CARDIOVASCULAR AND METABOLIC EFFECTS OF ANGIOGENESIS INHIBITORS

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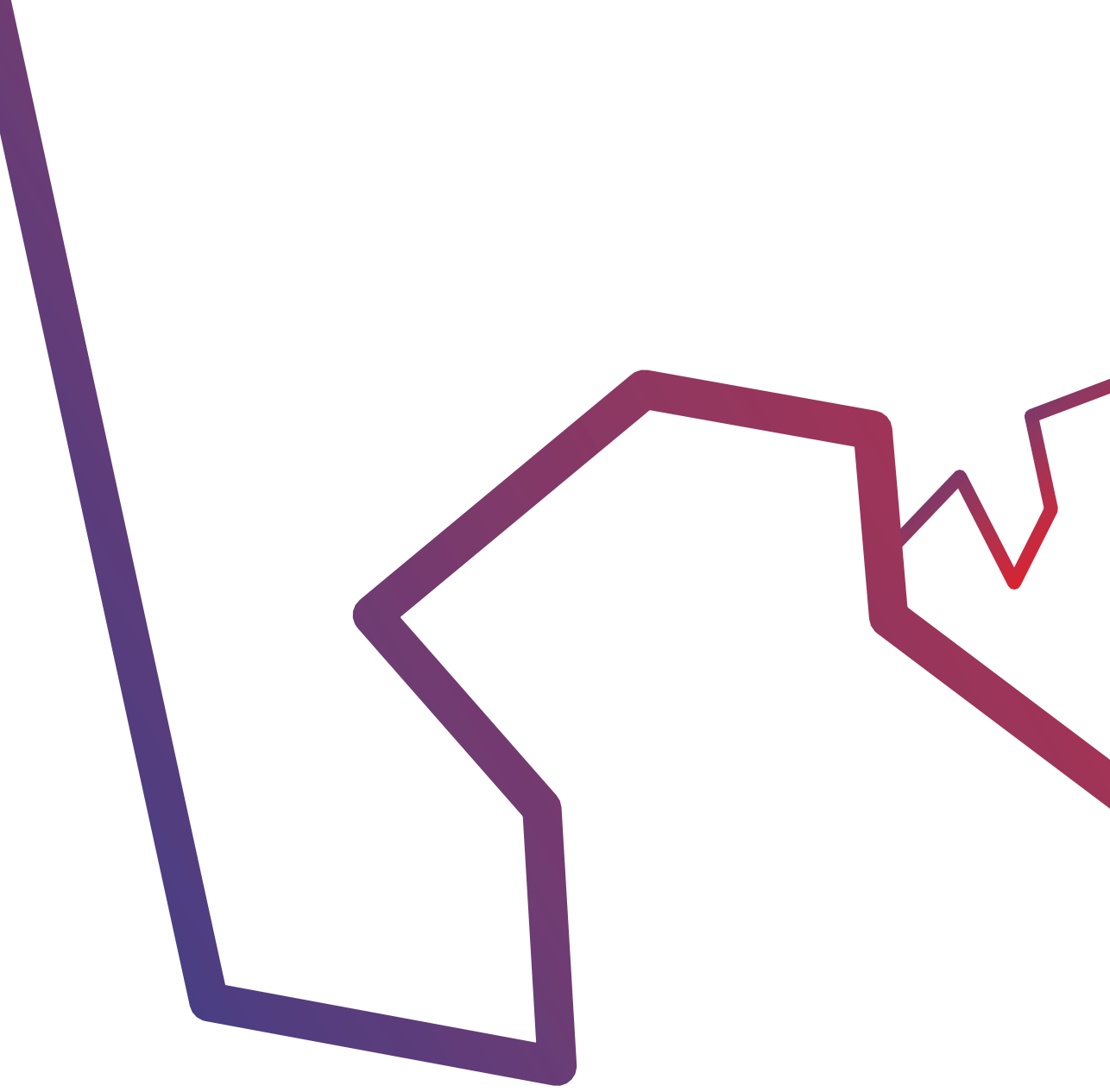


CHAPTER 1

Introduction

Chapter 1.1 Introduction and outline of thesis

Cancer is a leading cause of death in the Netherlands. Every year over 100.000 Dutch people are diagnosed with cancer. Treatment options include surgery, radiotherapy and systemic therapies such as chemotherapy, immune therapy, endocrine therapy and targeted therapy.

In this thesis the pathogenesis of adverse effects of angiogenesis inhibitors, a group of targeted agents, is studied.

Angiogenesis is the formation of new capillaries from endothelial cells of existing vasculature. This is an essential physiological process in wound healing, embryogenesis and the female reproduction system. It is, however, also essential for tumor growth. When a tumor exceeds a few millimeters in size as a consequence of hypoxia and nutrient deprivation an imbalance develops in angiogenesis stimulator- and inhibitor levels leading to an 'angiogenic switch'[1]. This process of angiogenesis is regulated by several growth factors and receptors. Vascular endothelial growth factor (VEGF) and its receptors play a key role. VEGF, a 45 kDA glycoprotein, is mainly secreted from mesenchymal, stromal and epithelial sources to act on endothelial cells. The angiogenic effects of VEGF are primarily mediated by VEGFreceptor-2 (VEGFR-2)[2]. In the seventies of the past century Folkman already proposed angiogenesis inhibition as a strategy for the treatment of malignancies. Several decades of research followed and in 2004 the first angiogenesis inhibitor, bevacizumab, was approved for the treatment of metastastic colon cancer in combination with chemotherapy[3].

Bevacizumab is an, intravenously administered, humanized monoclonal antibody directed against VEGF. It has been approved by the European Medicine Agency (EMA) for the treatment of metastatic colon cancer, metastatic breast cancer, non-small cell lung cancer, advanced or metastatic renal cancer, metastatic ovarian cancer,metastatic cervical cancer and glioblastoma. Meanwhile several other drugs including small molecule receptor tyrosine kinase inhibitors such as sunitinib have been developed. Sunitinib is approved for the treatment of metastatic renal cell carcinoma, gastrointestinal stromal tumor (GIST) and neuro-endocrine tumors of the pancreas. Sunitinib not only targets the VEGF-receptor, but also the platelet derived growth factor (PDGFR), stem cell factor receptor (c-KIT) , FMS-like tyrosine kinase-3 (FLT-3), glial cell-line derived neurotrophic factor receptor (RET) and colony stimulating factor 1 receptor (CSF1R) [27-30].

At the introduction of angiogenesis inhibitors no major side effects were expected. Under normal circumstances more than 99% of endothelial cells do not replicate and in healthy adults angiogenesis only seems to play a role in wound healing and the menstrual cycle[4,5]. However

unfortunately various side effects did occur in patients. One of the most reported side effects of VEGF-targeted therapies is hypertension. **Hypertension** occurs in 19% to 80% of patients, depending on the used definition of hypertension[6-8]. Even more patients experience a rise in blood pressure[9]. Several hypotheses for the pathogenesis of hypertension exist. First, it could be an effect on the endothelium as VEGF has been shown to induce synthesis or release of the vasodilator nitric oxide[10 11]. Inhibition of VEGF-signalling could result in a decrease in nitric oxide availability, which could lead to endothelial dysfunction, vasoconstriction and subsequent hypertension. Second, a possible effect on neurohumoral factors could be involved in the pathogenesis. Sunitinib has been shown to cause a rise in endothelin-1 after four weeks of treatment[12]. However no correlation between rise in blood pressure and endothelin-1 or other neurohumoral factors, such as aldosterone, renin or norepinephrine was reported. All clinical experiments into the pathogenesis of hypertension have been performed after several weeks of treatment, while the onset of hypertension in patients treated with VEGF-inhibition is usually within the first week. Thus, observed neurohumoral and endothelial changes could be a consequence rather than a cause of the rise in blood pressure.

Moreover in the last years concerns have been raised about the development of **heart failure**, reduction in left ventricular ejection fraction (LVEF) and the occurrence of cardiac ischemic events during treatment with sunitinib and other VEGF-targeted therapies[13-15]. Hypertension, a history of chronic heart failure and coronary artery disease are defined as predictors for the development of heart failure in patients treated with sunitinib[13,15,16]. Obesity, diabetes and insulin resistance are common in patients with renal cell carcinoma and are risk factors for the development of cardiac failure and hypertension. In several animal studies VEGF is reported to be the most important angiogenic factor in adipogenesis[17]. Moreover in patients inhibition of VEGF is associated with changes in body composition independent of gastrointestinal side effects and a drop in glucose concentrations [18,19]. These observations could be beneficial to patients, regarding the risk of cardiovascular events, however **excessive weight loss** and unexpected **hypoglycaemic events** are unwanted adverse events in the frail oncologic patient population. Looking beyond the boundaries of cancer treatment this effect on body composition and glucose levels are intriguing as they could be of interesting potential for the treatment of obesity or diabetes.

Aim and outline of this thesis

Drug-induced hypertension provides a unique opportunity to study the early phase of development of hypertension and cardiovascular toxicity. In this thesis, we describe a series of experiments focused on the early pathogenesis of cardiovascular and metabolic side effects of the VEGF-targeted therapies, bevacizumab and sunitinib, with the aim of providing insight into possible treatment or preventive strategies for patients experiencing these adverse effects and moreover to provide insight into the role of VEGF and other targeted growth factors in normal physiologic processes such as regulation of vascular tone.

In **chapter 2**, based on the observation that patients treated with angiogenesis inhibitors with previous ischemic heart disease are at risk of developing heart failure, we hypothesized that sunitinib may enhance cardiac vulnerability to ischemia which could contribute to the pathogenesis of sunitinib-associated heart failure. Therefore we exposed isolated human cardiac trabeculae to sunitinib and simulated ischemia and reperfusion in an in-vitro experiment. A better understanding of the pathogenesis of VEGFR inhibitors-related cardiotoxicity would allow us to identify those individuals who are at increased risk for developing heart failure.

In chapter 3, 4 and 5 we studied the pathogenesis of hypertension during treatment with angiogenesis inhibitors in animals, healthy volunteers and patients. In **chapter 3** the direct effect of scavenging VEGF from the arterial circulation in healthy volunteers on endothelial function was studied by arterial infusion of bevacizumab in order to investigate the role of VEGF in normal vascular physiology. In **chapter 4** a preclinical animal experiment was combined with a clinical study in metastastic renal cancer patients to study the effect on endothelial function after one week of sunitinib treatment. In **chapter 5** the early changes in aldosterone, renin and endothelin-1 levels were measured in a metastatic renal cancer patient group within one week after start of sunitinib treatment.

In chapter 6 and 7 we focus on the effect on body composition and glucose handling. Therefore in **chapter 6** the literature on VEGF inhibition and the effect on body composition was reviewed and in **chapter 7** the effect on insulin sensitivity and insulin clearance was studied by performing a hyperinsulinemic euglycemic clamp before and one week after start of sunitinib treatment in patients with metastatic renal cell carcinoma.

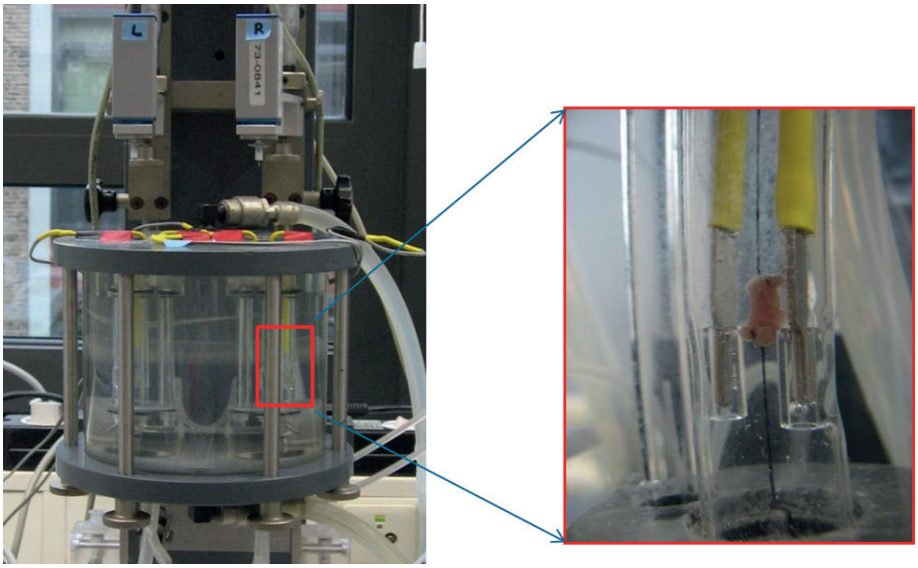
In the **chapter 8** the main findings and conclusions of the previous chapters are summarized and discussed and future perspectives are described.

Chapter 1.2 Applied methods

1. Atrial tissue *(chapter 2)*

During cardiac surgery with extracorporal bypass surgery the right auricle is incised to allow insertion of the cannules for the extracorporeal circulation. Some surgeons excise the right auricle at this timepoint. Therefore it is an unique opportunity to harvest human atrial tissue without exposing patients to unwanted risks. Patients were asked for informed consent prior to surgery. The method was based on the method described by Speechly-Dick[20]. After harvesting the atrial tissue it is immediately placed in a cold solution and transported to the laboratory. In the laboratory under magnification two atrial trabeculae are dissected and vertically suspended and linked to a force transducer in an organ bath *(figure 1)*. Once suspended, trabeculae are paced by field stimulation at 1 Hz by platinum electrodes which start contraction of the trabeculae. The force transducer registers the amount of force, rate and speed of contractions. By increasing field stimulation to 3 Hz and by changing the perfusate which passes through an artificial lung from an oxygen- and nutrition rich to oxygen- and nutrition deprived solution periods of ischemia and reperfusion can be simulated. Both trabeculae are suspended in a different organ bath, allowing the separate infusion of placebo or investigational product, but with the same conditions regarding the source of the tissue, perfusate and pacing allowing controlled and paired experiments.

FIGURE 1
Atrial trabeculae suspended in organ bath

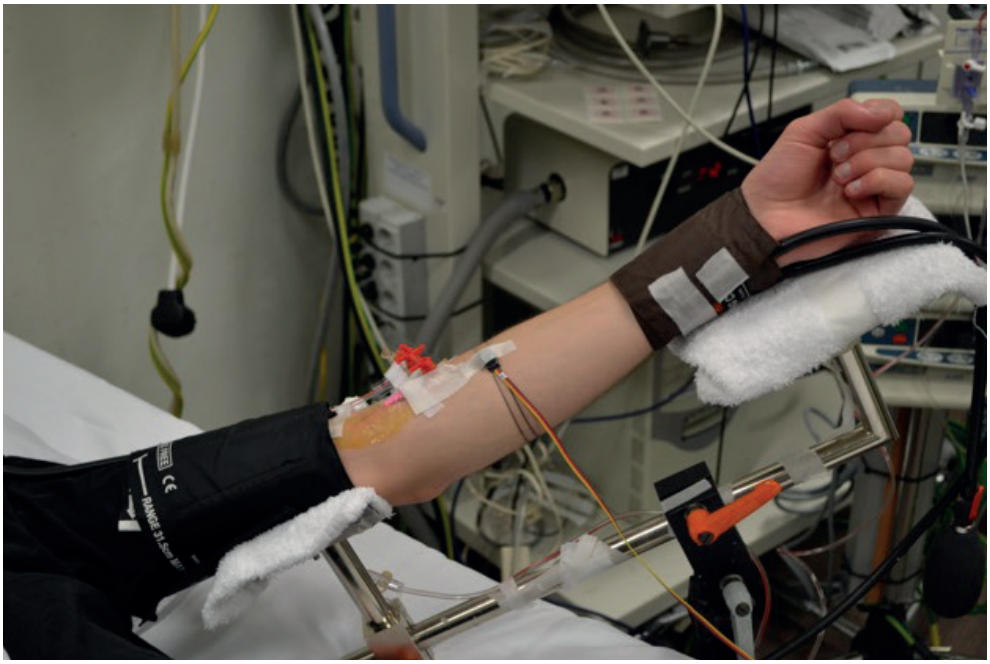


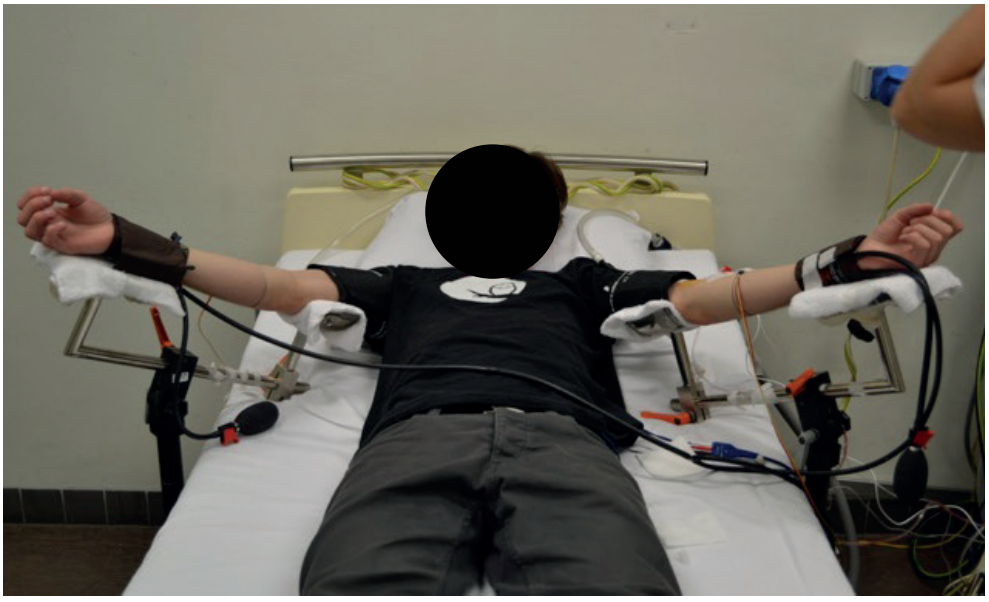
2. Venous occlusion plethysmography *(chapter 3 and 4)*

Venous occlusion plethysmography is a well validated technique to measure forearm blood flow (FBF) and the local vasomotor responses to the administration of drugs into the brachial artery. By recording the vasodilator response to an endothelium-dependent vasodilator, such as acetylcholine, and an endothelium-independent vasodilator, such as the nitric-oxide donor nitroprusside, we are able to assess endothelium-mediated vasodilation. Administration of drugs into the brachial artery results in high local concentrations in the forearm vascular bed, however after venous return into the systemic circulation, concentrations decrease because of the large volume of distribution and metabolism. Therefore there is only low systemic exposure to infused drugs preventing confounding systemic effects and reducing the chance of toxic side effects. The subject is placed on a bed and a wrist cuff, an upperarm cuff and a mercury-filled silastic strain gauge are applied to both arms (Figure 2). Inflation of the upper arm cuff to 40 mmHg during 6-10 heartbeats allows for unhindered arterial inflow but hampers venous return. The pooling of venous blood results in swelling of the forearm. The increase in diameter can be measured by the mercury-in-silastic strain gauges and the rate of this increase represents FBF. Forearm blood flow, under stable blood pressure, reflects the vascular tone in resistance vessels. The skin circulation of the hand is excluded, by inflation of the wrist cuff to 200mmHg, to restrict blood flow measurement to the skeletal muscle vascular bed as much as possible. Forearm blood flow is measured simultaneously in the experimental and non-experimental arm.

Endothelium function can be assessed by measuring changes in FBF in response to the infusion of different dosages of vasoactive substances. In the studies in healthy volunteers **(chapter 3)** we used 20 gauge arterial catheters to allow the measurement of arterial blood pressure for continuous check of the intra-arterial catheter position and for the detection of any relevant systemic effects. Furthermore, simultaneous measurement of arterial blood pressure allows for estimation of changes in forearm perfusion pressure and subsequent calculation of changes in vascular resistance. In the metastatic renal cancer patients **(chapter 4)** we used a smaller 27-gauge needle, without the possibility to measure arterial blood pressure, to prevent complications such as bleeding or thrombosis. Instead of forearm vascular resistance, the ratio of simultaneously measured FBF in experimental and non-experimental arm (FBF-ratio)was calculated in this study.

FIGURE 2
Venous occlusion plethysmography

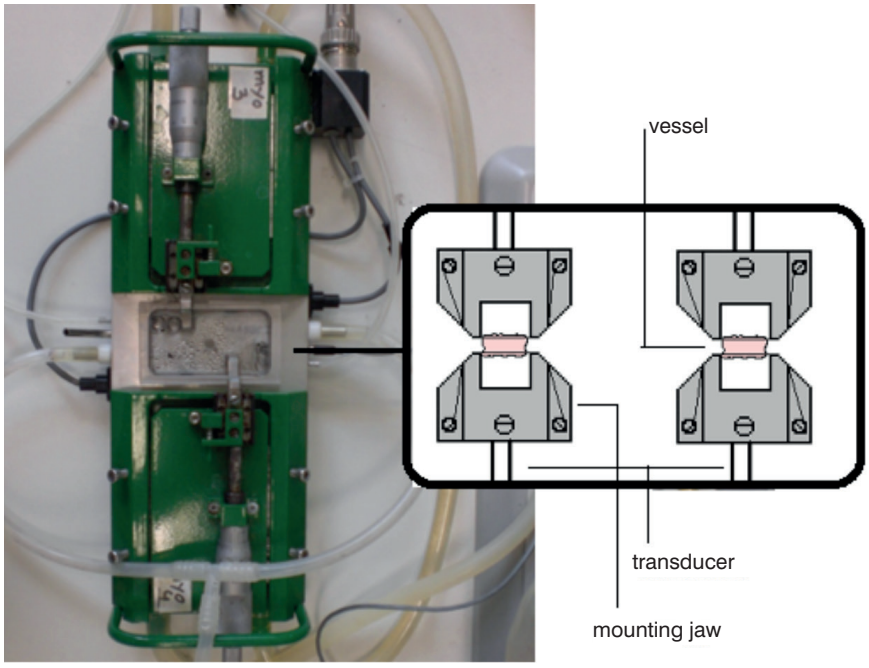




3. Wire myograph *(chapter 4)*

The myograph was first described by Mulvany and Halpern (1976). It provides a method to research the vasomotor properties of small proximal resistance arteries (100-400μm). Resistance vessels play a major role in the development of hypertension, as they regulate the peripheral vascular resistance. In this experiment rats are treated with placebo or sunitinib for one week and are subsequently euthanized. Post mortem, the intestine and mesenteric vessels are obtained from the rat and placed in a physiological salt solution. Small resistance arteries are cleared of other tissue (mainly fat) and dissected. The segment is placed on a 40 mm wire and attached to a mounting jaw of the Mulvany myograph in an organ bath. Then a second wire is inserted and attached to a second mounting jaw (Figure 3). From each rat 4 arteries are obtained and placed in pairs in 2 myographs. After normalization and equilibration different vasoactive substances can be added to the organ bath to measure the effect on the mounted vessels. The wires inside the vessels are connected to a transducer which can record the changes in contractile force of vascular smooth muscle cells.

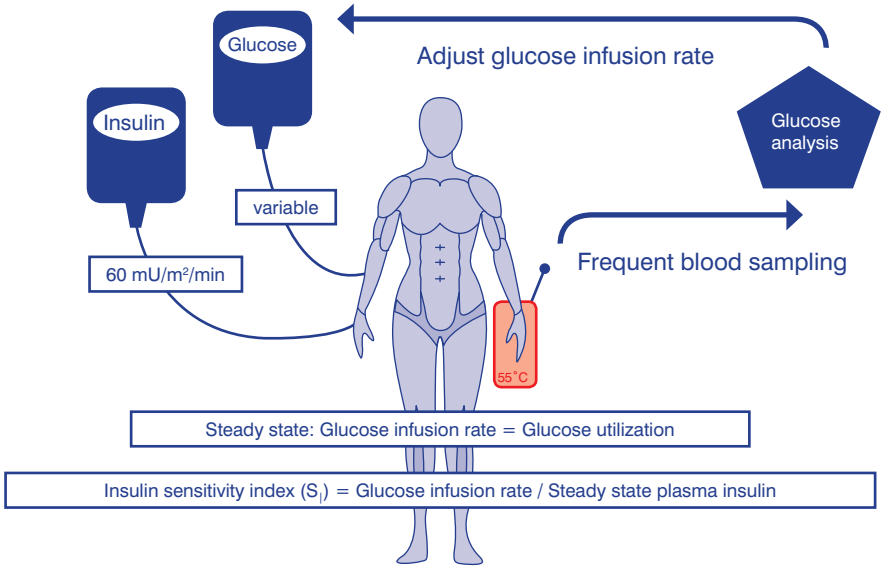
FIGURE 3
Mulvany myograph



4. Hyperinsulinemic euglycemic clamp *(chapter 7)*

The hyperinsulinemic euglycemic clamp, developed by deFronzo et al in 1979, is the gold standard for assessing insulin resistance in humans. After an overnight fast patients are placed on a bed. Two intravenous catheters are introduced. One is positioned in an anticubital vein for the infusion of insulin and glucose and the other is inserted in a dorsal vein of the hand of the opposite arm for blood glucose sampling. This hand is placed in a heated box, which opens arteriovenous shunts, reducing differences in glucose concentration between venous and arterial blood. Insulin is infused at a steady state throughout the entire experiment. After the start of the experiment blood glucose is sampled every 5 minutes. Depending on the blood glucose concentration the glucose infusion is adapted to reach a steady state concentration of blood glucose after 120 minutes. This method assumes that high doses of insulin will completely suppress hepatic glucose and pancreatic insulin production and that there is no change in blood glucose concentrations under steady-state conditions. Thereby the rate of glucose infusion is equal to the whole-body glucose utilization. This allows calculation of the insulin sensitivity index by dividing the glucose infusion rate by the steady state plasma insulin concentration.

FIGURE 4
Schematic view of the hyperinsulinemic euglycemic clamp



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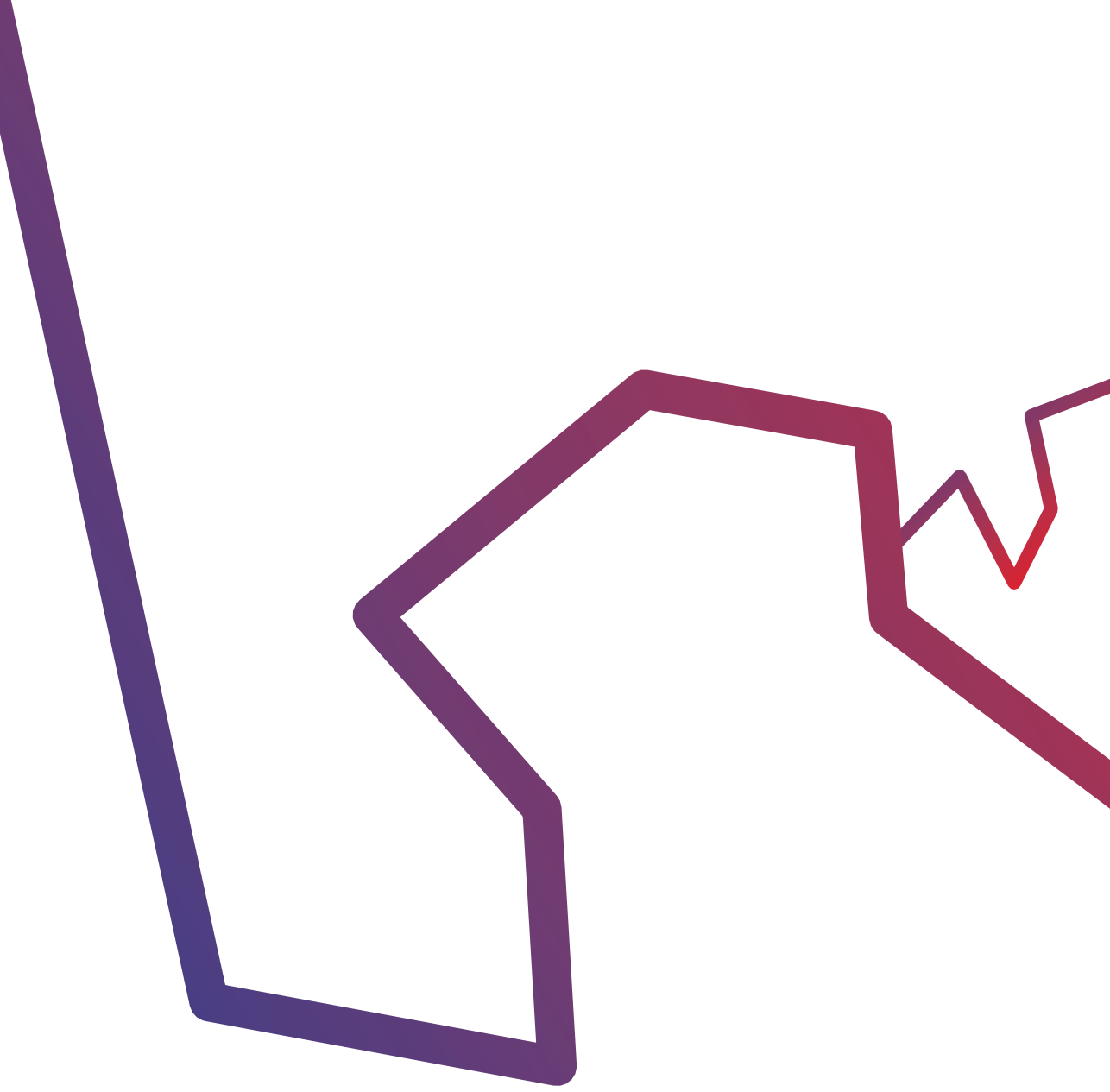

CHAPTER 2

Sunitinib does not attenuate contractile force following a period of ischemia in isolated human cardiac muscle

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Abstract

Aim

Concerns have been raised about the development of heart failure in patients treated for cancer with angiogenesis inhibitors, such as the tyrosine kinase inhibitor sunitinib. Patients with previous coronary artery disease and hypertension have an increased risk of developing heart failure. Therefore we studied the effect of sunitinib on the contractility of isolated human atrial trabeculae and the effect on recovery after ischemic stimulation.

Methods

After informed consent the atrial appendage of patients undergoing cardiac surgery was harvested and isolated trabeculae were placed in an organ bath with a force transducer. During electrical stimulation contractile force was measured during normal pacing or after simulated ischemia. Of each patient one trabecula was perfused with solvent and one with sunitinib.

Results

Contractile force (expressed as percentage of baseline force) declined over time to 57±8% and 73±20% after 150 minutes of stimulation for solvent and sunitinib treated trabeculae respectively (mean ± SE; n=8; $p>0.1$,). After simulated ischemia and reperfusion, contractile force was 40±6% in the control compared to 39±6% in the sunitinib treated trabeculae during the last final 5 minutes of reperfusion (n=12; $p>0.1$).

Conclusion

Sunitinib at low, but clinically relevant, concentrations does not have a direct effect on function of human atrial cardiomyocytes nor does it attenuate the recovery in contractile force of atrial cardiomyocytes after a period of ischemia. A direct and acute toxic effect on cardiomyocytes does not explain the development of heart failure in patients treated with sunitinib.

Introduction

Tyrosine kinase inhibitors (TKIs) are widely used in the treatment of cancer. Sunitinib, one of the most used angiogenesis inhibitors, is a multitargeted receptor TKI which is registered for the treatment of patients with metastatic renal-cell carcinoma (mRCC), gastrointestinal stromal tumors (GIST) and pancreatic neuroendocrine tumors.

Concerns have been raised about the development of heart failure, reduction in left ventricular ejection fraction (LVEF) and the occurrence of cardiovascular events during treatment with sunitinib and other vascular endothelial growth factor (VEGF)-targeted therapies[1-3]. In a recent meta-analysis of 5638 mRCC patients in 14 phase II-III trials with sunitinib the incidence of a heart failure was 3.6% [4]. In most phase I-III trials however patients with cardiovascular risk factors such as coronary artery disease (CAD) and patients with an older age are excluded. Therefore, these trials likely underestimate the real-life incidence of cardiotoxicity since these exclusion criteria are generally not taken into account in the daily clinical practice. Furthermore, it is questionable whether heart failure is always recognized in cancer patients with dyspnea and fatigue who present with several oncological pulmonary and general conditions.

In the few studies, observational or retrospective, which do not exclude patients with cardiovascular risk factors, an increased incidence of cardiac events, up to 33.8% in mRCC patients, is reported[5,6]. Hypertension, a history of CHF and CAD are defined as predictors for the development of heart failure in patients treated with sunitinib[1,3,7]. Chu et al describe that in a phase I/II study 75% of GIST patients with a history of CAD suffered a cardiovascular event versus 7% of those without CAD[1].

A better understanding of the pathogenesis of sunitinib-related cardiotoxicity would allow us to identify those individuals who are at increased risk for developing heart failure. Ideally this would be followed by the design of therapeutic or preventive interventions for this condition.

The exact mechanism of sunitinib induced cardiotoxicity is not known. It has been suggested that sunitinib-related cardiotoxicity may involve pathways different from VEGF[8,9] . *In vitro* studies show direct toxicity of sunitinib in cardiomyocytes and on contractile force in human atrial trabeculae, however this effect was observed at very high concentrations, up to 100- fold higher than therapeutic levels reached in plasma of patients [1,9,10].

In patients who developed heart failure during treatment with sunitinib, structural and functional abnormalities are seen in the mitochondria of cardiomyocytes[1,9]. Moreover, *in vitro,* sunitinib inhibits AMPK, an important kinase in maintaining metabolic homeostasis in the heart [9]. Based on

these studies and the observation that patients with previous CAD are at risk of developing heart failure we hypothesized that sunitinib may enhance cardiac vulnerability to ischemia which could contribute to the pathogenesis of sunitinib-associated heart failure. To explore this hypothesis we initiated a study on the ex-vivo effect of sunitinib on contractility of human cardiomyocytes at a clinically relevant sunitinib concentration, and moreover deprived atrial trabeculae from energy substrates (simulated ischemia) to investigate whether sunitinib-treated trabeculae attenuate contractile recovery after ischemia.

Methods

Patients

After approval of the protocol by the Institutional Review Board of the Radboud university medical center and prospective registration at *www.clinicaltrials.gov* (NCT01246778) 95 patients awaiting coronary artery bypass graft (CABG) and/or aorta valve replacement (AVR) agreed to participate. Written informed consent was obtained from all participants.

Patients with atrial arrhythmias, right ventricular failure, atrial enlargement or patients treated with theophylline, oral antiarrhythmics or sulphonylureas were excluded because these drugs may interfere with ischemia reperfusion outcome.

General experimental set up

The experimental set-up modified from Speechly-Dick et al as described by Riksen et al was used [11,12]. The right atrial appendage was harvested during cardiac surgery at the right atrial cannula insertion site before the introduction of the extracorporal circulation and immediately placed in cold modified Tyrode's solution (Nacl 118.5 mmol/l; KCl 4,8 mmol/l; $NaHCO_3$ 24.8 mmol/l; KH_2PO_4 1,2 mmol/l; $MgSO_4$ 1,44 mmol/l; $CaCl_2$ 1,8 mmol/l; glucose 10,0 mmol/l and pyruvate 10,0 mmol/l) on ice, which was continuously gassed with 95% oxygen and 5% CO_2. Two atrial trabeculae (diameter <1 mm; length >3 mm) were dissected, vertically suspended in an organ bath, and linked to a force transducer. Each trabecula was superfused with pre-oxygenated Tyrode's buffer (pO_2 500 to 600 mm Hg). Electrical field stimulation was performed in unstretched condition at 1 Hz using platinum electrodes placed on both sides of the trabeculae (pulse duration 60 ms; pulse current 40 mA). After 30 min of stimulation at unstretched conditions to allow recovery from transportation and preparation, trabeculae were gradually stretched over 10 min until optimal contractile force was achieved. At the end of 20 min of equilibration a baseline recording was performed during 5 minutes. Those trabeculae that failed to produce at least 0.2 g of developed force at start of equilibration were excluded (n=32).

For both experiments, sunitinib was extracted from clinically available sunitinib capsules (Pfizer, United Kingdom) by dissolving the content of capsules in DMSO. Final sunitinib concentration in superfusate was 32.4 ± 4.1 ng/ml (confirmed by LCMS in four random experiments included in this study). In patients the total plasma concentration of sunitinib and its active metabolite, including 90-95% protein binded drug , is 50 – 100 ng/ml. The active metabolite accounts for approximately 30% of plasma concentrations in patients. The concentration of DMSO in the organ bath did not exceed 0.01%.

Experiment 1: effect of sunitinib on contractility

Immediately after baseline recordings, for each patient (n = 8) the two trabeculae were randomly assigned to either DMSO (control) or sunitinib. Electrical field stimulation at 1 Hz was applied for the entire experiment of 150 minutes.

Experiment 2: effect of sunitinib on contractility after ischemia reperfusion

Immediately after baseline recordings for each patient (n = 12) the two trabeculae were randomly assigned to either DMSO (control) or sunitinib. After 20 minutes of superfusion with sunitinib or control, 60 minutes of simulated ischemia was started, which was accomplished by superfusing the trabeculae with substrate free modified Tyrode's solution (7.0 mM choline chloride substituted for glucose and pyruvate) and rapid pacing at 3 Hz. The superfusate was pumped into an artificial lung filled with 95% N_2/5% CO_2, which results in a low pO_2 of 10 to 20 mm Hg. Subsequently both trabeculae were subjected to 100 min of simulated reperfusion (pacing at 1 Hz, oxygen and nutrient containing superfusate as described above), still in the presence of either sunitinib or control.

Data recording and statistical analysis

All data are presented as mean ± SE. For each contraction of the trabeculae, we calculated developed force (difference between maximal tension during contraction and minimal tension during relaxation). This parameter was averaged for the last 5 minutes during equilibration (taken as baseline) and each subsequent 5-minute period. Data were expressed as percentage of baseline developed force. A paired Student's t-test was used to compare control with sunitinib treated trabeculae during the last 5 minutes of the protocol in both experiments. $P<0.05$ was considered significant.

Results

There were no significant differences in baseline characteristics between the groups in experiment 1 and 2 (table 1). In 52 patients atrial tissue was harvested; because of poor quality of trabeculae at start of the experiment 32 patients were excluded and therefore 20 patients were included in the analysis.

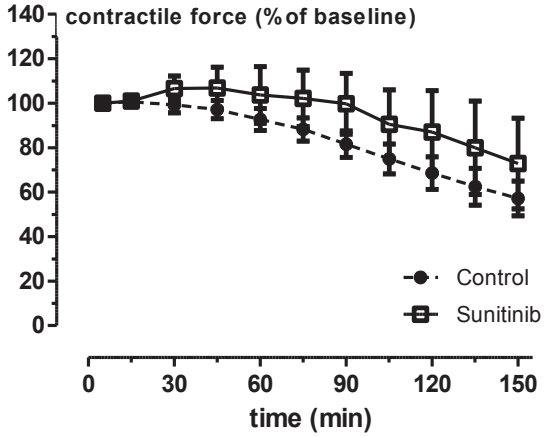
TABLE 1
Patient characteristics

Variable	Experiment	Experiment 2
Number	8	12
Females	3(37,5%)	3(25%)
Age (years)	62.6	62.1
Range	(37-75)	(40-77)
CABG	4 (50%)	6 (50%)
AVR	3 (37,5%)	6 (50%)
CABG+AVR	1 (12,5%)	
Medication		
Beta Blockers	7	6
ACE-inhibitors	3	5
AII-receptor antagonists	0	1
Ca-antagonists	1	3
Nitrate	4	4
Aspirin	6	7
Statin	5	6
Insulin	2	2

Experiment 1: effect of sunitinib on contractility

Baseline contractile force did not differ between trabeculae that were subsequently treated with control or sunitinib (0.50±0.1g and 0.51±0.05g; p=0.9). Contractile force (expressed as percentage of baseline force) declined over time to 57±8% and 73±20% after 150 minutes of stimulation for control and sunitinib treated trabeculae respectively (figure 1; p>0.1 for comparison between control and sunitinib treated cardiac muscle).

FIGURE 1
Effect of sunitinib on baseline contractile force (experiment 1)

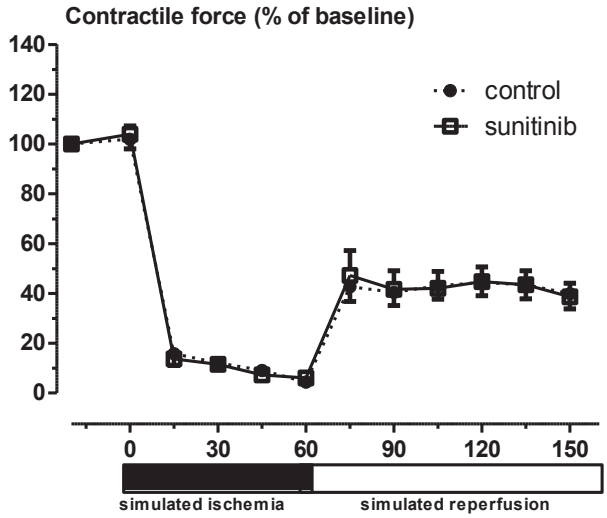


Experiment 2: effect of sunitinib on contractile force recovery after ischemia

Baseline contractile force did not differ between control and sunitinib treated trabeculae (0.54±0.1g and 0.51±0,05g; p=0.8). Ischemia reduced contractile force to a similar extent in trabeculae treated with control or sunitinib

Recovery was 40±6% in the control group compared to 39±6% in the sunitinib group during the last final 5 minutes of reperfusion (p>0.1).

FIGURE 2
Course of contractile force during simulated ischemia and reperfusion for trabeculae treated with control or sunitinib



Discussion

We studied the ex vivo effect of sunitinib on contractile force of human atrial trabeculae. This study provides for the first time data on the effect of sunitinib on human atrial contractile recovery after ischemia-reperfusion. At clinically relevant concentrations sunitinib did not have a direct effect on the function of human atrial cardiomyocytes nor did it attenuate the recovery in contractile force after a period of ischemia. These observations suggest that other actions of sunitinib are responsible for the clinical association of sunitinib therapy with heart failure.

In the literature, several mechanisms of sunitinib-induced heart failure have been proposed. These mechanisms can be divided in actions in the cardiomyocyte and actions on cardiac perfusion or hemodynamic loading conditions of the heart. Since we only studied the effects of sunitinib on isolated cardiac tissue, this discussion will focus on actions of sunitinib in cardiomyocytes.

Preclinical studies have suggested a direct toxic effect of sunitinib on cardiomyocytes. Hasinoff et al showed a dose dependent (2-5μM) increase of lactate dehydrogenase released from rat cardiomoyocytes treated with sunitinib[13,14]. Sunitinib exposure (0,1μM and higher) of Guinea

pig cardiomyocytes resulted in dose-dependent decreases in spontaneous contraction rate[15]. Moreover a direct toxic effect of sunitinib on human atrial trabeculae was observed by Rainer et al[10]. However these effects were only observed with supra-therapeutic concentrations of sunitinib. At a therapeutically more relevant concentration, Rainer et al did not observe an effect of sunitinib on contractile force during 30 minutes of exposure. In our experiment we prolonged the exposure time to optimize the uptake of sunitinib into the tissue, but we could still not detect an effect of sunitinib on contractile force.

Since clinical data suggest that cardiac ischemia increases the risk of sunitinib-induced heart failure [1,3,7], we hypothesized that sunitinib may aggrevate the consequences of ischemia-reperfusion injury of cardiomyocytes. This hypothesis is supported by various animal studies. Sunitinib inhibits the AMP protein Kinase (AMPK) pathway [9,14]. Moreover AMPK is known to have an important defensive role in cardiomyocytes against energy stress triggers[16]. It is also an important pathway to protect the heart against ischemia-reperfusion injury[17]. Apart from interference with the AMPK-pathway, sunitinib could reduce cardiomyocyte tolerance against ischemia-reperfusion injury by inhibiting PDGF- signaling [9]. In preclinical studies deletion of the PDGFr-β gene from cardiomyocytes leads to heart failure after exposure to overload[18]. Moreover in rats, PDGF seems to protect the heart when exposed to ischemic injury[19].

Our study shows, however, that sunitinib did not aggravate the functional consequences of ischemia followed by reperfusion, questioning the clinical relevance of these pathways in the development of sunitinib-induced heart failure.

Some limitations of our study should be mentioned. First, the onset of sunitinib induced heart failure is not very well studied and may vary between patients. Based on scarce retrospective data the time of onset of sunitinib-induced symptomatic heart failure differs from a mean of 22 days after initiation to a median time of 33.4 weeks[1,20]. In our study we only studied short term cardiac exposure of sunitinib. Therefore, based on our results we cannot exclude that longtime exposure to sunitinib does alter contractile recovery after ischemia. A second limitation is the use of atrial and not ventricular trabeculae. Hence we do not know whether ventricular cardiomyocytes may differ from atrial cardiomyocytes in their response to sunitinib. Third, we used sunitinib, but not its active metabolite, which could also play a role in development of cardiotoxicity.

In conclusion, our study suggests that direct myocardial toxicity is insufficient to explain sunitinib-associated heart failure. Therefore a combination of factors, such as structural or functional changes of the coronary vasculature, systemic hypertension or renal toxicity deserve more attention as potential causes of heart failure and volume overload.

Further exploration of the mechanisms of sunitinib-associated heart failure is essential for developing better strategies to prevent and treat angiogenesis-TKI induced heart failure, in particular since this class of drugs is increasingly used in frail and aged patients, who are at risk for developing heart failure.

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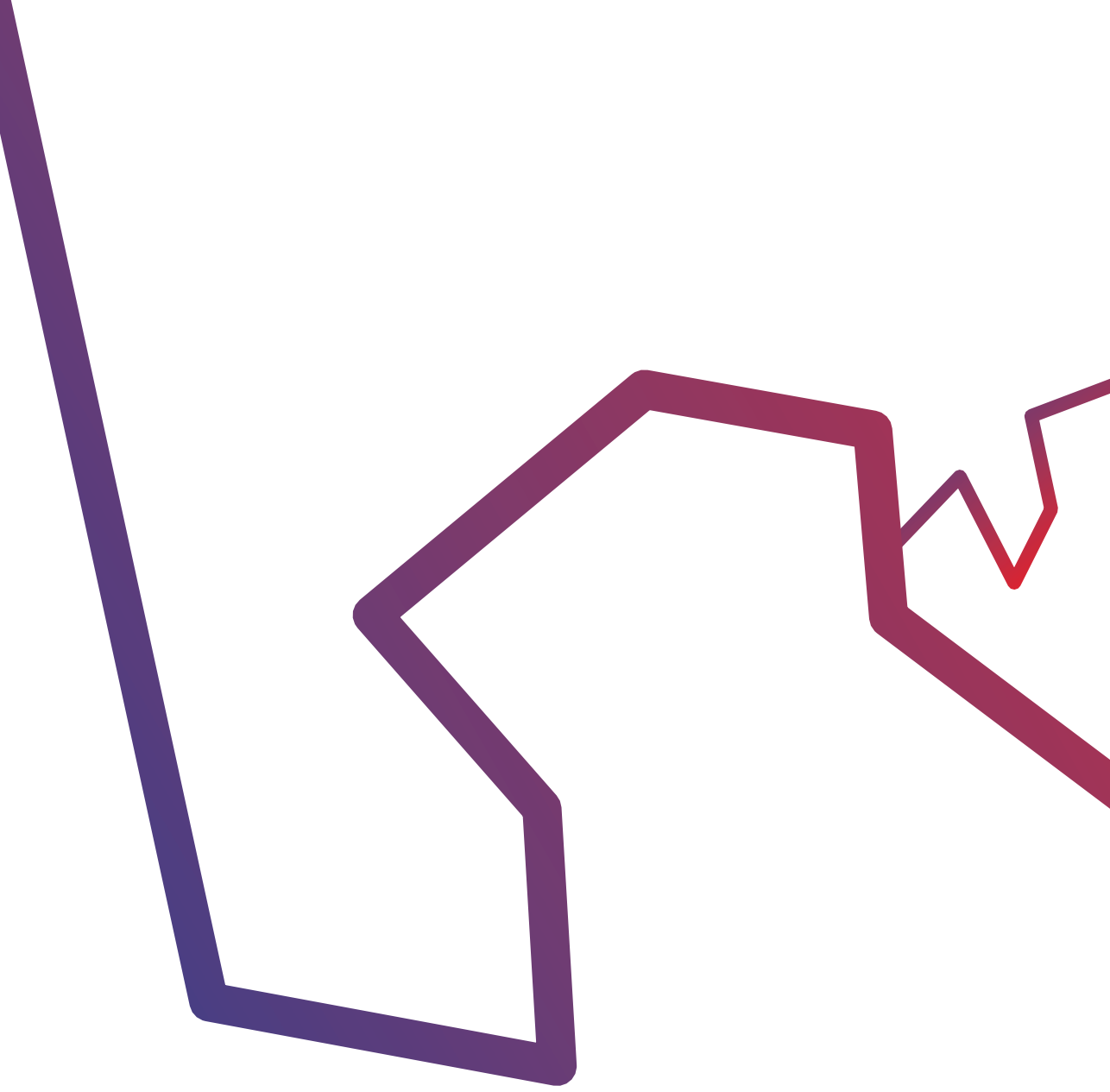
VASCULAR EFFECTS OF ANGIOGENESIS INHIBITORS



CHAPTER 3

The role of endogenous vascular endothelial growth factor in endothelium-dependent vasodilation in humans

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Abstract

Aim

Angiogenesis inhibitors have remarkably improved the outcome of patients with several types of cancer. Hypertension is the most reported side effect of angiogenesis inhibitors interfering with vascular endothelial growth factor signaling. In this study, we test the hypothesis that circulating vascular endothelial growth factor at physiological concentrations is essential to preserve normal endothelial control of vasomotor tone.

Methods and results

In seven healthy male volunteers, infusion of bevacizumab (monoclonal anti- vascular endothelial growth factor antibody) into the brachial artery for 15 minutes (144 μg/dl forearm volume/min) did not affect forearm vasodilator tone as measured with venous occlusion strain gauge plethysmography. In a separate group of twelve male volunteers, a similar bevacizumab infusion reduced the vasodilator response to two dosages of acetylcholine from (mean$\pm$SE) 440 $\pm$ 157% and 926 $\pm$ 252% to 169 $\pm$ 40% and 612$\pm$ 154 % ($p<0.05$). Finally, in a third group of 12 volunteers, bevacizumab did not alter the percentage increase in forearm blood flow during infusion of sodiumnitroprusside at dosages equipotent to acetylcholine.

Conclusion

Bevacizumab acutely and specifically reduced endothelium-mediated vasodilation at local concentrations that resemble plasma concentration after systemic exposure to bevacizumab. This observation suggests a physiological role for vascular endothelial growth factor in maintaining normal endothelial control of vasomotor tone. The role of the endothelium in the mechanism of bevacizumab-induced hypertension deserves further exploration.

Introduction

Angiogenesis, the formation of new capillaries from endothelial cells from existing vasculature, is essential for tumor growth. When tumor size exceeds a few millimeters an 'angiogenic switch' occurs as a consequence of hypoxia and nutrient deprivation[1]. Subsequently various pro-angiogenic factors are released by tumor cells to activate quiescent cells to promote vascular growth to the tumor. Vascular endothelial growth factor (VEGF) and its receptors play a key role in this process. However, also in normal physiology VEGF may play a role in vascular homoeostasis.

VEGF is an angiogenic growth factor which is mainly secreted from mesenchymal, stromal and epithelial sources to act on endothelial cells.. The angiogenic effects of VEGF are primarily mediated by VEGFreceptor-2 (VEGFR-2)[2].

In 1971 Folkman proposed angiogenesis inhibition as an alternative strategy for the treatment of malignancies[1]. Meanwhile several drugs that target angiogenesis in tumors have been developed, almost all of them directed to VEGF or its receptors. Bevacizumab (BVZ), a humanized monoclonal antibody selectively binding VEGF was the first VEGF inhibitor approved by the FDA.

BVZ in combination with chemotherapy has been approved for the treatment of metastatic colon carcinoma, unresectable or metastatic non-small cell lung cancer and glioblastoma and in combination with interferon alpha for metastatic renal cell carcinoma.

BVZ is generally considered to be safe and well tolerated but can be accompanied by a variety of side effects. An increased incidence of hypertension (defined as a blood pressure over 150/100 mmHg or a rise of 20 mm Hg in diastolic blood pressure) of up to 34% has been observed in BVZ-treated patients in clinical trials compared with up to 14% in those treated with standard therapy. The most serious reported adverse drug reactions are hemorrhage and arterial thrombo-embolism in patients treated with BVZ in combination with chemotherapy[3]. The most frequently observed adverse drug reactions across clinical trials in patients receiving BVZ are hypertension, fatigue or asthenia, diarrhea and abdominal pain[4].

Pharmacological doses of VEGF stimulate endothelial nitric oxide (NO) formation in preclinical models [5-7] and reduces blood pressure in animals and humans[8]. In mice, inhibition of VEGFR-2 rapidly increased blood pressure and reduced the expression of endothelial and neuronal NO synthases in the kidney. Moreover treatment with a NO antagonist abolished the effect of VEGFR-2 inhibition on blood pressure [2]. It is tempting to speculate that anti-VEGF treatment will

reduce endothelial NO release as the mechanism of BVZ-induced hypertension. However, the role of exposure of endothelial cells to endogenous VEGF has never been studied in humans in-vivo. Therefore, it is currently not known whether BVZ-treatment results in endothelial dysfunction.

Therefore, the aim of this study was to explore the effect of specific inactivation of endogenous VEGF by binding to its monoclonal antibody BVZ on baseline vasomotor tone and on endothelium-dependent vasodilation in humans in-vivo.

Materials and methods

Subjects

After approval of the protocols (NCT00929058, NCT01125943) by the Institutional Review Board of the Radboud University Nijmegen Medical Centre, a total of 31 healthy nonsmoking male volunteers were recruited after written informed consent. Female subjects were excluded to prevent the influence of menstrual cycle and related hormonal changes on vascular reactivity. Subjects were eligible if they had no clinical history of hypertension, hyperlipidemia, renal dysfunction or diabetes mellitus. On specific request from our institutional ethical review board, subjects at increased risk for malignancy (first-degree relatives with cancer presented before age of 50 years) and subjects with first degree relatives with premature atherosclerotic disease or recurrent (venous and/or arterial) thrombosis were excluded. All studies were performed according to institutional and Good Clinical Practice guidelines.

Study Design

General outline of the procedures

The experiments were performed after at least 24 hours of caffeine abstinence, in the morning after an overnight fast. Volunteers were studied in supine position in a temperature-controlled room ($23 \pm 1^{o}C$). At the start of each experiment a 20-gauge cannule (Angiocath; Becton Dickinson, Sandy, UT) was inserted into the brachial artery of the non-dominant arm for intra-arterial blood pressure measurement (Hewlett Packard, Böblingen, Germany) and infusion of drugs.

During intra-arterial infusion of saline (NaCl 0.9%) or drugs forearm blood flow (FBF) was measured at both arms simultaneously, using electrocardiogram-triggered venous occlusion mercury-in-silastic strain-gauge plethymography (Hokanson EC4, Hokanson, Inc). During all forearm blood flow (FBF) recordings the hand circulation was completely occluded using wrist

cuffs inflated to 200 mmHg. At least 30 minutes after the cannulation experiments were started. Drugs or solvent (either saline 0.9% or glucose 5%) were infused at a constant rate of 50 μl/dl forearm/ min.

In all three studies BVZ (Avastin, Roche, the Netherlands) was infused intra-arterially to separate direct local effects of VEGF inhibition from systemic actions, such as hypertension, that could indirectly interfere with endothelial function. The normally prescribed intravenous dosage for BVZ ranges from 5 mg/kg to 15 mg/kg once every two/three weeks. For our studies we aimed to achieve a local BVZ concentration that occurs systemically after an intravenous dose of 5 mg/kg. In a pharmacokinetic study after first administration of 5 mg/kg BVZ mean observed Cmax was 123.2 μg/ml[9].

Acetylcholine (ACH, Miochol, 20 mg dry powder, dissolved to its final concentration with saline 0.9%, Novartis, Greece) and nitroprusside (SNP, 50 mg dry powder, Spruyt Hillen, the Netherlands (dissolved to its final concentration with 5% glucose solution; Clinical Pharmacy, Radboud University Nijmegen Medical Centre) were used for the measurement of endothelium-dependent and endothelium-independent vasodilation respectively. Each dose of vasodilator was infused into the brachial artery for 5 minutes.

One week after completion of the experiment subjects received a questionnaire to evaluate late side effects.

Study 1: The acute vasomotor response to BVZ

Seven volunteers were recruited for this study. Forearm blood flow (FBF) was measured using venous occlusion strain gauge plethysmography. First, baseline FBF was recorded for 5 minutes. Subsequently, the vasomotor effect of BVZ was studied during a 15 minute infusion of BVZ (144 μg/dl forearm volume/min) in the brachial artery of the non-dominant arm. Thirty minutes after the BVZ infusion FBF was assessed again for studying late effects of BVZ infusion. During the last minute of 15 minutes of BVZ infusion venous blood was collected from both arms to measure local and systemic concentrations of BVZ.

BVZ concentration was measured with a 1-site enzyme-linked immunosorbent assay (ELISA developed by our laboratory. In short, 96-well plates (#655092, Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated overnight at 4 °C with 100 μl VEGF (0.50 μg/ml, Genentech Inc, San Francisco, CA), washed (96PW plate washer, TecanGroup Ltd., Männedorf, Switzerland), and then blocked with 150 μl Superblock (#37515, Pierce, Rockford, IL) during 30 minutes at ambient temperature, washed and further blocked with 300 μl BSA (#A-7906, Sigma Chemical,

St. Louis, MO) for 4 hours at ambient temperature. Subsequently, the plate was washed and standards (range 0-10 ng/ml Avastin, Roche, Basel, Switzerland), study samples and a reference sample were pipetted into the wells and the plate was incubated overnight at 4 °C. After washing, the plate was incubated with 100 μl Mouse anti-Human IgG (Fc) POD, (dilution 1:25,000, SouthernBiotech, Birmingham, U.K.) during 2 hr at ambient temperature and again washed. Thereafter incubation with 100 μl ready-to-use 3,3´, 5, 5´- tetramethyl-benzidine (TMB) (solution (Kem-En-Tec, Taastrup, Denmark) for 15-20 minutes took place for colour development. The reaction was stopped by addition of 0.5 M H_2SO_4 and optical density was measured at 450 nm in a Multiskan Ascent plate reader (Lab Systems, Oy, Helsinki, Finland). The analytical sensitivity, defined as the minimum BVZ concentration evoking a response significantly different from that of the zero calibrator, was 16 pg/ml. Plasma samples, diluted 4,000 – 24,000 fold, exhibited excellent parallelism. To six plasma samples known quantities of Avastin were added. The recoveries ranged in the plasma samples from 87% to 110% with a mean recovery of 98%. In each run the reference preparation, prepared from a pool of plasma from patients treated with BVZ, was used to monitor long-term performance of the assay. The concentration in reference preparation was 1670 ng/ml, the within-run coefficient of variation (CV) and the between-run CV amounted to 7.0% and 10.0%, respectively.

Study 2: The effect of BVZ on endothelium dependent vasodilation

In twelve evaluable volunteers the vasodilator response to ACH was studied in absence and presence of BVZ. The experiment started with measurement of baseline FBF during saline infusion during 5 minutes followed by two increasing dosages of ACH (0.5 and 2.0 μg/dl forearm volume/ min) 5 minutes each. After a washout period of 45 minutes measurements of FBF response to saline and the two increasing dosages of ACH were repeated during continuous infusion of BVZ during 15 minutes.

Subjects with less than a 100% increase in FBF in the absence of BVZ were excluded, because of non evaluable baseline response to ACH and replaced by newly recruited volunteers.

Study 3: The effect of BVZ on endothelium independent vasodilation

In a separate group of twelve evaluable volunteers the vasodilator response to SNP in absence and presence of BVZ was studied. First baseline FBF was measured during glucose infusion followed by two increasing dosages of SNP (0.06 and 0.2 μg/dl forearm volume/ min). After a washout period of 45 minutes measurements of FBF response to glucose and the two increasing dosages of SNP were repeated during continuous infusion of BVZ during 15 minutes.

Statistical Analysis

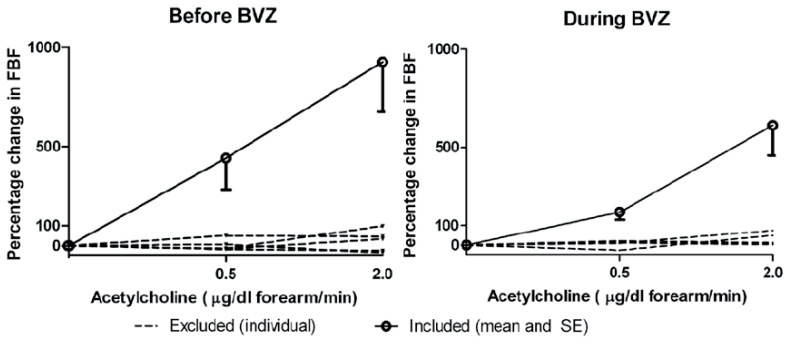
Statistical analysis was performed using the SPSS (version 16.0) software packages. Values are reported as mean$\pm$SE unless otherwise specified. Mean arterial pressure (MAP) was measured continuously during each recording of FBF and averaged per FBF registration. Drug-induced effects were expressed as percentage of change from preceding saline or glucose infusion. The percentage changes in FBF to each dosage of a vasodilator substance were averaged to one value for each vasodilator. These values were compared using a repeated measures ANOVA to assess the effect of BVZ. Before analysis, logarithmic transformation was performed to obtain a Gaussian distribution.

Results

In total 46 subjects signed informed consent. Five subjects were excluded based on medical history or medication use. Another 5 subjects withdrew consent for personal reasons prior to start of the study.

Five subjects were excluded after completion of the study protocol due to an absent response to ACH as was prespecified in our study protocol. In this study we choose healthy volunteers to rule out other factors influencing endothelial function such as medication, cardiovascular risk factors or underlying disease to assess the specific effect of BVZ on normal endothelial function. Subjects with an absent response to ACH measurement, may have other unknown factors that already altered endothelial function and interfere with the response to BVZ. The percentage change in FBF in these five individuals compared to the mean FBF in the included group before and during BVZ are depicted in figure 1. As shown in this figure, the lack of response to ACH was reproduced in the presence of BVZ. excluding regression to the mean as a potential confounder that could have been introduced by this exclusion. Thirty-one male subjects (23.9$\pm$1.2 years) were included in the analysis.

FIGURE 1
Percentage change in forearm blood flow (FBF; mL/dL forearm volume per minute) from baseline in response to acetylcholine represented as mean for the included group (n=12) and individual flows for the excluded individuals (n=5). BVZ indicates bevacizumab.



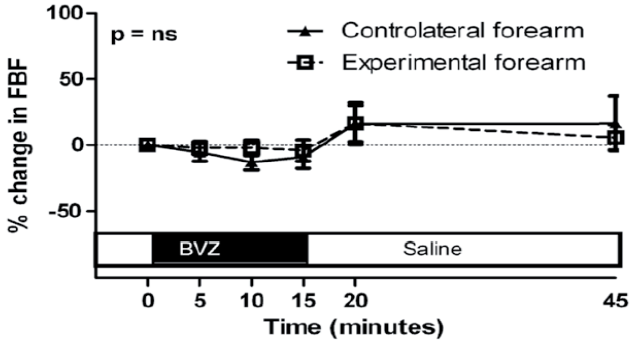
Study 1: The acute vasomotor response to BVZ

BVZ concentrations

At the end of the 15 minutes infusion of BVZ, BVZ reached a concentration of 136±13.2 µg/ml (n=7) in the experimental arm, resembling plasma concentration after systemic exposure to BVZ in patients treated with 5mg BVZ i.v./kg. BVZ concentration in the control arm was 8.3±0.8 µg/ml (n=7).

Intra-arterial BVZ did not alter baseline forearm blood flow (Figure 2). Blood pressure did not change either (baseline MAP:77.1±2.7mmHg; at the end of BVZ infusion:79.5±2.2mmHg).

FIGURE 2
Percentage change from baseline in forearm blood flow (FBF) in response to bevacizumab (BVZ) infusion in the experimental forearm and control forearm. No acute vasomotor response to infusion of BVZ was observed.



Study 2: The effect of BVZ on endothelium dependent vasodilation

In the experimental arm, forearm blood flow increased from 1.3±0.2 at baseline to 5.5±1.1 and 10.6±1.8 ml/dl/min for the two increasing dosages of ACH (0.5 and 2.0 μg ACH/dl forearm/ min) respectively. In the presence of BVZ, ACH increased forearm blood flow from 1.4±0.2 to 3.8±0.8 and 8.9±1.8 ml/dl/min respectively (figure 3). In the contralateral arm, forearm blood flow remained constant: 1.2±0.2, 1.1±0.2 and 1.2±0.2 ml/dl/min in the absence of BVZ and 1.1±0.3, 1.0±0.2 and 1.2±0.2 ml/dl/min in the presence of BVZ for baseline and two subsequent ACH doses respectively (figure 3). In the absence of BVZ, ACH increased FBF from baseline by 440±157% and 926±252%). In the presence of BVZ, ACH increased FBF from baseline by 169±40% and 612±154% respectively (n=12; $p < 0.05$ for the effect of BVZ, ANOVA for repeated measures on log-transformed data; figure 4) .

In the absence of BVZ, the percentage increase in forearm blood flow during sodium-nitroprusside (SNP) infusion was 270±45% (0.06 μg/dl/ min) and 671±162% (0.2 μg/dl/ min). In contrast to ACH, the vasodilator effect of SNP was not affected by simultaneous infusion of BVZ 248±64% and 679±156% (n=12; p>0,4; figure 4) . For the course in forearm blood flow, see figure 3.

FIGURE 3
Study protocol and actual forearm blood flow (FBF) in response to 2 increasing dosages of acetylcholine (ACH) and sodium nitroprusside (SNP). BVZ indicates bevacizumab.

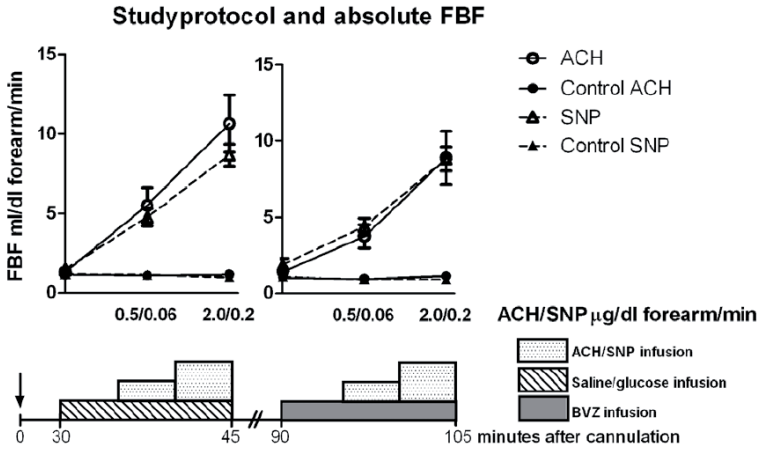
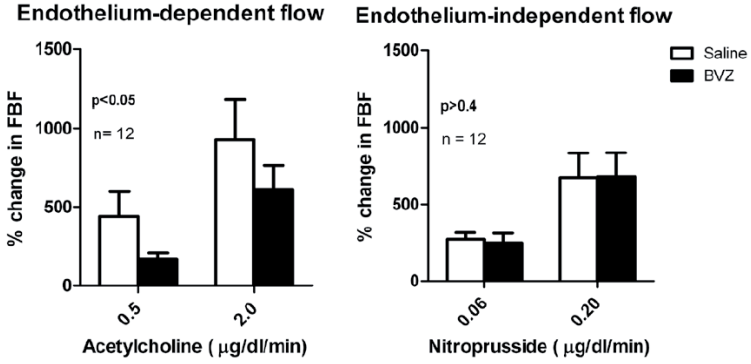


FIGURE 4
Increases in forearm blood flow (FBF) in response to 2 increasing dosages of acetylcholine and sodium nitroprusside, depicted as percentage change, from baseline. Error bars, SE of the mean. Statistical analysis was performed on the log-transformed data. BVZ indicates bevacizumab.



Blood pressure and heart rate did not change in response to the vasodilator agents (see table 1).

TABLE 1
Blood Pressure and Heart Rate

	ACH		SNP	
Infusion Period	MAP, mm Hg	Heart Rate, bpm	MAP, mm Hg	Heart Rate, bpm
Baseline	79.5 ±2.1	60 ±2	77.0 ±2.2	61 ±3
Vasodolator (low)	79.4 ±2.0	60 ±2	76.6 ±2.2	61 ±3
Vasodilator (high)	79.3 ±2.2	62 ±3	76.4 ±2.3	61 ±2
BVZ baseline	81.1 ±1.9	60 ±2	81.8 ±2.2	60 ±3
BVZ vasodilator (low)	81.0 ±1.9	62 ±3	81.0 ±2.1	60 ±3
BVZ vasodilator (high)	81.0 ±2.1	62 ±3	81.2 ±2.2	61 ±3

ACH indicates acetylcholine; BVZ, bevacizumab; MAP, mean arterial pressure; and SNP, sodum nitroprusside.

Side effects

No side effects of BVZ were reported during infusion or at 1-2 weeks after the experiment as evaluated by a questionnaire.

Discussion

In the present study, we show for the first time in humans that inactivation of circulating VEGF decreases endothelium dependent vasodilation within 15 minutes. This proves that circulating VEGF plays a role in maintaining normal endothelial control of vascular tone.

Previously, several clinical studies have shown decreases in endothelium dependent but also endothelium independent vasodilation during treatment with tyrosine kinase inhibitors targeting VEGF signaling[10, 11]. However these studies have been conducted after at least six weeks of anti-VEGF treatment when rise in blood pressure already had occurred. Hypertension by itself decreases endothelial function. Prolonged treatment with TKIs targeting the VEGF pathway has been reported to be associated with capillary rarefaction[11, 12]. Rarefaction increases vascular tone and interacts with both endothelium dependent and independent vasodilation. Rarefaction also occurs in idiopathic (essential) hypertensive patients[13, 14] and could therefore be a consequence rather than a cause of the rise in blood pressure that often occurs during treatment with these drugs. A recent study in swine showed an increase in blood pressure within a few hours after administration of sunitinib, a TKI targeting the VEGF pathway [15]. The fast onset of changes in

blood pressure makes rarefaction a less likely cause of VEGF targeting TKI-induced hypertension and suggests that rarefaction is a consequence rather than a cause of hypertension in this group of patients.

In this study, intra-arterial infusion of BVZ achieved a clinically relevant concentration of BVZ in the forearm while systemic exposure to this drug was low. This allowed us to study the local effect of selective VEGF deprivation without interference of systemic effects such as a rise in blood pressure. Bevacizumab selectively interrupts endogenous VEGF signaling. This contrasts with tyrosine kinase inhibitors such as sunitinib, which to do not only inhibit VEGF signaling but also the response to other growth factors such as PDGF or c-Kit. [11]. Finally, we studied healthy volunteers, without any co-medication that could interfere with vasomotor control such as chemotherapy [10]. Therefore, a strength and unique feature of our study is the specificity of the used pharmacological intervention.

We did not observe an effect of BVZ on vascular tone. This suggests that the effect on the muscle vascular bed is not sufficient to cause hypertension. However some remarks can be made about this conclusion. BVZ was infused for 15 minutes. The infusion time was based on previous investigations that showed a decrease in blood pressure in response to VEGF-infusion within a few minutes[16]. More importantly, time was limited by the allowed exposure of healthy volunteers to this drug. The given dose was high enough to reach local concentrations mimicking concentrations after systemic exposure in patients, but the continuous arterial influx of free non-bound VEGF could have resulted in some residual VEGF receptor stimulation in our experimental set-up and could therefore have underestimated the effect as observed during systemic therapy with BVZ. In addition, regional differences in the role of VEGF on baseline vascular tone may exist.

Several previous studies (Kappers et al) have shown that activation of the endothelin axis may be involved in the onset of hypertension during inhibition of VEGF signaling. The reported decrease in endothelium dependent vasodilation in our study could be due to diminished release of nitric oxide, as supported by the previously mentioned studies[6-9] . It has been previously shown that the inhibition of baseline NO can enhance the release of endothelin-1[17 18]. Thus, our observation provides a clue to a potential mechanism of increased endothelin-1 release in response to VEGF-targeted therapy.

Perspectives

Our observation does not exclude the possibility that BVZ increases vascular tone in other organs such as the kidney which is of particular interest with regard to the pathogenesis of hypertension. Our results indicate that local VEGF deprivation immediately reduces endothelium-dependent vasodilation, however, this study does not allow any conclusion on the mechanism of this altered endothelial function. Apart from a reduced release of NO, reduced formation of other endothelium-derived relaxing factors or an increased release of endothelium-derived contracting factors in response to acetylcholine may have contributed to this observation.

Conclusion

Our study is the first to investigate the acute effect of VEGF inhibition on vascular tone and endothelial function in healthy volunteers. It was performed in healthy volunteers to prevent a possible influence of previous and current medical treatment or disease on the vascular response to VEGF inhibition. Our study using a specific VEGF antibody suggests a role for circulating VEGF in normal endothelial control of vascular tone.

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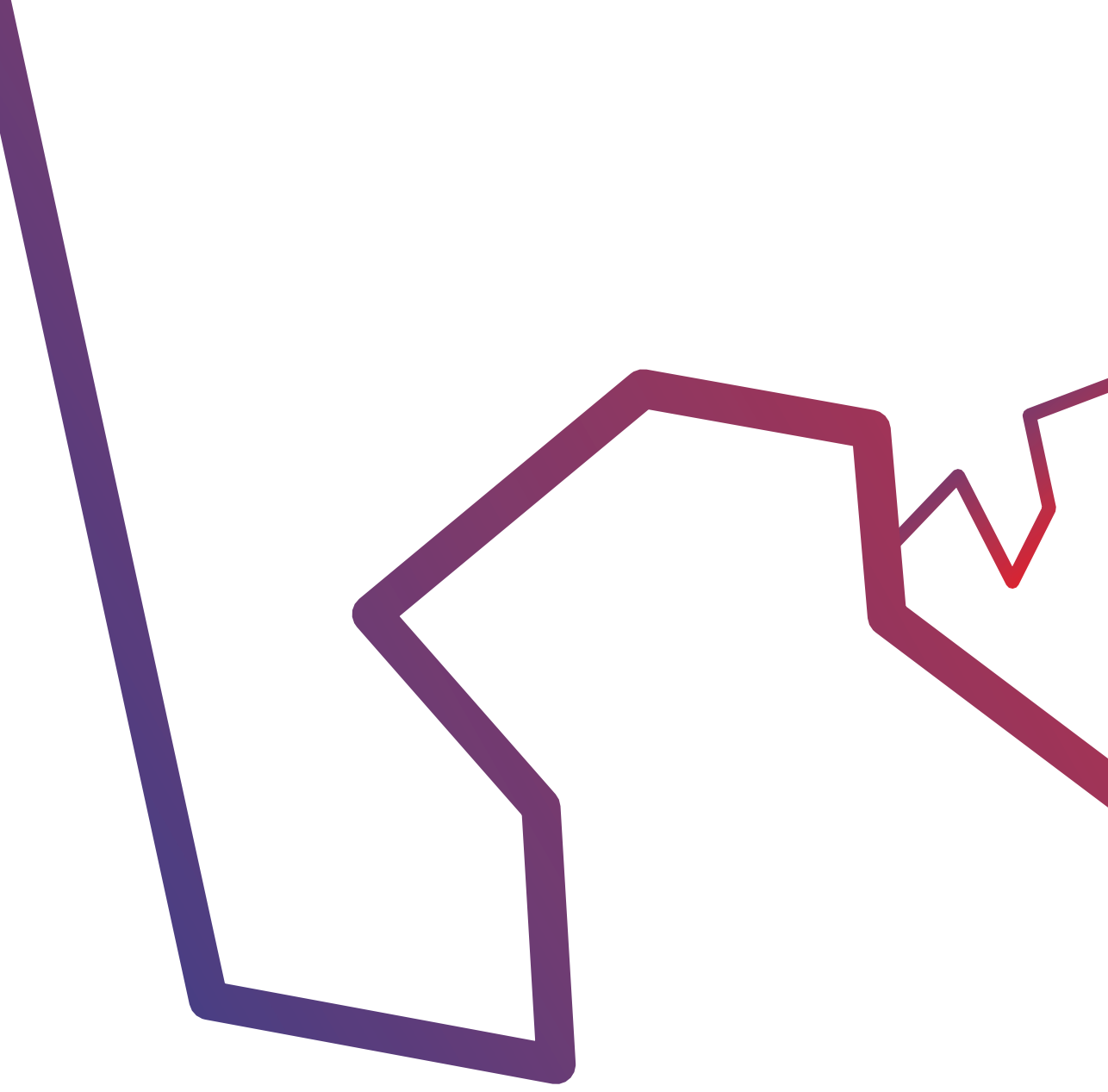

CHAPTER 4

Impaired endothelium-dependent vasodilation does not initiate the development of sunitinib associated hypertension

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Abstract

Tyrosine kinase inhibitors targeting angiogenesis have become an important part of the treatment of patients with several types of cancer. One of the most reported side effects of VEGFR targeted therapies is hypertension. In this study we hypothesized that the development of hypertension in patients treated with sunitinib, a multitargeted tyrosine kinase inhibitor, is preceded by reduced endothelium dependent vasodilation. Moreover we hypothesized that this endothelial dysfunction is a result of impaired nitric oxide release.In a placebo-controlled experiment we determined vascular responses in isolated mesenteric arteries of rats (n=26) after 7 days of sunitinib treatment. Sunitinib reduced endothelium dependent vasodilation, but not endothelium independent vasodilation. Moreover we observed that the difference in endothelium dependent vasodilation between controls and sunitinib-treated animals disappeared in the presence of L-NAME, a nitric oxide-antagonist. In patients with metastatic renal cell carcinoma, before and one week after start of sunitinib, the endothelium-dependent vasodilator response to intra-arterial acetycholine and the endothelium-independent vasodilator response to intra-arterial sodium nitroprusside was assessed with venous occlusion plethysmography. No changes in forearm bloodflow ratios were observed.. Mean arterial pressure did significantly increase from 101.9 ±3.8 mmHg to 106.1 ±2.6 mmHg after one week and further to 115.8 (±4.9) mmHg after two weeks of treatment.

Conclusion

In animals, this study confirms that exposure to high concentrations of sunitinib reduces endothelium-dependent vasodilation by reducing endothelial release of nitric oxide. In humans however, reduced endothelium-dependent vasodilation does not precede the development of hypertension in patients treated with sunitinib.

Introduction

Tyrosine kinase inhibitors (TKIs) targeting angiogenesis have become an important part of the treatment of patients with several types of cancer[1,2]. From the hypothesis of Folkman in 1971[3] about the need of angiogenesis for tumor growth to the introduction of the newest angiogenesis inhibitors more than 40 years have passed in which a lot of the therapeutic opportunities of targeting angiogenesis have become clear, but also showing several side effects of these treatments.

Sunitinib is an oral TKI, targeting VEGFR1-3, PDGFRA and PDGFRB, c-KIT , FLT3, CSF-1R, and RET. It is used widely as it has been approved for the treatment of metastatic renal cell carcinoma, gastrointestinal stromal tumor (GIST) and neuro-endocrine tumors of the pancreas.

One of the most reported side effects is hypertension. Hypertension occurs in 19% to 80% of patients[4-6]. Even more patients experience a rise in blood pressure[7]. Hypertension is correlated with improved progression free survival in patients with metastatic renal cell carcinoma treated with sunitinib and with bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF) [6,8].

The common pathway in the development of hypertension in the course of treatment with this class of drugs is VEGF. VEGF is an angiogenic growth factor, which is mainly secreted from mesenchymal, stromal and epithelial sources to act on endothelial cells. The angiogenic effects of VEGF are primarily mediated by VEGF receptor-2 (VEGFR-2). VEGF plays an important role in the proliferation of new blood vessels necessary for tumor growth[9,10]. VEGF and its receptors are also expressed in many normal tissues. Different studies show that exogenous VEGF causes hypotension[11]. In animal experiments, VEGF induces hypotension due to synthesis and/or release of nitric oxide (NO)[12]. In human umbilical vein endothelial cells treatment with VEGF resulted in both an acute (1 h) and chronic (>24 h) stimulation of NO production[13]. In mice, inhibition of VEGF receptor-2 rapidly increased blood pressure and reduced the expression of NO synthases in the kidney. Moreover, treatment with a NO-synthase antagonist abolished the effect of VEGF receptor-2 inhibition on blood pressure[14]. On the basis of this knowledge inhibition of VEGF in humans could result in a decrease in NO availability which could subsequently trigger the development of hypertension. In a previous study we have shown that selective scavenge of endogenous VEGF by intra-arterial infusion of bevacizumab decreases endothelium-dependent vasodilation acutely[15]. This supports the hypothesis that VEGF inhibition causes endothelial dysfunction and may cause hypertension by increasing vascular resistance in humans.

Besides the possible effects of VEGF inhibition on endothelial function some studies show that neurohormonal changes can play a role in the development of hypertension during treatment with angiogenesis inhibitors. For example in patients with mRCC and gastrointestinal stromal tumors administration of sunitinib was associated with a rise of plasma endothelin-1 (ET-1) concentration[16]. And other studies show that vascular rarefaction can occur during treatment with a small molecule angiogensis inhibitor[17]. In this study however we will focus on changes in endothelial function.

Previous studies in humans treated with VEGF-targeted therapies have shown a decrease in endothelial function after 6-10 weeks of treatment, however at that time point hypertension already occurred, so whether endothelial dysfunction precedes hypertension and is a cause rather than a consequence of the rise in blood pressure is unknown[17] Preclinical studies show that the occurrence of hypertension is accompanied by a decrease in urinary excretion of NO metabolites and reduced endothelium-dependent vasodilation in the coronary vasculature [16]. However, the exact role of NO in this sunitinib-induced endothelial dysfunction has not been studied before.

Therefore, we performed a preclinical and a clinical experiment. In sunitinib-treated animals and appropriate controls, endothelium-mediated vasodilation was assessed, as well as the role of NO in this vascular response. In a clinical study in mRCC patients who started treatment with sunitinib, we investigated whether the effect on endothelium-mediated vasodilation, as previously described, occurs before the onset of hypertension.

Material and Methods

Animals

The experimental protocol was approved by the Animal Experiments Committee of the Radboud university medical center and was performed according to the European guidelines on animal experiments. Male Wistar-Hannover rats (Harlan) were used in the experiments (n=28, 180-200g). Rats were housed in pairs in filter-top cages on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. Rats were randomly assigned to treatment for 7 days with either sunitinib 25 mg/kg or vehicle administrated by oral gavage. The content of sunitinib capsules, obtained from patients who discontinued therapy, was dissolved in hydrochloride (0.1 mmol/l) containing 0.5% polysorbate 80 and 10% polyethylene glycol 300 adjusted to pH 3.5-3.7 to a concentration of 12.5 mg/ml. On day 8 the rats were euthanized. Subsequently, the intestine and mesenteric vessels were removed and immediately immersed in

physiological salt solution (PSS, constituents in m M: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, EDTA 0.027, and glucose 5.5). Four to eight second order mesenteric resistance arteries (diameter 150–400 μm) per rat were dissected and mounted in a Mulvany wire myograph in PSS (Dual Wire Myograph model 400A; J.P. trading and the DMT multi-chamber wire myograph) and oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37°C. From the diameter-tension relation of the unstimulated vessels, the internal diameter was calculated and the vessels were set on the normalized diameter corresponding to 90% of the diameter at a transmural pressure of 100 mm Hg. Investigators were blinded for therapy allocation.

In at least two arteries of each rat the endothelium dependent vasorelaxation was determined after precontracting the arteries with U46619 (thromboxane A2 analog, Sigma-Aldrich, the Netherlands); after 25 minutes, a cumulative dose-response curve to acetylcholine (ACh, Sigma-Aldrich, the Netherlands) was obtained. In a subgroup of 14 rats, in at least two arteries from each rat, the endothelium independent vasorelaxation was determined by constructing a dose-response curve to the NO-donor sodium-nitroprusside (SNP, Riedel-de Haen, the Netherlands) after precontraction with U46619.

To determine the role of NO in the sunitinib-induced changes in endothelium dependent vasorelaxation, the ACh-induced relaxation was determined in a subgroup of 14 rats, in at least two arteries per rat, in the presence of the NO-synthase-antagonist L-NAME (10^{-4}M)(Sigma-Aldrich, the Netherlands).

Humans

After approval of the protocol (NCT01227213) by the Institutional Review Board of the Radboud university medical center a total of 10 patients with metastatic renal cell carcinoma (mRCC) starting treatment with sunitinib were recruited after written informed consent. Patients were eligible if they had a life expectancy of more than 12 weeks, a WHO performance status of 0-2 and no evidence of severe or uncontrolled diseases other than renal cell carcinoma. Patients treated with corticosteroids or oral anti-coagulants were excluded. A history of hypertension was not an exclusion criterium, but had to be controlled (<150/100 mmHg, the threshold for grade I toxicity according to the common terminology criteria of adverse events (CTCAE) version 3) before start of sunitinib. All studies were performed according to institutional and Good Clinical Practice guidelines. In the week before and one week after starting treatment with sunitinib (50 mg/day) the forearm blood flow (FBF) response to the endothelium-dependent vasodilator acetylcholine (ACh) and the NO-donor nitroprusside (SNP) was measured using venous occlusion plethysmography. Venous occlusion plethysmography is an well established method to measure changes in microcirculation and endothelial function[18,19]

The experiments were performed after at least 24 hours of caffeine abstinence, in the morning after an overnight fast and caffeine abstinence. Subjects were allowed to take their medication (including sunitinib) in the morning of the experiment with a cup of water. Subjects were studied in supine position in a temperature-controlled room (23±1°C). At the start of each experiment a 27-gauge needle (Braun Medical BV, Oss, the Netherlands) was inserted into the brachial artery of the non-dominant arm for intra-arterial administration of saline, ACh (Miochol, Thea Pharma NV, Zoetermeer, the Netherlands) and SNP (50 mg dry powder, Spruyt Hillen, the Netherlands; dissolved to its final concentration with 5% glucose solution; Clinical Pharmacy, Radboud university medical center).

During intra-arterial infusion of solvent (NaCl 0.9% for ACh and glucose 5% for SNP) or vasodilators, forearm blood flow (FBF) was measured at both arms simultaneously, using electrocardiogram-triggered venous occlusion mercury-in-silastic strain-gauge plethymography (Hokanson EC4, Hokanson, Inc). During all FBF recordings the hand circulation was completely occluded using wrist cuffs inflated to 200 mmHg. At least 30 minutes after the cannulation experiments were started. Drugs or solvent were infused at a constant rate of 50 μl/dl forearm/ min.

The experiment started with the measurement of baseline FBF during saline infusion during 5 minutes followed by 3 increasing dosages of ACh (0.5, 2.0 and 8.0 μg/dL forearm volume per minute) 5 minutes each. After a washout period of 30 minutes, FBF responses to glucose and 3 increasing dosages of SNP (0.06,0.2 and 0.6 μg/dL) were measured. Fifteen minutes after removal of the needle blood pressure was measured in the opposite arm in supine position using an automated blood pressure device (Welch Allyn Vital Signs 5300P).

Sunitinib measurement

Sunitinib concentrations were measured in the sunitinib-treated rats. In patients ,after analysis of the primary endpoints, sunitinib was measured in the plasma samples that were available after one week of treatment (n=6). Plasma samples were prepared according to the methodology adapted from Rodamer et al[20]. Plasma samples were deproteinized by addition of acetonitril containing Sunitinib-D10 (Toronto Research Chemicals inc,) as internal standard. Ten microliter of the supernatant is injected onto the LC-MS/MS system. Separation was performed with a Grace, VisionHT C18 column (1,5μm 50x2,0mm). The mobile phase, consisted of 66.6% acetonitril, 25% 20mM NH_4Ac pH 7.8, 8.3% methanol. For the mass spectrometric analysis, heated electrospray ionization (HESI) was operated at a spray voltage of +3kV, the capillary temperature and the vaporizer temperature were set at 250 and 325°C respectively. Argon was used as collision gas at a pressure of 1.5 mTorr. Positive ion mode was used with selected reaction monitoring (SRM) for the quantitation. The following SRM transitions were used: *m/z*

399.1(parent ion) to *m/z* 283.1 and 326.1 (both product ions) for sunitinib and *m/z* 409.4(parent ion) to *m/z* 283.2 and 326.2 (both product ions) for sunitinib-D10.

Statistical Analysis

Animals

Vascular contraction was determined as a percentage of precontraction (100%). The data are expressed as mean ± standard error of the mean; n indicates the number of animals. Values of the different arteries per rat were averaged. If less than two arteries of a rat in a single experiment met the predetermined quality criteria (precontraction to U46619 at least 8 mN) this rat was excluded from the analysis. Emax was defined as the contractile force at maximum relaxation to the vasodilator expressed as percentage of baseline. Comparisons between sunitinib treated and vehicle treated rats were made using the Mann-Whitney U test. P values < 0.05 were considered to be significant. Adequate curve fitting could not be achieved due to the attenuated relaxation in the sunitinib treated rats.

Humans

Data were analyzed using the SPSS (version 16.0) software packages. Values are reported as mean±SE unless otherwise specified.

Drug-induced effects were expressed as percentage change in FBF-ratio (infused arm/control arm) from preceding saline or glucose infusion. To this end, FBF ratios were calculated for each set of simultaneously measured FBFs separately. FBF-ratio's were averaged to one value for baseline (last 2 minutes) and for each vasodilator dose (last two minutes of each dose). For each dose the percentage change in FBF-ratio from baseline were calculated. After logarithmic transformation to obtain a Gaussian distribution, the effect of sunitinib was analysed using a repeated measures ANOVA (vasodilator dose and sunitinib therapy as within-subject factors). The effect of sunitinib on blood pressure was analysed using a paired student-T-test. Two-sided p-values < 0.05 were considered to indicate statistical significance.

Results

Animals

Body weights of vehicle and sunitinib treated rats were 265 ± 6 g (n=13) and 249 ± 8 g (n=13) ($p<0.05$) respectively. The diameter of the mesenteric resistance arteries used in this study was 272.6 ± 6.6 μm for vehicle rats (n= 55 vessels) and 250.1 ± 6.3 μm for sunitinib rats (n= 63 vessels) ($p<0.05$). Two rats, one in each group, died before day 7 for reasons not related to the treatment and were therefore excluded from the study protocol.

ACh-induced vasorelaxation

In 24 rats (12 on sunitinib) ACh induced vasorelaxation was analyzed. Maximum ACh-remaining vasoconstriction (Emax) in arteries precontracted with U46619 was significantly lower in control rats (11.1 ± 2% compared to sunitinib rats (35.4 ± 8.9%)($p<0.05$), see figure 1A.

ACh-induced vasorelaxation in the presence of L-NAME

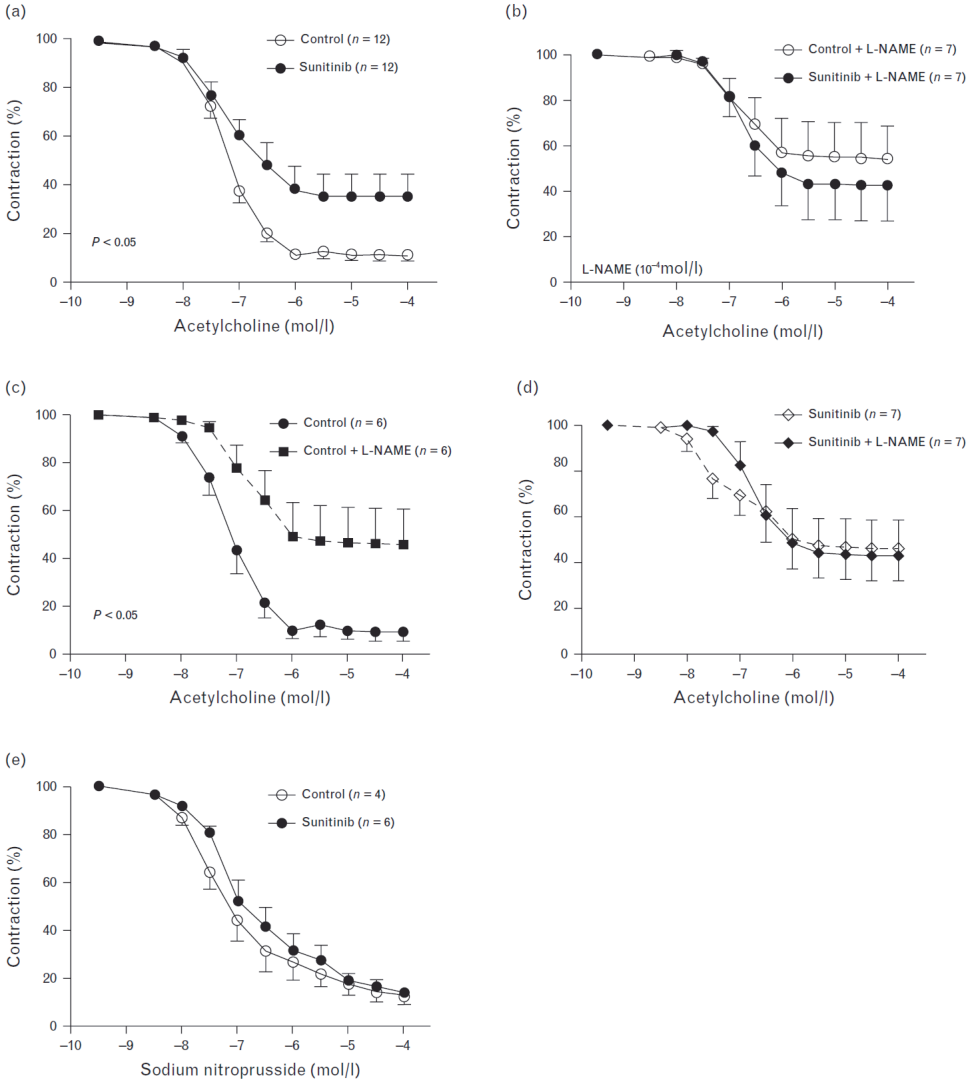
In a subgroup of 14 rats (7 on sunitinib) ACh relaxation was measured in the presence of the NO synthase antagonist L-NAME (10^{-4} M). In the control group L-NAME significantly attenuated the maximum response (Emax) to ACh (46.1 ± 14.2%) compared to ACh alone (9.5 ±3.6%) ($p<0.05$) in the same rats; figure 1C. In the sunitinib group L-NAME did not decrease the response to ACh (43.1 ± 14.6%) compared to ACh alone (46.4 ± 13.7) in the same rats (n=7); figure 1D. Emax values in response to ACH in presence of L-NAME were not significantly different between sunitinib and control groups. Thus, L-NAME significantly reduced the vasodilator response to ACh in the controls alone and not in the sunitinib treated rats. The difference in ACh-response between controls and sunitinib-treated animals disappeared after incubation of the vessels with L-NAME.

SNP-induced vasorelaxation

In a subgroup of 10 rats (6 on sunitinib) SNP induced vasorelaxation was measured. No differences were observed between groups; figure 1E.

FIGURE 1

Vascular responses in animal study. (a) Endothelium-dependent relaxation (ACH). (b) Endothelium-dependent relaxation (ACH) in presence of L-NAME. (c) Endothelium-dependent relaxation (ACH) with and without L-NAME in control rats. (d) Endothelium-dependent relaxation (ACH) with and without L-NAME treated rats. (e) endothelium-independent relaxation with SNP



Humans

10 patients signed informed consent and were included in the study. All subjects were male with a median age of 60 years (range 27-68 years); table 1. Six patients were already on antihypertensive medication before the inclusion in the study. The type of antihypertensive medication is shown in table 1. All patients had mRCC and sunitinib was the choice of treatment as decided by their oncologist.

TABLE 1
Baseline characteristics (mean ± SD)

Patient characteristics	Baseline	1 week	2 weeks
N	10	10	10
Age (mean ± SE)	55 ±12		
BMI (mean ± SE)	28.7 ± 5.7		
Hypertensive medication (n)	6	6	6
Calcium antagonist	4	4	4
Beta-Blocker	2	2	2
RAAS inhibitor	3	3	3
Thiazide diuretics	2	2	2
Blood Pressure > 140/90 mmHg	3	4	8
MAP (mmHg)	101.9 ± 12	106.2 ± 8.3	115.8 ±15.8
Systolic pressure (mmHg)	138.4 ± 14.6	140.3 ± 11.1	151.2 ± 20.8
Diastolic pressure (mmHg)	83.8 ± 12.7	89.1 ± 9.1	98.2 ± 14.7
Heart rate (bpm) (n=9)	67.6 ± 8.3	65.7 ± 9.4	NA
Baseline FBF (ml/dl)	1.6 ± 0.8	1.7 ±1.2	NA

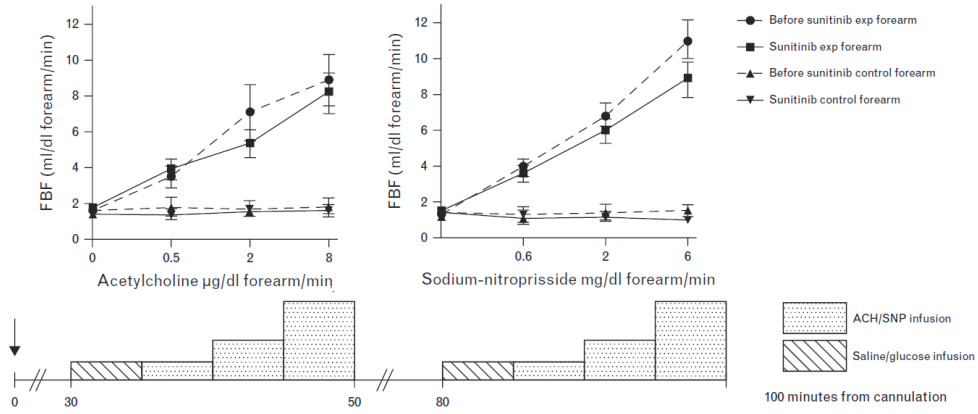
FBF, forearm blod flow; MAP, mean arterial pressure; RAAS, renin-angiotensin-aldostreine system

ACh-induced vasorelaxation

Before the start of sunitinib FBF increased in the infused forearm from 1.6±0.3 mL/dL at baseline to 3.5±0.6 mL/dL, 7.1±1,6 mL/dL and 8.9±1,4 mL/dL per minute for the 3 increasing dosages of ACh (0.5 and 2.0 and 8.0 μg ACh/dL forearm per minute), respectively. After one week treatment with sunitinib, ACh increased FBF from 1.7±0.4 ml/dl to 3.9±0.6, 5.4±0.8 and 8.2±1.1 mL/dL per minute, respectively (figure 2). In the absence of sunitinib, ACh increased median FBF-ratio (interquartile ranges) from baseline by 181 (49-321) %, 252 (103-885)% and 586 (191-1608)%. In the presence of sunitinib, ACh increased FBF ratio from baseline by 76 (25-388)%, 341 (25-454)% and 338 (24-1004)% respectively (n=10; *P>0. 1* for the effect of sunitinib,; figure 3). There is one outlier in the graph showing very high responses to both vasoactive substances (ACH and SNP) before the start of sunitinib. Exclusion of this outlier however does not change

the conclusion or interpretation of the experiments. For transparency we therefore included this patient in the analysis and graphs.

FIGURE 2
Study protocol and actual forearm blood flow in response to 3 increasing dosages of acetylcholine and sodium nitroprusside baseline and after 1 week of sunitinib treatment



SNP-induced vasorelaxation

Before the start of sunitinib FBF increased from 1.3 ±0.2 mL/dL at baseline to 4.0±0.4 mL/dL, 6.9±0.7 mL/dL and 11.0 ±1.2mL/dL per minute after three increasing dosages of SNP (0.6 and 2.0 and 6.0 µg SNP/dL forearm per minute), respectively. After one week of treatment with sunitinib, SNP increased FBF from 1.5±0.3mL/dL to 3.6±0.5mL/dL, 6.0±0.7mL/dL and 8.9±1.1mL/dL per minute, respectively (figure 3). In the absence of sunitinib, SNP increased median FBF ratio (interquartile ranges) from baseline by 183 (86-1288)%, 474(167-2039)% and 886 (357-4439)%, respectively. After one week of sunitinib, SNP increased median FBF ratio (interquartile ranges) from baseline by 202(128-399)%, 325(220-1171)% and 1050(269-2889)% (n=9; *P>0.1* for the effect of sunitinib; figure 4). In one patient it was not possible to perform the experiment with SNP due to repetitive dislocation of the needle.

FIGURE 3
Individual increases in forearm blood flow ratio (FBF-ratio) in response to 3 increasing dosages of ACh, depicted as percentage change from baseline. Below the horizontal axis in boxes the median FBF ratio and interquartile ranges. Statistical analysis was performed on the log-transformed data. Δ = patient 2

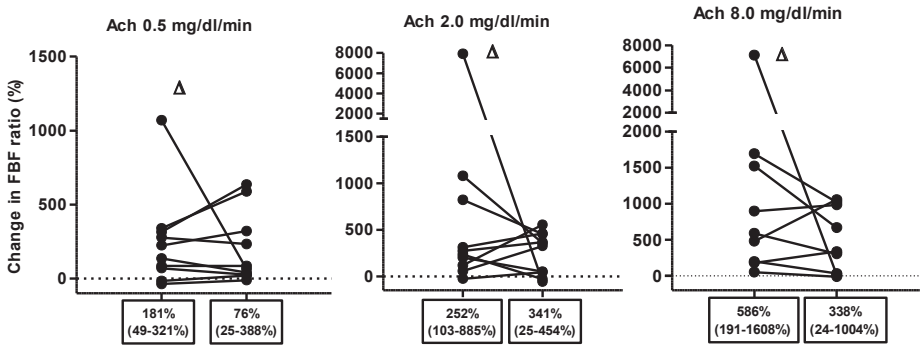
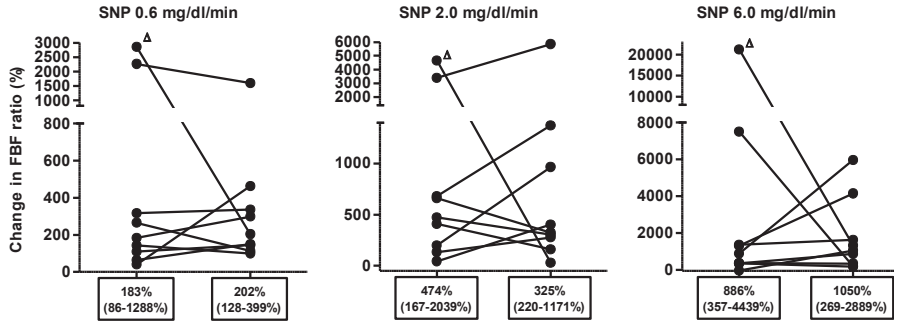


FIGURE 4
Individual increases in forearm blood flow ratio (FBF-rato) in response to 3 increasing dosages of SNP, depicted as percentage change from baseline. Below the horizontal axis in boxes the median FBF ratio and interquartile ranges. Statistical analysis was performed on the log-transformed data. Δ = patient 2



Blood pressure

In the 10 patients mean arterial pressure (MAP) before the start of sunitinib was 101.9±3.8 mmHg (mean ± SE). After one week of treatment with sunitinib the MAP increased to 106.2±2.6 mmHg ($p>0.1$,not significant). After two weeks of sunitinib treatment MAP was 115.9±4.9 mmHg a significant increase compared to baseline MAP ($p=0.02$) and compared to one week after start of sunitinib ($p=0.03$). MAP increased from baseline by more than 5 mmHg in two patients at week 1 and seven patients at week 2. After 2 weeks of treatment in total eight patients met the criteria of hypertension (> 140/90mmHg, International Society of Hypertension (ISH)), while all used the same antihypertensive medication as on baseline. At the visit at week 2, after recording of blood pressure, antihypertensive medication was changed or started by the treating oncologist to control hypertension.

Sunitinib concentrations

Total plasma sunitinib concentration in rats ($n=8$) was 134.4 ± 8.6 ng/ml after one week of treatment. In patients ($n=6$) after one week of treatment the mean concentration was 43.1 ± 8.1 ng/ml.

Discussion

Hypertension is an important side effect of VEGFR targeted therapies. In our studies we aimed to obtain insight in the early factors preceding and contributing to the development of hypertension. In the present study we focused on the effect of sunitinib on ACh-induced endothelium-dependent vasodilation as a reflection of endothelial function.

We show that in a sample of 10 patients who started sunitinib and in whom blood pressure increased in almost all at the end of 2 weeks of therapy, ACh induced endothelium-mediated vasodilation is not attenuated prior to the observed increase in blood pressure. Therefore impairment in endothelium-dependent vasodilation does not trigger sunitinib-induced hypertension.

In contrast to the human study, in rats treated for 7 days with sunitinib we did see a decreased endothelium-dependent vasorelaxation without a change in endothelium-independent vasorelaxation. Moreover in the rats we observed that the effect of sunitinib on ACh induced vasorelaxation was similar to the inihibitory effect of a NO-antagonist and that the difference in ACh-induced vasodilation between controls and sunitinib-treated animals disappeared in the presence of L-NAME. These observations are in agreement with previous studies showing the effect of VEGFR inhibition on NO synthase expression and NO-availability in pre-clinical studies[14,21].

In comparison with patients the sunitinib concentrations in our rats were higher and well above human therapeutic concentrations. Moreover previous studies have shown that rats treated with sunitinib 25mg/kg already have an increase of 20 mmHg in blood pressure after 2 days of treatment[22]. The observed endothelial dysfunction in rats could therefore be a consequence of hypertension rather than the preceding cause. This is supported by a previous clinical study conducted in patients after at least 6 weeks of treatment with a VEGFR inhibitor, when rise in blood pressure already had occurred, which showed a decrease in endothelium-dependent vasodilation[17]. This study is difficult to interpret however, since also endothelium-independent vasodilation was reduced by sunitinib suggesting that endothelium-independent actions of the angiogenesis inhibitor such as structural vascular changes. This is supported by our finding that the vascular diameter of the arterioles was smaller in sunitinib treated rats. However in our preclinical study it did not hamper endothelium independent vasodilation.

In a previous experiment in healthy volunteers we observed that bevacizumab, a monoclonal antibody against VEGF, does acutely attenuate the vasodilator response to ACh[15]. Selective interruption of VEGF signaling presumably has a different effect on vascular function than the less selective inhibition of multi-targeted kinase inhibitors such as sunitinib. This observation implies that vascular and hemodynamic effects induced by these TKIs are not solely dependent on interruption of VEGFR signaling but also on other targeted pathways .

Finally, some limitations of our studies should be mentioned. First, the number of included patients is rather limited and there is some variation in blood pressure and medication between patients before the start of the study. And moreover only male patients were included in this study. This is however a reflection of the general mRCC population[23] and given the invasive procedure in a fragile population we considered it not appropriate to extend the number of patients beyond the power calculation, which was based on previous studies in our center. Apart from the start of sunitinib no other medication was started or changed during the study period and blood pressure lowering medication at baseline did not prevent the occurrence of hypertension. In theory antihypertensive medication used by patients in our study could have reduced the impact of sunitinib on endothelial function, however blood pressure still increased in this patient group, not preceded by impaired endothelial function, indicating that the increase in blood pressure is not explained by an effect on endothelial function. Therefore co-medication did not hamper the interpretation of our study. We conducted this study to investigate early changes in endothelial function before the onset of hypertension. Our observations indicate that changes in endothelial function could play a role in sustaining or increasing the elevated blood pressure but not in the early development. Moreover, given the need to start active and standard treatment in patients with progressive metastatic disease, it was not deemed proportional to perform a placebo controlled study with the first aim of understanding the cause of hypertension

Perspectives

Future investigations should focus on other mechanisms than changes in endothelial function such as structural changes in the vascular bed or neurohormonal changes affecting the vascular system as a precursor of the development of hypertension.

Conclusion

In animals, this study confirms that exposure to high concentrations of sunitinib reduces endothelium-dependent vasodilation. As a novel finding, this study demonstrates that this endothelial dysfunction results from reduced endothelial release of NO. In humans however, reduced endothelium-dependent vasodilation does not precede the development of hypertension in patients treated with sunitinib. Therefore other factors should play a role as triggers for the development of hypertension.

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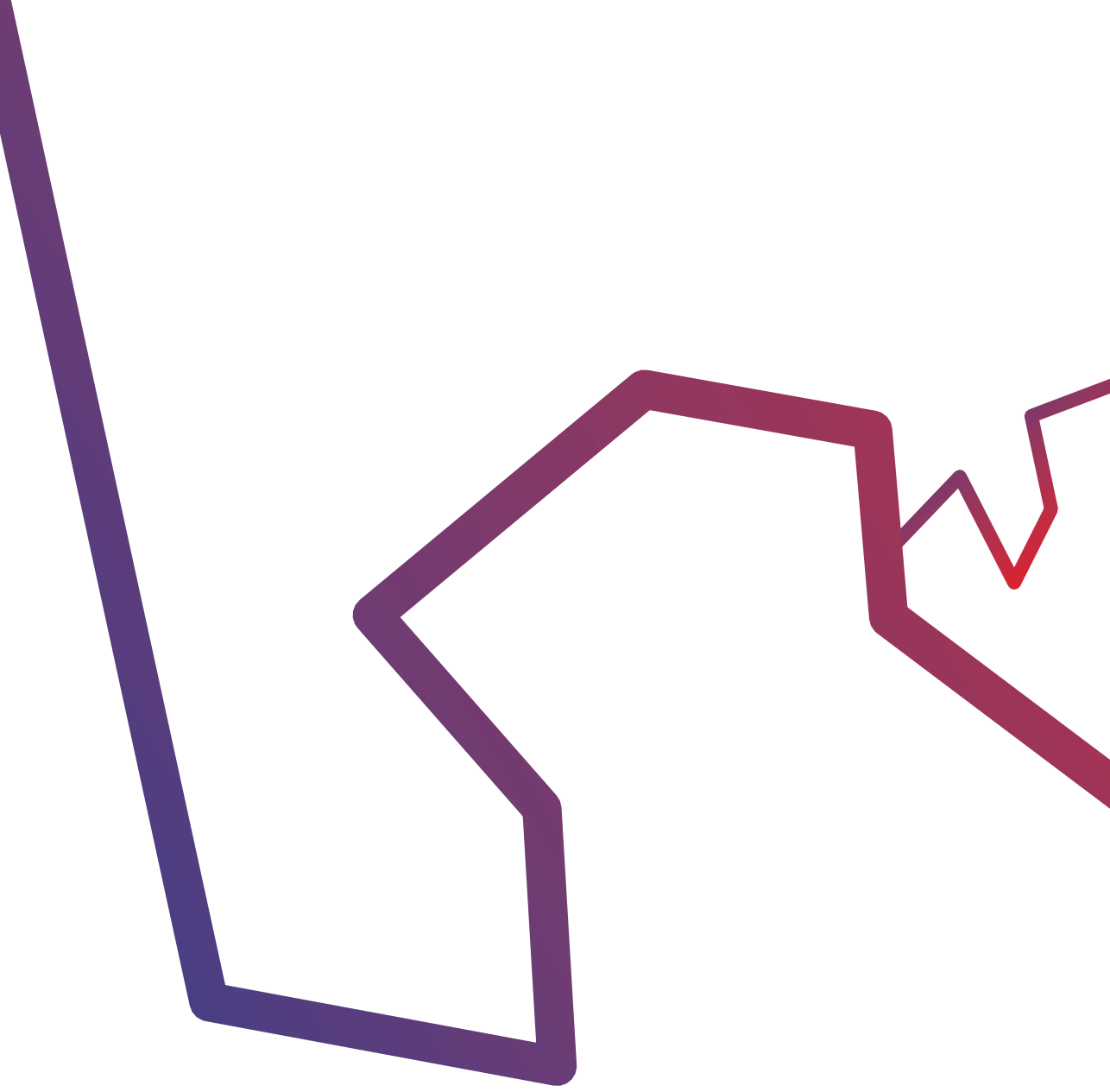


CHAPTER 5

Aldosterone may trigger the hypertensive response to sunitinib in patients with metastatic renal cell carcinoma

In preparation

A.M.J. Thijs, W.T.A. van der Graaf, J. Deinum, C.M. van Herpen, G.A. Rongen

Abstract

Introduction

Although hypertension is one of the most frequently reported side effects in patients treated with the VEGFR tyrosine kinase inhibitor sunitinib, its mechanism is still not well understood. It is unclear whether previously reported hormonal changes are a result or a cause of the rise in blood pressure. Therefore, we studied early effects of sunitinib on renin, aldosterone and endothelin plasma levels.

Methods

In 20 mRCC patients blood samples were obtained before and one week after the start of sunitinib 50mg per day, 4 weeks on-2 weeks off. Blood pressure (RR) was measured before, one and two weeks after the start of sunitinib.

Results

Eleven patients (55%) were on antihypertensive medication (AM) before the start of sunitinib. AM did not change in the first two weeks, except in one patient who needed an increase in dosage after 10 days. MAP increased from 100.1±2.4 at baseline to 104.6±2.0 at week 1 (not significant) and 110.1±3.4 mmHg at week 2 ($p<0.05$). Plasma endothelin increased from median 3.0 pg/mL (2.3-3.9) to 4.5 pg/mL (3.4-6,4) ($p<0.05$) at week 1 and aldosterone levels increased from median 0.25 nmol/L (0.18-0.30) to 0.39 nmol/L (0.24-0.53) ($p<0.05$) at week 1. Plasma renin was not significantly affected. The rise in MAP after two weeks was significantly correlated with the rise in aldosterone ($r^2= 0.56$; $p=0.01$) but not with endothelin ($r^2= 0.04$; $p=0.88$) at week 1.

Conclusion

Aldosterone/renin-ratio significantly increased after one week of sunitinib use, indicating an adrenal effect of sunitinib on aldosterone release. The rise in aldosterone is correlated with the increase in blood pressure after two weeks suggesting that aldosterone contributes to the early phase of hypertension in sunitinib-treated patients. Therefore antihypertensive medication targeting the aldosterone receptor, such as spironolactone, could be an option to treat sunitinib-induced hypertension.

Introduction

Tyrosine kinase inhibitors (TKIs) targeting the angiogenesis pathway have a key role in the treatment of several types of cancer(1, 2). Unfortunately these therapies are not without side effects. Sunitinib is an oral TKI, targeting VEGFR1-3, PDGFRA and PDGFRB, c-KIT , FLT3, CSF-1R, and RET. It is used widely as it has been approved for the treatment of metastatic renal cell carcinoma, gastrointestinal stromal tumor (GIST) and neuro-endocrine tumors of the pancreas.

A frequently reported side effect of VEGFR-TKIs is hypertension. Hypertension occurs in up to 80% of patients treated with sunitinib depending on the used definition for hypertension (3-5). Even more patients experience a rise in blood pressure(6). In patients with metastatic renal cell carcinoma, the anti-tumor effect of sunitinib, or other angiogenesis inhibitors, such as the monoclonal antibody against vascular endothelial growth factor (VEGF), correlates with the development of hypertension (5, 7, 8). At the same time, this hypertensive effect limits the use of these effective drugs as it may contribute to heart failure or cardiovascular events, which occur in up to 33% of the patients treated with blockers of VEGF-signaling(9). Therefore, it is important to treat sunitinib-induced hypertension promptly and to prevent organ dysfunction associated with its use.

The mechanism of VEGFR-TKI induced hypertension is not clear. Several studies regarding pre-eclampsia, a condition that is associated with the placental production of an endogenous scavenger of VEGF thus mimicking pharmacological VEGF-inhibition, have shown the involvement of the renin-angiotensin system in development of hypertension in pregnancy(10, 11). On the other hand, recent observational data in patients with pre-eclampsia suggest involvement of endothelin-1 as well, paralleled with suppression of the renin-angiotensin system(12). In contrast to pre-eclampsia, however, there is currently no evidence for the involvement of the renin-angiotensin-aldosterone system in the mechanism of sunitinib-induced hypertension. A previous study showed the activation of the endothelin system without significant change in aldosterone as measured after 4 weeks of treatment with sunitinib (13). In rodents, endothelin receptor antagonists reduce the increase in blood pressure associated with sunitinib therapy (14). So, currently endothelin seems more important than aldosterone in sunitinib-induced hypertension, providing a strong case for endothelin receptor antagonists to be explored as a treatment for sunitinib-induced hypertension and organ damage in humans. However, endothelin receptor antagonists are expensive and pharmacokinetic interactions with sunitinib can occur as they can affect CYP3A4 metabolism(15). In humans, hormonal changes early in the treatment with sunitinib, before the full hypertensive response has developed, are not yet known. These early neurohumoral changes are important as they better reflect the direct actions of sunitinib as

opposed to secondary neurohumoral changes that could result from changes in blood pressure. Since angiotensin II may induce endothelin release(16) we hypothesize that sunitinib activates the renin angiotensin system early after start of treatment and thereby serves as a trigger of hypertension and the increase in endothelin-1. We performed a clinical study in mRCC patients, who started treatment with sunitinib, to investigate the early changes in aldosteron, renine and endothelin and their correlation with the onset of hypertension.

Methods

After approval of the protocol by the Institutional Review Board of the Radboudumc and prospective registration at www.clinicaltrials.gov (NCT01227213) 20 patients with metastatic renal cell carcinoma in which sunitinib was the treatment of choice signed informed consent for one of two sub-studies. In the first 10 patients, the impact of sunitinib on endothelial function of the forearm microcirculation was studied(17). In the subsequent 10 patients, the effect of sunitinib on insulin-sensitivity was explored(18). In all these patients, plasma was sampled to measure renin, aldosterone and endothelin.

Patients using corticosteroids, oral anti-coagulants or any evidence of severe or uncontrolled diseases other than renal cell carcinoma were excluded. All patients had a WHO performance status 0-2 and a life expectancy of more than 12 weeks at time of inclusion.

Patients were followed during the first two weeks of treatment with sunitinib which was taken according to a 4 weeks 'on'/ 2 weeks 'off' regimen with a starting dose of 50mg per day. In case of adverse events, according to the judgment of the patient's oncologist, the dose could be decreased. If blood pressure increased above 140/90 mmHg antihypertensive treatment was initiated or adjusted.

Blood pressure was measured before the start of sunitinib (baseline) and one week (7-11 days; week 1) after the start of sunitinib in a quiet room in supine position after a period of at least 5 minutes rest using an automated device (Welch Allyn Vital Signs 5300P). At two weeks after the start of sunitinib blood pressure was measured at the outpatient clinic in sitting position using an automated device (Welch Allyn Vital Signs 5300P).

Blood samples for laboratory measurements were obtained from an intravenous line at baseline and week 1, in the morning after an overnight fast in supine position after a period of rest of at least 5 minutes.

Biochemical measurements

Plasma renin concentration was measured by an immunoradiometric assay (Cisbio), aldosterone by radioimmunoassay after extraction with dichloromethane and subsequent paper chromatography and endothelin (ET-1) by chemiluminiscent ELISA (Quantiglo®, R&D Systems;intra-assay coefficient of variation 4%).

Data analysis

Data are presented as mean±SEM or median and interquartile ranges as appropriate. Statistical analysis of blood pressure was performed by one way ANOVA followed by Tukey's multiple comparison testing. Biochemical measurements were analyzed by Wilcoxon rank sum test, considering not all values were distributed normally. For correlation analysis the non-parametric Spearman coefficient was used. $P < 0.05$ was considered significant. GraphPad Prism version 5.03 was used for all statistical analysis.

Results

Twenty mRCC patients signed informed consent and were included in this study. Subjects had a median age of 59 years (range 27-72 years); table 1. One female patient was included. Eleven patients were already on antihypertensive medication before the inclusion in the study. The type of antihypertensive medication is shown in table 1.

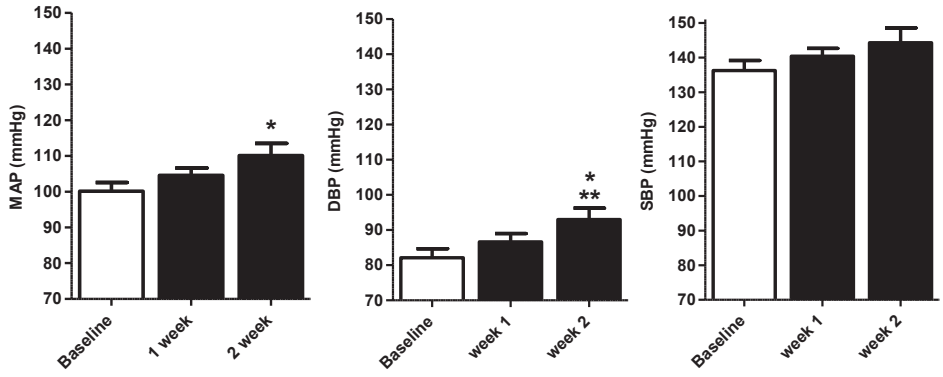
TABLE 1
Patient characteristics

Patient characteristics	Baseline	1 week	2 weeks
N	20	20	20
Age (median±range)	59 (27-72)		
BMI (mean±SE)	27.9 ± 1.0		
Hypertensive medication (n)	11	11	11
Calcium antagonist	5	5	5
Beta-blocker	4	4	4
RAAS inhibitor	6	6	6
Thiazide diuretics	4	4	4
Diastolic BP >90 and/or systolic BP >140 mmHg	6	10	13

Blood pressure

Mean arterial pressure (MAP) before the start of sunitinib was 100.1±2.4 mmHg (mean ± SE). After one week of treatment with sunitinib the MAP increased to 104.6±2.0 mmHg (not significant). After two weeks of sunitinib treatment MAP significantly increased from baseline to 110.1±3.4 ($p<0.05$). At week 2 MAP had increased from baseline by more than 5 mmHg in 13 patients (65%). Diastolic blood pressure (DBP) increased from 82.1±2.6 mmHg to 86.7±2.4 at week 1 (not statistically significant) and 93.0±3.2mmHg at week 2 ($p<0.05$ compared to baseline and week 1). Systolic blood pressure (SBP) did not change significantly After 2 weeks of treatment in total thirteen patients met the criteria (SBP > 140 mmHg or DBP > 90 mmHg) of hypertension , while all used the same antihypertensive medication as on baseline, except for one patient who had an increase in ramipril from 2.5mg to 5mg on day 10 to control hypertension. At the outpatient clinic at week 2, after recording of blood pressure, antihypertensive medication was changed or started by the treating oncologist to further control hypertension.

FIGURE 1
Blood pressure in response to sunitinib treatment *P<0.05 compared to baseline. **P< 0.05 compared to week 1.

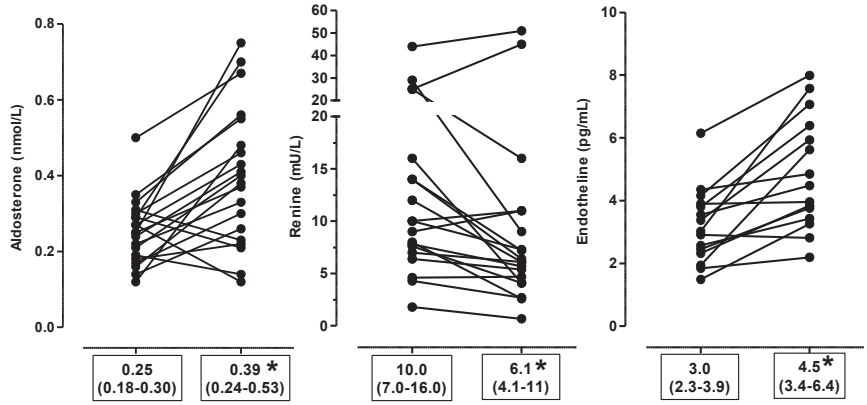


Biochemical measurements

In 80% of patients aldosterone increased after one week of treatment combined with a decrease in renin in also 80% of patients (16/20) (Figure 2). Endothelin was only measured in 15 patients, due to missing samples. Endothelin increased in 93% of patients (14/15) (figure 2).

5

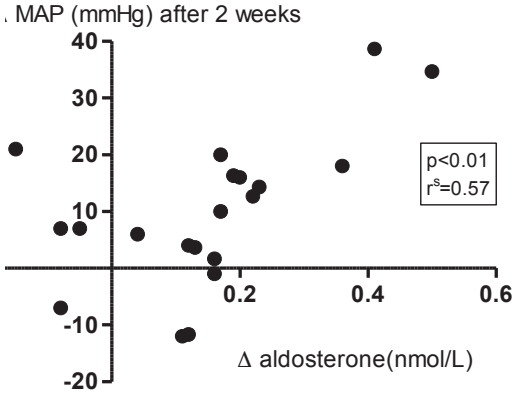
FIGURE 2
Individual values of biochemical measurements before and after 1 week of sunitinib treatment. In the boxes below the graphs median and interquartile ranges are depicted.
Aldosterone (n=20); renin (n=19); endothelin (n=15) *P<0.05 comparend to baseline



Correlation blood pressure and plasma aldosterone and endothelin

The rise in aldosterone at week 1 was correlated with the increase in blood pressure from baseline (both MAP and DPB) at week 2. This correlation was not found for the rise in endothelin and blood pressure (r^2= 0.04 ; p=0.88).

FIGURE 3
Correlation of delta aldosterone (baseline - week 1; n=20) with delta MAP at 2 weeks. Correlation of delta aldosterone with blood pressure analyzed in the subgroup of 15 patients, in whom endothelin was measured was also significant (P<0.01; r^2 =0.77)



Discussion

We showed, in a sample of 20 patients one week after start of sunitinib a significant rise in aldosterone and endothelin. Aldosterone, but not endothelin, was correlated with the subsequent increase in blood pressure after two weeks of treatment.

Aldosterone is the major mineralocorticoid, regulating electrolyte balance in the human body. It is known that overproduction of this hormone leads to hypertension and cardiac failure(19, 20). Two other clinical studies have not shown a rise in aldosterone during treatment with a VEGFR inhibitor (13, 21). An important difference between these studies and our study is the timing of sampling. We studied the effect on aldosterone after one week of treatment to study the factors

involved in the initiation of hypertension, while in the study of Kappers et al. blood samples were not taken until week 4, a time point at which hypertension already had occurred. As in our study Kappers et al reported a decrease in renin concentration after start of sunitinib. This is also supported by Curwen et al who showed a decrease in plasma renin activity in rats exposed to a VEGFR-inhibitor(22). This decrease in renin concentration could be caused by an increase in aldosterone. The etiology of the observed increase in aldosterone is not easily explained. In mice exposed to a VEGF-A antibody a rise in angiotensin II concentrations has been reported(23). As angiotensin-II stimulates the production of aldosterone via the angiotensin-II receptor this could explain the rise in aldosterone found in our study. However some other studies contradict these findings, as ACE-inhibition did not prevent hypertension in animals treated with a VEGFR inhibitor (14). Another possible mediator of the increased aldosterone concentration could be an increase in ACTH(24) or reduced clearance of aldosterone which in theory, could also result in a (transient) increase in aldosterone.

Besides the rise in aldosterone we observed a rise in endothelin after one week of treatment with sunitinib. This is in line with the findings of Kappers et al. after four weeks of sunitinib. (13). Endothelin-1, a protein produced by vascular endothelial cells, promotes vasoconstriction and is increased in hypertension(25, 26) In our study we did not find a correlation between the rise in endothelin and the rise in blood pressure. Therefore, we postulate that the rise in endothelin is secondary to the rise in aldosterone and/or blood pressure and that endothelin sustains rather than initiates the increase in blood pressure during sunitinib treatment. Several studies support this hypothesis, as aldosterone seems to up regulate endothelin-1 gene expression(27-29). In vitro VEGF stimulates the expression of prepro-endotheline-1 and secretion of endothelin-1 in human umbilical vein endothelial cells(30). Consequently, VEGFR-inhibition should decrease rather than increase plasma endothelin, as was indeed shown after start of sunitinib (13). Therefore, our observed increase in endothelin-1 does not seem to be a direct consequence of VEGFR inhibition, but should be secondary to other mechanisms such as increased plasma levels of aldosterone.

Finally, some limitations of our research should be mentioned. First, before the start of sunitinib already half of the patients included were on antihypertensive medication. Thirty percent of patients used an angiotensin converting enzyme inhibitor or an angiotensin-II receptor antagonist, which influence aldosterone release. However apart from the start of sunitinib no other medication was started or changed during the study period and blood pressure lowering medication at baseline did not prevent the development of hypertension. Furthermore, if anything, this medication should have blurred any relation between aldosterone and blood pressure. Therefore co-medication does not likely explain our observation. Second, non-specific time effects could have

confounded our observations. Given the need to start effective standard treatment in patients with progressive metastatic disease as soon as possible, it was not considered appropriate to perform a placebo controlled study with the first aim of understanding the pathogenesis of sunitinib-induced hypertension. Third, unfortunately our sampling method did not allow us to measure concentrations of angiotensin, which could have provided more insight into the renin-angiotensin system. For the same reason it was not possible to measure catecholamines to provide insight into the potential involvement of the sympathetic nervous system.

Perspectives

Future investigations could focus on mechanisms of sunitinib-induced aldosterone increase. Moreover, in patients treated with sunitinib, the application of aldosterone receptor antagonists for the treatment of sunitinib induced hypertension and their potential role in the prevention of cardiac damage should be studied.

Conclusion

Aldosterone/renin-ratio significantly increased after one week of sunitinib use, indicating an effect of sunitinib on aldosterone exposure. The rise in aldosterone is correlated with the increase in blood pressure after two weeks suggesting that aldosterone triggers hypertension in sunitinib-treated patients. Therefore, antihypertensive medication targeting the aldosterone receptor, such as spironolactone, could be an adequate treatment option to prevent or treat sunitinib-induced hypertension and related organ damage.

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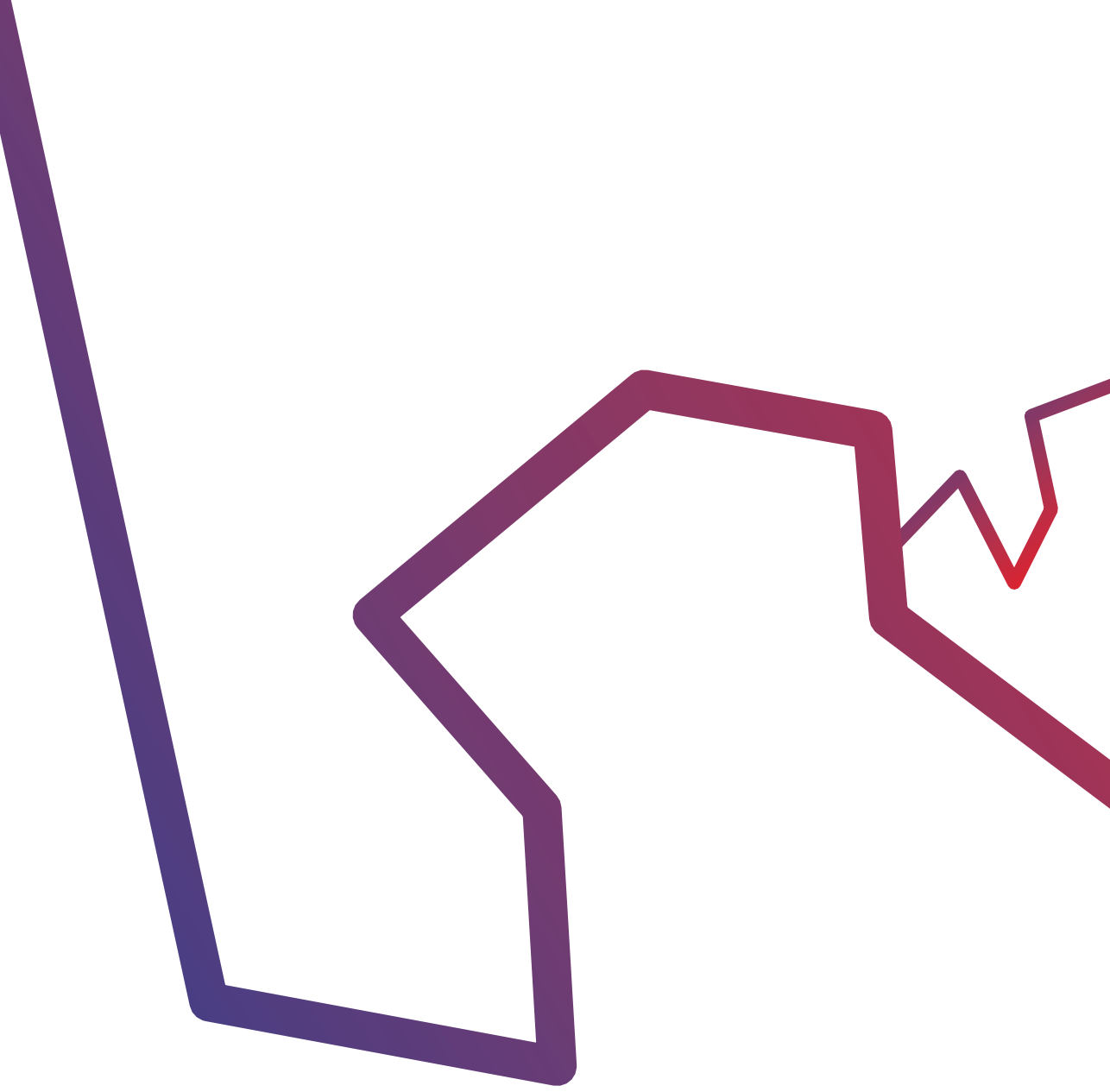
METABOLIC EFFECTS OF ANGIOGENESIS INHIBITORS



CHAPTER 6

Weight loss induced by tyrosine kinase inhibitors of the VEGF pathway

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Abstract

Weight loss, cachexia and sarcopenia are profound problems in the frail oncologic patient. With the development and increasing use of angiogenesis inhibitors in metastatic cancer patients, the question raises what their influence is on body weight and composition. Angiogenesis is not only important for the growth, development and metastatic potential of tumors, but also for physiologic processes in adipogenesis. A less known approach of angiogenesis inhibitors is their experimental use in obese models. This review focuses on the effects on body weight and composition of angiogenesis inhibitors, especially of those targeting the vascular endothelial growth factor (VEGF) pathway.

Introduction

Cancer, body weight and body composition are strongly interrelated. Weight loss occurs in 30-80% of cancer patients, with a severity depending on the type of tumor.[1] Weight loss is the result of an imbalance between energy intake and energy expenditure. Factors that contribute to decreased intake include anorexia, nausea and vomiting, constipation, diarrhea, pain, altered taste and depression. Besides this imbalance, cancer patients are at risk to develop cachexia. Cancer cachexia is a profound metabolic process characterized by breakdown of the muscles and abnormalities in fat and carbohydrate metabolism despite adequate nutritional intake. This debilitating and life-threatening paraneoplastic phenomenon is present in about 50% of cancer patients, most markedly in patients with lung or upper gastrointestinal cancers.[2] Furthermore, cancer patients are at risk to develop sarcopenia; a severe depletion of skeletal muscle. Sarcopenia is related to poor functional status, poor treatment response and reduced overall survival.[3;4]

Weight loss in cancer patients can be a direct effect of the disease but also be a side effect of treatment. Weight loss indeed is a common side effect of most types of anti cancer therapy.[5-8] However, clear insights in the prevalence and amount of weight loss due to the different anti cancer therapies are lacking. The degree of weight loss as an adverse event of anti cancer therapy can be scored according to the Common Toxicity Criteria Adverse Event (CTCAE criteria) [9]. These criteria score weight loss in grade 1 to 3, in which grade 1 is defined as 5-10% weight loss, without need for an intervention, grade 2 as 10-20% weight loss or nutritional support indicated and grade 3 is defined as weight loss of more than 20% compared to baseline body weight, or an indication for transparenteral nutrition or tube feeding. In study reports, little attention is paid to grade 1 and 2 toxicities. But grade 1 and 2 weight loss may already be substantial and clinically relevant, especially in the frail oncologic patient. As little as 5% weight loss alters measurable physiological parameters such as immune response, lung and cardiac function tests and autonomic autoregulation.[10] The mechanisms by which weight loss occurs during chemotherapy are not clear. Of course, anorexia, nausea, vomiting and diarrhea will contribute to weight loss during chemotherapy. Both weight loss prior to start as well as weight loss as an adverse event of chemotherapy seem to be an indicator for poor prognosis.(1) For example, patients with advanced ovarian cancer who suffered from weight loss during chemotherapy had a poorer overall survival compared to patients who gained weight.[11] However, not all cancer patients treated with chemotherapy lose weight, and some regain weight shortly after finishing their chemotherapy. Furthermore, some therapies, especially hormonal therapies, can provoke weight gain.[12-14]

With the development and increasing use of angiogenesis inhibitors, the question raises whether these targeted drugs also have such systemic adverse events of weight loss or changes in body composition. Angiogenesis is not only important for the growth, development and probability to metastasize of tumors, but also for physiologic processes in adipogenesis.[15] A less known, but interesting, approach of angiogenesis inhibitors is their (experimental) use in obesity. This review focuses on the effects on body weight and composition of angiogenesis inhibitors, especially of those targeting the vascular endothelial growth factor (VEGF) pathway. In one way this may be an unwanted side effect of anti cancer therapy but in another way it may be regarded as an innovative approach for the development of new treatment strategies of obesity.

Angiogenesis inhibitors and weight loss in cancer

Tumors are dependent on angiogenesis for growth and metastasis. They trigger the development of their own blood supply by disrupting the delicate balance of pro angiogenic and anti angiogenic factors. Pro-angiogenic gene expression is increased by physiological stimuli, such as hypoxia. Oncogene activation or tumor suppressor genes inactivation can tip the balance in favor of pro-angiogenic factors. Examples of pro angiogenic factors are vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) and placental growth factor (PlGF). VEGF and its receptors play a pivotal role in both normal and malignant angiogenesis. Activation of the VEGF pathway leads to endothelial cell activation, proliferation and survival. Moreover degradation of the basement membrane is necessary for endothelial cell migration and invasion, increased vascular permeability, and mobilization of endothelial progenitor cells (EPCs) from the bone marrow into the peripheral circulation.[16] VEGF is expressed in response to hypoxia, oncogenes or cytokines. Higher levels of VEGF (among other growth factors and cytokines) have been associated with more aggressive tumors, more accentuated weight loss and nutritional intake reductions concomitantly with higher resting energy expenditure compared to control patients.[17]

The VEGF pathway is considered to be the most important and best explored pathway in angiogenesis of tumors. Multiple treatment strategies, targeting VEGF as well as its receptor (VEGFR) and downstream signaling elements of this pathway, have been developed. Examples are bevacizumab, a monoclonal antibody against VEGF, used for the treatment of metastatic colorectal cancer and breast cancer and sunitinib and sorafenib, both VEGFR tyrosine kinase inhibitors used predominantly in the treatment of metastatic renal cell cancer.

Less is known about the effect of VEGF tyrosine kinase inhibitors (TKI) on body weight, since only severe weight loss is reported according to the CTCAE criteria.(Table 1) Even in case of the approved, and frequently applied, VEGFR TKIs (such as sunitinib, sorafenib and pazopanib),

data about weight loss are limited. Weight loss was reported for sorafenib (inhibitor of Raf-1, B-Raf, VEGFR, PDGFRβ, fms-like TK-3 and c-KIT) compared to placebo in patients with metastatic renal cell carcinoma.[18] In this study the sorafenib treated patients experienced significantly more diarrhea, without significant differences in nausea and anorexia.(18) Another study which combined sorafenib with interferon alpha in patients with metastatic renal cell cancer observed all grades weight loss in 63% of the patients.[19] In a placebo controlled trial with sorafenib in more than 600 hepatocellular carcinoma patients, significant more weight loss in the sorafenib treatment group was observed compared to the placebo.[20] In a phase II study of sunitinib (inhibitor of VEGFR1-3, PDGFRα and β, fms-like TK-3, KIT, colony stimulating factor receptor type 1 and neurotrophic factor receptor) in non small cell lung cancer (NSCLC), anorexia and decreased weight were reported together (gr 1-2: 30%, gr 3: 5%).[21] A phase I study which combined sunitinib with bevacizumab reported grade 1-2 weight loss in 12% of the participating patients.[22] Other trials using sunitinib did not report weight loss.[23;24]. In conclusion, in the majority of trials weight loss was not mentioned. In those trials that did report weight loss, the course of the weight loss after finishing study treatment was not mentioned nor was the effect of weight loss on quality of life, treatment outcome or prognosis.

An interesting potential new indication for treatment with a VEGFR inhibitor (especially sorafenib) is metastatic medullary, follicular and papillary thyroid cancer. In a phase II study with patients with medullary thyroid cancer, grade 1-2 weight loss was reported in 48% of the patients treated with sorafenib. [25]Another phase II study in which patients with metastatic papillary thyroid cancer were treated with sorafenib reported weight loss grade 1-2 in 58 to 89% of the patients and grade 3 weight loss in 5% of the patients.[26] The M.D. Anderson retrospectively reported their experience with sorafenib or sunitinib treatment in patients with dedifferentiated thyroid cancer. The result for weight loss and anorexia are presented together and were found in 20% of the patients.[27] Of further notice is the fact that hypothyroidism, causing weight gain instead of weight loss, is reported as an adverse event of both sorafenib and sunitinib in up to 85% of the metastatic renal cell cancer patients, with the requirement of replacement therapy in only a minority of them. [28;29]

To obtain more insights in the time course of weight loss we collected data from four phase I-II studies concerning approximately 70 patients who were treated with a tyrosine kinase inhibitor against VEGFR1-3. After 2 months of treatment we observed a mean weight reduction of 5.1% (range -15.8% to +5.2%), and at the end of study a mean weight reduction of 8.5% (range -23.8% to +5.2%). The number of patients who experienced a weight reduction of more than 5% was 45% after 2 months, and 69% at the end of the study. This significant weight loss was usually already present after 2-4 weeks of treatment. The weight loss was more than clinically

expected based on the limited number of patients complaining about anorexia, nausea and diarrhea complaints. Importantly, after discontinuation of the VEGFR tyrosine kinase inhibitors regain of body weight within a couple of weeks was observed. These observations suggest that treatment with VEGFR tyrosine kinase inhibitors is associated with disproportionate weight loss.

Apart from weight loss, changes in body composition have recently been reported.[30;31] In a subanalysis of the TARGET trial (phase III study comparing sorafenib to placebo in renal cell carcinoma patients) a significant loss of weight (-2.1 kg vs +0.8 kg, $p<0.01$) and skeletal muscle (-7.4 cm^2 vs -3.1 cm^2, $p=0.02$) was reported in the sorafenib group during the first 6 months of treatment. Changes in adipose tissue were similar but not significant ($p=0.3$).[30] After one year treatment with sorafenib, patients had lost 4.2 kg body weight, 12.1cm^2 of total muscle area and 33.1 cm^2 of adipose tissue area ($p<0.01$) as assessed on CT. Baseline sarcopenia was present in 52.5% of all patients, including 72% of the patients with a BMI<25 and in 34% of those with a BMI>25 kg/m^2. Women were more sarcopenic then men (65% and 48% respectively). After one year of treatment with sorafenib, 71% of patients (+18.5%) met the criteria for sarcopenia. (30) Low BMI and sarcopenia were associated with dose limiting toxicity of sorafenib.[31] Tumor progression and sorafenib are both potentially related to progressive loss of weight and muscle, however when correcting for tumor response on sorafenib treatment, no significant changes were found.[30]

Important adverse events of angiogenesis inhibitors are anorexia, nausea, stomatitis and diarrhea. By influencing energy intake and energy expenditure, all these adverse events can cause weight loss.[32] However, most of these adverse events are less common reported for angiogesis inhibitors compared to conventional chemotherapy. Moreover, edema and hypothyreodism are side effects of angiogenesis inhibitors that can contribute to weight gain. Therefore, it is hard to belief that only adverse events as anorexia, nausea, stomatitis and diarrhea are the explanation for the pronounced weight loss observed in cancer patients treated with angiogenesis inhibitors. This suggests that angiogenesis inhibitors may have a direct effect on body weight, beyond side effects and anti-tumor effects.

The role of angiogenesis in adipogenesis

Healthy adults have a stable mass of adipose tissue and the supporting vasculature is quiescent.[33] Adipose tissue can grow and regress during adult life. It has been hypothesized that this non-neoplastic adipose tissue growth is dependent on neovascularization.[34] Adipose tissue is highly vascularized, with an extensive capillary network nourishing each adipocyte.[35;36] Adipose tissue is considered being the largest endocrine gland because it produces free fatty acids, hormones, growth factors, and cytokines.[37] Several of these adipose tissue derived

products influence angiogenesis, with a delicate balance between pro-angiogenic factors, e.g. VEGF, fibroblast growth factor (FGF), IGF, tumor necrosis factor alpha (TNF-α), transforming growth factor (TGF-β), EGF, resistin and leptin, and anti-angiogenic factors, e.g. adiponectin, endostatin, thrombospondin 1 (TSP-1), and soluble VEGFR2.(15) As is the case in cancer, hypoxia in adipose tissue induces high levels of hypoxia-inducible transcription factor (HIF), which increase the expression of angiogenesis related factors including VEGF and downregulate several endogenous angiogenesis inhibitors.[38-40] A disturbance in this balance, either pathophysiologically or due to medical interventions, can cause changes in angiogenesis.

Compared to lean mice, nutritionally induced or genetically determined *ob*/*ob* obese mice have significantly larger subcutaneous and gonadal fat pads, accompanied by significantly higher blood content, increased total blood vessel volume and a high number of proliferating cells, which emphasizes the role of angiogenesis in the process of adipogenesis.[41] In several animal studies VEGF is the most important angiogenic factor in adipogenesis and inhibition of VEGF is associated with weight loss and metabolic changes. VEGF-A is highly expressed in rat adipose tissue and its expression increases significantly during adipocyte differentiation.[42-44] In rats, the omentum was found to have the greatest VEGF secretion and the omental adipocytes were the primary source of the VEGF. Incubation of omental adipocytes under hypoxic conditions induced an increase in VEGF expression.[45] These findings suggest an important connection between adipose tissue, VEGF and hypoxia.

Effects of VEGFR tyrosine kinase inhibitors on adipose tissue and in obesity

In a model with murine adipocytes implanted in dorsal skin chambers treatment with an antibody to the VEGFR-2 blocked the development into adipose tissue with inhibition of both angiogenesis as well as subsequent vessel remodeling.[46](Table 2) After 7 days of treatment, a significant lower vessel density compared to the control group was observed. Vatalanib (PTK787/ZK222584, a VEGFR tyrosine kinase inhibitor) treatment for four weeks of mice on a high fat diet resulted in a significant reduction of body weight and of subcutaneous and gonadal adipose tissue mass, without significant changes in blood vessel size and density.[47] Vatalanib also reduced adipose tissue development. No effect of vatalanib on blood glucose and insulin levels was found. In two earlier studies with vatalanib in nude mice on standard diet, no decrease in body weight was observed.[48;49] A third study, using vatalanib in a murine renal cell carcinoma model showed anti tumor activity as well as changes of bodyweight.[50] In another study, two weeks treatment with a monoclonal anti-VEGF antibody of *db*/*db* mice did not result in a significant difference in bodyweight, although the treated mice tended to gain less weight compared to the control group. At the cellular level, anti-VEGF treatment markedly inhibited formation of smaller differentiating adipocytes as well as formation of blood vessel sprouts and adipogenic/angiogenic cell clusters.

Also, the number of adipocytes was significantly reduced.[51] This effect on adipose tissue was also noticed in the patients treated with sorafenib for one year in the TARGET trial.[30]

The sarcopenia observed in patients treated with sorafenib may also be associated with the inhibition of VEGFR.[30] Inhibition of VEGFR by sorafenib in a variety of cells has been shown to result in downstream inhibition of PI3K, AKT, and mammalian target of rapamycin (mTOR). These elements are central to the activation of muscle protein synthesis by amino acids and other stimuli. The Akt/mTOR pathway is upregulated during hypertrophy and downregulated during muscle atrophy.[52;53] Phosphorylation of mTOR also results in the activation of amino acid transporters.[52;53] In this way, sorafenib treatment, has a direct inhibitory effect on protein synthesis and it limits the stimulating effect of amino acids.[54] Induction of muscle anabolism by physical activity occurs by pathways involving RAF, MEK, and MAPK/ERK kinases. This pathway is also inhibited by sorafenib.[55]

Although the observations listed above suggest that inhibition of angiogenesis may reverse obesity, angiogenesis inhibitors have not yet been applied to treat human obesity. One of the big hurdles to overcome is the observed severe side effect of sarcopenia, as are other frequently occurring adverse events which impair chronic use in obese patients. These adverse events include cardiovascular disorders[56;57], at which obese people already have an elevated risk. Furthermore, drug resistance may develop.[58] At last, knowledge about the long term adverse events of angiogenesis inhibitors is lacking. Nevertheless, the findings suggest that further research into this area is warranted.

Conclusion

Both in cancer and obesity, two totally distinct but major health problems, tyrosine kinase inhibition can be an important shared target for treatment.

Based on limited data in literature and own findings, we hypothesize that tyrosine kinase inhibitors may be an innovative approach for the development of new treatment strategies for obesity, one of the biggest threats to human health. Although the pathophysiological connection between angiogenesis and adipogenesis is well recognized, until now, only limited research has focused on treatment of obesity with angiogenesis inhibitors. VEGF(R) seems to play a central role in both angiogenesis as well as adipogenesis, although other targets of the described multityrosine kinase inhibitors can not be excluded to contribute also to these processes.

In case of cancer, weight loss has huge consequences for the frail oncologic patient, not in the least on quality of life. It is important to stress that tyrosine kinase inhibitors are more and more applied as chronic treatments in cancer patients. Better insights into the severity, the impact and the mechanism of weight loss due to angiogenesis inhibitors in cancer patients are essential for the development of preventive measures and treatment of this side effect.

TABLE 1.
Weight loss as adverse event of tyrosine kinase inhibitors in human studies.

Ref	Type of study	Target	Finding
[18]	Sorafenib vs placebo in mRCC patients	VEGFR, PDGFRβ, FLT-3, c-KIT	Sorafenib induced weight loss (all grades 10 vs 6%, grade 2; 5 vs 3% (significantly different), grade 3-4 <1 and 0%).
[19]	Sorafenib and IFN-α in mRCCpatients	VEGFR, PDGFRβ, FLT-3, c-KIT	All grades weight loss in 63% of the patients, grade 1 weight loss in 37%, grade 2 in 23% and grade 3 in 3% of the patients.
[20]	Sorafenib vs placebo in HCC patients	VEGFR, PDGFRβ, FLT-3, c-KIT	Significant more weight loss in sorafenib treated patients (all grades: 9% versus 1%, and grade 3: 2% versus 0%, respectively).
[31]	Sorafenib in HCC patients	VEGFR, PDGFRβ, FLT-3, c-KIT	Significant loss of weight and skeletal muscle in the sorafenib treated group during the first 6-12 months of treatment. . Low BMI and sarcopenia were associated with dose limiting toxicity of sorafenib.
[21]	Sunitinib in NSCLC patients	VEGFR1-3, PDGFRα/β, FLT-3, KIT	Anorexia and decreased weight (gr 1-2: 30%, gr 3: 5%).
[22]	Sunitinib and bevacizumab in patients with advanced solid cancer	VEGF, VEGFR1-3, PDGFRα/β, FLT-3, KIT	Grade 1-2 weight loss in 12% of the patients.
[23]	Sunitinib in RCC patients	VEGFR1-3, PDGFRα/β, FLT-3, KIT	No data on weight loss reported.
[24]	Sunitinib vs IFN-α in RCC patients	VEGFR1-3, PDGFRα/β, FLT-3, KIT	No data on weight loss reported.

TABLE 2.
Effects of angiogenesis inhibitors on adipose tissue and in obesity in preclinical studies.

Ref	Type of study	Target	Finding
[46]	Murine adipocytes implanted in dorsal skin chambers treatment with an antibody to the VEGFR-2	VEGFR2	Inhibition of the development of adipose tissue with inhibition of both angiogenesis and subsequent vessel remodeling.
[47]	Mice treated with valatinib and a high fat diet.	VEGFR1-3	Significant reduction of body weight and of subcutaneous and gonadal adipose tissue mass, without significant changes in blood vessel size and density.
[48;49]	Nude mice treated with valatinib and standard diet	VEGFR1-3	No decrease in body weight.
[50]	Murine renal cell carcinoma model treated with vatalanib	VEGFR1-3	Anti tumor activity as well as changes of bodyweight.
[51]	Monoclonal anti-VEGF antibody in *db*/*db* mice	VEGF	No significant difference in bodyweight. Inhibited formation of smaller differentiating adipocytes, blood vessel sprouts and adipogenic/angiogenic cell clusters. The number of adipocytes was significantly reduced.

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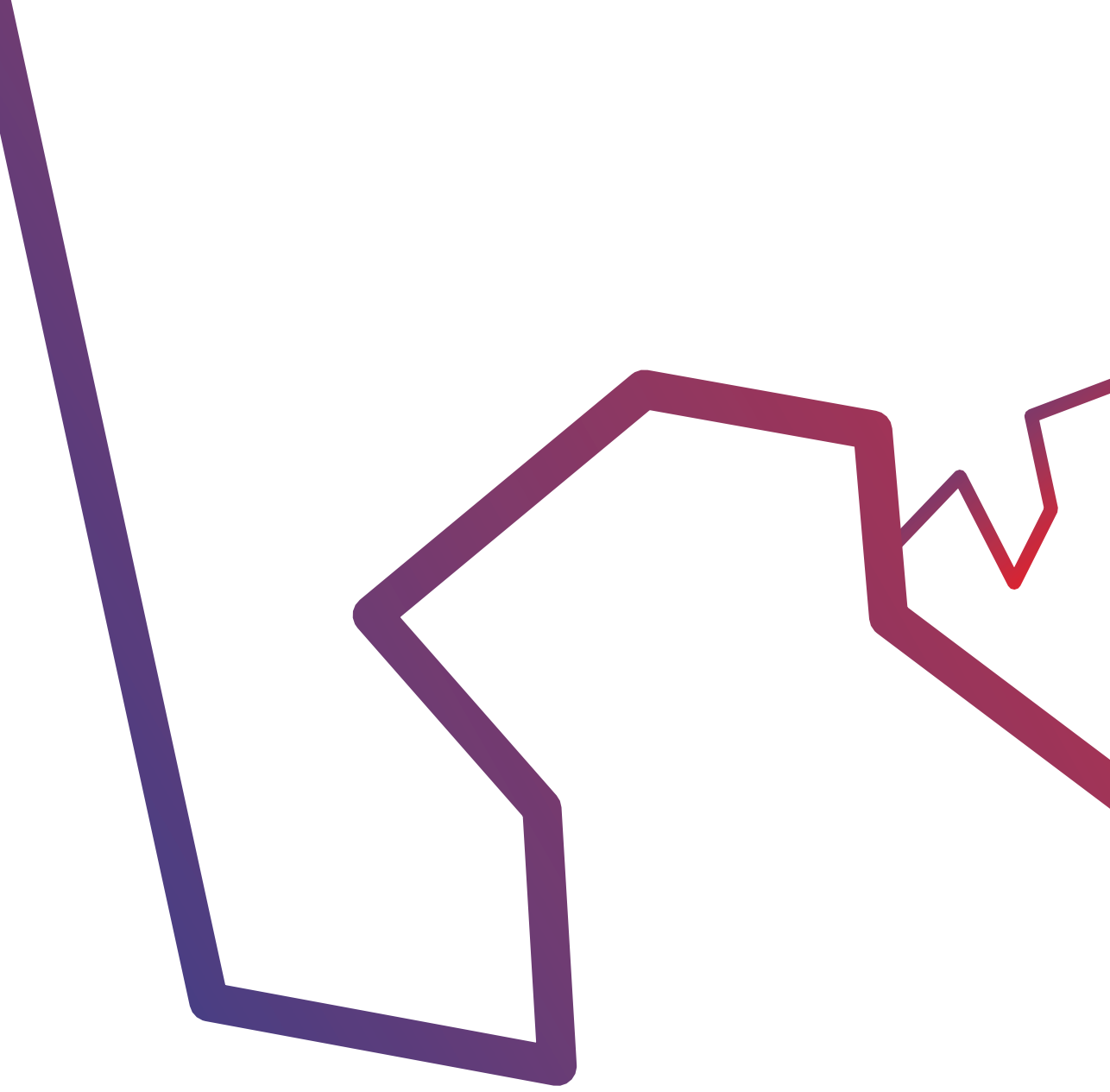

CHAPTER 7

The early effect of sunitinib on insulin clearance in patients with metastatic renal cell carcinoma

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Abstract

Aim

In patients with diabetes treated with sunitinib symptomatic hypoglycemias have been reported. To explore the mechanism of this adverse effect we performed a prospective study to investigate the effect of sunitinib on insulin concentration, insulin clearance and insulin sensitivity**.**

Methods

We studied the early effects of sunitinib on insulin sensitivity and insulin clearance with a hyperinsulinemic euglycemic clamp (insulin infusion rate 60 mU $\cdot$ m^{-2} $\cdot$ min^{-1}; steady state 90-120 minutes) in patients with renal cell carcinoma before and one week after start of sunitinib 50mg per day. Insulin sensitivity index (S_I) was defined as steady-state glucose disposal divided by the steady-state plasma insulin. Ten patients (one with diabetes, treated with metformin) were included in the study protocol.

Results

Steady-state insulin concentrations during the clamp increased after one week of sunitinib (from 128.9 $\pm$ 9.0 mU L^{-1} to 170.8 $\pm$ 12.8 mU L^{-1}, $p<0.05$; 95% CI on difference -64.3 to -19.6). The calculated insulin sensitivity index decreased from 0.22 $\pm$ 0.04 before to 0.18 $\pm$ 0.02 μmol kg^{-1} $\cdot$ min^{-1} $\cdot$ (mE/L)$^{-1}$ insulin ($p<0.05$; 95% CI on difference 0.07 to 0.08). As insulin infusion rate was similar for both clamps, the increased steady-state insulin concentration indicates reduced insulin clearance.

Conclusion

Sunitinib affects insulin clearance which could possibly lead to overexposure to insulin in patients using insulin or insulin-secretion stimulating agents.

Introduction

The development of tyrosine kinase inhibitors (TKIs) targeting the vascular endothelial growth factor (VEGF) have improved cancer treatment, however their use can be hampered by side effects. Sunitinib, a VEGFR-TKI, is currently used for the treatment of metastatic renal cell carcinoma (mRCC), gastrointestinal stromal cell tumors (GIST) and pancreatic neuroendocrine tumors.

The most reported side effects of sunitinib are hypertension, fatigue, hand-foot syndrome, and diarrhea [1, 2]. Several case reports and retrospective studies mention the occurrence of hypoglycemia during the treatment with sunitinib in patients with diabetes mellitus treated with a sulfonylureum derivate or insulin [3-6]. To explore the mechanism of this adverse effect we performed a prospective study to investigate the effect of sunitinib on insulin concentration, insulin clearance and insulin sensitivity**.**

Material and Methods

After approval of the protocol (NCT01227213) by the Institutional Review Board of the Radboud university medical center, a total of 10 mRCC patients with a indication to start sunitinib were recruited. All participants provided written informed consent. Patients were eligible if they had a life expectancy of more than 12 weeks, a WHO performance status of 0-2 and no evidence of severe or uncontrolled diseases other than renal cell carcinoma. Patients treated with corticosteroids or oral anti-coagulants were excluded. All studies were performed at the Radboud university medical center according to institutional and Good Clinical Practice guidelines. In the week before and one week (7-10 days) after starting treatment with sunitinib (50 mg/day), a 120 minutes hyperinsulinemic euglycemic clamp was performed.

The experiments were performed in the morning after an overnight fast. Patients took their medication in the morning of the experiment with a cup of water. Subjects were studied in supine position in a temperature-controlled room (23-24^{o}C). Before the start of the experiment blood samples were obtained for the measurement of fasting insulin and glucose. Two intravenous cannulae were inserted. One was positioned retrogradely into a dorsal vein of the hand that was placed in a plexiglass box, ventilated with heated air, for sampling of arterialized venous blood [7]. The second cannula was inserted in an antecubital vein of the contralateral arm for infusion of insulin and glucose. During the clamp insulin (Insulin aspart; Novorapid®; NovoNordisk, Bagsvaerd, Denmark; diluted in NaCl 0.9% to a concentration of 1 U ml^{-1}, with the addition of 2

ml whole blood per 50ml) was infused at a rate of 60mU $min^{-1}m^{-2}$ body surface area. Arterialized venous plasma glucose determinations were performed at 5-min intervals using an enzymatic-amperometric method (Biosen C-line GP+; EKF-diagnostic GmbH, Barleben, Germany). Plasma glucose was clamped at glucose 5.0mmol/L (i.e., 90mg/dL), to reach an euglycemic state, by a variable infusion of glucose 20% solution. The validity of the glucose clamp measurements of insulin sensitivity depends on achieving steady-state conditions. The t1/2 of insulin in plasma is 4-6 minutes, so after 30 minutes insulin levels already reach steady state, however glucose concentration and infusion should also reach steady state with a co-efficient of variation of <5%, therefore the period of 90-120 minutes of the clamp is as steady state. At 90 and 120 minutes after the start of the clamp, venous blood was sampled and after centrifugation the supernatant was stored at -80°C until the measurement of insulin in all samples of all patients by radio-immunoassay at the same time. Plasma insulin was assessed by an in-house radioimmunoassay (RIA) using 125I-labeled human insulin and anti-human insulin antiserum raised in guinea pig. Bound and free tracer were separated by sheep anti-guinea pig antiserum and precipitation by means of polytehylene glycol (PEG). The interassay coefficient of variation (CV) for the insulin measurement was 4.7% and the intra-assay coeffecient of variation was 9.7% at a level of of 34 mU/l.

Statistical Analysis

For calculation of the whole body glucose disposal (M-value) during the euglycemic clamp the following formula was used:

$$\text{M-value} = \frac{\text{Mean glucose infusion 90-120 min(mg/min)}}{\text{Weight (kg)}} \times \frac{1000}{(180\ \text{g/mol})}\ \mu\text{mol kg}^{-1}\,\text{min}^{-1}$$

The insulin sensitivity index [μmol kg^{-1} min^{-1} (mU L^{-1}) is a measure of insulin sensitivity in relation to the plasma insulin concentration during the steady state of the euglycemic clamp[8]. The insulin sensitivity index was calculated by dividing the *M* value by the mean insulin concentration during the euglycaemic clamp.

Differences in parameters before and after the start of sunitinib were statistically analysed using a parametric Student's t-test for paired observations. Data are presented as mean ± SEM. A value of $P < 0.05$ was considered to be statistically significant.

Results

Baseline characteristics

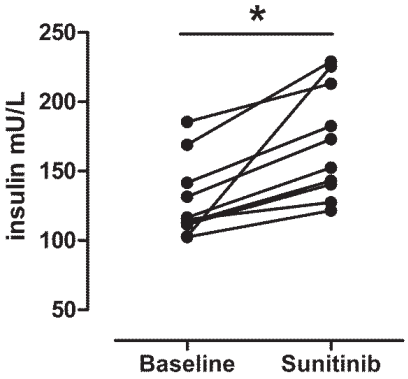
Between March 2012 and January 2013 ten patients signed informed consent and were included in the study. All patients had mRCC and sunitinib was the choice of treatment as decided by their oncologist. Mean age was 59(range 43-72) years. One patient had diabetes and used metformin. Sunitinib treatment for one week had no significant effect on fasting glucose, fasting insulin, BMI or blood pressure (Table 1). Creatinine increased significantly after start of sunitinib, however several studies show that the pharmacokinetics of insulin aspart are not affected by impaired renal function[9, 10].

TABLE 1
Baseline and clamp characteristics

Baseline characteristics (n=10)		
Age (mean + range) (years)	59 (43-72)	
Sex (male/female)	9/1	
BMI (± SE)	27.2 ± 1.1	
Diabetes (n)	1 (uses metformin)	
	Before start sunitib	**After 1 week of sunitinib**
Systolic blood pressure (mmHg)	134.0 ± 3.8	140.5 ± 3.1
Diastolic blood pressure (mmHg)	80.4 ± 3.5	84.2 ± 3.7
Glucose (fasting) (mmol/L)	5.1 ± 0.2	5.0 ± 0.2
Insulin (fasting) (mU/L)	13.8 ± 1.3	14.1 ± 2.7
Creatinine (mmol/L)	104.5 ± 10.0	122.2 ± 12.6*
Hyperinsulinemic euglycemic clamp	**Before start sunitinib**	**After 1 week of sunitinb**
Insulin (mU/L) at 90 minutes	127.0 ± 10.2	176.9 ± 13.7*
Insulin (mU/L) at 120 minutes	130.8 ± 8.4	164.7 ± 14.7*
Mean insulin mU/L	128.9 ± 9.0	170.8 ± 12.8*

All data presented as mean ±SE ; * P<0.05 compared to before start of sunitinib.
Conversion of glucose: mmol/L x 18.02 = mg/dL

FIGURE 1
Effect of sunitinib on individual insulin concentrations during steady state (90-120 min) of the hyperinsulinemic clamp before (baseline) and one week after the start of sunitinib treatment
*P < 0.02



Insulin sensitivity

During the hyperinsulinemic euglycemic clamp, glucose concentrations were stable but mean levels were not completely similar (before 4.84 ± 0.10 mmol L^{-1} and after one week of sunitinib 5.06 ± 0.09 mmol L^{-1}, P <0.05). The mean coefficient of variation for blood glucose during steady state was <5% in both experiments. Sunitinib treatment did not increase whole body glucose disposal (M-value) during the clamp (26.5 ± 3.7 μmol kg^{-1} min^{-1} before start vs. 28.5 ± 3.1 μmol kg^{-1} min^{-1}, P>0.05).

Mean plasma insulin concentrations (table 1) obtained during the steady state of the clamp were significantly higher after one week of sunitinib treatment (128.9 ± 9.0 mU L^{-1} versus 170.8±12.8 mU L^{-1}; p<0.02)(95% CI on difference -64.3 to -19.6) (Figure 1), indicating reduced clearance of insulin as insulin was infused at a fixed rate.

Before start of sunitinib, the patients were insulin resistant compared to previously studied lean healthy volunteers[11], as shown by a calculated insulin sensitivity index of 0.22 ± 0.04 μmol kg^{-1} min^{-1} per mU L^{-1}(95% CI on difference 0.07 to 0.08). After one week of sunitinib, the calculated insulin sensitivity index decreased to 0.18 ± 0.02 μmol kg^{-1} min^{-1} per mU L^{-1} (P <0.05) suggesting a decrease in insulin sensitivity.

Discussion

The present study demonstrates for the first time that sunitinib reduces insulin clearance in patients with mRCC. This conclusion is based on the higher steady state insulin concentrations during the clamp, where insulin is infused in a fixed infusion rate when subjects were treated with sunitinib.

The mechanism of the reduced insulin clearance during sunitinib treatment is not yet known. Insulin clearance is a complex phenomenon and depends on metabolic degradation, which is thought to be largely receptor mediated [12, 13]. The liver is the primary site for insulin clearance, removing 40-80% during the first portal passage [13]. The first step in insulin clearance is binding to its receptor on the cell membrane activating the tyrosine kinase pathway leading to internalization of insulin followed by degradation in lysosomes [14-16] [17]. In vitro studies have indicated that insulin internalization and insulin action are linked[18]. Thus insulin-induced insulin receptor activation not only mediates insulin actions but is also involved in its clearance. Sunitinib, has shown binding affinity with the insulin receptor and inhibits the insulin like growth factor type 1 receptor inducing ubiquitination and thereby degradation of this receptor [19, 20]. Therefore, it is likely that sunitinib can inhibit the kinase activity of the insulin receptor and consequently hamper insulin internalization and degradation.

Beside the effect on insulin clearance we observed a significant decrease in the calculated insulin sensitivity index, suggesting a decline in insulin sensitivity. However comparisons of insulin sensitivity during hyperinsulinemic euglycemic clamps are only valid if the same conditions are reached for all patients[21]. In our subjects the same insulin infusion rate was used throughout the experiment, however insulin concentrations were significantly higher after start of sunitnib and moreover glucose clamp levels differed significantly, therefore we cannot draw firm conclusions from these measurements regarding insulin sensitivity. It is intriguing that the potential effect of sunitinib on insulin sensitivity is congruent with its effect on insulin clearance, supporting the notion that both effects are mediated by interaction of sunitinib with the insulin receptor.

Some limitations to our study should be addressed. First, we did not observe an effect of sunitinib on fasting blood glucose and fasting insulin. However in our population of patients not treated with insulin or SU-derivatives, normal regulation of endogenous insulin secretion must have compensated the reduced insulin clearance by reduced pancreatic release of insulin. A similar study in patients with diabetes would be interesting to confirm the effect of sunitinib on insulin clearance in this population. However this is not feasible considering the size of the patient population and the needed sample size . Second, C-peptide concentration, as measure

of endogenous insulin production, was not measured. Under clamp conditions, C-peptide levels as marker of endogenous insulin production are typically suppressed[22, 23]. Third, creatinine increased significantly after start of sunitinib, however several studies show that the pharmacokinetics of insulin aspart are not affected by this level of impaired renal function[9, 10]. Fourth given the need to start active and standard treatment in patients with progressive metastatic disease, it was not deemed proportional to perform a placebo controlled study or to delay start of treatment to exclude a sequence/time effect with the first aim of understanding the cause of reported hypoglycemia.

Conclusion

In summary, one week treatment with sunitinib reduced insulin clearance in patients with metastatic renal cell carcinoma. In patients using insulin or insulin-secretion stimulating agents the effect of sunitinib on insulin clearance could in theory result in overexposure to insulin and thereby induce hypoglycemia. Further research into the effect of sunitinib on insulin levels in clinical practice should be performed to see whether the observed effect is of clinical relevance to patients.

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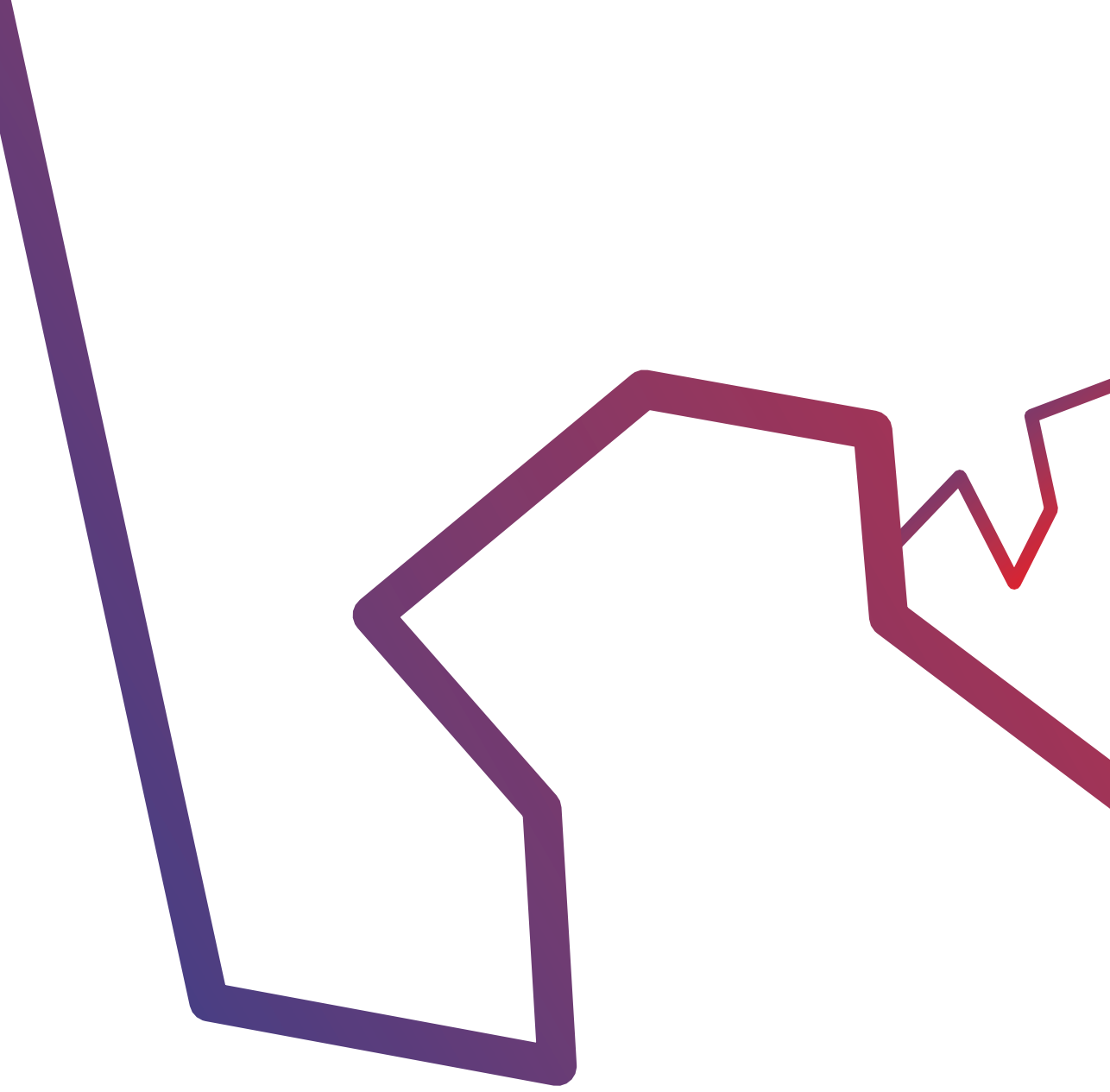

CHAPTER 8

Summary, general discussion and future perspectives

Summary

From the start of 21st century important progression has been made in understanding the pathophysiology of cancer. The discovery of several pathways, playing key roles in progression and metastatic potential of tumors have led to the development of a new type of drug therapy for the treatment of cancer. These new drugs are called targeted therapies, as in contrast to chemotherapy they target specific receptors and molecular pathways. One of the key pathways involved in tumor growth and spread is angiogenesis, the formation of new vessels. Vascular endothelial growth factor (VEGF) plays and important role in this process. During the development of these targeted agents, for example angiogenesis inhibitors, few toxic effects were expected as most of these pathways did not seem to play an important role in normal adult physiology. However after a decade of treatment with angiogenesis inhibitors in cancer patients we have learned that their use can be hampered by specific side effects.

This thesis focused on the effect of angiogenesis inhibitors on **cardiac function, blood pressure, body weight** and **insulin metabolism**.

Heart failure is reported as one of the side effects of angiogenesis inhibitors. Patients treated with these therapies have an increased risk (RR 2.78) of cardiac dysfunction(1). Especially patients with previous coronary artery disease have an increased risk of developing this serious adverse effect(2, 3). To get better insight in the eventual causality between the use of angiogenesis inhibitors and cardiac failure in **Chapter 2** an in vitro experiment is described, studying the effect of sunitinib on contractile force, and the effect of recovery after ischemic stimulation of human atrial trabeculae. Human atrial tissue was harvested during the incision of the right auricle during extracorporal bypass surgery, which provides a unique opportunity to collect human atrial tissue without exposing patients to unwanted risks. After isolation and connection to a force transducer in an organ bath contractile force of the human atrial trabeculae was measured under normal and ischemic conditions. Sunitinib at low, but relevant, concentrations did not have a direct effect on the function of human atrial cardiomyocytes nor did it hamper the recovery in contractile force after a period of ischemia. Therefore our experimental data do not support a direct effect of sunitinib on cardiac contractile force to cause the sunitinib-associated heart failure. Other actions of sunitinib such as structural or functional changes of the coronary vasculature, an increase in cardiac afterload by increasing blood pressure or renal toxicity deserve further investigation.

In **chapter 3, 4 and 5** the possible mechanisms for the initiation/development of **hypertension** in patients treated with VEGFR inhibitors are explored in various experiments, ranging from preclinical research in animals to clinical experiments in healthy volunteers and patients.

In **chapter 3** the role of vascular endothelial growth factor in maintaining vascular tone in normal physiology was studied in thirty-one healthy male volunteers using venous occlusion plethysmography. Bevacizumab, a monoclonal antibody directed against VEGF and also known for its ability to cause hypertension was infused into the brachial artery, achieving clinically relevant concentrations in the forearm, while achieving low systemic exposure. Bevacizumab did not directly affect forearm vasodilator tone. However bevacizumab did specifically reduce endothelium-mediated vasodilation, proving that circulating VEGF plays a role in maintaining normal endothelial control of vascular tone. This study did not elucidate the mechanism of this altered endothelial function.

To further explore the mechanism of altered endothelial function and to investigate the hypothesis that endothelial dysfunction initiates the start of hypertension during VEGF inhibition a preclinical and clinical experiment were performed in **chapter 4**. Sunitinib, a tyrosine kinase inhibitor targeting VEGFR and other tyrosine kinases was studied. After treatment with sunitinib or placebo for seven days rats were euthanized and thereafter the mesenteric arteries were mounted in a Mulvany wire myograph to assess endothelium-dependent (with acetylcholine) and endothelium-independent (with sodiumnitroprusside) vasorelaxation. Sunitinib reduced endothelium-dependent vasodilation, but not endothelium-independent vasodilation in this experiment. The difference in endothelium-dependent vasodilation between controls and sunitinib-treated animals disappeared in the presence of a nitric oxide antagonist, suggesting an effect of sunitinib on nitric oxide availability. In the clinical experiment ten patients with metastatic renal cell carcinoma starting first line treatment with sunitinib were included. Before the start and one week after the start of sunitinib endothelium-dependent and endothelium-independent vasodilation was assessed in the forearm using venous occlusion plethysmography. No changes in blood flow were observed, while mean arterial pressure significantly increased within one week after start of sunitinib and even more so after two weeks of treatment in these patients. In conclusion, in animals exposed to high concentrations of sunitinib endothelium dependent vasodilation is reduced, however this endothelial dysfunction does not precede the development of hypertension in patients treated with sunitinib. Other factors, such as neurohormonal changes, could play a role as trigger for the development of hypertension.

For that reason **chapter 5** reports the results of the measurement of aldosterone, renin and endothelin concentrations and blood pressure in twenty patients with metastatic renal cell carcinoma before and one week after start of sunitinib. Aldosterone and endothelin were significantly increased after one week and blood pressure was significantly increased after two weeks of treatment. We found the rise in aldosterone after one week, but not endothelin, correlated with the increase in blood pressure after two weeks, suggesting that aldosterone is a trigger for the development of hypertension in sunitinib treated patients.

Chapter 6 and **7** focus on **weight loss** and **insulin metabolism** of angiogenesis inhibitors.

In **chapter 6** the literature concerning the effect of angiogenesis inhibitors on body composition and adipogenesis was reviewed. Adipose tissue is highly vascularized and VEGF is the most important angiogenic factor in adipogenesis. In preclinical studies angiogenesis inhibitors reduce the number of adipocytes, suggesting that inhibition of angiogenesis may reverse obesity, however no studies using TKIs to treat obesity in humans exist. In cancer and obesity, both health problems with an increasing prevalence in the population, angiogenesis inhibition can be a target for treatment.

Previous studies describe the occurrence of hypoglycemias and even regression of diabetes during treatment with sunitinib, possibly by improving insulin sensitivity. Therefore **Chapter 7** describes the results of a hyperinsulinemic euglycemic clamp in ten patients with metastatic renal cell carcinoma before and one week after the start of sunitinib. Although we were not able to detect a definite effect of sunitinib on insulin sensitivity, sunitinib reduced insulin clearance as shown by an increase in insulin concentrations during the steady state of the hyperinsulinemic euglycemic clamp. We did not observe an effect on fasting glucose concentrations, however a normal regulation of endogenous insulin secretion must have compensated the effect on insulin clearance in our group of patients. In patients with diabetes using insulin or insulin-secretion stimulating agents the effect of sunitinib on insulin clearance could result in overexposure to insulin and thereby induce hypoglycemia.

General discussion

In this thesis several adverse effects of angiogenesis inhibitors were investigated. The first part of this thesis focused on the mechanisms causing cardiac failure and hypertension during angiogenesis inhibition. Hypertension is an adverse effect but also seems to be a biomarker for the antitumor effect of several angiogenesis inhibitors, which makes it of special interest for research. As can be concluded from the findings sunitinib-induced rise in blood pressure is preceded by an increase in aldosterone concentration. Moreover endothelial dysfunction may be a vascular consequence of VEGF-targeted therapies, but it does not precede the hypertension in VEGFR-TKI treated patients. In contrast to the decrease in endothelial function by scavenging VEGF in the arterial circulation in healthy volunteers and by exposing animals to high concentrations of sunitinib, in patients treated with sunitinib endothelial dysfunction did not occur before the development of hypertension. This difference between sunitinib and bevacizumab regarding the effect on endothelial function could be explained by the difference

in specificity between a solely VEGF scavenging antibody bevacizumab and the less specific tyrosine kinase inhibitor sunitinib, which also targets other pathways (such as PDGFR and c-KIT). Moreover the effect on endothelial function of bevacizumab was not enough to directly alter basal vascular tone, so therefore these observations are not incompatible with the conclusions regarding the timing in endothelial dysfunction and the onset of hypertension in chapter 4. Since endothelial dysfunction appeared to be a consequence rather than a cause of sunitinib-induced hypertension, we studied the early effects of sunitinib on aldosterone and endothelin. Aldosterone increased and precedes the development of hypertension as shown by the correlation between its increase and the rise in blood pressure. Endothelin also increased within one week after start of sunitinib, however this was not correlated with an increase in blood pressure. The rise in aldosterone could possibly initiate the release of endothelin-1, as in preclinical experiments it has been shown that aldosteron can trigger endothelin production(4, 5). In contrast VEGF stimulates the secretion of endothelin and therefore a direct effect of inhibition of VEGF on the rise in endothelin-1 is not expected(6). This should be investigated in further research. The increase in endothelin could explain the sustainment of hypertension, while after an initial rise in aldosterone shown in this thesis, aldosterone seems to normalize over time as reported at 4 weeks of treatment by Kappers et al. Regarding heart failure a direct effect of sunitinib on contractility of atrial trabeculae was not found, therefore other factors, such as altered preload or afterload or changes in microvasculature should be accountable for its development. The rise in aldosterone after start of sunitinib could also, besides the onset of hypertension, be involved in the development of cardiac failure. Aldosterone can induce the development of cardiac failure by inflammation, fibrosis of cardiomyocytes and treatment with aldosterone receptor antagonist showed to reduce mortality in patients with chronic heart failure(7, 8). Patients with primary hyperaldosteronism (Conn's disease) have a higher risk for development of left ventricular hypertrophy, atrial fibrillation, heart failure, stroke and myocardial infarction as compared with matched patients with essential hypertension(9). Hence the use of aldosterone receptor antagonists during treatment with angiogenesis inhibitors could provide an excellent way of preventing the development of hypertension and heart failure.

Recent preclinical observations suggest a role for inflammatory cells and VEGF produced by these cells in the regulation of blood pressure(10-12). These observations need further exploration in humans in-vivo and patients treated with VEGF-targeted therapy provide a window of opportunity to do so.

Furthermore from a literature review in this thesis we can conclude that angiogenesis inhibitors affect body composition and adipogenesis. In theory this could even provide therapeutic options for the treatment of patients with obesity, however side effects (next to the costs of these drugs),

as described in this thesis, will hamper the use of these type of drugs in patients already at risk of developing heart failure and hypertension.

In cancer patients weight loss has huge consequences for quality of life and future research should focus on the mechanism of weight loss during treatment with sunitinib to develop preventive measures for this toxic effect.

In the last part of this thesis we established that sunitinib affects the clearance of insulin, which could explain reported cases of hypoglycemias and remission of diabetes in literature. Based on this observation, we advice patients who are treated with insulin or sulfonylureum derivatives (which uncouples insulin secretion from glucose concentration) and who start sunitinib to check their fasting glucose levels frequently during the first weeks of sunitinib treatment.

Future perspectives

Learning from adverse effects: mechanism and opportunities

The introduction of angiogenesis inhibitors and targeted therapy in general has improved the outcome of several types of cancer. However adverse effects sometimes hamper their use. Future research in the interesting era of adverse effects is still warranted to optimize treatment.

For patients with cancer the development of targeted therapies has led to an improvement in progression free and overall survival, but also prolonged periods of treatment. In contrast to the conventional chemotherapy most of the targeted agents are administered orally in the outpatient setting. With prolonged duration of treatment the importance of toxic effects and their impact on quality of life is increasing. Minor adverse effects, as scored by the CTCAE criteria, can become a burden if affecting daily life and moreover dose reduction or interruptions can be needed possibly leading to a less effective treatment. Furthermore adverse effects can also cause major morbidity or even mortality if not recognized or treated in time. Identification of patients who are at risk for the development of side effects and the prompt administration of preventive measures or treatment is essential in optimizing treatment duration, safety and efficacy of targeted agents in cancer treatment. However some of the side effects of targeted therapies, such as hypertension in VEGFR targeted therapies, have been shown to be biomarkers of efficacy at least in renal cell carcinoma, which makes the approach to the prevention or treatment even more challenging(13).

Another reason to research the adverse effects of targeted therapy in cancer prompts us to leave the field of cancer treatment. Adverse effects of targeted therapies in patients with cancer

imply that these same pathways have an essential role in normal physiology. Research into the role of these pathways in normal physiology can provide insight into the pathogenesis of other diseases, such as hypertension or heart failure, and finally it can even open doors to explore therapeutic potential of these targeted pathways for the treatment of other diseases, such as diabetes, hypertension and obesity. In this regard, the results of this thesis justify future research to understand the role of tyrosine kinases in the regulation of insulin clearance and their role as a trigger of hypertension and its complications

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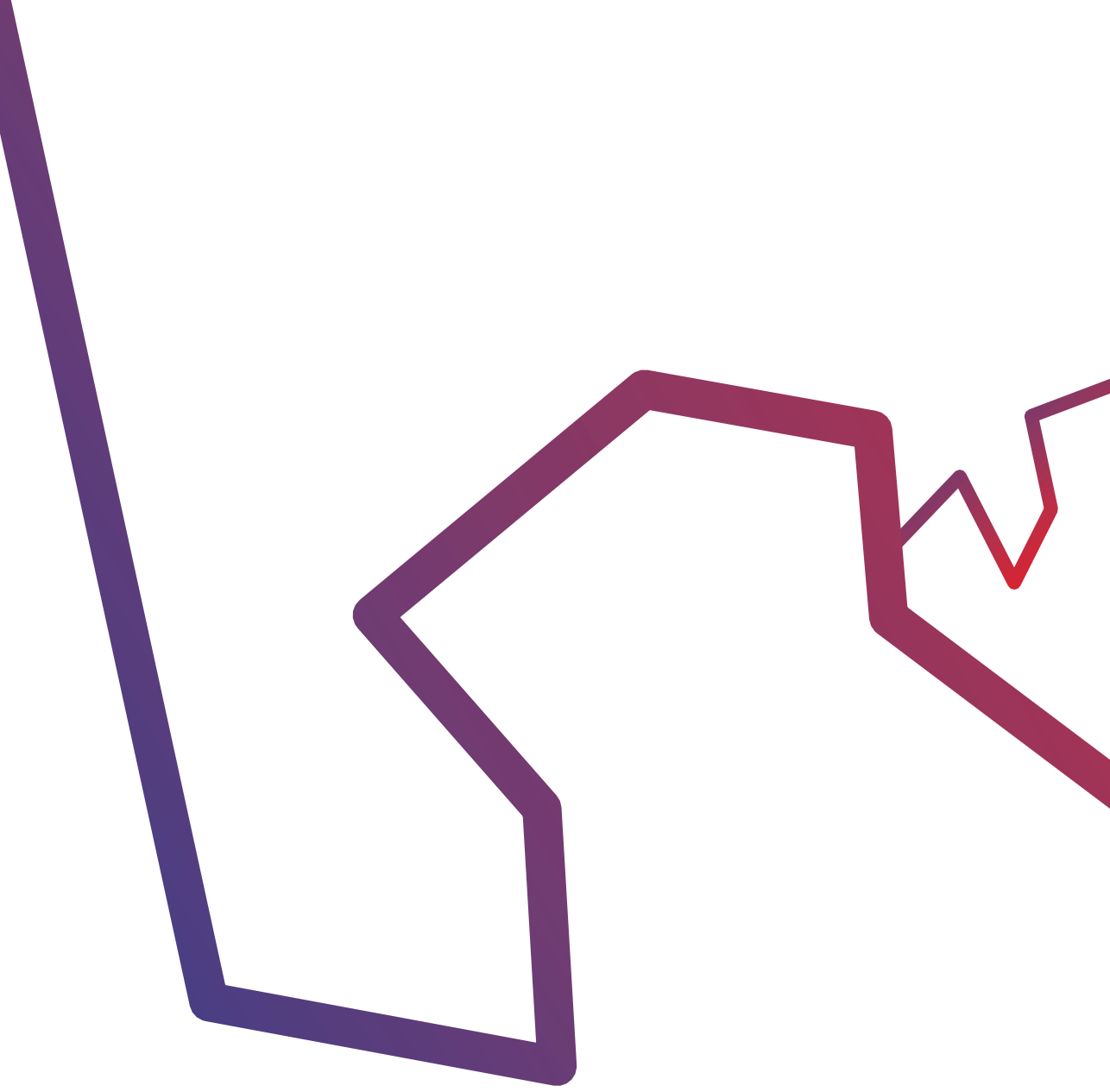

CHAPTER 9

Nederlandse samenvatting
List of publications
Dankwoord
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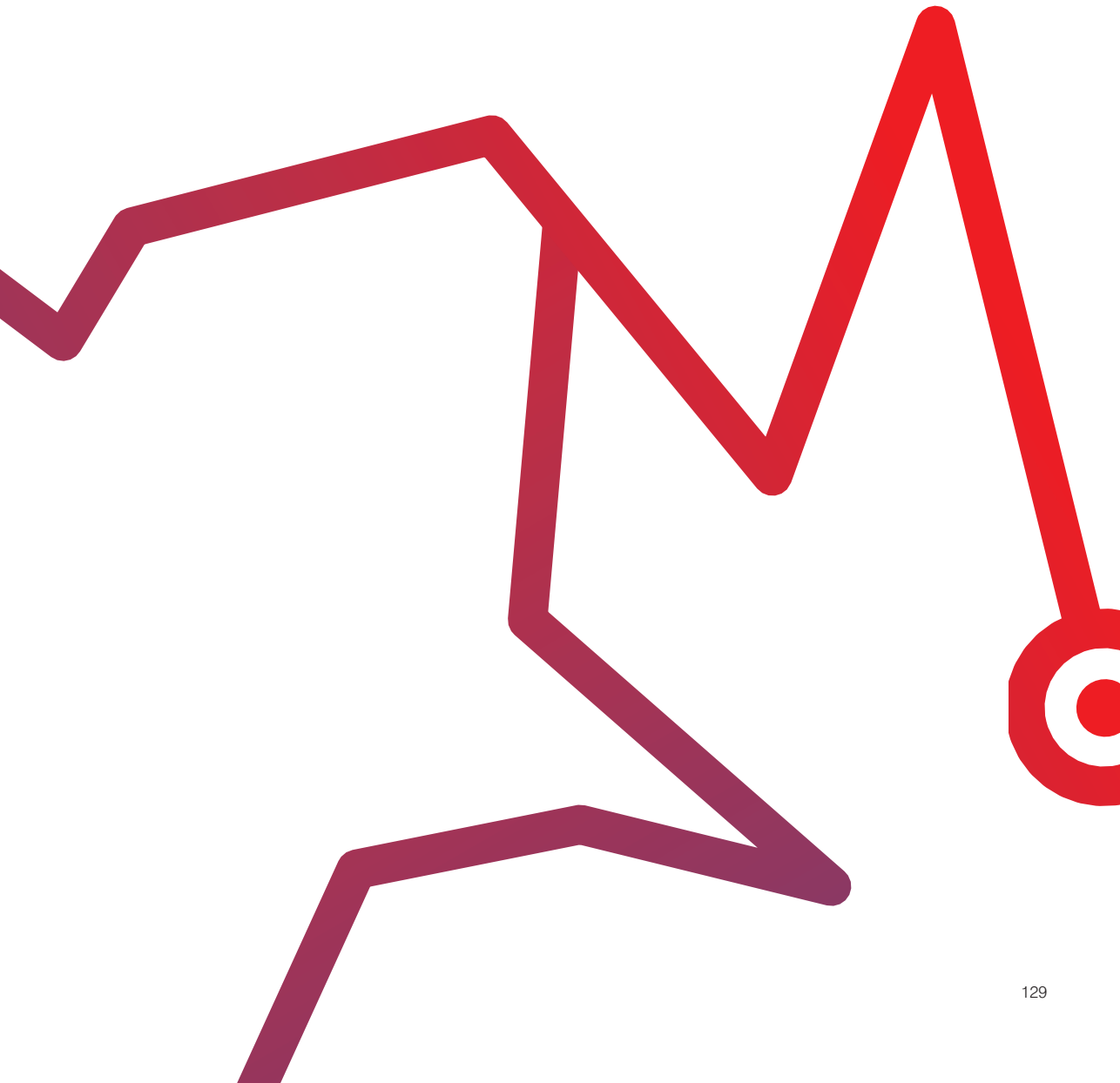

Nederlandse samenvatting

Vanaf het begin van de 21e eeuw is er een belangrijke vooruitgang geweest in het begrijpen van de ontstaanswijze en ontwikkeling van kanker. De ontdekking van verschillende processen en signaalwegen die een essentiële rol spelen in de groei en de verspreiding van kankercellen heeft geleid tot de ontwikkeling van een nieuw type geneesmiddelen voor de behandeling van kanker. Deze nieuwe medicijnen worden doelgerichte behandelingen (in het Engels 'targeted therapy') genoemd, omdat ze in tegenstelling tot de klassieke anti-kanker behandeling, gericht zijn op specifieke signaalwegen en/of aangrijpingspunten op de kankercel. Chemotherapie daarentegen is gericht op alle sneldelende cellen in het lichaam.

Eén van de processen die een belangrijke rol speelt in de groei en verspreiding van kanker is het vermogen van kankercellen om de aanmaak van nieuwe bloedvaten *(angiogenese)* te stimuleren. Hierdoor wordt de tumor voorzien van voldoende bouwstoffen en zuurstof. De tumor zorgt daarbij voor het vrijkomen van verschillende groeifactoren, met name de vasculaire endotheliale groeifactor (VEGF) die vervolgens aan de vasculaire endotheliale groeifactor receptor (VEGFR) bindt.

Dit proces van bloedvatnieuwvorming werd reeds in de jaren 70 van de vorige eeuw door de Amerikaanse onderzoeker Folkman beschreven. Na tientallen jaren van onderzoek werd in 2004 de eerste doelgerichte behandeling gericht op dit proces geïntroduceerd. Bevacizumab, een monoklonaal antilichaam gericht tegen VEGF, werd geregistreerd voor de behandeling van darmkanker en was daarmee de eerste remmer van de bloedvatnieuwvorming ofwel *angiogeneseremmer*. Inmiddels wordt bevacizumab ingezet in de behandeling van enkele andere soorten kanker en zijn er daarnaast meer dan veertig andere doelgerichte behandelingen geregistreerd.

Toen de angiogeneseremmers net werden geïntroduceerd was de verwachting dat er weinig bijwerkingen zouden zijn, omdat de behandeling doelgerichter op de kankercel afgestemd is dan bijvoorbeeld chemotherapie. Helaas traden er wel degelijk bijwerkingen op bij patiënten.

Dit proefschrift bestudeert een aantal van deze bijwerkingen van angiogeneseremmers, te weten de effecten op **hartfunctie**, het ontstaan van **hoge bloeddruk**, de veranderingen in het **gewicht** en **insuline metabolisme**.

De geneesmiddelen die worden gebruikt in de beschreven experimenten zijn bevacizumab, een monoklonaal antilichaam gericht tegen VEGF, dat via een infuus wordt toegediend, en

sunitinib, een angiogeneseremmer in tabletvorm, die behalve gericht tegen VEGF, ook andere signaalwegen (zoals PDGF en C-KIT) blokkeert.

Toegepaste methoden

De belangrijkste methoden die in dit proefschrift gebruikt worden zijn het *atriale hartspierweefsel model, veneuze occlusie plethysmografie,* de *mulvany myograaf* en de *hyperinsulinemische euglycemische clamp.*

In het *atriale hartspierweefsel model* (hoofdstuk 1, figuur 1) wordt humaan hartspierweefsel verkregen door incisie van het rechter hartoor voor het aansluiten van de hartlongmachine ten behoeve van ingrepen, waarbij tijdelijk het hart moet worden stilgelegd en de functie van hart- en longen moet worden overgenomen. Dit moment zorgt voor een unieke mogelijkheid om humaan hartspierfweefsel te verkrijgen zonder patiënten bloot te stellen aan extra risico's. Patiënten worden vooraf aan de operatie op de polikliniek om toestemming gevraagd. Na isolatie en verbinding van de hartspiertjes aan de krachttransducer in een orgaanbad kan de contractiele kracht onder normale omstandigheden en tijdens zuurstofgebrek worden gemeten en het effect van farmaca hierop worden bestudeerd.

Veneuze occlusie plethysmografie (zie figuur 2 in hoofdstuk 1) is een gevalideerde methode om bloeddoorstroming in de onderarm te kunnen meten in rust en in reactie op de toediening van verschillende medicijnen die worden toegediend in de slagader in de elleboogsplooi. De toediening in de elleboogsslagader zorgt ervoor dat er lokaal hoge concentraties van het geneesmiddel kunnen worden bereikt in de onderarm zonder dat de patiënt of gezonde vrijwilliger systemisch wordt blootgesteld aan toxische hoeveelheden, omdat na verspreiding van het geneesmiddel in het gehele lichaam de uiteindelijke concentratie zeer laag is. Om beide bovenarmen wordt een bloeddrukband aangebracht die herhaaldelijk wordt opgepompt tot 40mmHg om intermitterend kortdurend de bloeduitstroom te belemmeren, maar de instroom te behouden. De bloeddoorstroming wordt gemeten met kwik-gevulde bandjes die zeer nauwkeurig de toename in omvang, en dus toename van bloeddoorstroming, van de onderarm kunnen registreren. Deze bloeddoorstroming in de onderarm reflecteert de vaattonus in weerstandsvaten. Door infusie van acetylcholine kan men de endotheel-afhankelijke vaatverwijding meten en met sodiumnitroprusside de endotheel-onafhankelijke vaatverwijding.

Bij het gebruik van de *mulvany myograaf* (zie figuur 3 in hoofdstuk 1) worden de eigenschappen van zeer kleine weerstandsbloedvaatjes (100-400μm) bestudeerd. Deze kleine bloedvaten spelen een belangrijke rol in het ontstaan van hoge bloeddruk. Voor dit onderzoek worden deze bloedvaatjes vrij geprepareerd uit het vaatstelsel gelegen tussen de darmen van geofferde

ratten. Een klein segment van het bloedvat wordt vervolgens op twee draadjes geplaatst die zijn verbonden met meetapparatuur die de contractiekracht als reactie op verschillende prikkels kan meten.

In het laatste onderzoek wordt de *hyperinsulinemische euglycemische clamp* (zie figuur 4 in hoofdstuk 1) als methode toegepast. Dit is de gouden standaard voor het meten van insuline gevoeligheid in mensen. Bij deze methode wordt de bloedglucose vastgehouden ("clamp") op een bepaalde waarde tijdens toediening van insuline, door de infusie van glucose aan te passen. Gedurende het onderzoek dat twee uur duurt, wordt om de vijf minuten bloed afgenomen om de bloedglucosewaarde te bepalen en de hoeveelheid glucose-infuus hierop aan te passen. Na afloop van het experiment wordt berekend hoeveel glucose moest worden toegediend om bij een continue infusie van insuline de bloedglucose op peil te houden. Dit geeft daarmee de gevoeligheid van het lichaam voor insuline weer.

Samenvatting van de hoofdstukken

Hartfalen is beschreven als een van de bijwerkingen van angiogeneseremmers. Patiënten die worden behandeld met deze medicijnen hebben een verhoogd risico op de ontwikkeling van een verminderde hartfunctie. In het bijzonder patiënten met een eerdere vernauwing of blokkade in de kransslagaders hebben verhoogde kans op deze ernstige bijwerking. Om beter inzicht te krijgen in het verband tussen het gebruik van angiogeneseremmers en hartfalen wordt in **hoofdstuk 2** een *in vitro* experiment beschreven, waarbij het effect van sunitinib op contractiekracht van hartspierweefsel en het effect op het herstel van deze contractiekracht na een periode van zuurstofgebrek wordt gemeten. Sunitinib in lage, maar klinisch relevante, concentraties had geen direct effect op de functie van humane atriale hartspiercellen en tevens niet op herstel na een periode van zuurstofgebrek. Onze experimentele data ondersteunen niet de hypothese dat een direct effect van sunitinib op cardiale contractiekracht de oorzaak is voor het ontstaan van hartfalen bij het gebruik van angiogeneseremmers. Andere mogelijke oorzaken van hartfalen veroorzaakt door sunitinib zoals structurele of functionele veranderingen aan het coronaire vaatstelsel, een verhoging van de nabelasting van het hart door een verhoging van de bloeddruk of nierfalen dienen verder onderzocht te worden.

Hoge bloeddruk treedt op in 19% tot 80% van alle patiënten die worden behandeld met een angiogeneseremmer. In **hoofdstuk 3**, **4** en **5** worden mogelijke mechanismen voor het initiëren en ontwikkelen van hoge bloeddruk bij patiënten die worden behandeld met angiogeneseremmers onderzocht in verschillende experimenten, variërend van preklinisch onderzoek met dieren tot klinisch onderzoek met gezonde vrijwilligers en patiënten.

In **hoofdstuk 3** wordt de rol van de vasculaire endotheliale groeifactor (VEGF) op het behouden van een normale vaattonus bestudeerd in eenendertig gezonde vrijwilligers met veneuze occlusie plethysmografie. Bevacizumab werd toegediend in de slagader in de elleboogsplooi, waarbij klinisch relevant concentraties in de onderarm werden bereikt, en lage systemische concentraties. Bevacizumab had geen direct effect op de vaattonus in de onderarm. Er werd echter wel een specifiek effect gezien op de endotheel-afhankelijke vaatverwijding, daarmee bewijzend dat circulerend VEGF een rol speelt in het behouden van een normale endotheliale controle van de vaattonus. Deze studie verklaart nog niet het mechanisme achter deze verandering in endotheelfunctie.

Om dit mechanisme verder te onderzoeken en om de hypothese te toetsen dat een verstoring in de endotheelfunctie het ontstaan van een hoge bloeddruk initieert tijdens de behandeling met angiogeneseremmers werd een preklinisch en klinisch experiment uitgevoerd in **hoofdstuk 4**. In dit preklinische experiment werden ratten gedurende zeven dagen behandeld met sunitinib of placebo. Na zeven dagen werden de ratten geofferd en de mesenteriale (rondom de darmen) bloedvaten werden vrijgeprepareerd en opgehangen in een mulvany myograaf om de endotheel afhankelijke (met acetylcholine) en de endotheel onafhankelijke (met nitroprusside) vaatverwijding te meten. Sunitinib verminderde de endotheel afhankelijk vaatverwijding, maar niet de endotheel onafhankelijke vaatverwijding in dit experiment. Het verschil in endotheel afhankelijke vaatverwijding tussen de controle groep en de sunitinib behandelde groep verdween in de aanwezigheid van een stikstofoxide antagonist. Dit suggereert een effect van sunitinib op het stikstofoxide (NO) metabolisme. Stikstofoxide is een vaatverwijder, die normaal gesproken via het endotheel wordt vrijgegeven.

In het klinische experiment werden tien patiënten met recent gediagnosticeerde uitgezaaide nierkanker, waarvoor het plan was om te starten met behandeling met sunitinib, geïncludeerd in het onderzoek. Voor de start en een week na de start van de behandeling met sunitinib werd de endotheel afhankelijke en endotheel onafhankelijke vaatverwijding gemeten met veneuze occlusie plethysmografie. Er werden geen veranderingen in bloeddoorstroming geobserveerd, terwijl de bloeddruk wel significant steeg na een week behandeling met sunitinib en nog verder steeg na twee weken behandeling.

Concluderend werd in dieren, behandeld met hoge doseringen sunitinib, een vermindering gezien van de endotheelfunctie, maar deze verandering in endotheelfunctie gaat niet vooraf aan de ontwikkeling van hypertensie in patiënten behandeld met sunitinib. Andere factoren, zoals neurohormonale veranderingen, zouden een rol kunnen spelen in het uitlokken van de ontwikkeling van hoge bloeddruk.

Daarom beschrijft hoofdstuk 5 de resultaten van de meting van aldosteron, renine en endotheline concentraties bij twintig patiënten met uitgezaaide nierkanker net voor de start en een week na de start met sunitinib. Aldosteron en endotheline waren na een week significant verhoogd en de bloeddruk steeg significant na twee weken behandeling. De stijging in aldosteron, maar niet de stijging van endotheline, was gecorreleerd met de stijging van bloeddruk na twee weken. Dit suggereert dat aldosteron een trigger is voor de ontwikkeling van hypertensie in patiënten behandeld met sunitinib.

Hoofdstuk 6 en **7** gaan over het effect van angiogeneseremmers op **gewichtsverlies** en **insuline metabolisme**.

Hoofdstuk 6 geeft een overzicht van de wetenschappelijke literatuur over het effect van angiogeneseremmers op lichaamssamenstelling en de ontwikkeling van vetcellen. Vetweefsel is zeer rijk aan bloedvaten en VEGF is de belangrijkste factor in de ontwikkeling van bloedvaten tijdens de vorming van nieuw vetweefsel. In preklinische studies verminderen angiogeneseremmers het aantal vetcellen, dit zou suggereren dat remming van angiogenese overgewicht zou kunnen verminderen. Er zijn echter geen studies met mensen, die angiogeneseremmers met het doel om overgewicht te behandelen onderzoeken.

Eerdere studies beschrijven het optreden van hypoglycemieën (te lage bloedsuikers) en zelfs vermindering van diabetes gedurende de behandeling met sunitinib. Een van de oorzaken zou een verbetering in de gevoeligheid voor insuline kunnen zijn. Daarom worden in **hoofdstuk 7** de resultaten beschreven van een hyperinsulinemische euglycemische clamp bij tien patiënten voor- en een week na start van de behandeling met sunitinib. Ondanks dat we geen direct effect van sunitinib op insulinegevoeligheid konden detecteren, observeerden we wel een daling in de klaring van insuline gedurende de stabiele fase van de clamp. We zagen geen effect op de nuchtere glucose concentraties, maar dit kan verklaard worden, omdat de patiënten geen diabetes hadden en onder normale omstandigheden een vermindering in afgifte van insuline door de alvleesklier het effect van de verminderde insulineklaring zal compenseren. In patiënten met diabetes die insuline of insuline-secretie stimulerende medicijnen gebruiken kan het effect van sunitinib op de klaring van insuline mogelijk wel leiden tot een verhoogde blootstelling aan insuline en daarmee een te sterke daling van de bloedglucose.

Algemene beschouwing

In dit proefschrift werden verschillende bijwerkingen van angiogeneseremmers onderzocht. De eerste twee delen concentreerden zich op de mechanismen achter hartfalen en hypertensie tijdens de behandeling. Hypertensie is een bijwerking, maar daarnaast lijkt het ontwikkelen van

een hoge bloeddruk ook samen te gaan met het antitumor effect, het is dus een biomarker, waardoor onderzoek naar het ontstaan van deze bijwerking extra interessant is.

Uit dit proefschrift kan geconcludeerd worden dat de stijging van de bloeddruk door sunitinib wordt voorafgegaan door een stijging in de aldosteron concentratie. Daarnaast is endotheeldysfunctie een consequentie van de remming van VEGF, maar gaat het niet vooraf aan het ontwikkelen van hypertensie bij remming van de VEGF receptor door verschillende angiogenesremmers. In tegenstelling tot de vermindering in endotheelfunctie door het wegvangen van VEGF in de arteriële circulatie bij gezonde vrijwilligers en bij het blootstellen van dieren aan hoge doseringen sunitinib, zagen we geen verandering in endotheelfunctie bij patiënten met sunitinib vóór het optreden van een stijging in de bloeddruk. Het verschil tussen sunitinib en bevacizumab met betrekking tot het effect op endotheelfunctie kan wellicht verklaard worden door verschil in specificiteit tussen het specifiek wegvangen van VEGF en het minder specifieke effect van de tyrosine kinase remmer sunitinib die ook op andere signaalwegen (zoals PDGF en c-KIT) werkzaam is. Daarnaast zagen we ook in de studie met gezonde vrijwilligers dat het effect van bevacizumab op de endotheelfunctie niet sterk genoeg was om direct de vaattonus te beïnvloeden. De bevindingen in beide onderzoeken zijn dus niet tegenstrijdig met de conclusie met betrekking tot de timing van het optreden van endotheeldysfunctie en het optreden van hypertensie in hoofdstuk 4. Aangezien endotheeldysfunctie meer een gevolg dan een oorzaak van sunitinib-geïnduceerde hypertensie leek te zijn, onderzochten we de vroege effecten van sunitinib op aldosteron en endotheline. Aldosteron steeg en ging vooraf aan de ontwikkeling van hypertensie. Endotheline steeg ook na een week, echter er was geen relatie tussen de stijging en het ontstaan van hypertensie. De stijging in aldosteron kan mogelijk het vrijkomen van endotheline stimuleren, zoals in andere preklinische onderzoeken werd geobserveerd. Andere onderzoekers hebben beschreven dat VEGF de productie van endotheline juist stimuleert, dus dat een direct effect van de remming van VEGF op de stijging van endotheline niet is te verwachten. Dit zou verder onderzocht moeten worden. De stijging van endotheline kan het persisteren van de hoge bloeddruk verklaren, aangezien aldosteron na een initiële stijging zoals beschreven in dit proefschrift, na enkele weken normaliseert zoals beschreven in het onderzoek door Kappers et al.

Met betrekking tot hartfalen werd er geen direct effect van sunitinib op de contractiliteit van hartspierweefsel van de hartboezem geobserveerd. Andere factoren zoals een veranderde voor- of nabelasting van het hart of een verandering in de kleine bloedvaatjes moeten daarom verantwoordelijk zijn voor het ontstaan van hartfalen. De stijging in aldosteron na de start van sunitinib kan, behalve bij de initiatie van hypertensie, ook betrokken zijn bij de ontwikkeling van hartfalen. Eerdere onderzoeken laten namelijk zien dat aldosteron hartfalen kan induceren

door inflammatie en fibrose van hartspiercellen en behandeling met een aldosteron receptor antagonist laat een daling van de mortaliteit zien in patiënten met chronisch hartfalen. Daarnaast hebben patiënten met primair hyperaldosteronisme (ziekte van Conn) een hoger risico op ontwikkelen van linker ventrikel hypertrofie, boezemfibrilleren, hartfalen en een myocardinfarct in vergelijking met patiënten met essentiële hypertensie. Derhalve kan het gebruik van aldosteron receptor antagonisten tijdens de behandeling met angiogeneseremmers mogelijk een goede manier zijn om de ontwikkeling van hypertensie en hartfalen te voorkomen.

Verder concluderen we uit literatuuronderzoek dat angiogeneseremmers een effect hebben op lichaamssamenstelling en de ontwikkeling van vetweefsel. In theorie zou die zelfs mogelijkheden bieden voor de behandeling van patiënten met obesitas, echter de bijwerkingen, zoals beschreven in dit proefschrift, maar ook de hoge kosten, beperken de mogelijkheid van gebruik van deze medicijnen bij een groep patiënten met vooraf al een verhoogd risico op hartfalen en hypertensie.

In patiënten met kanker heeft gewichtsverlies gevolgen voor de kwaliteit van leven en verder onderzoek zal zich moeten richten op de het mechanisme en de preventie van gewichtsverlies tijdens de behandeling met sunitinib.

In het laatste gedeelte van dit proefschrift beschrijven we dat sunitinib de klaring van insuline beïnvloedt, wat een verklaring biedt voor de in de literatuur beschreven hypoglycemieën en vermindering in insuline behoefte bij nierkanker patiënten met diabetes tijdens de behandeling met sunitinib.

Toekomstperspectieven

Leren van bijwerkingen: mechanismen en mogelijkheden

De introductie van angiogeneseremmers en andere doelgerichte behandelingen heeft de prognose van verschillende soorten kanker verbeterd. Het gebruik wordt echter soms beperkt door de bijwerkingen. Toekomstig onderzoek naar de verschillende bijwerkingen is nog steeds noodzakelijk om de behandeling te optimaliseren.

Voor patiënten met kanker heeft de ontwikkeling van doelgerichte behandelingen geleid tot een verbetering in de progressievrije en algehele overleving, maar ook langere periodes van behandeling. In contrast tot de conventionele chemotherapie worden de meeste doelgerichte behandelingen oraal in de poliklinische setting gegeven. Met een verlengde behandelperiode wordt de impact van bijwerkingen op de kwaliteit van het leven groter. Milde bijwerkingen kunnen een last worden als ze het dagelijks leven beïnvloeden en soms zijn daardoor dosisaanpassingen

of onderbrekingen van de behandeling nodig, waardoor de behandeling ook mogelijk minder effectief wordt. Verder kunnen bijwerkingen leiden tot ernstige morbiditeit of zelfs mortaliteit als ze niet tijdig herkend of behandeld worden. Herkenning van patiënten die een verhoogd risico hebben op het ontwikkelen van bijwerkingen en het tijdig inzetten van preventieve maatregelen of behandeling is essentieel in het optimaliseren van behandelduur, veiligheid en effectiviteit van doelgerichte behandelingen van kanker. Echter kunnen sommige bijwerkingen, zoals hypertensie tijdens angiogeneseremming, ook dienen als biomarker voor het effect van de behandeling, wat de preventie en behandeling van deze bijwerking nog uitdagender maakt.

Een andere reden om onderzoek te doen naar bijwerkingen van doelgerichte behandelingen ligt niet binnen het gebied van kankerbehandeling. Bijwerkingen van doelgerichte behandelingen bij patiënten met kanker impliceren dat dezelfde signaalwegen ook binnen de fysiologie een rol moeten spelen. Onderzoek naar de rol van deze signaalwegen in de normale lichaamsfuncties kan inzicht geven in het ontstaan van andere ziektes, zoals hoge bloeddruk en hartfalen, en uiteindelijk zelfs deuren openen naar potentieel gebruik van dezelfde signaalwegen voor de behandeling van andere ziektes, zoals diabetes, hypertensie en overgewicht. In dit kader rechtvaardigen de resultaten van dit proefschrift verder onderzoek naar de rol van de verschillende signaalwegen in de regulatie van insuline en hun effect op hoge bloeddruk en de bijbehorende complicaties.

List of publications

1. The early effect of sunitinib on insulin clearance in patients with metastatic renal cell carcinoma
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2. Impaired endothelium-dependent vasodilation does not initiate the development of sunitinib-associated hypertension
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3. Sunitinib does not attenuate contractile force following a period of ischemia in isolated human cardiac muscle
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8. Survival of the probiotic L. plantarum 299v and its effects on the faecal bacterial flora, with and without gastric acid inhibition
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   Dig Liver Dis 2005 Jan; 37(1):44-50

Bedankt!

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Curriculum Vitae

Annemarie Thijs werd geboren op 5 oktober 1979 te Maastricht. Ze behaalde haar VWO-diploma in 1997 aan het Stedelijk Gymnasium te Breda. Na een jaar in Honduras op het Liceo Catolico Cristo Rey in Siguatepeque begon ze in 1998 aan haar opleiding Geneeskunde aan de Universiteit Maastricht en behaalde in 2004 cum laude haar artsexamen. Na haar artsexamen werkte ze een half jaar als arts-assistent op de afdeling interne geneeskunde in het Amphia ziekenhuis te Breda. In 2005 werd ze aangenomen voor de opleiding interne geneeskunde in het Radboudumc te Nijmegen onder supervisie van (prof. Dr. J.W.M van der Meer en Prof. Dr. J. de Graaf en later prof. dr.J.W.A. Smit). Van 2005 tot 2008 werkte zij als AIOS interne geneeskunde in het Jeroen Bosch Ziekenhuis in 's-Hertogenbosch (opleider: dr. P. Netten). Daarna zette zij haar opleiding voort in het Radboudumc. In 2009 onderbrak zij haar opleiding om aan het onderzoek te werken, dat in dit proefschrift beschreven staat, dit onder begeleiding van prof. Dr. Winette van der Graaf (Radboudumc, Medische Oncologie), prof. Dr. Gerard Rongen (Radboudumc, Farmacologie-Toxicologie) en dr. Carla van Herpen (Radboudumc, Medische Oncologie). In de periode van 2009 tot 2015 werd dit onderzoek afgewisseld met de opleiding tot internist en tevens de differentiatie Medische Oncologie en de differentiatie Klinische Farmacologie. In februari 2015 werd haar opleiding tot internist-klinisch farmacoloog afgerond en in september 2015 volgde tevens de registratie tot internist-oncoloog. Na voltooiing van haar specialisatie werkt zij vanaf 1 oktober 2015 als internist in het Catharina ziekenhuis in Eindhoven.

Tijdens haar onderzoeksperiode ontving zij voor het onderzoek beschreven in hoofdstuk 3 van dit proefschrift een prijs voor het beste abstract op de Internistendagen 2012, de Takeda-Gert van Montfrans hypertensieprijs 2013 van het Nederlands Hypertensie Genootschap en de NVKFB (Nederlandse vereniging voor Klinische Farmacologie en Biofarmacie) TOP-publicatie prijs 2014. Voor hoofdstuk 4 kreeg ze de prijs voor best poster EACPT (European Association of Clinical Pharmacology and Therapeutics) 2013 in Geneve, Zwitserland. En voor het onderzoek beschreven in hoofdstuk 7 van dit proefschrift ontving zij een ESMO (European Society of Medical Oncology) travel grant 2014.

Annemarie woont samen met Sander van de Waarsenburg. Samen hebben zij twee kinderen, Noor (2007) en Maud (2010).