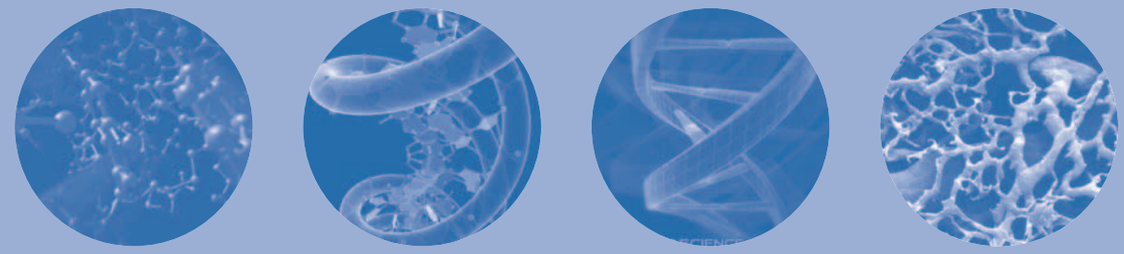
Novel biomarkers for cancer detection and prognostication



Novel biomarkers for cancer detection and prognostication Niven Mehra

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Novel biomarkers for cancer detection and prognostication

Nieuwe biomarkers voor de detectie van tumoren en voorspelling
van het verloop van de ziekte
(met een samenvatting in het Nederlands)

Proefschrift

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General introduction

The need for cancer biomarkers for treatment selection and surrogate endpoints

Effectivity of cytotoxic chemotherapeutic treatment regiments has historically been defined on select cancer patient populations, and endpoints have generally been based on tumor shrinkage by imaging techniques, pathological and clinical evaluation. However with recent developments in genomic technology, cancer biology and the introduction of targeted therapies, patient selection and endpoints require modification. A new era in the field of oncology is in progress based on prospective hypothesis driven translational studies, in which the presence of molecular biomarkers determines patient treatment eligibility, and the kinetics of these molecular biomarker may serve as surrogate endpoints.

A biomarker is a substance present in a tumor, or produced by the tumor and host, that can be used for differentiating neoplastic from normal tissue based on measurements in body fluids, secretions, cells, and/or tissues. Most commonly, a biomarker is thought of as a biologic measurement that represents the disease quantitatively or in activity, which goes up when the disease progresses or relapses, and goes down when the disease is into remission. Of vital importance for this biomarker, is that (the kinetics of) this substance is more easily measured, more quickly observed, and demonstrates enhanced sensitivity or specificity over established clinical decision tools.

Another type of biomarker is a marker for treatment selection. Most cancer treatments benefit only a minority of the patients to whom they are administered. Improved molecular tools are needed to select treatment modalities for individual patients based on their molecular tumor patterns, to select optimal therapeutic interventions. Being able to predict which patient is most likely to benefit from a certain (targeted) treatments would not only save this patient from unnecessary toxicity and inconvenience and thus increase quality of life, but also reduce over-treatment and decrease the exorbitant health care costs within the oncological setting.

Prognostic biomarkers may be considered in the clinical management of a patient, to be used as decision aids in determining whether a patient should receive adjuvant treatment modalities or how aggressive that therapy should be. Predictive biomarkers are generally used to make more specific choices between different treatment options.

Although there is a large quantity of literature on prognostic and predictive factors, there is still a lack of validated molecular markers for measuring biological activity which is needed to help oncologist's decision making to include patients for targeted pathways. In case of many angiogenesis inhibitors, such as Bevacizumab, or vascular disrupting agents (VDA) for which the exact therapeutic target is known, predictive biomarkers needed to establish eligibility for patients or surrogate markers of treatment efficacy have to thus far not been validated. The clinical testing of these agents is currently hampered by the lack of robust biomarkers of biological activity.

The development of a non-invasive biomarker that has robust diagnostic, predictive or prognostic value is the Holy Grail of clinical research, and the basis for translational research performed in this thesis.

Outline of this thesis

The focus of research in this thesis is the development and validation of prognostic and predictive biomarkers that may aid the oncologist in patient selection and which may serve as surrogate markers of treatment efficacy and treatment outcome. The main objective was that these biomarkers were to be non-invasive, and easily measured in the peripheral blood of cancer patient. To achieve material for study, we collected peripheral blood of patients visiting the outpatient ward of the Department of Oncology and patients enrolled in clinical study programs, and stored patient plasma, peripheral blood cells and RNA/DNA in a blood bank together with clinical patient information, including established endpoints and tumor markers. This blood bank contained a myriad of possible biomarkers for hypothesis-driven research; peripheral blood mononuclear cells, circulating endothelial cells, proteins and nucleic acids.

In this thesis we used a variety of approaches for biomarker discovery; in **Part I** we assessed whether we could identify a non-invasive surrogate markers of angiogenesis, as new vessel formation plays critical roles in the growth and metastatic spread of tumors. Moreover, many agents targeting the vascular endothelial growth factor (VEGF) pathway are currently being evaluated in clinical trials, however robust surrogate endpoints of biological activity or biomarkers for patient selection are still lacking for this assessment. Circulating endothelial cells shed from the angiogenic tumor microenvironment (Chapter 3), and endothelial progenitor cells mobilized by the tumor from the bone-marrow (Chapter 4) are evaluated as surrogate markers of angiogenesis and are studied further as marker for the assessment of optimal biological dose and surrogate endpoint during treatment with phosphokinase C-inhibitor Enzastaurin, an anti-angiogenic targeting agent in combination with gemcitabine and cisplatin based chemotherapy (Chapter 5).

In **Part II** we addressed the question whether extracellular nucleic acids present in the plasma of patients with cancer, could be quantified using real-time amplification for use as prognostic biomarker (reviewed in Chapter 7). These circulating nucleic acids are largely derived by necrosis or apoptosis of the tumor. Most studies have evaluated whether these circulating nucleic acids could be used as diagnostic marker, however, as this biomarker likely reflects tumor activity we investigated whether circulating nucleic acid may serve as a prognostic biomarker. Every tumor cell that releases nucleic acids (due to necrosis or apoptosis) will have multiple gene copies of mitochondrial genes compared to genomic genes, as cells contain hundreds of mitochondria compared to one nucleus. Therefore we addressed whether mitochondrial gene amplification had elevated sensitivity and specificity as biomarker compared to genomic nucleic acids. We collected plasma from patients with advanced prostate caner and patients with benign prostate hyperplasia and assessed the strength of mitochondrial nucleic acids as prognostic biomarker compared to established prognostic variables such as prostate specific antigen (PSA) and hemoglobin count (Chapter 8), and as pan-tumor marker in patients with prostate, head and neck, kidney and colorectal cancer (Chapter 9). Due to discrepancies in the literature on applicability of circulating nucleic acids as prognostic or predictive factor, we demonstrate that methodology, sample preparation and storage strongly affect results (Chapter 10).

In **Part III** we use a different approach of "discovery-based research" analyzing proteomic

expression patterns in serum of renal cancer patients (introduction in Chapter 11). Surface Enhanced Desorption Ionisation-Time of Flight Mass Spectrometry (SELDI-TOF MS) has resulted in a serum proteome wide search of expression signatures for cancer diagnostics in a variety of cancers such as ovarian, breast, prostate, colorectal cancer. However, promising at first proteomics is criticized because of the lack of reproducibility between institutes and research groups. Therefore we first investigated the reproducibility of SELDI-TOF MS in two Dutch institutes using identical assay procedures of two previous published studies on patients with renal cell carcinoma (Chapter 12). Next, we investigated whether we could identify a serum expression signature that could predict patient outcome prior to anti-tumor treatment, and which was an independent predictor when compared to the gold standard of survival prediction, the extended Memorial Sloan-Kettering Prognostic Factors Model for survival (Chapter 13).

This thesis is framed by a general discussion on the three chapters, by means of a debate on a number of controversies in the literature concerning this thesis (Chapter 14).

PART 1 - Angiogenesis

INTRODUCTION:

Circulating endothelial (progenitor) cells as surrogate markers of tumor angiogenesis, vascular damage and optimal dose assessment for anti-vascular agents

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Abstract

Targeting tumor growth by angiogenesis inhibitors and vascular-disrupting agents represents a revolutionary modification for cancer treatment. Since the first approval of the targeting antibody against the angiogenic cytokine VEGF bevacizumab, approval of sunitinib and sorafenib many clinical trials with numerous anti-angiogenic agents are ongoing. However, there is still a dire need for surrogate markers of biological activity, as the optimal biological activity of these drugs on their targeted pathways cannot be assessed by maximum tolerable dose (MTD), for their mode of effect is expected to be primarily cytostatic rather than cytotoxic. In this introduction we give a brief overview of the range of surrogate markers, which can be used at present or in the near future to aid the oncologists in pruning and redefining optimal dosing of anti-angiogenic agents in patients. We will focus more closely on circulating endothelial (progenitor) cells as surrogate marker of angiogenesis, treatment response and outcome, as this modality is the subject of this thesis. Discovery of reliable and early surrogate markers of angiogenesis and biological activity will consequently improve the success rate of biological agents being implemented in the clinical setting.

Introduction

It is well established that the formation of new blood vessels is a key regulator in the growth of many solid and hematopoietic tumors (1). First a range of oncogene activated mutations are needed to transform dormant and poorly vascularized tumors into tumors with the capability to attract and form new blood vessels (2;3). Endothelial cell proliferation and reorganization in early tumor lesions leading to new blood vessel formation and recruitment of neighboring vessels, is termed the 'angiogenic switch', and represents a transition from a ±1-2 mm^3 tumor to large and metastasizing deadly tumors. The term angiogenesis represents the process of blood vessel formation from the pre-existing surrounding vasculature. Vasculogenesis, in contrast, first believed to occur only during embryologic development, is the term for when endothelial precursor cells (angioblasts) migrate and differentiate in response to local cues (such as growth factors and extracellular matrix) to form new blood vessels (4). These vascular trees are then reorganized and extended through angiogenesis. Recently, however, it was realized that vasculogenesis can also occur in the adult organism. Bone marrow-derived circulating endothelial precursor cells were identified and reportedly able to contribute, albeit to varying degrees, to neovascularization, such as during tumor growth, or to the revascularization process following trauma, e.g. after cardiac ischemia.

Targeting tumor progression through inhibition of vascular proliferation

The endothelium has become a very promising target for experimental targeted therapies in cancer, for a variety of reasons. First, angiogenesis is very infrequent in the adult, with an endothelial doubling time of approximately 120 days. The expected toxicities and side effects will be minimal, except during times of wound healing, inflammation, ischemia, and pregnancy. Second, as the tumor endothelium is thought more genomically stable than tumor cells themselves, resistance mechanisms for anti-vascular therapies are unlikely to arise within one lifespan of endothelial cells. Third, combining anti-angiogenic agents with conventional cytotoxic agents or radiation therapy may result in additive or synergistic anti-tumor effects. Fourth, normalization of the tumor endothelium may result in better delivery of the chemotherapeutic agent. Fifth, the broad applicability of these treatments to many tumor types makes endothelial cells an ideal target.

Vascular targeting drugs: the pitfalls from bench to bedside

It has taken approximately 30 years since the rationale for anti-angiogenesis was first proposed, and the first angiogenesis targeting agent to be FDA-approved as cancer treatment in humans. Agents targeting the vascular endothelial growth factor (VEGF) pathway, such as the monoclonal antibody bevacizumab, have been proven effective in lung (5), breast (6;7), colorectal (8) and renal cell cancer (9). There are currently many different clinical trials with angiogenesis inhibitors, and agents specifically targeting the tumor vasculature (vascular-disrupting agents), which are summarized in Table 1.

Table 1

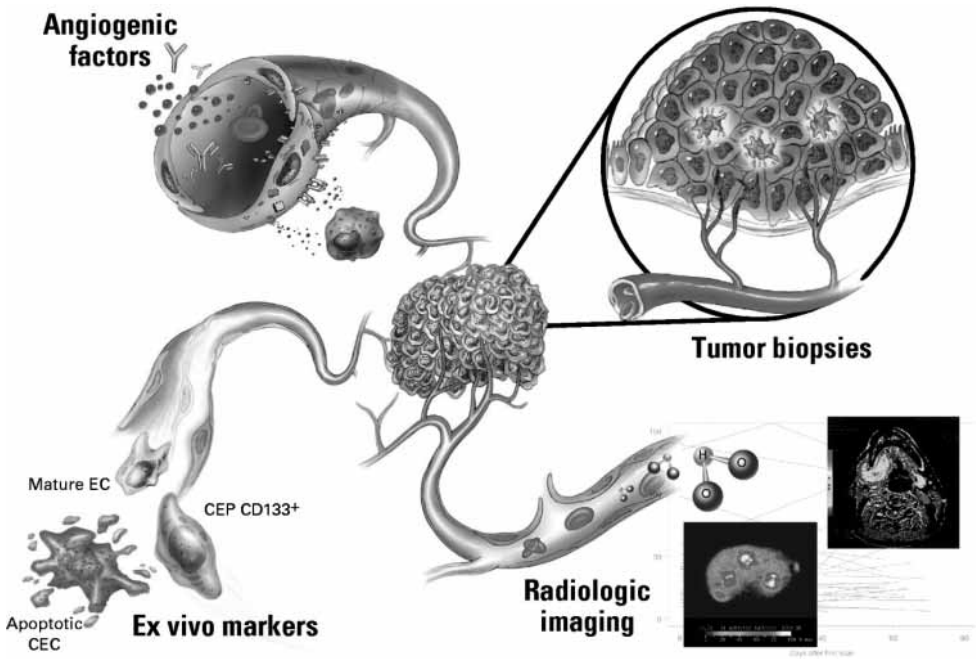
Mechanism	Drug	References
Inhibition of endothelial proliferation	ABT-510	(87-89)
	Combretastatin A4 Phosphate	(90;91)
	Lenalidomide	(92;93)
	LY317615 (Enzastaurin)	(NR)
	Soy Isoflavone	(94)
	Thalidomide	(95-98)
Inhibition of angiogenic cytokines	AMG-706	(NR)
	Anti-VEGF Antibody Bevacizumab	(7-9;99-103)
	AZD2171	(NR)
	Bay 43-9006 (Sorafenib)	(104)
	PI-88	(NR)
	PTK787/ZK 222584 (Valatinib)	(105)
	SU11248 (Sunitinib malate)	(106-108)
	VEGF-Trap	(NR)
	XL184	(NR)
	ZD6474	(109)
Inhibition of endothelial-specific signaling	ATN-161	(110)
	EMD 121974 (Cilenigtide)	(111)
Non-specific mechanism	Celecoxib	(112-114)

Adapted from http://www.nci.nih.gov/clinicaltrials/developments/anti-angio-table
Abbreviations: (NR), no published reports in phase I-III

Although several angiogenesis inhibitors are now approved, in the past decade there were also high profiled inhibitors that have failed to live up to their promise. This is due to a variety of reasons. First, animal models do not directly translate to human studies. Most preclinical studies are carried out on tumors grown subcutaneously, which is not a common site for tumor growth in humans. Fewer studies are performed with spontaneous or orthotopically grown tumors, which may more accurately reflect the human situation. Also in-vitro studies often use very aggressive, and highly proliferative (tumor cell turnover in order of days) human tumors as xenografts, however slow-growing tumors are also more typical of tumors found in humans. Second, patients having received anti-angiogenic agents rarely have partial or complete responses. This outcome is most likely explained by the fact that anti-angiogenic agents are cytostatic and chemo-and radiotherapeutic treatment modalities are cytotoxic agents. Therefore, assessing tumor response based on tumor shrinkage may not reflect the biological activity of these drugs. However, there may be a difference between agents specifically blocking a growth factor (e.g., antibodies) and a kinase inhibitor which generally is not completely specific and may inhibit multiple targets. This latter group may benefit

from MTD treatment. As a consequence, anti-angiogenic trial design strategies require modification. Phase-I studies need to investigate the optimal biological dose (OBD), rather than the MTD for these highly specific drugs. Phase-II studies may select disease stabilization or time to progression as end-point, rather than tumor regression. Third, monotherapy of angiogenesis inhibitors may not prove to be the best approach, and registered single agents are possibly more successful using a combinatorial approach. Tumors are highly adaptive; when one angiogenic cytokine is blocked (e.g. VEGF), tumors may rely on other angiogenic cytokines (e.g. bFGF, PlGF, PDGF) to counter hypoxia. It is therefore more conceivable that cancer therapy will need to be combinatorial, to target multiple pathways simultaneously. Fourth, no validated surrogate markers of angiogenesis inhibitors (to assess optimal biological activity) are currently available. Various experimental imaging strategies and surrogate markers are been investigated and are now more commonly incorporated into clinical studies with angiogenesis inhibitors (Figure 1), and of which circulating endothelial cells (CECs), endothelial progenitor cells (EPCs) are investigated more closely in the following sections of this thesis.

Figure 1



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Circulating endothelial cells with mature and stem-cell like phenotype

In the peripheral blood surrogate markers of angiogenesis and vessel damage may exist; circulating cells with an endothelial phenotype. A distinction has been made in CEC and EPC; however these cells are not completely different entities. It is thought that following vessel damage or at times of angiogenesis, bone-marrow residing angioblasts-like cells may differentiation into endothelial progenitor cells and these cells are mobilized into the peripheral blood. Removed from the bone-

marrow protective niches these circulating cells down-regulate their stem cell markers and differentiate into more mature endothelial cells (10;11). Therefore it has been postulated that CECs and EPCs may be seen as same cells but at varying time point along the differentiation span of the angioblast. An elegant study using fluorescence in situ hybridization on sex chromosomes of circulating endothelial like cells in patients following bone marrow transplantation from a sex-mismatched recipient, demonstrate that most circulating endothelial cells are shed from the vasculature and a minority is bone marrow derived (12). However the functionality differed between both entities. Based on the markers used, circulating cells with endothelial characteristics are denoted as CEC or EPC, however at present no consensus exists on which markers are solely expressed on CEC/EPC. Because of the gradualism of endothelial-gene expression on differentiation EPC, it likely will be impossible to completely differentiate between CEC and EPC. Below we will review the literature by both types of circulating endothelial cells in the peripheral blood.

CEC reflect a more mature endothelial cell, as they express a variety of endothelial markers, but no detectable progenitor or stem cell markers presently known. CECs have been identified and studied in various pre-clinical models (13-18). CECs can also be detected in the circulation of cancer patients (19;20) and in a variety of other physiological (pregnancy, menstruation) and pathological circumstances, such as vasculitis (21), sickle cell anemia (22), cytomegalovirus (23) and rickettsial infection (24;25), and myocardial infarction (26;27). In patients with cancer it has been suggested that these cells most likely originate from the angiogenic cancer vasculature, however partly also from cytokine activated non-cancerous vasculature and the bone-marrow. Therefore CEC are being studied as surrogate marker for optimal dose assessment in clinical trials with biological agents, and as predictors of disease-free survival and therapeutic efficacy. An overview and results of clinical studies incorporating circulating endothelial cells as surrogate marker of antivascular drug activity, response or survival is given in Table 2.

A fraction of the circulating cells with endothelial characteristics (still) expresses stem-cell and progenitor markers. These endothelial progenitors cells (EPCs) are immature bone marrow-derived endothelial cells which can be recruited by hypoxia and cytokines (28-30) to enter the circulation and can incorporate into sites of hypoxia, ischemia and/ or active neovascularization, leading to (collateral) vessel formation and revascularization (31-34). It has therefore been proposed that endothelial progenitor cells are directly involved in the repair of injured endothelium. The functional role attributed to EPC led to a series of studies in which the numbers and properties of these cells have been characterized and studied on in various disease states associated with disordered endothelial function. It has been shown in patient studies that fewer circulating endothelial progenitor cells are present in patients with atherosclerotic disease (35), diabetes mellitus (36;37), cardiac transplant vasculopathy (38), in-stent restenosis (39), myocardial infarction (40), ischemia (41;42), and fewer EPC are associated with increased risk of death from cardiovascular causes (43). In pre-clinical studies, inhibition of EPC mobilization resulted in retardation of tumor growth (14;44), supporting the importance of bone marrow-derived EPCs in tumor angiogenesis. Furthermore, MTD dosing of cytotoxic chemotherapy lead to robust mobilization of EPCs few days after the end of a cycle of drug administration, and the development of drug resistant tumors (17). Vascular disrupting agents (VDAs) also lead to acute EPC mobilization from the bone marrow followed by incorporation into the angiogenic tumor vasculature (45). These studies underscore the importance of EPC contribution to tumor growth, during and after certain anticancer therapies, and demonstrate the rationale for

Table 2

Study	Marker	Method	Outcome	Reference
Breast cancer (n=13)	CD45-, P1H12+ CD31+ CD34+ CD133-	FACS	decrease after tumor resection	(20)
Breast cancer (n=81) receiving metronomic chemotherapy	CD45- P1H12+ CD133-	FACS	after two months patients with high (apoptotic) CECs show increased PFS and OS	(80)
Rectal cancer (n=5) receiving BV with radiation and chemotherapy	CD45- ?	FACS	high dose BV reduced CECs in 3/5 patients	(64)
Myeolodysplastic syndrome	CD45-, P1H12+ CD31+ CD34+ CD105+ CD133-	FACS	increased compared to controls, correlation with MVD, no association with survival	(115)
Myelodysplastic syndrome and acute leukemias	CD45- P1H12+ CD31+ CD133-	FACS	decrease in CECs at dose ? 175 mg/m2 in 4/5 patients, in 3 patients with decreased blasts	(82)
Breast cancer (n=9)	CD146	PCR	increase in CECs compared to controls, correlation with FACS analysis of CECs	(116)
GIST (n=73)	CD45- CD31+ CD146+ CD133-	FACS	patients with PR or SD>6 months all had elevated CECs 6-20d after SU11248	(81)
B-cell lymphoma (n=32)	VE-cadherin	PCR	decreasing trend after chemotherapy ± anti-CD20 antibody	(53)
Breast cancer (n=30)	Tie-2	PCR	elevation compared to controls	(62)
Mixed patients solid tumors (n=112)	CD146+	Beads	elevation in progressive cancer patients	(19)
Phase I study (mixed; n=xx) with vascular targeting agent ZD6126	CD146+	Beads	significant increase 4 hours following ZD6126 no relation with dose escalation	(84)
Phase I study (mixed; n=28) with PKC inhibitor enzastaurin HCl	CD146+	Beads	significant increase 2-4 hours following GC chemotherapy, high baseline poor prognosis	(UR)

Methods used are flow cytometry (FACS), real-time mRNA amplification (PCR), or anti-body based capture, isolation and quantification of circulating cells (Beads). Abbreviations: BV, bevacizumab; PFS, progression free survival; OS, overall survival; PKC, protein kinase C; MVD, mean vessel density, UR, unpublished result.

metronomic dosing and anti-angiogenic regiments together with MTD treatment of chemotherapy or VDAs. Bone marrow progenitor cells have also been linked to the initiation of metastasis; progenitor cells expressing endothelial growth factor receptor 1 home to tumor-specific pre-metastatic sites and provide a permissive niche for incoming metastasizing tumor cells. Blockage of bone marrow derived progenitor cells by antiangiogenic treatment abrogated mobilization and blocked tumor metastasis (11). In humans, recent evidence supports a contribution of EPCs to tumor growth, response to treatment and outcome. Bone-marrow derived EPCs directly contribute to tumor angiogenesis by incorporation into the angiogenic vasculature (46-48), and recent evidence also suggests cytokine release by EPCs to directly stimulate local angiogenesis (49-51). An overview of the emerging studies on EPCs in human cancer is given in Table 3.

To understand the relative contribution of CEC and EPC to prognosis it is important to analyze the phenotype of these cells. Several methods are used to do so. Below you will find an overview of these methods.

Characterization of circulating endothelial cells

In the past years various techniques have been studied and validated for the extraction, isolation and/or quantitation of circulating endothelial cells from the peripheral blood. As CEC are present in very low cell numbers when compared to total number of circulating cells, enrichment using EC-specific cell-surface markers is needed.

One such enrichment method uses surface marker P1H12/ CD146 specifically present on endothelial cells. Peripheral blood is incubated with a murine antibody against human CD146, previously conjugated to magnetic Dynabeads carrying goat antimouse immunoglobulin. After binding of antibody with cell surface marker CD146, the CEC can magnetically be separated from the hematopoietic peripheral blood cells, washed and stained with additional markers, and quantified using fluorescence microscopy by their distinctive fluorescence, binding of ≥10 beads and their size of 20-50 µm (Figure 2). Using this validated methodology, Mediterranean spotted fever was evaluated by analyses of Rickettsia conorii infected CEC (24), activated CEC were demonstrated in sickle cell anemia (22), and elevated numbers were evaluated in ANCA-positive vasculitis (21).

Figure 2A

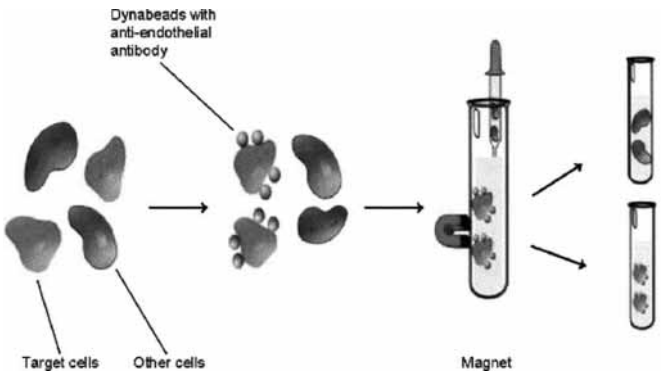

Figure 2B

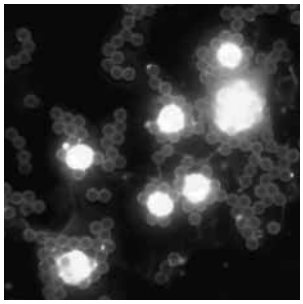

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Table 3

Study	Marker	Method	Outcome	Reference
Breast cancer (n=81)	CD133+	FACS	no correlation with outcome	(80)
Gastric and breast cancer		CFU	not increased compared to healthy controls	(56)
Cervical cancer (n=10)	CD34+ VEGRF2+	FACS	inversely correlated with intratumoral oxygen tension	(117)
Myeolodysplastic syndrome	CD45-, P1H12+ CD31+ CD34+ CD133+	FACS	increased compared to controls, no association with survival	(115)
Colorectal, RCC, H&N, prostate (n=131)	CD133+	NASBA	correlated with survival, elevated in bone metastasis	(83)
Breast cancer (n=30)	CD133+	PCR	non-significant elevation compared to controls	(62)
Phase I study (mixed; n=28) With enzastaurin HCl	CD133+	NASBA	significant decrease 8-24 hours following GC chemotherapy, increase between cycles	(UR)
Lung cancer (n=53)	CD34+ VEGFR2+	FACS	decrease in responders, high pretreatment numbers related to decreased OS	
	CD133+ and CD34+	PCR	not significantly altered, no relation with OS	(47)
Myeolofibrosis with myeloid metapasia (n=110)	CD34+ CD133+ VEGFR2+	FACS	increased compared to controls, elevation in early stage of the disease	(118)
Hepatocellular cancer		CFU	increased compared to liver cirrhosis and healthy controls	(119)

Methods used are flow cytometry (FACS), real-time mRNA amplification (PCR and NASBA), and quantification of endothelial progenitor colony forming units (CFU). Abbreviations: OS, overall survival, UR, Unpublished result.

Another method uses flow cytometric analyses to evaluate CEC numbers in the peripheral blood (20;52). Flow cytometry has the advantage of using more than one cell-surface or intracellular markers specific for endothelial cells, and negative selection markers, to enhance qualitative CEC enumeration. Most commonly used markers are endothelial-specific markers such as CD31, P1H12/CD146, endoglin/CD105, and VCAM-1/CD106. In the peripheral blood of breast cancer and patients with lymphoma, it was demonstrated that elevated CECs were present compared to controls, and after surgical resection of the tumor or after clinical remission CEC numbers decreased (20).
Real-time quantitative reverse transcription-PCR is another method currently available to study endothelial-specific RNA copies present in the peripheral blood mononuclear cells.
Endothelial-specific genes VE-cadherin, Tie-2 and VEGFR-2 have been evaluated in a patient group with mixed hematological and solid malignancies (53).

Characterization of circulating endothelial progenitor cells

In a landmark study putative endothelial progenitors cells were isolated from human peripheral blood by magnetic bead selection on the basis of cell surface antigen expression of VEGFR-2 (54). Cultured cells gradually gave rise to endothelial cell cobblestone-morphological colonies with stem-cell like proliferative characteristics (expressing stem-cell marker CD34 and VEGFR-2), which incorporated into the vasculature of rabbits undergoing neovascularization due to hindlimb ischemia. Adaptation of this technique led to a method of quantifying the number of adherent cobblestone-morphological colonies on fibronectin coated culture plates (colony forming units, CFU), as a measure of EPC present in the peripheral blood (55-57). Recently, elevated EPC were found by quantifying CFU in patients with hepatocellular carcinoma (58).

Other more feasible and direct methods for evaluating the number of EPC have been performed using flow cytometric analyses, by adding a stem-cell marker to the CEC markers mentioned previously. Two commonly used stem-cell markers are CD34 and CD133. CD133 is a novel hematopoietic stem cell marker, whose function is unknown, and is expressed on EPC. CD133 expression is rapidly downregulated as progenitors differentiate (59). By flow cytometry increased levels of EPC have been found to be present in myeodysplastic disease (60) and infantile hemangioma (61), and recently in patients bearing lung cancer (47). Real-time RT-PCR on endothelial progenitor-specific gene CD133 has also been successfully used to investigate its presence in cancer (53;62).

After mobilization from the bone marrow, stem cell and progenitor receptors are rapidly lost, and it is therefore important to keep in mind that the number of quantified circulating EPC in most studies are likely underestimated.

Other techniques for the assessment of changes in angiogenesis/and or tumor blood vessels

Visualizing the tumor vasculature

With anti-angiogenic therapy, evaluation of tumor size alone is inadequate. As anti-angiogenic agents exert their effect on the tumor vasculature, direct analysis of the endothelial tumor vasculature has been performed by serial tumor biopsy with histopathological staining and quantification of the vasculature (mean vascular density, MVD) and the viability of endothelial cells (63;64). Studies with anti-angiogenic agents to date demonstrate that tumor biopsies are of limited value and this technique is too invasive and impractical for implementation in large clinical trials. Therefore more focus has been drawn on imaging techniques to visualize or monitor changes in the tumor vasculature during therapy (65-67). The imaging of blood flow by CT, MRI, and ultrasound allow repeated monitoring in a clinical setting.

A number of imaging modalities are available, and adaptable, which may be sensitive and specific enough to investigate changes in the tumor macrovasculature, pinpoint sites of angiogenesis, determine amount of blood vessel growth, characterize functional abnormalities of vessels, and assess vascular heterogeneity. At present the resolution of CT, MRI, and ultrasonography imaging is still insufficient to monitor the microvasculature. CT imaging with

contrast agents has been performed as surrogate marker of biological drug efficacy, to define the intravascular compartment, including blood flow, blood volume, mean fluid transit time, and capillary permeability (64). Contrast-enhanced ultrasound can identify vascular features in tumors at different levels of resolution (40-200 micron vessel diameter), and can generate an index of blood flow, blood volume or vascularity within the tumor (68). Color flow-doppler has already been used to characterize solid tumors in humans (69;70). MR imaging can define both blood volume as blood vessel permeability, using dynamic enhancement of blood pool contrast agents, such as gadolinium (69;71-73). In studies the amount of contrast uptake correlates well with MVD (74). Furthermore, PET imaging can be used to evaluate blood flow and volume, as well as tumor metabolism in anti-angiogenic trials (75). PET imaging with inhalation of carbon monoxide labeled 11C or 15O, can be used to calculate blood flow within tumors, with spatial resolutions of ±2mm. Radiolabeled fluoromisonidazole is currently being used to assess the effect of tumor hypoxia on treatment outcome (76), and the efficacy of anti-angiogenic agents in clinical trials. More detailed information on imaging techniques is reviewed by McDonald et al. (77).

Circulating angiogenic factors as surrogate markers

Angiogenic growth factors produced by tumor or surrounding stromal cells diffuse towards nearby quiescent pre-existing blood vessels, or spread through the vasculature, and bind unique receptors located on endothelial cells and their precursors. The best studied cytokines and cognate receptors are of the VEGF family (VEGFR-1/flt-1, VEGFR-2/flk-1/KDR, VEGFR-3/flk-1/KDR, VEGFR-4/neuropilin-1), FGF family (bFGF/FGF-R), PDGF (PDGF-A/PDGFR-α, PDGF-B/PDGFR-β), and the angiopoietins (ang-1/Tie2, ang-2/Tie2), reviewed in (78). The cytokines involved in blood vessel formation comprise a complex organization of many pro- and anti-angiogenic factors in equilibrium, with numerous positive and negative relationships. Nevertheless, after optimal anti-angiogenic (metronomic) dosing of cyclophosphamide VEGF levels strongly decrease (79), elevated urine VEGF levels after topoisomerase II inhibitor treatment predict relapse, and VEGF serum kinetics aided in establishment of OBD of bevacizumab in phase II studies (9). However further clinical validation of VEGF and other cytokines as surrogate marker of treatment efficacy has to be further established.

Conclusion

Drugs interfering with the vasculature, such as anti-angiogenic or vascular targeting agents, are an important emerging field in targeted cancer therapy. It has taken only 15 year since the VEGF gene was cloned and the FDA approval of bevacizumab for first-line cancer treatment. However, advances in drug discovery have not been paralleled by identification of reliable surrogate markers of biological activity or angiogenesis. At the present time, angiogenesis and anti-angiogenic drug activity are monitored by mean vascular density and immunohistochemical staining of biopsies of skin or tumor, circulating levels of angiogenic cytokines, and by monitoring of angiogenesis by a variety of imaging techniques. However, these monitoring techniques are invasive, not reliable enough, or still in need of clinical validation.

Circulating endothelial (progenitor) cells are potentially ideal biological markers for monitoring angiogenesis, anti-angiogenic treatment response and relapse (47;64;80-84). CECs are more mature endothelial cells expressing a variety of endothelial but no precursor markers most likely sloughed of the angiogenic tumor vasculature or are possibly bone-marrow derived. Monitoring of

CECs could be used to assess efficacy of endothelial damage by anti-angiogenic and vascular disrupting agents, or metronomic dosage of chemotherapeutic agents. Exploratory studies on CECs in human cancer has been assessed only since ±5 years, and results indicate that these cells (a) are increased in cancer patients, (b) are increased in progressive disease, (c) increase directly after cytotoxic and vascular disrupting agents, (d) decrease after removal of the tumor (e), decrease after anti-angiogenic treatment, (f) and elevated apoptotic/viable CEC ratio indicate better overall and disease-free survival following treatment. These results demonstrate that CECs may also be used to assess optimal anti-angiogenic dose of novel agents in phase-I studies, however many of these clinical studies are currently still being performed.

EPCs represent both early endothelial progenitor cells, which may express an endothelial precursor marker but few to none endothelial markers, and late endothelial progenitor cells, which express endothelial markers with low or no expression of an endothelial precursor marker. Recent evidence suggest that EPCs are (a) strongly elevated in subsets of human tumors, (b) may incorporate into angiogenic tumor vasculature, (c) home to tumor sites and support local angiogenesis by release of cytokines, (d) are mobilized by VEGF and other angiogenic cytokines, (e) are mobilized by maximum tolerable dosing of chemotherapy in contrast to metronomic dosing, (f) are associated with metastasized disease, (g) are increased in non-responding patients compared to responders, (h) and are associated with poor prognosis and survival. Pre-clinical results indicate that endothelial progenitor cells are specifically more sensitive to anti-angiogenic agents, compared to mature endothelial cells (57), and might therefore be a sensitive biological marker to assess efficacy of anti-angiogenic therapies in-vivo. Recent finding also suggest that the recruitment of bone marrow-derived angiogenic cells provide a potential mechanism of escape to some anti-angiogenic or general anti-cancer treatment strategies (45).

Most imaging techniques still have to become more refined to image the microvasculature and tumors deep within the human body with high resolution and specificity (77). Initial results from trials with angiogenesis inhibitors with above imaging techniques suggest that changes in vascular permeability, vascular volume fraction and metabolism can be detected in tumors soon after therapy begins. However, these changes do not always predict clinical efficacy, as indicated by improved disease-free or overall survival. Therefore, until further advances in clinical utility, more effective surrogate markers of angiogenic inhibition are needed.

The discovery of robust surrogate markers of angiogenesis and anti-angiogenic drug efficacy, combined with optimized trial design, will create a platform for bringing anti-angiogenic cancer therapy into standard oncology practice. Approaches to image or monitor angiogenesis are currently still under clinical investigation, and in the following chapter of this thesis circulating endothelial (progenitor) cells are the subject of study.

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Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients

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Abstract

Purpose: There is accumulating evidence from preclinical studies that circulating endothelial cells (CECs) play an important role in neovascularization and tumor growth. The role of CECs in human cancer progression is sparsely investigated. We therefore analyzed CECs in peripheral blood of cancer patients. In addition, we correlated CEC levels in these patients with plasma levels of cytokines that are known to mobilize CECs in experimental models.

Material and Methods: Viable CECs were isolated, quantified and cultured from cancer patients' whole blood by using magnetic beads coupled to an antibody directed against CD146, a pan-endothelial marker. Viable cells were visualized by calceinAM staining. Positive staining for specific endothelial cell markers (i.e. von Willebrand factor, CD31, VEGF-receptor-2) was used to confirm the endothelial phenotype.

Results: Cancer patients with progressive disease (n=95) had on average 3.6-fold more CECs than healthy subjects (n=46, $p<0.001$). Patients with stable disease (n=17) had CEC numbers equal to that circulating in healthy subjects ($p=0.69$). A subset of *in vitro* cultured CECs incorporated into endothelial layers and formed colonies. Plasma levels of cytokines that are thought to mobilize CEC from the bone marrow [VEGF, PlGF, SDF-1α, and SCF (n=71)] did not correlate with CEC amounts. The levels of viable CECs in cancer patients were modified by G-CSF treatment and chemotherapy.

Conclusion: In progressive cancer patients, the amount of CECs is increased. These CECs are viable and may contribute to vessel formation. The number of CECs is influenced by anti-cancer treatment.

Introduction

The role of angiogenesis in tumor growth is well-established [1]. New blood vessels are formed by endothelial cells (ECs) derived from preexisting vessels in the tumor's microenvironment. Recently it has been shown that bone marrow-derived endothelial progenitor cells (EPCs) and hematopoetic stem cells (HSC) mobilized by tumor or ischemia induced signals may also contribute to neovascularization through a process called vasculogenesis [2;3]. In animal models EPCs were shown to be incorporated in areas of new vessel formation [4] and the combination of HSCs and EPCs was essential for tumor growth [5].

In patients, an increase in circulating endothelial cells (CECs) has been associated with several clinical syndromes (myocardial infarction [6], infectious vasculitis [7], ANCA-associated vasculitis [8], sickle cell crisis [9], kidney transplant rejection [10]) or interventions (CABG, burn injury [11], VEGF165 gene therapy [12], statin treatment [13]). In contrast, a decrease in CEC numbers is found in subjects with increasing cardiovascular risk profile[14], in patients at risk for coronary artery disease [15] or in patients with type 2 diabetes mellitus[16]. In cancer patients, Mancuso et al. [17] detected increased amounts of CECs using flow cytometry. CEC numbers decreased in patients with lymphoma after complete remission following chemotherapy, and after mastectomy in breast cancer patients.

It is generally believed that ECs appear in the peripheral blood either due to release from the bone marrow in response to tissue ischemia regulated through cytokines [5;18], or due to shedding

from activated or damaged (tumor)vessels [19]. It has yet to be elucidated whether CECs are mobilized in a similar fashion in human subjects.

Here we report a quantitative and functional analysis of viable CECs obtained from the peripheral blood of cancer patients. We show that levels of CECs are increased in patients with progressive disease. CEC subpopulations are able to incorporate into endothelial monolayers. In addition, we show that CEC levels are modulated by chemotherapy and granulocyte colony stimulating factor (G-CSF). We did not find a correlation between the number of CECs and the levels of VEGF, PlGF, SDF-1α, or SCF, cytokines that mobilize EPCs. Whether CECs may serve as a target for anti-cancer therapy warrants further studies.

Patients and Methods

Patients

After obtaining informed consent, blood samples were collected from patients visiting the outpatient clinic of the department of Medical Oncology or patients hospitalized in the University Medical Center in Utrecht, the Netherlands. Unless otherwise stated, patients did not receive concurrent anti-cancer therapy. Progressive disease (n=95) was defined as radiologically documented progressive cancer (>25 % increase in tumor size comparing tumor size prior to and after the blood sampling date), or as clear clinical evidence of disease progression. Tumor types included head and neck cancer (n=10), colon (n=13), prostate (n=25), gastric (n=3), esophagus (n=3), renal cell (n=6), breast (n=10), melanoma (n=3), ovarian (n=5), cervix (n=2), carcinoid (n=3), glioma (n=2), and 10 patients with other tumor types. Of progressive cancer patients, 30 patients had received prior cytotoxic chemotherapy, mean time from chemotherapy to sampling was 335 days, median 113 days, range 1 to 1718 days. Tumor types from patients with stable disease (n=17) included renal cell (n=3), prostate (n=3), colon (n=2), adenoid cystic (n=2), thyroid (n=3) and 4 patients with other tumor types. Stable disease was defined as no increase in tumor size on radiological follow up when comparing tumor size prior to and after the blood sampling date. In prostate cancer patients, tumor stratification of stable or progressive disease was also done guided by PSA level. Of stable cancer patients, 10 patients had received prior cytotoxic chemotherapy, mean time from chemotherapy to sampling was 397 days, median 103 days, range 7 to 1722 days. Healthy volunteers (n=46) served as controls.

Isolation of CEC from whole blood

The isolation of CEC was performed according to a previously reported and validated methodology [6-9;20]. Briefly, to isolate endothelial cells, peripheral blood was incubated with magnetic beads (Dynal M450 IgG1, Dynal AS, Oslo, Norway) that had been previously conjugated to a monoclonal antibody directed against CD146, SendoI (Kordia Life Sciences, Leiden, the Netherlands). SendoI specifically recognizes all lineages of endothelial cells (not hematopoetic or epithelial cells) in peripheral blood of human subjects [21]. CD146 is a cell surface receptor involved in cell migration and maintenance of tissue organization [22]. When human blood samples were spiked with Human Umbilical Vein Endothelial Cells (HUVEC) these cells could be retrieved in excess of 90% with a clear preservation of cell morphology. Dynabeads without secondary antibody did not bind cells. Peripheral blood from subjects was drawn in a siliconized tube containing 1 mg/mL EDTA by venapuncture. The

first 3-5 mL were discarded to avoid contamination of ECs traumatically released from the punctured vessel wall. One milliliter whole blood was diluted 1:3 with NaCl 0.9% and incubated for 30 minutes with 20 µL (=2.8 x106) antibody-coupled magnetic beads at 4°C on a roller bench. Unbound cells were removed by magnetic separation using a MPC-L magnet (Dynal AS, Oslo, Norway). Beads-bound cell fraction was rinsed with PBS-BSA 0.1%. To quantify viable ECs the isolated cells (in a final volume of 100 µL medium containing 20% fetal bovine serum) were fluorescently stained with CalceinAM (Molecular Probes, Eugene, Oregon), and quantified in a Nageotte hemocytometer using a fluorescence microscope (Leica, Rijswijk, the Netherlands). CECs were clearly distinguishable from other cells by their distinct fluorescence, the binding of >10 beads, and a size of 20-50µm. Reported CEC numbers are the mean of a duplicate quantification. Selected aliquots of beads-bound CECs were cytospun (for 5 min at 500 rpm) on glass slides, fixated with acetone and dried for >4 hours. Subsequent phenotypic analysis was carried out with the primary antibodies against CD31 (PECAM, Santa Cruz Biotechnology, Santa Cruz, CA), von Willebrand factor (DAKO, Glostrup, Denmark), and VEGF-receptor 2 (Santa Cruz Biotechnology, Santa Cruz, CA). For fluorescent staining, secondary antibodies conjugated to TRITC (DAKO, Glostrup, Denmark) or FITC (Southern Biotechnology Ass. Inc., Birmingham, AL) were used. Slides were mounted with vectashield (Vector laboratories, Burlingame, CA) for maintenance of fluorescence. Beads-bound HUVEC were used as positive control.

Outgrowth of CECs

When attempting to monoculture CD146+ cells, no growth was seen. Therefore, an endothelial feeder layer was used for co-culture of CECs to provide a permissive environment for attachment and outgrowth. In order to be able to distinguish between the CECs and the feeders, CECs were fluorescently labeled with CFDA-SE (carboxy-fluorescein diacetate succinimidyl ester, Molecular Probes, Leiden, the Netherlands) prior to adding them to the feeders. CFDA-SE is a marker that is used for cell tracing [23;24]. This dye is retained within cells after cell division, making it an excellent marker for follow up of mixed cell cultures. Labeling was performed according to the manufacturer's protocol. To provide a feeder-layer for the isolated CEC, HUVEC or $HMEC_1$ (human dermal microvascular endothelial cells [25]) were used. The $CD146^+$ cells isolated from 1 mL peripheral blood (typically 100 to 1000 cells) were co-cultured with HUVEC or $HMEC_1$ ($7x10^4$/well) in a 6-well plate (Costar, Cambridge, MA) pre-coated with fibronectin (12.5 µg/mL, Sigma-Aldrich BV, Zwijndrecht, the Netherlands). All EC cultures were performed in EGM-2 endothelial growth medium (Bio Whitacker, Walkersville, MD, USA). The CFDA-SE positive fluorescent CECs attached on the fibronectin coated surface within a few hours between the feeder-layer endothelial cells and spread out. After 4-7 days, colonies of isolated cells were formed within the sparse feeder layer. Colonies were defined as clusters consisting of ⩾ three green fluorescent attached spindle-shaped cells. Co-cultured isolated endothelial cells and feeder EC stained uniformly positive for DiI-ac-LDL (1,1'dioctadecyl-3,3,3',3'-tetramethylindo-carboxyanine-labeled acetylated low-density lipoprotein, Sanbio BV, Uden, the Netherlands), confirming the endothelial phenotype of expanding isolated cells.

Analysis of cytokine levels in plasma

In platelet-poor citrated plasma of patients and volunteers we measured levels of vascular endothelial cell growth factor (VEGF), placental growth factor (PlGF), stem cell factor (SCF) and stromal cell-derived factor 1α (SDF-1α) using commercially available ELISA kits (R&D systems, Abingdon, Oxon, UK), following manufacturer's guidelines. Samples were measured in duplicate. Obtained optical density (OD) values were plotted against standard curves generated on the ELISA

plate with correlation coefficient of >0.99. Samples with an OD value below the lowest value on the standard curve were set at 0.

Statistical analysis

All results were analyzed by applying SPSS software (version 10.0.5). Statistical differences with a p-value <0.05 were considered significant. Analysis was done, when appropriate, by Mann-Whitney test, (un)paired student's t-test and Pearson's correlation analysis. Data are presented as mean ± standard error of the mean (SEM).

Results

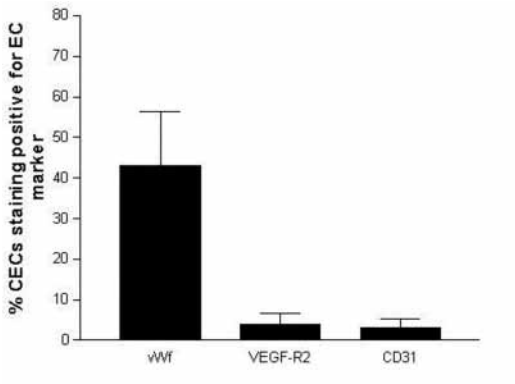
Quantification of CECs

CECs isolated from peripheral blood of cancer patients using an endothelium specific antibody (SendoI, antiCD146) coupled to Dynabeads were easily recognized and quantified (figure 1B, inset). Characterization of the primary isolated CECs after cytospin preparation by immunofluorescence revealed that subsets of the CECs were positive for expression of the EC markers von Willebrand factor, VEGF receptor-2 and CD31(figure 1A). Mean levels of viable CECs were 3.3-fold higher in cancer patients (n=112, mean 399 ± 36 CEC/mL) than in healthy volunteers (n=46, mean 121 ± 16 CEC/mL; p=0.001 by Mann-Whitney test). Because cancer patients had variable levels of CECs we determined whether disease progression was associated with an increase in CECs. Patients with progressive disease (n=95, 438 ± 65 CEC/mL) had more CECs as compared to healthy volunteers (n=46, p<0.001 by Mann-Whitney test, figure 1B). Patients with stable disease (n=17, 179 ± 61 CEC/mL, figure 1B) showed amounts of CEC comparable to healthy volunteers (p=0.69 by Mann-Whitney test). Longitudinal follow-up of patients not receiving anti-cancer treatment yielded a variation of ~30% in CEC levels. No difference in CEC amounts was found between male (319 ± 51 CEC/mL and female subjects (241 ± 28 CEC/mL, p=0.18 by t-test). There was a significant difference in age between volunteers and cancer patients (average 38 years in volunteers vs. 57 years in patients, p<0.001 by t-test), but there was no change in CEC number with increasing age (Pearson's correlation coefficient r=0.089, p=0.182). When using 250 CEC/mL as a cutoff value, CECs had a predictive value for progressive cancer of 92 %. Sensitivity of the test was 44%, and specificity 91%. In the studied progressive cancer patients CECs were increased in all different tumor types (figure 1C), reaching statistical significance in five out of six reported tumor types (tested by Mann-Whitney test).

Cytokines and CECs

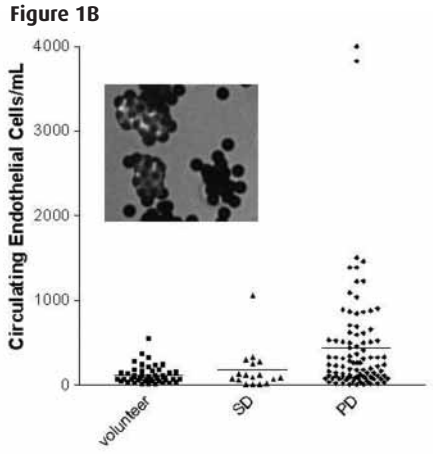
We next addressed the question whether levels of cytokines that are known to be involved in the mobilization of progenitor cells from the bone marrow correlated with CEC. From 71 subjects (51 cancer patients and 20 healthy volunteers) plasma levels were measured for VEGF, PlGF, SDF-1α, and SCF. Cancer patients had increased plasma levels of PlGF compared to healthy volunteers (cancer: 31.2 ± 7.5 pg/mL vs. volunteers: 5.2 ± 2.9 pg/mL, p=0.01 by Mann-Whitney test). Other tested cytokines showed no differences between cancer patients and volunteers. Levels of VEGF, PlGF, SDF-1α and SCF did not significantly correlate with CEC levels in tested individuals.

Figure 1A



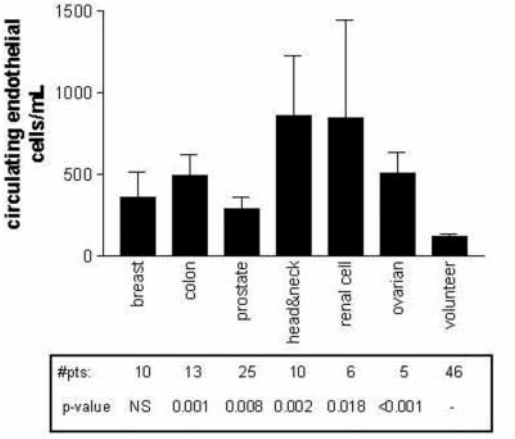
Average percentage of CECs that stain positive by immunofluorescence for endothelial markers after isolation with antiCD146-coupled magnetic bead isolation. vWf, von Willebrand factor; VEGF-R2, vascular endothelial growth factor receptor 2

Figure 1B



CEC amounts in peripheral blood of cancer patients with progressive disease (PD) or stable disease (SD) and healthy volunteers (healthy). Inset Appearance of CECs under fluorescence microscope. Small particles attached to cells are immunomagnetic beads used for isolation.

Figure 1C

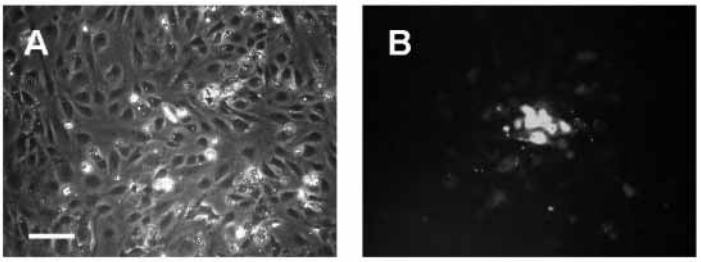


CEC amounts are increased in progressive cancer patients irrespective of primary tumor.

Outgrowth of circulating endothelial cells in culture

After quantification we assessed whether these viable CECs were functional. CECs adhered within four to six hours to fibronectin-coated culture dishes in cocultures with feeder-layer endothelial cells. Four to seven days after seeding, between 5 and 70% of the isolated CECs (recognized through CFDA-SE positivity) formed colonies (figure 2A,B). Fluorescence was less bright in cells at the periphery of the colony, consistent with the property of fluorescent CFDA-SE to divide equally to two daughter cells after division. All colonies formed in culture were acLDL-DiI positive (data not shown). This procedure was performed with CECs from 30 patients with similar results. The percentage of isolated CECs that formed colonies was strongly dependent on the donor. No increase in colony formation per seeded CEC could be assessed in patients with progressive disease (data not shown).

Figure 2

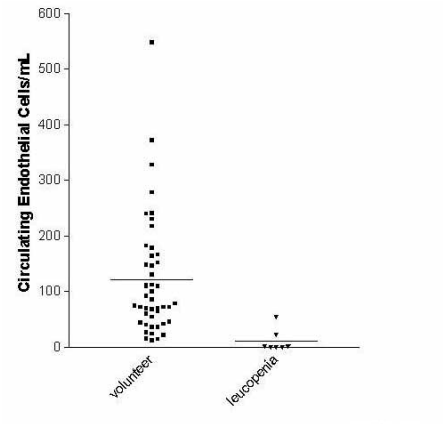


Coculture of a colony of CECs (green fluorescently labeled with CFDA-SE) between HUVEC. Small black spots are immunomagnetic beads, used for isolation (A, phase contrast; B, green fluorescence). Scale bar, 100 µm.

Chemotherapy and growth factor treatment affect the levels of circulating endothelial cells

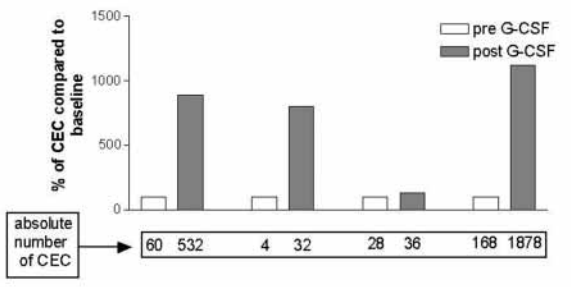
To address the question whether CEC levels could be modulated by chemotherapy, cancer patients were monitored for levels of CECs during leucopenia in the weeks after the chemotherapy (defined as <1x109 leucocytes/L). The CEC levels became almost undetectable in leucopenic patients (figure 3A; n=7, 11 ± 8 CEC/mL, p=0.0001 by Mann Whitney for difference with healthy volunteers). Cytotoxic chemotherapy with taxanes (n=7, 175mg/m^2 and n=1 200 mg/m^2) resulted in rapid (2-4 hours after infusion) increase in CEC numbers for 7 out of 8 tested patients. The average CEC number prior to therapy was 24 ± 6 CEC/mL. After treatment, numbers increased to 104 ± 27 CEC/mL (p=0.011, by paired t-test).

Figure 3A



Leucopenia after chemotherapy results in almost complete disappearance of CECs, in comparison to healthy volunteers.

Figure 3B



G-CSF treatment induces increased amounts of CECs in cancer patients receiving chemotherapy.

We also analyzed CECs in four patients receiving chemotherapy with the support of G-CSF. Patients' primary tumors were seminoma testis (n=1), non-seminoma testis (n=2) and small cell lung cancer (n=1). Prior to, and 3-8 days after, initiation of the G-CSF treatment CECs were quantified in the peripheral blood of these patients (figure 3B). In 3 out of 4 patients CEC amounts increased

8-9 fold. The fourth patient manifested only a minor increase in the number of CECs after G-CSF treatment. G-CSF treatment of healthy individuals, treated to mobilize stem cells for allogeneic bone marrow transplantation, resulted in similar high CEC numbers (n=15, mean 1736 ± 631 CEC/mL, $p<0.0001$ for difference with untreated healthy volunteers, by Mann-Whitney test).

Discussion

We show here that patients with progressive cancer display increased levels of endothelial cells present in the peripheral blood when compared to patients with stable disease or healthy volunteers. These CECs were viable, with a subpopulation able to adhere to an endothelial layer and proliferate in a coloniform fashion. No correlation was found between CEC levels and plasma levels of cytokines that are thought to mobilize CEC from the bone marrow.

Isolated CECs probably originated from three sources: the mature blood vessels, the tumor vasculature and the bone marrow. Not all viable CD146 positive cells express detectable amounts of generally accepted endothelial markers. The putative mixed population of mature and progenitor cells may account for a variable expression of mature endothelial markers. A heterogeneous EC-specific antigen expression is also seen in freshly isolated endothelial precursor cells [26;27]. But the expression of CD146 in circulating cells is restricted to cells of endothelial origin[21], whereas other markers (CD31, acLDL-DiI, Ulex-lectin) may crossreact with other cells, such as monocytes [28]. To distinguish between mature and precursor endothelial cells, CD133, a progenitor cell marker, can be used [3]. Interpretation of CD133 expression should be done with caution, though, because the marker is lost very rapidly upon differentiation from an anchor-independent EPC released from the bone marrow to a CEC that is able to incorporate into a vessel. The viability and growth capacity of the CECs in our patients and the low expression of mature EC markers are compliant with the assumption that at least a subset is derived from a progenitor pool in the bone marrow.

Some of the CECs found in peripheral blood from cancer patients may be shed from the (tumor) vessel wall, as observed in vascular injury syndromes [6-8]. Tumor (endothelial) cells may be blood-borne by mechanical stress caused by chaotic blood flow and proteolysis of the subendothelial matrix to facilitate EC migration during tumor angiogenesis. This leads to exposure of tumor cells to the flowing blood, resulting in so-called mosaic vessels, which consist of both endothelial and tumor cells [19]. CEC shedding may also occur in distant non-tumor-derived vessels, e.g. due to activation of the quiescent endothelial cells by tumor-induced increases of cytokine levels. The large variation in CEC outgrowth we observed on feeder-endothelium may reflect the variable contribution of shed, non-proliferating versus mobilized, proliferating CECs in different cancer patients.

Not all progressive cancer patients have increased CEC numbers in their peripheral blood. The growth speed of the tumor and its vasculature, the number, location and size of potential metastases, and the residence time of CECs in the circulation may influence the presence of CECs in peripheral blood. Our data suggest that factors other than primary tumor type determine CEC numbers. Finally, risk factors for cardiovascular co-morbidity may also confound our results [14].
An important question is whether CECs may serve as a target for the treatment of cancer. The absolute number of CECs in the peripheral blood is low (about 1-10 ECs per 10^6 mononuclear cells from full blood). Moreover, the contribution of bone marrow-derived EPCs to tumor progression in

humans is still controversial. However, preclinical models provide solid evidence that bone marrow derived CECs play a role in the maintenance and formation of blood vessels. EPCs can differentiate into mature endothelial cells in response to a variety of angiogenic growth factors produced either by the tumor cells, vessel wall ECs, vascular non-endothelial cells (pericytes, smooth muscle cells), inflammatory cells, bone marrow stromal cells, hematopoetic stem cells, or the EPCs themselves. A hallmark of EPCs is that they have a high proliferative potential [29]. EPCs have the potential to incorporate into regenerating vessels during ischemia [4;30]. They have clinical significance in restoring circulation [27;31] and in improving cardiac function after myocardial infarction [32]. Bone marrow derived endothelial and hematopoietic progenitor cells can fully support tumor growth, as shown in a bone marrow transplant model with Id1+/- Id3-/- compound mutant mice that lack stress angiogenesis and are tumor-resistant [5]. Id proteins are helix-loop-helix DNA binding proteins interfering with transcription factor binding. After receiving wild type bone marrow or VEGF-mobilized stem cells, tumor (vessel) growth was completely restored in Id1+/- Id3-/- compound mutant mice, resulting in the death of the host. Because CECs home to sites of angiogenesis they make excellent vehicles for anticancer treatment. Mice transplanted with modified bone marrow cells (containing ECs) loaded with truncated soluble VEGF receptor-2 exhibited diminished tumor growth [33]. Furthermore, injection of human CD34+ cells (including EPCs) into NOD/SCID mice with non-Hodgkin's lymphoma significantly increased tumor growth [34]. In a different model system selective tumor homing and subsequent anti-melanoma effect could be established by intravenous injection of endothelial cells over-expressing interleukin-2 [35]. Promising reports with therapeutic injection of bone marrow cells or bone marrow stem cells in patients with critical limb ischemia [36] or myocardial ischemia [37;38] provide initial evidence that also in human subjects therapeutic vasculogenesis may play a role. And our correlative data of CEC increase with tumor growth and CEC disappearance during leucopenia support a possible role for vasculogenesis in human cancer.

Reported absolute numbers of CECs in literature vary considerably, ranging from 1 to 10.000 CECs per milliliter of peripheral blood [6;8;9;17]. This is probably due to different definitions of CECs and the various techniques applied for the quantification of CECs. Consensus of definitions and quantification techniques is essential to allow comparisons.

In a murine model, Heissig et al. showed mechanisms by which endothelial progenitor cells and hematopoietic stem cells (HSC) are mobilized into the peripheral blood [39]. Several plasma factors (VEGF, PlGF, SDF-1α) induce MMP-9-mediated SCF/sKitL shedding from bone marrow stroma, essential for the migration of progenitor cells of endothelial and hematopoietic origin to the vascular niche of the bone marrow, subsequent differentiation, and mobilization into the circulation. In humans, increased plasma levels of VEGF are reported to correlate with CEC numbers in peripheral blood (after CABG and burn injury [11], myocardial infarction [40] and in cancer patients [17]) but the other factors in either plasma did not correlate with CEC mobilization [40] or were not tested. To see if this mobilization model could be applicable to our patient group we measured cytokines in the plasma. Although one of the factors that is known to mobilize stem/progenitor cells, PlGF, was elevated in cancer patients, no correlation was found between PlGF or any of the other factors tested and CECs. Several previously observed factors may account for these findings, such as a limited half-life of cytokines in the plasma [41] or variable correlations between local and systemic cytokine levels, resulting in absent [40;42] or inverse [43] correlation between stimulus (cytokine) and effect (i.e. CECs) in peripheral blood.

CEC levels increased in the hours following infusion of cytotoxic taxane-based chemotherapy. One possible explanation for this phenomenon would be that taxanes interact with cytoskeletal microtubules in mitotic angiogenic ECs in the vessel wall [44] resulting in detachment of tumor ECs into the circulation. Several vascular targeting agents act through interference with the tubulin cytoskeleton [45]. These effects are apparent within 24 hours. We are currently investigating the use of CECs as markers of vascular damage and treatment efficacy during treatment with tubulin interacting vascular targeting agents [46].

We found that in patients with active cancer, G-CSF treatment induced CEC increase, probably by mobilization from the bone marrow. These cells may home to sites of active angiogenesis, such as the tumor microenvironment. That colony-stimulating factor-induced EC homing is a biologically relevant phenomenon was shown in models for ischemia [30], tumor growth [4;47] and graft fallout endothelialization [48], in which growth factor treatment resulted in increased vessel growth. Our findings indicate that the use of G-CSF in cancer patients may have biological effects beyond reducing neutropenia and its complications. Further studies are warranted to investigate the effect of G-CSF and subsequent mobilization of CEC, which may enhance tumor angiogenesis in cancer patients, potentially leading to worse prognosis.

In conclusion, our findings demonstrate that CECs are increased in progressive cancer patients. The CECs are viable and subsets have a capacity to proliferate. The level of CECs can be modified both positively and negatively depending on the agents used. Further studies are warranted to determine whether CECs may serve as a target for anti-cancer therapy or as a biosensor of anti-(vascular) cancer treatment response.

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Progenitor marker CD133 mRNA is elevated in peripheral blood of cancer patients with bone metastases

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Abstract

Purpose: We examined whether RNA expression of CD133, a surface molecule expressed on progenitors from hematopoietic and endothelial lineages, and CD146, a pan-endothelial marker, are increased in the blood of cancer patients and whether these factors correlate with patient characteristics and are predictive factors of survival.

Experimental design: We developed a real-time quantification method (NASBA), to determine expression of CD146 and CD133 mRNA in the peripheral blood mononuclear cells of 131 progressive cancer patients, 37 healthy volunteers and five patients having received G-CSF. Overall survival and other clinicopathological parameters were obtained. Cox Proportional Hazards studies were performed.

Results: We show that patients with metastatic disease have a significant increase in CD133 mRNA ($p=0.03$), specifically patients with bone metastasis ($p<0.001$). Cancer patients with high CD133 mRNA expression, using a defined cut-off value, demonstrate a decreased survival compared to patients with low or undetectable CD133 expression (21% vs 45% cumulative survival, respectively, after 20 months; $p=0.01$). Among patients with metastasis to the bone cumulative survival was 22%, compared to 61% for patients with high or low CD133 levels ($p=0.004$). Multivariate analysis demonstrated that CD133 expression is an independent predictor for overall survival in patients with bone metastases. CD146 mRNA was not increased in patients with cancer, nor did it correlate with clinical parameters or survival.

Conclusion: CD133, but not CD146, mRNA expression is increased in cancer patients with metastatic disease, specifically with bone metastasis. In addition, CD133 mRNA expression appears to be an independent prognostic factor for overall survival.

Introduction

Blood vessel growth is critical for the progression and spread of tumors. Tumor vessel growth can arise by sprouting of pre-existing vessels or by incorporation and differentiation of bone marrow-derived stem cells into angiogenic vessels. Several studies suggest that these processes may be monitored in the peripheral blood by the quantification of endothelial and endothelial progenitor cells.

Circulating endothelial cells (CEC) are increased in patients with progressive cancer (1-4). These CEC's may originate from the bone marrow or may be shed from the tumor vasculature. Pre-clinical data show a significant increase of mature CEC's in the blood of tumor-bearing mice and a correlation of CEC numbers with tumor size (5). CEC levels can be altered by chemotherapeutic and anti-angiogenic treatment (6;7). The bone marrow-derived endothelial progenitor cells (EPC) express stem cell and endothelial markers and can be recruited by hypoxia and cytokines (8-12) and subsequently incorporate into sites of active neovascularization (11;13;14). Experiments with endothelial progenitor cells in mice indicate that these cells contribute to tumor angiogenesis and that inhibition of EPC mobilization results in retardation of tumor growth (6;12;15-18). Recent data also indicate that hematopoietic bone marrow-derived progenitor cells facilitate metastasis by initiating the pre-metastatic niche (19).

Therefore CEC's, and bone marrow-derived hematopoietic and endothelial progenitor cells are being evaluated as surrogate markers of blood vessel growth, tumor progression, and response markers for anti-angiogenic therapy in clinical trials (20-22). The clinical relevance of increased EPC's in human tumor-angiogenesis is, however, still controversial. Firstly, there are no reports of increased EPC's in the peripheral blood of patients with solid tumors, even though an increase has been reported in patients with myelodysplastic disorders (23), multiple myeloma (24) and infantile hemangioma (25). Secondly, increased CD133-positive cells have not been implicated as a predictor of poor prognosis in patients with cancer. Nevertheless, a recent study with six cancer patients indicated that EPC do contribute to tumor neo-angiogenesis. Bone-marrow derived progenitor cell differentiation into tumor endothelium was found to vary from 1 to 12% (14). This study indicates that there may be large differences in the contribution of EPC's in human cancers and that xenograft models may overestimate the role of EPC's in tumor neovascularization.

We hypothesized that increased CD133 mRNA or CD146 mRNA expression in the peripheral blood compartment correlates with patient survival. In order to test this hypothesis we developed a real-time mRNA quantification assay, based on Nuclear Acid Sequence Based Amplification (NASBA) technology for high throughput analysis of CD133, a marker of bone marrow-derived precursor cells and of CD146, a pan-endothelial cell marker. This method is highly reproducible and can be performed on samples that have been frozen and therefore may be a good alternative for the flow cytometry (1;5-7;25-28) and magnetic bead assays (3;29-32) for monitoring CEC, and hematopoietic and endothelial progenitor levels in clinical trials.

We quantified these markers in the peripheral blood mononuclear fraction of 131 patients with solid tumors and 37 healthy controls. Our results show a significant increase of CD133 mRNA in patients with metastasis to the bone, regardless of their primary tumor. We further observed that CD133 mRNA expression correlates with clinical characteristics and high expression is associated with a greater risk of death.

Patients and methods

Characterization of study patients and healthy volunteers.
Between February 2003 and August 2005, blood samples were collected from new or relapsed patients visiting the outpatient clinic of the Department of Medical Oncology, or patients hospitalized at the University Medical Center in Utrecht, The Netherlands.

Tumor types are summarized in Table 1. In addition, five healthy subjects, who volunteered as bone marrow donor and were receiving G-CSF, were included as in vivo positive controls for CD133 mRNA (33). Blood samples were also acquired from six cancer patients before, during and after infusion of gemcitabine and cisplatin (0, 2, 4, 8, 24 and 48 hours) for in vivo positive controls of CD146 mRNA. A previous study revealed that infusion of gemcitabine and cisplatin increased the numbers of CD146-positive cells found in peripheral blood (unpublished result). All prostate cancer patients had hormone refractory disease and were on maintenance hormonal therapy. Other cancer patients were either naïve for cancer treatment or did not receive chemo- or radiotherapeutic treatment for at least one month before blood collection. Healthy subjects of similar age (n=37) served as controls.

Table 1 - Characteristics of 131 untreated cancer patients

Age (years)	
Mean ± SD	63.5 ± 9.8
Range	31 - 88
Sex	
Male	107 (82%)
Cancer patients	
Renal cell carcinoma	43 (34%)
Prostate cancer	34 (26%)
Head and neck cancer	27 (21%)
Colorectal cancer	27 (21%)
Metastasis	
No	14
Yes	111
Bone	50 (38%)
Liver	27 (21%)
Lung	37 (28%)
Lymph node	36 (28%)
Other	28 (20%)
Unknown	6
Survival (months)	
Median	12.5
Interquartile range [25-75%]	5.3 - 27.6

Abbreviations: SD, standard deviation

The following variables were obtained from the medical records of the 131 patients: sex, age, previous anti-tumor treatment, location of metastasis (by radiological tests and physical examination within 1 month of blood draw), hemoglobin and leucocyte levels, platelet count, alkaline phosphatase, lactate dehydrogenase, and PSA levels. Patient survival was noted after a median observation period of 16 months (range 3-33 months).

Blood collection and isolation of nucleic acids.

Peripheral blood mononuclear cells were isolated from blood of patients, using a Vacutainer CPT Cell Preparation Tube with Sodium Citrate (Becton Dickinson, Mountain View, California), according to the manufacturer's recommendations. The mononuclear cells were washed once in serum-free RPMI medium (Gibco BRL, Invitrogen, Breda, The Netherlands) and resuspended in 520 µl serum free medium. 15 µl of PBMC's in medium were diluted 5-fold and analyzed using the Cell Dyn 1700 (Abbott Laboratories, Abbott Park, Illinois) to establish white cell counts, and red blood cell and platelet contamination. 500 µl PBMC's in medium were added to 4500 µl L6 lysis buffer (containing guanidine thiocyanate, Triton X-100, EDTA and Tris-HCl), aliquoted per ml, and stored directly at –80°C until further use.

For nucleic acid isolation we added approximately 300 000 MNC's from the lysed-PBMC solution and filled the volume to 1 ml with lysis buffer. The nucleic acid now present in the lysis buffer was further purified with the method described by Boom et al. (34). The isolated nucleic acid was eluted in 50 µl elution buffer. Usually a dilution was made such that the equivalent of 10,000 cells/5 µl was used as input in NASBA amplification reactions.

Nucleic acid sequence-based amplification (NASBA)

For the quantification of CD133 and CD146 mRNA we used a one-tube, real-time detection and quantification method based on nucleic acid sequence-based amplification (NASBA) (35-37). For patient samples U1A DNA was also amplified as internal control for the cell input, amplification and isolation for each sample.

Standard NASBA nucleic acid amplification reactions were performed in a 20 µl reaction volume and contained: 40 mM Tris-pH 8.5, 90 mM KCl, 12 mM $MgCl_2$, 5 mM dithiotreitol, 1 mM dNTP's (each), 2 mM rNTP's (each), 0.2 µM primer P1 (Invitrogen, Breda, The Netherlands), 0.2 µM primer P2 (Invitrogen, Breda, The Netherlands), 0.05 µM molecular beacon (Eurogentec, Maastricht, The Netherlands), 375 mM sorbitol, 0.105 µg/µl bovine serum albumin, 6.4 units AMV RT (Seikagaku, Chiyoda-ku Tokyo, Japan), 32 units T7 RNA polymerase (Invitrogen, Breda, The Netherlands), 0.08 units RNase H (Gibco BRL, Invitrogen, Breda, The Netherlands) and input nucleic acid. For the amplification of RNA, the mixture (without the enzymes) was heated to 65°C for 3 minutes in order to denature any secondary structure in the RNA and to allow the primers to anneal. In the case of DNA, 2 units of *Msp* I (40 U/µl, Roche Diagnostics Nederland BV, Almere, The Netherlands) were added and the mix was incubated at 37°C for 15 minutes, followed by denaturation at 95°C for 3 minutes. After cooling the mixtures to 41°C the enzymes were added. The amplification took place at 41°C for 90 min in a thermostated fluorimeter (RetinAlyzer Primagen, Amsterdam, The Netherlands). The molecular beacons used in these experiments are labeled with 6-fluorescine (6-FAM) (fluorescent label) at its 5′ end and with {[4-(dimethylamino)phenyl] azo}benzoic acid (DABCYL) (quencher) at its 3′ end. Primer and beacon sequence of CD146, CD133 and U1A are shown in table 2.

Table 2 - Primer and beacon sequences

Name	Primer	Sequence 5′-3′
CD133	P1	*AATTCTAATACGACTCACTATAGGG*AAGAACAGGGATGATGTTGGGTCTCA
	P2	TTTCAAGGACTTGCGAACTCTCTTGA
	MB	**CGATCC**AAGGACAAGGCGTTCACA**GGATCG**
CD146	P1	*AATTCTAATACGACTCACTATAGGGAGAGGGG*CTAATGCCTCAGATCGATGTA
	P2	CCCCCGTCTCGTAAGAGCGAA
	MB	**CGATCC**TTAAGTCAGATAAGCTCCC**GGATCG**
U1A	P1	*AATTCTAATACGACTCACTATAGGG*AGAGGCCCGGCATGTGGTGCATAA
	P2	TGCGCCTCTTTCTGGGTGTT
	MB	**CGCATGC**TGTAACCACGCACTCTCCTC**GCATGCG**

The T7 promoter moiety of primer P1 sequences is shown in italics, the stem sequences of the molecular beacon probes are shown in bold.

The reaction mixtures were excited at 485 nm and fluorescence was measured at 530 nm and the fluorescent signal of the molecular beacon probe was measured every 45 seconds. Readings were normalized to the background of a reaction mixture containing TE (10mM Tris, 1 mM EDTA) instead of template.

The amount of target sequence present in samples was calculated using a standard curve generated from in vitro RNA (CD133 and CD146) or plasmid DNA (U1A) standards that indicated the

relation between time-to-positivity (TTP) and input amount. The standards were used in serial dilutions ranging from $5x10^5$ to 67 copies (CD133), $1x10^6$ to 100 copies (CD146), or $3x10^5$ to 500 (U1A) copies per reaction mixture. Human umbilical endothelial cells (HUVEC) and the immortalized human microvascular endothelial cell-line (HMEC-1) (38) were used as positive control for CD146 mRNA. The NT-2 teratocarcinoma cell line (39) was used as a positive control for CD133 mRNA. The technical lowest detection for the CD133 assay (67 copies in vitro RNA) corresponds to 25 spiked NT-2 cells. The minimal technical detection for the CD146 assay (100 copies in vitro RNA) corresponds to 33 NT-2 cells.

All amplifications were performed in duplicate. The average of these duplicate amplifications was considered as the value for the sample. The concentration of target sequence in the samples was expressed as log copies per reaction mixture. If the difference between duplicate amplifications was > 0.5 log value, the amplification for that sample was repeated.

Cell Culture and spike experiment

The NT-2 teratocarcinoma cell line was cultured in RPMI medium (Gibco BRL, Invitrogen, Breda, The Netherlands), supplemented with penicillin, streptomycin, glutamate and 5% fetal calf serum. HMEC-1 was cultured with M199 (Gibco BRL, Invitrogen, Breda, The Netherlands), supplemented with penicillin, streptomycin, glutamate, 10% fetal calf serum and 10% human serum. Cells were grown in 75 cm^2 flasks to confluence, trypsinized and split 1:3. Three days prior to the flow cytometry experiments medium was replaced with phenol free medium to reduce autofluorescence.

For the spike experiments NT-2 and HMEC-1 cells were resuspended as single cells in PBMC's and quantified using a Bürker-Turk hemacytometer. For the NASBA experiments, 0, 10, 30,100, 300 or 1000 cells were spiked in 100 µl serum free RPMI medium containing 200 000 PBMC's. Subsequently cells were lysed in 900 µl L6 buffer and frozen at -80°C.

For flow cytometry an equivalent of the spiked cells in the NASBA assay were added to $5x10^5$ or $1x10^6$ cells. 200 000 events were acquired for analysis (FACS Calibur, Becton Dickinson, San Jose, California). For NT-2 and HMEC-1 spike experiments the CD133-2 APC conjugated antibody (Miltenyi Biotec, Bergisch-Gladbach, Germany) and the P1H12 PE conjugated antibody (Chemicon, Temecula, CA) were used. The results were analyzed using CellQuest software (BD Biosciences, San Jose, California).

CEC quantification

For CEC isolation 0, 10, 30, 100 or 300 HMEC-1 cells were spiked in 1 ml peripheral blood and diluted with 3 ml physiological salt. The isolation of CECs from whole blood was performed as previously described (3;29;31;32;40). Briefly, magnetic beads (Dynal M450 IgG_1, Dynal AS, Oslo, Norway) conjugated to an anti-CD146 monoclonal antibody (Kordia Life Sciences, Leiden, The Netherlands) bind CD146-expressing cells which allows magnetic separation from unbound, CD146-negative cells. Viable, CD146-positive cells can subsequently be quantified by counting CalceinAM (Molecular Probes, Eugene, Oregon) fluorescently labeled cells in a Nageotte hemacytometer.

Statistical analysis

Median copy numbers of CD133 and CD146 mRNA and interquartile ranges were compared between patients with different cancer types and controls. The same was done comparing median copy numbers between patients with and without metastases at different sites, and patients of different sex. Differences were tested using the Mann-Whitney test.

To estimate correlations between CD133 and CD146 mRNA copy number and age, leucocyte and hemoglobin levels, platelet count, alkaline phosphatase, lactate dehydrogenase and prostate specific antigen the Spearman's correlation coefficient was used.

A Receiver Operating Characteristic (ROC) was constructed by calculating the proportion of positive tests among those who died ('sensitivity') and the proportion of positive tests among those who survived ('1-specificity') for each cut-off point of CD133 levels. The cut-off point with the combination of highest sensitivity and specificity was 200 copies (sensitivity and specificity of 64% and 71%, respectively, in patients with bone metastasis). This cut-off point was used in all subsequent analyses.

Differences in survival between cancer patients with CD133 levels below and above 200 copies were evaluated according to the method of Kaplan and Meier. Kaplan-Meier curves were truncated when fewer than five patients are left at risk in one or both arms of the curve. Differences in Kaplan-Meier curves were tested using the Log-rank statistic.

To identify independent factors influencing survival, univariate and multivariate risk factor assessments were performed using the Cox Proportional Hazards model. This was first done for the total group of patients and then for patients with bone metastases only. Hazard ratios with their corresponding 95% confidence intervals were estimated. Variables that showed a p-value <0.10 in the univariate analysis were selected and entered into a multivariate model.

The laboratory parameters hemoglobin, leucocyte and platelet count were not determined in four patients, lactate dehydrogenase and alkaline phosphatase not in 39 and 27 cases, respectively. Correlative testing and Cox Proportional Hazards analysis for these variables were performed with fewer than 131 cases.

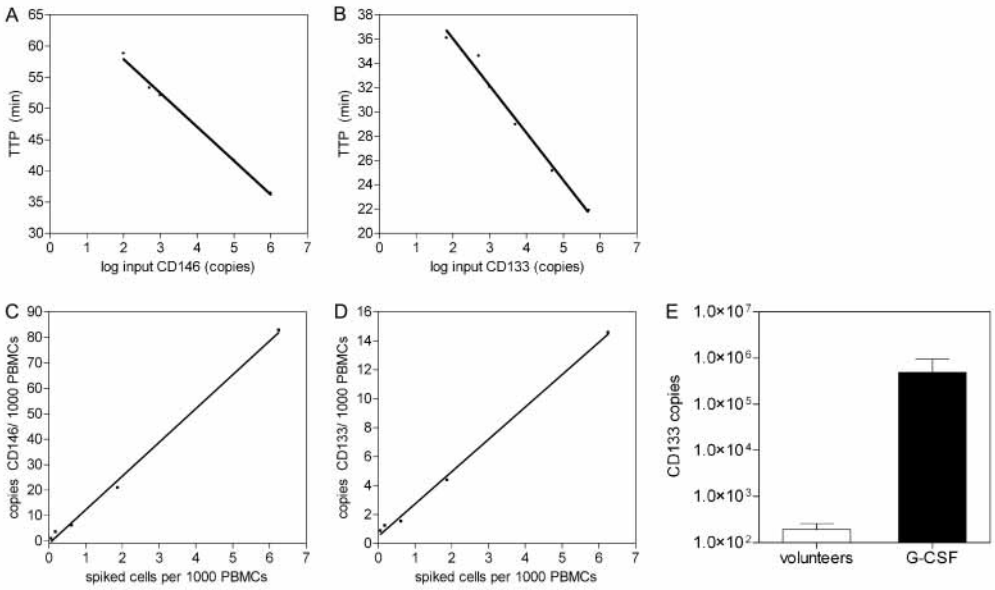
All analyses were performed using SPSS software (version 12.0.1) and p-values below 0.05 (two-sided) were considered statistically significant. All error bars depicted are standard errors of the mean.

Results

Validation of CD133 and CD146 real-time mRNA amplification assay

In vitro translated RNA of CD146 and CD133 cDNA was added at different concentrations to a background of nucleic acids from mononuclear cells to achieve a standard curve for input in relation to time to positivity (TTP; Figure 1A and B). The sensitivity and linearity of the CD133 assay was tested using NT-2 teratocarcinoma cells spiked in a background of human PBMC's (Figure 1C). There was a strong linear correlation between input and copies CD133 measured (r=0.99, P<0.001) in PBMC's. The

Figure 1A, 1B, 1C, 1D, 1E



Validation of CD133 and CD146 real-time mRNA amplification. CD133 (A) and CD146 (B) standard curve with in vitro translated RNA. NT-2 cells (C) or HMEC-1 (D) cells spiked in a background of PBMC's. CD133 mRNA measured in PBMC's of healthy volunteers (n=37) and healthy subjects treated with granulocyte-colony stimulating factor (G-CSF; n=5), Mann-Whitney Test P=0.0001 (E).

sensitivity of the test was approximately 0.05 cells per 1000 MNC's or 50 cells per ml peripheral blood (assuming approximately $1x10^6$ lymphocytes and monocytes per ml peripheral blood). The sensitivity and linearity of the CD146 assay was determined by measuring HMEC-1 cells spiked in a background of human PBMC's. The CD146 assay had a strong linear correlation between input and CD146 copies measured (r=1.00, P<0.001; Figure 1D). The sensitivity for the CD146 assay was approximately 0.05 cells per 1000 PBMC's or 50 cells per ml peripheral blood.

Next we compared the sensitivity of CD146 and CD133 mRNA quantification to other commonly used detection assays such as flow cytometry and the magnetic beads-assay. NT-2 cells were quantified by flow cytometry and NASBA after they were spiked in a background of human PBMC's. The sensitivity of both assays was comparable (0.18 cells per 1000 PBMC's for the NASBA assay and 0.15 cells per 1000 PBMC's for flow cytometric quantification). We found very strong correlation between quantification of spiked cells using NASBA and flow cytometry (r=0.96, P<0.001).

HMEC-1 cells were spiked in peripheral blood or PBMC's and were quantified subsequently by magnetic bead extraction, FACS and NASBA. There was a strong correlation between endothelial cell quantification using magnetic bead isolation and NASBA (r=0.96, P<0.001), and between FACS analysis and NASBA (r=0.99, P=0.005). Using magnetic bead isolation, the lower limit of detection is approximately 10 spiked EC's per ml blood, which is approximately 15-fold more sensitive than NASBA and flow cytometry.

In vivo validation of real-time mRNA amplification assays

To assess our assay in vivo, we analyzed CD133 expression in the PBMC's of five healthy subjects who volunteered as bone marrow donor and had received granulocyte-colony stimulating factor (G-CSF) for stem cell mobilization (Figure 1E). There was a 2500-fold increase in CD133 mRNA expression in the PBMC's of the bone-marrow donors compared to healthy controls ($p<0.001$).

To evaluate the CD146 assay in vivo, we isolated CD146-positive endothelial cells from six patients receiving gemcitabine and cisplatin using an antibody against CD146 bound to magnetic beads, and quantified all viable EC's by counting calcein-AM positive rosetted bead-bound cells under a fluorescence microscope. We subsequently measured CD146 mRNA expression in the PBMC's and found a significant correlation ($r=0.89$, $P=0.019$) between these two methods.

Bone metastasis is a determinant for high CD133 expression

The mononuclear fraction of peripheral blood from 131 cancer patients and 37 volunteers was tested on CD133 mRNA, CD146 mRNA and U1A DNA as internal reference for cell input, amplification and isolation. Levels of CD133 and CD146 were detectable in respectively 45% and 16% of the cancer patients and in 38% and 22% of the controls, respectively. There was no significant difference in expression of CD133 or CD146 mRNA between the total cancer patient group and the controls ($P=0.395$ and $P=0.311$ respectively). However, when analyzed according to tumor type, patients with prostate cancer had statistically significantly increased CD133 mRNA levels compared to healthy controls (median 154 copies with interquartile (IQ) range 0-1088 and median 0 copies with IQ range 0-281, respectively; $p=0.033$). Patients with renal cancer, head and neck and colorectal cancer did not have an increase in CD133 expression when compared to healthy controls (CD133 results shown in Table 3). Further analysis revealed that bone involvement was related to high CD133 levels in the peripheral blood. Patients with bone metastasis (n=50) had significantly elevated CD133 levels compared to patients without bone metastasis (median 163 copies with IQ range 0-721 and median 0 with IQ range 0-41, respectively; $p=0.0002$). As most

Table 3 - CD133 RNA copies according to tumor type and site of metastasis

tumor type	reference	median CD133 copy number + IQ-range tumor type	% subjects with CD133 copy > detection limit	median CD133 copy number + IQ-range reference	% subjects with CD133 copy > detection limit	comparison CD133 copies between tumor type and reference, P (MW)
all cancers (n=131)	controls (n=37)	0 [0-265]	45%	0 [0-281]	38%	0.40
RCC (n=43)	controls (n=37)	0 [0-270]	49%			0.45
prostate (n=34)	controls (n=37)	153 [0-1088]	61%			0.03
H&N (n=27)	controls (n=37)	0 [0-208]	37%			0.94
CRC (n=27)	controls (n=37)	0 [0-76]	37%			0.62
site of metastases						
all metastases (n=110)	no metastases (n=14)	53 [0-323]	51%	0 [0-15]	20%	0.03
bone (n=50)	no bone (n=81)	163 [0-721]	63%	0 [0-41]	37%	0.0002
liver (n=27)	no liver (n=103)	0 [0-76]	37%	28 [0-310]	50%	0.09
lung (n=37)	no lung (n=93)	0 [0-197]	41%	18 [0-435]	50%	0.96
lymph (n=36)	no lymph (n=94)	0 [0-584]	42%	0 [0-243]	49%	0.15
other (n=20)	no other (n=110)	0 [0-297]	35%	0 [0-245]	49%	0.98

Abbreviations: IQ, interquartile range [25-75%]; MW, Mann-Whitney Test

patients with bone metastasis were prostate cancer patients (n=32) we also analyzed patients with renal cell carcinoma, head and neck and colorectal cancer with metastasis to the bone separately (n=18), and similarly found a significant increase in comparison with patients without bone lesions (P=0.034).

CD133 mRNA expression correlates with markers of disease progression.

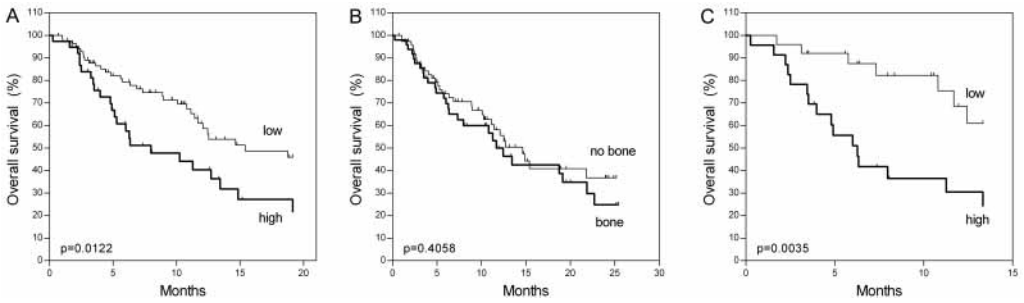
There was no relationship of CD146 or CD133 mRNA expression with patient age or sex. There was a negative correlation between CD133 and hemoglobin level (r=-0.21 with P=0.019, n=126), but none with leucocyte levels, platelet count, lactate dehydrogenase, or alkaline phosphatase. In the 34 patients with prostate cancer we found a positive correlation between number of CD133 copies and prostate specific antigen (r=0.37, P=0.040). CD146 mRNA expression had no relation with any laboratory parameter tested.

High CD133 mRNA expression is associated with decreased survival

Patients had a median follow-up of 16 months after blood draw. Of the 131 patients, 64 patients were alive, 62 patients died due to disease progression, 3 patients had a non-cancer related death, and 2 patients were lost to follow-up.

Kaplan-Meier estimates of overall survival using a cut-off of 200 copies CD133 mRNA per 10000 copies U1A DNA, showed a significant decrease (P=0.012) in survival in patients with high CD133 (more copies than the cut-off) compared to patients with low CD133 expression (lower or undetectable levels). Median survival for patients with low CD133 expression was 15.4 months while patients with high CD133 levels had a median survival of 8.0 months (Figure 2A). After approximately 20 months of follow-up the cumulative survival of patients with low and high CD133 copies was 45% and 21%, respectively. The survival curves of patients with or without metastases to the bone were roughly overlapping (Figure 2B).

Figure 2A



Patients with elevated CD133 mRNA expression have decreased survival. The Kaplan-Meier survival curves for the 131 cancer patients evaluated according to dichotomized CD133 mRNA expression (>200 copies or "high" versus <200 copies or "low") (A). Survival curves for all 131 cancer patients evaluated according to the presence or absence of bone metastasis (B). Survival curves of the 50 patients with bone metastasis, evaluated according to high or low CD133 mRNA expression (C).

Many patients with bone metastasis had increased CD133 mRNA expression compared to healthy volunteers. The range of CD133 mRNA expression varied from 0 to approximately 1000 copies. We evaluated whether survival in this group was also affected by high or low CD133 mRNA expression.

Table 4 - Results of univariate and multivariate Cox Proportional Hazards Analysis

Univariate	all patients			bone metastasis		
Variable	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
Sex	1.19	0.63-2.24	0.58	3.30	0.75-14.55	0.12
Age	1.00	0.97-1.03	0.90	0.98	0.93-1.03	0.45
Hemoglobin	0.58	0.47-0.72	<0.0001	0.63	0.45-0.88	0.006
Leucocytes	1.09	1.02-1.18	0.02	1.03	0.93-1.14	0.56
Platelets+	1.06	0.96-1.18	0.26	1.10	0.80-1.51	0.57
Lactate dehydrogenase+	1.07	1.04-1.10	<0.0001	1.33	0.10-1.79	0.05
Alkaline phosphatase+	1.46	1.20-1.77	0.0002	1.59	1.14-2.23	0.007
Treatment-naive	0.40	0.24-0.67	0.0004	0.41	0.18-0.93	0.03
Metastasis	1.03	0.59-1.81	0.92			
Bone metastasis	1.22	0.74-2.01	0.44			
CD133 copies (<200; >200)	1.77	1.06-2.94	0.03	2.90	1.33-6.32	0.007
Multivariate	all patients			bone metastasis		
Variable	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
Hemoglobin	0.73	0.54-1.00	0.05	0.70	0.36-1.37	0.30
Leucocytes	1.01	0.87-1.18	0.86			
Lactate dehydrogenase+	1.07	1.04-1.11	<0.0001	1.61	1.03-2.53	0.04
Alkaline phosphatase+	1.16	0.82-1.63	0.40	0.96	0.56-1.63	0.87
Treatment-naive	0.38	0.18-0.79	0.009	0.71	0.12-4.14	0.70
CD133 copies (<200; >200)	1.83	0.80-4.18	0.15	9.73	1.08-87.49	0.04

All recorded clinical characteristics were entered in a univariate analysis together with CD133 mRNA expression, dichotomized as high (>200 copies) versus low (≤200 copies). All variables included in the multivariate model showed a p<0.10 in the univariate analysis. In patients with metastasis to the bone, high CD133 copies were identified as an independent predictor of overall survival. Abbreviations: HR, hazard ratio; CI, confidence interval; + Parameter divided by 100 units

After approximately 13.5 months the cumulative survival was 61% in the patients below the cut-off, as compared to 22% of patients above the cut-off (P=0.004; Figure 2C).

CD133 expression is an independent predictor for survival

In the upper part of Table 4, hazard ratios are presented for patient characteristics as determined in the univariate analysis. When the total group of cancer patients was evaluated low hemoglobin levels, high levels of leucocytes, lactate dehydrogenase and alkaline phosphatase, naivety for cancer treatment and a high number of CD133 copies were associated with increased mortality (p<0.10) and included in the multivariate model. When only patients with bone metastases were evaluated, the same patient characteristics, except for leucocytes were selected for inclusion in the multivariate model.

In the multivariate models (lower part Table 4), only lactate dehydrogenase and naivety for cancer treatment appeared independent, statistically significant predictors of survival (hazard ratio (HR) 1.07 per 100 units and 0.38, respectively; 95% confidence interval (95% CI), 1.04 to 1.11 and 0.18 to 0.79, respectively), whereas hemoglobin levels showed a borderline effect (p=0.051). In patients with bone metastases, high CD133 mRNA expression (at least 200 copies mRNA per 10 000 copies U1A DNA) and lactate dehydrogenase were both significant and independently related to survival. In this group, the hazard ratio for death in patients with high CD133 mRNA expression was 9.73 (95% CI: 1.08-87.50), while the hazard ratio for lactate dehydrogenase was 1.61 per 100 units (95% CI: 1.03 to 2.53).

Discussion

Endothelial cells and endothelial precursor cells are considered hopeful candidates for monitoring angiogenesis, tumor growth, and treatment response in cancer patients. We therefore developed a real-time NASBA, to quantitatively monitor the mRNAs of stem cell marker CD133 and the pan-endothelial cell marker CD146 in the mononuclear fraction of cancer patients. NASBA is a 96-well based method of nucleic acid amplification highly receptive to standardization and currently a FDA-approved method to determine viral load in HIV-1 infected patients (36;37;41). We show that CD133 mRNA expression is increased in patients with metastasis, more specifically bone metastasis, independent of primary tumor type. Furthermore, an exploratory analysis showed that high levels of CD133 mRNA are associated with decreased survival in patients with bone metastasis. CD146 mRNA was not increased in this group of cancer patients nor showed a relationship with clinical parameters tested.

Experimental data have clearly established a role for bone marrow-derived progenitors in tumor progression (12;15;17;18). Recently, VEGFR1-positive hematopoietic progenitor cells have also been linked to the regulation of metastasis (19). A subset of these VEGFR1-positive cells was CD133 positive. Clusters of these VEGFR1-positive cells in human tissues prepare for the arrival of metastatic tumor cells. These findings indicate that progenitor cells may play a role at different levels of disease progression: initiation of metastasis and disease progression. However, despite the convincing preclinical evidence, there are few clinical studies demonstrating a role for progenitor cells. Tumors of six patients who developed cancer after having received bone-marrow transplantation from donors of the opposite sex, showed varying (1-12%) incorporation of EPC's in the tumor vasculature (14).

A large number of human studies have measured CEC and EPC numbers in the circulation as a surrogate marker for tumor progression (1-4;20;24-26;42;43). Only few studies have investigated CD133 as a marker in cancer patients. Sussman et al. measured with real-time PCR mRNA of CD133, VEGFR-2, Tie-2, and VE-Cadherin in the peripheral blood cells of 19 patients with breast cancer and 11 controls and only found a significant increase in Tie-2 mRNA in patients with infiltrating carcinomas as opposed to benign disease (44). Rabascio et al. assessed the same parameters as Sussman et al. in the blood of 84 cancer patients (comprising 7 different types of cancer) and 14 controls. They found that VE-cadherin was the only marker that was increased in cancer patients. VE-cadherin was particularly increased in hematological malignancies (2). Neither study shows a significant increase in CD133 mRNA. These findings are in agreement with our results for the total cancer group, but we did find a significantly increased CD133 mRNA in cancer patients with bone metastasis. A possible explanation for the negative results on progenitor cell detection in patient studies are probably caused by the assay detection limit. The amounts of CEC's present in cancer patients by flow cytometry are reported to be between 3,900-100,000 cells/ml (1;45-47). A PCR-based assay of CEC's described the lowest level of detection to be approximately 500 cells per ml blood (4). EPC levels are two-orders of magnitude lower than CEC's, constituting approximately between 0-500 cells/ml, in untreated patients and controls (1;46). Rabascio and Sussman do not mention the detection limit of CD133-positive cell number in their PCR-based assay. In our spike experiments we could accurately quantify 50 spiked cells expressing CD133 or CD146 per ml of blood, and the lowest limit of detection of our NASBA was 25 and 33 cells, respectively.

We hypothesize that the close proximity of cancer to the bone marrow can directly recruit and mobilize CD133-positive endothelial and hematopoietic progenitors to facilitate tumor growth. Our findings are also in agreement with the novel concept that bone marrow-derived progenitors facilitate metastasis. In an exploratory analysis we found that among patients with bone metastasis those with high CD133 copy numbers had a significantly shorter median survival than patients with low CD133 copy numbers. Whether high CD133 copy number is correlated with enhanced growth of primary tumor or metastatic lesions, and/or enhanced tumor-angiogenesis, needs to be established in large, prospective and controlled clinical trials.

CD133 expression also has been demonstrated on human cancer stem-cells (48;49). This subpopulation of cancer cells is present in cancer tissue and has not been demonstrated in patients' blood. It is not possible to ascertain which specific cell type is positive for CD133 of CD146, as real-time mRNA quantification is performed on a mixed population of PBMC's and does not allow typing of individual cells. This is a limitation of real-time mRNA quantification assays, compared to other quantification methods such as flow cytometry and the magnetic bead assay. A recent flow cytometric study indicates that all bone-marrow CD133-positive cells co-express hematopoietic stem-cell marker CD34 (50). Preliminary results from our laboratory showed that 85% of CD133-positive cells by flow cytometry also co-expressed hematopoietic marker CD34 (Mehra et al. unpublished data). However, using mRNA amplification it is not possible to exclude the possibility that CD133 mRNA detected in our study might be also expressed by a different cell type than endothelial or hematopoietic precursors.

In our study CD146 mRNA expression did not correlate with presence of cancer, survival or clinical data. Recently, Fürstenberg et al. published an increase of CD146 mRNA using real-time PCR in 11 patients with newly diagnosed breast cancer (4). While sensitivity of both assays is in the same order of magnitude, the discrepancy between our and their study could be explained by the difference in tumor type: breast cancer versus patients with cancers of the kidney, prostate, head and neck, colon and rectum.

In summary, we have shown that CD133 levels are increased in patients with metastases, specifically bone metastases. Exploratory analysis of the relation between levels of CD133 and survival provides a rationale to pursue CD133 as a potential marker of cancer spread and progression. Real-time mRNA amplification of CD133 is a reliable and easy method that may be used to monitor large patient groups. Further validation of these markers requires homogenous, large patient populations with access to clinical data and adequate follow-up.

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Phase I pharmacokinetic and pharmacodynamic study of the oral protein kinase C β-inhibitor enzastaurin in combination with gemcitabine and cisplatin in patients with advanced cancer

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Abstract

Purpose: Enzastaurin targets the PKC and PI3K/AKT pathways to reduce tumor angiogenesis and cell proliferation and to induce cell death. A phase I trial was conducted to evaluate the feasibility of combining enzastaurin with gemcitabine and cisplatin.

Methods: Patients with advanced cancer received a 14-day lead-in treatment with oral enzastaurin followed by subsequent 21-day cycles of daily, oral enzastaurin (350 mg qd to 500 mg bid) combined with gemcitabine on days 1,8 (1000 or 1250 mg/m^2) and cisplatin on day 1 (60 or 75 mg/m^2). Circulating endothelial cell (CEC) numbers and CD146 and CD133 mRNA expression were evaluated as pharmacodynamic markers.

Results: Thirty-three patients (median age 58) were enrolled in 7 dose levels. The maximum tolerated dose was not identified. Two dose-limiting toxicities (grade 2 QTc interval prolongation and grade 3 fatigue) were reported. Other toxicities included grade 3/4 neutropenia (3/6 patients) and thrombocytopenia (1/6 patients), and grade 3 leukopenia (2 patients) and fatigue (5 patients). Enzastaurin bid ($\geqslant$250 mg) resulted in more discontinuations and low-grade toxicities. In the combination, enzastaurin exposures decreased slightly but remained above the target of 1400 nM while gemcitabine/cisplatin exposures were unaltered. Three patients (9.1%) had partial responses and 13 (39.4%) had stable disease. Measurement of CEC numbers and CD146 and CD133 mRNA expression did not contribute to decision-making on dose escalation.

Conclusions: Recommended phase II dose is enzastaurin 500 mg qd, gemcitabine 1250 mg/m^2, and cisplatin 75 mg/m^2. This regimen is well tolerated with no significant alterations in the pharmacokinetic parameters of any drug.

Introduction

Angiogenesis, or formation of new vasculature, is essential for the continued growth of tumors (1-4). Hence, various antiangiogenic agents, especially those targeting vascular endothelial growth factor (VEGF) signaling, are currently being tested for cancer treatment (5,6).

Enzastaurin, an acyclic bisindolylmaleimide (7), is a potent selective serine/threonine kinase inhibitor that affects the cellular signaling pathways essential for growth, proliferation and apoptosis (8).

Selective inhibition of the protein kinase C (PKC) pathway with enzastaurin affects *in vitro* and *in vivo* angiogenesis, since PKC, an important regulator of cell growth, also mediates the VEGF-receptor signaling cascade (9-11). Enzastaurin also induces tumor cell apoptosis and reduces proliferation by inhibiting the PI3K/AKT pathway (12).

In preclinical pharmacological studies in rats and dogs, enzastaurin showed antitumor and antiangiogenic activity (13,14) and was well tolerated. In some dogs given high daily doses (exposures higher than those expected to occur in most patients), QT and QTc prolongation was observed after 5 weeks, and cataracts were seen after 13 weeks (personal communication, Horton N, February, 2006). Monotherapy in humans was well tolerated up to 700 mg,15 and no significant

toxicities were observed in a phase I study of cancer patients (16). Moreover, the oral bioavailability and pharmacokinetics of enzastaurin enables a dose schedule that results in chronic continuous exposure, the most promising strategy for antiangiogenic drug use (17).

Based on preclinical studies showing significant antitumor effect when antiangiogenic drugs were added to cytotoxic chemotherapy (18,19), and since the toxicity profiles of enzastaurin and cytotoxic agents are nonoverlapping, we initiated a phase I study of enzastaurin and gemcitabine/cisplatin to determine the recommended phase II dose and to evaluate safety, pharmacokinetics, and pharmacodynamic biomarkers.

In targeted therapies, biomarker development for treatment efficacy is essential (20), since the relation between toxicity and antitumor effect is less defined (21-24). Quantification of circulating endothelial cells (CECs) and endothelial (progenitor)-specific mRNA expression (CD133 and CD146) in the peripheral blood mononuclear cells (PBMCs) have been recognized as possible biomarkers of cancer activity (4,25-30). Because enzastaurin inhibits VEGF signaling, we hypothesized that angiogenesis-related biomarkers may be useful to evaluate treatment efficacy during enzastaurin administration.

Methods

Patients' eligibility criteria

A phase I dose-escalating study of enzastaurin in combination with gemcitabine/cisplatin was performed at the Antoni van Leeuwenhoek hospital/Netherlands Cancer Institute (Amsterdam, the Netherlands) and at the University Medical Center Utrecht (Utrecht, the Netherlands). Eligibility criteria included: histologically or cytologically diagnosed advanced or metastatic cancer as defined by the Response Evaluation Criteria in Solid Tumors (31); World Health Organization performance status of 0-2; ⩾18 years of age; predicted life expectancy ⩾3 months; and adequate renal, hepatic, and haematopoietic function. Patients must have discontinued all previous anticancer or other investigational therapies (except for luteinizing hormone-releasing hormone analog therapy for men with hormone-refractory prostate cancer) ⩾4 weeks before study entry, or 6 weeks in case of pretreatment with mitomycin-C or nitrosoureas or nonsteroidal antiandrogens therapy. Patients must have recovered from the acute effects of prior therapy. Exclusion criteria included: hearing loss and/or neuropathy (⩾grade 2 per common toxicity criteria [CTC]); symptomatic central nervous system metastasis; concomitant acute infection or leukemia; baseline electrocardiogram (ECG) abnormalities, including QTc prolongation (>450 msec for males or >470 msec for females); pregnancy; inadequate use of contraception; and inability to swallow capsules. The Medical Ethics Committee of both hospitals approved the study protocol and all patients had to give written informed consent.

Treatment plan and study design

During the first cycle (14-day duration), patients received oral enzastaurin (25 or 100 mg capsules) once daily (qd). In subsequent 21-day cycles, oral enzastaurin was given daily with gemcitabine as a 30-minute infusion on day 1 and 8 and cisplatin as a 3-hour infusion on day 1. Patients received pre- and post-hydration according to institutional guidelines. The starting dose

level was enzastaurin 350 mg, gemcitabine 1000 mg/m^2 and cisplatin 60 mg/m^2 Beginning at dose level 5 (enzastaurin 250 mg, twice daily [bid], gemcitabine 1250 mg/m^2 and cisplatin 75 mg/m^2), gemcitabine and cisplatin were not escalated, and enzastaurin was administered bid due to the less than proportional increase in plasma exposures in a previous study (17). If escalation proceeded beyond dose level 6, the increases in enzastaurin doses were determined based on data from the previous cohort(s). Patients were to receive 7 cycles of therapy unless disease progression or unacceptable toxicity occurred. Patients could continue after the planned 7 cycles if their investigator deemed it beneficial.

To begin a dose level, 3 patients were treated. If no patient experienced a dose-limiting toxicity (DLT) during the first 15 days of combination therapy, escalation occurred to the next dose level. A DLT was defined as any nonhematologic CTC grade 3/4 toxicity excluding nausea, vomiting and electrolytes; absolute neutrophil count <0.5 x 10^9/L for >7 days or <1.0 x 10^9/L with fever; or platelets <10.0 x 10^9/L. If 1 of the initial 3 patients had a DLT, 3 additional patients were enrolled to the same dose level. If at any time, 2 or more patients in a dose level had a DLT, accrual ceased and this was considered the maximum tolerated dose (MTD). The recommended phase II dose was defined as one dose level below that at which 33% of patients within a cohort experienced DLT. In the absence of MTD, the phase II dose would be identified as the dose which achieved the therapeutic plasma concentration of 1400nM of enzastaurin and its metabolites (based on IC_{90} of 70nM and plasma protein binding of 95%).

Patient evaluation

Before enrollment and during treatment, patients were assessed by physical examination, radiological scans for tumor measurement, tumor measurement of palpable or visible lesions, hematology and serum chemistry, ECG, and a slit lamp ocular examination. Toxicities were graded according to the CTC version 2.0 (32). Post-study follow-up included hematology, serum chemistry, ECG measurements, and a slit lamp ocular examination.

Pharmacokinetic studies

For enzastaurin analysis, heparinized blood samples were collected on day 14 of cycle 1; predose and 1, 2, 4, 6 and 8 hours post-dose and on day 1 of cycle 2; prior to gemcitabine infusion; and at 1, 2, 4.5, 8, 10-12, and 23-25 hours post-infusion. Analysis was performed by Advion Biosciences (NY, USA) using a validated liquid chromatography with tandem mass spectrometry analytical method. The lower limit of quantification was 0.5 ng/mL. Pharmacokinetic parameters were determined using noncompartmental methods (WinNonlin V4.1). For gemcitabine analysis, heparinized blood samples were collected on day 1 of cycle 2 at predose, 15, 30, and 35 minutes, and 1, 1.5, 2, 4.5, 8, 10-12, 23-25, and 47-49 hours after start of the infusion. Analysis of gemcitabine and its metabolite 2′2′-difluoro-2′-deoxy-uridine (dFdU) were performed at the Slotervaart Hospital (Department of Pharmacy and Pharmacology), using high-performance liquid chromatograph with ultraviolet detection as previously described (personal communication, van Maanen, 2002). For cisplatin analysis, heparinized blood samples were collected at predose, 5 and 30 minutes, and 1, 1.5, 3, 4, 7.5, 9.5-11.5, 22.5-24.5, and 46.5-48.5 hours after start of infusion. Sample pre-treatment was performed as previously described (33). A validated method using graphite furnace atomic absorption spectrometry was applied to determine the total and ultrafilterable platinum plasma concentrations (34). Cisplatin concentration-time data were analyzed for pharmacokinetic parameters

using noncompartmental methods with WinNonlin Pro 5.0.1(Pharsight, Mountain View, CA). Gemcitabine concentration-time data were analyzed using nonlinear mixed effect modeling.

Quantification of surrogate angiogenesis markers from whole blood

For the isolation and quantification of CEC biomarkers in PBMCs, blood was drawn on day 1, cycle 1 (predose), day 8 and day 1 of cycle 2 and cycle 3, prior to gemcitabine infusion and at 2, 4, 8, 24, and 48 hours after start of infusion (no 48 hour sample in cycle 3). Viable CECs were isolated and quantified as described previously, using CD146 antibody-labeled immunomagnetic beads (35-39). Further, the relative CEC content and the endothelial and hematopoietic progenitor cell content in PBMCs were quantified by nucleic acid sequence-based amplification of CD146 and CD133 mRNA as previously described (27).

Statistical analysis

The (un)paired student's t-test and analysis of variance were applied. Bivariate correlations between CECs or copies of mRNA and patient variables were performed by calculation of the Pearson correlation coefficient or Spearman's rho. Data are either presented as mean values ± standard error of the mean, or as mean or median values with range. P-values of <0.05 were considered significant. All analyses were performed using SPSS software version 12.0.1.

Results

Patient characteristics

Patient characteristics of the 33 patients enrolled in the study (May 2002-March 2005) are presented in Table 1. The most common diagnoses were adenocarcinoma of the pancreas and melanoma. All patients were Caucasian.

Dose Administration

A total of 162 cycles were initiated, and the median number of cycles per patient was 5 (range, 1-12). Eleven patients were treated for $\geqslant 7$ cycles. Table 2 describes patient accrual and dosage escalation. More patients in the bid cohorts than in the qd cohorts discontinued from the study in the early cycles. Overall, the reasons for discontinuation were disease progression (52%), lack of clinical benefit (18%), adverse events (27%), or other reasons (3%).

Maximum Tolerated Dose

Two DLTs were recorded, both for patients in the bid cohorts. A CTC grade 2 QTc interval prolongation (possibly related to enzastaurin and leading to discontinuation) was recorded for a patient in dose level 6. This patient had a fluctuating QTc interval at study entry, but the QTc interval was within the protocol limits. The other DLT (grade 3 fatigue) was reported by a patient in dose level 7 who discontinued from the study during cycle 1. Because there was not a dose level in which two or more patients experienced a DLT, the MTD was not identified. Therefore, the recommended phase II dose was based on tolerability and the ability to achieve therapeutic plasma concentrations.

Table 1 - Patient characteristics

	No. of Patients	% of Patients
Total number	33	
Male/female	22/11	67/33
Median age (range)	58 (38-79)	
Tumor types		
Esophagus	2	6
Pancreas	10	30
Ovary	1	3
Prostate	1	3
Kidney	2	6
Cholangiocarcinoma	1	3
Melanoma	7	21
Mesothelioma	2	6
Adenocarcinoma of unknown primary	3	9
Head and neck	1	3
Breast	1	3
Urothelium cancer	1	3
Carcinoma amp vater	1	3
ECOG performance status		
0	19	58
1	13	39
2	1	3
Prior therapy		
Surgery	17	52
Chemotherapy	11	33
Radiotherapy	9	27
Hormonal therapy	1	3

ECOG=Eastern Cooperative Oncology Group

Table 2 - Dose levels and DLTs

Dose Level	Enzastaurin, mg	Gemcitabine, mg/m^2	Cisplatin, mg/m^2	N	DLT
1	350	1000	60	4*	None
2	350	1250	60	3	None
3	350	1250	75	3	None
4	500	1250	75	4†	None
5	2 x 250	1250	75	3	None
6	2 x 350	1250	75	7‡	1 G2 QTc**
7	2 x 500	1250	75	9§	1 G3 fatigue**

*1 patient added after a patient discontinued before DLT analysis.
†1 patient added to replace a patient who was unavailable for PK analysis
‡3 patients were added when DLT occurred. The seventh patient was added when a patient discontinued from the study during cycle 1 and did not receive GC treatment.
§3 patients were added when DLT occurred. Another 3 patients were added to replace those in the cohort who were not evaluable for safety analysis due to discontinuations.
**DLT occurred during cycle 1 (enzastaurin monotherapy)
DLT=dose-limiting toxicity; QTc=Q-T interval corrected for heart rate

Table 3 - Main hematological toxicities observed during treatment with enzastaurin in combination with GC chemotherapy*

Toxicity	Grades 1	2	3	4	Total
	N (%)	N (%)	N (%)	N (%)	N (%)
Anemia	1 (3)	7 (21)	1 (3)	0	9 (27)
Leukocytopenia	0	2 (6)	2 (6)	0	4 (12)
Neutropenia	0	1 (3)	6 (18)	3 (9)	10 (30)
Thrombocytopenia	0	1 (3)	6 (18)	1 (3)	8 (24)
Creatinine	0	0	1 (3)	0	1 (3)
Hyperglycemia	0	0	1 (3)	0	1 (3)
Hypoatremia	1 (3)	0	0	0	1 (3)
Hypomagnesemia	0	2 (6)	0	0	2 (6)
Increased ALT	11 (33)	1 (3)	0	0	12 (36)
Increased AST	8 (24)	1 (3)	0	0	9 (27)

*Enzastaurin monotherapy did not result in any hematologic toxicity.

Table 4 - Main non-hematological toxicities observed during enzastaurin, or in combination with GC chemotherapy

Toxicity	Grades 1	2	3	4	Total
	N (%)	N (%)	N (%)	N (%)	N (%)
Gastrointestinal toxicity					
Nausea	17 (52)	10 (30)	1 (3)	0	28 (85)
Vomiting	12 (36)	8 (24)	1 (3)	0	21 (64)
Diarrhea	5 (15)	1 (3)	1 (3)	0	7 (21)
Constipation	0	0	2 (6)	0	2 (6)
Other	5 (15)	0	0	0	5 (15)
Neurological toxicity					
Sensory neuropathy	1 (5)	0	0	0	1 (5)
Ototoxicity					
Tinnitus	0	8 (24)	0	0	8 (24)
Other toxicities					
Febrile neutropenia	0	0	1 (3)	0	1 (3)
Alopecia	3 (9)	3 (9)	0	0	6 (18)
Left ventricular function	0	0	1 (3)	0	1 (3)
Fatigue	6 (18)	7 (21)	5 (15)	0	18 (55)
Urine color change	5 (15)	0	0	0	5 (15)

Toxicity

Generally, no direct enzastaurin-related side effects were noted. Grade 3/4 hematologic toxicities (Table 3) were noted across all dose levels and were considered to be related to gemcitabine/cisplatin, resulting in a week's delay of the second gemcitabine/cisplatin cycle. These toxicities were reversible. Four patients had study-related, grade 4 hematologic toxicity (Table 3). Grade 3 maximum toxicity was recorded in anemia, creatinine, and hyperglycemia for 1 patient (3%) each. Grade 4 nonhematologic toxicities were not observed (Table 4). Other toxicities were non-frequent and of little discomfort. More grade 1 and 2 toxicities were reported by patients in the bid cohorts than in the qd cohorts. These low-grade toxicities led to several discontinuations.

Table 5 - Summary of enzastaurin and total analyte exposure following enzastaurin dosing in cycle 1 and 2 in all dose cohorts

Geometric mean (%CV)						
		Once Daily Dosing		Twice Daily Dosing		
		350 mg	500 mg	250 mg	350 mg	500 mg
Enzastaurin	Css,av (nM) Cycle 1	855 (196) n=8	1340 (94.7) n=4	1490 (42.1) n=3	1790 (172) n=7	2140 (176) n=6
	Css,av (nM) Cycle 2	771 (161) n=6	1430 (115) n=5	973 (48.3) n=3	1620 (162) n=5	1160 (182) n=4
Total analyte (Enzastaurin and its metabolites)	Css,av (nM) Cycle 1	1690 (145) n=8	2450 (76.6) n=4	2850 (28.4) n=3	3460 (133) n=7	4310 (128) n=6
	Css,av (nM) Cycle 2	1840 (116) n=6	2780 (96.7) n=5	2160 (26.8) n=3	3350 (119) n=5	2740 (125) n=4

Css,av=Average steady-state concentration; N=number of subjects for PK analysis

Pharmacokinetics

Data from 30 patients were available for pharmacokinetic evaluations of enzastaurin across all the dose levels. Exposures in each dose group were compared between cycles 1 and 2 (Table 5). Data indicated that the pharmacokinetics of enzastaurin were not altered when administered in combination for 350 mg qd, 500 mg qd, and 350 mg bid. Compared to cycle 1, there was a lower mean exposure for 250 mg bid and 500 mg bid in cycle 2.

Plasma concentrations of gemcitabine decreased quickly after termination of infusion and were generally below the minimum quantification limit of the assay within 2 hours (data not shown). Plasma dFdU concentrations generally reached a maximum at termination of the gemcitabine infusion at approximately 30 minutes after start of infusion, decreased slowly and remained quantifiable at 48 hours after start of infusion.

When platinum was analyzed the median terminal half-life was 83 hours (range 43-261) for total platinum (data not shown). The maximum concentration occurred at or near the end of the 3 hour intravenous infusion of cisplatin. The geometric mean (%CV) for the area under the curve (AUC) was 54 (±50) μg·h/mL for total platinum. The geometric mean (%CV) of total platinum plasma clearance and volume of distribution at steady state was 12.0 (±40.1) mL/min and 79.3 (±26.8) L, respectively. The pharmacokinetic results for gemcitabine and cisplatin in combination with enzastaurin are comparable to historical, single-agent, pharmacokinetic data of gemcitabine (personal communication, Musib L., August, 2006) and cisplatin (40) suggesting that enzastaurin does not influence either platinum elimination or gemcitabine pharmacokinetics.
Based on the safety and pharmacokinetic data, the recommended phase II dose is enzastaurin 500 mg qd, gemcitabine 1250 mg/m^2, and cisplatin 75 mg/m^2.

Treatment response

Data from 9 patients was unavailable due to discontinuation (because of adverse events) prior to tumor assessment; thus, the remaining 24 patients were evaluable for response. Three patients from dose levels 3 (pancreatic cancer), 5 (head and neck cancer) and 6 (ovarian cancer) had partial responses. The duration of response was 3.5 months, 5.6 months, and 5.8 months, respectively. Thirteen patients reported a best response of stable disease, 6 of whom were stable for ⩾5 months. Progressive disease was the best response for 8 patients, and there were no complete remissions.

Angiogenesis-related biomarkers

Data was available from 28 patients for CEC quantification and 21 patients for CD133 and CD146 mRNA quantification. For the analysis of CECs, the non-parametric Mann-Whitney test and Wilcoxon signed rank test were used. CEC numbers and CD146 and CD133 mRNA expression did not change significantly during enzastaurin monotherapy (Figure 1A-C). Furthermore, enzastaurin did not influence the change in CECs and CD146 mRNA expression after treatment with gemcitabine and cisplatin. CEC numbers and CD146 mRNA expression in PBMCs was increased to maximum levels over baseline after a median of 4 hours after gemcitabine/cisplatin infusion in the first and second

Figure 1A

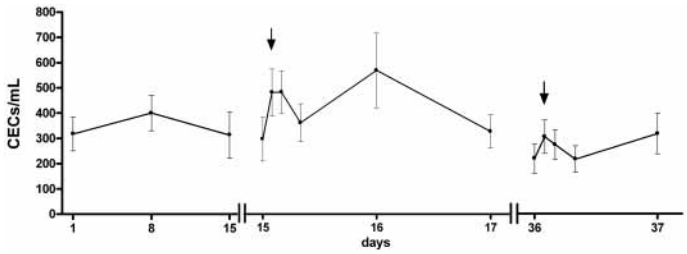


Figure 1B

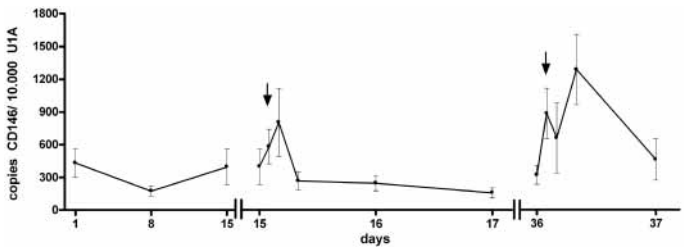
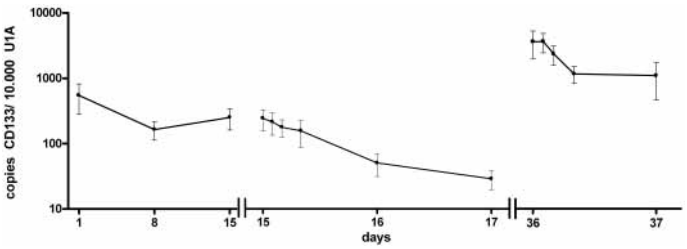


Figure 1C



Absolute CEC numbers (A), CD146 (B) and CD133 (C) mRNA expression during 14 days enzastaurin lead-in followed by enzastaurin + gemcitabine/ cisplatin chemotherapy (2 cycles). Patients are grouped according to enzastaurin dose. Note truncations on x-axis at day 15; between day 17 and day 36.

cycles of the combination treatment. CEC numbers increased significantly 2 hours after gemcitabine infusion in the first cycle (P=0.003) and with borderline significance (P=0.05) in the second cycle of gemcitabine/cisplatin treatment. CD146 mRNA expression was significantly increased 2 hours after gemcitabine infusion during the first (P=0.04) and second cycle of gemcitabine/cisplatin treatment (P=0.04). CD133 mRNA expression in PBMCs decreased directly after infusion of chemotherapy and was significantly decreased at 8, 24, and 48 hours after the gemcitabine/cisplatin treatment (P=0.03, P=0.002, and P=0.0003, respectively). The second cycle of gemcitabine/cisplatin also led to a significant CD133 mRNA decrease, 24 hours after infusion (P=0.008). A 10-fold median increase in CD133 mRNA expression between the first and second gemcitabine/cisplatin cycles (P=0.0003) was observed.

Discussion

In this clinical study we assessed toxicity and safety of oral enzastaurin (qd or bid) combined with gemcitabine/cisplatin and evaluated the pharmacokinetic profile of the three drugs. Both monotherapy and the combination were generally well tolerated at the tested doses. Adding enzastaurin to gemcitabine/cisplatin chemotherapy did not seem to influence the known hematologic toxicity profile of gemcitabine/cisplatin. No grade 3/4 bone marrow, hepatic, or ocular toxicity was reported. Pharmacokinetics revealed that cisplatin and gemcitabine exposures were not altered when given in combination with enzastaurin. Exposures of enzastaurin did not change with altered dosing or when combined with gemcitabine/cisplatin. Safety and pharmacokinetics data enabled dose escalation to continue. Due to less than dose proportional increase in enzastaurin exposures in a previous study, enzastaurin qd was changed to bid. The bid regimen resulted in slightly greater plasma exposures but was also associated with more low-grade toxicities, suggesting that bid dosing was less tolerable than the qd dosing. In light of the chronic daily dosing of enzastaurin, qd dosing is preferred for further studies.

Enzastaurin inhibits the PKC family (8,41,42) and the PI3K/AKT pathway (12) to inhibit tumor cell growth and tumor induced angiogenesis. Blocking the PKC, pathway results in abrogation of VEGF-induced angiogenesis, vascular function, and vascular permeability (43,44). Preclinical studies show significant antitumor effect of adding antiangiogenic drugs to cytotoxic chemotherapy (29,45). An antiangiogenic drug may normalize tumor vessel perfusion, which enables better delivery of chemotherapy to the tumor cells (30). Furthermore, because both treatment modalities have a different target, the toxicity profiles are not expected to overlap.

Pharmacodynamic markers that identify biological efficacy are highly needed to select an optimal dose of angiogenesis inhibitor (46). CECs are increased in cancer patients during disease progression and in the hours following cytotoxic chemotherapy (24). *In vitro* toxicity data shows that IC_{50} of enzastaurin for endothelial precursor cell outgrowth is about 12-fold lower than IC_{50} for stationary or exponentially growing human umbilical vein endothelial cells (6 μM) (14). (L.V.Beerepoot, unpublished data) Because ablation of CECs affects tumor growth and because inhibitors of angiogenesis (partly) seem to elicit their antitumor effect through inhibition of (VEGF-mobilized) CEC incorporation into tumor vasculature (47-50) the kinetics of CECs during antiangiogenic treatment may be a promising and easily accessible marker to assess efficacy of this class of drugs. However, the methodology to measure CEC and progenitor cells vary and their quantification is not

a validated assay. Furthermore, very little is known about changes in these CECs during conventional chemotherapy. Our data should therefore be considered exploratory.

In this study, the baseline amounts of CECs reflect the heterogeneity of the patient group that was studied. Single-agent enzastaurin had no effect on any of the angiogenesis biomarkers analyzed. However, a semi-acute increase in the biomarkers was seen in the hours following gemcitabine/cisplatin infusion. Our assays did not allow an assessment of the source of the observed increase which may be detachment of CECs from the (tumor) vessel wall or recruitment from the bone marrow (39,50-52). The increased CD133 mRNA levels after chemotherapy may point to a rapid mobilization of progenitor cells from the bone marrow. It is tempting to speculate that these progenitor cells have an impact on treatment outcome and this deserves further study. In spite of interesting observations, in this exploratory analysis CEC or CD133 and CD146 mRNA levels did not prove to be a useful pharmacodynamic marker that influenced decision-making on dose escalation.
In conclusion, combination therapy of enzastaurin with gemcitabine/cisplatin chemotherapy is feasible and safe. Its toxicity profile seems comparable to that of gemcitabine/cisplatin chemotherapy alone. Enzastaurin addition did not increase the toxicity of gemcitabine/cisplatin chemotherapy. The combination regimen did not alter the exposures of individual drugs. The recommended phase II dose is enzastaurin 500 mg qd, gemcitabine 1250 mg/m^2, and cisplatin 75 mg/m^2.

For pharmacodynamic purposes, repeated quantification of CECs and amplification of endothelial (progenitor) mRNAs as part of a clinical trial is feasible. Exploration of the full potential of CEC biomarkers as a possible surrogate marker of treatment effect of enzastaurin warrants further study.

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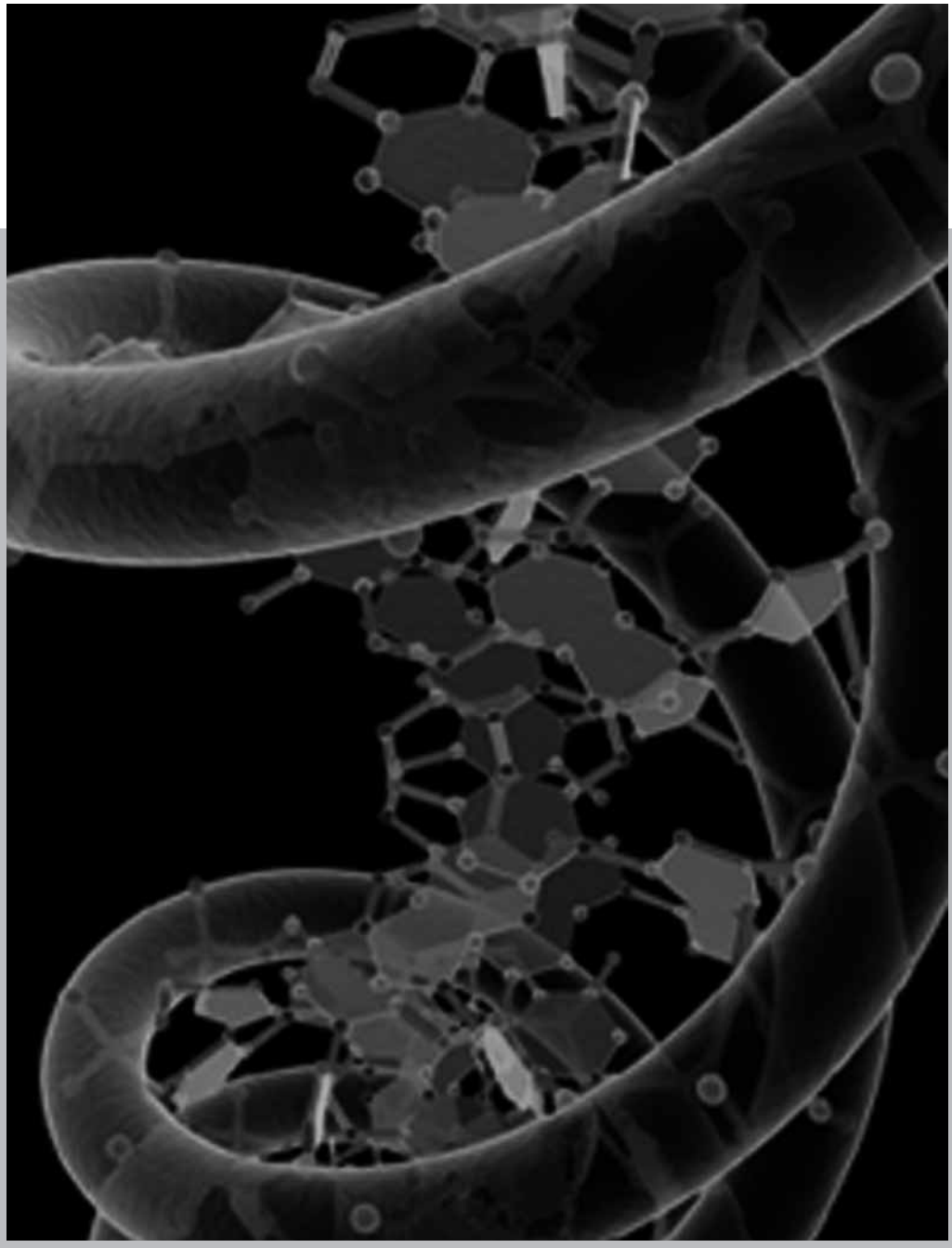
PART 2 - Circulating nucleic acids



Amplification of circulating nucleic acids as predictor of prognosis or treatment outcome in cancer patients

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Abstract

The finding that cell-free nucleic acids can be quantified from the body fluids of cancer patients has ignited research to evaluate these nucleic acids as potential biomarkers. It is now accepted that stable tumor-derived RNA and DNA is present in virtually all extracellular compartments. Furthermore, these nucleic acids could be isolated and analyzed in a qualitative or quantitative manner. Most of these reports demonstrate an elevation in cell-free (tumor-derived) DNA and RNA in the peripheral blood of cancer patients compared to patients with benign disease or healthy subjects. Amplification of circulating (tumor-derived) nucleic acids at cancer diagnosis and during follow-up after anti-cancer treatment has been assessed in relatively fewer studies. These studies indicate that tumor-specific or total levels of nucleic acids could be a valuable tool in clinical cancer management as it is possible to predict response to treatment, prognosis, relapse or survival in cancer patients.

In this review we provide a comprehensive overview of the recent studies that have isolated and quantified the amount of nucleic acids or amplified tumor-specific changes in nucleic acids present in the peripheral blood of cancer patients, to assess whether these circulating nucleic acids can be used as novel tumor marker and as prognostic factor.

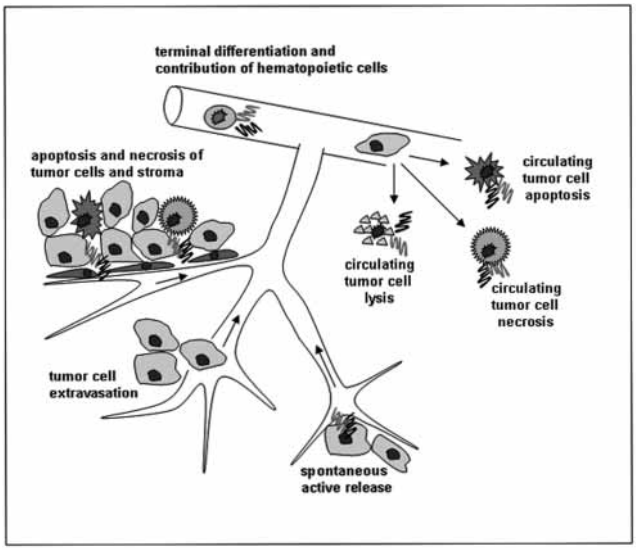
A historical perspective on circulating nucleic acids

Since the discovery of DNA by Miescher and his pupil Altman in 1869, it was thought that nucleic acids were only present within the cell compartment. In 1948, however, Mandel and Metais detected measurable quantities of circulating DNA in the plasma of both patients and healthy persons (1). This is remarkable as it came only a few years after the discovery of DNA as the material of inheritance. Until the 1970s most studies on circulating DNA were done on sera of patients with systemic lupus erythematosus and rheumatoid arthritis. However, in 1977, the first publication appeared in the field of oncology (2). It was suggested that plasma or serum DNA could be used as a vital tool for the assessment of cancer therapy, since levels of circulating DNA decreased after successful treatment. In 1989, Stroun et al. suggested that the circulating DNA in cancer patients originates from their tumors (3). This hypothesis was proven correct when tumor-associated oncogene mutations were amplified from circulating DNA isolated from plasma of patients with leukemia (4) and pancreatic cancer (5). Following these reports, an increasing number of publications have shown the presence of circulating tumor-associated nucleic acids in patients with many different types of cancer (4-10). In addition, other types of nucleic acids have been detected in the circulation of cancer patients, such as RNA (9), mitochondrial DNA and RNA (11), and viral DNA (12). These nucleic acids have been found to be present in plasma, serum, saliva (13), broncho-alveolar fluid (14), urine (15) of patients with cancer.

Circulating nucleic acids are released from hematopoietic cells, cancer cells in situ and circulating tumor cells

When nucleic acids are no longer present in the cell but dispersed in body fluids or in the circulation, they are called circulating nucleic acids. It is known that circulating nucleic acids can be present in healthy individuals as well as in patients with various diseases, but the exact origin of these freely circulating nucleic acids is incompletely understood. It has been suggested that circulating DNA in healthy individuals has a hematopoietic origin and is derived from lymphocytes or other nucleated cells (16). In patients with cancer, a substantial percentage of the circulating nucleic acids are derived from tumor cells in-situ and possibly from circulating tumor cells, tumor stroma, and endothelial cells and pericytes within the tumor.

Figure 1



Potential mechanisms of extracellular nucleic (NA) acid release into the peripheral blood (PB). Intratumoral necrosis and apoptosis are likely mechanisms for nucleic acids release into the PB. Other possible mechanisms include tumor cell extravasation, followed by circulating tumor cell apoptosis, necrosis or lysis, with direct release of their NA into the PB. Tumor cells may also actively secrete parts of their genome into the PB, without affecting tumor cell vitality, or prior to impeding cell death. A small contribution to basal level of NA in the PB is likely to originate from hematopoietic cells and terminal differentiation. Tumor cells are depicted in blue (apoptotic tumor cells in dark blue, necrotic tumor cells in light blue), hematopoietic cells in pink and stromal cells in red. DNA and RNA are shown in green and black.

There are potentially different mechanisms how nucleic acids may end up extracellularly. The mechanism of cellular release of nucleic acids may differ between healthy and diseased individuals and possibly even between different forms of cancer. Stroun et al. excellently reviewed the different mechanisms of how DNA may end up in the blood plasma. This can also be extrapolated to other forms of circulating nucleic acids, such as RNA, mitochondrial (mt)DNA, and mtRNA (17). The proposed mechanisms include lysis of circulating tumor cells, necrosis and apoptosis of cancer cells, spontaneous or active release from cells, and terminal differentiation (Figure 1). Most probably, the presence of the circulating DNA in plasma and serum is not being caused solely by one mechanism. An update based on recent literature and findings in our laboratory is given below.

Lysis of circulating cancer cells or micrometastases releases nucleic acids in the bloodstream. However, this is most likely not the only source of nucleic acids as calculations indicate that for the DNA concentrations found in plasma, approximately 1,000-10,000 cancer cells per ml would have to be present. Few studies found positive correlations between circulating tumor cells and levels of circulating (tumor) DNA/RNA (18). This indicates a limited contribution of lysed circulating tumor cells to the totality of circulating nucleic acid levels. Already in early cancer lesions, before the presence of tumor cells in the circulation, detectable levels of tumor DNA have been found (19), which indicate that another mechanism is responsible for the release of nucleic acids in peripheral blood.

Necrosis of the tumor is another hypothesis for the presence of circulating nucleic acids in the blood of cancer patients. Tumor necrosis already takes place in the earliest stages of tumor outgrowth, when cancer cells have not gained the ability to attract blood vessels from the pre-existing vasculature to the tumor. This is in line with the fact that circulating nucleic acids are found in early-stage cancers.

A potential application of circulating nucleic acids is the prediction of a response to treatment. Any treatment that induces tumor necrosis should lead to an increase in the level of circulating

nucleic acids. Knowledge of the kinetics of the release and clearance of nucleic acids following anti-cancer treatment is imperative. Inadequate time points have led to erroneous conclusions. A pioneering study demonstrated a decrease in cell-free DNA in 66-90% of cancer patients receiving radiotherapy (2), and therefore conclusions have been made that cell-death was an unlikely mechanism for the elevation of nucleic acids in the circulation. Recent evidence points out that the first hours and days post-treatment are more informative, as increases in cell-free DNA are found, which disappear well within the first week following cytotoxic treatment (20-22). Furthermore, in conditions associated with increased cell death or tissue destruction, such as hepatitis, septicemia (23), and trauma (24), an increase in plasma DNA is found. Therefore, accumulating evidence suggests that necrosis plays an important role in the detection of tumor-derived nucleic acids in plasma of cancer patients. However, as reported earlier, mechanisms causing release of nucleic acids from the cell may vary between and within cancer types. A study with NSCLC patients revealed that circulating DNA levels in plasma did not associate with necrosis or lymphoid infiltration of the tumor (25).

Several other observations led to the hypothesis that apoptotic cancer cells are the predominant origin of the circulating DNA in the plasma or serum of cancer patients. Endonuclease activation is a characteristic feature of apoptosis. This degrades genomic DNA at internucleosomal linker regions and produces mono- and oligo- nucleosomal fragments of ±180bp. Electrophoresis of DNA from plasma and serum of cancer patients also showed a ladder pattern as seen in apoptotic cells (26). Another study showed that both 180 base-pair fractions, and larger DNA fractions possibly associated with necrosis, were found in the blood stream of cancer patients (27). The appearance of a cell-death ELISA kit that quantitatively detected circulating nucleosomes in plasma or serum led to investigating apoptosis as a possible tumor marker (28-32). In 96 stage 0-III breast cancer patients, significant elevated nucleosomes in the plasma were found, and using a defined cutoff, an association between high nucleosomal DNA and decreased relapse-free survival was found (28). Another report showed an increase in nucleosomal DNA in the serum and to a lesser degree in plasma in the days following radiotherapeutic and radiochemotherapeutic treatment (33). In this study they found strong correlations between β-globin DNA, their marker for total levels of circulating DNA, and nucleosomal DNA, suggesting a relation between circulating DNA and apoptotic cell-death. Comparable studies demonstrated that the kinetics of nucleosomes following treatment were an early marker to predict response (30;34;35). It has been suggested that a significant proportion of the circulating DNA is bound to protein molecules such as nucleosomes (33). Based on these findings it is likely that both necrosis and apoptosis are important mechanisms leading to elevated levels of circulating nucleic acids in cancer.

The hypothesis that DNA is spontaneous or actively released by the tumor (or lymphocytes in healthy individuals) has been studied since the early 70s. Cells can spontaneously release a nucleoprotein complex from which the DNA subsequently is released (36). In cultured lymphocytes it was shown that of newly synthesized DNA a selective portion of the genome was preferentially excreted, and also that after DNA release the cells remained functional (37). There was no association between DNA excretion by lymphocytes and apoptosis. It has been suggested that the typical DNA ladder pattern following apoptosis may be also the seen in spontaneously released DNA (38). Others have indicated that DNA is released in the medium of non-proliferating cancer cells prior to cell-death (39). Studies in our laboratory indicate a relation between impeding cell-

death and levels of extracellular mtDNA and mtRNA in the medium of cultured endothelial cells and various tumor cell-lines. After serum starvation, but well before cell-death, we saw a dramatic increase of mitochondrial nucleic acids being excreted into the medium, making it very likely that the nucleic acids in these cases originated from spontaneous release from tumor cells as well as from non-cancerous cells (20).

Not only tumor cells but non-malignant cells in the tumor environment may release nucleic acids into the circulation. Terminal differentiation of erythrocytes, keratinocytes and megakaryocytes may also contribute to the level of circulating nucleic acids in cancer patients. Every day the same amount of cells will terminally differentiate as the amount of cells that go into apoptosis. After terminal differentiation, the fragmented DNA is pushed out of the nucleus but the cell remains functional. Enhanced cellular turnover and subsequent elevated terminal differentiation in patient with cancer may contribute to the increased baseline levels of these circulating nucleic acids. However, other mechanisms discussed above are more likely to contribute to the bulk of circulating nucleic acids in patients with cancer.

Circulating (tumor-derived) nucleic acids as a diagnostic marker for cancer

Many studies have investigated whether circulating (tumor-derived) nucleic acids can be used as diagnostic marker to differentiate between healthy subjects, and subjects with various (benign/early) forms of cancer. Circulating nucleic acids were found to be a powerful marker in discriminating between these groups (25;40;41), however in most studies the properties or levels of these circulating nucleic acids were retrospectively assessed. A large number of studies also indicate that circulating nucleic acids are increased in various forms of non-cancerous disease (24;42-46), and therefore cannot be used accurately to identify patients with cancer. A large multi-center prospective study with over 1000 patient samples analyzed, have quantitatively assessed levels of circulating nucleic acids in individuals who were later diagnosed with various forms of cancer or other pathologies. The conclusion of this study was that circulating DNA levels are a non-specific marker for cancer screening in the population (43). Another study investigated both quantitative and qualitative characteristics of the plasma DNA in over 300 patient samples, and also concluded that plasma DNA levels were not specific enough for cancer screening, although qualitative assessments should be further assessed as screening tool for cancer (47). Qualitative studies testing a panel of tumor-specific alterations in certain genes, such as in the p53 (48), K-ras (5), and VHL gene (49), might provide a specific test for certain forms of cancer, and thereby a very attractive non-invasive diagnostic tool for population screening. For example, methylation of p16 is highly specific for presence of cancer (50), however sensitivities vary between 10 and 90%, depending on tumor type. K-*ras* mutations may also only be used as tumor marker in approximately 50% and 70% of colorectal cancer and pancreatic cancer, respectively. Therefore amplification of certain alterations may be highly specific, but present markers will not have broad applicability. Other alterations, such as mutations in p53 gene are essential in the pathogenesis of almost all tumors; however detection is cumbersome as a variety of silencing mutations are spread over various exons of the p53 gene. Furthermore, the fraction of tumor-derived DNA in plasma was found to vary between 3 and 93% of total DNA levels (27). This may undermine certain qualitative studies, such as on loss of heterozygosity (LOH) analysis, which requires at least 20% of tumor DNA of the total circulating DNA (51). Although data are promising, prospective qualitative studies on alterations in circulating nucleic acids in healthy individuals (at risk) have not been performed thus far.

Total levels of circulating nucleic acids are a predictor of prognosis, treatment outcome and survival

Leon and colleagues published the first study on the level of free DNA in the serum of 173 patients with cancer using a radioimmunoassay to quantify total double strand DNA (2). They demonstrated an increase in DNA levels in approximately 50% of cancer patients as compared to healthy subjects. Also higher DNA levels were found in the serum of patients with metastatic disease in comparison with non-metastatic disease, but no correlation was found between DNA levels and size or location of the primary tumor. Leon et al. first suggested that persistent high or increasing DNA levels in the circulation may signal a relapse. Since this hallmark paper, other studies have quantified total DNA levels or PCR-amplified certain "household" genes as a measure of total DNA or RNA levels. In Table 1, we have summarized the relevant literature. We have limited the overview to studies where circulating nucleic acid levels were evaluated as marker for prognosis, treatment outcome, and survival probability. Fournie et al. quantified total amounts of DNA in the plasma of 68 patients with lung cancer (39). They showed that DNA levels were increased in stage IV as compared to stage I-III lung cancer, and demonstrated a correlation between DNA levels and the serum markers neuron-specific enolase (NSE) and lactate dehydrogenase (LDH). In univariate analysis, DNA levels, NSE and LDH were significantly associated with patient survival, but not in multivariate regression analysis. Survival curves using DNA levels as dichotomous variable (cut-off 100 ng/ml) showed a median survival of ±1.9 months and ±8.3 months for patients with lower (n=56) or higher (n=9) plasma DNA levels than the cut-off. Sozzi et al. evaluated the kinetics of plasma DNA in 81 disease free, surgically resected lung cancer patients, using a DNA DipStick assay, which measures total dsDNA (52). At baseline, higher levels of circulating DNA did not correlate with relapse-free survival. However, in follow-up plasma circulating DNA levels showed a trend toward reduction in relapse-free individuals (n=35), while plasma DNA increased 2-

Table 1 - total levels of circulating nucleic acids

nucleic acid	gene	method	tumor type(s)	reference(s)
DNA				
total DNA				
	dsDNA	32^P	lung	(39)
	dsDNA	DNA Dipstick	lung	(52)
	ß-globin	PCR	breast	(41;68)
	GAPDH	PCR	lung	(69)
	hTERT	PCR	lung	(25)
mt DNA	16S rRNA	PCR	prostate, breast	(11;20)
nucleosomal DNA	-	ELISA	lung, pancreas, colorectal, breast cervical	(21;22;28;30;31)
viral DNA	EBV	PCR	gastric, naspopharynx, lymphoma	(12;66;70;71)
	HPV	PCR	cervical, head and neck	(72;73)
RNA				
mt RNA	COX1	PCR	prostate, breast	(11;20)

Abbreviations: ds, double strand; mt, mitochondrial; EBV, Epstein Barr virus;

20 fold in patients who relapsed (n=4). In an exploratory study Holdenrieder et al. demonstrated a strong elevation of nucleosomes in lung cancer patients compared to other cancer types (34). In a later prospective study they investigated the nucleosomal DNA kinetics in serum during two cycles of chemotherapy (31). Pre-therapeutic levels of circulating nucleosomes did not distinguish responders from non-responders; however, the baseline values of nucleosomes before the start of the second and third cycle were significantly lower for patients with response than non-responding patients ($p<0.0001$). Of particular interest are the kinetics during the first week of therapy: non-responders displayed a larger increase post-chemotherapy compared to responders, and nucleosomes dropped less efficiently until day 8. These kinetics are hard to rationalize as efficient cytotoxic treatment would suggest higher cell-death and thus higher nucleosomal levels in serum. This may be explained, as the authors discuss, by the fact that patients with metastatic disease showed a poorer response than stage III patients. Therefore nucleosome levels after cytotoxic treatment may reflect tumor mass and/or metastasized disease. Nevertheless, the area under the curve (AUC) of nucleosome levels until day 8, were predictive for outcome ($p=0.005$). Clearly, understanding the kinetics of the release and clearance of nucleic acids is essential to select reliable time points for further studies. To illustrate this, we have found an increase in the level of circulating nucleic acids in the plasma of responding patients in the first hours following infusion with cytotoxic chemotherapy; however these elevations disappeared within 24-48 hours (20).

In our laboratory we have focused on the amplification of mitochondrial nucleic acids in plasma and its prognostic value. As a single cell can contain hundreds to thousands of mtDNA copies as opposed to two copies of genomic DNA, we hypothesized that amplification of mt nucleic acids would increase the sensitivity and/or specificity as tumor marker. We quantified mtDNA and mtRNA levels in plasma of 75 prostate cancer patients and studied an association between cell-free mt nucleic acids and patient survival. We showed that elevated pre-therapeutic levels of plasma mt nucleic acids were indicative for a significant decreased two-year survival (35% vs. 73% cumulative survival for mtDNA and 21% vs. 73% for mtRNA). In multivariate analysis with cell-free mt nucleic acids, metastasis status, PSA doubling time, and hemoglobin levels, mtRNA appeared a strong independent predictor of 2-year survival. Similar results were obtained in breast cancer patients (20). Most importantly, we showed that elevated mtRNA levels, above the normal range found in healthy subjects, can be used as prognostic marker in prostate, head and neck, kidney and colorectal cancer (unpublished data), providing a rationale for use as general tumor marker. All these studies have investigated pre-therapeutic levels of mt nucleic acids in advanced cancer patients. However, we have preliminary evidence that shows an elevation of mt nucleic acids as early as two hours following cytotoxic chemotherapy in responders but not in non-responders (unpublished data). Large prospective follow-up studies are currently being performed with mt nucleic acids as predictive marker.

Presence and levels of tumor-specific alterations in nucleic acids as novel tumor marker

In contrast with the measurement of total circulating DNA levels, qualitative amplification techniques can specifically determine levels of tumor-derived DNA. Amplification of tumor-specific nucleic acids present in the plasma may therefore be seen as an important non-invasive approach to obtain specific information about the tumor. The limitation of this approach is that tumorigenesis of one tumor type may be the result of different oncogenic alterations, and broad applicability to other tumor types will not be possible. Some examples of specific tumor-associated genetic alterations that can be detected are microsatellite alterations, allelic imbalance, translocation,

mutations in oncogenes, methylation of promoter regions of tumor suppressor genes, and the presence of viral genes. Many studies have amplified these changes in the DNA/RNA and assessed the presence as (early) diagnostic marker for cancer (48;50;53-56). However, far fewer studies correlated presence and levels of mutated DNA/RNA with patient characteristics, outcome and survival. Table 2 gives an overview of the literature of tumor-specific alterations in circulating DNA and RNA as prognostic marker.

Table 2 - tumor specific (alterations in) circulating nucleic acids

nucleic acid	alteration	gene(s)	tumor type(s) reference(s)
DNA			
mutation	K-ras	pancreas	(57)
	p53	breast, lung	(58;59;74;75)
LOH	Microsatellite markers	breast, lung, melanoma, oral[1]	(52;58-60;76-79)
rearrangement	CDRIII	lymphoma, leukemia	(8)
methylation	APC	esophagus	(80)
	$P14^{ARF}$	bladder	(81)
	P16	breast, colorectal	(10;59;82;83)
	MGMT	melanoma, glioblastoma multiforma	(63;84)
	RASSF1A	breast	(61-63;84)
	14-3-3σ	lung	(64)
	MYOD1	cervical	(85)
	ER	melanoma	(86)
RNA			
tumor specific regulation	CK19	colon, breast	(18;65)
	Mammaglobin	breast	(18)
	CEA	colon	(65)
	MAGE-3, MUC-18, p97 and tyrosinase	melanoma	(87)

[1]tumors of the oral cavity, pharynx, larynx
Abbreviations: LOH, loss of heterozygosity

Castells et al. studied the mutational status of the K-ras oncogene in pancreatic cancer (80-90% caused by mutations in the K-ras gene) and showed that patients with mutant-type K-ras exhibited a shorter median survival, and the presence of plasma K-ras mutations was a strong independent prognostic factor (57). Frickhofen et al. studied rearranged immunoglobin (Ig) heavy chain DNA present in the plasma of patients with acute lymphoblastic leukemia and non-Hodgkin's lymphoma, which was identical to products amplified from DNA extracted from the tumor cells of these patients (8). Treatment of patients with cytotoxic chemotherapy was followed by rapid clearance of rearranged Ig DNA in the plasma of responding patients and persisting tumor-derived DNA was correlated with non-responders and relapsing patients. Silva et al. studied plasmas of breast cancer

patients prior to mastectomy for LOH of five markers with a reported high rate of alteration, the mutational status of the p53 gene, and methylation of $p16^{INK4a}$ (58;59). They found that plasmas of ±20% of patients showed persistence of the same molecular alterations 4-6 weeks after mastectomy compared to the alterations found prior to the operation. Persistence of these tumor-derived plasma DNA was related to poor-prognosis parameters such as vascular invasion, lymph node metastasis (>3 positive lymph nodes) and higher histological grade. The presence of tumor DNA alterations in plasma prior to mastectomy were with borderline significance associated with differences in disease-free survival (P=0.05) and with borderline significance as independent prognostic marker in multivariate analysis (P=0.08). The presence of LOH in the plasma of melanoma patients was also related to an increased risk of death and poor survival (60). Muller et al. have clearly demonstrated the clinical significance of scrutinizing circulating DNA for promoter methylation associated with poor patient outcome; they found that RASSF1A DNA methylation and APC DNA methylation status in pre-therapeutic sera were strong independent prognostic markers for outcome (61) They also demonstrated that RASSF1A methylation status during adjuvant tamoxifen therapy is indicative for responsiveness to treatment, relapse and outcome (62). In stage IV melanoma patients RASSF1A status also correlated with therapeutic response of biochemotherapy and overall survival (63). In another elegant report the promoter region of 14-3-3σ, a gene responsible for G_2 cell cycle checkpoint control in response to DNA damage is studied. Cell-lines with reduced or absent 14-3-3σ (due to promoter methylation or p53 inactivation) are more sensitive to cell death by radiation or chemotherapy therapy. Ramirez et al. have demonstrated that methylated 14-3-3σ in the serum of NSCLC patients receiving platinum based chemotherapy is an independent prognostic factor for survival and indicates responsiveness to chemotherapy (64).

The presence of tumor-specific RNA has been studied to a lesser extent. Silva et al. demonstrate that epithelial tumor RNA's correlate with advanced stage in colon and breast cancer (18;65) and are also associated with circulating colon cancer cells.

Conclusion

It is clear that circulating nucleic acids potentially contain a huge assortment of molecular markers, and in the coming decade research most likely will lead to the development of non-invasive and highly specific diagnostic and follow-up markers in cancer management. Reports discussed in this review have clearly indicated that a substantial fraction of circulating nucleic acids in the body fluids of cancer patients are tumor derived, as amplification of tumor-specific alterations in plasma highly match the alterations found in the tumor or metastases. We conclude that highly promising biomarkers are present in circulating nucleic acids and may be implemented into routine clinical use within a couple of years from now.

The amplification of circulating viral DNA implicated in the etiology of certain (subsets of) cancer, has proven to be a very sensitive and specific tumor marker for the follow-up of patient after treatment, and has been successfully implemented as surrogate marker in phase-II studies (66). Detection of nucleosomal DNA or total levels of DNA are easy and straightforward assays, which may become important predictive markers for chemo- or radiotherapeutic treatment modalities. The amplification of genetic alterations which are highly tumor-specific also has clinical utility.

However, as previously stated, the limitation of this approach is that tumorigenesis of one tumor type may be the result of different oncogenic alterations. Other areas of interest in nucleic acid amplification for instance, are non-invasive testing of mutational tumor status on circulating nucleic acids, for e.g. eligibility for specific targeted therapies. Furthermore, the presence of poor prognostic gene signatures can be assessed by gene expression profiling using circulating RNA. These techniques might especially be of importance for identification of molecular signatures of primary tumors of unknown origin. Amplification of tumor-specific alterations or quantification of total levels of circulating nucleic acids as predictive factor for insensitivity or relapse following cancer treatment, or in follow-up after treatment, may individualize life assessment estimation and therapeutic decision making.

Of major importance for the quantification of total levels of circulating nucleic acids (e.g. DNA, RNA, mitochondrial DNA/RNA, and viral DNA/RNA) as a standardized tumor marker, is further methodological work to identify time-frames for blood collection and storage to assess the influence of different blood-collection, nucleic acid isolation and amplification protocols on levels of nucleic acid quantification. Refinement and standardization of sample collection and storage will have to lead to new collaborative multicenter studies to assess circulating nucleic acids as novel tumor marker in comparison with current standards of choice. As tumor-specific alterations are detected in the serum or plasma with varying percentages between studies, and even between centers within one study, these variations therefore also reflect differences in protocol and sensitivity of amplification. Therefore standardization and strict validation of protocols are direly needed.

Furthermore many questions still need to be answered about how nucleic acids enter the blood and other body fluids. Based on the collection of literature, it is most likely that tumor necrosis and apoptosis comprises the bulk of circulating nucleic acids; however, active secretion of parts of their genome and RNA by viable cancer cells (prior to cell-death), clearly demonstrated in vitro, cannot be excluded. Whether circulating nucleic acids are functional in immune suppression (39), are involved in the blood-borne transfer of genetic tumor characteristics into the genome of non-tumor cells (“genometastasis”,(67)), or may have other beneficial purposes in tumorigenesis or progression has to be further elucidated.

Nevertheless, the quantification of (tumor-specific) nucleic acids is most promising as non-invasive molecular tumor marker for therapeutic monitoring during cancer treatment and as follow-up marker. The performance of circulating nucleic acids as quantitive tumor marker is currently being validated in large prospective studies, and its clinical utility will be finally become transparent.

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Circulating mitochondrial nucleic acids have prognostic value for survival in patients with advanced prostate cancer

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Abstract

Purpose: Advanced prostate cancer represents a heterogeneous disease entity with differences in clinical behavior, response to therapy and survival. We assessed whether we could distinguish poor from good prognosis patients at presentation in our clinic, by means of quantifying circulating cell-free mitochondrial (mt) and genomic nucleic acids in plasma.

Experimental design: We collected plasma from 75 prostate cancer patients and 14 subjects with benign disease. Nucleic acids were isolated and mtDNA (16S ribosomal RNA), mtRNA (cytochrome c oxidase subunit 1) and genomic DNA (U1A DNA) transcripts were quantified by real-time amplification. An association between cell-free nucleic acids and metastasis, PSA doubling time, and hemoglobin levels was determined. Multivariate Cox Proportional Hazard and survival estimation studies were performed.

Results: We demonstrate that elevated mtDNA and mtRNA levels are present in plasma of prostate cancer patients with a poor 2-year survival (P=0.02 and P=0.003, respectively). Cancer patients with high plasma mt nucleic acids, using a calculated optimal cut-off point, demonstrate a decreased survival compared to patients with low levels (35% vs. 73% cumulative survival for mtDNA and 21% vs. 73% for mtRNA). Multivariate analysis indicates that mtRNA is an independent predictor of 2-year survival.

Conclusion: Quantification of plasma mt nucleic acids may be used to recognize patients with a poor prognosis. In advanced prostate cancer patients mtRNA appeared the strongest predictor of overall survival and an independent prognostic factor for cancer-related death. Amplification of mt nucleic acids shows increased sensitivity and specificity over genomic DNA as diagnostic and prognostic marker in prostate cancer patients.

Introduction

Prostate cancer is the most common cancer among men, with approximately 230,000 new annual cases in the United States alone. About 10 to 20 percent of men with prostate cancer present with metastatic disease, and in many others, metastases develop despite treatment with surgery or radiotherapy. For the group with distant metastasis 5-year survival is 34% and, consequently, treatment is predominantly palliative (1). Advanced prostate cancer embodies a heterogeneous disease entity with varying clinical behavior, and therefore distinguishing the population at high-risk for treatment failure and death would allow for better disease prognostication and patient decision-making.

Quantification of circulating plasma nucleic acids has been suggested as a diagnostic marker for cancer (2-5), although recent studies have focused on the quantification of nucleic acids before and during treatment in previously diagnosed cancer patients. A decrease in genomic nucleic acids in plasma appears to be related with a response to treatment (6-9) and/or an association with disease-free and overall survival (9-11). Elevated circulating nucleic acids in plasma of cancer patients is considered to originate from lysis (12), apoptosis (13;14), necrosis (15), and/or spontaneous active release from (circulating) tumor cells (13;16). It has been shown that alterations in the circulating DNA match the mutations present in the tumor, indicating that at least

part of these elevated circulating nucleic acids originate from tumor cells (17). The nucleic acids present in plasma are bound to the surface of blood cells (18;19), proteins (20), nuclear complexes (21), or apoptotic bodies (22), which enhance stability and provide protection from nucleases. Recent evidence has also shown the presence of stable RNA in the plasma, serum, and saliva of cancer patients (22-24).

Most reports have focused on quantification of genomic nucleic acids in plasma, but plasma may also contain extrachromosomal mt nucleic acids. A single cell can contain hundreds to thousands of mtDNA copies as opposed to two copies of genomic DNA. Although mtDNA levels previously have been quantified in plasma of healthy volunteers (25), no reports measured mtDNA in the plasma of cancer patients. One recent report quantified mtDNA in the saliva of patients with head and neck cancer and healthy controls and found a significant increase of mtDNA levels in cancer patients, and a correlation between mtDNA and tumor stage (26).

In this report we assessed whether we could use real-time quantification of mt nucleic acids in plasma to determine prognosis in advanced prostate cancer patients at presentation in our outpatient clinic. Here we provide evidence that mt nucleic acids are increased in prostate cancer patients with a poor prognosis, and that measuring mt nucleic acids can be used to evaluate survival probabilities, independent of current prognostication factors.

Material and methods

Characterization of study patients and healthy volunteers.

Blood samples were collected from advanced prostate cancer patients visiting the outpatient clinic of the Departments of Medical Oncology and Urology, University Medical Center in Utrecht, the Netherlands from October 2001 to July 2003. The study was approved by the hospital Ethics Committee and written informed consent was obtained from all patients. Male control subjects (n=14) were patients referred by general practitioners to the Urology outpatient clinic with the differential diagnosis of prostate cancer, and whom were later diagnosed having benign prostate hyperplasia, prostatitis, or benign urethral or bladder pathology. Patient follow-up ended July 2005.

The following patient characteristics were recorded at time of blood collection: age, presence of metastasis, hemoglobin count, and PSA. The median referral PSA in the prostate cancer group was 31.0 ng/ml (interquartile range 3.4 to 556.5), and in the benign group 1.5 ng/ml (interquartile range 0.7 to 2.8). PSA doubling time (PSAdt) was calculated by assuming first-order kinetics and by using two additional PSA measurements after their initial visit, separated by a minimum of three months, or until the patients started chemotherapeutic and/or radiotherapeutic treatment. An increase in PSA was defined as more than 0.2 ng/ml change from previous measurement. Dichotomized PSAdt (< or > 3 months PSAdt) was used as a surrogate end-point for prostate cancer-specific mortality, as previously described (27). PSAdt was retrospectively determined for 59/75 patients. Most prostate cancer patients had hormone refractory prostate disease.

Plasma isolation

After blood collection with BD Vacutainer tubes with sodium citrate (BD Biosciences, Mountain View, California), the samples were directly placed on ice. Plasma was isolated from blood of patients within two hours by centrifugation at 1700 rcf for 15 minutes. The plasma was carefully removed, without disturbing the buffy coat, aliquoted and stored at –80°C until further use. The plasma was spun once before storage (defined as one-spin plasma). We analyzed the batch of samples collectively after a median of 26.6 months storage (interquartile range from 23.2 to 34.5 months). The samples were thawed at room temperature and centrifuged at 3400 rcf for 15 minutes to remove aggregates formed in the freeze-thawing process before nucleic acid isolation.

NASBA amplification

For the quantification of mitochondrial and genomic nucleic acids, we used a one-tube, real-time detection and quantification method based on NASBA. Nucleic acids were isolated from 100 µl of the supernatant plasma, and standard NASBA nucleic acid amplification reactions were performed as previously described (28). The amplified mtDNA transcript encodes 16s rRNA, and the mtRNA transcript encodes cytochrome c oxidase subunit 1 (CO*X1*). In selected primer regions no mutations are described for prostate cancer (29).

COX1 NASBA primer sequences are AATTCTAATACGACTCACTATAGGGAGAGGAGACACCTGCTAGGTGTAA (primer P1, T7 underlined), GGTGCCCCCGATATGGCGTTC (primer P2) and **CGATCC**AAGGACAAGGCGTTCAC-A**GGATCG** (molecular beacon, stem sequence shown in bold). 16s rRNA primers are ATTCTAATACG-ACTCACTATAGGGAAGAACCGGGCTCTGCCATCTTAA (primer P1, T7 underlined), GTAATCCAGGTCGGTTTCTA (primer P2) and **CGTACG**TGATATCATCTCAACTTAGTAT**CGTACG** (molecular beacon, stem sequence in bold). The amplified genomic transcript encodes U1A. The primers selected for this gene are reported (28). To avoid (co)amplification of mtRNA in the mtDNA amplification assay, primers were designed to span an RNA splice-site with one primer in the 3' end of 16S rRNA and the other in the tRNA just downstream of the mitochondrial transcription terminator. As *Msp I* digestion and denaturation was not performed in the mtRNA amplification assay, mtDNA could not be amplified.

A dilution series of target sequence (plasmid containing mtDNA and genomic target sequence and in vitro translated RNA for mtRNA target sequence) was amplified and the time points at which the reactions became positive (the time to positivity, TTP) were plotted against the input amounts of nucleic acids. This way a calibration curve was created that could be used to determine the amount of target sequence present in patient plasma samples. The average of duplicate amplifications was considered as the value for a sample. If the difference between duplicate amplifications was > 0.5 log value, the amplification for that sample was repeated. Due to repeated differences between duplo measurements of both mtDNA as mtRNA, three out of 89 samples were excluded from further analysis. For genomic U1A DNA measurements 83 out of 89 samples were available.

Cell culture

PC-3 prostate cancer cells (ATCC, Rockville, MD) were cultured in RPMI 1640 medium (Gibco BRL, Invitrogen, Breda, The Netherlands), supplemented with penicillin, streptomycin, glutamate and 5% fetal calf serum. For generation of PC-3 cells devoid of mitochondria, cells were cultured in the presence of 50 ng/ml ethidium bromide, 50 µg/ml uridine and 100 µg/ml pyruvate. Approximately 300,000 cells were lysed in L6 lysis buffer and frozen at -80 degrees Celsius until nucleic acid isolation and quantification.

Statistical Analysis

For differences in median copy number of mtDNA and mtRNA between survivors and non-survivors after a two year follow-up period, patients with and without metastasis, patients with high or low PSAdt and patients with cancer or benign disease, the non-parametric Mann-Whitney test was used. For correlative testing of mtDNA copies, mtRNA copies, patient age, PSA and hemoglobin levels, the Spearman's correlation coefficient was used.

A ROC curve was constructed to discriminate survivors from non-survivors, and to discriminate patients with cancer from patients with benign disease, using mtDNA or mtRNA copy numbers. For cancer prognostics and diagnostics the cut-off value with the combination of highest sensitivity and specificity was selected and used in all subsequent analyses. To give an idea of mtDNA and mtRNA as strong predictors of survival, we calculated survival prediction results of dichotomous mtDNA and mtRNA based on a variety of cutoffs. The results indicated that the choice of cutoff does not influence the relevance of mitochondrial DNA and RNA as predictors of patient outcome. Differences in survival between cancer patients with mt nucleic acids below or above selected cut-off values were evaluated according to the method of Kaplan and Meier. The Log-rank statistic was used to assess differences between both arms of the survival curves.

To identify independent factors influencing survival, multivariate risk factor assessment was performed using the Cox Proportional Hazards model. Variables included in the model were presence of metastasis, hemoglobin count, PSAdt and mtDNA or mtRNA. The laboratory parameters hemoglobin, PSA and PSAdt were not determined in 21, 7 and 16 cases, respectively, therefore correlative testing and Cox Proportional Hazards analysis for these variable were performed on fewer than 75 prostate cancer cases.

All results were analyzed using SPSS software (version 12.0.1). Error bars depicted are standard errors of the mean. Interquartile ranges shown are from 25th to 75th percentile. P-values below 0.05 (two-sided) were considered significant.

Results

Validation of real-time nucleic acid sequence-based amplification assay for mtDNA and mtRNA transcripts

We first assessed linearity of the assay by creating a calibration curve of the mtDNA and mtRNA target sequence. The transcripts encoding 16s rRNA and cytochrome c oxidase subunit 1 (COX1) were quantified and a standard curve of mtDNA and mtRNA input in relation to time to positivity (TTP) was generated (Figure 1A). We next tested the specificity of our assay by quantification of mt nucleic acids of the PC-3 prostate cancer cell-line treated with the intercalating agent ethidium bromide, which at low concentrations preferentially inhibits the synthesis of mtDNA, mtRNA and mt proteins (30). After 4 weeks of culture no mt nucleic acids were detectable (Figure 1B). The growth rate and viability in respiratory-deficient PC-3 cells was not affected when the cells were cultured in medium supplemented with pyruvate and uridine to enable ATP generation through glycolysis (not shown).

Figure 1A

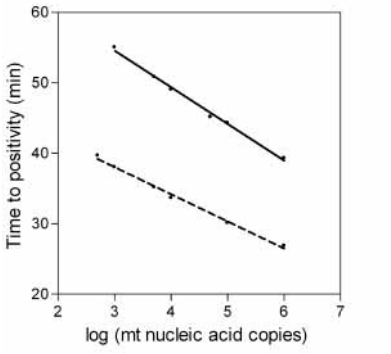
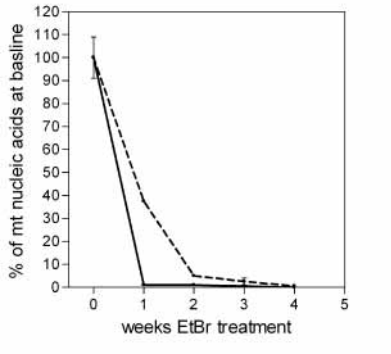


Figure 1B



Validation real-time amplification mt nucleic acids. Calibration curve of the transcripts encoding 16s rRNA (mtDNA, dotted line) and COX1 (mtRNA, solid line) (A). PC-3 prostate cancer cells were cultured with ethidium bromide for 4 weeks and every week mtDNA (dotted line) and mtRNA (solid line) transcripts were quantified (B).

Relating mitochondrial and genomic nucleic acids with clinical and laboratory parameters

We isolated nucleic acids from the plasma of 89 patients with prostate cancer and benign disease (patient characteristics are shown in Table 1), and quantified the transcripts encoding 16s rRNA, COX1 and U1A DNA. In Table 2 the median copy numbers are shown for mitochondrial and genomic nucleic acids in all samples measured. Median copies of mtDNA and mtRNA in plasma were respectively ±3000 and ±700 fold higher than genomic DNA. We then evaluated whether extracellular mtDNA in plasma was related with extracellular mtRNA, and found a strong correlation (n=86; r=0.73 with P<0.001). However, no significant correlation was found between genomic and mitochondrial nucleic acids. Subsequently, we examined the relation between mtDNA and hemoglobin count (n=58; r=-0.29 with P=0.028) and PSA levels (n=74; r=0.27 with P=0.021). MtRNA was also significantly correlated with PSA levels (n=74; R=0.31 with P=0.007) but not with hemoglobin count (n=58; r=-0.25 with P=0.064). There was no significant relation between mt nucleic acids in plasma and patient age (n=69; r=-0.20 with P=0.10 for mtDNA and r=-0.16 with P=0.19 for mtRNA). There were also no significant differences in levels of mtDNA or mtRNA in patients with metastasized disease compared to patients with locally advanced cancer (P=0.31 and P=0.26, respectively), between patients with androgen dependent or independent prostate cancer (P=0.67 and P=0.86, respectively) or between patients with high or low PSAdt (P=0.09 and P=0.89, respectively). No significant associations were found between plasma U1A DNA and the clinical and laboratory variables tested (data not shown).

High plasma mitochondrial nucleic acids in poor prognosis patients

We determined whether we could recognize patients with poor prognosis at presentation in our outpatient clinic by quantification of circulating cell-free mt nucleic acids in plasma. After a median follow-up time of 28.4 months after inclusion, 57% of all patients with advanced prostate cancer had died due to progression of their tumor. We assessed whether poor prognosis patients, based on two-year follow-up data, had elevated mitochondrial nucleic acids. At presentation in our outpatient clinic, non-survivors had a 3.8 fold increase in mtRNA compared to survivors (P=0.003; non-survivors 16,038 copies median, interquartile range from 5,097 to 48,544 copies and survivors 4,183 copies median, interquartile range 2,269 to 8,579 copies). Plasma mtDNA levels were also increased in non-survivors, with a 2.6 fold increase compared to survivors (P=0.02; non-survivors 61,590 copies

Table 1 - Clinical characteristics of 75 prostate cancer patients

Characteristics	No.	(%)
Benign prostate disease	14	(15.7%)
Prostate cancer	75	(84.3%)
Metastasis		
Yes	51	(68.0%)
No	24	(32.0%)
Hormone refractory		
Yes	61	(81.3%)
No	14	(18.7%)
Survival		
Alive	26	(34.7%)
Deceased tumor-related	43	(57.3%)
Deceased non-tumor related	1	(1.3%)
Unknown/ lost to follow-up	5	(6.7%)
Survival (months)		
Median	28.1	
Interquartile range [25-75%]	8.9-35.7	

Table 2 - Genomic and mitochondrial copy number in patient samples

Sample	genomic DNA	mtDNA	mtRNA
All			
Median	10.5	34,938	7,822
Interquartile	1.1-25.6	16,304-80,502	2,891-20,788
% detectable	82%	100%	100%
Benign disease			
Median	4.3	19,037	3,161
Interquartile	0.6-24.5	13,515-24,744	2,284-7,340
% detectable	85%	100%	100%
Prostate cancer			
Median	12.8	49,193	9,321
Interquartile	1.2-25.7	18,683-109,485	3,507-25,435
% detectable	83%	100%	100%

median, interquartile range from 30,765 to 189,131 copies and survivors 23,275 copies median, interquartile range from 14,372 to 59,249 copies). In contrast, plasma U1A DNA levels were not increased in poor prognosis patients (P=0.71).

Survival prediction based on levels of plasma mitochondrial nucleic acids

We subsequently assessed whether we could discriminate survivors from non-survivors based on dichotomized mtDNA and mtRNA variables. The AUC ROC, and sensitivity and specificity for selected mtDNA and mtRNA cut-off points are shown in Table 3. We assessed whether patients with plasma nucleic acid levels above 26,000 copies mtDNA and 10,500 copies mtRNA had significant differences in 2-year survival compared to patients with lower levels. Kaplan-Meier estimates showed significant differences for patients both with high and low mtDNA and mtRNA (Figure 2A and B). Cumulative 2-year survival for patients with elevated mtDNA levels was 35%, while survival of patients with low levels was 73%. Cumulative survival after 2 years for patients with high and low mtRNA levels was 21% and 73%, respectively. Median survival for patients with elevated mtDNA and mtRNA was only 13.1 and 11.8 months, respectively.

Table 3 - Selected prognostic cut-off values in prostate cancer patients.

Type	NA	cut-off	sensitivity	specificity	PPV	NPV	AUC	95%CI
Prostate	mtDNA	26,000	80%	58%	65%	73%	0.70	0.58-0.81
	mtRNA	10,500	69%	79%	77%	71%	0.72	0.60-0.83

Abbreviations: NA nucleic acid; PPV positive predicting value; NPV negative predicting value.

Figure 2A

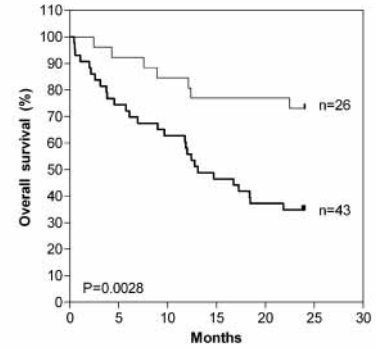
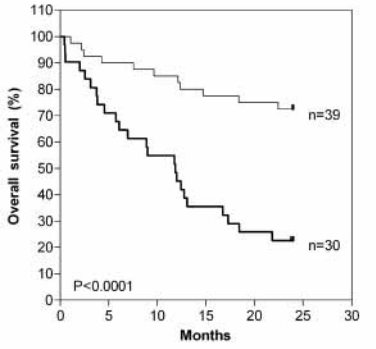


Figure 2B



Kaplan-Meier estimates of overall survival. Cumulative 2-year survival of prostate cancer patients with high (bold line) and low (faint line) circulating cell-free mtDNA (A) and mtRNA (B) levels at presentation.

Mitochondrial RNA is an independent predictor of survival

We evaluated whether circulating plasma mtDNA or mtRNA were predictors of patient survival and whether they had additive value when combined with established prognostic markers. We analyzed the relationship of metastasis status, PSAdt, and hemoglobin count with patient outcome in our advanced cancer population, and found that all variables were significantly associated with cancer-related death by univariate analysis. Dichotomized mtDNA and mtRNA were assessed together with these variables in a multivariate model, to establish the independent properties of mtDNA and mtRNA in prostate cancer prognostics. In addition, we evaluated whether mtDNA or mtRNA was the stronger predictor. When testing mtRNA against all variables, hemoglobin count, PSAdt, and mtRNA were all significant independent predictors of survival (HR 0.45 with 95% CI of 0.26-0.80, HR 0.32 with 95% CI of 0.13-0.81 and HR 2.87 with 95% CI of 1.09-7.57, respectively; Table 4). When testing mtDNA, only hemoglobin count and PSAdt were significant independent predictors for two-year survival (Hazard ratio (HR) 0.41 with 95% confidence interval (CI) of 0.23-.074 and HR 0.26 with 95% CI of 0.10-0.68, respectively).

Comparison of plasma mtDNA, mtRNA and genomic DNA levels in prostate cancer patients and control subjects

Next, we analyzed plasma from patients with benign disease of the prostate and urinary tract as control subjects. The median mtDNA copies per 100 µl plasma for prostate cancer patients were 49,193 (interquartile range 18,683 to 109,485 copies) and for benign controls 19,037 copies (interquartile range 13,515 to 24,744). Median mtRNA copies were 9,321 (interquartile range 3,507 to 25,435 copies) and 3,161 (interquartile range 2,284 to 7,340 copies) for prostate cancer patients

Table 4 - Multiple Cox Proportional Hazard analysis.

	overall survival		
Variable	HR	95% CI	P
Metastasis	1.89	0.41-8.79	0.42
Hemoglobin count	0.41	0.23-0.74	0.003
PSAdt	0.26	0.10-0.68	0.007
mtDNA[1]	2.58	0.83-8.07	0.10
Metastasis	1.84	0.40-8.57	0.44
Hemoglobin count	0.45	0.26-0.80	0.006
PSAdt	0.32	0.13-0.81	0.02
mtRNA[2]	2.87	1.09-7.57	0.03

Abbreviations: PSAdt, prostate specific antigen doubling time; HR, hazard ratio; CI, confidence interval.1mtDNA cut-off 26.000 and 2mtRNA cut-off 10.500 copies

and benign controls, respectively. When we compared the levels of nucleic acids in cancer patient plasma and the 14 benign controls, we found a significant increase of both mtDNA and mtRNA (Mann-Whitney, P=0.005 and P=0.029, respectively), but not for U1A DNA (P=0.25). We assessed how well mtDNA or mtRNA plasma levels could discriminate prostate cancer from benign disease by ROC analysis. The AUC ROC for mtDNA based diagnosis of prostate cancer patients was 0.76 (95% confidence interval (CI) 0.63-0.90) and for mtRNA 0.70 (95% CI 0.55-0.85). Selected cut-off points were for mtDNA 26,000 copies and for mtRNA 5,400 copies. Sensitivity and specificity for identification of prostate cancer from subjects with prostatitis, benign prostate hyperplasia and other benign disorders of the urogenital organs was 67% and 92% for mtDNA and 63% and 77% for mtRNA, respectively.

Discussion

Of the patients who died within two years after presentation, 80% had elevated levels of mtDNA and almost 70% had elevated mtRNA copies in their plasma compared to survivors. Cumulative 2-year survival was markedly decreased for patients with high levels of circulating mt nucleic acids. We therefore consider circulating mt nucleic acids as a novel prognostic marker for advanced cancer patients.

In the first report evaluating circulating nucleic acids (31), the authors found a 90% decrease in plasma DNA of patients following radiotherapy, and persistent high or increasing plasma DNA was associated with poor prognosis. Other groups have since reported an association of high plasma DNA levels with poor prognosis or a lack of response to treatment (6;9). Mitochondrial DNA encodes for proteins of the respiratory chain and cells can contain hundreds to thousands of mtDNA copies per cell, instead of 2 copies genomic DNA. Here we show that amplification of mt nucleic acids in plasma has an advantage over genomic nucleic acids. We report that elevated levels of mt nucleic acids measured at presentation in our clinic were already predictive for patient outcome. Whether mt nucleic acids can also be used as a follow-up marker during therapy is currently being assessed in a prospective study.

As it has been demonstrated that nucleic acids can be released from cells actively or passively (cell death), one would speculate that both RNA and DNA end up extracellular. Previous reports have demonstrated that white blood cells possess nucleic acid-binding receptors on their outer membranes, internalize and degrade nucleic acids into oligonucleotides (32). In this study we find a correlation between mtDNA and mtRNA copies, and between mt nucleic acids and PSA levels. This may indicate that the elevated nucleic acids have a direct relationship with tumor-cell burden (33), and/or that para-neoplastic characteristics, such as enhanced protease activity, may cause a decrease in the nucleic acid binding capacity to circulating blood cells (18). However, retrospective studies are exploratory and may not reflect true associations, and should therefore be treated with caution. Nevertheless, mtRNA is a stronger predictor of patient outcome than mtDNA, and only mtRNA is an independent prognostic factor in multivariate analysis. Whether mtRNA stability is specifically enhanced in poor prognosis patients has yet to be determined.

In the first publication on circulating plasma nucleic acids in 1977, the authors describe an increase of DNA in the plasma of cancer patients compared to healthy controls (31). Since then, several groups have demonstrated an increase in circulating plasma nucleic acids in cancer patients, and demonstrate a sensitivity and specificity of 85% and 73% in identifying prostate cancer from benign disease (3). However, a similar study could not confirm the potential value of plasma nucleic acids as a diagnostic tool in prostate cancer screening (34). Also quantification of circulating plasma RNA does not seem to have enough discriminatory power as a screening tool for cancer (35). In this study we show that prostate cancer patients have an approximately 3-fold increase in mtDNA, and a 2-fold increase in mtRNA copy number compared to the benign controls. However, the sensitivity and specificity is not sufficient to warrant further studies as a diagnostic tool.

To conclude, we demonstrate a strong increase in plasma mt nucleic acids in prostate cancer patients with poor prognosis. Quantification of mt nucleic acids in plasma could be a valuable predictor of prognosis in advanced hormone-refractory relapsed prostate cancer patients and their potential should be further evaluated in a large prospective multicenter study.

Acknowledgements

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Circulating mitochondrial ribonucleic acid identifies poor prognosis in prostate, head and neck, kidney and colorectal cancer patients

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Abstract

Background: Previous studies have demonstrated a role for circulating plasma DNA and RNA as a possible diagnostic and prognostic marker. No studies have investigated the diagnostic and prognostic significance of mitochondrial (mt) nucleic acids as pan-tumor marker in the plasma of different human cancers.

Experimental design: We collected plasma from 198 cancer patients (prostate, head-and-neck, renal, and colorectal cancer) and 40 healthy subjects. Nucleic acids were isolated and mtDNA (16S ribosomal RNA) and mtRNA (cytochrome c oxidase subunit 1) transcripts were quantified by real-time amplification. Mt nucleic acids were assessed as discriminatory marker for cancer, and as prognostic marker based on standardized cut-off points.

Results: We demonstrate that mtRNA, but not mtDNA, is increased in plasma of cancer patients compared to healthy subjects (p=0.001 and p=0.32, respectively). High mtRNA copies in plasma could discriminate between survivors and non-survivors (cut-off 75th percentile cancer patients, p=0.02). We found no significant differences in survival based on mtDNA copies.

Conclusion: We found increased mtRNA copies in plasma of 198 cancer patients compared to healthy controls. All standardized cutoffs for mtRNA could discriminate between good and poor prognosis patients, independent of cancer type. Elevation of plasma mtRNA, but not mtDNA, is a poor prognostic factor.

Introduction

The peripheral blood of cancer patients contains a vastness of unexplored potential cancer markers. A possible marker, which has been under scrutiny for the last decade is the presence of elevated circulating nucleic acids. Highly stable DNA and RNA can exist extracellularly in bodily fluids in healthy and in diseased individuals (1). Low amounts of circulating nucleic acids are present in healthy individuals, and most likely originate from hematopoietic blood cells (2). However, in cancer patients an increase in nucleic acids is found which may be released through apoptosis, necrosis, lysis, and also through spontaneous release by tumor cells (3). The alterations in the circulating nucleic acids match alterations present in the tumor, indicating that these elevated circulating nucleic acids (partly) originate from tumor cells (4-6).

Plasma DNA and RNA has initially been suggested as a diagnostic marker for cancer, but now is also studied as follow-up marker during treatment in previously diagnosed cancer patients. As circulating nucleic acids in plasma are partly tumor related (7), it is possible to identify tumor-specific alterations in a non-invasive way (8-12). In addition, the level of circulating nucleic acids and their kinetics may have significance as tumor marker. Increased levels of cell-free nucleic acids prior to treatment are associated with a poor prognosis, and a decrease in nucleic acids in plasma after treatment appears to be related with a response (13-15) and/or an association with disease-free and overall survival (15).

Recently also extrachromosomal mitochondrial nucleic acids have been quantified, as opposed to genomic nucleic acids. Using real-time amplification the level of mtDNA in plasma of healthy

subjects (16) and both mtDNA and mtRNA in cancer patients (17) have been quantified. In the latter study it was shown that quantification of mt nucleic acids had increased sensitivity and specificity as diagnostic and prognostic marker over genomic nucleic acids. It is unclear whether the findings in prostate cancer may be translated to other tumor types, and whether mtDNA/RNA may be seen as a pan-tumor marker.

The amount of contradictory literature on circulating nucleic acid levels as tumor marker (18;19) and a variety of methodologies (20-24) clearly indicate that protocols on blood handling, plasma isolation and storage all may greatly influence the validity of circulating nucleic acids as prognostic or diagnostic marker. An important item is the centrifugation of plasma to remove large particles (e.g. platelets and fragments or complexes that contain nucleic acids). Previously, it has been suggested that the measurement of nucleic acids in so called two-spin plasma has an improved sensitivity and specificity over one-spin plasma. Therefore, using two-spin plasma, we assessed in a large study with four different cancer types whether mt nucleic acid quantification could be used for cancer patient detection and prognostication.

We extracted nucleic acids from 238 plasma samples, comprising healthy volunteers, prostate, head and neck, kidney, and colorectal cancer, and amplified mtDNA (16S ribosomal RNA) and mtRNA (cytochrome c oxidase subunit 1) transcripts. We demonstrate that mtRNA is elevated in the plasma of cancer patients compared to healthy subjects, and that an elevation of mtRNA discriminates poor prognosis patients from patients with a better prognosis, independent of tumor type.

Material and methods

Characterization of study patients and healthy volunteers

Blood samples were collected from 198 cancer patients visiting the outpatient clinic of the Departments of Medical Oncology, University Medical Center in Utrecht, the Netherlands from February 2003 to September 2005. All cancer patients were untreated, new or relapsed patients, and comprised cancers of the prostate, head and neck, kidney and colorectum. The study was approved by the hospital Ethics Committee and written informed consent was obtained from all patients. Plasma of 40 healthy, age controlled subjects served as controls. Patient follow-up ended June 2006.

The following patient characteristics were recorded at time of blood collection: age, gender, presence of metastasis, hemoglobin, leukocyte and platelet count and comorbidity, and in a subset of patients, lactate dehydrogenase, alkaline phosphatase, PSA and CEA.

Plasma isolation

After blood collection with CPT Vacutainer tubes with sodium citrate (BD Biosciences, Mountain View, California), plasma was isolated from blood of patients within two hours by centrifugation at 1900 rcf for 30 minutes. The plasma was carefully removed without disturbing the buffy coat, transferred to a 15 ml tube, and centrifuged a second time at 3400 rcf for 15 minutes at 4°C to remove possible cellular contamination, large aggregates, and eliminate platelets from the plasma (defined as two-spin or platelet-poor plasma). The plasma was subsequently aliquoted and stored

at –80°C until further use. We analyzed the batch of samples collectively after a median of 26.1 months storage (interquartile range from 14.7 to 32.5 months). The samples were thawed at room temperature and briefly spun on a table-top centrifuge before nucleic acid isolation.

Nucleic acid isolation and NASBA amplification

Nucleic acids were isolated from 100 Ìl plasma using a silicon extraction protocol, as previously described by Boom et al. (25). For the quantification of mitochondrial and genomic nucleic acids, we used a real-time detection and quantification method based on NASBA (17). The amplified mtDNA transcript encodes 16s rRNA, and the mtRNA transcript encodes cytochrome c oxidase subunit 1 (COX1). Primers sequences are previously reported. The average of duplicate amplifications was considered as the value for a sample. If the difference between duplicate amplifications was > 0.5 log value, the amplification for that sample was repeated.

Statistical Analysis

For differences in median copy number of mtDNA and mtRNA between two or more groups, the non-parametric Mann-Whitney test or Kruskal-Wallis test was used. Linear regression and correlative (Spearman Test) analysis was used to identify variables which may influence mtDNA and mtRNA copy number in plasma.

To assess the mtDNA and mtRNA as a general tumor marker, we calculated cutoffs according to a defined procedure and tested for their power to discriminate between survivors and non-survivors. This was done as follows:

1. For cancer prognostication, the cutoff values at the 95th percentile of the 40 healthy volunteers was calculated (cutoff for mtRNA 949 and mtDNA 9159 copies) and defined as cutoff to detect poor prognostic cancer patients, given the fact that a specific pan-tumor prognostic detection marker demands elevated copies above levels found in healthy subjects.
2. To identify discriminatory cutoffs in a similar way for prognostication of poor prognostic patients within the cancer group, the quartiles (25th and 75th quartile) and the median copies found in the 198 investigated cancer patients were determined as cutoff (the 75th percentile cutoff for mtRNA was 1643 and mtDNA 6073 copies).

Differences in survival between cancer patients were evaluated using selected cut-off value according to the method of Kaplan and Meier. The Log-rank statistic was used to assess differences between both arms of the survival curves.

All results were analyzed using SPSS software (version 12.0.1). Error bars depicted are standard errors of the mean. Interquartile ranges shown are from 25th to 75th percentile. P-values below 0.05 (two-sided) were considered significant.

Results

Evaluation of mitochondrial DNA and RNA level in prostate, head and neck, kidney and colorectal cancer

By using serial dilutions of plasmid containing the mtDNA transcript and in-vitro translated RNA containing the mtRNA transcript, a standard curve was generated by which we could calculate the

copy number present in plasma samples by their time to positivity. Nucleic acids were isolated from platelet-poor citrate plasma of 40 healthy controls, 73 prostate, 28 head and neck, 27 renal and 30 colorectal cancer patients. MtDNA transcripts encoding 16s rRNA, and mtRNA transcripts encoding cytochrome c oxidase subunit 1 (COX1) were amplified by real-time amplification. In all cases (100%), it was possible to calculate mtDNA and mtRNA copy number present in the plasma of healthy controls and patient samples. Median mtRNA concentration present in the plasma of cancer patients was 431 copies and 252 copies in controls (p=0.0007; Table 1). Median mtDNA copy number was 1701 and 1708 copies, respectively (p=0.34). MtRNA levels were strongly elevated in cancers of the prostate (median 470 copies; p=0.0009), head and neck (median 572 copies; p=0.002) and kidney (median 802 copies; p=0.0007), but not in colorectal cancer (median 198 copies; p=0.80). In contrast, mtDNA copies were only significantly increased in patients with kidney cancer (median copies 3312; p=0.02). When we evaluated whether mtRNA could be used as diagnostic cancer marker, after exclusion of the colorectal cancer group, we found an area under the Receiver Operator Characteristic curve of 0.71 (95% confidence interval from 0.63 to 0.79).
Next, relationships between mt nucleic acids and blood parameters or tumor markers were studied, however no correlations were found.

Table 1 - Plasma mtDNA and mtRNA concentrations for controls and cancer patients

		mtDNA			mtRNA		
	n	median	range	p-value*	median	range	p-value*
Controls	40	1708	363-3036		252	56-443	
Cancer	158	1701	191-6073	0.34	431	160-1643	0.0007
Prostate	73	1125	136-5813	0.71	470	148-1647	0.0009
Head&neck	28	1244	82-9519	0.80	572	201-1585	0.002
RCC	27	3312	1681-15122	0.02	807	210-4388	0.0007
Colorectal	30	1649	234-4244	0.63	198	103-520	0.80

* compared to healthy controls, Non-Parametric test

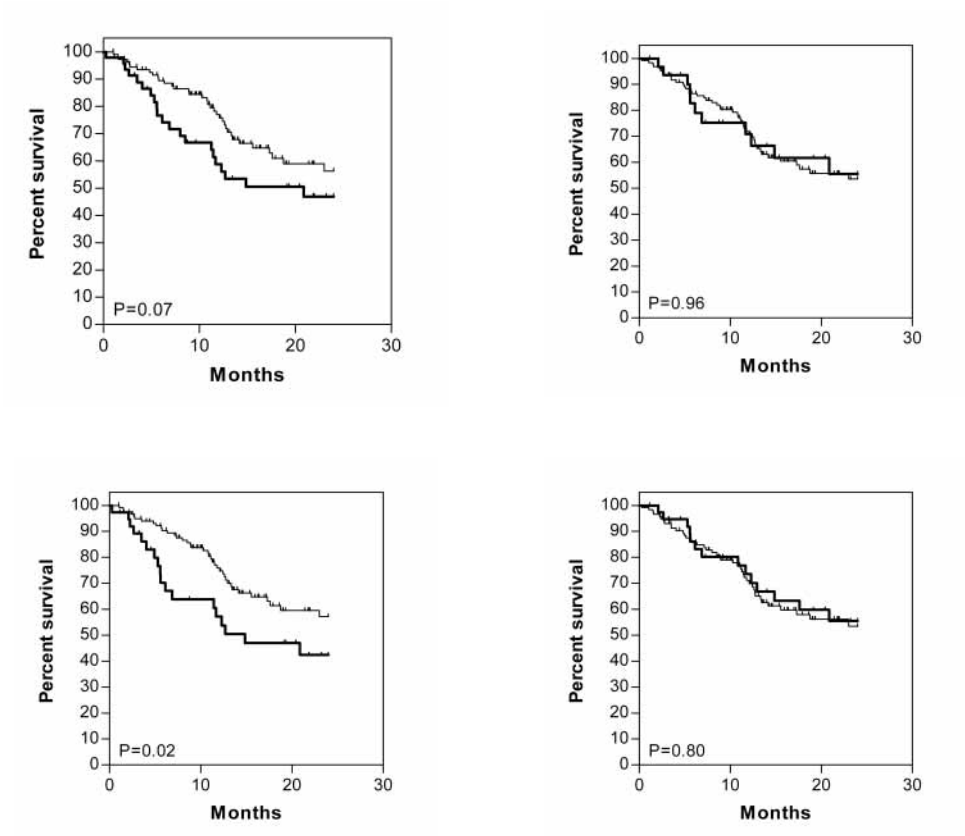
Mitochondrial RNA copies can predict survival, independent of tumor type

To determine whether mtRNA and mtDNA had prognostic value, patients were followed for a median of 30.6 months after blood collection (interquartile range from 15.9 to 31.1 months), and tumor-related death and survival was noted. Median survival of all cancer patients was 15.6 months (95% confidence interval 11.3 to 19.9 months).

We first assessed the *diagnostic* power of mtRNA or mtDNA for cancer patient prognostication. To detect cancer patients associated with a poor outcome, mtRNA and mtDNA were dichotomized at the 95th percentile of the range found in healthy controls, and "low" and "high" were defined as mt nucleic acids copies below or above the 95th percentile, respectively. Patients with high mtRNA had a decreased survival (median survival 14.9 months) compared to patients with low mtRNA (median survival not reached; Log rank 3.21 with P=0.07; Figure 1A). However, elevated mtDNA did not discriminate patients on survival (Figure 1B).

Next we assessed within the group of 198 cancer patients whether patients with mtRNA or mtDNA copies higher than median, or patients higher than the 75th percentile of the range found in the 198 cancer patients, had worse prognosis compared to patients with lower concentrations.

Figure 1A, 1B, 1C, 1D



Kaplan-Meier survival estimates of 198 patients with cancer analyzed according to plasma mtRNA and mtDNA. Cut-off values were based on the 95th percentile of mtRNA (A; top left) and mtDNA (B; top right) copies in plasma of healthy controls. The 75th percentile of the range found in cancer patients were also used as dichotomous variable to assess survival for mtRNA (C; bottom left) and mtDNA (D; bottom righta). Bold lines indicate patients above cutoff and faint line indicate patients below cutoff.

Using the defined cutoff at the median found in the cancer patients, we found a decreased survival of patients with elevated copies (data not shown). The patients with highest mtRNA copies (above 75th percentile) also showed significant decreased survival, when compared to the patients with lower copy number (Log rank 5.05 with P=0.02; Figure 1C). However, mtDNA could not predict survival using both these cutoffs (data not shown for median copy number; 75th percentile depicted in Figure 1D).

Discussion

In this study, we used real-time amplification to quantify levels of mitochondrial nucleic acids in the plasma of four different types of cancer, and evaluated diagnostic and predictive power of these potential tumor markers. We demonstrate a strong elevation of mtRNA copies in patients with prostate, head and neck and renal cancer. Patients with mtRNA copies above the normal range found in healthy controls, showed poorer survival after a two-year follow-up period.

There are only few studies evaluating circulating mt nucleic acids as potential tumor marker, as apposed to genomic nucleic acids. Suggestions have been made that real-time amplification of mt nucleic acids might yield increased sensitivity or specificity over genomic nucleic acids, as 100-1000 copies of a mt gene is present compared to 2 copies found in genomic DNA (26). Most studies which have quantified genomic plasma nucleic acids have shown large variations between different cancers (27). This limits the use of defined cut-off values of a specific cancer type for diagnostics and/or prognostication and prohibits broad applicability. Here, we demonstrate that mt RNA is particularly increased in poor prognosis patients with prostate, head and neck, kidney, and colorectal cancer, and identification of these patients is possible using a defined cut-off value. Even though colorectal cancer patient as group did not have significantly increased mt nucleic acids as compared to healthy subjects, patients with mtRNA concentrations above the 95th percentile found in healthy subjects demonstrated a decreased survival (cumulative survival for low and high levels 61% compared to 30% after 24 months follow-up, data not shown). This is the first report demonstrating the use of cell-free (mitochondrial) nucleic acids as a prognostic marker independent of cancer type.

Detection of elevated genomic nucleic acids has been suggested as diagnostic and prognostic marker for cancer, but as often has been disputed in other studies. These inconsistencies may be caused by a number of reasons; not all the markers chosen (e.g. ß-globin, GAPDH, APP, hTERT) might reflect total nucleic acid level, patient selection and stage differ between studies, and methodological protocols vary for blood collection (blood tubes, lag-time between plasma isolation), centrifugation speed, plasma collection (one vs. two-spin), nucleic acid isolation and detection methods (real-time amplification vs. colorimetric, radioimmunoassay and UV absorbance). Therefore, standardization of the above is essential for definitive a conclusive comparisons between studies.

Previous reports indicate that for optimal discrimination between cases and controls, plasma subjected to only one spin is inferior than plasma which is spun twice (16;18). Therefore we banked plasmas of patients participating in this study according to a two-spin protocol, with the second centrifugation at 3400 rcf to diminish platelet contamination in plasma. We demonstrate that mtRNA, but not mtDNA is increased in cancer patients, which is not in accordance with our previous study using one-spin plasma. We have demonstrated an impressive discrimination of survivors and non-survivors based on both mtDNA and mtRNA in 78 advanced prostate cancers (17). In present study including 73 advanced prostate cancers, the prognostic power of mt nucleic acids was reduced, which most likely is attributable to using two-spin instead of one-spin plasma, however the cause is not entirely clear. First, if cellular contamination in one-spin plasma would have caused higher mitochondrial nucleic acids, it would not explain the prognostic significance of mtDNA and mtRNA. As it has been shown that platelets contain ±10 copies mtDNA and <1 copy of mtRNA (28)(and personal communication with M. de Baar), this could explain the decrease in plasma mtDNA, while not visibly influencing mtRNA copy number, as we now have used platelet-poor plasma. However, peripheral blood cells (i.e. leukocyte and platelet count) did not show any relationship with mt nucleic acids or survival, therefore we believe cellular contamination to be a not very probable reason. Second, another source of nucleic acids, specifically of circulating DNA, are large protein-nucleic acids complexes or particles, such as nucleosomes (13), which may be spun down at high centrifugation (3400 rcf), without affecting RNA levels. Whether this may also be the case for mtDNA has to be further investigated, nevertheless, mtRNA remains a prognostic marker in this study with two-spin plasma.

In exploratory analysis we found lower plasma mtRNA levels in females compared to males (data not shown), which is in accordance with Yin et al. who indicate differential mitochondrial presence between both sexes in healthy as well in tumor tissue (29). In patients with type 2 diabetes decreased mtDNA content has been shown in peripheral blood mononuclear cells (30). Analysis also indicates a decreased concentration of cancer patients with type 2 diabetes in plasma (data not shown). For further studies with mt nucleic acids as prognostic marker in cancer these confounders might have to be adjusted for.

Concluding, this is the first report to demonstrate circulating nucleic acids as prognostic marker, independent of tumor type. An elevation above the normal range may be used to distinguish patients with poorer prognosis. Further prospective studies are necessary to address the clinical significance of circulating mt nucleic acids as prognostic marker.

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Studies on free circulating mitochondrial nucleic acids in plasma of healthy subjects and cancer patients prior to and following anti-cancer treatment

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Abstract

Purpose: Cell-free mitochondrial (mt) DNA and RNA are present in the blood of healthy subjects and cancer patients. We determined the stability and kinetics of mitochondrial nucleic acids in peripheral blood and in cell culture, assessed kinetics during anti-tumor treatment, and correlated plasma levels with patient prognosis.

Experimental design: Blood samples were collected from healthy controls and cancer patients prior to treatment. After blood collection the mt nucleic acids levels were quantified by real-time amplification of mtDNA (encoding18S rRNA) and mtRNA (encoding COX1). Differences in plasma levels between different blood processing protocols were studied. Cell culture experiments were performed to assess the level of cell-free mtDNA and mtRNA in medium and correlated with cell-death and/or spontaneous release. Kinetics were assessed directly and 24 and 48 hours after infusion of cisplatin/gemcitabine combination chemotherapy. Furthermore, the levels of mtDNA and mtRNA in breast cancer patients prior to treatment were associated with patient outcome.

Results: Circulating mtDNA and mtRNA are more abundantly present in the plasma than genomic nucleic acids. When plasma is isolated from peripheral blood four hours after blood draw, an increase is seen in mtDNA and mtRNA levels (±1.8 and ±1.5 fold), however once plasma is isolated their levels are stable up to four hours at room temperature. MtDNA is related with cell-death (r=0.87), however after serum starvation an increase is seen in both mtDNA and mtRNA levels, independent of cell-death. Both mtDNA as mtRNA levels directly decrease post-infusion of chemotherapy, with lowest levels at 48 hours after infusion. High levels of both mtDNA as mtRNA in breast cancer patients prior to treatment, is indicative for a poor outcome.

Conclusion: For the real-time amplification of cell-free mt nucleic acids, plasma, standardization of blood processing is essential for the control of variability and reproducibility. The kinetics of mt nucleic acid release is suggestive for spontaneous release prior to cell death. Inhibition of cell proliferation and induction of cell-death by chemotherapy rapidly decreased levels of mtDNA and mtRNA in plasma, and high levels at presentation were associated with poor survival probabilities. Amplification of plasma mtDNA and mtRNA after standardized blood-processing is a promising follow-up marker in cancer management.

Introduction

During the process of tumorigenesis, cancer progression and metastasis formation, increasing amounts of cell-free nucleic acids are detected in various body fluids. These nucleic acids can be shed into the blood stream as a consequence of necrosis and/or apoptosis of the tumor, tumor microenvironment, circulating tumor cells or may even be released actively by cancer cells (1). Nucleic acids can readily be isolated from plasma, and amplified for tumor-specific DNA or RNA sequences (2;3). Multiple studies have shown that circulating nucleic acids show identical aberrations e.g. alterations in microsatellite markers (4), mutations (5) or aberrant methylation profiles (6) as DNA from the tumor itself. Also the RNA expression profiles in plasma may identify malign signatures matching intratumoral gene expression that could be used for cancer detection or as predictive marker (7). Beside amplification of specific sequences, studies have also focused on the quantification of the total amount of circulating nucleic acids in blood, which can be related to

the presence of cancer e.g. prostate (8), breast (9) and lung cancer (10-12). Furthermore, total circulating levels or tumor-specific nucleic acid sequences decline as a consequence of anti-tumor treatment and an increase during follow-up is associated with relapse (13-15).

In most of the studies described above, genomic DNA markers have been used. We however developed assays for the quantification of extrachromosomal circulating mitochondrial DNA (mtDNA) and RNA (mtRNA), as cells can contain hundreds of mitochondria with multiple copies of DNA and RNA. We hypothesized that the quantification of mitochondrial nucleic acids might be a more sensitive surrogate tumor marker than genomic nucleic acids. In a previous study, we demonstrated that levels of mitochondrial nucleic acids, but not genomic DNA, in the plasma of patients with advanced prostate cancer were an independent prognostic factor to identify patient outcome (16).

These results led us investigate cell-free mitochondrial nucleic acid kinetics in the peripheral blood in this study. It is still largely unknown how the different steps involved in sample preparation influence the amount of circulating mtDNA and mtRNA. Recent methodological papers were published, where it was shown that the delayed separation of plasma from blood cells, freezing and thawing of plasma, storage of plasma, centrifugation, choice of anticoagulant and the method of extraction have an influence on the amount of free-circulating genomic DNA (17-20).

This paper describes a series of methodological experiments to determine time-frames for sample preparation, sample processing and storage conditions for free-circulating mitochondrial nucleic acids. Furthermore, we assessed whether mitochondrial nucleic acids were also spontaneously released in the medium of cultured cell-lines, or whether it was related with cell death. Finally, we studied levels of mitochondrial nucleic acids in the plasma of cancer patients before and after treatment with gemcitabine and cisplatin chemotherapy, and assessed whether elevation of mitochondrial nucleic acids correlated with patient prognosis in untreated advanced breast cancer patients.

We conclude that mitochondrial nucleic acids have to be rapidly isolated from peripheral blood, however are stable in plasma over 4 hours at room temperature. Mitochondrial nucleic acids in plasma decrease directly after effective anti-tumor treatment, and high levels correlate with a poor patient prognosis. Quantification of mitochondrial nucleic acids in plasma is an easy and reproducible assay, and a promising pan-tumor marker for the identification of patients with poor prognosis.

Material and methods

Characterization of study patients and healthy volunteers

Blood samples were collected from untreated patients with breast cancer, either newly diagnosed or relapsed patients visiting the outpatient clinic of the Departments of Medical Oncology, University Medical Center in Utrecht, the Netherlands. Inclusion of patients started in October 2001 and ended in July 2003. Patient follow-up ended July 2005. Furthermore blood was collected from 12 patients with a variety of tumors (prostate n=1, melanoma n=5, pancreas n=4, kidney n=1, and ovary n=1).

This blood was sampled before and 2, 4, 8, 24, and 48 hours post-infusion of combination chemotherapy, consisting of gemcitabine (1250 mg/kg^2) and cisplatin (60 or 75 mg/kg^2). The study was approved by the hospital Ethics Committee and written informed consent was obtained from all patients.

Plasma isolation

Peripheral blood was drawn from healthy subjects and breast cancer patients with Vacutainer tubes containing sodium citrate (Becton Dickinson, Mountain View, CA). After blood collection the samples were directly placed on ice and plasma was isolated within two hours by centrifugation at 1700 rcf for 15 minutes at 4°C. The plasma was carefully removed, without disturbing the buffy coat, aliquoted and stored at –80°C until further use (defined as one-spin plasma). After thawing, breast cancer plasmas were subjected to an extra centrifugation at 3400 rcf for 15 minutes to remove aggregates and complexes formed during the freeze-thawing process. Blood was drawn from the 12 cancer patients prior and during chemotherapeutic treatment using a Vacutainer Cell Preparation Tube with sodium citrate (Becton Dickinson, Mountain View, CA). The tubes were centrifuged for 30 minutes at room temperature at 2800 rcf, the supernatant plasma was transferred to a 15 mL tube, followed by a second centrifugation step at 3400 rcf for 15 minutes at 4°C. Plasma was aliquoted and stored at –80°C until further use (2-spin plasma).

Nucleic acid isolation

100 µl of plasma or supernatant was added to a 1.5 ml eppendorf tube containing 900 µl L6 lysis buffer, containing guanidine thiocyanate, Triton X-100, EDTA and Tris-HCl. The nucleic acids now present in the lysis buffer were further purified with the method described by Boom et al (21). The isolated nucleic acids were eluted in 50 µl Tris/HCl.

Real-time nucleic acid amplification

Standard nucleic acid sequence-based amplification (NASBA) reactions were performed as previously described (16). To achieve quantification, a dilution series of target sequence (plasmid containing mtDNA target sequence and in vitro translated RNA for mtRNA target sequence) was amplified and the time points at which the reactions became positive (the time to positivity, TTP) were plotted against the input amounts of nucleic acids. All amplifications were performed in duplicate. The average of these duplicate amplifications was considered as the value for this sample. If the difference between duplicate amplifications was > 0.5 log value, the amplification for that sample was repeated.

Methodological experiments

For the stability of mitochondrial nucleic acids in peripheral blood at room temperature, blood was drawn and kept at room temperature for 0, 4, 24, 48 and 72 hours. Plasma was isolated and divided in two aliquots, the first which was directly frozen, and a second which was subjected to a second centrifugation at 3400 rcf before storage of the supernatant at -80ºC. To test the stability of mitochondrial nucleic acids in plasma, plasma was isolated and then kept at room temperature for 0, 15, 30, 60, 120, 480 minutes before storage. To test the effect of centrifugation on levels of mitochondrial DNA or RNA, plasma was isolated and aliquoted. One aliquot was directly frozen, the others were subjected to an extra centrifugation at 1700, 3400, 6800, 10 000, 16 100 rcf before storage. To test the effect of freeze-thawing on the stability of mitochondrial nucleic acids, we

isolated directly isolated plasma and quantified mtDNA and mtRNA, or subjected the plasma to one or two freeze-thaw cycles. To test the effect of possible platelet contamination in the plasma of patients on mitochondrial nucleic acid copies, platelets were isolated from peripheral blood as previously described (22) for spike experiments. In brief, after discarding the first 5 mL, blood was drawn in 50-mL syringes containing 0.11 mol/L sodium citrate, acid citrate, and glucose (ACD) and centrifuged at 170g for 20 minutes. After separating the platelet-rich plasma (PRP), PRP was centrifuged for 10 minutes at 1500g and resuspended in M199. A dilution series of $1x10^7$ to $\pm 2x10^4$ platelets were spiked directly into lysis buffer and stored at -80ºC until nucleic acids isolation and amplification.

Cell culture experiments

The endothelial cell-line HMEC-1 (ATCC, Rockville, MD) was cultured in M199 medium (Gibco BRL, Invitrogen, Breda, The Netherlands), supplemented with penicillin, streptomycin, glutamate and 10% fetal calf serum. Cells were plated in 6-well plates and grown until 90% confluence. The medium was refreshed (time point 0 hours) and subsequently removed from 2-wells after 24, 48, 72 and 96 hours of culture. The medium was subsequently centrifuged in 15 mL tubes at 1300g for 15 minutes at room temperature, and 750 µL medium was directly frozen at -80ºC until nucleic acid amplification. The cell pellet was resuspended in medium 1:1 diluted with Trypan Blue. Trypan blue positive and negative cells and were quantified using a Bürker-Turk hemacytometer, and expressed as numbers of cells/mL. After 96 hours, the cells were washed with PBS, and serum starved for 6 hours with M199 medium containing no fetal calf serum. After serum starvation, the medium was removed, centrifuged and frozen, and from the cell-pellet total and Trypan blue positive and negative cells were again quantified.

Statistical Analysis

For comparison of the median copy number of mtDNA and mtRNA between survivors and non-survivors, the non-parametric Mann-Whitney test was used. A Receiver Operating Characteristic (ROC) curve was constructed to discriminate survivors from non-survivors using mtDNA or mtRNA copy numbers. A cut-off was selected as previously described (16), and differences in survival between cancer patients with mt nucleic acids below or above selected cut-off values were evaluated according to the method of Kaplan and Meier. The Log-rank statistic was used to assess differences between both arms of the survival curves. All results were analyzed using SPSS software (version 12.0.1). Error bars depicted are standard errors of the mean. Interquartile ranges shown are from 25^{th} to 75^{th} percentile. P-values below 0.05 (two-sided) were considered significant.

Results

Nucleic acid-sequence based amplification of mitochondrial nucleic acids in plasma. Figure 1 shows typical calibration curves obtained by the amplification of a dilution series of plasmid DNA containing the mtDNA sequence encoding 16sRNA, and in-vitro translated RNA containing the mtRNA sequence encoding cytochrome c oxidase subunit 1 (COX1). By plotting the amount of input copies against their time to positivity (TTP), a standard curve was constructed, that could calculate the amount of target sequence present in a plasma sample by interpolation of their TTP. Validation of the NASBA assay was has been previously reported (16). To assess the levels of mitochondrial nucleic acids in the plasma of healthy subjects in relation to genomic DNA, we quantified mtDNA,

Figure 1A

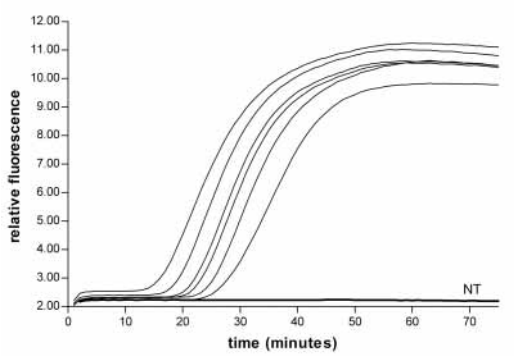
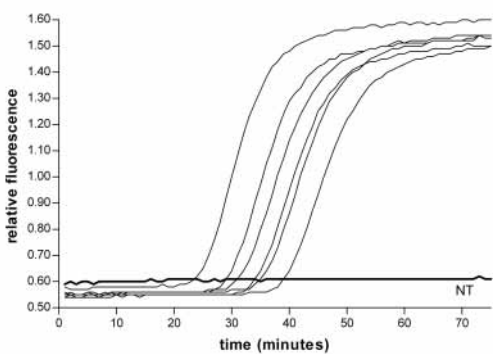


Figure 1B



Typical calibration curve obtained with plasmid mtDNA (A) and in-vitro translated mtRNA (B). Shown on the x-axis is the relative fluorescence of molecular beacon 6-carboxyfluorescin specific for mtDNA (A) and mtRNA (B), and on the x-axis the time in minutes. Relative fluorescence was measured every 60 seconds. For mtDNA, the standards used in serial dilutions ranged from 1x106 copies to 500 copies (corresponding to the curves from left to right), and a sample containing no template (NT). For mtRNA, the standards used ranged from 1x106 copies to 1000 copies (from left to right), and a sample containing no template.

mtRNA and U1A DNA, which is a common housekeeping gene. Circualting mtRNA was ±17 times more abundantly present and mtDNA was ±330 times more abundantly present when compared to U1A DNA copies in plasma (data not shown).

Stability of whole blood and plasma at room temperature

It has been suggested that hematopoietic cells can spontaneously secrete nucleic acids into the extracellular space, but this process may also be associated with necrosis and/or apoptosis. Whatever the mechanism responsible for the presence of cell-free nucleic acids, it was important to assess whether a lag time between blood collection and centrifugation would affect the level of mtDNA and mtRNA in plasma. An increase in time would be expected if there where a relation with necrosis or apoptosis of hematopoietic cells, however the kinetics of active release from blood cells is largely unknown. To study these hypotheses, we drew five blood tubes from three healthy volunteers. From each volunteer one tube was used to directly isolate plasma, while the other tubes were stored for 4, 24, 48 or 72 hours at room temperature before plasma isolation. After plasma isolation, nucleic acids were isolated and mtDNA and mtRNA copies were quantified (Figure 2A). An increase is seen in both mtDNA and mtRNA plasma copies after 4 hours at room temperature following blood collection (mtDNA at 0 hours 99350 ± 27996 copies, 4 hours 181486 ± 57115 copies; mtRNA at 0 hours 64822 ± 12176 copies, 4 hours 99301 ± 37700 copies), followed by a less dramatic slope of increase until the 48 hour time point (mtDNA 24 hours 404838 ± 126834 copies, 48 hours 391956 ± 52092 copies; mtRNA 24 hours 134198 ± 43675 copies, 48 hours 155238 ± 44364 copies). From the 48 to 72 hour time point, a decrease is seen in the amount of mitochondrial nucleic acids present in the plasma (mtDNA 72 hours 247182 ± 55747 copies; mtRNA 72 hours 86087 ± 2878 copies). Because the mitochondrial nucleic acid kinetics might be due to particles containing extracellular bound nucleic acids or large nucleic acid-protein complexes, we performed an experiment with a second centrifugation at 3400 rcf, to possibly remove these larger nucleic acid complexes. In this experiment, the levels of both mtDNA and mtRNA were ±100-fold lower, and there was a clear linear increase in time up till the 72 hour time point (Figure 2B).

Figure 2A

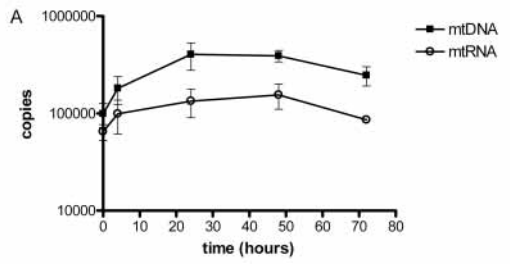


Figure 2B

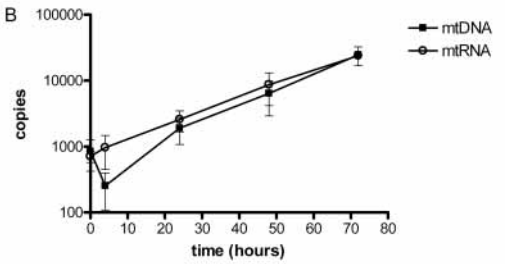
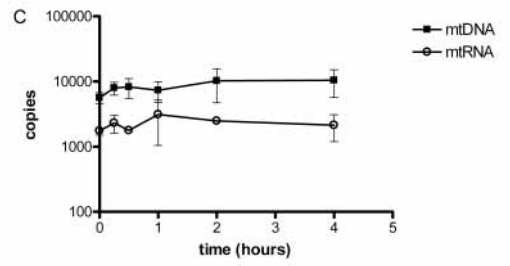


Figure 2C



Levels of mitochondrial nucleic acids in blood and plasma incubated at room temperature. Peripheral blood was drawn in tubes containing sodium citrate and left at room temperature for 0, 4, 24, 48 and 72 hours before centrifugation, nucleic acid isolation and amplification. Plasma was subject to only one spin (A) or two spins (B). After peripheral blood was drawn and plasma was isolated, the stability of mitochondrial nucleic acids in plasma was assessed. Nucleic acids were quantified directly and after 15, 30 minutes, 1, 2 and 4 hours incubations at room temperature (C).

We further investigated whether the time between plasma isolation and further processing would also influence mitochondrial nucleic acid copy numbers. Therefore plasma was isolated from four healthy donors and incubated for 15 minutes, 30 minutes, 1, 2, and 4 hours at room temperature (Figure 2C). No significant changes were detected in levels of mitochondrial nucleic acids during the 4 hours at room temperature.

The amount of centrifugation steps affects the mitochondrial nucleic acids copy number

Next we investigated how the one or two-spin protocol affects the level of mitochondrial nucleic acids in plasma. For this experiment plasma was isolated from two healthy subjects, and the plasma was isolated and the supernatant was subjected to no extra centrifugation or either a second centrifugation at 1700, 3400, 6800, 10 000 or 16 100 rcf. Nucleic acids present in the one-spin plasma compared to two-spin plasmas were ±30-fold higher (mean increase mtDNA 28.7 ± 6.4 copies and mtRNA 30.2 ± 12.0 copies). One-spin mtDNA samples had an average of $7.2x10^5$, while two-spin samples had an average $3.8x10^4$ copies ($P<0.001$; Figure 3A). One-spin mtRNA samples had an average of $3.9x10^5$, while two-spin samples had an average $1.5x10^4$ copies ($P<0.001$; Figure 3B). Between the two-spin mtDNA samples there were significant differences between the centrifugation speeds ($P=0.014$; highest circulating mtDNA of $7.0x10^4$ copies with 6800 rcf). However, for the two-spin mtRNA samples there were no significant differences between different centrifugation speeds ($P=0.76$). Consequently, a second centrifugation step, and less strongly the speed of the second centrifugation, affects the mitochondrial nucleic acid level in plasma.

Figure 3A

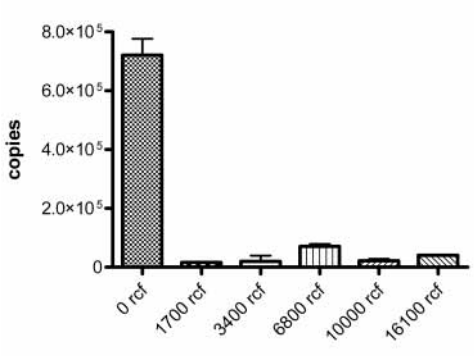
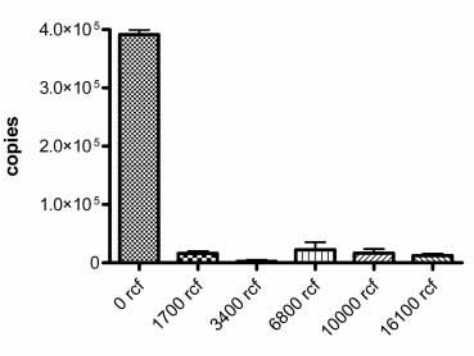


Figure 3B

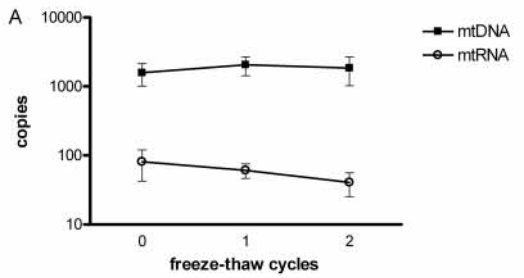


Effect of centrifugation on mitochondrial nucleic acids in plasma. Plasma was isolated by one centrifugation step at 1700g, and subjected to a second spin at increasing speed. MtDNA (A) and mtRNA (B) were isolated and amplified from the supernatant of these 2-spin plasmas (A).

Freezing and thawing of plasma

To investigate the effect of repeated freezing and thawing on the stability of isolated mitochondrial nucleic acids, nucleic acids were isolated from the plasma of 4 healthy donors. One aliquot was immediately used to determine the amount of mtDNA and mtRNA. A second aliquot was stored at -80°C and subjected to two cycles of freezing and thawing, before mtDNA and mtRNA were quantified (Figure 4). No significant changes were seen after one or two freeze-thaw cycles in comparison to fresh isolation (P=0.89 and P=0.56, respectively).

Figure 4



Stability of mitochondrial nucleic acids after two freeze-thaw cycles. Plasma was subjected to 0, 1 and 2 freeze-thaw cycles before mitochondrial nucleic acids were isolated and amplified (A).

High platelets count may influence mitochondrial nucleic acid copies in plasma

As high levels of platelets are present in the blood and strongly elevated platelet numbers can be present in cancer patients, we studied the possibility that platelets or platelet particles containing nucleic acids might affect the mitochondrial nucleic acid levels we quantify and call cell-free or circulating. Therefore we isolated platelets from peripheral blood and made a dilution range of $1x10^7$ to $2x10^5$ platelets/ mL directly in lysis buffer. We quantified the mitochondrial nucleic acids in lysis buffer, and found high mtDNA and mtRNA copy number when as few as $2x10^5$ platelets/ mL were directly spiked into the lysis buffer (figure 5).

Figure 5A

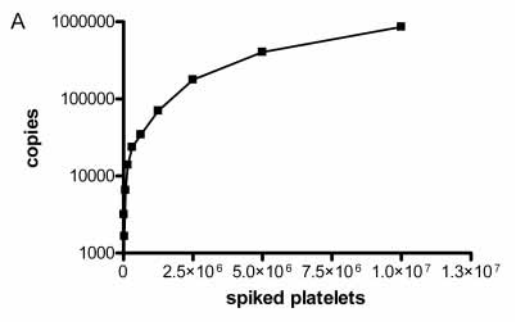
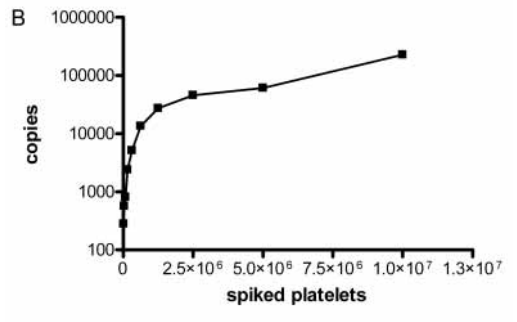


Figure 5B



Effect of platelet contamination in plasma on mitochondrial nucleic acid copies. Increasing amount of platelets were spiked directly in lysis buffer or platelet poor plasma and frozen. After thawing mtDNA copies (A) and mtRNA copies (B) were isolated and quantified.

Cells in culture secrete mitochondrial nucleic acids into the medium

The endothelial cell-line HMEC-1 was cultured until 90% confluence. These cells display contact inhibition, induce cell-cycle arrest and show typical endothelial cobblestone appearance with high confluence. The medium was refreshed, and subsequently every 24 hours the amount of mtDNA and mtRNA in the plasma was quantified, and correlated with the amount of total and dead cells present in the medium (Figure 6). There were increasing levels of mtDNA copies in the medium with the length of incubation, but a decrease in mtRNA copies. A correlation was found between mtDNA and Trypan blue positive cells (r=0.87 with p=0.06), however none with mtRNA (r=-0.33 with P=0.61). The cells were subsequently serum starved for 6 hours, which led to an 86-fold increase in mtRNA and a 1.9-fold increase in mtDNA, without any increase in Trypan blue positive cells.

Figure 6A

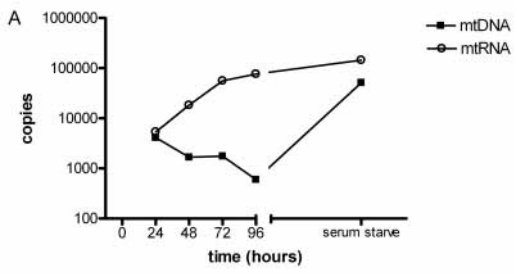
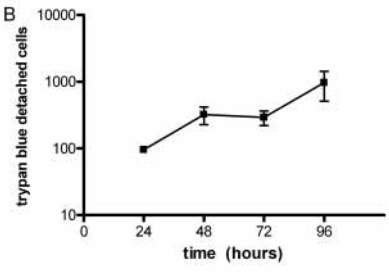


Figure 6B



Mitochondrial nucleic acids are secreted in the medium of non-cancerous cells. The endothelial cell line HMEC-1 was cultured for 96 hours, and every 24 hours medium was removed and stored for nucleic acid, isolation and quantification. Subsequently cells were serum starved for 6 hours (A). Also the numbers of detached trypan blue positive HMEC-1 cells were quantified (B).

Mitochondrial nucleic acids in plasma decrease after chemotherapeutic treatment

We next evaluated the levels of mitochondrial nucleic acids before and directly following cytotoxic chemotherapy in 12 patients with various types of cancer. Infusion of gemcitabine and cisplatin arrests tumor growth and induces apoptosis; therefore a direct increase would be seen in mitochondrial nucleic acids if there were a correlation with cell death *in-vivo*. Before infusion, and 2, 4, 8, 24 and 48 hours post-infusion blood was drawn, plasma was isolated, and mitochondrial nucleic acids were quantified (Figure 7). At baseline, before infusion, there were large differences in mtDNA and mtRNA, which might reflect tumor heterogeneity (8736 ± 6424 copies and 32071 ± 27761 copies, respectively). After infusion of cytotoxic chemotherapy, a decrease in average copy numbers of mtDNA in the medium was detected, with lowest average copies at 48 hours post-infusion. For mtRNA, a rapid decrease was seen with almost undetectable levels at 4 hours post-infusion. Between 4-8 hours post-infusion a brief increase in mean mtRNA levels is seen, however this increase is again undetectable at the 48 hour time point.

Figure 7A

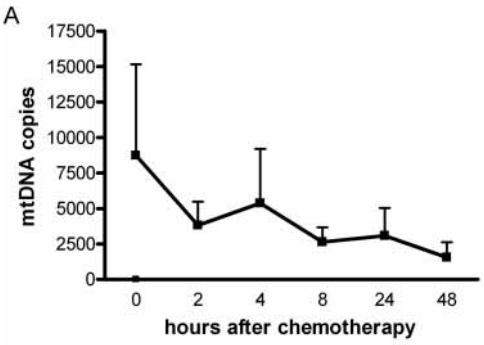
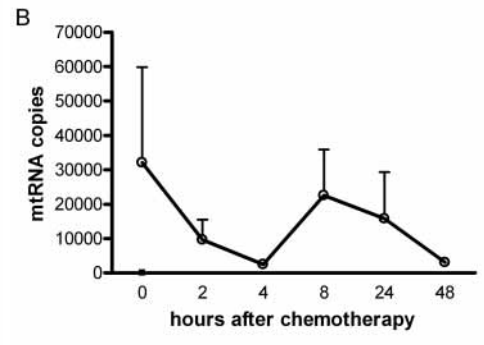


Figure 7B



Effect of chemotherapy on mtDNA and mtRNA levels in plasma. Patients were treated with a combined regiment of gemcitabine and cisplatin chemotherapy, and after 2, 4, 8, 24, and 48 hours after induction blood was drawn and mtDNA copies (A) and mtRNA copies (B) were isolated and quantified.

High mtDNA copies in plasma indicate poor survival probability in breast cancer patients.

Blood was drawn from 36 advanced breast cancer patients prior to chemotherapeutic treatment. Clinical characteristics are shown in Table 1. We wanted to evaluate whether baseline levels of mitochondrial nucleic acids could predict response to treatment. Therefore patients were followed for a median of 28.0 months. Of the 36 patients 18 patients died within our observation period due to cancer progression, and 18 patients were still alive. Breast cancer patients had a median of 141.408 copies mtDNA and 14.395 copies mtRNA (interquartile range from 51.867 to 343.570 copies and from 46.501 to 227.263 copies, respectively). Both mtDNA as mtRNA were strong predictors for survival in advanced breast cancer patients (AUC ROC of 0.74 with 95% CI of 0.57-0.92 and 0.71 with 95% CI of 0.53-0.88, respectively). We evaluated whether we could retrospectively predict which patients had a good or poor prognosis, based on high or low level of mitochondrial nucleic acids. When optimal cutoff values were selected for breast cancer patients, differences in overall survival between high and low copies of both mitochondrial nucleic acids were highly significant (Figure 8). Cumulative survival for high and low levels of mtDNA was 8% versus 74%

Table 1 - Clinical characteristics of 36 advanced breast cancer patients.

Characteristics	No.	(%)
Benign breast lesions	10	(21.7%)
Breast cancer	36	(78.3%)
Metastasis (present)		
No	14	(61.1%)
Yes	24	(38.9%)
Survival		
Alive	18	(50.0%)
Deceased tumor-related	18	(50.0%)
Survival (months)		
Median	23.7	
Interquartile range [25-75]	0 – 10.59	

Figure 8A

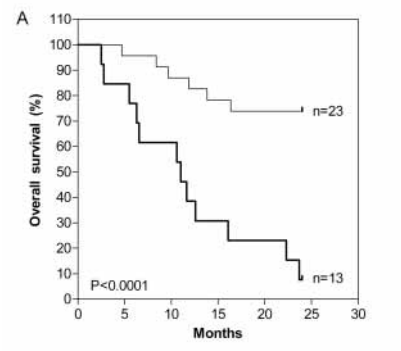
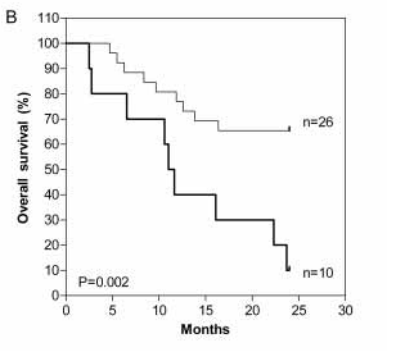


Figure 8B



Kaplan-Meier estimates of overall survival after 2-year follow-up. Patients with high mtDNA (A) and high mtRNA (B) had a decreased survival.

(Log rank 17.6, P<0.0001), and high and low levels of mtRNA was 10% versus 65% for mtRNA (Log rank 9.5, P<0.002) after a two year follow-up period.

Discussion

Recent evidence indicates that the quantification of cell-free mitochondrial deoxyribonucleic and ribonucleic acids in plasma may be an important indicator of the presence of cancer, response to treatment or patient survival. However, recent methodological articles on stability and reproducibility of cell-free genomic DNA and RNA in plasma, indicate that blood processing protocols (20;23-25), storage time (24;26;27), nucleic acid isolation and quantification protocols (28), may all in lesser or greater effect alter levels, variance and reproducibility of a quantitative measurement. As this is of great importance in the design of plasma isolation protocols in clinical studies with molecular DNA or RNA markers, we assessed in this study the stability and levels of mtDNA and mtRNA in whole blood and plasma, and the effect of freeze-thawing and possible contaminants such as platelets herein.

When peripheral blood is drawn from a patient, many processes could occur ex-vivo which may elevate the amount of circulating nucleic acids in the blood, such as necrosis, apoptosis, or enhanced spontaneous release from hematopoietic cells or circulating tumor cells. Two studies indicate that the amount of amplifiable genomic DNA from plasma is relatively stable when the blood is approximately left for 6 hours at room temperature before centrifugation and plasma isolation (24;25). Our experiments on the other hand indicate that storage of blood for 4 hours at room temperature, prior to centrifugation, does increase the level of mtDNA and mtRNA. This increase is ±1.8 and ±1.5 fold average for mtDNA and mtRNA levels, respectively, as compared to direct isolation. After 24 hours this increase is even ±4.1 and ±2.1 fold increased, respectively. However we clearly show that once the peripheral blood has been centrifuged and plasma has been isolated, the level of mitochondrial nucleic acids remains stable for at least 4 hours at room temperature. This may suggest that the elevation in cell-free mitochondrial nucleic acids in plasma are actively secreted, or either originating from cell death, and therefore it should be advised to isolate the plasma from the hematopoietic blood compartment as soon as possible after blood draw (maximum 2 hours). Furthermore we show through experiments with differential centrifugation speed that the levels of mitochondrial nucleic acids are ±30 fold higher in one-spin plasma compared to 2-spin plasma. From a diagnostic point of view, two-spin plasma might yield higher specificities (29), however for follow-up and prognostication this is less obvious and will have to be assessed in future studies. Furthermore we show that platelets could contribute to the amount of mtDNA and mtRNA copies in plasma. Our calculations show that each platelet contains ±48 copies mtDNA and ±10 copies mtRNA. Previously it has been shown that platelets contain ±7 copies mtDNA per cell (30), and <1 copies of mtRNA (personal communication with Michel de Baar). A possible explanation could be that the isolated platelets were contaminated with PBMCs (1 PBMC in every 1790 platelets, meaning that in our dilution range of platelets also 5587 to 11 PBMCs/ mL were present). Whether PBMCs contributed to the mtDNA and mtRNA levels in lysis buffer is unknown. In our experiments we spiked large amounts of platelets into lysis buffer, however, platelets numbers in plasma collected from patient samples are much lower. In three patients we evaluated the amount of platelets in peripheral blood, and in one and two-spin plasma samples (data not shown). We can deduce from these experiments that especially in one-spin samples, platelets or platelet-particles could contribute to mtDNA and mtRNA copy number. Then again, these data are inconsistent with patient data; in the breast cancer patients in this study, we did not find significant correlations between platelet count in the peripheral blood and mtDNA or mtRNA copies in plasma ($r=0.15$ and $r=0.33$ in 29 patients).

Another question we wanted to answer was whether mitochondrial nucleic acids were spontaneous secreted into the medium or associated with cell-death. From previous cell culture experiments it can be concluded that genomic DNA is actively secreted into the medium, in non-proliferating cells prior to cell death (31). Our experiments indicate a relationship of mtDNA, but not mtRNA copies, with Trypan blue positive cells in the medium, however the extracellular mtDNA could be secreted prior to cell death. Evidence indicative for the latter was seen when cells were serum starved. An elevation in both extracellular mtDNA and mtRNA was seen, while no increase in dead cells was seen. Experiments with various tumor cell-lines showed similar release of mitochondrial nucleic acids, as reported by Li et al. for genomic DNA (31).

Especially of interest is the amplification and quantitation of specific-sequences or total levels of nucleic acids as response and follow-up marker during anti-tumor treatment. In recent study by Gautschi et al. was demonstrated that decreasing levels of hTERT during treatment was highly indicative for responders, while non-responders showed stable or increasing levels. In an early study radiotherapeutic treatment also show decreased levels of total DNA in plasma (32), however a recent study demonstrates the opposite kinetics after radiotherapeutic and radiochemotherapeutic treatment (28). We assessed in two-spin plasma, whether the kinetics of mtDNA or mtRNA changed during combination chemotherapy acutely and 24 and 48 hours after the infusion of chemotherapy. Already in the first hours post-infusion an average decrease is seen in both mtDNA and mtRNA in the plasma of these patients. This is in accordance with the suggestion that cell-free nucleic acids are not entirely derived from cell death, but more so from active release of tumor cells. When tumor cell proliferation is inhibited or cell-death is induced a decrease might be seen. These hypotheses are still speculative and have to be further substantiated.

A recent report in breast cancer patients shows no significant difference in overall survival based on quantitation of plasma DNA (33). We specifically demonstrate high levels of plasma mtDNA and mtRNA in patients with poor two-year survival. Using an optimal cut-off point we could highly discriminate the survivors from the non-survivors, as previously reported for prostate cancer. Most studies which have quantified genomic plasma nucleic acids have shown large variations between different cancers, therefore restricting the use of defined cut-off values in one patient set for cancer diagnostics (34). We too found that the predefined cut-off value for prognostication in breast cancer could not be extrapolated to our previous data on prostate cancer. It has been shown that mtDNA content may vary between organs and tissues (35), which may explain results in baseline levels of plasma of mitochondrial nucleic acids in prostate and breast cancer.

In conclusion, this study demonstrates isolation and quantitation of mitochondrial nucleic acids in clinical studies has to be carried out according to strict protocol, concerning time frames of isolation. Furthermore we demonstrate that mitochondrial nucleic acids are decreased on average directly after infusion of anti-cancer treatment, which may be based on reduced activity of active tumor cell release. We demonstrate that using optimal cut-off points per cancer type it is possible to identify patients with poor-prognosis for possible additional treatment of more scrutinizing follow-up.

Acknowledgements

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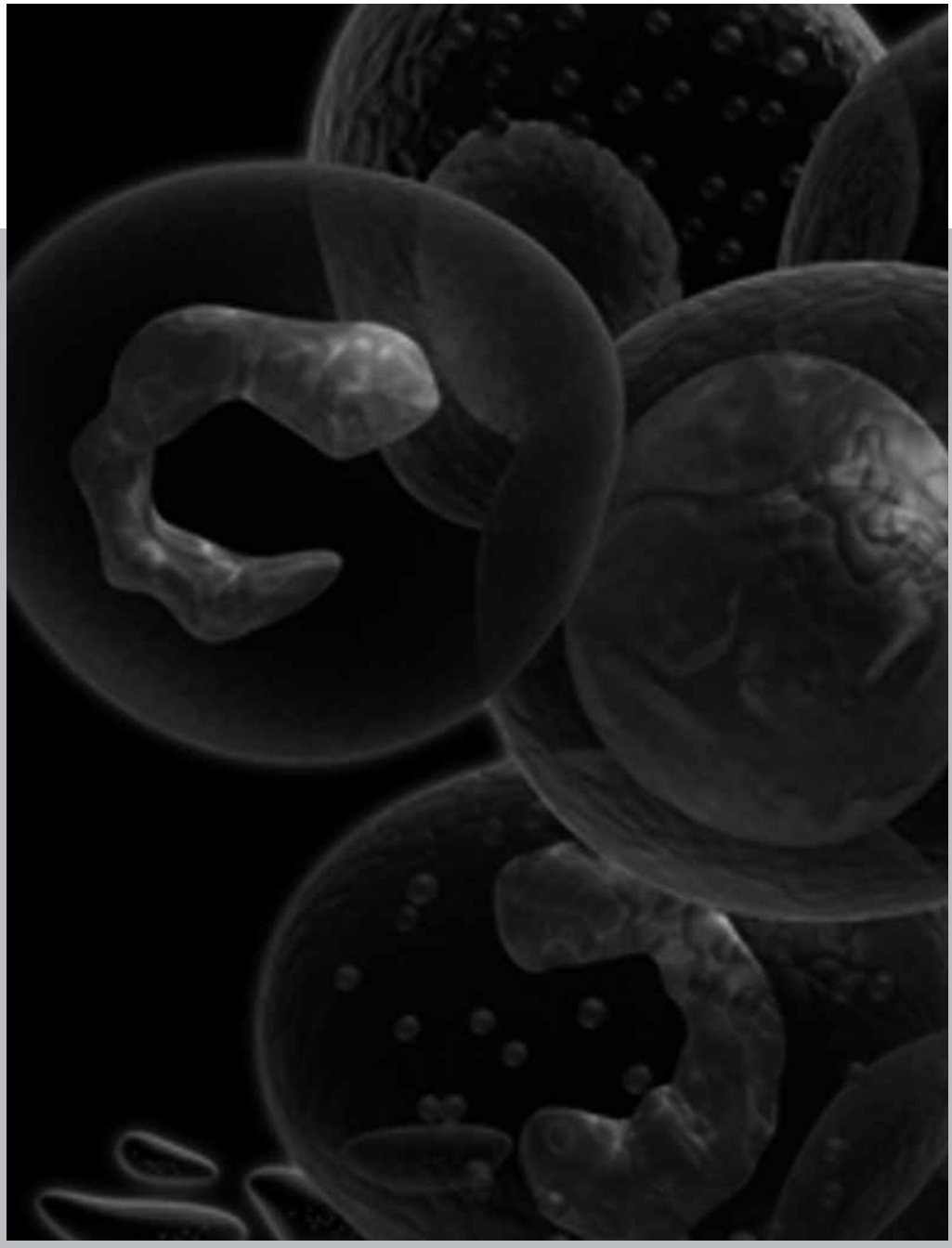
PART 3 - Proteomics



Protein Profiling by Mass Spectrometry as Approach for Diagnostic and Prognostic Cancer Biomarker Discovery

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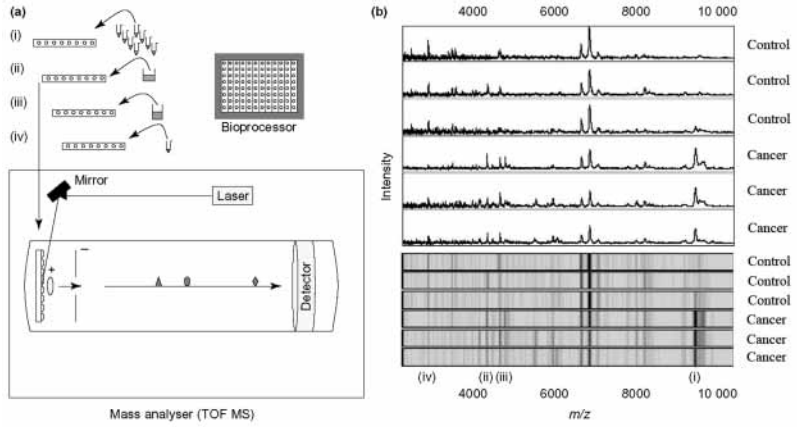
Introduction

Serum proteome analyses allow for simultaneous assessment of hundreds of proteins by mass spectrometry (MS) techniques (1), of which surface-enhanced desorption ionization time-of-flight (SELDI-TOF) MS is a key technology. A protein profile within a complex mixture like serum can be analyzed by application of microliters of sample to an array spot, upon which proteins bind by the addition of a binding buffer on affinity capture chromatography surface based on their physiochemical characteristics. After the application of an energy absorbing matrix for the absorption of laser energy, the emitted laser light causes the large proteins complexes to disassemble and to become positively ionized. These positively charged proteins are separated over an electric field due to protein size and amount of charge. The time of flight is proportional to the protein mass per charge. The SELDI-TOF mass spectrum is commonly depicted by their mass to charge ratio on the x-axis, and their protein abundance as intensity on the y-axis (Figure 1). Mass spectrometry currently represents the most important analytical proteomic tool, as this method is capable of identifying proteins and peptides with relative ease, and can perform multiparametric analysis of complex biological samples (2).

Figure 1



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The use of plasma, serum or other bodily fluids presents an easily accessible source of material, for standardization of sample collection and repeated measurements. However, it is known that inter- and intra individual changes in serum can depend on sex, hormone level, nutrition state, or inflammation, and can change the protein profile drastically. Furthermore, biomarkers involved in the genesis and progression of cancer must be present at high enough level to be detected by MS. Table 1 demonstrates the approximate concentration of biomarkers identified by SELDI-TOF MS and classical cancer biomarkers in serum. Although the use of MS on bodily fluids at present still represents an arduous challenge, it may prove a suitable approach to identify diagnostic cancer markers for population screening or biomarkers to predict prognosis in cancer patients.

SELDI-TOF MS for cancer diagnostics

Serum proteomics by SELDI-TOF MS has resulted in the publication of numerous articles in clinical oncology, differentiating by dissimilar protein profiles the presence of cancer from health. Petricoin et al. has pioneered the use of MS as a diagnostic tool in ovarian cancer (3). In a five-center case control study, three biomarkers were later identified which had enhanced sensitivity for early ovarian cancer detection over that of CA125 alone (4). Also in breast cancer a sensitivity of 93% for all cancer patients and a specificity of 91% was demonstrated for all controls using three selected biomarkers (5), again higher detection than the current diagnostic tumor marker CA15.3. Numerous publications have also identified diagnostic markers for cancers of the prostate (6), head and neck (7), pancreas (8), kidney (9), nasopharynx (10), colon (11), and lung (12).

Table 1

Biomarker	Approximate concentration[a]	Biomarker for cancer type	Reference
Serum proteins			
Albumin	$600x10^6$	-	(38)
Immunoglobins	$30x10^6$	-	(38)
C-reactive protein	$4x10^4$	-	(38)
SELDI-TOF MS			
Apolipoprotein A1	$40x10^6$	Ovarian, prostate, renal	(4;9;39)
Transthyretin (prealbumin) fragment	$6x10^6$	Ovarian	(4)
Inter-α-trypsin inhibitor fragment	$4x10^6$	Ovarian	(4)
Serum amyloid A protein	$20x10^6$	Renal, nasopharyngeal, ovarianprostate, pancreas	(8-10;40-42)
Haptoglobin-a-subunit	$1x10^6$	Ovarian, renal	(9;43)
Vitamin D-binding protein	$10x10^6$	Prostate	(44)
α_1-Antitrypsin	$10x10^6$	Pancreatic	(8)
α_1-Antichymotrypsin	$5x10^6$	Pancreatic	(8)
Classical tumor markers			
Alpha-fetoprotein	150	Hepatoma, testicular	(38)
Prostate specific antigen	140	Prostate	(38)
Carcinoembryonic antigen	30	Colon, lung, breast, pancreatic	(38)
Human choriogonadotropin	20	Testicular, choriocarcinoma	(38)
Human choriogonadotropin-β subunit	2	Testicular, choriocarcinoma	(38)

[a] [pmol/liter]
This table is modified and expanded from (13)

How come that at present SELDI-TOF MS is not yet being evaluated as standard diagnostic marker for use in population screening? Recently, important concerns were raised on the validity of serum proteomic pattern analysis by mass spectrometry for early cancer diagnosis (13-16). First of all, strong discriminatory peaks separating individuals with cancer from healthy controls or patients with benign disease were identified to represent acute-phase proteins, produced by the liver, diseased organs and inflammatory cells as a reaction to cancer. These proteins are present in extremely high abundance, precluding their release from small tumor tissue. Many of these acute-phase proteins were also found to be present in patients with inflammatory disease or shown not to be different when analyzed by a biochemical test. Second, validation of initial studies by

independent groups demonstrated incapability to (completely) reproduce previous results (reviewed in(13)), or even reanalyzing original data indicated erroneous methodology in sample collection between controls and patients and incorrect analysis (17). Possible explanations for the inability to reproduce results performed by other research groups are the possibility that the serum may contain such huge numbers of discriminatory proteins between cancer and non-cancer, that the chance finding the same discriminatory protein signature between different patient populations is very small. Second, methodological differences such as different chips to immobilize the candidate proteins, with different binding characteristics e.g. hydrophilic, hydrophobic, cationic, anionic and immobilized-metal affinity capture chromatography surface, may be causative for irreproducible results. Furthermore, the protein chip may not have the capacity to bind quantitatively all proteins present in the sample, and sample processing prior to chip binding also may influence outcome e.g. serum dilution and albumin depletion.

At present, consensus in the proteomic field exists for improved regulations on trial design, sample storage, analysis and validation to ensure comparability in results between institutes, and to prevent systemic bias and overfitting of data. However, lack of reproducibility of previous results by independent laboratories still remains a crux dilemma in the proteomic field. For renal cell carcinoma, two reports have been published on diagnostic biomarkers found by SELDI-TOF MS (9;18). However, no overlap in discrimatory biomarkers were found between these two studies, most probably because of differences in methodology. To address this hypothesis, we performed a collaborative study to reproduce results performed by the two studies on renal cancer patients, by using identical methodologies (Chapter 12).

SELDI-TOF MS for cancer prognostics

SELDI-TOF MS studies have mainly been directed at cancer diagnostics and not at predicting clinical outcome. Only a few reports have investigated whether proteomic MS can identify biomarkers predicting responsiveness to treatment, disease progression or survival. Two independent reports using MS on glioma tissue identified differences in protein expression between high- and low grade glioma (19) and discovered protein patterns able to independently predict patient survival (20). Proteomic analyses on tumor tissue in node-negative breast cancer patients identified cytosolic ubiquitin and ferritin light chain as prognostic biomarkers (21).

The drawback of proteomic methods such as MS on cancer tissue is that tumor biopsies are invasive, not routinely performed, and complex sample preparation such as microdissection may be necessary. Efforts were made to identify biomarkers predictive of cancer outcome from the bodily fluids of cancer patients. In patients with stage I-III breast carcinoma, proteomic changes in plasma were examined by SELDI-MS in response to chemotherapy (22). The authors attempted to identify predictive biomarkers from the plasma prior and during treatment that could predict final tumor response, but none were discovered. Several other reports did demonstrate the validity to ascertain predictive biomarkers from the peripheral blood of patients with cancer. In melanoma patients after lymph-node dissection, discriminatory MS fingerprint proteins correctly predicted the occurrence of disease progression in 80% of cases (23). In neuroblastoma patients the intensity of serum amyloid A was discriminative between good and bad prognosis patients (24). In a retrospective study of high-risk breast cancer patients a 40-protein signature model had a positive and negative predicting value for metastatic relapse of 84% and 81% (25). The ability to use SELDI as surrogate serum

marker of sentinel lymph node evaluation was also evaluated (26). In sentinel lymph node positive and negative samples, 81% and 77% samples, respectively, were correctly classified. Using serum from patients with acute lymphoblastic leukemia (ALL) before initiation of cytotoxic therapy, the authors demonstrated the possibility predict recurrence of ALL with 84% to 94% and negative predictions of 62% to 74% (27).

In these initial clinical studies on SELDI prognostics, most authors did not demonstrate whether their models were attractive for eventual clinical implementation by testing their discriminatory models as independent predictors of prognosis with other staging systems. Furthermore, in all above mentioned studies, serum samples originated from one institute, therefore sample processing could bias these studies, and extrapolation to other institutes may not be possible.

No MS reports have evaluated discriminatory prognostic or predictive signature models in the management of patients with renal cell cancer. Patients with advanced disease have a poor prognosis, with few other solid tumor cell types showing such uniform resistance to cytotoxic chemotherapy agents (28). High dose interleukin-2 demonstrated a 14% partial or complete response rate (29), and other cytokine regiments such as interferon alpha demonstrated similar or lower response rates (30). Overall median survival after progression after cytokine therapy is only 12 months (31). Elucidation of the VHL protein function and pathways, has led to targeted therapies in this highly resistant malignancy, such as sunitinib (32), erlotinib and bevacizumab (33), and sorafenib (34). Currently no biomarkers are present that may assess patient eligibility for certain targeted therapies, or identify patients with poor prognosis for adjuvant treatment modalities prior to cytokine therapy failure. In this thesis, we addressed the validity of using SELDI-TOF MS to identify poor prognosis fingerprint patterns present in the serum of pre-treatment renal cancer patients in two independent institutes. Furthermore we evaluated whether these novel discriminatory biomarkers were independent markers when compared to the predictive Memorial Sloan-Kettering Survival model (35), which were recently extended and validated (36).

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Validation of SELDI-TOF MS serum protein profiles for renal cell carcinoma in new populations

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Abstract

Currently, no suitable biomarker for the early detection or follow-up of renal cell carcinoma (RCC) is available. We aimed to validate previously reported potential serum biomarkers for RCC obtained with Surface Enhanced Laser Desorption Ionisation-Time of Flight Mass Spectrometry (SELDI-TOF MS) in our laboratory using distinct patient populations. Two sets of sera from RCC patients and healthy controls (HC) were gathered from different institutes and analysed according to published procedures. The first set (40 RCC, 32 HC) consisted of mainly pre-surgery samples from patients with disease stages I-IV. The second set (26 RCC, 27 HC) were mostly sera from patients with stage-IV disease, drawn after nephrectomy. Only the increased expression of the previously found serum amyloid-α (SAA) peak cluster could be validated in a similar RCC patient subset in both our populations in two independent analyses. It was seen both in early- and late-stage disease and in pre- and post-surgery samples. These results were also confirmed by ELISA. Other previously identified biomarker candidates (mass-to-charge ratio's (m/z) 3 900, 4 107, 4 153, 5 352 and 5 987) proved difficult to reproduce upon duplicate analysis. Modification of the analytical protocol for these markers resulted in their detection, but we did not achieve satisfactory classification of patients and controls with these alleged biomarkers in any of our two sample sets. Instead, two new peaks (m/z 4 289 and 8 151) were identified with better performance (sensitivity and specificity ±65-90%) for separating patients from controls in the first sample set. Concluding, only the SAA peak cluster was validated as a robust RCC biomarker candidate, which is present in a specific subset of these patients, regardless of disease stage or nephrectomy status. In addition, two new peaks were seen which might prove useful as biomarkers, provided these are validated in new populations.

Introduction

Renal cell carcinoma (RCC) is difficult to diagnose at early stages due to a lack of clear clinical symptoms. When symptoms do occur, about 30% of patients already have metastatic disease. In addition, a similar part of patients with resection of localised disease will have a recurrence (1). Therefore, a need remains for reliable markers for diagnosis and follow-up of RCC, preferably in easy-accessible body fluids. Surface Enhanced Laser Desorption Ionisation-Time of Flight Mass Spectrometry (SELDI-TOF MS) (2;3) is being increasingly used to search for new and better tumour markers in (serum) protein profiles, e.g. for ovarian (4), breast, (5) prostate (6) and colorectal cancer (7). Its appeal lies in the ease with which a multitude of samples can be analysed with a minimum of sample preparation in a single SELDI-TOF MS analysis. Indeed, SELDI-TOF MS has also been performed to identify biomarker proteins for early detection of RCC. Two studies describe protein profiling of serum with SELDI-TOF MS (8;9). Tolson et al. (8) reported a peak cluster at a mass-to-charge ratio (m/z) of approximately 11 000 from serum amyloid α-1 and variants, which was 100% specific for RCC compared to healthy controls, although not very sensitive (detection in 8 of 25 patients). Five m/z values of 3 900, 4 107, 4 153, 5 352 and 5 987 were reported by Won et al. (9) in a classification tree separating RCC from healthy and non-RCC urologic disease, with sensitivities en specificities ranging from 80 to 100%.

Although these results are promising, concerns have risen regarding reproducibility of protein profiles and the nature of detected biomarkers (10-17). For the same cancer types, different biomarkers have been found by different research groups (13;18). Yet, reproducible protein profiles

can hardly be presumed when using different assay procedures or sample handling, or when samples from a patient population with other characteristics are used (19). To ensure that supposed biomarker proteins are not due to chance or bias and are robust enough for detection in different laboratories, validation with an independent sample set originating from a different institute, but handled using standard analytical procedures, is imperative. Yet, only few groups have performed such validation. Attempts to validate biomarker proteins for ovarian and breast cancer with samples from other institutes have been reported, although not all with similar success (4;20;21). For prostate cancer a biomarker validation project is ongoing (22;23), also standardising analyses among different laboratories. However, the RCC serum protein profiles in the two above-mentioned studies have not been validated in other laboratories or with new populations, leaving the question of their validity and robustness. Thus, instead of starting a new search for RCC biomarkers using SELDI-TOF MS, we attempted to move forward and validate the ones postulated by previous studies. We applied the previously developed analytical protocols (8;9) to two distinct sample sets of patients and controls from two different institutes and assessed robustness and validity of reported RCC serum protein profiles.

Materials and methods

Chemicals

All used chemicals were obtained from Sigma, St. Louis, MO, USA, unless stated otherwise.

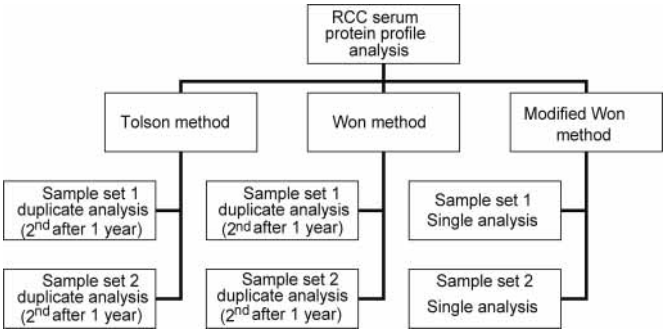
Patient samples

Our first set of samples (set 1) consisted of sera from 40 patients with renal cell carcinoma and 32 healthy controls (HC) obtained from the Netherlands Cancer Institute, in Amsterdam, The Netherlands. The second set (set 2) consisted of sera from 26 patients with renal cell carcinoma and 27 healthy controls obtained at the University Medical Centre in Utrecht. All serum samples originated from a serum bank, where they had been collected according to institutional protocols. Sample collection was performed with individuals' informed consent after approval by the institutional review boards.

Protein profiling

The two serum sample sets were analysed at separate time-points in our laboratory. Analyses for both sample sets were repeated after storage at –20°C for a year. A scheme of the experimental set-up is shown in Figure 1. For quality control we analysed two separate pools of HC sera from set 1 and set 2 respectively on seven chips in each analysis. Assay procedures were identical to those reported (8;9), using CM10 chips with a weak cation exchange chromatography and as matrix a 50% solution of sinapinic acid (SPA) for Tolson's analysis and a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) for Won's analysis. Only the albumin depletion for Tolson's method was performed slightly different, using Cibacron Blue spin columns from a different manufacturer (Ciphergen Biosystems Inc., Freemont CA, USA). Briefly, serum was denatured and depleted from albumin according to manufacturer's instructions. Equivalent quantities of denatured serum to Tolson's were loaded onto these columns, taking into account the different binding capacity for albumin. Protein chips were analysed using the PBS-IIc ProteinChip Reader (Ciphergen Biosystems). Data acquisition parameters in our experiments were optimised for detection of the reported

Figure 1



Experimental set-up. Two separate samples sets were analysed according to two previously published methods . In addition, a modified version of one of the methods was used to yield better peaks (see Results section).

biomarkers. Using Tolson's method, spectra were collected to 80 kDa with focus mass kept at 8 000 Da according to reported procedures. M/z values for the detected proteins were calibrated externally with a standard peptide mixture (Ciphergen Biosystems) containing [Arg8] vasopressine (1 084.3 Da), somatostatine (1 637.9 Da), dynorphine (2 147.5 Da), ACTH (2 933.5 Da), insulin , β-chain (bovine) (3 495.9 Da), insulin (human recombinant) (5 807.7 Da), and hirudin (7 033.6 Da).

Statistics and bioinformatics

Data were analysed with the ProteinChip Software package, version 3.1 (Ciphergen Biosystems). Per sample set, all acquired spectra were compiled and analysed as a whole. Spectra were baseline subtracted and normalised to the total ion current from 1 500 Da to the spectrum's end. Spectra with normalisation factors above 2.00 or lower than 0.50 were excluded from clustering. Biomarker Wizard (BMW) software (Ciphergen Biosystems) was used for peak clustering. Peaks were autodetected when occurring in at least 30% of spectra and with first and second pass signal-to-noise (S/N) of 5 and 2 respectively, in a 0.3% cluster mass window. In case of no autodetection of peaks of interest, peak detection was performed manually and the BMW applied including these user-detected peaks. Mean peak intensities between groups were compared by non-parametric statistical tests with $p < 0.01$ defined as statistically significant. The mean coefficient of variation (CV) in each sample set was calculated with replicate peak intensities from the quality-control samples. Biomarker Patters Software (BPS; Ciphergen Biosystems) was used to generate classification trees for patients and controls.

Protein identification

The identity of proteins of interest was elucidated using immunocapture with appropriate antibodies. For confirmation of the 11-kDa peak identities the same mouse monoclonal antibody to serum amyloid-α (SAA) was used as reported (Abcam Ltd., Cambridge, UK). In short, antibody was coupled to protein A ceramic HyperD beads (Pall/Biosepra, Saint-Germain-en-Laye, France) and washed thrice with phosphate buffered saline (PBS). After a 30-min incubation of serum diluted in PBS, the unbound fraction was collected. Beads were washed five times with PBS and once with

deionised water. Finally, bound proteins were eluted from the beads with 0.1 M acetic acid. Unbound fractions as well as eluates were profiled both on gold chips or NP20 chips and on the original CM10 chip surface.

Measurement of serum SAA levels

Results of protein profiling for SAA were validated by ELISA using a commercially available kit (Tridelta Development Ltd, Maynooth, Ireland). All sera were analysed in duplicate according to manufacturer's instructions.

Results

Patient samples

Patient and sample characteristics are summarised in Table 1. Two distinct patient populations were gathered: In our first sample set, most samples had been drawn before surgery and patients had mostly early stage disease, as was the case for the samples in the published studies. In contrast, many samples from set 2 were from patients with metastatic stage-IV disease who had undergone nephrectomy. This enabled us to assess the abundance of the previous markers across several disease stages and the influence of primary tumour resection on their presence. No group of patients with other urological diseases was included in our analysis. Sample collection procedures differed between the institutes regarding clotting time and storage temperature (Table 1).

Table 1 - Patient and sample characteristics of sample sets 1 and 2 compared to those of Won and Tolson. Means (SD) for patient and sample age.

	Sex			Patient age (years)		RCC [1]) stage	Surgical status	Sample handling	Sample age (years)	
	RCC	*HC*	*Non-RCC disease*	*RCC*	*HC*				*RCC*	*HC*
Set 1	25 M 15 F	20 M 12 F	-	57.1 (11.0)	56.1 (8.79)	16 IV 4 III 16 I/II 4 unknown	33 pre-surgery 5 post-surgery 2 unknown	30 min coagulation at RT. Centrifugation at 1900 g. Storage at –30°C.	2.94[2] (0.56)	1.90 (0.034)
Set 2	19 M 7 M	7 F 20 F	-	63.7[3] (10.2)	42.9 (11.1)	24 IV 2 II/III	8 pre-surgery 18 post-surgery	2-6h coagulation at RT+ overnight at 4°C. Centrifugation at 1500 g. Storage at –80°C.	1.05 (0.55)	1.28 (0.21)
Tolson	15 M 10 F	26 matched	-	59.3 (14.6)	matched	6 IV 1 III 7 II 11 I	25 pre-surgery	Overnight coagulation at 4°C. Serum supernatant collected. Storage at –70°C.	NR	NR
Won	15 M+F	6 M+F	15 M+F	NR	NR	NR	15 pre-surgery	NR	All collected in same period	

1) Abbreviations: F: female, HC: healthy control, M: male, NR: not reported, RCC: renal cell carcinoma, RT: room temperature.

2) $p < 0.000$ independent samples t-test.

3) $p < 0.000$ non-parametric Mann-Whitney U-test.

Protein profiling: Tolson method

In the first analysis we obtained optimal spectra using laser intensity 142 and detector sensitivity 7, collecting an average of 165 laser shots per spot. For the second analysis a year later, acquisition parameters had to be re-optimised to laser intensity 155 and detector sensitivity 8, while other parameters were kept constant. On both occasions, protein patterns were highly reproducible within and between the sample sets and the two analyses. Mean peak CV's for the quality-control sera from each sample set were 20% and 30% for set 1 and 2 respectively, based on the 11 most prominent peaks common to both sample sets. Few samples had to be excluded due to aberrant normalisation factors (Table 2). Comparison of spectra from the duplicate analysis with the one from Tolson shows the presence of the same peaks (Figure 2). Results of the duplicate analysis were highly congruent, except for the 9.2-kDa peak, which was seen more frequently in the second analysis. The 11-kDa peaks were present in both our sample sets in a roughly similar patient subset as Tolson's (Table 2). In two healthy controls from set 1 and one from set 2 this peak was also visible, although with S/N < 2 (Figure 3). None of the excluded spectra showed the 11-kDa peaks. The peak at 10.85 kDa, also indicated as a potential biomarker by Tolson, was found in very few of our patients (Table 2). Also for some other (non-biomarker) peaks the frequencies we found were lower than reported, such as for the 9.2-kDa peak from haptoglobin in the first analysis and for the peaks at 15.8 and 15.1 kDa (β- and α-globin). The relative abundances of Tolson's biomarkers in our patients in relation to their surgery status and disease stage are summarised in Table 3.

When applying the BMW application to these spectra, the 11-kDa peaks came up as most significantly different in both analyses and both sample sets ($p < 0.000$). However, since these peaks are only occur in a subset of RCC patients, their sensitivity for RCC detection is limited. Therefore we also evaluated other discriminating peaks that were present in all patients and/or controls. Peaks at 8.7, 14.1, 17.3 and 28 kDa were consistently decreased in patients in both

Table 2 - Comparison of peak detection in Tolson's samples and in our set 1 and 2. Peaks were considered present when having a S/N ⩾2.

Peak detection in number of patients/controls						Peak identity [1]
	Sample set 1 (max 40/32)		*Sample set 2 (max 26/27)*		*Sample set Tolson (max 25/26)*	
	1st analysis	*2nd analysis*	*1st analysis*	*2nd analysis*		
Excluded from analysis	0/1	4/1	2/2	1/0	N.A.	
9.2 kDa	4/2 [2]	14/13	1/2 [2]	14/13	15/12	Haptoglobin 1-α
10.85 kDa [3]	1/0 [4]	3/0	1/0 [4]	1/0	16/6	Unknown
11.4 kDa [3]	10/0	11/0	4/0	5/0	7/0	des-RS SAA-1
11.5 kDa [3]	18/0	19/0	5/0	7/0	8/0	des-R SAA-1
11.68 kDa [3]	18/1	20/1	6/0	7/0	8/0	SAA-1
13.7 kDa	30/29	28/29	15/25	17/26	25/26	Transthyretin
15.1 kDa	1/0	1/0 [5]	1/5	0/0 [5]	25/26	α-Globin
15.8 kDa	19/14	13/11	11/16	14/11	25/26	β-Globin
28.0 kDa	39/31	36/31	24/25	25/27	25/26	Apolipoprotein A-I

1) Abbreviations: SAA-1: serum amyloid α1; R: arginine; S: serine

2) When using a cut-off of S/N >1, detected in 14 RCC patients and 9 controls from sample set 1 and 18 patients and 17 controls from sample set 2

3) Tolson biomarker candidate

4) When using a cut-off of S/N >1, detected in 4 RCC patients in set 1 and 4 RCC patients in set 2

5) When using a cut-off of S/N >1, detected in 1 RCC patient and 2 HC in set 1 and 2 RCC patients and 1 HC in set 2.

Figure 2A

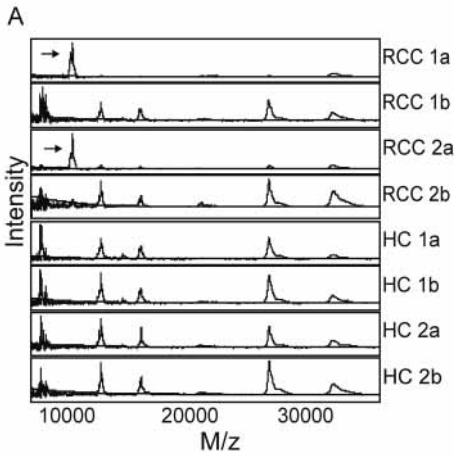
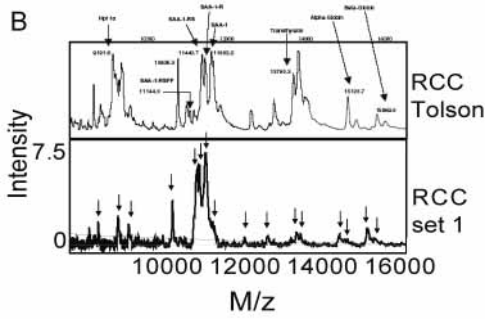


Figure 2B



Spectra parts generated according to Tolson's procedure. A: Samples from set 1 and set 2 are shown. The 11-kDa peaks are visible in a subset of the RCC sera (arrow), but not in those from HC. B: Comparison of one of our spectra with the one reported by Tolson et al . Nearly all peaks present in the Tolson spectrum were visible in our spectrum (arrows), although some with lower peak intensities, which is possibly due to the fact that the published spectrum was acquired with extra high laser intensity for peaks > 10 kDa. Tolson spectrum reprinted by permission from Macmillan Publishers Ltd: [Lab Invest] (8) , copyright 2004.

Table 3 - Abundances of Tolson peaks (peak height "averages" of duplicate analysis and concentrations measured by ELISA) and patient characteristics.

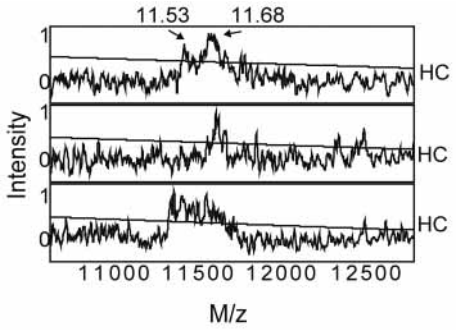
	Patient ID	Abundance of peaks of interest			Surgical status	Disease stage
		10.85 kDa	*11-kDa cluster*	*SAA concentration (µg/ml)*		
Set 1	RCC 364	---	++	50.9	Pre-surgery	II
	RCC 367	+/-	+++	67.0	Pre-surgery	IV
	RCC 368	---	+	3.97	Pre-surgery	I
	RCC 369	+/-	+++	2.35	Pre-surgery	IV
	RCC372	-	++	74.5	Pre-surgery	IV
	RCC 373	-	+++	75.6	Pre-surgery	IV
	RCC 374	++	+++	72.8	Pre-surgery	IV
	RCC 375	---	++	50.5	Pre-surgery	IV
	RCC 376	+/-	++	49.2	Pre-surgery	IV
	RCC 377	--	+++	36.7	Pre-surgery	IV
	RCC 378	+/-	+++	71.8	Pre-surgery	I
	RCC 379	+/-	+++	67.5	Pre-surgery	I
	RCC 381	-	+++	89.0	Pre-surgery	II
	RCC 387	+/-	++	43.7	Pre-surgery	I
	RCC 388	---	+	15.2	Pre-surgery	I
	RCC 390	---	+	33.1	Unknown	IV
	RCC 392	-	+++	61.0	Pre-surgery	IV
	RCC 393	---	++	9.59	Pre-surgery	IV
	RCC 397	---	+	36.3	Pre-surgery	III
	RCC 399	++	---	11.4	Post-surgery	Unknown
	RCC 403	+	---	7.50	Pre-surgery	I
Set 2	RCC 94	+/-	+++	3.24	Pre-surgery	IV
	RCC 96	---	+++	61.1	Pre-surgery	IV
	RCC 99	---	+	44.9	Pre-surgery	IV
	RCC 100	++	++	53.2	Post-surgery	IV
	RCC 109	--	++	50.9	Pre-surgery	IV
	RCC 114	---	+	7.61	Post-surgery	IV
	RCC 116	---	+++	94.0	Pre-surgery	IV

analyses and both sample sets ($p < 0.000$, except for 14.1 kDa in set 2: $p < 0.03$). Classification trees were constructed with BPS, but none of the above-mentioned discriminatory peaks, nor any other peaks yielded trees with suitable sensitivity and specificity for both sample sets (data not shown).

Protein profiling: Won method

Figure 3 shows spectra from the optimisation of laser intensity and detector sensitivity for the Won peaks at 3.90, 4.10, 4.15, 5.35 and 5.99 kDa. In the first analysis we obtained optimal spectra using laser intensity 135 and detector sensitivity 7, collecting an average of 165 laser shots per spot. Again, for the second analysis re-optimisation of acquisition parameters was needed, resulting in laser intensity 128 and detector sensitivity 7 keeping other parameters constant. On both occasions, protein patterns were highly reproducible across the sample sets and the two analyses. However, many spectra were "empty" and had to be excluded from further data analysis (Table 4). We noted that this was related to the presence of a glassy layer on the chip spot for these samples. This layer is probably due to lack of a washing step with water in the procedure, leaving salt remnants on the chip that cause ion suppression. Due to this, only two quality-control samples from set 1 and three from set 2 were assessable. Mean peak CV's of the 7 most prominent peaks in these spectra common to both sample sets were 60% and 23% for set 1 and 2 respectively. Our peak intensities for the peaks of interest were markedly lower than the intensity values reported by Won, except for the peak at 4.10 kDa (Table 5, original procedure and Figure 4). Varying acquisition parameters did not result in higher intensities for these peaks. As a result, only the 4.10-kDa peak met the criterion of a S/N > 5 for clustering and the others had to be manually detected. Although

Figure 3



Detection of the 11-kDa cluster in healthy controls (HC) from sample set 1 and 2. The straight line represents the noise level.

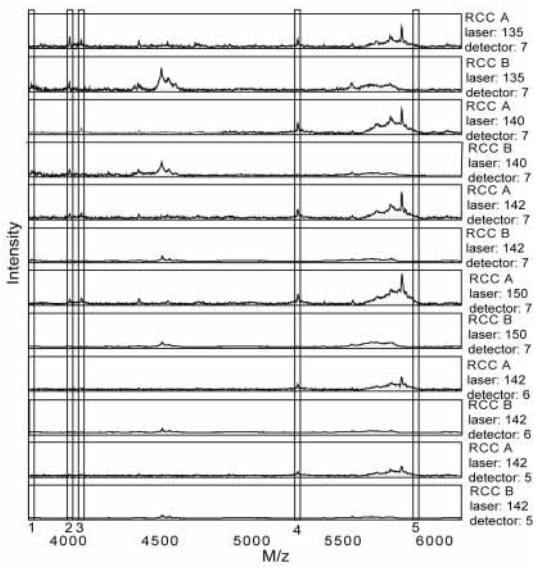
Table 4 - Spectra from the Won method included for data analysis.

	Assessable RCC/total RCC		Assessable HC/total HC	
	Set 1	*Set 2*	*Set 1*	*Set 2*
Original procedure, 1st analysis	20/40	18/26	12/32	17/27
Original procedure, 2nd analysis	13/40	11/26	14/32	13/27
Modified procedure	33/40	25/26	26/32	24/27

the mean peak intensities from the two analyses were comparable, there were some differences between the first and second analysis regarding the group with the highest mean peak intensity and the observed p-values. None of the Won peaks was significantly different at the 0.01-level in our analyses (Table 5, original procedure). Some of the peaks did show a (non-significant) expression difference between patients and controls, but this was only observed in one of the analyses (m/z 4 097, 5 348), or was discordant with the expression difference reported by Won (m/z 5 350, 5 991). The lack of an expression difference in patients and controls for m/z 4 153 was in agreement with Won's result, however.

In an attempt to obtain better intensities for the peaks of interest, we also used a slightly modified assay for these samples (single analysis). Instead of 2% 2-mercaptoethanol in the denaturation solution, 2% dithiotreïtol (DTT), a more effective reductant, was used and the protease inhibitor PMSF was left out. Instead of saturated CHCA, a 50% SPA solution in 50% ACN + 0.5% TFA was used as energy absorbing matrix. We have applied this matrix solution successfully for masses below 15 kDa in other experiments, among which the Tolson procedure. SELDI-TOF MS acquisition parameters were optimised for these conditions to laser intensity 142 and detector sensitivity 7. From Figure 5 it can be seen that with the modified method the same samples yielded higher peak intensities for m/z 3 901, 4 107, 4 153, 5 352 and 5 987, with reproducible spectra among sample set 1 and 2. In addition, many more peaks were present in the mass spectrum than with the original procedure, and few spectra did not meet normalisation criteria (Table 4). Two significant differences in agreement with Won's result could now be seen at m/z 5 996 and 3 894 in set 1. However, the found increase in RCC patients of a peak at 4 161 Da in set 2 (p = 0.004, Table 5, modified procedure) was not according to Won's results.

Figure 4



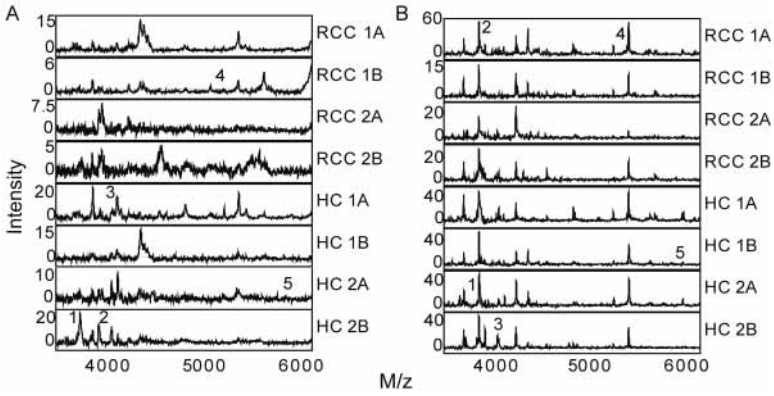
Optimisation of SELDI-TOF MS acquisition parameters for detection of the Won peaks (1-5). Two RCC samples were used for optimisation (A and B). Both laser intensity and detector sensitivity were optimised separately.

Table 5 - Observed peak expression differences detected in our sample sets with Won's method (original procedure) and a slightly modified procedure. Mean (SD) peak intensities for the RCC and HC groups are given.

Reported by Won			Observed Set 1 [1]				Observed Set 2 [1]			
m/z (Da)	HC peak intensities	RCC peak intensities	m/z (Da)	P [2]	HC peak intensities	RCC peak intensities	m/z (Da)	P [2]	HC peak intensities	RCC peak intensities
			original procedure (duplicate)				*original procedure (duplicate)*			
3901	22.9 (5.38)	14.4 (5.67)	3901	0.186	1.24 (0.570)	1.08 (0.67)	3904	0.792	2.13 (1.60)	1.84 (1.01)
			3897	0.409	2.35 (2.35)	1.72 (2.24)	3897	0.750	1.46 (1.47)	1.26 (0.650)
4107	29.4 (7.09)	6.48 (7.40)	4100	0.119	3.56 (2.34)	2.33 (1.97)	4104	0.322	7.74 (6.39)	5.54 (4.95)
			4101	0.409	13.7 (9.24)	17.4 (10.1)	4097 [3]	0.052	13.7 (7.38)	8.32 (4.33)
4153	9.19 (6.75)	8.44 (4.50)	4155 [3]	0.907	2.96 (3.09)	2.33 (1.58)	4159 [3]	0.146	1.21 (1.11)	1.66 (1.17)
			4157 [3]	0.072	4.29 (6.30)	4.22 (2.44)	4163 [3]	0.339	1.96 (1.16)	1.68 (1.84)
5352	16.5 (3.65)	12.2 (4.37)	5357	0.559	0.962 (0.567)	0.892 (0.665)	5353	0.291	1.64 (1.15)	2.43 (1.97)
			5350	0.022	1.76 (1.46)	3.08 (1.45)	5348 [3]	0.099	3.11 (2.34)	1.68 (1.36)
5987	7.70 (4.14)	4.82 (4.93)	5983	0.111	0.919 (0.480)	0.671 (0.488)	5991	0.056	0.517 (0.489)	1.05 (0.951)
			5996	0.593	1.17 (0.661)	1.15 (0.939)	5991	0.750	1.53 (0.990)	1.38 (0.944)
	Classification tree difference		*modified procedure*				*modified procedure*			
3901	↑ in RCC vs. non-RCC		3894 [3]	5.19*10-05	9.01 (4.10)	4.81 (2.13)	3883	0.383	5.33 (2.57)	4.89 (1.70)
4107	↑ in HC vs. RCC		4098	0.171	39.2 (11.5)	35.4 (13.8)	4098	0.226	59.0 (9.9)	56.4 (11.8)
4153	↑ in non-RCC vs. RCC		4160 [3]	0.543	9.99 (8.77)	8.10 (5.90)	4161	0.004	8.04 (3.70)	11.3 (3.6)
5352	↑ in HC vs. non-RCC		5364	0.346	0.862 (0.635)	0.666 (0.658)	5361	0.803	1.95 (2.50)	1.39 (1.03)
5987	↑ in non-RCC vs. RCC		5996 [3]	3.49*10-05	1.60 (0.54)	0.961 (0.501)	5995	0.476	1.87 (1.10)	1.65 (0.81)

1) Values for the first and second analysis respectively.
2) Non-parametric Mann-Whitney U-test.
3) Expression concordant with Won's result.

Figure 5



Classification tree reported by Won et al. The primary node includes the peak at 4 107 Da, which roughly separates between patients (left brach) and controls (right branch). Reprinted with permission from Wiley-VCH verlag GmbH & Co KG.

To assess whether the many patients who had undergone nephrectomy in sample set 2 could be the cause of the lack of significant expression differences, only data from the combined pre-surgery sera in set 1 and 2 were analysed (41 RCC and 59 HC). After normalisation, 49 (22 RCC, 27 HC) and 39 (15 RCC, 24 HC) spectra from the two analyses with the original procedure and 85 (35 RCC, 50 HC) spectra from the modified procedure were assessable. None of the peaks of interest were discriminative in the presurgery samples with the original procedure. Only in the second analysis we saw a slight difference at m/z 4 159 (p = 0.09, intensity HC 3.89 vs. RCC 4.33), which was not in agreement with Won's result, however. In data from the modified assay, the peak at 4 097 Da was seen slightly, but significantly decreased in patients compared to controls (intensity 44.8 vs. 52.6, p = 0.003). Although an expression difference at m/z 5 996 and 3 894 was still present, significance was lost in this analysis (p = 0.11 and p = 0.12).

Lastly, we looked for other discriminative peaks than those reported by Won. Few significant differences between the RCC and control groups were found using the original procedure. A significant peak at 8 597 Da was observed, which was decreased in RCC patients (p < 0.000) both with the original and modified procedure using either the combined samples from set 1 and 2, or only the pre-surgery ones. Considering the pre-surgery samples only, in both analyses following the original procedure consistent expression differences were seen for m/z 2 826 (p = 0.01 and p = 0.09 respectively) and 4 291 (p = 0.04 and p = 0.01). However, with the modified procedure many more peaks differed significantly, notably m/z 4 289, 3 960, 8 151, 6 198, 3 163, 8 707, 4 303, 6 456 (p < 0.000 in the combined sample sets and in the pre-surgery samples).

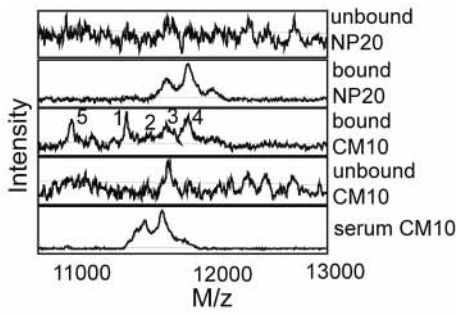
Generating classification trees, we found some with reasonably good classification of RCC and healthy persons. The (non-significant) Won peaks at 4 097 and 5 348 Da in the second original analysis did not come up as tree classifiers, nor did the significant m/z's (5 996 and 3 894) in the data from the modified method (data not shown). Yet, from these data a tree could be built using m/z 4 289 as single classifier with sensitivity 73.3% and specificity 66.0% upon 10-fold cross-validation. Also, m/z 8 151 showed promising performance as a single classifier (cross-validation sensitivity 90.6%, specificity 81.5%). However, these peaks were good classifiers only for sample

set 1. We also investigated whether the significantly different 4 097-Da peak in the pre-surgery serum samples using the modified procedure was a useful classifier. Ten-fold cross validation with this m/z as single classifier yielded limited sensitivity and specificity of 62% and 59%. The other significant m/z's we found by clustering were no classifiers or did not yield trees with reasonable sensitivity and specificity (data not shown).

Protein identification

The identity of the 11-kDa cluster was confirmed by using a serum amyloid-α mouse monoclonal antibody (Abcam Ltd., Cambridge, UK). A similar peak cluster as the one in the profiling experiment was seen in the bound fraction of this antibody, both on NP20 and on CM10 chips (Figure 6). The identities of the discriminating peaks at 14.1 and 17.3 kDa in the Tolson analysis and that of 8 597 Da in the Won analysis could be deduced from the protein profiling results and were confirmed by similar immunocapture experiments. Both these discriminating Tolson peaks were lost with the Won analysis. Although highly similar, these methods differ in the addition of a reducing agent to the denaturation buffer in the latter procedure. A peak cluster around 14 kDa has been described, identified as transthyretin and several isoforms, among which a glutathionylated form at 14.1 kDa (24). Similarly, from the Swiss-Prot database (http://www.expasy.org/) it is known that a 17-kDa homodimer of apolipoprotein A-II is formed by disulfide linkage of two monomers. With the Won procedure, reduction would result in loss of the glutathion group for the 14.1-kDa peak to form a mass of 13.7 kDa and the formation of two 8.5-kDa monomers from the 17-kDa dimeric protein, which was indeed the case in our results. Therefore, we performed immunocapture with a transthyretin rabbit polyclonal antibody (Abcam Ltd., Cambridge, UK) and an apolipoprotein A-II goat polyclonal antibody (Chemicon Europe Ltd., Hampshire, UK). Thus, the identity of glutathionylated transthyretin was confirmed, as was that of the apolipoprotein A-II fragment missing a terminal glutamine residue in one chain of the dimer (Tolson method, 17.3 kDa) and in the monomer (Won method, 8 597 Da).

Figure 6



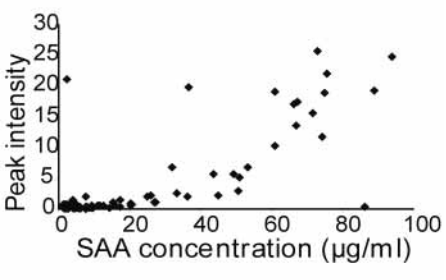
Spectra parts generated with Won's original procedure (A) and with a slightly modified one (B). The same samples in set 1 and set 2 are shown for either assay. Peak intensities for the peaks of interest at 3 901, 4 107, 4 153, 5 352 and 5 987 Da (peaks 1-5), are higher in the modified procedure.

Measurement of serum SAA levels

We observed good agreement between the results of SELDI-TOF MS protein profiling and ELISA regarding the abundance of SAA (Figure 7). It is also clear from this figure that protein profiling is less sensitive than ELISA for the detection of SAA. Similar to Tolson's result, SAA levels below 20

µg/ml were generally not detectable with SELDI-TOF MS in our analyses. However, in several RCC samples the 11-kDa peaks were detectable despite a measured SAA concentration below 10 µg/ml (Table 3). Most HC had SAA levels below 10 µg/ml, except for 6 samples with levels between 10-25 µg/ml.

Figure 7



Identification of the serum amyloid-· (SAA) peak cluster by immunocapture. The spectra parts successively show protein profiling on NP20 chip of the serum fraction that has not bound to the SAA-1 antibody, the fraction that has bound to this antibody on NP20 chip and on CM10 chip, the unbound serum fraction on CM10 chip, and the original protein profile of whole serum on CM10 chip. Peaks 1-3 represent des-RS SAA-1 (11.4 kDa), des-R SAA-1 (11.5 kDa) and SAA-1 (11.68 kDa) respectively. The peak at 11.9 kDa (4) may be another post-translationally modified form of SAA-1. The fifth peak at 10.8 kDa may be the same as found by Tolson, although it was not reported to be a form of SAA-1.

Discussion

Serum protein profiling of renal cell carcinoma was performed to assess the validity and robustness of previously reported biomarker proteins for this type of cancer. We were able to detect the discriminating cluster of serum amyloid-α peaks described by Tolson et al. in about the same subset of RCC patients in both sample sets. Furthermore, the protein profiling result was validated with an ELISA for SAA. The observed expression difference between patients and controls was detected despite differences in sample handling and patient characteristics compared to Tolson's samples. Moreover, it was stably present a year after the first analysis. This indicates SAA is a robust biomarker candidate for RCC, which is also supported by the fact that we found it not only with the assay from Tolson, but also with both the original and modified assay from Won, implying that albumin depletion does not influence the presence of this peak cluster. However, for some other peaks we found markedly lower frequencies in both analyses, such as for the postulated biomarker of 10.85 kDa. Since we did detect it in some of the patients, it is unlikely that the lower frequency is due to the analytical procedure, but more probable, these differences reflect real biological variation in patients. The co-presence of this peak with the SAA peaks as reported by Tolson could not be confirmed in our populations. Furthermore, its postulated prognostic value suggested by its presence in four out of six patients with metastatic disease in Tolson's population does not seem to hold, as most of our metastatic patients did not have this peak.

Since we found a similar number of patients with the SAA peak cluster as Tolson, i.e. SAA levels > ±20 µg/ml, this possibly represents a specific sub-population of RCC patients. As can be concluded from Table 3, disease stage or surgical status do not account for this distinction, nor seems the abundance of this peak correlated with disease stage. A study by Le at al. (25) described a high sensitivity and specificity for the presence of SAA and isoforms in prostate cancer patients suffering from bone metastases, but this does not explain the abundance of these peaks in some of our

patients with early-stage disease. The cluster of SAA peaks has also been mentioned in several other reports. In a study in ovarian cancer it was found in about half of the patients (26) and a study in nasopharyngeal cancer (27) showed an increase of SAA in ±70% of these patients and in ±40% of lung cancer patients. These peaks are thus not specific for RCC, but might reflect a process that occurs only in a subset of these cancer patients. Despite that we found some other expression differences in both sample sets, these were not large enough for good classification of patients and controls. Furthermore, both a decrease of a 17.3-kDa peak (possibly des-glutamine apolipoprotein A-II) and glutathionylated transthyretin have been described in the sera from patients with other cancers before (24;28-30) and thus they seem not specific for RCC either. Yet, these proteins might have a role as markers in disease or therapy monitoring.

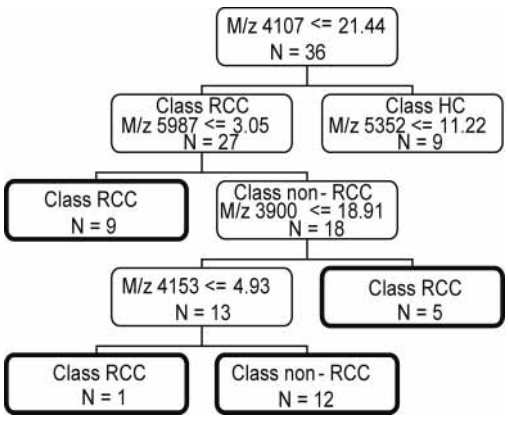
We experienced difficulties in the analysis with the Won procedure. Many spectra were not assessable, possibly because of ion suppression caused by salt remnants on the chip. Moreover, peak intensities for the peaks of interest were generally much lower than those reported, despite optimisation of MS acquisition parameters, and Won's discriminating peaks were not readily detectable. Thus, this analytical protocol seems less robust than e.g. Tolson's. Furthermore, only non-significant expression differences were observed with this procedure, but only in sample set 2, which was the least comparable to Won's. The only similarity with Won's data for this procedure was a lack of an expression difference for m/z 4 153. A slightly modified method, including a better reductant and omission of protease inhibitor in the denaturation buffer, as well as a different energy-absorbing matrix generally improved peak detection. Moreover, much fewer spectra suffered from ion suppression. Although also in this modified procedure a final washing step with water was lacking, the use of a different matrix might have precluded ion suppression effects. Using this method, the observed peak differences proved reflective of the patient characteristics: only in sample set 1, most comparable to Won's, we found two of the previously described expression differences. Yet, in the combined pre-surgery sera from set 1 and set 2, these expression differences became statistically insignificant. Possibly, other patient characteristics than nephrectomy status are responsible for this discrepancy, e.g. disease stage. Indeed, all pre-surgery sera in set 2 were from patients with metastatic disease, whereas in set 1 these included 20 patients with RCC stage I-III. In addition, with this modified method only for the combined pre-surgery sera we found a peak at 4 097 Da higher in controls compared to patients, as reported by Won. However, the difference in mean peak intensities between patients and controls was only slight, a marked difference compared to Won's result. Therefore, despite the observed expression difference, this peak was not identified by the BPS as a main classifier for the classification tree, nor was it able to split patients and controls correctly when manually selected as the main classifier. Thus, we could not reproduce the classification performance of this peak that was suggested by Won's classification tree.

For a peak at 4 161 Da we found an expression difference in sample set 2 with the modified procedure, but not in set 1, nor in the combined pre-surgery sera. This protein (increased in non-RCC disease vs. RCC according to Won's classification tree) might be a specific inflammatory protein that is neither deregulated in RCC nor in health, which is concordant with the similar mean peak intensities for patients and controls mentioned by Won (8.442 ±4.496 and 9.192 ±6.746 respectively (9)). The increase of this peak in RCC that we saw in sample set 2 might be due to differences in patient characteristics or sample handling compared to Won's samples. Interestingly, a more recent report on serum protein profiling for RCC also describes the increase of a peak at m/z

4 151 in RCC patients compared to controls (31). This peak was observed under similar assay conditions and thus may well be the same protein as Won's. Its performance to detect RCC in stage I was reported lower than for later stages (31). This could explain why we only saw a significant difference in sample set 2 (mostly stage–IV disease) and not in set 1 or the pre-surgery sera (mainly early-stage samples), and, if Won and co-workers also evaluated earlier disease stages, why they did not find this difference.

Altogether, some of Won's postulated biomarkers seem prone to lack of reproducibility (at least with the original method) and lack of validity. Although differences with our results might be caused by different sample handling conditions or other disease stages, this at least stresses the necessity of careful control of these parameters if the Won proteins were to be used as biomarkers. Furthermore, the few assessable spectra with the original procedure may have hampered our power to detect the expression differences described previously. However, the 4 107-Da peak splitting patients and controls in the first node of Won's classification tree is only based on a total of six controls and 15 patients, which is even less than our assessable individuals. With the modified method we could evaluate many more spectra, but the expression difference at m/z 4 107 that we found for the pre-surgery samples was not large enough for correct classification of patients and controls (Figure 8). Thus, the classification tree reported by Won might well be the result of overfitting of data. For, five peaks to fit a population of 36 samples in three groups is a rather high number, since peaks in the lower branches of a classification tree are discriminative for successively lower numbers of samples, thus more likely influenced by outliers or artefacts in the data. Possibly, the other discriminative m/z's at 4 289 and 8 151 that we found with this procedure have more potential as biomarkers for RCC. A decrease of a peak at 4 289 Da has been mentioned for several other cancers (32;33), one group attributing this mass to a fragment of inter-α trypsin inhibitor heavy chain 4 (33). Also, peaks with an m/z similar to 8 151 have been reported as biomarker candidates (6;34). However, the consistent decrease of these peaks in RCC remains to be established in new sets of pre-surgery serum samples including patients with early-stage disease. In addition, their identity and their specificity for RCC or cancer in general must be addressed.

Figure 8



Correlation of serum levels of SAA as measured by ELISA with peak intensities of the 11.68-kDa peak from SELDI-TOF MS protein profiling.

Considering our goal to validate previously reported biomarkers for RCC, lack of robustness was the first hurdle to take, especially for the Won analysis. It is unlikely that chip characteristics, (manual) sample processing or instrument integrity were the cause of this, since the spectra that were assessable with both the original and modified analytical protocol from Won, and those from the Tolson protocol did show reproducible profiles across the sample sets and the duplicate analyses. More probably, critical steps in the analytical protocol were the cause, such as the use of a good reductant (DTT) and suitable energy-absorbing matrix (SPA). Furthermore, duration of all steps might prove critical: as DTT reduces full-length haptoglobin, resulting in the release of the 9.2-kDa 1-α chain, the lack of detection of this peak in the first Tolson analysis may have been caused by incomplete reduction of the full-length protein. Because of this potential sensitivity of the technique to such subtle changes, the structural characterisation of any postulated biomarkers and development of more quantitative assays are needed for routine clinical analyses. The second hurdle in validation is related to the law of large numbers. Even when peaks are reproducibly detected, failure to validate expression differences between patients and controls may still occur as a result of limited sample size: too few samples could result in insufficient statistical power to identify a significant difference for the alleged biomarkers. Alternatively, statistically significant differences found previously could rather be the result of chance or bias, in which case former data were overfitted. These problems seem likely to have occurred with Won's result, which was based on very few samples. However, we cannot rule out that this was also the case for our data, since our sample size was still limited.

It might be argued that the only expression difference we could validate was that of the acute phase protein SAA, and that this is due to the extreme increase of this protein during acute phase reactions, which makes detection with SELDI-TOF MS more feasible. However, the change in protein expression detectable with SELDI-TOF MS does not so much depend on the size of this change, as on the concentration range in which it occurs. As for all equilibrium reactions, the binding of proteins such as SAA to the chromatographic chip surface describes an S-curve. If an expression difference occurs in the linear range of this S-curve, a rather small increase can result in binding of much more protein and thus a large change in SELDI-TOF MS peak intensity. Alternatively, a small change in peak intensity might represent a large expression difference, when this happens in the plateau regions of this S-curve. Unfortunately, in complex matrices such as serum, one can never be sure when to be in the linear or plateau range, due to competition for binding sites from other proteins (which causes a shift of the S-curve to the right). Furthermore, the more abundant proteins, often acute phase proteins, compete and bind preferably to the chip, causing limited sensitivity of SELDI-TOF MS for less abundant proteins compared to antibody assays such as ELISA. Thus, the ability to detect expression differences with SELDI-TOF MS depends more on the abundance of the protein under investigation than on the extent of the expression difference.

Concluding, we show that, depending on the analytical protocol, some of the alleged RCC biomarkers were difficult to reproduce. From the detectable candidate biomarkers, only the increased expression in RCC of SAA and variants could be unambiguously validated in our populations, with potential specificity for a certain subset of cancer patients. However, two newly identified biomarker candidates merit further evaluation in new sample sets.

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SELDI-TOF MS serum protein profiling predicts poor prognosis in renal cancer patients

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Abstract

Background: Approximately 30% of patients present with metastasized disease and only 15-25% of patients respond to anti-tumor treatment. Surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) may identify protein signatures in the serum proteome of RCC patients that discriminate between poor and good prognosis patients.

Methods: In this study we analyze protein profiles in the serum of 57 renal cancer patients (2% stage I, 12% stage II, 7% stage III and 79% stage IV patients according to the American Joint Committee on Cancer) and 59 healthy controls. Denatured serum samples were incubated on CM10 ProteinChip arrays and analyzed using the PBS-IIC ProteinChip Reader. Clinical data was collected and the extended Memorial Sloan-Kettering Prognostic Factors Model for survival was calculated. Ratios discriminating between RCC cases and controls were selected to generate a predictive multi-protein model. Univariate and multivariate Cox Proportional Hazard analyses were performed. Protein masses were identified.

Results: In RCC serum samples we identified ion masses predictive for patient survival, and built a protein-model consisting of five signature peaks with m/z ratios of 2944, 3331, 6457, 6654, and 9201 Da, that could correctly identify poor prognosis patients with sensitivity and specificity of 80% and 76% for 1-year survival. Cumulative 1-year survival was 93% for low-risk patients, compared to 48% for high-risk patients (P=0.0001, Log-rank test). Multivariate analysis indicated that our model was an independent predictor of survival.

Conclusion: SELDI-TOF MS can be used to assess the prognosis of RCC patients independent of present prognostic factor models.

Introduction

Kidney cancer accounts for approximately 3% of all adult malignancies. Peak incidence of renal cell cancer (RCC) is from 50-70 years, with a male to female ratio of 6:1 (1). Kidney cancer can grow to very large sizes without causing any complaints, and when symptoms such as hematuria, abdominal pain or discomfort occur it is commonly associated with an advanced stage of the disease (2). Response rates in patients with metastatic RCC remain at 15-25% for immune based therapies, and approximately 2-40% for small molecules or antibodies, such as sorafenib, sunitinib, erlotinib and bevacizumab (3). RCC is currently the most lethal of all urological cancers with a corresponding 5-year survival rate of 9% in metastatic disease (4).

Although treatment outcome is improving due to the development of various targeted therapies, adequate selection of patients for these treatment approaches is important. SELDI-based protein MS has mostly been limited to diagnostics (5-9), while many other potential implementations of SELDI-TOF MS in the oncology field are practically untried. Gene expression profiles of tumor tissues allow the possibility to select patients on a particular gene expression signature out of a homogenous population of cancer patients, whom would benefit from adjuvant treatment to gain complete remission or increase disease free survival (10;11). Subsequently, proteomic-based research evaluating protein expression in high-throughput tissue arrays originating from RCC tumor biopsies or resection material have identified new protein markers as independent predictors of survival

together with established molecular and clinical predictors (12). Furthermore, experimental data in breast cancer cell lines using MALDI-TOF MS with artificial neural network (ANN) algorithms identified proteomic fingerprint patterns indicative for responsiveness or resistance to chemotherapy (13). Other studies have used mass spectrometry to identify protein patterns in tumor tissue which predict clinical outcome in patients with diverse cancers (14-16).

Profiling of proteins in body fluids, such as urine, plasma or serum to predict patient outcome would be an attractive and non-invasive approach for utilization in the clinic. An example that supports this concept is a proteomic study in the plasma of 31 neuroblastoma patients, where the authors demonstrated that high levels of the protein serum amyloid alpha-1 strongly correlated with prognosis (17). Other recent reports demonstrate the feasibility to correctly assign progressors from non-progressors in melanoma patients after complete lymph node dissection (18) and to predict metastatic relapse and prognosis in post-operative high-risk breast cancer patients (19). These studies underscore the value of identifying proteomic fingerprints in patient blood to predict responsiveness to treatment and survival.

In this study we perform SELDI-TOF mass spectrometry on 116 serum samples from two different institutes. Here we report the identification of a multi-protein signature that can distinguish poor from good prognosis renal cancer patients, and is an independent predictor of survival when assessed together with the Memorial Sloan-Kettering Prognostic Factors Model. Our results show the validity to use SELDI-TOF MS to identify high-risk RCC patients.

Materials and methods

Blood collection.

A pooled serum sample set was analyzed from two different institutes in the Netherlands. The first set consisted of blood samples collected from 37 patients with RCC and 32 healthy controls from the Netherlands Cancer Institute, in Amsterdam (Institute 1). The second set consisted of 20 patients with RCC and 27 healthy controls from the University Medical Center in Utrecht (Institute 2). The serum handling protocol differed between both institutes, as the Netherlands Cancer Institute had a 30-minute coagulation step, a 30-minute centrifugation step at 1900g and storage at -30°C, while University Medical Center Utrecht had a 2-6 hours coagulation at room temperature followed by overnight coagulation at 4°C, centrifugation for 15 minutes at 1500g and storage at -80°C. Inclusion of the first patient set was between January 2001 and April 2003, the second set between April 2003 and October 2004. Samples were collected by approval of both institutional ethical boards, and informed consent was obtained from each subject before blood collection.

Clinical characteristics.

Cancer patients included were untreated patients before nephrectomy or relapsed patients with metastatic disease. The following variables were obtained from the medical records of the 57 patients: sex, age, the 1997 classification of TNM stage, American Joint Committee on Cancer (AJCC) stage, Karnofsky performance status, time from diagnosis to treatment with interferon-α, prior radiotherapeutic treatment, presence of hepatic, lung and retroperitoneal nodal metastases, hemoglobin count, lactate dehydrogenase and serum calcium. The extended Memorial Sloan-Kettering

Prognostic Factors Model (extended Motzer criteria) for survival was calculated, and using these criteria, patients were classified into 3 risk categories based on the number of prognostic factors (20;21). Patient survival was assessed at 12 and 36 months after blood collection, after a median observation period of 31 months (interquartile range 19 – 41 months).

Protein profiling.

Assay procedures were based on those reported by Won et al. (9). In short, 2 µL of serum were denatured with 3 µL of 20 mM Tris-HCl, pH 7.5 containing 9.5 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 2% dithiotreïtol (DTT). After mixing, 45 µl of 50 mM sodium acetate pH 4.0, containing 0.05% Triton X-100 (binding buffer) were added. CM10 chips were equilibrated twice with 350 µL of binding buffer on a platform shaker for 5 min. Fifty µl of the diluted serum was added to each well and incubation performed for 1 h. The array was washed with 350 µl of binding buffer, rinsed with a 1:20 dilution of binding buffer and air dried. A 50% solution of sinapinic acid in 50% acetonitrile (ACN) + 0.5% triflouroacetic acid (TFA) was used for energy absorbing matrix. Two times 0.5 µl of this solution was applied to the spots. All protein chips were analysed using the PBS-IIC ProteinChip Reader (Ciphergen Biosystems). M/z values were calibrated externally with a standard peptide mixture (Ciphergen Biosystems) containing [Arg8] vasopressine (1084.3 Da), somatostatine (1637.9 Da), dynorphine (2147.5 Da), ACTH (2933.5 Da), insulin β-chain (bovine) (3495.9 Da), insulin (human recombinant) (5807.7 Da), and hirudin (7033.6 Da).

Protein identification.

Tentative identities of proteins of interest were based on protein masses and further elucidated using immunocapture with appropriate antibodies. Fifty microliters of 0.1 µg/µL antibody in phosphate buffered saline (PBS) were coupled to 2 µL of protein A ceramic HyperD beads (Pall/Biosepra, Saint-Germain-en-Laye, France) for 30 min on a platform shaker. Beads were subsequently washed 3 times 5 min with 200 µL PBS. Ten µL of serum were diluted to 50 µL with PBS and applied to the beads for one hour. The unbound fraction was collected. The beads were then subjected to 5-min washes with, respectively, 200 µL PBS, five times a 200 µl-mixture of 50 mM Tris pH 7.5/ 0.2% CHAPS/ 0.5 M NaCl, 200 µL PBS and deionized water. Finally, bound proteins were eluted from the beads for 10 min with 15 µL 0.1 M acetic acid. Unbound fractions as well as eluates were profiled on gold chips or NP20 chips using two times 1 µL of a 50% SPA solution in 50% ACN + 0.5% TFA as energy absorbing matrix. In addition, whole serum, bound and unbound fractions were profiled on CM10 chips using the original assay procedures.
Since no antibody for the α-chain of the haptoglobin molecule was available, indirect evidence for the identity of haptoglobin-α1 fragment was provided by haptoglobin phenotyping as described previously (22).

Statistics and bioinformatics.

Data were analyzed using the ProteinChip Software package, version 3.1 (Ciphergen Biosystems) and SPSS software (version 12.0.1). P-values below 0.05 (two-sided) were considered significant. All acquired spectra were compiled and analyzed as a whole. Spectra were baseline subtracted and normalized to the total ion current from 1500 Da to the spectrum's end. Spectra that had normalization factors higher than 2.00 or lower than 0.50 were excluded from analysis.

Differences in peak intensities of m/z ratios between healthy controls and RCC patients, or between dichotomous clinical variables, were calculated using the non-parametric Mann-Whitney

Test. For correlative testing of protein masses with clinical characteristics and the extended Memorial Sloan-Kettering Prognostic Factors Model the Spearman's correlation coefficient was used.

Univariate Cox Proportional Hazard Analysis was used to identify predictive m/z ratios in relation to survival ($P<0.05$). For the generation of a predictive multi-protein model, all m/z ratios were dichotomized and included by forward selection, starting with the strongest ratio. We minimalized data fitting for our patient population by using the median intensity of all cancer patients to standardize dichotomization of m/z ratios, instead of cut-offs based on maximum separability between two groups. The multi-protein model with highest predictive power contained five m/z ratios and was not improved by the addition of any of the other significant predictive ratios. The model was dichotomized in 'low-risk', defined as 0-3 m/z ratios above the median intensity cut-off, and 'high-risk', defined as 4-5 ratios above the cut-off value, based on the combination of the highest sensitivity and specificity as predictive factor for survival.

Cox's Proportional-Hazard analysis was used to assess whether our proteomic model was an independent predictive model when compared to the extended Memorial Sloan-Kettering Prognostic Factors Model for survival. Life estimation was evaluated according to the method of Kaplan and Meier and Log-rank statistic was used for comparison of two survival curves.

Results

Baseline characteristics and differences between institutes.
Serum samples were collected from 57 renal cancer patients and 59 control subjects, of which 38 (66.7%) and 27 (47.5%) were of male sex, respectively. Median age of the cancer patients was 59.3 years (range 22.8 to 80.1 years) and volunteers 52.4 (range 22.4 to 71.5). The characteristics of the 57 patients included in this study are shown in Table 1. Most patients from Institute 1 were newly diagnosed patients prior to nephrectomy, while the patient population from Institute 2 consists of relapsed metastasized AJCC stage IV patients. There was no difference in patient survival between Institute 1 and 2 ($P=0.70$).

Protein profiles in patient sera correlate with prognostic factors model.
Mass spectra generated by the ProteinChip reader were first baseline subtracted and then normalized. 19 of the 116 serum profiles were excluded from analysis as they did not meet normalization inclusion criteria. Protein profiling revealed 32 significantly decreased and 6 significantly increased m/z ratios in the remaining 48 renal cancer patients compared to the 49 healthy subjects (Table 2). This set of 38 m/z ratios was selected for further analysis with the Memorial Sloan-Kettering Prognostic Factors Model (also called the Motzer criteria (20), which were recently extended and validated by Mekhail et al. (21)). In short, the extended Motzer criteria were calculated by the presence or absence of seven validated prognostic factors, and patients were classified into 3 risk categories predictive for survival. We identified an association between 11 m/z ratios and the extended Motzer criteria, of which the strongest are m/z 6457, 6861, 6654 and 3331 ($r=-0.53$ with $P=0.0001$, $r=-0.50$ with $P=0.0003$, $r=-0.48$ with $P=0.001$ and $r=-0.44$ with $P=0.002$, respectively). The median intensity for m/z ratio 6457 is 24.4 with interquartile range 17.1-30.1 in favorable risk patients, 18.6 with interquartile range 11.2-24.9 in intermediate risk patients, and 7.6

Table 1 - Baseline characteristics of 57 untreated renal cancer patients

	Institute 1		Institute 2	
Characteristics	**No. (%)**		**No. (%)**	
Renal cell cancer	37	20		
Clear-cell carcinoma	25	(68%)	12	(60%)
Non-clear cell	4	(11%)	2	(10%)
Mixed papillary/ clear-cell	1	(3%)	1	(5%)
No PA histology performed	7	(19%)	5	(25%)
Primary tumor				
No resection	33	(89%)	7	(35%)
Resection	4	(11%)	13	(65%)
AJCC stage				
1	1	(3%)	0	(0%)
2	7	(19%)	0	(0%)
3	3	(8%)	0	(0%)
4	26	(70%)	20	(100%)
Metastasis				
No	10	(27%)	0	(0%)
Yes	24	(65%)	20	(100%)
Unknown	3	(8%)	0	(0%)
Metastasis location				
Bone	6	(16%)	2	(10%)
Liver	3	(8%)	4	(20%)
Lung	15	(41%)	13	(65%)
Lymph node	8	(22%)	3	(15%)
Mediastinal	2	(5%)	4	(20%)
Other	4	(11%)	6	(30%)
Follow-up time				
Median, months	38.7		13.3	
Interquartile range	33.3-43.7		10.4-25.6	
Survival				
Median, months	24.1		15.4	
Interquartile range	10.2-x		12.71-x	

Table 2 - Peaks (m/z) with discriminating power (P<0.05) between cancer patients and controls

***m/z* (Da)**	**±**	**P**	***m/z* (Da)**	**±**	**P**
2,745.9	-	0.0006	4,320.2	-	0.005
2,759.1	-	0.004	4,602.6	+	0.01
2,872.9	-	0.01	4,705.0	+	0.004
2,944.2	-	0.03	4,798.0	+	0.02
2,966.4	-	0.006	5,072.5	-	0.0001
3,163.1	-	0.0001	6,198.2	-	0.0001
3,221.7	-	0.01	6,441.0	-	0.008
3,321.8	-	0.01	6,456.7	-	0.0003
3,330.9	-	0.01	6,654.3	-	0.01
3,786.3	-	0.04	6,846.0	-	0.004
3,889.7	-	0.04	6,861.0	-	0.002
3,897.0	-	0.02	8,151.9	-	<0.0001
3,941.1	-	0.03	8,579.1	-	<0.0001
3,960.5	-	<0.0001	8,707.5	-	0.0003
4,077.9	-	0.009	9,200.9	+	0.02
4,097.6	-	0.02	9,426.2	+	0.01
4,276.3	-	0.009	13,733.7	-	0.002
4,289.3	-	<0.0001	15,136.4	-	0.04
4,303.4	-	<0.0001	18,384.2	+	0.002

* Increase or decrease in m/z ratio between cancer patients compared to controls.

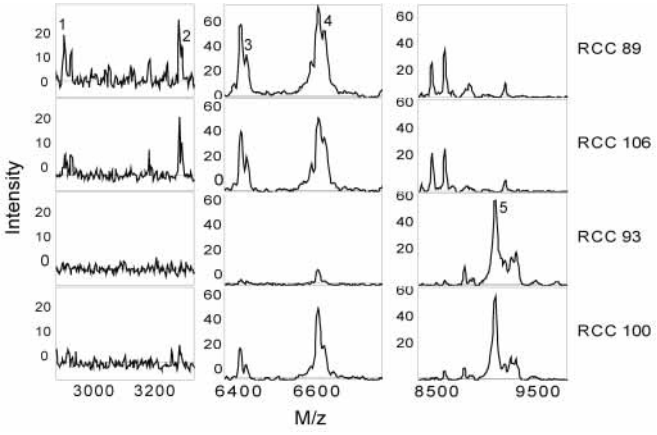
with interquartile range 4.3-16.8 in poor risk patients according to the extended Motzer criteria. The median intensity for m/z ratio 6861 is 7.5 with interquartile range 5.1-8.5, 4.8 with interquartile range 3.7-7.7 and 2.9 with interquartile range 1.2-5.0, the median intensity for m/z ratio 6654 is 33.8 with interquartile range 28.5-41.4, 29.5 with interquartile range 15.6 and 34.8, and 18.4 with interquartile range 8.9-30.6, and the median intensity for m/z ratio 3331 is 7.7 with interquartile range 6.3-10.2, 6.4 with interquartile range 2.5-9.7 and 4.1 with interquartile range 3.4-6.5 for patients with favorable, intermediate and poor risk, respectively.

Generation of a multi-protein signature model to predict survival.

We subsequently analyzed whether any of the discriminatory m/z ratios between cancer patients and healthy subjects had an association with survival. By univariate Cox Proportional Hazards Analysis we identified 12 ratios predictive of patient survival. To assess whether SELDI-TOF MS could be used to identify the patients with good or bad prognosis, a multi-protein model was built based on these 12 dichotomized ratios. Because of our relative sparse dataset, dichotomization based on maximum separability between survivors and non-survivors could cause overfitting our data. To minimalize this problem we dichotomized ratios by taking the median intensity of all 48 cancer patients as cut-off value. The multi-protein model with best predictive power for survival, generated using forward selection, contained five of the 12 m/z ratios (being m/z 2944, 3331, 6457, 6654 and 9201). Representative spectra of these ratios in our SELDI-TOF MS model are depicted for four patients with good or poor survival (Figure 1).

A Receiver Operating Characteristic (ROC) was constructed by calculating the proportion of positive tests among those who died (sensitivity) and the proportion of positive tests among those who survived (specificity) for the continuous m/z ratio. By evaluating the area under the ROC (AUC ROC) as measure of predictive power, the AUC ROC of the five-protein model was higher than the AUC ROC of all individual predictive continuous m/z ratios (data not shown). In the SELDI-TOF MS protein model for survival prediction, low-risk for death was defined as 0-3 m/z ratios above the median intensity, and high-risk as 4-5 m/z ratios above the median intensity.

Figure 1 - MS-spectra of predictive SELDI-TOF MS model.



Zoomed regions of the mass spectra are shown for five ion signals incorporated in the predictive model for two patients with long-term survival (RCC 89 and 106) and two patients with poor survival (RCC 93 and 100). Ratios depicted are m/z 2944 (1), 3331 (2), 6457 (3), 6654 (4) and 9201 Da (5).

Kaplan-Meier plots of 1-year survival.

Figure 2A

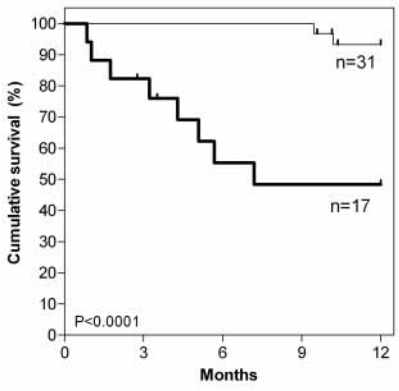
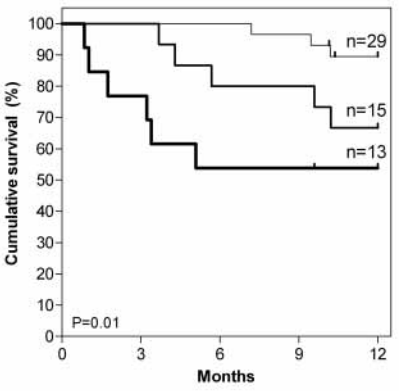


Figure 2B



SELDI-TOF MS protein signature model, consisting of 5 signature peaks m/z 2944, 3331, 6457, 6654, and 9201 Da (A). Extended Memorial Sloan-Kettering Prognostic Factors Model (B).

Serum protein profiles discriminate between good and poor prognosis RCC patients.

After a median follow-up period of 31 months after blood collection, 28 of the 57 patients died due to cancer progression (of which 10 patients died within 12 months after inclusion), 1 patient had a non-tumor related death and 28 patients were alive. We assessed how well our multi-protein signature could identify patients with poor prognosis from a heterogeneous patient population. Based on our proteome signature model, the AUC ROC for 1-year survival was 0.78 with P=0.007 for identifying poor prognosis patients. The sensitivity, specificity, positive and negative predictive value of our signature was 80%, 76%, 47% and 94%, respectively. The 1-year survival for low-risk patients was 93%, compared to 48% for high-risk patients (Figure 2A; log rank 15.1, P=0.0001). Next, we assessed whether the protein-model could also predict 3-year survival. The AUC ROC, sensitivity, specificity, positive and negative predictive value of our protein-model was 0.69 with P=0.03, 54%, 83%, 77% and 65%, respectively. The 3-year survival for low and high-risk patients was 61% and 8%, respectively (Data not shown; log rank 18.42 with P<0.0001). Because the SELDI-TOF MS model was constructed on a pooled patient population from two independent institutes with different serum handling protocols, we had to validate whether our model was still predictive for survival when tested separately on patients from these two institutes. Using the SELDI-TOF MS

Table 3 - Univariate and multivariate analysis for survival

	univariate			multivariate		
Variable	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
Extended Motzer criteria						
Favorable risk	1.0	-	-	1.0	-	-
Intermediate risk§	3.55	[0.85-14.86]	0.08	0.58	[0.09-3.79]	0.57
Poor risk§	6.86	[1.71-27.56]	0.007	2.65	[0.57-12.25]	0.21
Prognostic protein-model	12.04	[2.85-56.09]	0.002	10.03	[1.85-54.21]	0.007

§ Comparison with favorable risk patients (reference)

predictive protein model it was possible to discriminate survivors from non-survivors, independent of institute (data not shown; Institute 1 cumulative 1-year survival for low and high-risk patients 90% and 40%, log rank 10.55 with P=0.001; Institute 2 cumulative 1-year survival for low and high-risk patients was 100% and 57%, log rank 6.06 with P=0.01).

Proteomic model is an independent predictor of survival.

In our patient dataset the extended Memorial Sloan-Kettering Prognostic Model and our predictive proteomic model were both significant predictors of 12-month survival by univariate analysis (Table 3). The 1-year survival plot according to the extended Motzer criteria is depicted in Figure 2B (log rank 9.17 with P=0.003).

We evaluated whether our multi-protein model was an independent predictor of 1-year survival when tested together with the extended Motzer criteria in multivariate analysis. The multi-protein model was the only significant independent predictor of cancer-related death (Table 3). The hazard ratio (HR) of cancer-related death for the multi-protein model was 10.0 (95% confidence interval (CI) from 1.85 to 54.21). When we assessed the multi-protein model for 3-year survival in multivariate analysis, our model also was the only significant independent predictor, with a HR of 4.77 (95% CI of 1.95 to 11.69 with P=0.001; data not shown).

Protein identification.

Based on protein masses, the respective tentative identities of peaks 3331, 6654 and 6457 Da were doubly and singly charged apolipoprotein C-I and its (singly charged) fragment without the N-terminal threonine and proline. An immunocapture experiment with an apolipoprotein C-I monocolonal antibody (Abcam Ltd, Cambridge, UK) was performed to confirm these identities. For analysis, three sera from patients with high peaks at 3331, 6654 and 6457 Da were pooled (pool 'high'), as well as three sera from patients with low peaks at these m/z's (pool 'low'). Immunodepleted serum showed lower peak intensities for these peaks than whole serum, although the binding capacity, especially in pool 'high' was not sufficient to remove all protein (Figure 3A and B). In the bound fraction, peaks at these m/z 's were readily visible, with higher intensities for 'high' than for 'low'.

The tentative identity of the 9201-Da peak was haptoglobin-α1 fragment. The mass at 4603 Da could correspond to a doubly charged haptoglobin-α1 molecule, since its peak intensity highly correlated with that at 9201 Da. No haptoglobin antibody for the α-fragment of the molecule was available, so indirect evidence for the identity of the 9201-Da peak was provided by phenotyping of haptoglobin. The haptoglobin protein exists in three phenotypes, all containing the haptoglobin-β chain. The difference lies in the linkage of this β chain by disulfide bridges with either two α1 chains (phenotype 1-1), or one α1 chain and a variable number of α2 chains (phenotype 1-2), or only a variable number of α2 chains (phenotype 2-2). If the postulated identity of haptoglobin-α1 fragment is correct, its peak intensities from SELDI-TOF MS should correlate with the phenotype. That is, there should be no peak at 9201 Da in patients with phenotype 2-2, and the peak should be highest in patients with phenotype 1-1. Firstly, a reduction experiment was performed to assess whether the appearance of the peaks at 4603 and 9201 could be explained by the presence of DTT in the denaturation buffer, as this reduces disulfide bridges between the β and α chains of haptoglobin (Figure 3C). Three sera from patients with high peaks were pooled (pool 'high'), as well

Protein identification.

Figure 3A

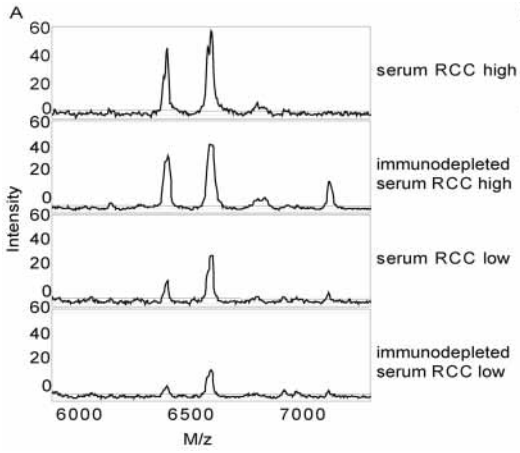


Figure 3B

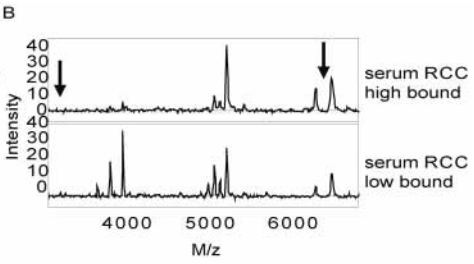
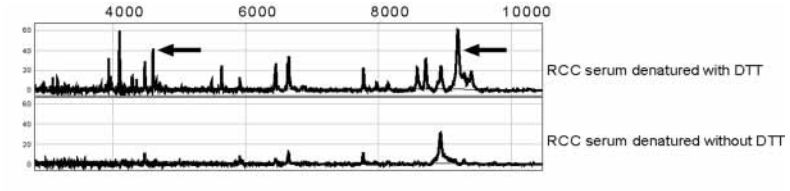
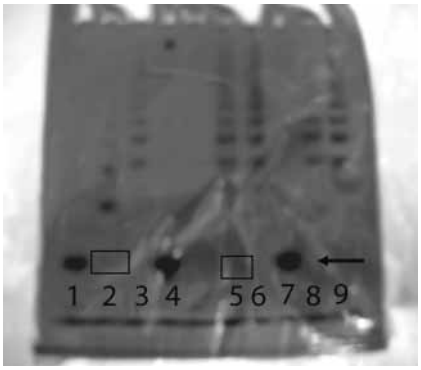


Figure 3C





Identification of apolipoprotein C-I by immunocapture (panel A and B). A: Peaks at 6.4 and 6.6 kDa are decreased in the RCC sera of pool 'high' (±20%) and 'low' (±45%) after immunodepletion with apolipoprotein C-I antibody. These correspond to apolipoprotein C-I (6.6 kDa) and its fragment without the N-terminal Thr-Pro (6.4 kDa). B: In the fractions of pool 'high' and 'low' bound to the antibody two peaks at 6.4 and 6.6 kDa are visible (arrow). A very small peak from the doubly charged molecule (3.3 kDa) is also present. All spectra represent samples profiled on CM10 chips according to the original protocol.

Identification of haptoglobin-α1 chain (panel C and D). C: When serum was denatured with the reductant DTT in the denaturation buffer (original analysis), peaks at 4.6 and 9.2 kDa are clearly visible in the spectrum (arrows). Without DTT these peaks disappear, indicating that they are the result of reduction, which disrupts disulfide bridges such as those present between haptoglobin α and β chains. D: Haptoglobin phenotyping showed that in samples 1, 4, and 7 only a clear band of the haptoglobin α1 chain is present (arrow; phenotype 1-1), in samples 2 and 5 this is a faint band (phenotype 1-2), and in samples 3, 6, 8 and 9 there is no band of the α1 chain (phenotype 2-2). Samples 1, 4, 7: high intensities at 9201 and 4603 Da. Samples 2, 5, 8: intermediate intensities at 9201 and 4603 Da. Samples 3, 6, 9: low intensities at 9201 and 4603 Da.

as three sera from patients with no peaks (pool 'low'). Pooled samples were profiled on CM10 chips using the original procedure, but with or without DTT in the denaturation buffer. Without DTT, neither in the spectrum of the pool 'high', nor of the pool 'low' peaks at 4603 and 9201 Da were visible, contrary to the result with DTT. Subsequently, the same six samples were phenotyped separately, in addition to 3 samples with intermediate peak intensities for the m/z's of interest. Patients with high peak intensities at 9201 and 4603 Da had phenotype 1-1, whereas patients with no peaks h-ad phenotype 2-2. Some of the patients with intermediate peak intensities had phenotype 1-2 and some 2-2 (Figure 3D). Thus, haptoglobin phenotype correlated with peak intensities at 9201 and 4603 Da.

Discussion

We have performed SELDI-TOF mass spectrometric analyses on serum samples of patients with renal cancer and characterized protein profiles that were able to predict their clinical outcome. Key ions in our model strongly correlated with established tumor prognostication factors. Using our proteomic signature, we could identify patients with a poor 1- and 3-year survival out of a heterogeneous population of renal cancer patients. In our analysis the proteomic model was a strong and independent predictor of survival when tested against the extended Memorial Sloan-Kettering Prognostic Factors Model for survival. We therefore provided proof of concept that in renal cancer SELDI-TOF MS can generate a serum proteomic signature that may be used as a prognostication tool to distinguish poor prognosis patients from patients with long-term survival.

Our predictive signature model contained five mass-to-charge ratios, with a strong predictive estimation of survival probabilities. Two of the three ions corresponding to apolipoprotein C-I, namely the doubly charged apolipoprotein C-I and its singly charged fragment without the N-terminal threonine and proline, were also significantly associated with established prognostic factors such as tumor size, nodal status, and the extended Motzer criteria. Mian et al. demonstrated the feasibility to correctly differentiate stage I from IV AJCC in 88 of 96 melanoma patients, based on the presence of serum fingerprint proteins (17). In renal cancer, further assessment of serum fingerprint patterns with established prognostic factors will have to be evaluated prospectively in a large uniform population.

Apoliproteins A-I and II are decreased in the serum of patients with ovarian (23) and prostate cancer (24), respectively, and have been used in SELDI-based cancer diagnostics. However, few reports have demonstrated decreased expression of apolipoprotein C (apoC) in cancer (25;26). Here, we specifically find 2 variants of apoC-I with strong association with survival; decreased apoC-I is associated with worse tumor and nodal grade, and decreased survival. Another identified protein in our model is haptoglobin-α1 fragment, which is increased in a variety of human cancers (27;28). Haptoglobin is a glycoprotein mainly secreted by the liver and involved as hemoglobin scavenger, however it may also be produced by other tissues such as tumor cells. Haptoglobin is involved in immune suppression in cancer (29), facilitating cell migration (30) and angiogenesis (31). We now also implicate presence of a 9.2 kDa haptoglobin-α1 fragment in reduced survival of renal cancer patients. The last protein fragment in our model, a 2.9 kDa fragment, has been described previously using SELDI-MS in serum (32), but has not yet been identified.

Most SELDI-TOF MS studies have been directed at cancer diagnosis and not at predicting clinical outcome. Only a few reports have investigated whether proteomic MS can identify biomarkers predicting responsiveness to treatment, disease progression or survival. Three reports demonstrate the validity of finding predictive biomarkers in the blood of patients with cancer (17-19). In all three studies, samples and controls originated from one center, therefore extrapolation to other institutes may not be possible. We have used pooled sample sets from two institutes with completely different sample handling protocols, and performed the analysis at different time points. When we evaluated our predictive model on patients of both institutes separately, we could still strongly distinguish survivors from non-survivors. The ions strongly associated with clinical outcome in our non-uniform sample set are most likely robust predictors of prognosis, as differences between the sampling procedures may have aided a more stringent selection of biomarkers with a broad applicability.

Irrespective of differences in blood sampling, large variations in SELDI-based mass spectrometric results (between different research groups), are most likely attributable to different assay procedures (33). In our search for a prognostic profile of patients with RCC, we used two assay procedures previously reported for the detection of patients with renal cancer. The first procedure was a slight adaptation of the method used by Won et al. (9) and the second was identical to the one described by Tolson et al. (22). We could identify the strongest biomarkers of survival prediction using the first assay procedure, and there was no overlap in biomarkers found between the two procedures. One of the differences that may explain these findings was albumin-depletion prior to protein profiling in the procedure described by Tolson, which resulted also in depletion of some of the markers in our multi-protein model.

In conclusion, we show that RCC patients' blood contains an assortment of biomarker proteins that taken together can independently predict patient outcome. We are currently evaluating in a separate prospective study whether these m/z ratios are of prognostic value in renal cell cancer management.

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GENERAL DISCUSSION

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General Discussion

The goal of this discussion is to put the results of the three chapters into broader perspective by defining a number of ongoing controversies when interpreting our results with those of others. Per chapter important questions will be put forward and will be debated.

PART I

Approximately 10^{13} endothelial cells are lining the blood vessels, and are generally non-dividing terminal differentiated cells. In the absence of disease affecting the vasculature it would be rational to find none or a sporadic endothelial cell in the circulation (1). However, in patients with vascular disease, circulating endothelial cells were found to be present within the peripheral blood cells (2;3). Based on this reasoning, a elevation in endothelial cells in the circulation was also demonstrated in other conditions with vascular trauma occurred (4-6), acute ischemia (7), and blood vessel remodeling (8;9). Most likely these cells were shed from the damaged or angiogenic microvasculature, and shortly circulated in the peripheral blood until lysis, apoptosis, necrosis or removal by organs such as the liver or spleen. In mouse models and later in patients with cancer it was shown that circulating endothelial cells were associated with presence of tumor (9) and progressive disease (8). It has been suggested that the activated angiogenic endothelium was causative for many endothelial cells to be shed into the peripheral blood. Therefore it had been put forward that these CEC might be an excellent surrogate marker of angiogenesis and possibly the highly sought after marker for biological dose assessment for the many vascular targeting agents currently being tested.

However it also became evident that among the circulating endothelial cells, also CECs were present with stem-cell like qualities (10). Using fluorescence in situ hybridization (FISH), in patients having received bone marrow transplants from patients of the other sex, it was shown that ±95% of CECs were of recipient origin, and ±5% were bone-marrow derived (11). It has been postulated that these endothelial progenitor cells (EPC) contributed to post-natal vasculogenesis due to ischemia, and angiogenic repair of damaged or atherosclerotic vasculature (12). In a latter study it was shown in with six heterogeneous cancer patients whom received sex-mismatched bone marrow, and later developed cancer, that EPCs also contribute to tumor neo-angiogenesis, albeit in varying contribution from 1 to 12% (13). This was in contrast with previous literature using various xenograft models that demonstrate EPC contribution to up to 50% of the tumor endothelium. Furthermore, inhibition of EPC mobilization and incorporation results in smaller tumors and enhanced survival (14;15). Therefore, the contribution of endothelial progenitor cells to tumor angiogenesis has been subject of a controversial debate, for definitive evidence on EPC contribution to enhanced tumor growth and decreased survival in humans is lacking. Data is also lacking whether EPCs can be used for biological dose assessment in human studies.

CAN CECs AND EPCs BE USED TO DETERMINE OPTIMAL BIOLOGICAL DOSE?

At the preclinical level CECs and EPCs have been used to determine the optimal biological dose (OBD) of various anti-vascular agents. In a dose-escalation study with a blocking antibody against VEGFR-2, the OBD was established by monitoring the dose-dependent decrease in EPC level. This dose induced lowest levels of EPCs matched by the greatest decrease in tumor volume, and escalation of the dose did not alter EPC levels further (16). Most other studies also demonstrated

striking correlations between EPCs and the OBD of the anti-angiogenic or vascular disrupting agents tested (17-23). Dual effects have been seen with anti-angiogenic drugs, where during treatment increases were found in levels of CECs and paralleled inhibition of EPC mobilization (24).

To address the question whether quantification of CEC and EPC levels in clinical studies could be used to monitor biological activity and identify the optimal biological dose, we have performed experiments in Part I of this thesis. Using the magnetic bead assay we evaluated whether CECs could be used as surrogate marker for anti-angiogenic treatment, in a phase I study with PKC-inhibitor Enzastaurin in combination with cycles of gemcitabine and cisplatin based chemotherapy (Chapter 6). The baseline CEC levels correlated well with patient ECOG scores, and baseline levels were predictive of the length a patient was included in the trial until discontinuation (unpublished data). However, during the first 14 days of Enzastaurin monotherapy no significant changes in CEC levels were found. Possibly no biological active dose was assessed for Enzastaurin during the period of monotherapy, or Enzastaurin did not cause shedding of endothelial cells into the peripheral blood (cytostatic on endothelial cells). We can conclude that immunomagnetic bead-extracted CEC were not helpful in assessing OBD during the first 14 days of Enzastaurin monotherapy. In contrast, two hours after chemotherapeutic infusion (maximum after a median of 4 hours), a significant peak in CECs were found, which normalized to pre-infusion levels after approximately 1-2 days. Using real-time amplification of endothelial CD146 mRNA similar kinetics were found. This CEC elevation showed dose-dependency for the chemotherapeutic regiments (unpublished data). Therefore we can conclude that chemotherapy-induced cytotoxicity results in elevated CEC in the peripheral blood.

During Enzastaurin monotherapy also no significant changes in EPC levels could be shown. Amplification of stem cell marker CD133 demonstrated an ±100 fold increase in mRNA levels after the first gemcitabine/cisplatin cycle according to mean tolerable dosing regiments (MTD), which is in accordance with another recent clinical study (25). Chemotherapeutic infusion causes a direct decrease in CD133+ cells and further decrease until at least 48 hours after infusion. Therefore, MTD chemotherapy does seem to have an anti-angiogenic effect, at least in the beginning of each chemotherapy cycle, however, this effect is abrogated because the rebound of CD133+ EPCs after the break period. After VDA treatment in mice, vascular shutdown and subsequent damage led to a rapid mobilization and incorporation of EPCs into the viable tumor rim. The authors demonstrate that combinatorial treatment of VDAs with anti-angiogenic agents abrogates this vascular damage induced-EPC mobilization, and subsequently the regrowth of the viable tumor rim. These results suggest that the addition of an anti-angiogenic agent to MTD chemotherapy schedules would prevent the EPC mobilization caused by these schedules, and therefore would decrease the between-cycle regrowth of the tumor due to inhibition of EPC mobilization and incorporation. Exploratory analysis of our data clearly indicates a dose dependent (synergistic) effect of Enzastaurin after the first cycle of chemotherapy; post infusion we find a strong and significant correlation ($r=-0.66$ with $P=0.005$) between Enzastaurin dose and CD133 expression (unpublished data). How higher the anti-angiogenic dose of PKC inhibitor Enzastaurin, how lower the CD133+ cell rebound in the peripheral blood during the break period between MTD dosing. Further exploratory analysis demonstrated that CD133 levels after one and two weeks of treatment with Enzastaurin, and during the first and second cycle of gemcitabine/cisplatin was predictive of the amount of days a patients would be included in the trial until discontinuation; how higher CD133 expression the fewer days included in the trial (unpublished data). Due to the relative few patient numbers and

lack of objective tumor response, no definitive data could be acquired on the establishment of OBD for Enzastaurin using quantification of CEC or EPCs.

DO IMMUNOMAGNETIC ISOLATED CECs INCLUDE BONE-MARROW DERIVED CELLS?

In Chapter 4 we assessed CEC levels in the peripheral blood of patients with cancer, and demonstrated for the first time that these CD146+ CECs were only increased in patients with progressive disease (8). These CD146+ CECs had limited proliferative capacities ex vivo, which is in conclusion with a recent report that CD146+ cells isolated by magnetic beads stain uniformly negative for stem cell marker CD133 (26). A recent report using FACS analysis however demonstrated that a small subset of CD133+ bone marrow progenitors can express surface marker CD146 (27), however functionally most CECs quantified by our magnetic bead isolation most likely originated from the angiogenic mature microvasculature.

An interesting preclinical report demonstrated that shortly after treatment with VDAs, which cause rapid shutdown of the tumor vasculature and subsequent massive central tumor necrosis, EPC are mobilized into the peripheral blood (28). These EPCs home to the angiogenic viable tumor rim and contribute to enhanced tumor angiogenesis and regrowth of the tumor. In the Enzastaurin study we did not further characterize the CD146+ CECs by (immuno)histochemical or functional assays, e.g. whether these cells demonstrated virtually unlimited ex vivo expansion, or by staining with CD133 stem cell marker. However, real-time amplification of stem cell marker CD133 did not show similar kinetics as magnetic bead extracted-CEC or CD146+ mRNA after chemotherapeutic infusion. This would be expected when the majority of CD146+ mRNA and magnetic bead extracted-CEC elevation was caused by bone marrow-mobilized CD146+ EPCs. Therefore the bulk of magnetic bead extracted-CECs most likely represent mature endothelial cells, shed due to direct chemotherapeutic cytotoxicity.

DID WE CHOOSE THE RIGHT MARKER TO ASSESS CECs?

CD146 is considered a panendothelial-specific marker, and its presence has also been demonstrated on certain subsets of CD34+ endothelial progenitors. Using the above mentioned isolation method of CEC by CD146 antibodies conjugated with magnetic beads (3-8;29;30), we and other groups have clearly demonstrated the possibility to isolate and quantify endothelial cells from the peripheral blood. By immunofluorescence microscopy it is possible to distinguish endothelial cells by size (20-50 µm), and high CD146 expression (>10 binding CD146+ beads). The enumeration of CECs by immunomagnetic isolations necessitates considerable experience, expenditure and has to be performed on fresh material and takes approximately 3 hours to perform. Therefore we evaluated whether a real-time amplification assay for CD146 would provide similar results, for this assay can be performed on frozen material and is highly reproducible, easily amenable to standardization and less time consuming (31). There was an excellent correlation in the enumeration of spiked endothelial cells in the peripheral blood between real-time amplification and immunomagnetic isolation (r=0.96) and between real-time amplification and flow cytometry (r=0.99). However, the immunomagnetic isolation method was ±5 fold more sensitive assay for detection. A recent study demonstrated reasonable agreement between the flow cytometric quantification of CECs (CD45-CD34+ CD146+) and the immunomagnetic method for CEC quantification in whole blood, especially amongst patient groups (32). Another clinical study investigated CECs by real-time quantification using CD146, and by using flow cytometry (a panel of CEC markers) in patients with breast cancer.

Both methods showed approximately equivalent results (r=0.67). CD146 mRNA expression correlated with VEGF, and was elevated in cancer compared to healthy controls. In our phase I study with Enzastaurin combined with gemcitabine/cisplatin, we also found similar CECs kinetics between the immunomagnetic and real-time quantification assay after vessel trauma induced by the infusion of cytotoxic chemotherapy. However, in our real-time amplification study using CD146 we did not find elevated expression in cancer patients compared to controls. Furthermore, CD146 mRNA expression was not correlated with clinical characteristics, nor was a prognostic factor. Recent evidence using flow cytometry in rectal cancer patients enrolled in a phase I trial with bevacizumab, demonstrated that in the peripheral blood of these cancer patients CD45+ hematopoietic cells also expressed CD146. This CD146 expression was primarily detected on a subset of CD3+CD4+ lymphocytes (33), and the authors conclude that CD146 as sole marker for the enumeration of CECs inadequate. Using immunomagnetic bead isolation and subsequent quantification of CECs, the EC can physically be seen, and small cells containing one or few bound beads (possibly CD3+CD4+ lymphocytes) are not quantified as CECs. However as real-time PCR based amplification assays are performed on a mixed population of PBMCs, including activated T-cells, individual typing of cells is not possible. CD146 mRNA expression has to be further evaluated as biomarker for CECs, but evidence from this thesis suggests higher success rate as follow-up marker during therapy, than as static marker of angiogenesis.

ARE ENDOTHELIAL PROGENITOR CELLS A PROGNOSTIC BIOMARKER?

The significance of CD133+ cells as a prognostic marker was further evaluated in Chapter 5, in a large group of patients (n=131) with cancers of the kidney, prostate, head and neck and colorectum (31). We demonstrate that a baseline elevation of CD133 expression in these progressive cancer patients was already indicative for a poorer outcome. Multivariate Cox Proportional Hazard analyses demonstrated that CD133 mRNA expression was an independent prognostic factor in patients with metastases to the bone, however significance was not reached for the entire population (p=0.04 and p=0.15, respectively). Chapter 5 was the first report of a biomarker of vasculogenesis that in a clinical study was demonstrated to be an independent prognostic factor of patient survival. CD133 expression however, was not an independent predictor of survival in the entire population. This may be explained by preclinical studies which have shown that the contribution of EPCs to cancer vessels depends on the cancer type and grade (34). This contribution varies significantly depending on the organ site (35). In our study we show that indeed more CD133+ cells are mobilized in patients with metastases to the bone, thus EPC contribution in human cancers might also be dependent on organ site. However, within the patient population with bone metastases, there was a large heterogeneous variation in level of CD133 mRNA present in the peripheral blood. This might be due to the differentiation status of the metastasized tumor; large differences were found in the contribution of EPCs to tumor angiogenesis between well-differentiated and poorly-differentiated prostate adenocarcinomas (34). Lastly, other studies demonstrate that the contribution of EPCs to cancer vessels is dependent on the stage of the tumor. In a spontaneous hepatocellular carcinoma mouse model it was shown that EPCs have a supportive role in late-stage tumor angiogenesis (36), a concept which has recently indirectly been clinically supported in patients with unresectable (late-stage) and respectable (early-stage) hepatocellular carcinoma (37). However conflicting studies, e.g. in prostate cancer demonstrate exclusively a contribution of EPCs into the early, first steps of tumor vascularization (34).

Mobilization of endothelial progenitor cells from the bone marrow and incorporation into tumor vessels require complex interplay between tumor and its microenvironment, and between chemokines and chemokine-receptors. In our study we can conclude that the localization of the tumor is primarily an important predictor of EPC mobilization, however further assessment within this heterogeneous cancer group will identify whether grade, stage or an underlying pro-angiogenic molecular fingerprint are key determinants for EPC mobilization into the peripheral blood.

PART II

Nucleic acids are primarily present within the cell compartment, but are also dispersed into most body fluids of cancer patients (Chapter 7). Potential mechanisms of nucleic acid release into the circulation are lysis, necrosis, apoptosis and active release by cells (38). In the 70s, the first report demonstrated a strong elevation of circulating nucleic acids in the peripheral blood of cancer patients (39), and was later shown to be largely tumor derived by amplification of tumor specific alterations (40;41). The nucleic acids are bound tot the surface of blood cells (42;43), proteins (44), circulating nuclear complexes (45) and apoptotic bodies, which enhance stability and provide protection from nucleases. The presence of stable tumor DNA, viral DNA, mitochondrial DNA (mtDNA), and recently found (mt)RNA in plasma, serum, saliva, broncho-alveolar fluid and urine has ignited research to evaluate these nucleic acids as potential biomarkers. Recent findings suggest that circulating nucleic acids may be implemented as potential diagnostic or prognostic marker in the near future.

ARE MITOCHONDRIAL NUCLEIC ACIDS A MORE EFFECTIVE BIOMARKER THAN GENOMIC NUCLEIC ACIDS?

Two previous studies have evaluated the use of circulating genomic plasma DNA as diagnostic marker for prostate cancer. Using the β-globin gene, it was possible to distinguish prostate cancer from patients with benign prostate hyperplasia (BPH), using a defined cutoff, with sensitivity and specificity of 85% and 73%, respectively (46). A second study using the APP gene as marker for total levels of circulating DNA, also demonstrated increased levels of genomic DNA in prostate cancer patients compared to healthy individuals, however not when compared to patients with BPH (47). Currently, no study has evaluated the use of circulating plasma RNA as diagnostic or prognostic marker in prostate cancer.

To address the above question we isolated nucleic acids from the plasma of 75 prostate cancer patients and 14 subjects with benign disease of the urological tract (Chapter 8). We amplified genomic DNA (gene U1A), genomic RNA (U1A mRNA), mtDNA (16S ribosomal RNA) and mtRNA (cytochrome oxidase subunit 1 mRNA) and assessed whether mt or genomic nucleic acids were a better diagnostic marker or a stronger predictor of patient outcome. We show that mtDNA was detectable in all cancer patients and controls, however genomic DNA only in ±82%. Furthermore, levels of mtDNA were ±3000 fold higher than genomic DNA. Also mtRNA was detectable in all cases, however genomic RNA was undetectable using our procedures. When comparing the diagnostic qualities of circulating nucleic acids for prostate cancer diagnosis, mtDNA and mtRNA levels were significantly increased in the plasma of prostate cancer patients compared to patients with benign disease, however genomic DNA was not. We therefore conclude that with our markers chosen, mt nucleic acids have increased specificity identifying prostate cancer from benign subjects,

opposed to the use of genomic DNA. Also for the assessment of patient prognosis, we show that levels of mt nucleic acids strongly predict patient outcome prior to anti-cancer treatment, however genomic DNA does not.

A cell can contain tens to hundreds of mitochondria, and therefore can contain hundreds to thousands of mtDNA copies, as opposed to two copies of genomic DNA. Also mRNA levels of mt genes are likely to be elevated compared to mRNA levels of genomic house-keeping genes. We have demonstrated that mt nucleic acids are more easily quantified compared to genomic nucleic acids, and our results demonstrate enhanced sensitivity and specificity as biomarker opposed to genomic DNA. The discrepancies in the two previously published reports may be due to methodological variations in plasma collection and storage, isolation, amplification efficiencies, or due to selection of the of gene choice as marker for total DNA levels. However, both the β-globin and APP gene are not shown to be amplified in prostate cancer, therefore more likely variation in sample collection (between controls and patients), or other methodological differences and amplification efficiencies are responsible for the opposing results. In conclusion, amplification of mt nucleic acids seems to have elevated prognostic value apposed to amplification of genomic nucleic acids.

IS THE USE OF ONE- OR TWO-SPIN PLASMA BENEFICIAL FOR THE USE AS CANCER BIOMARKER?

In the literature it is shown that in normal controls mean circulating DNA levels in plasma and serum are under ±100 ng/mL in healthy controls, and elevated over 100 ng/mL in cancer patients. However these studies are hard to compare due to methodological variations which heavily affect the concentration of circulating DNA. For instance serum shows strongly elevated concentrations over plasma, and weaker associations are found in serum compared to plasma with respect to patient survival (48). As serum DNA levels was found to be related to patients' leukocyte counts, conclusions were made that the excess DNA found in serum is likely to be liberated in vitro during the clotting process (49;50). Furthermore, as it is demonstrated that a fraction of circulating DNA may be bound to the extracellular membrane of peripheral blood cells, proteins, nucleosomes, and other cellular fragments, it is clear that other methodological variations, such as relative centrifugational force used for spinning plasma, and the use of one- or two times centrifuged samples (one-spin and two-spin), also will affect plasma concentration of circulating DNA. Recently, a study compared the use of one-spin and two-spin plasma samples for the amplification of circulating DNA in cancer patients (47). Results demonstrate a three fold increase in mean DNA concentrations in one-spin compared to two-spin samples. These observations are possibly the result of a technical error, occurring when the plasma is removed too closely to the buffy layer, resulting in the contamination of cellular DNA. However, depending on the relative centrifugational force of the seconds spin (which is generally higher in most literature), a depletion of protein-, nucleosomal- or other fragment-bound DNA may also occur. Importantly, only significant differences between cancer patients and healthy subjects were found in the two-spin fraction.

For cancer diagnosis, it therefore seemed the case that two-spin plasma was superior to one-spin samples. In our initial study in prostate cancer (Chapter 8), we demonstrated in one-spin samples a slightly lesser sensitivity and specificity for detecting prostate cancer using mtDNA and mtRNA, compared to a prior published study using the β-globin gene for circulating total levels of DNA which used two-spin plasma. Therefore we embarked on a second study, where we evaluated the diagnostic and prognostic characteristics of circulating mt nucleic acids in prostate, head and neck,

kidney and colorectal cancer patients in two-spin plasma (Chapter 9). When we directly compare the results of both studies using the prostate cancer patient group (which were comparable patient populations), we found remarkable differences. First, in the two-spin study plasma mtDNA concentration was not significantly increased compared to controls, compared to the one-spin study. Second, in the two-spin study mRNA demonstrated a stronger and more significant elevation, compared to the one-spin study. Third, for prognostics two-spin samples proved inferior as opposed to one-spin samples. These results are difficult to comprehend, for if cellular contamination in one-spin samples would have been causative for an elevated concentration of circulating nucleic acids, no strong relationship would be expected with patient prognosis. Furthermore in our study, no relationship was found between patients' leukocyte and platelet count with mt nucleic acids in plasma. Therefore, it is more likely that in two-spin samples, with a high relative centrifugational force for the second spin, a source of predictive nucleic acids is depleted, such as large protein-nucleic acid complexes or particle-bound mt nucleic acids. However, these suggestions have to be proven correct in further investigational studies. However, we can conclude from work in this thesis that amplification of mt nucleic acids from one-spin plasma shows enhanced power as predictive biomarker over two-spin plasma. Amplification of mt nucleic acids for cancer diagnostics demonstrated poorer diagnostic power of mtDNA in samples from the two-spin study, however comparable power for mtRNA between both studies.

CAN MITOCHONDRIAL NUCLEIC ACIDS BE USED AS PAN-TUMOR BIOMARKER

Previous reports on quantitive levels of genomic nucleic acids in plasma show large variations in plasma concentrations between different types of cancers (47;51), limiting the use of a defined cutoff value for cancer diagnostics and possibly prognostics. We have shown that quantification of mt nucleic acids demonstrates enhanced sensitivity and specificity over genomic nucleic acids. However, it is unclear whether elevated mt nucleic acids serve as pan tumor diagnostic marker or as predictive marker. Therefore we quantified plasma levels of circulating mt nucleic from the (two-spin) plasma of four different types of cancers, and evaluated diagnostic and predictive power of mt nucleic acids (Chapter 9). We demonstrate that elevated mtRNA concentrations, using a defined cutoff above the 95th percentile of concentrations found in healthy controls, indicate a worse prognostic outcome. This is the first report demonstrating the use of circulating nucleic acids as a prognostic marker, independent of tumor type. Using one-spin plasma we also assessed survival estimation on defined cutoff values of circulating mt nucleic acids concentration for prostate cancer and breast cancer patients. However, we found that the defined cutoff value for prognostication in breast cancer could not be extrapolated to prostate cancer. A possible explanation for this finding is that the breast and prostate cancer samples were not isolated and quantified collectively (but as groups separately), which may induce variation in baseline levels. However, a more likely explanation is varying mtDNA (and thus mtRNA) content between different organs and tissue. In Table 1 (Chapter 9), also variations in mt nucleic acid concentrations are shown for the four groups of cancers in the two-spin samples, showing significant variations between cancer subtypes. It is thus likely that variations in mt nucleic acids concentrations exist, and are attributed to mt content of varying tissues and organs. However with strict standardization of methodological procedures and collective measurements, in two-spin plasma samples mt nucleic acids remain a pan-tumor prognostic marker.

PART III

Mass spectrometry (MS) has been used as a discovery tool for the identification of novel protein biomarkers present in biological fluids and cancer cells. Petricoin introduced a novel MS approach to assess a profile of peaks (without knowledge of their identify), as a discriminator between health and diseased states, by using powerful bioinformatic algorithms. This report gained considerable public as was demonstrated that a specific fingerprint protein profile was detectable within the serum proteome of women with ovarian cancer. This pattern could be used to discriminate neoplastic from non-neoplastic disease with a sensitivity of 100% and specificity of 95%, and therefore justified implementation as a screening tool for women with ovarian cancer in the general population (52). Enthousiasm was tempered by the publication of another article, that reanalyzed the dataset from Petricoin's group, and demonstrated that many discriminating peaks were more likely experimental artifacts caused by different methodological collection of patient and control samples (53). Following this initial report, many following studies also identified protein profiles, which could discriminate various forms of cancer from healthy individuals with high sensitivity and specificity, however most of these report could not be validated by independent research groups. These controversies and following international debate have lead to the standardization of experimental procedures, and the need for validation of discriminatory patterns found in one research institute to another independent institute.

CAN PREVIOUSLY IDENTIFIED BIOMARKERS FOR RENAL CANCER BE VALIDATED IN AN INDEPENDENT STUDY?

When we embarked on our MS studies to identify proteomic profiles specific for early detection of renal cell carcinoma (RCC), there where two recent reports which claimed to have identified discriminatory serum biomarkers. A study by Tolson et al. demonstrated the presence of ±11 kDa peaks identified as full length serum amyloid 1-α and two serum amyloid 1-α protein fragments (54). The second study by Won et al. identified five proteins of 3900, 4107, 4153, 5352 and 5987 Da, that could separate RCC from healthy and non RCC samples (55). In both reports different assay procedures were used, that may be accountable for the different discriminatory markers found between the studies. Before any of these potential biomarkers can be implemented, validation studies have to be performed using another patient population and subsequent analysis in an independent institute. In our study we applied the reported analytical methodologies of both studies on two distinct sample sets of patients from two different institutes, to address the validity and robustness of these potential RCC biomarkers.

We were able to validate serum amyloid 1-α as RCC biomarker in both of our patient populations, even though they differed in RCC stage and serum sample handling. This indicates that serum amyloid 1-α is robust biomarker candidate for RCC. Tolson identified the serum amyloid 1-α cluster in 8/25 RCC patients (sensitivity 32%) with 100% specificity. In our dataset we also show that the presence of serum amyloid 1-α is very specific (100% specificity for fragment of 11.5 kDa), however sensitivities remain low (sensitivity of 19% and 45%, respectively for sample set 1 and 2; Table 2 of Chapter 12).

For the validation of the biomarkers described by Won et al., we had significant more difficulties. Using the original procedure, many spectra were not assessable; however optimization of the analytical procedure improved peak detection. Only in sample set 1, which was a similar patient

population as in the report by Won et al., we could demonstrate two of the five biomarkers. Furthermore, differences in mean peak intensities between patients and controls were not as strong as reported. Therefore, we conclude that the biomarkers identified by Won et al. lack reproducibility and robustness as marker (reasons are further elaborated in the discussion of Chapter 12).

In five different reports in prostate cancer, only two of the many discriminatory masses appear in more than a single publication (56-60). Also of the many potential biomarkers identified from the serum proteome of patients with ovarian cancer, only a few of the identified peaks are consistent in more than one report (52;53;61-64). As we point out, reproducible protein profiles can hardly be presumed when using different assay procedures or sample handling. Standardization of these procedures is of dire importance as the range of commercially available chips display different binding characteristics, depending on their hydrophilic, hydrophobic, cationic, anionic, or immobilized-metal affinity capture chromatography surface. Furthermore, other analytical steps have proven to be crucial in (re)producing select protein biomarkers, such as albumin depletion, the use of a good protein reductant, varying denaturation buffers and quantity and quality of subsequent washing steps. Lastly, we demonstrate that varying patient populations between studies (tumor stage) also may account for differences in protein profiles found. Using the biomarker serum amyloid 1-α, we show varying sensitivities for the detection of patients with RCC between distinct patient populations.

CAN MASS SPECTROMETRY ALSO BE APPLIED FOR THE IDENTIFICATION OF PROGNOSTIC PROTEOMIC PATTERNS?

As previously stated, diagnostic biomarkers of cancer must be at high enough level to be detected by MS. In Table 1 of Chapter 11, we show that the concentration of potential MS biomarkers in serum is at much higher concentration than that of classical tumor markers. Many of these MS biomarkers represent acute-phase proteins, produced by the liver, diseased organs and inflammatory cells as reaction to cancer. The biomarker concentration precludes release from small tumor tissue. It is however clearly demonstrated that many host-derived acute-phase proteins are tumor specific (specifically protease-cleaved fragments of these proteins) and can be used as cancer diagnostic marker; however the question whether these biomarkers can also be applied as prognostic proteomic marker has been studied at a far lesser extent. Up to date, no report has identified prognostic or predictive proteomic biomarkers in serum for RCC. This has been reported in a handful of reports for other cancers (65-71), however mostly small patient numbers were used and these studies derived their samples from only one institute. Therefore sample processing could bias these studies and extrapolation to other institutes may not be possible.

We addressed the title question in two independent sample sets, consisting of distinct patient populations; patient prior to nephrectomy and in patients with metastasized disease. In Chapter 13 We demonstrate the validity to identify robust prognostic biomarkers that are able to predict survival probabilities independent of institute, tumor stage and sample processing methodology. We have identified two variants of apolipoprotein C, haptoglobin-α1 fragment, and a yet unidentified 2.9 kDa fragment, all having strong association with survival.

We used two analytical methodologies to investigate potential prognostic biomarkers. The first was the modified method used by Won et al. (55), the second was identical to the one described by

Tolson et al. (54). No overlap was found in prognostic biomarkers between both analytical methods, therefore assay procedures also strongly influence prognostic biomarker identification. The strongest and most robust biomarkers were identified using the method described by Won et al. In our search for diagnostic biomarkers for cancer (Chapter 12), we only identified serum amyloid albumin-α as overlapping biomarker using both methods. Serum amyloid albumin-α has also been shown to be a robust diagnostic biomarker in a variety of cancers (54;57;61;63;66;72;73), and a prognostic biomarker in neuroblastoma (66) and prostate cancer patients (60). However, in RCC serum amyloid albumin-α was not identified as prognostic marker. No overlap in putative prognostic biomarkers identified in other cancers (65-71) were found. Therefore apolipoprotein C variants, haptoglobin-α1 fragment, and the 2.9 kDa fragment may prove specific prognostic biomarkers for RCC. Of importance is a validation of these markers in yet another dataset of patients, using the patient population in Chapter 13 as training set, and the new population as test set. This study is currently being performed and will ascertain whether our putative prognostic biomarkers may be implemented as biomarker to identify patients with poor prognosis. Enrollment of these patients in adjuvant treatment modalities, or eligibility for targeted therapies would increase response rates and median survival for this highly resistant disease entity.

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Summary in English

Chapter 1 gives a general introduction on biomarkers and describes the need for novel non-invasive molecular and cellular biomarkers, due to recent developments in cancer biology and the introduction of targeted therapies.

Chapter 2 describes the outline of this thesis. In chapter 3 to 6 molecular and cellular markers for angiogenesis are evaluated for their prognostic value and as surrogate marker of antiangiogenic activity. In chapter 7 to 10 circulating mitochondrial nucleic acids are assessed as novel molecular diagnostic and prognostic marker. Using a proteomic approach in chapter 11 to 13 serum protein profiles are assessed as diagnostic and prognostic marker in patients with renal cancer.

PART I – ANGIOGENESIS

Chapter 3 is an introductory chapter regarding the importance of tumor angiogenesis for cancer growth and metastatic spread, and describes the need for the identification and validation of surrogate markers of angiogenesis. Many agents targeting the angiogenic pathway are currently being evaluated in early patient trials, however assessment of effectivity of these agents is currently hampered as no robust surrogate marker of biological drug activity and optimal dosing levels currently exist. Furthermore it is not possible to select for susceptibility for angiogenic treatment for the lack of predictive and prognostic angiogenic biomarkers. The focus in this chapter is on circulating endothelial cells shed from the angiogenic vessels, and on endothelial stem cells mobilized from the bone marrow compartment. These cells are evaluated as possible surrogate markers of angiogenesis and vasculogenesis, and as prognostic marker in the following chapters.

Chapter 4 assesses levels of circulating endothelial cells in the peripheral blood of patients with cancer. Preclinical studies have demonstrated that these cells play an important role in neovascularization and tumor growth; however in human cancer these cells have not been associated with progressive disease. Viable circulating endothelial cells were isolated from the blood of cancer patients by magnetic beads conjugated to pan-endothelial antibody CD146, and quantified ex-vivo. Their endothelial phenotype was confirmed by additional staining of specific anti-endothelial antibodies. A subset of these isolated endothelial cells incorporated into endothelial layers and displayed characteristic endothelial morphological colonies. Patients with progressive disease had significantly increased levels compared to healthy controls, and patients with stable disease. The number of these cells are influenced by chemotherapeutic treatment modalities, however did not correlate with cytokines thought to mobilize endothelial progenitor cells from the bone marrow. This manuscript is the first human study to implicate an association of elevated levels of circulating endothelial cells with cancer progression.

Chapter 5 examines whether endothelial cells or endothelial progenitor cells are a prognostic marker in human cancer. Instead of quantifying the number of circulating endothelial (progenitor) cells by magnetic bead extraction (as performed in Chapter 4) we assessed whether ribonucleic acid amplification of endothelial and progenitor markers are predictive factors for survival in human cancer patients. Therefore we developed a real-time quantification method to determine expression

of CD146 and CD133 mRNA in the peripheral blood mononuclear cells of progressive cancer patients. CD133 mRNA is expressed by progenitors from hematopoietic and endothelial lineages, and these cells are shown to contribute to cancer neo-angiogenesis in preclinical studies. Patients with metastatic disease, especially bone metastases, demonstrate significant increased CD133 mRNA expression. Importantly, patients with elevated levels above a defined cutoff, show decreased survival after an approximately two year follow-up period. Multivariate analysis identified CD133 expression as independent predictor for overall survival in patients with bone metastases. Our nucleic acid amplification assay is designed for high throughput analysis of multiple patient samples, reproducible and highly specific, therefore ideal for implementation in large patient studies. This study for the first time implicates elevated levels (expression) of endothelial and hematopoietic progenitors as poor prognostic marker, independent of tumor type studied.

Chapter 6 investigates in a phase I trial the feasibility of combining Enzastaurin with gemcitabine and cisplatin. Enzastaurin targets the protein kinase C pathway, which results in inhibition of angiogenesis in part by blocking the VEGF-receptor signaling cascade. Furthermore Enzastaurin inhibits the P13K/AKT pathways, resulting in inhibition of tumor cell proliferation and tumor cell apoptosis. Circulating endothelial cells and endothelial progenitor cells were assessed as biomarker optimal biological assessment of Enzastaurin and treatment efficacy. The recommended Enzaustaurin dose for further evaluation in phase II studies is 500 mg once daily based on tolerability and pharmacokinetic characteristics. Maximum tolerable dosing of gemcitabine and cisplatin did significantly alter the levels of the biomarkers studied. Circulating endothelial cells and CD146 mRNA levels showed similar kinetics and increased to maximum levels over baseline after a median of four hours after infusion in the first and second gemcitabine/cisplatin cycle. CD133 mRNA expression decreased directly after infusion of gemcitabine/cisplatin chemotherapy in both cycles. A ten-fold median increase of CD133 mRNA expression was observed between the first and second cycle. The biological markers circulating endothelial cells, CD146 and CD133 mRNA expression were not useful in assessing the optimal biological dose level. The repeated quantification of circulating endothelial cells and amplification of endothelial (progenitor) mRNA's as pharmacodynamic biomarker in clinical trials was shown to be feasible. Further study is necessary to investigate the full potential of these biomarkers as surrogate markers of treatment effect or as predictive biomarker in angiogenesis targeting therapies.

PART II: CIRCULATING NUCLEIC ACIDS

Chapter 7 gives an overview on the studies on circulating nucleic acids as prognostic or predictive biomarker in human cancer. Tumor-derived RNA and DNA has been isolated and quantified in virtually all body fluids from cancer patients in a quantitative and qualitative manner. Quantitative amplification of circulating nucleic acids detects certain house-keeping genes and is considered a measure of total circulating nucleic acid levels. Detection of microsatellite alterations, allelic imbalance, translocations, mutations in oncogenes, methylation of promoter regions or tumor suppressor genes, and the presence of viral genes are considered qualitative amplifications of circulating tumor-derived nucleic acids. This introduction describes the possible mechanisms of nucleic acid release into the peripheral blood and body fluids, and gives a detailed overview of studies incorporating qualitative and quantitative assessment of circulating nucleic acids for cancer prognostication.

Chapter 8 addresses the question whether quantitive assessment of circulating mitochondrial nucleic acids have increased sensitivity and specificity over genomic nucleic acids as prognostic marker in patients with cancer of the prostate. Each cancer cell generally contains only two copies of a genomic gene, while over a hundred of mitochondrial gene copies are present. To test our hypothesis, we isolated and amplified genomic and mitochondrial RNA and DNA from 75 progressive and untreated cancer patients, and tested for an association with clinically poor prognostic laboratory parameters and survival. We show that significant increased levels of mitochondrial RNA and DNA are present in patients with prostate cancer, compared to patients with benign disease of the prostate. Furthermore, we demonstrate that high levels are associated with significantly poorer survival after 20 months follow-up period. This however was not the case for genomic nucleic acids. Mitochondrial ribonucleic acids were shown to be an independent prognostic marker, when compared to existing prognostic markers used in the clinic. We provide novel evidence that mitochondrial nucleic acids are increased in prostate cancer patients, and are strongly associated with survival probabilities, independent of current prognostication factors.

Chapter 9 tests the hypothesis whether elevated mitochondrial nucleic acid levels can be used as general tumor marker in patients with a variety of cancer types. We collected plasma from 198 cancer patients, comprising cancers of the prostate, head and neck, kidney, colon and rectum. We demonstrate that mitochondrial ribonucleic acids are increased in the plasma of the combined group of cancer patients, compared to healthy control subjects. Elevated mitochondrial ribonucleic acids could significantly discriminate between patients with poor and good prognosis, independent of cancer type. This is the first report to demonstrate circulating nucleic acids as prognostic tumor marker, independent of the tumor type.

Chapter 10 is a methodological study on the stability and kinetics of mitochondrial nucleic acids in peripheral blood, cell culture, and during treatment with anti-tumor drugs. We provide evidence that mitochondrial nucleic acid level may be released from the peripheral blood cells, unless plasma is rapidly isolated. Mitochondrial nucleic acids are released from healthy and tumor cells in-vitro prior to impeding cell death, however rapidly decrease post-infusion of chemotherapeutic treatment regiments in-vivo. This may suggest that circulating mitochondrial nucleic acids from apoptotic or necrotic tumor cells in-vivo are rapidly cleared, or that active release of nucleic acids is inhibited by anti-tumor treatment. We demonstrate that high levels of mitochondrial nucleic acids are a poor prognostic marker in patients with breast cancer. Results from the experiments performed in this chapter indicate that blood processing protocols, storage time, nucleic acids isolation protocols may all in lesser or greater effect alter levels, variance and reproducibility of a quantitative measurement.

PART III: PROTEOMICS

Chapter 11 is a general introduction in mass spectrometry for the identification of cancer-specific proteomic patterns in patient blood. An overview on the recent literature on mass spectrometry as an early diagnostic cancer test is given, and the lessons we learned from these early studies are extensively discussed. Furthermore, mass spectrometry may possibly also be used as tool to predict responsiveness to treatment, disease progression or survival. The need for novel diagnostic and prognostic tumor markers for patients with renal cancer is discussed, and provides insight in the rationale behind chapter 12 and 13.

Chapter 12 is a validation study of potential serum biomarkers for renal cell cancer patients. Two reports using different analytical methodologies have identified putative biomarkers for the detection of renal cancer, however both these studies have not been validated by an independent institute in a new patient population. We were able to reproducibly detect the putative biomarker serum amyloid-· in both studies, indicating serum amyloid-· as robust biomarker. However the expression differences between patients and controls were not large enough for good classification, and thus as early detection tool. We experienced difficulties reproducing results from one study using the published analytical protocol, however slight adaptation of this protocol resulted in better peak detection and reproducibility. The analytical protocol from the other study seemed more robust. We identified two new peaks which might prove useful as biomarkers, but have to be validated in new populations.

Chapter 13 investigates whether mass spectrometry may identify protein signatures in the serum proteome of patients with renal cancer that predict prognosis. Discriminatory protein profiles between patients and controls were identified and used to generate a predictive model. This model could accurately separate patients with poor prognosis from those with good prognosis, and appeared an independent predictor of survival when tested with the golden standard of prognostication for patients with renal cancer. This study demonstrates the validity of mass spectrometry as novel prognostic tool in renal cell cancer. The predictive protein model remains to be validated in a prospective study in a new patient population, which is currently being performed.

Chapter 14 is a general discussion on the findings in part one to three, and puts the results into broader perspective by defining a number of ongoing controversies when interpreting our results with similar and opposing results by others.

Summary in Dutch

(Samenvatting in het Nederlands)

Wanneer bij een celdeling mutaties optreden in genen die betrokken zijn het bij het reguleren en controleren van de celdeling, kan dit uiteindelijk ontaarden in een kankergezwel. Vroege ontdekking van kanker is erg belangrijk. Hoe eerder kanker wordt ontdekt, hoe groter de kans is op een minder ingrijpende behandeling. Bovendien is bij vroegtijdige ontdekking van kanker de kans op langdurige overleving vaak groter.

Naarmate het kankerproces voortschrijdt, zullen er ook mutaties optreden waardoor nieuwe bloedvaten aangelegd kunnen worden naar de tumor in ontwikkeling (angiogenese). Ook ontstaan mutaties zodat ontaarde cellen het omliggende weefsel binnen kunnen dringen (invasie) en zich los kunnen maken uit hun omgeving en kunnen terechtkomen in andere plaatsen in het lichaam waar ze verder uitgroeien tot een tumor (metastasering).

In dit proefschrift wordt onderzoek gedaan naar nieuwe tumor markers, die mogelijk geïmplementeerd kunnen worden voor de detectie van kanker, of als voorspellende marker voor het verloop van de ziekte. Momenteel worden veel nieuwe anti-kanker medicamenten getest bij patiënten, echter ontbreken vaak tumor markers om het effect van de behandeling op niet-invasieve wijze te kunnen monitoren, en vroegere voorspelling te geven wanneer een behandeling faalt in vergelijking met reeds bestaande methoden.

In deel 1 van dit proefschrift wordt onderzoek gedaan naar mogelijke markers die gebruikt kunnen worden om het proces van angiogenese te kunnen volgen.

De vorming van nieuwe bloedvaten wordt geïnduceerd door de afscheiding van zogenaamde angiogenetische groeifactoren. De groeifactoren stimuleren endotheelcellen, de cellen aan de binnenkant van de bestaande bloedvaten tot deling en uiteindelijk tot vertakking van bloedvaten naar een kanker toe. Tevens zorgen deze angiogenetische factoren ervoor dat er uit het beenmerg specifieke stamcellen gemobiliseerd worden, die door het bloed getransporteerd worden naar plekken waar angiogenese plaatsvindt. Deze cellen worden endotheel voorlopercellen genoemd en dragen mogelijk ook bij tot bloedvatvorming. Angiogenese is noodzakelijk bij de groei van kankercellen omdat bouwstoffen, afvalstoffen en zuurstof anders te ver moeten diffunderen tussen de expanderende kanker en een bestaand bloedvat. Zo kan een beginnende tumor slechts een kubieke millimeter (ongeveer een aantal miljoen cellen) groot worden vanwege een tekort aan zuurstof, energie of bouwstoffen. Dit leidt ertoe dat een kankergezwel stopt met groeien vanwege sterfte van cellen die voor diffusie te ver verwijderd zijn van bestaande bloedvaten. Door de aanmaak van angiogenetische factoren en de vorming van nieuwe bloedvaten (door lokale groei en beenmerg afhankelijke groei) kan hij wel groeien. Specifieke medicamenten (zoals bevacizumab) worden ontwikkeld om de aanmaak of gebruik van angiogenetische factoren door tumorcellen te kunnen verhinderen. Op die manier zou de ontwikkeling van een tumor gestopt kunnen worden.

Veel van deze nieuwe medicamenten worden nu getest in klinische onderzoeken bij kankerpatiënten, helaas bestaan er geen goede markers om het biologische effect van deze medicamenten te onderzoeken. Veel van deze medicamenten hebben geen bijwerkingen, en daarom is de optimale biologische dosis bij de mens niet goed in te schatten. Veelal wordt gebruik gemaakt van radiologisch onderzoek gecombineerd met bestaande tumor markers en klinisch patiënt onderzoek. Helaas vertellen deze testen vaak pas laat of een behandeling effectief is, en heeft men vaak geen inzicht of de optimale biologische dosis van deze anti-angiogenetische medicamenten wordt gegeven.

Daarom is het belangrijk een niet-invasieve, surrogaat markers van bloedvatvorming te vinden, die bij voorkeur uit een simpele bloedtest kunnen worden bepaald en een correcte weergave geven van het proces van bloedvatvorming in een tumor. In dit proefschrift hebben we onderzocht of circulerende bloedvatcellen, of endotheel cellen, uit het bloed van kanker patiënten geïsoleerd kunnen worden. We hebben gevonden dat deze cellen inderdaad aanwezig zijn, en in aantal verhoogd zijn bij patiënten met progressieve ziekte (hoofdstuk 4). Tevens hebben we aangetoond dat endotheel voorlopercellen uit het beenmerg verhoogd zijn bij patiënten met uitgezaaide kanker, en dat verhoogde aantallen van deze cellen geassocieerd zijn met een slechte overleving (hoofdstuk 5). Deze vinding heeft het ons mogelijk gemaakt te bekijken of deze surrogaat markers ook gebruikt kunnen worden om een de optimale dosis van een nieuw anti-angiogenetische medicament te onderzoeken, of een mogelijke marker is voor vroege therapierespons (hoofdstuk 6). Helaas waren er geen veranderingen te zien in de hoeveelheid endotheel (voorloper) cellen gedurende de behandeling met dit medicament, en was het niet mogelijk een optimale biologische dosering te bepalen. Verder onderzoek moet duidelijk maken wat de waarde is van deze mogelijke markers van angiogenese.

In deel 2 van dit proefschrift wordt gekeken naar een ander soort tumor marker. Kankercellen kunnen ongebreideld delen, maar bij veel van deze celdelingen vindt er echter ook veel sterfte van de cellen plaats (door gecontroleerde celdood/apoptose en ongecontroleerde celdood/necrose). Het genetische materiaal van deze cellen blijkt voor een deel in de bloedbaan terecht te komen en kan daaruit bepaald geïsoleerd en gekwantificeerd worden. We laten zien dat het bepalen van de hoeveelheid mitochondriaal (celorganellen in de cel, die fungeren als energiecentrales) genetisch materiaal mogelijk is uit het bloed van gezonden en kanker patiënten. We tonen aan dat de hoeveelheid van dit genetische materiaal in het bloed verhoogd is bij kankerpatiënten, en dat verhoogde aantallen kopieën hiervan geassocieerd is met een zeer slechte overleving (hoofdstuk 8 en 9). We laten echter ook zien dat niet alleen celdood oorzakelijk is voor hogere hoeveelheden genetisch materiaal in het bloed; kankercellen kunnen ook mogelijk actief hun genetisch materiaal uitscheiden in de bloedbaan (hoofdstuk 10). De functie hiervan is nog allerminst duidelijk. Na behandeling met anti-kanker medicamenten wordt een daling gezien van de hoeveelheid genetisch materiaal in de bloedbaan. Verder onderzoeken moeten blijk geven of deze daling gecorreleerd is met een behandel respons en toegepast kan worden als nieuw tumor marker.

In deel 3 van dit proefschrift wordt gekeken of massaspectrometrie gebruikt kan worden om nieuwe markers voor kanker detectie of ziektevoorspelling te vinden. Massaspectrometrie is een techniek die onder andere gebruikt kan worden voor de identificatie, kwantificatie en profilering van eiwitten (fragmenten) in zeer kleine hoeveelheden van biologische vloeistoffen zoals bloed.

Het is mogelijk door middel van massaspectrometrie eiwitprofielen te vinden die diagnostisch zijn voor kanker. We hebben onderzocht of we twee bestaande studies konden reproduceren door de gevonden diagnostische eiwitten voor niercel kanker te kwantificeren middels massaspectrometrie in een nieuwe groep patiënten (hoofdstuk 12). We hebben aangetoond dat dit niet mogelijk is voor alle gevonden eiwitten, mogelijk vanwege verschillende analytische methodieken en verschillende patiënten populaties. Tevens hebben we bestudeerd of er eiwitprofielen te vinden waren die prognostische waarde hadden. In niercel kanker was er een profiel te vinden die het mogelijk maakte patiënten te rangschikken op slechte en goede overleving (hoofdstuk 13). Dit eiwitprofiel wordt in een nieuwe studie gevalideerd, zodat deze mogelijk toegepast kan worden als klinisch voorspellende marker.

PART I

ANCA	anti-neutrophil cytoplasmic antibodies
AUC	area under the curve
bFGF	basic fibroblast growth factor
bid	bis die; twice daily
CEC	circulating endothelial cell
DLT	dose-limiting toxicity
CV	coefficient of variation
CABG	coronary artery bypass graft surgery
CFDE-SE	carboxyfluorescein diacetate, succinimidyl ester
CRC	colorectal cancer
CTC	common toxicity criteria
dFdU	2'2'-difluoro-2'-deoxy-uridine
DiI-ac-LDL	1,1'dioctadecyl-3,3,3',3'-tetramethylindo-carboxyanine-labeled acetylated low-density lipoprotein
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
EPC	endothelial progenitor cell
FACS	fluorescence activated cell sorting
GC	gemcitabine/ cisplatin
G-CSF	granulocyte colony stimulating factor (filgrastim)
H&N	head and neck
HR	hazard ratio
HUVEC	human umbilical vein endothelial cell
HMEC1	human microvascular endothelial cell
IQ	interquartile
MMP	matrix metalloproteinase
MNC	mononuclear cell
MTD	maximum tolerated dose
NOD/ SCID	non-obese diabetic/ severe combined immunodeficiency disease
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PlGF	placental growth factor
RCC	renal cell cancer
SDF-1·	stromal cell-derived factor 1 alpha
SCF	stem cell factor or soluble kit-ligand
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vWf	von Willebrand factor
qd	quaque die; every day

PART II

APP	amyloid precursor protein
AUC	area under the curve
CEA	carcinoembryonic antigen
CI	confidence interval
COX1	cytochrome c oxidase subunit 1
CPT	cell preparation tubes
dsDNA	double strand DNA
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
$HMEC_1$	human microvascular endothelial cell
HR	hazard ratio
hTERT	human telomerase reverse transcriptase
Ig	immunoglobulin
LDH	lactate dehydrogenase
LOH	loss of heterozygosity
mt	mitochondrial
NA	nucleic acid
NASBA	nucleic acid sequence based amplification
NSCLC	non-small cell lung cancer
NSE	neuron-specific enolase
PB	peripheral blood
PCR	polymerase chain reaction
ROC	receiver operating characteristics
PSA	prostate-specific antigen
PSAdt	prostate-specific antigen doubling time
RCC	renal cell cancer
rcf	relative centrifugal force
rRNA	ribosomal RNA
TTP	time to positivity
UV	ultraviolet

PART III

ACN	acetonitrile
ACTH	adrenocorticotropic hormone
AJCC	American joint committee on cancer
apoC	apolipoprotein C
BMW	biomarker wizard
BPS	biomarker patterns software
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
CV	coefficient of variation

HC	healthy control
HR	hazard ratio
kDA	kilodalton
MALDI-TOF	matrix-assisted laser desorption/ionization
MS	mass spectrometry
m/z	mass-to-charge ratio
PBS	phosphate buffered saline
PMSF	phenylmethanesulfonyl fluoride
RCC	renal cell carcinoma
SELDI-TOF	surface enhanced laser desorption ionisation-time of flight
SAA	serum amyloid-α
S/N	signal-to-noise ratio
SPA	sinapinic acid
TFA	trifluoroacetic acid
TNM	classification of malignant tumours (by tumorsize, nodal status, and metastasis)

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Curriculum Vitae

Niven Mehra was born in Rotterdam, the Netherlands, on June 21st 1977. In 1995 he graduated from the Melanchthon College, in Rotterdam, and started his medical studies at the University of Utrecht. In 1999 he enrolled in a scientific research project on the role of Macrophage Inhibitory Factor in prostate cancer metastasis, at the VAMC institute in Florida, USA, under supervision of Dr. Meyer-Siegler. During his internships in 2001 and 2002, he started working on a research elective on angiogenesis inhibitor ABT526, a peptide homologue of Thrombospondin-1, in the laboratory headed by Prof.. Dr. E.E. Voest. After finishing his medical training in 2002, he continued his scientific research on this dissertation under supervision of Prof. Dr. E.E. Voest. "In 2006, he was admitted to carry out his residency program of Internal Medicine (head Internal Medicine Prof. Dr. E. van der Wall) in Hospital Gelderse Vallei, in Ede (under supervision of Dr. R. Heijligenberg).

Niven Mehra werd geboren te Rotterdam, op 21 juni 1977. In 1995 voltooide hij de middelbare school aan het Melanchthon College, te Rotterdam, en begon aan zijn studie geneeskunde aan de Universiteit van Utrecht. In 1999 verrichte hij in de Verenigde Staten wetenschappelijk onderzoek naar de rol van Macrophage Inhibitory Factor in prostaat kanker metastasering, aan het VAMC instituut te Florida, onder supervisie van Dr. Meyer-Siegler. Vervolgens verrichtte hij wetenschappelijk onderzoek in het kader van een verlengde keuze co-schap aan angiogenese remmer ABT526, een peptide analoog van thrombospondine-1, in 2001 en 2002 in het laboratorium van Prof. Dr. E.E. Voest. In 2002 verkreeg hij zijn artsen bul, en startte aan het huidige promotie onderzoek. In 2006 wordt hij aangenomen tot de opleiding interne geneeskunde (opleider Prof. Dr. E. van der Wall), en start hij als arts assistent in ziekenhuis de Gelderse Vallei, te Ede (opleider Dr. R. Heijligenberg).

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