately 1 μM in size [43]. Similar to CEC, increased numbers of EMP have been observed in diseases with markedly vascular involvement, including cardiovascular disease, connective tissue disease, cancer, and thrombotic diseases [44-47], and their number in the blood is also considered to reflect endothelial injury [48]. Possibly, enumeration of both CEC and EMP might give more information on endothelial health than each parameter alone.

## Conclusion
Endothelial cells, whether being endothelial cells *in situ*, CEC, or EPC, play a crucial role in normal physiology and pathophysiology. Quantification and characterization of these cells has already been demonstrated to be of clinical importance. Future studies should be conducted on well defined, large groups in prospective studies, and most

important, rely on a well validated assay, so that that question about the true clinical relevance of CEC can finally be answered.

## REFERENCES


1. 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.
2. Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost. 2005;93:228-235.
3. 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.
4. 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.
5. 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.
6. 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.
7. 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.
8. Strijbos MH, Kraan J, Sleijfer S, Gratama JW. Potential biomarkers in metastatic gastrointestinal stromal tumors. Clin Cancer Res. 2007;13:6244.
9. 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.
10. Duda DG, Cohen KS, di Tomaso E, et al. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J Clin Oncol. 2006;24:1449-1453.
11. 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.
12. 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.
13. Beerepoot LV, Mehra N, Linschoten F, et al. Circulating endothelial cells in cancer patients do not express tissue factor. Cancer Lett. 2004;213:241-248.
14. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol. 2004;15:139-145.
15. 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.
16. 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.

17. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis. Lancet. 2003;361:206-210.
18. 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.
19. Burrows FJ, Derbyshire EJ, Tazzari PL, et al. Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy. Clin Cancer Res. 1995;1:1623-1634.
20. Hladovec J, Jirka J, Prerovsky I, Malek P. Decrease of endothelaemia during immunosuppression. Biomedicine. 1976;25:204-206.
21. Agapitov AV, Haynes WG. Role of endothelin in cardiovascular disease. J Renin Angiotensin Aldosterone Syst. 2002;3:1-15.
22. Shankar A, Loizidou M, Aliev G, et al. Raised endothelin 1 levels in patients with colorectal liver metastases. Br J Surg. 1998;85:502-506.
23. Spinella F, Rosano L, Di Castro V, Natali PG, Bagnato A. Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. J Biol Chem. 2002;277:27850-27855.
24. Wulfing P, Kersting C, Tio J, et al. Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. Clin Cancer Res. 2004;10:2393-2400.
25. Asham E, Shankar A, Loizidou M, et al. Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. Br J Cancer. 2001;85:1759-1763.
26. Salani D, Rosano L, Di Castro V, et al. ABT-627, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma growth in vitro. Clin Sci (Lond). 2002;103 Suppl 48:318S-321S.
27. Ngo CV, Picha K, McCabe F, et al. CNTO 859, a humanized anti-tissue factor monoclonal antibody, is a potent inhibitor of breast cancer metastasis and tumor growth in xenograft models. Int J Cancer. 2007;120:1261-1267.
28. Furuya M, Ishida J, Inaba S, et al. Impaired placental neovascularization in mice with pregnancy-associated hypertension. Lab Invest. 2008;88:416-429.
29. Grundmann M, Woywodt A, Kirsch T, et al. Circulating endothelial cells: a marker of vascular damage in patients with preeclampsia. Am J Obstet Gynecol. 2008;198:317 e311-315.
30. Landburg PP, Elsenga H, Schnog JB, Duits AJ. Increased Serum Levels of Anti-Angiogenic Factors Soluble fms-Like Tyrosine Kinase and Soluble Endoglin in Sickle Cell Disease. Acta Haematol. 2008;120:130-133.
31. Landburg PP, Teerlink T, van Beers EJ, et al. Association of asymmetric dimethylarginine with sickle cell disease-related pulmonary hypertension. Haematologica. 2008;93:1410-1412.
32. Powers RW, Catov JM, Bodnar LM, Gallaher MJ, Lain KY, Roberts JM. Evidence of endothelial dysfunction in preeclampsia and risk of adverse pregnancy outcome. Reprod Sci. 2008;15:374-381.
33. Schnog JB, Duits AJ, Muskiet FA, ten Cate H, Rojer RA, Brandjes DP. Sickle cell disease; a general overview. Neth J Med. 2004;62:364-374.

34. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 2005;106:2923-2924.
35. Smadja DM, Mauge L, Susen S, Bieche I, Gaussem P. Blood outgrowth endothelial cells from cord blood and peripheral blood: angiogenesis-related characteristics in vitro'-a rebuttal'. J Thromb Haemost. 2008.
36. Aoudjit F, Vuori K. Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. J Cell Biol. 2001;152:633-643.
37. Solovey A, Gui L, Ramakrishnan S, Steinberg MH, Hebbel RP. Sickle cell anemia as a possible state of enhanced anti-apoptotic tone: survival effect of vascular endothelial growth factor on circulating and unanchored endothelial cells. Blood. 1999;93:3824-3830.
38. 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.
39. Gasparini G, Longo R, Toi M, Ferrara N. Angiogenic inhibitors: a new therapeutic strategy in oncology. Nat Clin Pract Oncol. 2005;2:562-577.
40. Timmermans F, Plum J, Yoder MC, Ingram DA, Vandekerckhove B, Case J. Endothelial progenitor cells: Identity defined? J Cell Mol Med. 2008.
41. Timmermans F, Van Hauwermeiren F, De Smedt M, et al. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arterioscler Thromb Vasc Biol. 2007;27:1572-1579.
42. Tesse A, Martinez MC, Meziani F, et al. Origin and biological significance of shed-membrane microparticles. Endocr Metab Immune Disord Drug Targets. 2006;6:287-294.
43. Nguyen M, Arkell J, Jackson CJ. Active and tissue inhibitor of matrix metalloproteinase-free gelatinase B accumulates within human microvascular endothelial vesicles. J Biol Chem. 1998;273:5400-5404.
44. Bernal-Mizrachi L, Jy W, Jimenez JJ, et al. High levels of circulating endothelial microparticles in patients with acute coronary syndromes. Am Heart J. 2003;145:962-970.
45. Combes V, Simon AC, Grau GE, et al. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. J Clin Invest. 1999;104:93-102.
46. Koga H, Sugiyama S, Kugiyama K, et al. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. J Am Coll Cardiol. 2005;45:1622-1630.
47. Toth B, Nieuwland R, Liebhardt S, et al. Circulating microparticles in breast cancer patients: a comparative analysis with established biomarkers. Anticancer Res. 2008;28:1107-1112.
48. Shantsila E, Blann AD, Lip GY. Circulating endothelial cells: from bench to clinical practice. J Thromb Haemost. 2008;6:865-868.
49. 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.

50. 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.
51. Bull TM, Golpon H, Hebbel RP, et al. Circulating endothelial cells in pulmonary hypertension. Thromb Haemost. 2003;90:698-703.
52. Shaffer RG, Greene S, Arshi A, et al. Flow cytometric measurement of circulating endothelial cells: the effect of age and peripheral arterial disease on baseline levels of mature and progenitor populations. Cytometry B Clin Cytom. 2006;70:56-62.

# CHAPTER 11

SAMENVATTING

**Vasculaire biomarkers in de oncologie**

De vasculaire intima bestaat uit ca. $1.2 * 10^{12}$ endotheelcellen. Het endotheel vormt niet uitsluitend een barrière tussen het bloed en de organen, maar is een dynamische structuur met een groot aantal functies. Zo zijn endotheelcellen van groot belang voor de regulatie van de vasomotor tonus, voor hemostase, voor vocht- en elektrolytentransport. Zij spelen tevens een grote rol in de regulatie van inflammatie en angiogenese. De kwantificatie van specifieke endotheliale eigenschappen, zoals de mate van activatie, dysfunctie, proliferatie, of de mate van vaatschade kan van groot klinisch belang zijn.

Zeker in de oncologie zou de kwantificering van vaatschade relevante informatie kunnen opleveren. De structuur en fysiologie van tumorvasculatuur verschilt sterk van de normale vasculatuur. Ze zijn fragiel, hebben een verhoogde permeabiliteit voor een groot aantal moleculen, en zijn verminderd gevoelig aan vassopressoren en dilatatoren. Ook de normale vasculatuur van kankerpatiënten raakt gemakkelijk aangetast, wanneer de door de tumor gesecreteerde matrix-metalloproteinasen het vasculaire basale membraan degraderen. Dit is noodzakelijk voor de extravasatie en metastasering van maligne cellen. Als de mate van endotheelschade effectief zou correleren met de mate van metastasering, zou een surrogaatmerker voor vaatschade een prognostische waarde kunnen hebben.

Naast een mogelijke rol als prognostische marker, zou een surrogaatmerker voor vaatschade van belang kunnen zijn in de vroegtijdige identificatie van patiënten. Bij patiënten met een goede respons op antivasculaire therapie met bijvoorbeeld monoklonale antistoffen of receptor tyrosine kinaseremmers, zou een duidelijke toename van vaatschade kunnen duiden op een gunstig effect van de behandeling. Bij patiënten zonder duidelijke respons kan de arts beslissen de behandeling te stoppen en zo de patiënt onnodige toxiciteit besparen.

Echter, het implementeren van CEC als routinebepaling in een klinische setting wordt vooralsnog gehinderd door ten eerste het zeer lage aantal CEC in de circulatie, en ten tweede de onenigheid over het juiste immunofenotype en de meest geschikte detectietechniek. Dit proefschrift gaat over het detecteren van CEC, de voor- en nadelen van de huidige beschikbare technieken en over de mogelijke toepassingen van CEC als surrogaatmerker voor vaatschade.

**Hoofdstuk 1** geeft een algemene introductie en beschrijft de doelstellingen van dit proefschrift.

In **hoofdstuk 2** worden diverse CEC detectietechnieken en hun voor- en nadelen besproken. Er wordt ook een overzicht gegeven van de mogelijke toepassingen van CEC als surrogaatmerker in de oncologie.

In **hoofdstuk 3** valideren wij een frequent gebruikt detectie assay dat gebaseerd is op flow cytometrie. Met dit assay worden klinisch relevante resultaten beschreven. Echter, het aantal CEC dat gedetecteerd wordt met dit assay staat mee staat

in sterk contrast tot eerdere studies. Op basis van de resultaten van deze studie hebben wij besloten om alle vervolgstudies uit te voeren met het CellSearch™ assay, ontwikkeld door Immunicon (tegenwoordig Veridex, Raritan, NJ).

Dit assay gebruikt immunomagnetische verrijking door middel van een CD146-gebonden monoklonale antistof, gevolgd door image cytometrie. De geïsoleerde CD146 positieve cellen worden gekleurd met fluorochroom gelabelde monoklonale antistoffen tegen de endotheliale merker CD105 en de leukocytenmerker CD45, en met de kernkleuring DAPI. CEC worden gedefinieerd als intacte cellen, minimaal 4 µm in diameter, met een $DAPI^+$, $CD146^+$, $CD105^+$, $CD45^-$ immunofenotype. In een studie met 40 gezonde donoren vonden wij met dit assay 0-20 CEC/mL, wat vergelijkbaar is met andere gevalideerde assays. Ook de eerdere gerapporteerde toenames van CEC aantallen bij patiënten met gemetastaseerde kanker en met sikkelcel anemie konden wij reproduceren met het CellSearch™ assay.

In **hoofdstuk 4** wordt bestudeerd wat het effect is van leeftijd en geslacht op het aantal CEC in het bloed bij gezonde vrijwilligers en bij patiënten met gemetastaseerde kanker. Hiernaast wordt ook onderzocht of er een correlatie is tussen het aantal CEC en plasmaconcentraties van diverse endotheliale proteïnen.

In **hoofdstuk 5** wordt onderzocht of de CEC gedetecteerd met het CellSearch™ van het vaatbed of van beenmerg afkomstig zijn. Wij beschrijven hoe door kunstmatig vaatschade op te wekken door bloedvatcannulatie, de vasculaire oorsprong van CEC gedetecteerd met het CellSearch™ wordt bevestigd.

In **hoofdstuk 6** bekijken we in het bloed van gezonde donoren, kankerpatiënten en in navelstrengbloed of een semikwantitatieve PCR voor de endotheel gerelateerde proteïnen CD31, CD144, CD146 en vWF een sneller en goedkoper alternatief zou kunnen zijn voor het kwantificeren van vaatschade. De hoofdstukken 7, 8, en 9 beschrijven of CEC ons al dan niet informeren over de aanwezigheid en ernst van vaatschade bij verschillende ziektes.

In **hoofdstuk 7** wordt de correlatie bestudeerd tussen CEC en een groot aantal traditionele en nieuwe serum- en plasmamerkers bij patiënten met sikkelcel anemie. Ook werd er gezocht naar een relatie tussen het aantal CEC en het aantal aangetaste organen bij deze patiënten.

**Hoofdstuk 8** beschrijft een gelijkaardig onderzoek bij preeclampsie.

In **hoofdstuk 9** wordt bij patiënten met hormoonrefractaire prostaatkanker onderzocht of het aantal ofwel de verandering in CEC, het aantal circulerende tumorcellen en serumconcentraties van de angiogene proteïnen Tissue Factor en Endotheline 1 van voorspellende waarde zijn voor een respons op behandeling met docetaxel.

**Hoofdstuk 10** geeft een overzicht van hoe de resultaten van dit proefschrift onderzoekers en artsen in de toekomst mogelijk verder kunnen helpen met het bestuderen van vaatschade.

# ABBREVIATIONS

| | |
|---|---|
| 7-AAD | 7-aminoactinomycin D |
| Ac-LDL | Acetylated low density lipoprotein |
| ACS | Acute chest syndrome |
| ADMA | Asymmetrical dimethylarginine |
| ALAT | Alanine aminotransferase |
| Ang-1 | Angiopoietin 1 |
| Ang-2 | Angiopoietin 2 |
| APC | Allophycocyanin |
| ASAT | Asparate aminotransferase |
| B7-H3 | Co stimulatory molecule B7, homologue 3 |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CEC | Circulating endothelial cells |
| CMOIC | Cells meeting our immunophenotypic criteria |
| DAPI | 4',6-diamidino-2-phenylindole |
| DBP | Diastolic blood pressure |
| Dil | 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate |
| DNA | Deoxyribonucleic acid |
| DRAQ5 | 1,5-bis {[2-(methylamino)ethyl]amino}-4,8-dihydroxy anthracene-9,10-dione |
| EDTA | Ethylenediaminetetraacetic acid |
| EPC | Endothelial progenitor cells |
| ELISA | Ezyme-linked immunosorbent assay |
| EM | Electron microscopy |
| F | Female |
| FCS | Fetal calf serum |
| FGF | Fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| Fli-1 | Friend leukemia virus integration 1 |
| Flt-1 | Fms-like tyrosine kinase 1 |
| FSC | Forward light scatter |
| GGT | Gamma glutamyl transpeptidase |
| HELLP | Hemolysis, elevated liver tests, low platelets |
| HUVEC | Human umbilical vein endothelial cells |
| ICAM-1 | Intercellular adhesion molecule 1 |
| IHC | Immunohistochemistry |
| ILP | Isolated limb perfusion |
| LDS-751 | Laser dye styryl-751 |
| M | Male |
| MA | Microalbuminuria |
| mAb | Monoclonal antibody |
| mL | Milliliter |

| | |
|---|---|
| MelCAM | Melanoma associated cellular adhesion molecule |
| MVD | Microvessel density |
| mRNA | Messenger ribosomal nucleic acid |
| NA | Not applicable |
| ng | Nanograms |
| NS | Not significant |
| PB | Peripheral blood |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PDGF | Platelet derived growth factor |
| PE | Phycoerythrin |
| PECAM-1 | Platelet/endothelial cell adhesion molecule 1 |
| PerCP | Peridinin-chlorophyll-protein Complex |
| pg | Picograms |
| PHT | Pulmonary hypertension |
| PI | Propidium iodide |
| PlGF | Placenta-like growth factor |
| PSI | Pounds per square inch |
| RET | Retinopathy |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SBP | Systolic blood pressure |
| SCD | Sickle cell disease |
| SDF-1 | Stromal cell derived factor 1 |
| SDMA | Symmetrical dimethylarginine |
| SSC | Sideward light scatter |
| TGF-β1 | Transforming growth factor β1 |
| TIE-2 | Tunica internal endothelial cell kinase 2 |
| TNF | Tumor necrosis factor α |
| UEA-1 | Ulex europaeus lectin 1 |
| ULC | Skin ulcera |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VDA | Vascular disrupting agents |
| VEGF | Vascular endothelial growth factor |
| VWF | Von Willebrand Factor |
| VWF:Ag | Von Willebrand Factor antigen |
| WF:RCo | Von Willebrand Factor ristocetin cofactor |

# REFERENCES USED IN THIS THESIS


1. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. Obstet Gynecol. 2002;99:159-167.
2. ACS. American Cancer Society: Cancer Statistics 2007 Presentation. Atlanta; 2008.
3. Agapitov AV, Haynes WG. Role of endothelin in cardiovascular disease. J Renin Angiotensin Aldosterone Syst. 2002;3:1-15.
4. Aird WC. Endothelium as an organ system. Crit Care Med. 2004;32:S271-279.
5. Alvarez DF, Huang L, King JA, ElZarrad MK, Yoder MC, Stevens T. Lung microvascular endothelium is enriched with progenitor cells that exhibit vasculogenic capacity. Am J Physiol Lung Cell Mol Physiol. 2008;294:L419-430.
6. Anargyrou K, Terpos E, Vassilakopoulos TP, et al. Normalization of the serum angiopoietin-1 to angiopoietin-2 ratio reflects response in refractory/resistant multiple myeloma patients treated with bortezomib. Haematologica. 2008;93:451-454.
7. Armstrong AJ, Garrett-Mayer ES, Yang YC, de Wit R, Tannock IF, Eisenberger M. A contemporary prognostic nomogram for men with hormone-refractory metastatic prostate cancer: a TAX327 study analysis. Clin Cancer Res. 2007;13:6396-6403.
8. Asakura H, Jokaji H, Saito M, et al. Plasma levels of soluble thrombomodulin increase in cases of disseminated intravascular coagulation with organ failure. Am J Hematol. 1991;38:281-287.
9. Asham E, Shankar A, Loizidou M, et al. Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. Br J Cancer. 2001;85:1759-1763.
10. Azen S, Xiang A, Lapuerta P, Ryutov A, Buckley J. Comparison of Predictive Accuracy of Neural Network Methods and
Cox Regression for Censored Survival Data.
11. Babich V, Meli A, Knipe L, et al. Selective release of molecules from Weibel-Palade bodies during a lingering kiss. Blood. 2008;111:5282-5290.
12. Bagnato A, Rosano L. The endothelin axis in cancer. Int J Biochem Cell Biol. 2008;40:1443-1451.
13. Bagnato A, Spinella F, Rosano L. The endothelin axis in cancer: the promise and the challenges of molecularly targeted therapy. Can J Physiol Pharmacol. 2008;86:473-484.
14. Bardin N, George F, Mutin M, et al. S-Endo 1, a pan-endothelial monoclonal antibody recognizing a novel human endothelial antigen. Tissue Antigens. 1996;48:531-539.
15. Bardin N, Moal V, Anfosso F, et al. Soluble CD146, a novel endothelial marker, is increased in physiopathological settings linked to endothelial junctional alteration. Thromb Haemost. 2003;90:915-920.
16. Baumann MU, Bersinger NA, Mohaupt MG, Raio L, Gerber S, Surbek DV. First-trimester serum levels of soluble endoglin and soluble fms-like tyrosine kinase-1 as first-trimester markers for late-onset preeclampsia. Am J Obstet Gynecol. 2008;199:266 e261-266.

17. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol. 2004;15:139-145.
18. 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.
19. Belcher JD, Bryant CJ, Nguyen J, et al. Transgenic sickle mice have vascular inflammation. Blood. 2003;101:3953-3959.
20. Benjamin RS, Choi H, Macapinlac HA, et al. We should desist using RECIST, at least in GIST. J Clin Oncol. 2007;25:1760-1764.
21. Berthold DR, Pond GR, de Wit R, Eisenberger M, Tannock IF. Survival and PSA response of patients in the TAX 327 study who crossed over to receive docetaxel after mitoxantrone or vice versa. Ann Oncol. 2008;19:1749-1753.
22. Bicknell R, Harris AL. Mechanisms and therapeutic implications of angiogenesis. Curr Opin Oncol. 1996;8:60-65.
23. Biguzzi E, Franchi F, Bucciarelli P, Colombo M, Romeo R. Endothelial protein C receptor plasma levels increase in chronic liver disease, while thrombomodulin plasma levels increase only in hepatocellular carcinoma. Thromb Res. 2007;120:289-293.
24. Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost. 2005;93:228-235.
25. Boehme MW, Galle P, Stremmel W. Kinetics of thrombomodulin release and endothelial cell injury by neutrophil-derived proteases and oxygen radicals. Immunology. 2002;107:340-349.
26. Boffa MC, Karmochkine M. Thrombomodulin: an overview and potential implications in vascular disorders. Lupus. 1998;7 Suppl 2:S120-125.
27. Bongers TN, de Maat MP, van Goor ML, et al. High von Willebrand factor levels increase the risk of first ischemic stroke: influence of ADAMTS13, inflammation, and genetic variability. Stroke. 2006;37:2672-2677.
28. Boos CJ, Lip GY, Blann AD. Circulating endothelial cells in cardiovascular disease. J Am Coll Cardiol. 2006;48:1538-1547.
29. Brown KA, Didion SP, Andresen JJ, Faraci FM. Effect of Aging, MnSOD Deficiency, and Genetic Background on Endothelial Function. Evidence for MnSOD Haploinsufficiency. Arterioscler Thromb Vasc Biol. 2007.
30. Buckstein R, Kerbel RS, Shaked Y, et al. High-Dose celecoxib and metronomic "low-dose" cyclophosphamide is an effective and safe therapy in patients with relapsed and refractory aggressive histology non-Hodgkin's lymphoma. Clin Cancer Res. 2006;12:5190-5198.
31. Bull TM, Golpon H, Hebbel RP, et al. Circulating endothelial cells in pulmonary hypertension. Thromb Haemost. 2003;90:698-703.
32. Canbakan B, Keven K, Tutkak H, Danisman N, Ergun I, Nergizoglu G. Circulating endothelial cells in preeclampsia. J Hum Hypertens. 2007;21:558-563.
33. Cao Y, Liu Q. Therapeutic targets of multiple angiogenic factors for the treatment of cancer and metastasis. Adv Cancer Res. 2007;97:203-224.
34. Celik I, Surucu O, Dietz C, et al. Therapeutic efficacy of endostatin exhibits a biphasic dose-response curve. Cancer Res. 2005;65:11044-11050.

35. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. N Engl J Med. 1995;332:1317-1322.
36. Clancy RM. Circulating endothelial cells and vascular injury in systemic lupus erythematosus. Curr Rheumatol Rep. 2000;2:39-43.
37. Cockell AP, Learmont JG, Smarason AK, Redman CW, Sargent IL, Poston L. Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. Br J Obstet Gynaecol. 1997;104:235-240.
38. Cohen SJ, Punt CJ, Iannotti N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol. 2008;26:3213-3221.
39. Corcoran TB, O'Shea A, Engel A, Shorten GD. The influence of propofol on P-selectin expression and nitric oxide production in re-oxygenated human umbilical vein endothelial cells. Acta Anaesthesiol Scand. 2006;50:348-354.
40. Cortelezzi A, Fracchiolla NS, Mazzeo LM, et al. Endothelial precursors and mature endothelial cells are increased in the peripheral blood of myelodysplastic syndromes. Leuk Lymphoma. 2005;46:1345-1351.
41. 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.
42. Curran SD, Muellner AU, Schwartz LH. Imaging response assessment in oncology. Cancer Imaging. 2006;6:S126-130.
43. 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.
44. de Jong S, Teerlink T. Analysis of asymmetric dimethylarginine in plasma by HPLC using a monolithic column. Anal Biochem. 2006;353:287-289.
45. Del Papa N, Colombo G, Fracchiolla N, et al. Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. Arthritis Rheum. 2004;50:1296-1304.
46. Desideri G, Ferri C. Endothelial activation. Sliding door to atherosclerosis. Curr Pharm Des. 2005;11:2163-2175.
47. di Tomaso E, Capen D, Haskell A, et al. Mosaic tumor vessels: cellular basis and ultrastructure of focal regions lacking endothelial cell markers. Cancer Res. 2005;65:5740-5749.
48. 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.
49. Dignat-George F, Sampol J, Lip G, Blann AD. Circulating endothelial cells: realities and promises in vascular disorders. Pathophysiol Haemost Thromb. 2003;33:495-499.
50. Ding S, Li C, Lin S, et al. Comparative evaluation of microvessel density determined by CD34 or CD105 in benign and malignant gastric lesions. Hum Pathol. 2006;37:861-866.
51. Ding YT, Kumar S, Yu DC. The role of endothelial progenitor cells in tumour vasculogenesis. Pathobiology. 2008;75:265-273.

52. Dixon GL, Heyderman RS, van der Ley P, Klein NJ. High-level endothelial E-selectin (CD62E) cell adhesion molecule expression by a lipopolysaccharide-deficient strain of Neisseria meningitidis despite poor activation of NF-kappaB transcription factor. Clin Exp Immunol. 2004;135:85-93.
53. Duda DG, Cohen KS, di Tomaso E, et al. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J Clin Oncol. 2006;24:1449-1453.
54. Duda DG, Cohen KS, Scadden DT, Jain RK. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc. 2007;2:805-810.
55. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. Faseb J. 2003;17:984-992.
56. Duits AJ, Rodriguez T, Schnog JJ. Serum levels of angiogenic factors indicate a pro-angiogenic state in adults with sickle cell disease. Br J Haematol. 2006;134:116-119.
57. Duits AJ, Schnog JB, Lard LR, Saleh AW, Rojer RA. Elevated IL-8 levels during sickle cell crisis. Eur J Haematol. 1998;61:302-305.
58. Ellis LM. Mechanisms of action of bevacizumab as a component of therapy for metastatic colorectal cancer. Semin Oncol. 2006;33:S1-7.
59. Elshal MF, Khan SS, Raghavachari N, et al. A unique population of effector memory lymphocytes identified by CD146 having a distinct immunophenotypic and genomic profile. BMC Immunol. 2007;8:29.
60. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 2005;106:2923-2924.
61. Ema M, Yokomizo T, Wakamatsu A, Terunuma T, Yamamoto M, Takahashi S. Primitive erythropoiesis from mesodermal precursors expressing VE-cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. Blood. 2006;108:4018-4024.
62. Erdbruegger U, Haubitz M, Woywodt A. Circulating endothelial cells: a novel marker of endothelial damage. Clin Chim Acta. 2006;373:17-26.
63. Escudier B, Eisen T, Stadler WM, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med. 2007;356:125-134.
64. Eskens FA, Verweij J. The clinical toxicity profile of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) targeting angiogenesis inhibitors; a review. Eur J Cancer. 2006;42:3127-3139.
65. Feldman DR, Motzer RJ. Novel targets and therapies for metastatic renal cell carcinoma. Oncology (Williston Park). 2006;20:1745-1753; discussion 1756.
66. Flaherty KT. Sorafenib in renal cell carcinoma. Clin Cancer Res. 2007;13:747s-752s.
67. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285:1182-1186.
68. Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. Ann Surg. 1972;175:409-416.
69. Folkman J. Tumor angiogensis: role in regulation of tumor growth. Symp Soc Dev Biol. 1974;30:43-52.

70. Folkman J. Angiogenesis--retrospect and outlook. Exs. 1992;61:4-13.
71. Fonsatti E, Sigalotti L, Arslan P, Altomonte M, Maio M. Emerging role of endoglin (CD105) as a marker of angiogenesis with clinical potential in human malignancies. Curr Cancer Drug Targets. 2003;3:427-432.
72. 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.
73. Furstenberger G, von Moos R, Senn HJ, Boneberg EM. Real-time PCR of CD146 mRNA in peripheral blood enables the relative quantification of circulating endothelial cells and is an indicator of angiogenesis. Br J Cancer. 2005;93:793-798.
74. Garlanda C, Dejana E. Heterogeneity of endothelial cells. Specific markers. Arterioscler Thromb Vasc Biol. 1997;17:1193-1202.
75. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol. 2006;8:1223-1234.
76. George DJ, Halabi S, Shepard TF, et al. Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. Clin Cancer Res. 2001;7:1932-1936.
77. George DJ, Regan MM, Oh WK, et al. Radical prostatectomy lowers plasma vascular endothelial growth factor levels in patients with prostate cancer. Urology. 2004;63:327-332.
78. George F, Brisson C, Poncelet P, et al. Rapid isolation of human endothelial cells from whole blood using S-Endo1 monoclonal antibody coupled to immuno-magnetic beads: demonstration of endothelial injury after angioplasty. Thromb Haemost. 1992;67:147-153.
79. George F, Poncelet P, Laurent JC, et al. Cytofluorometric detection of human endothelial cells in whole blood using S-Endo 1 monoclonal antibody. J Immunol Methods. 1991;139:65-75.
80. Georgiou HD, Namdarian B, Corcoran NM, Costello AJ, Hovens CM. Circulating endothelial cells as biomarkers of prostate cancer. Nat Clin Pract Urol. 2008;5:445-454.
81. Gerber HP, Ferrara N. Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. Cancer Res. 2005;65:671-680.
82. Gerdes VE, Kremer Hovinga JA, Ten Cate H, Brandjes DP, Buller HR. Soluble thrombomodulin in patients with established atherosclerosis. J Thromb Haemost. 2004;2:200-201.
83. Gilbert JS, Ryan MJ, LaMarca BB, Sedeek M, Murphy SR, Granger JP. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. Am J Physiol Heart Circ Physiol. 2008;294:H541-550.
84. Glowinska-Olszewska B, Urban M, Tolwinska J, Peczynska J, Florys B. [Correlation analysis between biochemical and biophysical markers of endothelium damage in children with diabetes type 1]. Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw. 2005;11:221-227.

85. Goessens BM, van der Graaf Y, Olijhoek JK, Visseren FL. The course of vascular risk factors and the occurrence of vascular events in patients with symptomatic peripheral arterial disease. J Vasc Surg. 2007;45:47-54.
86. Goon PK, Boos CJ, Lip GY. Circulating endothelial cells: markers of vascular dysfunction. Clin Lab. 2005;51:531-538.
87. 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.
88. Gouault-Helimann M, Josso F. [Initiation in vivo of blood coagulation. The role of white blood cells and tissue factor (author's transl)]. Nouv Presse Med. 1979;8:3249-3253.
89. Gougos A, St Jacques S, Greaves A, et al. Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukemic cells, and syncytiotrophoblasts. Int Immunol. 1992;4:83-92.
90. Grant DS, Williams TL, Zahaczewsky M, Dicker AP. Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). Int J Cancer. 2003;104:121-129.
91. Gross HJ, Verwer B, Houck D, Recktenwald D. Detection of rare cells at a frequency of one per million by flow cytometry. Cytometry B Clin Cytom. 1993;14:519-526.
92. Grundmann M, Woywodt A, Kirsch T, et al. Circulating endothelial cells: a marker of vascular damage in patients with preeclampsia. Am J Obstet Gynecol. 2008;198:317 e311-315.
93. Haider S, Knofler M. Human tumour necrosis factor: physiological and pathological roles in placenta and endometrium. Placenta. 2009;30:111-123.
94. Halabi S, Small EJ, Kantoff PW, et al. Prognostic model for predicting survival in men with hormone-refractory metastatic prostate cancer. J Clin Oncol. 2003;21:1232-1237.
95. Hamberg P, Verhagen PC, de Wit R. When to start cytotoxic therapy in asymptomatic patients with hormone refractory prostate cancer? Eur J Cancer. 2008;44:1193-1197.
96. Haubitz M, Woywodt A. Circulating endothelial cells and vasculitis. Intern Med. 2004;43:660-667.
97. Hauser PV, Collino F, Bussolati B, Camussi G. Nephrin and endothelial injury. Curr Opin Nephrol Hypertens. 2009;18:3-8.
98. Hinnen P, Eskens FA. Vascular disrupting agents in clinical development. Br J Cancer. 2007;96:1159-1165.
99. Hofmann S, Grasberger H, Jung P, et al. The tumour necrosis factor-alpha induced vascular permeability is associated with a reduction of VE-cadherin expression. Eur J Med Res. 2002;7:171-176.
100. Hotchkiss KA, Ashton AW, Mahmood R, Russell RG, Sparano JA, Schwartz EL. Inhibition of endothelial cell function in vitro and angiogenesis in vivo by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol Cancer Ther. 2002;1:1191-1200.

101. Hrachovinova I, Cambien B, Hafezi-Moghadam A, et al. Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. Nat Med. 2003;9:1020-1025.
102. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med. 2004;350:2335-2342.
103. Ishii H, Uchiyama H, Kazama M. Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. Thromb Haemost. 1991;65:618-623.
104. 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.
105. Jain RK. Antiangiogenic therapy for cancer: current and emerging concepts. Oncology (Williston Park). 2005;19:7-16.
106. Jerkic M, Rivas-Elena JV, Prieto M, et al. Endoglin regulates nitric oxide-dependent vasodilatation. Faseb J. 2004;18:609-611.
107. Johnston MG, Hay JB, Movat HZ. The modulation of enhanced vascular permeability by prostaglandins through alterations in blood flow (hyperemia). Agents Actions. 1976;6:705-711.
108. Joras M, Poredos P, Fras Z. Endothelial dysfunction in Buerger's disease and its relation to markers of inflammation. Eur J Clin Invest. 2006;36:376-382.
109. Kaaja R. Predictors and risk factors of pre-eclampsia. Minerva Ginecol. 2008;60:421-429.
110. Kabbinavar F, Hurwitz HI, Fehrenbacher L, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol. 2003;21:60-65.
111. Kamat AA, Kim TJ, Landen CN, Jr., et al. Metronomic chemotherapy enhances the efficacy of antivascular therapy in ovarian cancer. Cancer Res. 2007;67:281-288.
112. Keeney M, Gratama JW, Chin-Yee IH, Sutherland DR. Isotype controls in the analysis of lymphocytes and CD34+ stem and progenitor cells by flow cytometry--time to let go! Cytometry B Clin Cytom. 1998;34:280-283.
113. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. Lancet. 2006;367:1066-1074.
114. 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.
115. Kimura K, Hashiguchi T, Deguchi T, et al. Serum VEGF-As a prognostic factor of atherosclerosis. Atherosclerosis. 2006.
116. Kohandel M, Kardar M, Milosevic M, Sivaloganathan S. Dynamics of tumor growth and combination of anti-angiogenic and cytotoxic therapies. Phys Med Biol. 2007;52:3665-3677.
117. Kozuka K, Kohriyama T, Nomura E, Ikeda J, Kajikawa H, Nakamura S. Endothelial markers and adhesion molecules in acute ischemic stroke--sequential change and differences in stroke subtype. Atherosclerosis. 2002;161:161-168.

118. 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;17:223-233.
119. Kramer I, Lipp HP. Bevacizumab, a humanized anti-angiogenic monoclonal antibody for the treatment of colorectal cancer. J Clin Pharm Ther. 2007;32:1-14.
120. Lalich M, McNeel DG, Wilding G, Liu G. Endothelin receptor antagonists in cancer therapy. Cancer Invest. 2007;25:785-794.
121. Lechner D, Kollars M, Gleiss A, Kyrle PA, Weltermann A. Chemotherapy-induced thrombin generation via procoagulant endothelial microparticles is independent of tissue factor activity. J Thromb Haemost. 2007.
122. Li C, Guo B, Wilson PB, et al. Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. Int J Cancer. 2000;89:122-126.
123. Lin SM, Wang YM, Lin HC, et al. Serum thrombomodulin level relates to the clinical course of disseminated intravascular coagulation, multiorgan dysfunction syndrome, and mortality in patients with sepsis. Crit Care Med. 2008;36:683-689.
124. Maio M, Altomonte M, Fonsatti E. Is it the primetime for endoglin (CD105) in the clinical setting? Cardiovasc Res. 2006;69:781-783.
125. Malyszko J, Malyszko JS, Mysliwiec M. Endothelial cell injury markers in chronic renal failure on conservative treatment and continuous ambulatory peritoneal dialysis. Kidney Blood Press Res. 2004;27:71-77.
126. 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.
127. 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.
128. Mancuso P, Rabascio C, Bertolini F. Strategies to investigate circulating endothelial cells in cancer. Pathophysiol Haemost Thromb. 2003;33:503-506.
129. Marioni G, Ottaviano G, Giacomelli L, et al. CD105-assessed micro-vessel density is associated with malignancy recurrence in laryngeal squamous cell carcinoma. Eur J Surg Oncol. 2006;32:1149-1153.
130. Martin TA, Watkins G, Lane J, Jiang WG. Assessing microvessels and angiogenesis in human breast cancer, using VE-cadherin. Histopathology. 2005;46:422-430.
131. Maynard SE, Min JY, Merchan J, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003;111:649-658.
132. McEver RP. Adhesive interactions of leukocytes, platelets, and the vessel wall during hemostasis and inflammation. Thromb Haemost. 2001;86:746-756.
133. McMaster MT, Zhou Y, Fisher SJ. Abnormal placentation and the syndrome of preeclampsia. Semin Nephrol. 2004;24:540-547.
134. Meinardi MT, Gietema JA, van der Graaf WT, et al. Cardiovascular morbidity in long-term survivors of metastatic testicular cancer. J Clin Oncol. 2000;18:1725-1732.
135. Menon C, Ghartey A, Canter R, Feldman M, Fraker DL. Tumor necrosis factor-alpha damages tumor blood vessel integrity by targeting VE-cadherin. Ann Surg. 2006;244:781-791.

136. Mezentsev A, Merks RM, O'Riordan E, et al. Endothelial microparticles affect angiogenesis in vitro: role of oxidative stress. Am J Physiol Heart Circ Physiol. 2005;289:H1106-1114.
137. Miller KD. E2100: a phase III trial of paclitaxel versus paclitaxel/bevacizumab for metastatic breast cancer. Clin Breast Cancer. 2003;3:421-422.
138. Moroni G, Del Papa N, Moronetti LM, et al. Increased levels of circulating endothelial cells in chronic periaortitis as a marker of active disease. Kidney Int. 2005;68:562-568.
139. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med. 2007;356:115-124.
140. Muller-Delp JM. Aging-induced adaptations of microvascular reactivity. Microcirculation. 2006;13:301-314.
141. Munoz R, Shaked Y, Bertolini F, Emmenegger U, Man S, Kerbel RS. Anti-angiogenic treatment of breast cancer using metronomic low-dose chemotherapy. Breast. 2005;14:466-479.
142. 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.
143. Myatt L, Webster RP. Vascular biology of preeclampsia. J Thromb Haemost. 2009;7:375-384.
144. Nadar SK, Al Yemeni E, Blann AD, Lip GY. Thrombomodulin, von Willebrand factor and E-selectin as plasma markers of endothelial damage/dysfunction and activation in pregnancy induced hypertension. Thromb Res. 2004;113:123-128.
145. 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.
146. Ngo CV, Picha K, McCabe F, et al. CNTO 859, a humanized anti-tissue factor monoclonal antibody, is a potent inhibitor of breast cancer metastasis and tumor growth in xenograft models. Int J Cancer. 2007;120:1261-1267.
147. Nieda M, Nicol A, Denning-Kendall P, Sweetenham J, Bradley B, Hows J. Endothelial cell precursors are normal components of human umbilical cord blood. Br J Haematol. 1997;98:775-777.
148. 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.
149. Nuver J, Smit AJ, Sleijfer DT, et al. Microalbuminuria, decreased fibrinolysis, and inflammation as early signs of atherosclerosis in long-term survivors of disseminated testicular cancer. Eur J Cancer. 2004;40:701-706.
150. 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.
151. Offersen BV, Borre M, Overgaard J. Quantification of angiogenesis as a prognostic marker in human carcinomas: a critical evaluation of histopathological methods for estimation of vascular density. Eur J Cancer. 2003;39:881-890.
152. Ohlin AK, Larsson K, Hansson M. Soluble thrombomodulin activity and soluble thrombomodulin antigen in plasma. J Thromb Haemost. 2005;3:976-982.

153. Olmos D, Arkenau HT, Ang JE, et al. Circulating tumour cell (CTC) counts as intermediate end points in castration-resistant prostate cancer (CRPC): a single-centre experience. Ann Oncol. 2009;20:27-33.
154. Ozdogu H, Sozer O, Boga C, Kozanoglu L, Maytalman E, Guzey M. Flow cytometric evaluation of circulating endothelial cells: a new protocol for identifying endothelial cells at several stages of differentiation. Am J Hematol. 2007;82:706-711.
155. Panorchan P, George JP, Wirtz D. Probing intercellular interactions between vascular endothelial cadherin pairs at single-molecule resolution and in living cells. J Mol Biol. 2006;358:665-674.
156. Pastorino F, Brignole C, Marimpietri D, et al. Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy. Cancer Res. 2003;63:7400-7409.
157. Patterson DM, Rustin GJ. Vascular damaging agents. Clin Oncol (R Coll Radiol). 2007;19:443-456.
158. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med. 1994;330:1639-1644.
159. Pluda JM. Tumor-associated angiogenesis: mechanisms, clinical implications, and therapeutic strategies. Semin Oncol. 1997;24:203-218.
160. Pries AR, Kuebler WM. Normal endothelium. Handb Exp Pharmacol. 2006:1-40.
161. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. J Histochem Cytochem. 2006;54:385-395.
162. Quilici J, Banzet N, Paule P, et al. Circulating endothelial cell count as a diagnostic marker for non-ST-elevation acute coronary syndromes. Circulation. 2004;110:1586-1591.
163. 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.
164. Rademaker-Lakhai JM, Beerepoot LV, Mehra N, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral protein kinase C beta-inhibitor enzastaurin in combination with gemcitabine and cisplatin in patients with advanced cancer. Clin Cancer Res. 2007;13:4474-4481.
165. Rana S, Karumanchi SA, Levine RJ, et al. Sequential changes in antiangiogenic factors in early pregnancy and risk of developing preeclampsia. Hypertension. 2007;50:137-142.
166. Ratain MJ, Eckhardt SG. Phase II studies of modern drugs directed against new targets: if you are fazed, too, then resist RECIST. J Clin Oncol. 2004;22:4442-4445.
167. Rini BI, Rathmell WK. Biological aspects and binding strategies of vascular endothelial growth factor in renal cell carcinoma. Clin Cancer Res. 2007;13:741s-746s.
168. Romani AA, Borghetti AF, Del Rio P, Sianesi M, Soliani P. The risk of developing metastatic disease in colorectal cancer is related to CD105-positive vessel count. J Surg Oncol. 2006;93:446-455.
169. Rosenblatt JI, Hokanson JA, McLaughlin SR, Leary JF. Theoretical basis for sampling statistics useful for detecting and isolating rare cells using flow cytometry and cell sorting. Cytometry B Clin Cytom. 1997;27:233-238.

170. 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.
171. Salani D, Rosano L, Di Castro V, et al. ABT-627, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma growth in vitro. Clin Sci (Lond). 2002;103 Suppl 48:318S-321S.
172. Salmaggi A, Eoli M, Frigerio S, Ciusani E, Silvani A, Boiardi A. Circulating intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and plasma thrombomodulin levels in glioblastoma patients. Cancer Lett. 1999;146:169-172.
173. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006;355:2542-2550.
174. Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J Cell Sci. 2005;118:771-780.
175. 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.
176. Schimming R, Hunter NR, Mason KA, Milas L. [Inhibition of tumor neo-angiogenesis and induction of apoptosis as properties of docetaxel (taxotere)]. Mund Kiefer Gesichtschir. 1999;3:210-212.
177. Schnog JB, Kater AP, Mac Gillavry MR, et al. Low adjusted-dose acenocoumarol therapy in sickle cell disease: a pilot study. Am J Hematol. 2001;68:179-183.
178. Schnog JB, Mac Gillavry MR, van Zanten AP, et al. Protein C and S and inflammation in sickle cell disease. Am J Hematol. 2004;76:26-32.
179. Schnog JB, Rojer RA, Mac Gillavry MR, Ten Cate H, Brandjes DP, Duits AJ. Steady-state sVCAM-1 serum levels in adults with sickle cell disease. Ann Hematol. 2003;82:109-113.
180. Schnog JJ, Hovinga JA, Krieg S, et al. ADAMTS13 activity in sickle cell disease. Am J Hematol. 2006;81:492-498.
181. Schnog JJ, Lard LR, Rojer RA, Van der Dijs FP, Muskiet FA, Duits AJ. New concepts in assessing sickle cell disease severity. Am J Hematol. 1998;58:61-66.
182. Schrage WG, Eisenach JH, Joyner MJ. Ageing reduces nitric-oxide- and prostaglandin-mediated vasodilatation in exercising humans. J Physiol. 2007;579:227-236.
183. Schuch G, Heymach JV, Nomi M, et al. Endostatin inhibits the vascular endothelial growth factor-induced mobilization of endothelial progenitor cells. Cancer Res. 2003;63:8345-8350.
184. 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.
185. Serjeant GR. The emerging understanding of sickle cell disease. Br J Haematol. 2001;112:3-18.
186. Seynhaeve AL, Eggermont AM, ten Hagen TL. TNF and manipulation of the tumor cell-stromal interface: "ways to make chemotherapy effective". Front Biosci. 2008;13:3034-3045.

187. Shankar A, Loizidou M, Aliev G, et al. Raised endothelin 1 levels in patients with colorectal liver metastases. Br J Surg. 1998;85:502-506.
188. Shantsila E, Blann AD, Lip GY. Circulating endothelial cells: from bench to clinical practice. J Thromb Haemost. 2008;6:865-868.
189. Shchors K, Evan G. Tumor angiogenesis: cause or consequence of cancer? Cancer Res. 2007;67:7059-7061.
190. Shih IM. The role of CD146 (Mel-CAM) in biology and pathology. J Pathol. 1999;189:4-11.
191. 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.
192. 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.
193. 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.
194. Spinella F, Rosano L, Di Castro V, Natali PG, Bagnato A. Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. J Biol Chem. 2002;277:27850-27855.
195. St-Jacques S, Forte M, Lye SJ, Letarte M. Localization of endoglin, a transforming growth factor-beta binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. Biol Reprod. 1994;51:405-413.
196. 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.
197. 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.
198. Strijbos MH, Kraan J, Lamers CHJ, Sleijfer S, Gratama JW. Quantification of circulating endothelial cells by flow cytometry. Clin Cancer Res. 2009;In press.
199. Strijbos MH, Rao C, Schmitz PI, et al. Correlation between circulating endothelial cell counts and plasma thrombomodulin levels as markers for endothelial damage. Thromb Haemost. 2008;100:642-647.
200. Svatek RS, Karam JA, Roehrborn CG, Karakiewicz PI, Slawin KM, Shariat SF. Preoperative plasma endoglin levels predict biochemical progression after radical prostatectomy. Clin Cancer Res. 2008;14:3362-3366.
201. Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med. 2004;351:1502-1512.
202. Targher G, Bertolini L, Zoppini G, Zenari L, Falezza G. Increased plasma markers of inflammation and endothelial dysfunction and their association with microvascular complications in Type 1 diabetic patients without clinically manifest macroangiopathy. Diabet Med. 2005;22:999-1004.
203. Teerlink T. Determination of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine in biological samples by HPLC. Methods Mol Med. 2005;108:263-274.

204. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst. 2000;92:205-216.
205. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. J Immunol Methods. 2007.
206. Torsney E, Charlton R, Parums D, Collis M, Arthur HM. Inducible expression of human endoglin during inflammation and wound healing in vivo. Inflamm Res. 2002;51:464-470.
207. Tschudi MR, Barton M, Bersinger NA, et al. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. J Clin Invest. 1996;98:899-905.
208. Urban M, Wojtkielewicz K, Glowinska B, Peczynska J. [Soluble thrombomodulin--a molecular marker of endothelial cell injury in children and adolescents with obesity]. Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw. 2005;11:73-77.
209. Van De Wiele C, Oltenfreiter R, De Winter O, Signore A, Slegers G, Dierckx RA. Tumour angiogenesis pathways: related clinical issues and implications for nuclear medicine imaging. Eur J Nucl Med Mol Imaging. 2002;29:699-709.
210. Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med. 2006;12:642-649.
211. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J Cell Biol. 1984;99:2034-2040.
212. Wallez Y, Vilgrain I, Huber P. Angiogenesis: the VE-cadherin switch. Trends Cardiovasc Med. 2006;16:55-59.
213. Walsh DA. Pathophysiological mechanisms of angiogenesis. Adv Clin Chem. 2007;44:187-221.
214. Wang HY, Yang YM, Zhuang Y, Chen HN, Wan YL, Huang YT. The effect of celecoxib on tissue factor expression in pancreatic cancer cells. Chin Med J (Engl). 2007;120:1753-1756.
215. Wang Y, Klijn JG, Zhang Y, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet. 2005;365:671-679.
216. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol. 1993;143:401-409.
217. Widemann A, Sabatier F, Arnaud L, et al. CD146-based immunomagnetic enrichment followed by multi-parameter flow cytometry: a new approach to count circulating endothelial cells. J Thromb Haemost. 2008.
218. 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.
219. Woywodt A, Goldberg C, Scheer J, Regelsberger H, Haller H, Haubitz M. An improved assay for enumeration of circulating endothelial cells. Ann Hematol. 2004;83:491-494.

220. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis. Lancet. 2003;361:206-210.
221. Wright TJ, Leach L, Shaw PE, Jones P. Dynamics of vascular endothelial-cadherin and beta-catenin localization by vascular endothelial growth factor-induced angiogenesis in human umbilical vein cells. Exp Cell Res. 2002;280:159-168.
222. Wulfing P, Kersting C, Tio J, et al. Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. Clin Cancer Res. 2004;10:2393-2400.
223. Xu GB, Luo LH, Lu XG. [Detection of thrombomodulin in both plasma and tissue extracts of cancer patients and its clinical significance]. Zhejiang Da Xue Xue Bao Yi Xue Ban. 2003;32:141-144.
224. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med. 2003;349:427-434.
225. Yao G, Liu ZH, Zheng C, et al. Evaluation of renal vascular lesions using circulating endothelial cells in patients with lupus nephritis. Rheumatology (Oxford). 2008;47:432-436.
226. Yipp BG, Hickey MJ, Andonegui G, et al. Differential roles of CD36, ICAM-1, and P-selectin in Plasmodium falciparum cytoadherence in vivo. Microcirculation. 2007;14:593-602.
227. Yu J, May L, Milsom C, et al. Contribution of host-derived tissue factor to tumor neovascularization. Arterioscler Thromb Vasc Biol. 2008;28:1975-1981.
228. Zhang H, Park Y, Wu J, et al. Role of TNF-alpha in vascular dysfunction. Clin Sci (Lond). 2009;116:219-230.
229. Zhang SJ, Zhang H, Wei YJ, et al. Adult endothelial progenitor cells from human peripheral blood maintain monocyte/macrophage function throughout in vitro culture. Cell Res. 2006;16:577-584.
230. Zheng PP, Hop WC, Luider TM, Sillevis Smitt PA, Kros JM. Increased levels of circulating endothelial progenitor cells and circulating endothelial nitric oxide synthase in patients with gliomas. Ann Neurol. 2007.
231. Zhu Y, Sun Y, Xie L, Jin K, Sheibani N, Greenberg DA. Hypoxic induction of endoglin via mitogen-activated protein kinases in mouse brain microvascular endothelial cells. Stroke. 2003;34:2483-2488.
232. Zondor SD, Medina PJ. Bevacizumab: an angiogenesis inhibitor with efficacy in colorectal and other malignancies. Ann Pharmacother. 2004;38:1258-1264.
233. Zoppini G, Targher G, Zamboni C, et al. Effects of moderate-intensity exercise training on plasma biomarkers of inflammation and endothelial dysfunction in older patients with type 2 diabetes. Nutr Metab Cardiovasc Dis. 2006;16:543-549.

# PUBLICATIONS BASED ON THE STUDIES IN THIS THESIS

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

Circulating endothelial cells in oncology: pitfalls and promises.
Strijbos MH, Gratama JW, Kraan J, Lamers CH, den Bakker MA, Sleijfer S.
Br J Cancer. 2008 Jun 3;98(11):1731-5. Epub 2008 May 27. Review.

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

Circulating endothelial cells: a potential parameter of organ damage in sickle cell anemia? Strijbos MH, Landburg PP, Nur E, Teerlink T, Leebeek FW, Rijneveld AW, Biemond BJ, Sleijfer S, Gratama JW, Duits AJ, Schnog JJ; CURAMA study group. Blood Cells Mol Dis. 2009 Jul-Aug;43(1):63-7. Epub 2009 Apr 7.

On The Origin of (CD105+) Circulating Endothelial Cells. Strijbos MH, Verhoef C, Gratama JW, Sleijfer S. Thromb Haemost. 2009 Aug;102(2):347-51.

Peripheral Blood mRNA Levels Of The Endothelium-Associated Markers CD31, CD144, CD146 And Von Willebrand Factor Do Not Serve As Surrogate Markers For Circulating Endothelial Cells. Strijbos MH, van Krimpen BA, Debets JE, Kraan J, Sleijfer S, Gratama JW, Lamers CHJ. *Submitted for publication.*

Levels Of Circulating Endothelial Cells In Normotensive And Preeclamptic Pregnancies. Strijbos MH, Snijder CA, Lamers CHJ, Gratama JW, Duvekot JJ. *Submitted for publication.*

Early Changes In Circulating Endothelial Cells, Circulating Tumour Cells And Serum Levels Of Tissue Factor, In Particular Combined, Predict Overall Survival In Castration Resistant Prostate Cancer Treated With Docetaxel. Strijbos MH, Gratama JW, Schmitz PIM, Rao C, Onstenk W, Doyle GV, Miller C, de Wit R, Terstappen LWMM, Sleijfer S. *Submitted for publication.*

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# CURRICULUM VITAE

De schrijver van dit proefschrift werd op 14 april 1976 geboren in Weert. In 1995 behaalde hij het VWO diploma aan de Philips van Horne Scholengemeenschap te Weert. In 1996 begon hij aan de kandidaturen van de studie geneeskunde aan het Limburgs Universitair Centrum te Diepenbeek, België. De doctoraatsfase werd gevolgd aan de Katholieke Universiteit Leuven, de coschappen aan de Erasmus Universiteit Rotterdam. Na het behalen van het artsendiploma in april 2005, volgde aansluitend het onderzoek in het Erasmus MC – Daniel den Hoed Kliniek. Dit proefschrift is hiervan het resultaat. In 2009 startte hij met de opleiding tot internist aan het Universitair Ziekenhuis Gent. Momenteel is hij werkzaam als arts-assistent inwendige ziekten in het Jan Palfijn Ziekenhuis te Merksem, onder supervisie van dr. Jan Vanwelden.