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Pharmacogenetics of sunitinib in metastatic renal cell carcinoma

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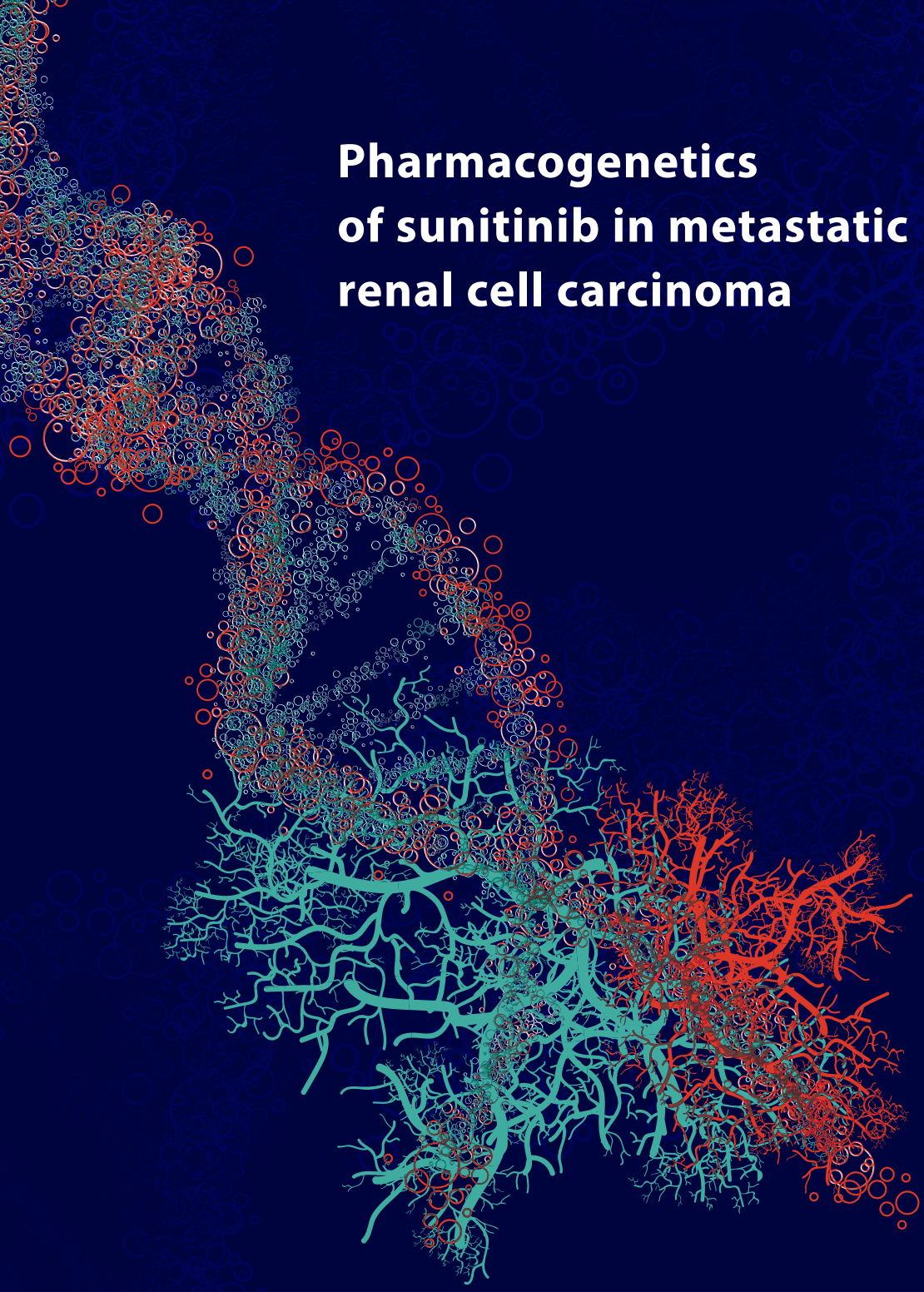

Meta Diekstra

Pharmacogenetics of sunitinib
in metastatic renal cell carcinoma

Meta Diekstra

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Pharmacogenetics of sunitinib

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Opgedragen aan mijn ouders

"He who loves practice without theory is like the sailor who boards ship without a rudder and compass and never knows where he may be cast."

Leonardo da Vinci, 1452-1519

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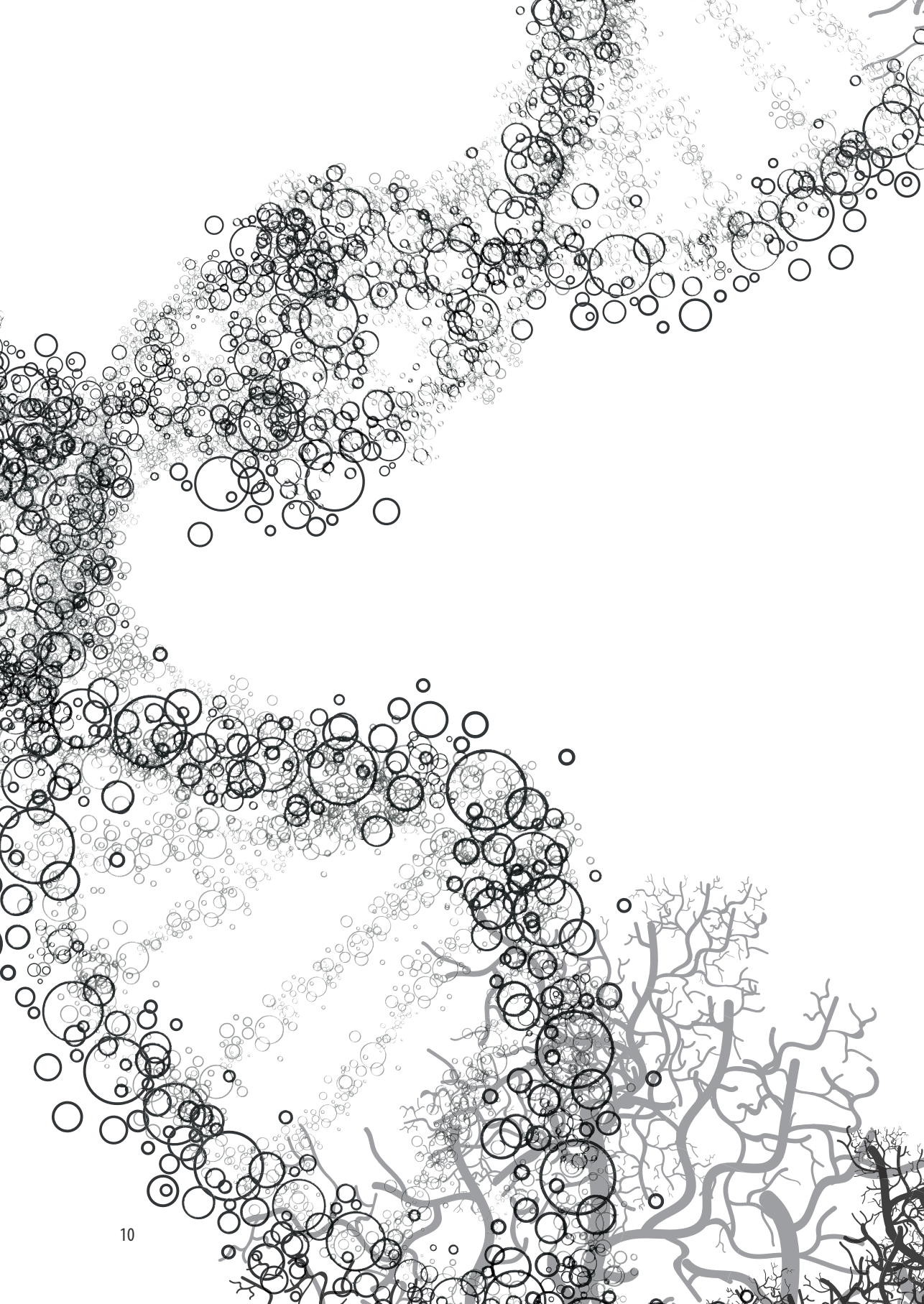
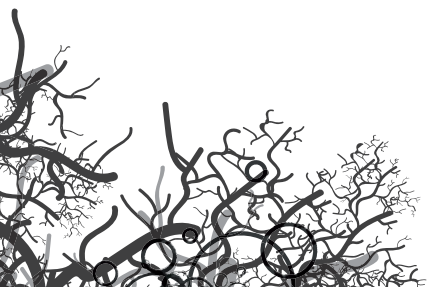


CHAPTER 1

General introduction



Renal cell carcinoma: epidemiology, pathology and risk factors

The most common type of kidney cancer (>90%) is renal cell carcinoma (RCC) which comprises about 2-3% of all malignancies in adults.[1,2] RCC typically occurs in people aged between 60 and 70 years old and is more common in men than in women.[1-3] In 2013, worldwide 350,000 people were diagnosed with RCC with more than 140,000 deaths. In the Netherlands 2,327 new cases were diagnosed in that year.[3,4] The age-adjusted incidence of RCC seems to have increased in the last couple of years with a higher incidence in developed countries compared to developing countries. The higher RCC incidence could be explained by enhanced use of imaging techniques such as ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI).[2,3] In most cases tumors are small and non-metastatic at first presentation, but approximately 17% of RCCs are metastasized at first diagnosis. Preferred sites of metastasis are lung, liver, bone, brain, and only rarely adrenal glands or the contralateral kidney.[1-3]

RCC is a heterogeneous tumor encompassing a wide variety of tumor types with clear cell RCC as the most common histological subtype (≈75%). Less common are the non-clear cell types: papillary type I (5%), papillary type II (10%), chromophobes (7%), medullary, collecting duct, and translocation renal tumors (**Figure 1**).[1-3] Moreover, multiple subtypes can be distinguished within a tumor. Non-clear cell subtypes have a poor prognosis in contrast to clear cell RCCs that show improved survival outcomes.[5]

Risk factors for RCC are smoking, obesity, hypertension or hereditary causes.[1-3] A mutation in the von Hippel-Lindau (*VHL*) gene is the most frequent hereditary cause (>80% of the clear cell RCCs). This causes a dysfunction of the VHL protein which prevents VHL to form a complex with hypoxia inducible factor α (HIF-α) so that HIF-α cannot be destroyed by proteasome degradation. Consequently, HIF-α accumulates in the cell inducing the secretion of proangiogenic factors, including the vascular endothelial growth factor (VEGF), that allow tumors to grow easily through oxygen supply by blood vessel formation.[1,3,6]

RCC treatment options in the era of targeted therapy

First and foremost, before any treatment is being considered and if RCC is discovered at an early stage, surgical removal is the standard of care with the aim to cure the disease, provided that the patient has a good performance status. Minimally invasive techniques such as nephron sparing surgery (partial nephrectomy), exposure to extreme temperatures (cryoablation or radiofrequency ablation) are used rather than radical nephrectomy (removal of the entire kidney) to prevent chronic kidney disease and other morbidity.[1-3] Elderly patients with a poor prognosis or comorbidity and diagnosed with (localized) RCC with a tumor size <40 mm, should be under active surveillance by regularly imaging. Especially in asymptomatic patients, this period of observation before starting treatment is essential. In about 20% of the cases, small renal tumors may turn

out to be benign; slowly growing and self-limiting tumors, and only in 1 to 2% of the cases metastatic disease was reported.[1-3]

In case of metastases or if surgery is not deemed feasible, treatment options will be more palliative than curative. Radiotherapy can offer pain relief in bone or brain metastases and chemotherapy has little or no effect.[1-3] The current drug arsenal against metastatic renal cell carcinoma (mRCC) is large and continues to expand. For a long time cytokines (interleukin-2 and interferon-alpha) were the only available treatment options with limited efficacy and substantial side effects. Since 2005 the number of drugs against kidney cancer has been growing with the arrival of targeted therapies that act on small proteins present in signal transduction pathways in and around the tumor. Targeted therapies that act in the tumor cell and inhibit the mammalian target of rapamycin (mTOR) are the so-called mTOR inhibitors everolimus and temsirolimus and cause a decreased expression of HIF-α. Temsirolimus is used in patients with a poor prognosis but is less and less prescribed, while everolimus is indicated as second-line treatment after VEGF-targeted therapy for good or intermediate prognosis. An alternative treatment option is the monoclonal antibody bevacizumab used in combination with interferon-alpha, which functions in between endothelial cells and tumor cells targeting VEGF and preventing it from binding to its receptor. Especially the (multi)targeted tyrosine kinase inhibitors (TKIs) showed to be very successful and target receptors on the endothelial cell with an anti-angiogenic effect (**Figure 1**).

Sorafenib was the first TKI approved for the treatment of RCC.[7] Sunitinib entered the market in 2006. In the pivotal phase III trial of sunitinib in mRCC, it was shown that sunitinib significantly improves progression-free survival (PFS) and objective response rate compared to the former standard choice of therapy interferon-alpha ($P<0.001$) and an almost significant improvement in overall survival (OS) was seen for sunitinib ($P=0.051$).[8] Later, in 2009, pazopanib was approved for mRCC treatment and meanwhile many more TKIs were being investigated, such as axitinib.[9,10] The targeted therapies as mentioned above have been proven effective in clear cell RCC. For non-clear cell RCC however, less is known about the efficacy of anti-VEGF therapies and mTOR-inhibitors.[5]

Sunitinib pharmacology

The work described in this thesis focuses on sunitinib. Sunitinib is administered orally, most often given as 50 mg in a 4 week on/2 week off schedule. Dose reduction can be applied in steps of 12.5 mg where a continuous regimen of 37.5 mg is also applied. The main pharmacodynamic targets of sunitinib are VEGF-receptors type 1, 2 and 3, platelet-derived growth factor receptors (PDGFRs) α and β, Fms-related tyrosine kinase 3 (FLT3), and cytokine receptor Kit (c-KIT).[8] Sunitinib is mainly metabolized by cytochrome P450 (CYP)3A4 to its active metabolite SU12662, and inactive metabolites. Both sunitinib and

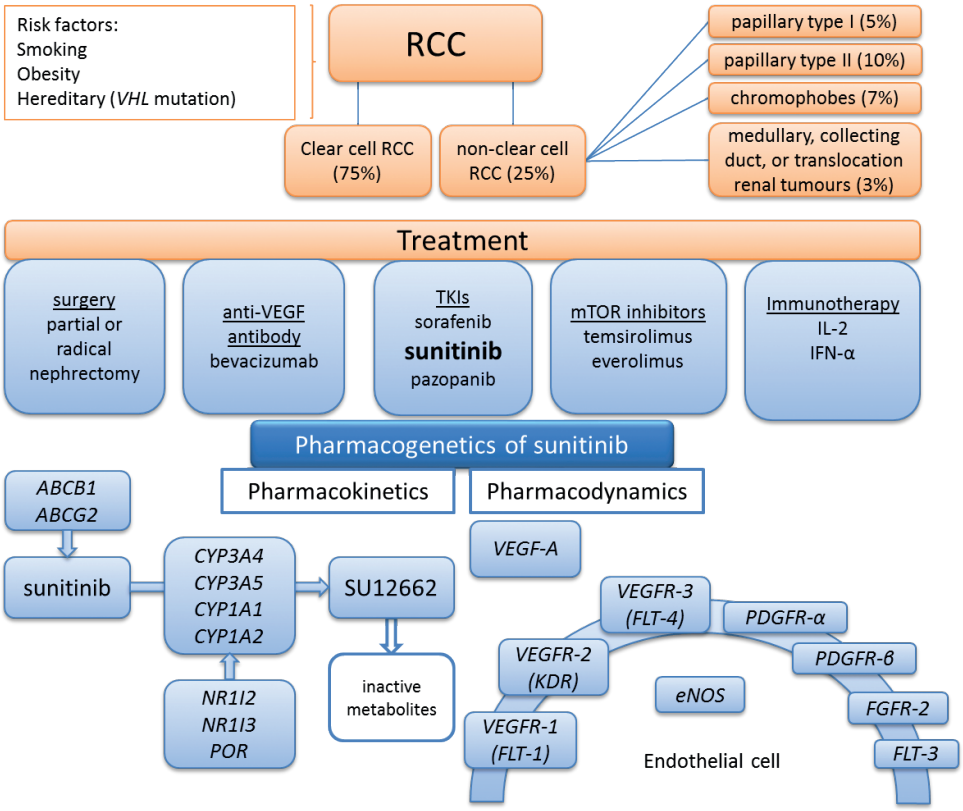


Figure 1: Overview of RCC risk factors, tumor types, and treatment options with a focus on sunitinib and pharmacogenetics of sunitinib in mRCC.

SU12662 embody the total amount of active drug in plasma.[11-13] Furthermore, CYP3A5, CYP1A1 and CYP1A2 could be involved in sunitinib metabolism. The pregnane X receptor (NR1I2), the constitutive androstane receptor (NR1I3) and P450 oxidoreductase (POR) can influence CYP3A4 expression or activity.[14,15] Other important modulators of sunitinib pharmacokinetics (PK) are the efflux transporters ATP-binding cassette (ABCB1) and the breast cancer resistance protein (ABCG2).[16] Patients show a high variability in PK of sunitinib and SU12662 and respond very differently to drugs with large differences in toxicity and efficacy.[12,13,17-19] Most common side effects are fatigue, hand-foot syndrome (HFS), hypertension, mucositis, hair or skin discoloration and hypothyroidism. About 30% of RCC patients on sunitinib required a dose reduction for any of these adverse events.[13,17-19]

Despite successful advances in RCC treatment since the advent of targeted therapy, we have not yet reached our goal. Interpatient variability in response is large and the great difficulty is that we cannot predict an individual's response to sunitinib, which is highly essential in order to give patients an optimally effective and safe treatment and to efficiently use the available drug arsenal without adjustments afterwards. For that reason, we need specific biomarkers that can predict the individual treatment response with regard to efficacy and toxicity of sunitinib.

Pharmacogenetics of sunitinib

It is plausible that genetic variability in genes involved in the PK or pharmacodynamics (PD) of a drug are important determinants of a person's response to the drug with respect to adverse events and efficacy. Pharmacogenetics thoroughly explores this hypothesis. Previous pharmacogenetic studies used the candidate gene approach to select small genetic variations, namely Single Nucleotide Polymorphisms (SNPs), located in genes thought to be related to PK and PD of sunitinib. PK related genetic variants in genes coding for CYP enzymes, drug efflux transporters or influencers of CYP3A activity have been identified to be associated with toxicity or efficacy of sunitinib in mRCC.[17-19,20-21] SNPs in *CYP1A1*, *CYP3A5*, *ABCB1*, *ABCG2*, and *NR1I3* showed an association directly with toxicity or with dose reductions due to toxicity.[17,18] Furthermore, SNPs in *CYP3A5*, *ABCB1, NR1I2* and *NR1I3* have been associated with PFS on sunitinib.[19] Regarding PD, SNPs associated with sunitinib toxicity or efficacy were located in genes encoding receptors on the endothelial cells or endothelial nitric oxide synthase (eNOS). SNPs in *VEGF-A*, *VEGFR-1*, *VEGFR-2 VEGFR-3, FLT3,* and *eNOS* have been associated with adverse events (particularly hypertension), PFS or OS on sunitinib (**Figure 1**).[19,20,21] Yet, no genetic biomarker has been implemented for clinical use and little is known about the mechanisms underlying possible SNP effects. Therefore, the objective of this thesis is to investigate whether pharmacogenetic testing of SNPs prior to treatment initiation may possibly predict the individual response to sunitinib in mRCC and therefore benefit the patient's wellbeing.

Aim and outline of thesis

The general aim of this thesis was to study the predictive ability of germline pharmacogenetic markers on toxicity and efficacy outcomes of sunitinib in mRCC patients. This research is part of a European collaborative project on "TArgeted therapy in Renal cell cancer: GEnetic and Tumor related biomarkers for response and toxicity" (EuroTARGET) funded by the European Commission under the Seventh Framework Programme (FP7).[22]

Chapter 2 systematically reviews studies on SNPs (pharmacogenetics) but also gene expression or epigenetic biomarkers (pharmacogenomics) as predictors of TKI treatment

outcome in mRCC containing studies from 2005 to 2015. The quality of included studies is discussed and the prospect for clinical implementation of study results or the need for follow-up studies.

Previously, SNPs have been associated with toxicity or efficacy outcomes on sunitinib, but it is not clear whether these SNPs are also causally related to PK of sunitinib.[17-19] In **Chapter 3** it is studied whether previously associated SNPs located in genes involved in the metabolism or absorption of sunitinib influence the clearance of sunitinib and its active metabolite SU12662.

In **Chapter 4** a pooled dataset is formed by including patients from previous pharmacogenetics analyses on sunitinib performed in the United States, Spain and the Netherlands.[17-19,20,21] The aim of this study is to investigate whether SNPs associated with toxicity and efficacy on sunitinib in earlier exploratory studies could be confirmed in a larger group of 333 mRCC patients. To this end, 22 significantly associated SNPs ($P<0.05$) from previous exploratory studies are selected and retested in this study for association with sunitinib treatment outcomes.

At the American Society of Clinical Oncology (ASCO) annual meeting in 2012, Urun *et al*. reported that SNP rs4646437G>A in *CYP3A4* had a protective effect on sunitinib related toxicity as shown in a cohort of 159 mRCC patients.[23] In **Chapter 5** the relationship between rs4646437 in *CYP3A4* and sunitinib toxicity or efficacy is further investigated in a large cohort of mRCC patients.

A set of relatively new SNPs (reported from 2011 onwards) in *CYP3A4*, *NR1I2*, *POR*, *IL8*, *IL4-R*, *IL13*, *HIF1A* and *MET* were reported as potential prognostic or predictive markers for TKI treatment outcome.[24-30] With an exploratory nature, **Chapter 6** evaluates whether these SNPs play a role in sunitinib toxicity or efficacy outcomes in a large cohort of sunitinib treated mRCC patients.

In **Chapter 7** SNP rs34231037 in *VEGFR-2* (*KDR*) is analyzed in a large series of clear cell mRCC patients treated with sunitinib for the possible effect on response and PFS. This study objective was based on a Genome Wide Association Study (GWAS) of Maitland *et al*. in which this SNP was identified as a possible predictor for variations of soluble VEGFR2 (sVEGFR2) in the blood and sVEGFR2 levels at baseline and after 4 weeks of pazopanib treatment.[31]

A better understanding of the relationships between sunitinib exposure, the pharmacological response, and the clinical outcomes is vital to find predictive biomarkers for treatment outcome. **Chapter 8** describes a nonlinear mixed-effects population PK/PD model that is built by means of blood sampling data of sunitinib treated patients diagnosed with either mRCC or colorectal carcinoma (CRC) and a group of 12 healthy volunteers.[32] The aim of this study is to evaluate the predictive performance of this model including PK parameters, blood pressure, sVEGFR-2, sVEGFR-3, and SNP genotypes.

Subsequently, the applicability of the model to predict clinical outcomes on sunitinib treatment is explored.

Due to the rapid developments in genetic testing technologies, possibilities to test more than one SNP at a time become easily accessible. In **Chapter 9** about 1 million SNPs are tested for the association with sunitinib toxicity or efficacy in a GWAS that allows less biased hypothesis-free testing for associations as compared to the candidate gene approach used in the aforementioned chapters.

This thesis closes with a general discussion and future perspective on the topic in **Chapter 10** and a summary written in English and Dutch in **Chapter 11**.

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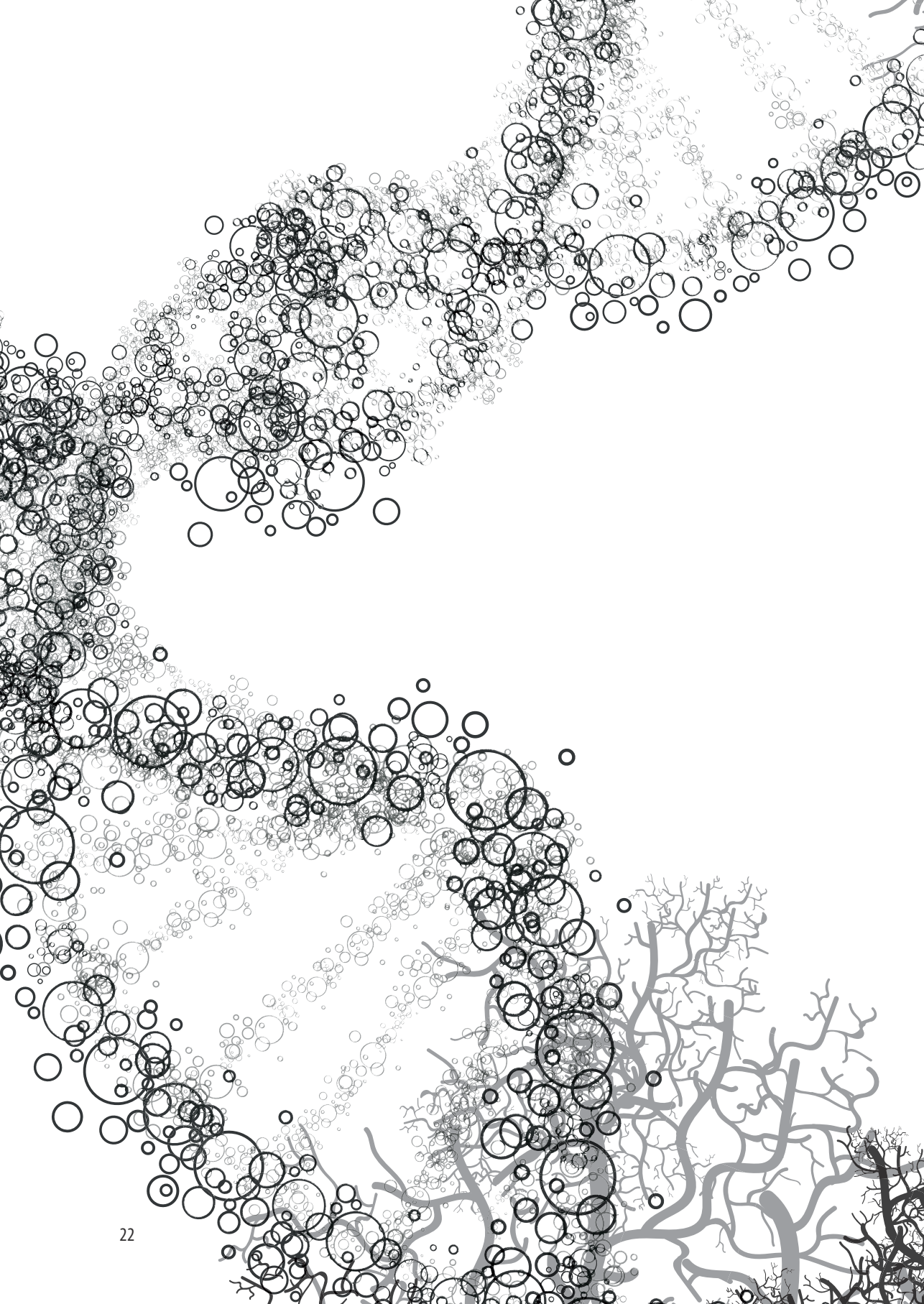

CHAPTER 2

A decade of pharmacogenomics research on tyrosine kinase inhibitors in metastatic renal cell cancer: a systematic review

Diekstra MH, Swen JJ, Gelderblom H, Guchelaar H-J.

Exp Rev of Mol Diagn. 2016;16(5):605-618.

ABSTRACT

Objective The individual response to targeted tyrosine kinase inhibitors (TKIs) in the treatment of metastatic renal cell cancer (mRCC) is highly variable. Outlined in this article are findings on potential biomarkers for TKI treatment outcome in mRCC and an evaluation of the status of clinical implementation.

Methods Articles were selected by two independent reviewers using a systematic search in five medical databases on renal cell carcinoma, TKIs, and pharmacogenetics.

Results Many researchers have focused on predictive biomarkers for treatment outcome of targeted therapies in mRCC patients. Attempts to explain differences in efficacy and toxicity of TKIs by use of genetic variants in genes related to the pharmacokinetics and pharmacodynamics of the drug have been successful.

Conclusion Most findings on potential biomarkers have not been validated and therefore biomarker testing to guide choice of therapy and dose in mRCC is not yet feasible.

INTRODUCTION

Using pharmacogenomics in our endeavor to personalize mRCC treatment

Renal cell cancer (RCC) represents ~3% of cancer in adults (approximately 170,000 people worldwide per year). RCC typically occurs in adults aged 50-70 years and is more common in men than in women. For many years, cytokine therapies (interleukin-2 and interferon-alpha) were the only available treatment options with limited efficacy and considerable toxicity.[1,2] In the last decade, multiple targeted agents for the treatment of metastatic RCC (mRCC) have become available, namely: the multitargeted oral tyrosine kinase inhibitors (TKIs) with antiangiogenic activity. Of the first generation TKIs, sorafenib was approved for the treatment of mRCC in 2005, followed by sunitinib (2006) and pazopanib (2009). The first-generation TKIs target the vascular endothelial growth factor (VEGF) pathway by inhibition of the vascular endothelial growth factor receptors (VEGFR-1, 2, and 3) but also inhibit many other targets. Sorafenib, sunitinib, and pazopanib all target the platelet-derived growth factor receptor (PDGFR) α and/or β and the c-Kit protein (c-Kit). Additionally, sunitinib and sorafenib inhibit FMS-like tyrosine kinase 3 (Flt-3) and glial cell line-derived neurotrophic factor receptor (REarranged during Transfection) tyrosine kinases. Furthermore, sunitinib inhibits colony-stimulating factor 1 receptor, and sorafenib specifically inhibits the RAF kinases (C-RAF and B-RAF).[3-6]

Starting 2012, the second-generation TKIs emerged with an improved potency and selectivity, such as axitinib. Many of these second-generation TKIs were assessed for the treatment of mRCC, such as tivozanib, cediranib, and cabozantinib.[2-5,7,8] Axitinib predominantly blocks the VEGF receptors, with a 50-450 times higher potency than the first-generation TKIs and less off-target activity, which could explain the larger therapeutic window and fewer side effects.[2,7]

Increased exposure to sunitinib is associated with improved survival outcomes but also an increased risk for adverse events.[3,5] Sunitinib revealed an improvement in progression-free survival (PFS) and objective response rate compared to interferon-alpha ($P< 0.001$) and showed a tendency towards an improved overall survival (OS) ($P=0.051$).[3] For several years, sunitinib has been considered the first-choice treatment for mRCC. However, based on the results of the PISCES trial (a randomized, controlled, double-blind, cross-over trial assessing treatment preference for pazopanib versus sunitinib in patients with metastatic renal cell carcinoma) and the larger COMPARZ trial (a phase 3 study of pazopanib versus sunitinib in the treatment of locally advanced and/or metastatic renal cell carcinoma)[9,10] indicating noninferiority for pazopanib with regard to toxicity, patient preference, and quality of life, both pazopanib and sunitinib are prescribed as first-line treatment for mRCC.[9,10]

The individual response to TKIs is highly variable in mRCC patients: some experience severe toxicities for which dose reductions or even cessation of therapy is needed, while others show no response at all. Previous studies identified single nucleotide polymorphisms (SNPs) in genes encoding enzymes or transporters related to pharmacokinetics (PK) and pharmacodynamics (PD) of TKIs which have been associated with toxicity and efficacy of TKIs.[11,12] Also cytokines and antiangiogenic factors have been studied as potential predictors for TKI treatment outcome in mRCC patients. Ultimately, biomarker testing prior to start of therapy may result in providing the individual patient the most effective treatment with the least possible side effects. Several review articles on pharmacogenomics in mRCC have been published[11-14], but only one with a systematic approach.[14] In our current systematic review article, we strictly focus on the predictive value of biomarkers (as opposed to the prognostic value), especially SNPs (pharmacogenetics biomarkers) and only in TKI treatment. Here, we outline the findings from currently available pharmacogenomics studies on potential predictive biomarkers for mRCC treatment outcome. In addition, we evaluate the quality of the selected studies, clarify the status on implementation of these predictive biomarkers into the clinic, and give recommendations as to what the focus of attention should be in future studies.

METHODS

Protocol

A systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines.[15] Prior to the literature search, a systematic review protocol was prepared following the PRISMA-P 17-item checklist (**Supplementary document 1**)[15] and registered at the international prospective register of systematic reviews (PROSPERO; CRD42015016509).[16]

Systematic literature search

Together with an experienced librarian, we performed a systematic search in 5 medical databases: MEDLINE, EMBASE (OVID version), Web of Science, The Cochrane Library, and Cochrane Central Register of Controlled Trials (CENTRAL). This search comprised three topics: renal cell carcinoma, TKIs (e.g. sunitinib, sorafenib), and pharmacogenetics (**Supplementary document 2**). All databases were searched for keywords on these items taking into account the terminological and technical differences between these databases. Various synonyms and related terms for all subjects were used. The search was performed on 14 January 2015. Results were limited to articles in the English language and from the year 2004 onwards. Meeting abstracts were excluded. From

our initial search up to the moment of our final selection of articles, new studies may have appeared. To cover articles published after 14 January 2015, the initial search was repeated in MEDLINE on 31 May 2015.

Selection process and data extraction

Articles were first selected by title, then by abstract, and finally relevant data were extracted systematically from included full text articles. Selection of included articles was performed by two independent reviewers (MD, JS). In case of disagreement, a third reviewer (HJG) made the final decision. References were eligible when genetic biomarkers (i.e. SNPs, genetic variants, gene/protein expression) were investigated in relation to TKI treatment and mRCC. Review articles, preclinical studies (animal models and in vitro studies), phase I or II clinical trials, and papers discussing pathology, prognosis, tumor biology, or somatic mutations (e.g. Von Hippel-Lindau mutations) were excluded. Accordingly, articles were excluded if nontreatment-related biomarkers were studied or if no TKI response was assessed, because these generally consider prognostic biomarkers.

Extracted data were first author, year of publication, demographics (age, sample size, sex, ethnicity), sample material and origin (i.e. germline DNA from blood, serum, or other), methods, tumor type, drug information (i.e. drug name, treatment line, dose), evaluated biomarkers, endpoints, and statistical approach. Articles resulting from the secondary search on 31 May 2015 were subject to an identical selection process. Using cross-references of the included articles from our systematic search, relevant articles were added to our final selection. Clinical validity (the robustness of the statistical association between a pharmacogenetic variant and outcome of drug therapy) and clinical utility (a test's health-care value) of the included articles was evaluated in a descriptive manner.[17,18]

RESULTS

Search results

Our primary search identified 968 articles (**Supplementary document 2**). The secondary search identified 30 additional articles. After selection by title, abstract, and full text, a total of 54 articles were available for evaluation in this review. **Figure 1** represents a flowchart on the study selection process. The extracted data from the included articles are presented in **Table 1** and **Supplementary document 3**.

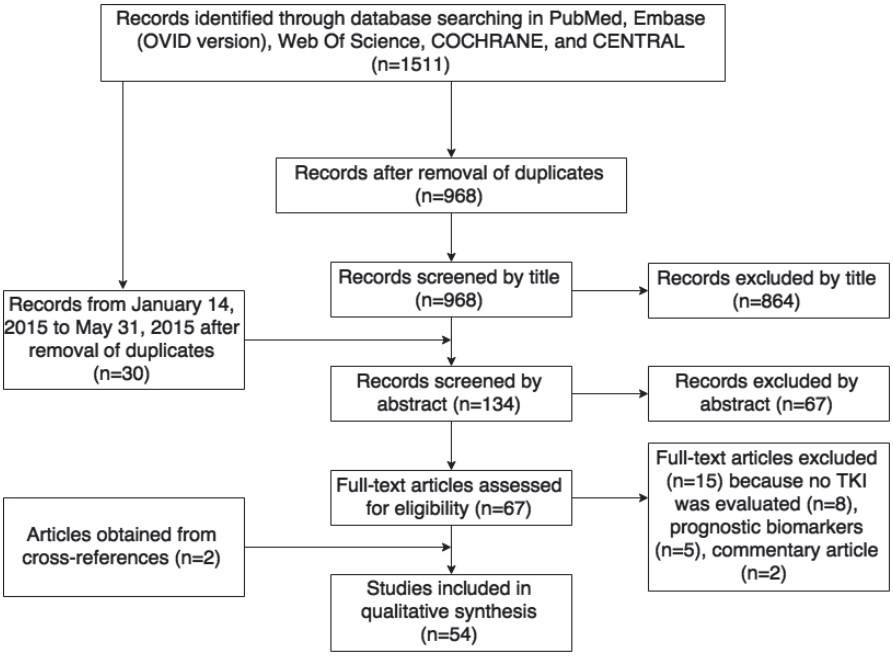

Figure 1 Flowchart of study selection process. Our original search on the 14th of January 2015 resulted in a total of 968 articles (**Supplementary document 2**). For the first selection by title, there was a disagreement between the two independent reviewers on a number of 114 titles (12%). After consensus was reached with the last review author (HJG), this resulted in a number of 104 articles which was complemented with 33 additional articles of the search from January 14 to May 31, 2015. A total of 134 articles was available for the second selection step based on abstract reading. Consensus had to be reached for 20 of the 134 abstracts (15%) and resulted in exclusion of 67 articles. Consequently, a number of 67 articles underwent full text review and data extraction. Finally, 15 articles were excluded for data extraction because no treatment was evaluated (n = 8), prognostic biomarkers were investigated (n = 5) or these were no original articles but commentaries (n = 2) and using cross-references, we included two additional studies.[37,69] Ultimately, a total of 54 articles was included in our systematic review.

SNPs associated with TKI treatment outcome in mRCC patients

From 2007 up till now, several genetic variants have been assessed for their possible association with the treatment outcome of sunitinib, pazopanib, sorafenib, or axitinib. These SNPs have been selected according to the candidate gene approach based on the knowledge on PK and PD of the drug. The reported findings in this field are outlined below and summarized in **Table 1** and **Figure 2**.[19-44]

Table 1 Reported SNP associations ($P \leq 0.05$) with TKI treatment outcome in mRCC from 2009 to2015.

First Author, year	TKI	No. of patients	Ethnicity	Associated SNPs	Study endpoint	Effect size	95%CI	*P* value
van Erp *et al.*, 2009 [19]	SUN	219	white: 93.6%	*CYP1A1* rs1048943	Leukopenia	OR:6.24	1.20-32.42	*P*=0.029
				FLT3 rs1933437		OR:0.36	0.17-0.77	*P*=0.008
				NR1I3 (rs2307424, rs2307418, rs4073054) CAG haplotype		OR:1.74	1.02-2.96	*P*=0.041
				VEGFR2 rs2305948	Any toxicity > grade 2	OR:2.39	1.02-5.60	*P*=0.046
				ABCG2 (rs55930652, rs2622604)		OR:0.38	0.17-0.83	*P*=0.016
				CYP1A1 rs1048943	Mucosal inflammation	OR:4.03	1.24-13.09	*P*=0.021
				ABCB1 (rs1045642, rs1128503, rs2032582) TTT haplotype	HFS	OR:0.39	0.16-0.94	*P*=0.035
Garcia-Donas *et al.*, 2011 [20]	SUN	89 (efficacy)	mostly European	*VEGFR3* rs307826	PFS	HR:3.57	1.75-7.30	*P*=0.0079
				VEGFR3 rs307821		HR:3.31	1.64-6.68	*P*=0.014
		95 (toxicity)		*CYP3A5* rs776746	dose reductions due to toxicity	HR:3.75	1.67-8.41	*P*=0.022
Beuselinck *et al.*, 2014 [21]	SUN	96	95% CAU	*ABCB1* rs1125803	Time-to-dose-reduction (TTDR)	-	-	*P*=0.012
				ABCB1 rs2032582		-	-	*P*=0.024
Mizuno *et al.*, 2010 [22]	SUN	5	JAP	*ABCG2* rs2231142	exposure and toxicity	-	-	*P*<0.05
Mizuno *et al.*, 2012 [23]	SUN	19	JAP	*ABCG2* rs2231142	Dose-adjusted AUC	-	-	*P*=0.02
Miura *et al.*, 2014 [24]	SUN	1	JAP	*ABCG2* rs2231142	grade 3 and 4 toxicity	-	-	-
Eechoute *et al.*, 2012 [25]	SUN	255	91% CAU	*VEGFA* (rs699947, rs833061, rs2010963) ACG haplotype	SBP	-	-	*P*=0.014
					MAP	-	-	*P*=0.036
					Grade 3 hypertension	OR:0.59	0.34-1.03	*P*=0.031
				eNOS rs2070744	Grade 3 hypertension	OR:2.62	1.08-6.35	*P*=0.045
Kim *et al.*, 2012 [26]	SUN	63	CAU	*VEGFA* rs2010963	prevalence of hypertension	OR:13.62	3.71-50.04	*P*=0.05
				VEGFA rs2010963	duration of hypertension	21%	12%-29%	*P*=0.02
				VEGFA rs3025039 + *VEGFR2* rs2305948	OS	HR:3.18	1.12-8.99	*P*=0.03
Kim *et al.*, 2013 [27]	SUN	65	Asian (Korea)	*ABCG2* rs2231142	grade 3 or 4 thrombocytopenia	OR:9.90	1.16-infinity	*P*=0.04
					grade 3 or 4 neutropenia	OR:18.20	1.49-222.09	*P*=0.02
					grade 3 or 4 HFS	OR:28.46	2.22-364.94	*P*=0.01
Takayoshi *et al.*, 2015 [28]	SUN	1	probably JAP	*ABCB1* (rs1045642, rs1128503, rs2032582)	Severe toxicity	-	-	-
Teo *et al.*, 2015 [29]	SUN	25	CHI = 86.1% (other Malay or Indian)	*ABCB1* rs1045642	sunitinib exposure	76.81 vs 56.55 ng/ml		*P*=0.03
					all-grade rash	RR:3.00	1.17-7.67	*P*<0.05
					all-grade mucositis	RR:1.60	1.10-2.34	*P*<0.05
					disease progression	RR:4.57	1.08-19.42	*P*=0.05

Table 1 Continued

First Author, year	TKI	No. of patients	Ethnicity	Associated SNPs	Study endpoint	Effect size	95%CI	*P* value
Diekstra *et al.*, 2015 [30]	SUN	333	96% white	*CYP3A5* rs776746	dose reductions	OR:2.0	1.0-4.0	*P*=0.039
				ABCB1 (rs1128503, rs2032582, rs1045642)	PFS	HR:1.9	1.3-2.6	*P*=0.000275
van der Veldt *et al.*, 2011 [31]	SUN	136	95.6% CAU	*CYP3A5* rs776746	PFS	HR:0.27	0.08-0.89	*P*=0.032
				NR1I3 (rs2307424, rs2307418, rs4073054) CAT haplotype		HR:1.76	1.11-2.79	*P*=0.017
				ABCB1 (rs1045642, rs1128503, rs2032582) TCG haplotype		HR:0.52	0.29-0.95	*P*=0.033
Scartozzi *et al.*, 2013 [32]	SUN	84	CAU (Italy)	*VEGFA* rs833061	PFS OS	HR:0.71 HR:0.69	-	*P*=0.0197 *P*=0.0011
				VEGFA rs2010963	PFS	HR:0.19	-	*P*=0.0201
				VEGFR3 rs6877011	PFS OS	HR:0.35 HR:0.39	- -	*P*<0.0001 *P*<0.0001
Motzer *et al.*, 2014 [33]	SUN	202	88% CAU	*VEGFA* rs699947	PFS, OS, objective response or time-to-tumor progression	no significant correlations		
				VEGFA rs1570360				
				VEGFR3 rs448012				
				VEGFR3 rs307821				
				VEGFR3 rs307826				
Beuselinck *et al.*, 2013 [34]	SUN	88	94% CAU	*ABCB1* rs1128503	PFS	HR:0.464	0.234-0.918	*P*=0.027
				NR1I3 rs4073054		HR:1.864	1.082-3.210	*P*=0.025
				VEGFR3 rs307821		HR:1.981	1.060-3.702	*P*=0.032
				FGFR2 rs2981582		HR:2.669	1.094-6.511	*P*=0.031
				NR1I2 rs2276707		HR:2.978	1.012-8.761	*P*=0.047
				ABCB1 rs1128503	OS	HR:0.415	0.193-0.894	*P*=0.025
				NR1I3 rs4073054		HR:1.927	1.046-3.549	*P*=0.035
				VEGFR3 rs307821		HR:2.265	1.202-4.268	*P*=0.011
				NR1I3 rs2307424		HR:1.913	1.006-3.636	*P*=0.048
				VEGFR3 rs307826		HR:2.223	1.187-4.163	*P*=0.013
Beuselinck *et al.*, 2014 [35]	SUN	91	93% CAU	*VEGFR1* rs9582036	Response Rate (RR)	0% vs 46%		*P*=0.028
					OS	HR:0.249	0.078-0.799	*P*=0.008
				VEGFR1 rs9554320	PFS	HR:2.713	1.321-5.575	*P*=0.005
Mizuno *et al.*, 2014 [36]	SUN	19	JAP	*ABCG2* rs2231142	Oral clearance (CL/F)	Improvement of PK model: ΔOFV=8.59 (*P*=0.003)		
Diekstra *et al.*, 2014 [37]	SUN	114		*CYP3A4* rs35599367	Sunitinib clearance	-22.5%	-	*P*=0.01
				CYP3A5 rs776746	SU12662 clearance	+28.0%	-	*P*=0.04
				ABCB1 rs2032582	Sunitinib clearance	+18.0%	-	*P*=0.02
Xu *et al.*, 2010 [38]	PAZ	116 (exploratory) 130 (replication)	white	*UGT1A1* rs8175347 (*28) TA7 homozygotes	Bilirubin levels	OR:13.1	5.3-32.2	$P=4.5\times10^{-5}$
Xu *et al.*, 2011 [39]	PAZ	115 (exploratory) and 128 (replication)	white	*HFE* rs2858996 + rs707889 (in strong LD)	ALT levels	OR:39.7	2.2-703.7	*P*=0.006

Table 1 Continued

First Author, year	TKI	No. of patients	Ethnicity	Associated SNPs	Study endpoint	Effect size	95%CI	*P* value
Xu *et al.*, 2011 [40]	PAZ	397	84% white	*IL8* rs1126647	PFS	HR:1.8	1.2-2.7	*P*=0.009
				HIF1A rs11549467		HR:1.8	1.1-3.1	*P*=0.03
				HIF1A rs11549467	Response rate (RR)	RR:30% vs. 43%		*P*=0.02
				NR1I2 rs3814055		RR:37% vs. 50%		*P*=0.03
				VEGFA rs833061		RR:33% vs. 51%		*P*=0.02
Motzer *et al.*, 2013 [41]	SUN or PAZ	719 (SUN: N=350, PAZ: N=369)	Europe, North America, Asia, Australia	*UGT1A1* rs8175347 and rs4148323 (**28*,**37* and **60*)	Bilirubin levels on sunitinib	OR:4.51	1.26-16.11	*P*=0.024
					Bilirubin levels on pazopanib	OR:3.65	1.31-10.16	*P*=0.012
Xu *et al.*, 2015 [42]	PAZ or SUN	3 substudies: 186 pts (PAZ) 690 pts (353 PAZ + 337 SUN) 88 (SUN)	87% white 62% white 98% white	*IL8* rs1126647	OS	HR:1.32	1.15-1.52,	$P=8.8\times10^{-5}$
Tsuchiya *et al.*, 2013 [43]	SOR	33	JAP	*ABCC2* rs717620	high-grade skin rash	35 vs 0%	-	*P*=0.032
				*HLA-A*24*		-	-	*P*=0.049
Escudier *et al.*, 2015 [44]	AXI and SOR	305 (AXI: N=159, SOR: N=146)	94.1% white	*VEGFR2* rs2071559	PFS sorafenib	2.223	1.268-3.897	*P*=0.0053
					OS sorafenib	2.584	1.390-4.803	*P*=0.0027

ABCB1: ATP-binding cassette member B1; ABCG2: ATP-binding cassette member G2; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AXI: axitinib; CAU: Caucasian; CC: clear cell renal cell carcinoma; CHI: Chinese; GIST: gastrointestinal stromal tumor; JAP: Japanese; ΔOFV: delta Objective Function Value; OS: overall survival; PAZ: pazopanib; PFS: progression-free survival; PK: pharmacokinetic; SOR: sorafenib; SUN: sunitinib.

Pharmacogenetics to predict sunitinib toxicity

The first pharmacogenetic study on sunitinib tested for associations of genetic polymorphisms with toxicity endpoints ($P<0.05$) in a cohort of 219 patients with mRCC (N=159), gastrointestinal stromal tumor (GIST) (N=50), or other tumors (N=10). Van Erp *et al.* observed an increased risk for leukopenia in *CYP1A1* rs1048943, and *FLT3* rs1933437 and absence of CAG in the *NR1I3* haplotype (rs2307424, rs2307418, rs4073054). *CYP1A1* rs1048943 was associated with mucosal inflammation, and presence of TTT in *ABCB1* (rs1045642, rs1128503, rs2032582) was associated with HFS. Presence of the TT copy in *ABCG2* (rs55930652, rs2622604) and *VEGFR2* rs2305948 was associated with any toxicity >grade 2.[19]

Later, in a group of 95 mRCC patients, Garcia-Donas *et al.* reported that the *CYP3A5* A-allele (*CYP3A5*1*) of rs776746 was associated with dose reductions due to toxicity

(adjusted $P<0.05$).[20] In a study of Beuselinck *et al.*, an increased time-to-dose reduction was seen for the TT genotype patients of rs1128503 and rs2032582 in *ABCB1* compared to C-allele and G-allele carriers, respectively.[21]

Mizuno *et al.*[22,23] observed a case of severe haematological toxicity in an RCC patient with the AA genotype of rs2231142 in *ABCG2*. This prompted a study in 5 patients (1 variant homozygous, 3 heterozygous, and 1 wild-type homozygous) in which they observed an elevated sunitinib exposure for AA carriers.[22] The variant allele A is common in Asians (~30%), but rare in Caucasians (~10%) and African Americans (~5%). In a subsequent study with 19 Japanese patients, only 1 patient had the AA genotype of rs2231142 in *ABCG2*.[23] It was reported that this SNP was associated with an increase in systemic exposure to sunitinib, possibly causing thrombocytopenia and hypertension.[23] Miura *et al.*[24] followed with a case report in 2014 describing a patient with mRCC who developed severe grade 3 and 4 adverse events on sunitinib, namely fever, thrombocytopenia, transaminase elevation, hypoxia, and pulmonary edema. Again, the AA genotype of rs2231142 in *ABCG2* was suggested to explain these toxicities in Asians.

Hypertension is a common adverse effect of sunitinib. Eechoute *et al.* identified genetic biomarkers by using the pathway approach using data from 255 patients with clear cell mRCC (N=167), non-clear cell mRCC (N=22), an unknown type of RCC (N=4), GIST (N=53), or another tumor type (N=9).[25] An ACG haplotype in *VEGF-A* (rs699947, rs833061, rs2010963) was associated with elevations in systolic blood pressure (SBP) and mean arterial pressure (MAP). The same haplotype in *VEGF-A* and presence of the C-allele of rs2070744 in *eNOS* showed a tendency to develop grade 3 hypertension.[25] Similarly, Kim *et al.*[26] evaluated 63 sunitinib-treated mRCC patients for the association of SNPs in *VEGF* or *VEGF-R* with hypertension and clinical outcome. The GG genotype of SNP rs2010963 in *VEGF* was associated with a greater chance for the occurrence and duration of hypertension.[26]

Sunitinib-induced toxicities in relation to pharmacogenetic determinants were studied by Kim *et al.*[27] in a number of 65 Korean mRCC patients. Compared to C-allele carriers, the AA genotype of rs2231142 in *ABCG2* had an increased risk for grade 3 or 4 thrombocytopenia, neutropenia, and HFS.[27] In addition, a case report of Takayoshi *et al.*[28] reported a 65-year-old woman with mRCC who showed severe toxicities and was found to have the TTT haplotype on *ABCB1* (rs1045642, rs1128503, rs2032582). It was thought that the severe toxicities as observed in this patient could be associated with a high sunitinib exposure, probably explained by the *ABCB1* genetic polymorphism.[28]

Teo *et al.*[29] reported an association analysis in a Chinese population of 25 patients to investigate the effect of SNPs on both PK and clinical outcome (i.e. toxicity and response). The variant CC genotype of rs1045642 in *ABCB1* was associated with an increased risk for rash and mucositis as compared to T-allele carriers ($P<0.05$). No association with sunitinib treatment outcome was observed for rs776746 in *CYP3A5*.[29]

In order to verify the value of earlier reported associations, Diekstra *et al.*[30] pooled patients from exploratory studies in The Netherlands, Spain, and the United States[19,20,25,26,31], resulting in a sample size of 333 mRCC patients. Data confirmed the association of *CYP3A5*1* with the need for dose reductions ($P<0.05$) as presented earlier by Garcia-Donas *et al.*[20,30]

Pharmacogenetics to predict sunitinib efficacy

In a follow-up paper to van Erp *et al.*[19,31], the same group tested a comparable set of SNPs for associations with sunitinib efficacy. In a population of 136 mRCC patients, they observed that *CYP3A5*1*, presence of the TCG copy in *ABCB1* (rs1045642, rs1128503, rs2032582), or absence of the CAT copy in *NR1I3* (rs2307424, rs2307418, rs4073054) were associated with an improved PFS. Carriers of the favorable genetic profile (at least an A-allele of *CYP3A5*, a TCG copy in *ABCB1*, or a missing CAT copy in *NR1I3*) showed an improved PFS and OS compared to the disadvantageous genetic profile carriers.[31]

With regard to SNPs in *VEGF* and *VEGF-R*, Garcia-Donas *et al.* reported SNPs rs307826 and rs307821 in *VEGF-R3* to be associated with a decrease in PFS in a study of 89 mRCC patients.[20] Kim *et al.*[26] showed that carriers of the combination of CC and GG genotypes of rs3025039 in *VEGF-A* and rs2305948 in *VEGF-R2*, respectively, showed a worse OS compared to noncarriers.[26] In another study of *VEGF* and *VEGF-R* SNPs in a group of 84 mRCC patients, Scartozzi *et al.*[32] report that carriers of the favorable SNP genotypes of rs833061, rs6877011, rs2010963, and rs699947 showed a better overall response rate compared to the unfavorable genotype carriers.[32] By using a similar approach with a slightly different SNP set, Motzer *et al.*[33] report conflicting results. They could not identify any significant associations of SNPs in *VEGF-A* or *VEGFR3* with efficacy outcomes in sunitinib treatment (PFS, OS, objective response, or time-to-tumor progression).[33]

Beuselinck *et al.* assessed 16 SNPs in 10 genes for their relation with efficacy endpoints in 88 mRCC patients treated with sunitinib.[34] SNP rs1128503 in *ABCB1*, rs4073054 in *NR1I3*, and rs307821 in *VEGF-R3* were associated with PFS and OS. For PFS only, rs2981582 in *FGF-R2* and rs2276707 in *NR1I2* showed an association. For OS only, rs2307424 in *NR1I3* and rs307826 in *VEGF-R3* were associated ($P<0.05$).[34] In a separate study on 91 subjects, the CC genotypes of rs9582036 in *VEGF-R1* had a lower response rate and a decreased survival (PFS and OS) compared to A-allele carriers.[35] Furthermore, a decreased PFS was observed for the AA genotype of rs9554320 in *VEGF-R1* compared to C-allele carriers.[35] Teo *et al.*[29] reported that the CC genotype of rs1045642 in *ABCB1* was associated with progression of the disease as compared to T-allele carriers ($P<0.05$)[29] in 25 Chinese patients.

In the aforementioned study of Diekstra *et al.*, presence of CGT in the *ABCB1* haplotype (rs1128503, rs2032582, rs1045642) was confirmed to be associated with an improved PFS ($P<0.001$) as previously observed by van der Veldt *et al.*[30,31]

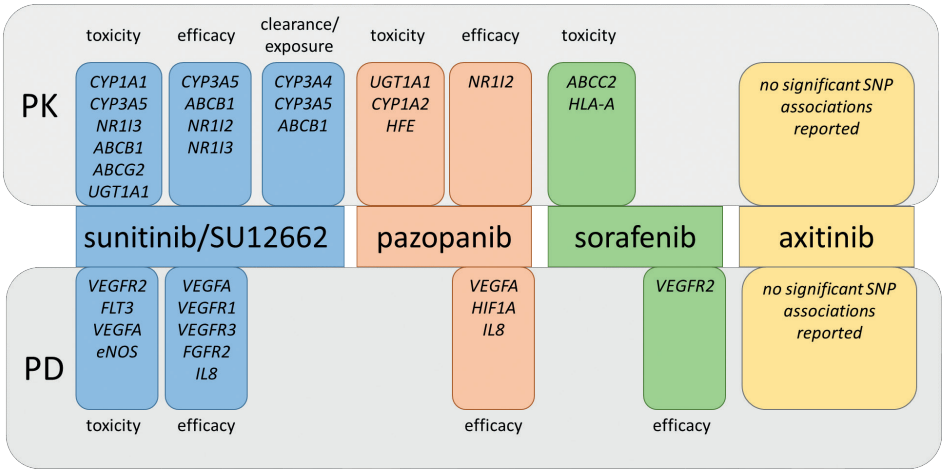

Figure 2 Main results on associations of SNPs involved in pharmacokinetics or pharmacodyna-mics of TKIs with clinical treatment outcome in mRCC.

An increased risk for common sunitinib toxicities was seen for SNPs in *CYP1A1*, *NR1I3*, *ABCB1* and *ABCG2*. Furthermore, *CYP3A5*1* was associated with dose reductions due to toxicity and an increased time-to-dose-reduction (TTDR) was seen for *ABCB1* SNPs. Associations with the clearance of sunitinib and/or SU12662 were observed for SNPs in *CYP3A5*, *ABCB1* and *CYP3A4*. A higher exposure to sunitinib and progressive disease was reported for rs1045642 in *ABCB1* in Chinese patients. For efficacy, *CYP3A5*1* and SNPs in *ABCB1*, *NR1I2* and *NR1I3* were associated with PFS. Associations of *CYP3A5*1* with the need for dose reductions and of the *ABCB1* haplotype with PFS were confirmed in a larger study.[30] Regarding pharmacodynamics, SNPs in *VEGFR2*, *FLT3*, *VEGF-A* and *eNOS* were associated with sunitinib toxicities and with hypertension in particular. Associations with either response rate, PFS and/or OS were observed for SNPs in *VEGF-A*, *VEGF-R1*, *VEGF-R3* and *FGF-R2*.[19-44] For pazopanib, only articles from the group of Xu *et al.* were included in our search. The main findings were that *UGT1A1*28*, *UGT1A1*60* and *CYP1A2* (rs762551) were associated with bilirubin levels and SNPs in *HFE* were associated with increased ALT levels. Additionally, SNPs in *IL8*, *HIF1A*, *NR1I2* and *VEGFA* were associated with either PFS or response rate. For patients using either sunitinib or pazopanib, *UGT1A1* SNP genotypes *28, *37 and *6 showed an increased risk for hyperbilirubinemia and rs1126647 in *IL8* was associated with OS. Only two pharmacogenetic studies on sorafenib were found in which genetic polymorphisms in *ABCC2* and *HLA-A* were associated with high-grade skin rash and rs2071559 in *VEGF-R2* was associated with PFS and OS. For axitinib, no associations were reported.[40-44]

Pharmacogenetics to predict sunitinib PK

To investigate the effect of genetic variants on the PK of sunitinib, Mizuno *et al.* performed a population PK study in a set of 19 patients which identified the AA genotype of rs2231142 in *ABCG2* to be a predictor for increased clearance of sunitinib.[36] In the study of Teo *et al.*[29], including 25 patients of Chinese origin, the variant CC genotype of rs1045642 in *ABCB1* was associated with a higher exposure to sunitinib as compared to T-allele carriers ($P<0.05$).[29] Diekstra *et al.*[37] tested a selection of the earlier associated SNPs related to the PK of sunitinib for possible association with clearance of sunitinib and/or its active metabolite (SU12662). Fourteen SNPs were tested in a sample of 114 patients with RCC (N=69), neuro-endocrine tumors (N=14), GIST (N=8), or another solid tumor type (N=23). A 28% increase in clearance of SU12662 was observed for the AG genotype of rs776746 in *CYP3A5*. Furthermore, the TT variant genotype of rs2032582 in *ABCB1* showed an 18% increase in clearance of sunitinib ($P<0.05$). For T-allele carriers of rs35599367 in *CYP3A4* (*CYP3A4*22*), a 22.5% decreased clearance was observed ($P<0.01$).[37]

Pharmacogenetics to predict pazopanib toxicity

Fewer studies are available on pharmacogenetics of pazopanib, since pazopanib was approved 3 years after sunitinib. Pazopanib and sunitinib share most common toxicities, although for pazopanib hepatotoxicity with elevations of alanine transaminase levels in particular is a common adverse effect.[38] In 2010, Xu *et al.*[38] investigated 28 SNPs for their relation with levels of alanine aminotransferase (ALT) and bilirubin in pazopanib-treated mRCC patients from an exploratory cohort (N=116) and a replication cohort (N=130). In the exploratory cohort, SNPs rs8175347 (*UGT1A1*28*), rs4148323 (*UGT1A1*60*), and rs762551 in *CYP1A2* were associated with bilirubin levels, and none of the SNPs showed an association with ALT levels. In the replication cohort, only the TA-repeat polymorphism of *UGT1A1*28* was associated with hyperbilirubinemia. The TA7 homozygote patients developed more hyperbilirubinemia compared to other genotypes, probably due to a reduced expression of UGT1A1. It was suggested that this is not likely to affect pazopanib exposure nor toxicities, since pazopanib is not glucuronized by UGT1A1.[38] In roughly the same patient cohorts (N=115 explorative and N=128 for replication), Xu *et al.* studied 9308 SNPs in 282 candidate genes. For rs2858996 and rs707889 in *HFE*, which are in strong linkage disequilibrium, the TT genotypes were associated with increased ALT levels.[39]

Pharmacogenetics to predict pazopanib efficacy

In a subsequent study, Xu *et al.*[40] investigated a slightly different set of 27 SNPs in candidate genes (SNPs in *IL-8* and *HIF1A*) to test whether these are related to pazopanib efficacy. In a cohort of 397 RCC patients, a decreased PFS was observed for TT variant

carriers of rs1126647 in *IL8* compared to wildtype AA. A worse PFS and response rate were observed for AG carriers of rs11549467 in *HIF1A* compared to wild-type GG. A reduced response rate was also observed for TT carriers of rs3814055 in *NR1I2* compared to wild-type CC and for wildtype CC of rs833061 in *VEGFA* compared to TT genotypes.[40]

Pharmacogenetics to predict clinical outcome of sunitinib and pazopanib

In some of the included studies, a combined genetic association analyses was performed on patients who are either treated with sunitinib or pazopanib. Motzer *et al.*[41] investigated the *UGT1A1* polymorphisms as reported by Xu *et al.*[38] in 719 patients from the COMPARZ trial: a phase III, randomized, clinical trial comparing pazopanib versus sunitinib in mRCC treatment.[10,41] Subjects with an expected reduction in function of UGT1A1 based on the SNP genotypes of rs8175347 and rs4148323 (*28, *37, and *6) showed increased levels of bilirubin at baseline and had a higher risk for developing hyperbilirubinemia on treatment with either sunitinib or pazopanib. None of the *UGT1A1* SNPs showed an association with elevated ALT levels.[41] More recently, Xu *et al.*[42] examined three separate patient cohorts of 186 patients on pazopanib in the first cohort, 690 patients using either pazopanib (N=353) or sunitinib (N=337) in the second cohort, and the third cohort comprised 88 patients on sunitinib. The variant T allele of SNP rs1126647 in *IL8* was associated with an inferior OS in every separate cohort ($P<0.05$). In a meta-analysis of the association results in the combined data set of 964 patients, this SNP was also associated with a worse OS.[42]

Pharmacogenetics to predict clinical outcome of sorafenib or axitinib

Few studies are available on pharmacogenetics of sorafenib in mRCC and axitinib. In a study of 55 Japanese mRCC patients, Tsuchiya *et al.*[43] reported that the CC genotype of rs717620 in *ABCC2* compared to the CT genotype and the *HLA-A*24* allele were both associated with an increased risk for sorafenib-induced high-grade skin rash. In 2015, Escudier *et al.*[44] reported a set of 305 mRCC patients (N=159 axitinib and N=146 sorafenib), in which 15 SNPs were investigated in *VEGF-A*, *VEGF-R1*, *VEGF-R2*, and *HIF-1A* for the association with blood pressure or efficacy outcomes. It was observed that no significant association was reported for any of the SNPs with treatment outcome on axitinib. For sorafenib treatment, it was found that SNP rs2071559 in *VEGF-R2* was associated with PFS and OS ($P<0.01$), but the sensitivity/specificity analysis on this SNP showed a result of <80%. Therefore, it cannot be considered as a predictive SNP for survival outcome on sorafenib in mRCC.[44]

Other associated biomarkers with TKI treatment outcome in mRCC patients

In addition to SNPs as potential biomarkers, many other biomarkers including histological subtypes, molecular subtypes, protein expression, immuno-expression, and microRNA were evaluated for potential association with TKI treatment outcome. Results are presented in **Supplementary document 3**.[45-72]

Histological and molecular subtypes in sunitinib treatment

In a case report by Choueiri *et al.*[45], a 49-year-old woman initially diagnosed with clear cell mRCC was later confirmed to have an Xp11.2 translocation RCC histological subtype which generally involves the *TFE3* gene. This patient developed severe HFS on sorafenib and, therefore, switched to sunitinib on which she showed a partial response followed by a durable response for more than 2 years.[45] This could indicate that the Xp11.2 translocation histological subtype is a biomarker that predicts a more favorable efficacy and toxicity profile on sunitinib compared to sorafenib. In a recent communication, Beuselinck *et al.*[46] showed that molecular subtypes of clear cell mRCC can predict the response to sunitinib with regard to response rate, PFS, and OS. These tumor subtypes are characterized based on chromosome copy-number aberrations, methylation status, and gene mutations in von Hippel-Lindau and the polybromo-1 protein (PBRM1).[46]

Protein expression and immunoexpression in sunitinib treatment

With regard to protein expression, Paule *et al.*[47] investigated molecular biomarkers in primary tumors of 23 mRCC patients. Herein, levels of VEGF soluble isoforms VEGF121 and VEGF165 were associated with sunitinib response, and the ratio of VEGF121/VEGF165 was associated with prognosis. Gruenwald *et al.*[48] investigated another type of soluble markers and reported circulating endothelial cells (CEC) to be elevated within 28 days after start of sunitinib treatment in patients with a PFS above the median.[48] Later, in 2012, expression levels of the chemokine receptor CXCR4 were associated with objective response rate and survival on sunitinib as communicated by D'Alterio *et al.*[49] In a report of Garcia-Donas *et al.*[50], protein expression was investigated in 67 sunitinib-treated mRCC patients. A high expression of HIF2A and PDGFRB proteins showed an improved response to sunitinib, and VEGFR3 expression was associated with PFS. Furthermore, expression of HIF2A and VEGF-A or EGLN3 mRNA content were associated with OS.[50] Minardi *et al.*[51] showed that VEGF expression was correlated with distant metastasis-free survival (DMFS) and OS in mRCC patients, but showed no association with regard to sunitinib treatment.[51] In a set of 50 Japanese RCC patients, Sato *et al.*[52] investigated the Extracellular Matrix MetalloPRoteinase Inducer (EMMPRIN), a cell-surface glycoprotein that belongs to the immunoglobulin superfamily encoded by a gene localized to 19p13.3. High expression

of EMMPRIN was seen for sunitinib-treated patients and sunitinib-resistant cells.[52] In the above-mentioned study of Motzer *et al.*[33], baseline levels of angiopoietin-2 and matrix metalloproteinase were associated with tumor response, and HIF-1α expression was associated with PFS.[33] The expression of phosphorylated VEGF-R2 was associated with PFS and OS in mRCC patients by Del Puerto-Nevado *et al*.[53] Gambini *et al.*[54] reported a case of a 60-year-old man with complete remission after 1 year on sunitinib. For this patient, a significant expression of neuronspecific enolase was observed together with the presence of pancreatic metastases. The hypothesis is that these neuroendocrine markers could predict the response to TKIs.[54]

Considering immunoexpression, Mikami *et al.*[55] studied a subset of 25 Japanese mRCC patients on sunitinib. Herein, a high expression of the cancer stem cell marker CD44 was associated with a poor treatment outcome and responsible for developing sunitinib resistance. Also, the combination of high expression of TNF-α and CD44 was associated with a decreased PFS.[55] Dornbusch *et al.*[56] evaluated the immunoexpression of tumor protein markers in 42 patients with mRCC. They concluded that HIF-1a, CA9, Ki67, CD31, pVEGFR1, VEGFR1 and -2, pPDGFR-a and -b were associated with the response to sunitinib ($P<0.05$).[56]

Micro RNA in sunitinib treatment

Some studies focused on microRNA (miRNA) as a possible predictor for sunitinib treatment outcome. miRNAs are noncoding RNAs that regulate gene expression.[57] Gámez-Pozo *et al.*[57] associated the expression of miRNA combinations (e.g. hsa-miR-141, hsa-miR-31, hsa-miR-125a-5p) in peripheral blood of RCC patients with poor or prolonged response to sunitinib.[57] Also, Berkers *et al.*[58] showed that miRNA was related to the response to sunitinib as tested in 20 mRCC patients: a significantly lower expression of miRNA-141 was observed for poor responders.[58] On miRNA expression of miRNA, Prior *et al.*[59] identified miRNA-942, miR-628-5p, miR-133a, and miR-484 as predictors for sunitinib efficacy (i.e. time to progression, OS, and resistance). It was postulated that high levels of miRNA-942 will stimulate the secretion of matrix metalloproteinase-9 and VEGF and thereby effectuate resistance to sunitinib.[59]

Pazopanib

Other than SNPs, our literature search identified no associations of biomarkers with pazopanib treatment outcome in mRCC. Choueiri *et al.*[60] studied biomarkers along the VHL/HIF-1a/HIF-2a axis but reported no significant associations with pazopanib activity in patients with advanced clear-cell RCC.[60]

Sorafenib

Mean VEGF plasma levels showed a significant increase in response to sorafenib, while plasma proteins VEGF-R2 and tissue inhibitor of metalloproteinase 1 (TIMP-1) were decreased in response to sorafenib as was observed by Peña *et al.*[61] Feng *et al.*[62] showed that high levels of circulating cell-free DNA (cfDNA) were associated with a poor response to sorafenib in 18 Chinese mRCC patients. Aziz *et al.*[63] reported that a high microvessel area was associated with a better response on sorafenib; smaller primary tumors were observed (P=0.005).[63] Another study showed that miRNA-30a is predictive for sorafenib efficacy.[64] Zheng *et al.*[64] explain that miRNA-30a inhibits its targeted gene Beclin-1, and consequently, the cytotoxic potential of sorafenib will increase.[64]

Potential biomarkers from analyses including patients using different therapies

Some of the included articles evaluate more types of TKIs in the same analysis or in combination with other therapies in mRCC. In a cohort of 282 mRCC patients, Doberstein *et al.* showed that the expression of the L1 cell adhesion molecule was predictive both for OS and for therapy resistance in mRCC treatment with either sunitinib, rapamycin, or cisplatin.[65] Furuya *et al.*[66] studied 66 Japanese patients (26 received IFN-alpha and 21 patients received concomitant sorafenib as a second-line treatment) in which significantly higher serum levels of the IFN-alpha receptor messenger RNA were seen for patients with a better response and longer OS on IFN-alpha treatment with or without the addition of sorafenib.[66] Sharpe *et al.*[67] observed a significant reduction in vessel density (CD31), phospho-S6K expression, PDL-1 expression, and FOXP3 expression, but also a significant increase in cytoplasmic FGF-2, MET receptor expression in vessels, Fuhrman tumor grade, and Ki-67 in 85 patients who use either sunitinib or pazopanib. In addition, progression occurred later in patients with high levels of Ki-67 and CD31.[67]

Yamada *et al.*[68] studied 27 cytokines as possible predictive biomarkers for TKI treatment in 13 mRCC patients treated with sunitinib (N=8), axitinib (N=4), or sorafenib (N=1). The plasma granulocyte macrophage colony-stimulating factor level was higher in patients who showed a partial response compared to patients with stable disease or progressive disease (P<0.05). Although not significant, higher IL-6 levels were observed in patients with progressive disease (P=0.141).[68] Interestingly, Yamada *et al.* refer to a paper of Tran *et al.*[69] who describe that for patients receiving pazopanib, high IL-6 was associated with an improved PFS compared to placebo (P=0.009).[69]

Peters *et al.*[70] examined epigenetic biomarkers, namely the methylation status in primary tumor tissues in a cohort of 18 patients treated with either sunitinib (N=12), sorafenib (N=4), axitinib (N=1), or bevacizumab (N=1), as a first-line treatment. A decreased PFS and OS were observed for the hyper methylated status of CST6 and LAD1.

For the prediction of therapy failure on first-line therapy, sensitivity and specificity were determined and resulted in a specificity of 1.0 and a sensitivity of 0.73 for LAD1 methylation.[70] Dubrowinskaja *et al.*[71] of the same research group studied the same patient cohort and showed that a higher methylation status of the Neurofilament Heavy polypeptid gene was related to a decrease in OS.[71]

Finally, Choudhury *et al.*[72] investigated 48 patients on molecular subtypes of clear-cell RCC for their association with response to either sunitinib or pazopanib as a first-, second-, or third-line treatment. Using a multigene assay (8 genes: CXCL5, EFNA5, EMCN, LAMB3, PLG, PRAME, RARRES1, SLC6A19), clinical prognostic subtypes were identified, which were shown to be predictive for response and survival on TKI treatment.[72]

DISCUSSION

This systematic review outlines the available literature on associations of SNPs and other biomarkers with clinical treatment outcome of TKIs in mRCC. The most promising biomarkers on predicting TKI outcome are related to PK (genes encoding CYP450 enzymes, CYP450 regulators, or drug efflux transporters) and PD (genes encoding VEGF or VEGF receptors and interleukins).[19-44] For sunitinib, SNPs in *CYP1A1*, *CYP3A5*, *CYP3A4*, *NR1I2*, *NR1I3*, *ABCB1*, *ABCG2*, *VEGF-A*, *VEGFR1*, *VEGFR2*, *VEGF-R3*, *FGF-R2*, *FLT3*, *eNOS*, *UGT1A1*, and *IL8* were related to toxicity, efficacy, clearance, or drug exposure. Moreover, the potential of SNPs in *CYP3A5* and *ABCB1* to predict sunitinib related outcomes was confirmed in a large set of patients. With regard to pazopanib, SNPs in *UGT1A1*, *CYP1A2*, and *HFE* were associated with either bilirubin levels or ALT levels, and SNPs in *IL8*, *HIF1A*, *NR1I2*, and *VEGFA* were associated with efficacy outcomes on pazopanib. The association of rs1126647 in *IL8* with OS was confirmed in two independent data sets including patients treated with either sunitinib or pazopanib.[40,42] Sorafenib toxicity and efficacy were related to genetic polymorphisms in *ABCC2*, *HLA-A*, and the only large study (N>300) available on sorafenib treatment showed that rs2071559 in *VEGF-R2* was related to efficacy. For axitinib, no SNP associations were reported.[19-44] Other reported biomarkers include proteins (e.g. VEGF), epigenetic markers (miRNA or methylation), and histological or molecular subtypes.[45-72] Clinical implementation of these biomarkers remains a difficult issue, since most studies present nonvalidated findings from retrospective cohort studies, effect sizes are rather small, heterogeneity between studies is large, sample sizes are limited, and above all, no consensus has been reached on the interpretation of these findings in terms of clinical utility on guiding individual dosing regimens of TKIs.[17,18]

Our systematic approach minimizes the risk that we have missed relevant articles. Moreover, we only focused on TKIs (excluding other mRCC treatments) to ensure

that similar mechanisms and drug targets are compared. A meta-analysis is generally preferred to easily summarize the findings of all studies and subsequently value the clinical utility of associated biomarkers. However, in this case, a meta-analysis would be misjudging because of the large heterogeneity in study design, sample sizes, ethnicities, study endpoints, tested SNPs, evaluated treatments, and statistical approach. Sample sizes range from 1 to 451, in which the number of tested SNPs varied between 1 and 6852 SNPs.[22,39] Furthermore, publication bias has very likely occurred and obscures a clear conclusion from a meta-analysis. In this review, we compared study results on the drugs of interest, sample sizes, ethnicity differences, and effect sizes with confidence intervals and *P*-values. One of the limitations in the studies in this field is that biomarkers are typically selected with a significance threshold value of $P<0.05$. However, often a large number of hypotheses are tested in the same data set, increasing the chance for false-positive associations.[73] Another important issue is the difference in allele frequencies of SNPs among ethnicities. For example, SNP allele frequencies in *CYP3A5* and *ABCB1* differ between Caucasian and Asian patients. Furthermore, co-medication during TKI treatment and total exposure to the TKI was not taken into account in most studies, while drug-drug interactions may be especially important with regard to the effects on PK and PD. These matters have an important impact on the clinical validity and should be taken into account before assessing the possibilities on clinical implementation.

The SNPs tested in most of the included studies were selected according to the candidate gene approach. On PK, the results show that SNPs in genes coding for CYP enzymes, CYP regulators, and drug efflux transporters (ABCB1, ABCC2, and ABCG2) seem to play an important role in sunitinib treatment outcome, especially *CYP3A5* and *ABCB1* SNPs.[19-21,29,31,34,37] In some of the articles on sunitinib, the intermediate endpoints clearance and exposure were investigated and confirmed the hypotheses that SNPs in *CYP3A4*, *CYP3A5*, and *ABCB1* influence drug exposure (**Figure 2**).[22,29,36,37] Sunitinib and pazopanib are metabolized by CYP3A4 and probably CYP3A5 but are not known to be metabolized by UGT1A1.[38,41,74] Pazopanib inhibits UGT1A1 in vitro. In pazopanib-treated patients, SNPs in *UGT1A1* may cause a reduced expression of UGT1A1 and, together with the inhibitory effect of pazopanib, would result in hyperbilirubinemia. For sunitinib, however, this enhanced effect on inhibition does not apply.[38,41] Since sunitinib and pazopanib are both metabolized by the same CYP enzymes and are substrates for ABCB1 and ABCG2, one could expect to find the same SNPs in these PK-related genes to be related to pazopanib outcomes. Only for a SNP in *NR1I2*, a relation with response rate on pazopanib was observed.[40] The articles in this review have not tested pazopanib toxicity outcomes for these SNPs related to PK. However, the effects on total drug exposure resulting from changes in CYP enzyme activity may be very different for sunitinib and pazopanib. Including both sunitinib- and pazopanib-treated patients in this type of

analyses is not very reasonable[41,42], since sunitinib is converted to an active metabolite with similar activity and potency and a longer half-life, while pazopanib only has less active metabolites. In addition, the extent of absorption for pazopanib and sunitinib may be very different (due to substrate specificity, for example), and its role in the total PK of the TKI is unclear. Changes in genes encoding efflux transporters can, therefore, be more important for sunitinib than for pazopanib. Regarding pharmacodynamics of sunitinib, SNPs in *eNOS*, *IL8*, *VEGF-A*, and *VEGF-R1,2,3* present promising biomarkers for toxicity or survival on sunitinib.[19,20,25,26,32,34,35] However, results on *VEGF-A* and *VEGF-R1,2* or *3* genes are contradictory for both toxicity and efficacy outcomes since also negative associations were reported.[30,33] In a recent analysis of our group, we associated SNPs in *IL8* and *IL13* with sunitinib toxicities (hypertension, leukopenia, and any toxicity >grade 2) and referred to the findings of Xu *et al.*[40,42,75] Here, it was carefully hypothesized that IL8 and VEGF can be related in their effect on sunitinib-induced toxicities.[75] For sorafenib, only one study with a significant sample size is available that reported a SNP in *VEGF-R2* to be a potential predictor for efficacy (PFS and OS).[44] This effect was explained by a change in the promoter, subsequently altering transcription factor-binding and thereby the expression of VEGF-R2, but no clear downstream effect was presented.[44] In addition to SNPs, other types of biomarkers are potentially important. Results on epigenetics (miRNA and DNA methylation status) may influence gene expression and SNP effects, and results on protein markers can provide useful insight into mechanisms involved in SNP effects.[45-72] The associated SNPs and other biomarkers are different between TKIs, and therefore, we cannot assume a class effect pharmacogenetics in mRCC. While many targets, metabolizing enzymes, and drug transporters of the TKIs are comparable, significant differences in mechanisms of action can still exist.

A randomized clinical trial (RCT) is considered the hallmark of evidence to show utility for a biomarker in a clinical setting. However, an RCT is not critical for clinical application. It has already been proven that genetic substudies of RCTs in cancer are sufficient evidence to implement pharmacogenetic testing as is illustrated by testing *EGFR* for erlotinib and *(K)RAS* for cetuximab and panitumumab.[76] Funakoshi *et al.*[14] assessed the level of evidence (LOE) of associated biomarkers by arranging the articles into different categories.[77,78] Categories A and B represent the highest LOE with prospective RCT addressing biomarker questions and prospective studies not primarily designed to address biomarker questions. Categories C and D represent the lowest LOE with prospective and retrospective observational studies.[14,77,78] In this field, these categories would be a false premise since no randomized studies and few prospective studies (category A or B) are available on pharmacogenetic biomarker studies in mRCC, which means only category C and D studies with a low LOE are left.

Gillis *et al.*[18] mention that both clinical validity and clinical utility should be considered in translating the findings into a practical method to improve clinical practice. There is a lack of consensus on the definition of clinical utility. However, Gillis *et al.* are confident that validation of the associated biomarker in an independent replication cohort is the best evidence of clinical utility.[18] Janssens *et al.* consider a pharmacogenetic test useful when it is 'sufficiently predictive for clinically important phenotypes, contributes to improved health outcomes, and is economically feasible to implement in community-based practice settings'.[17] Clinical application is very likely for the reported SNP associations of *CYP3A5* and *ABCB1*, which reflect a considerably high predictive value since these SNPs have been associated with sunitinib PK[37], associations on clinical outcome are confirmed[20,30,31], and these findings are further supported by the exposure-effect relationship on sunitinib treatment.[7] However, no genetic biomarkers for clinical outcome on TKIs in mRCC have been implemented yet in any of the innovative guidelines such as the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG).[79,80]

In conclusion, a decade of pharmacogenomics research on TKIs in mRCC patients has revealed *CYP3A5*1* and *ABCB1* as the most promising biomarkers for sunitinib treatment outcome. Implementation of pre-emptive pharmacogenetic testing will become likely when confirmed in independent cohorts preferably in prospective studies.

In the near future, results from genome-wide association studies from discovery and validation sets are expected. Many more SNPs in different genes will be investigated in the genome-wide association studies as compared to the candidate-gene approach that is used in most of the currently available studies. This could mean that earlier discovered SNPs will be affirmed, but these results may also identify additional genetic variants as biomarkers for outcome of TKI treatment in renal cell cancer. Additionally, we think that it is essential to further study both immunological and angiogenic factors (both protein expression and genetic polymorphisms) on prognosis and anti-VEGF treatment outcome in mRCC. Besides the effect on VEGF inhibition, sunitinib and other TKIs may influence the immune system, but we need a better understanding on the mechanisms involved.[42,69,75] Anti-angiogenic therapy may result in a further induction of the immunosuppressive environment of the RCC tumor.[81] Future findings may indicate that we need a combination therapy to treat both the immunogenic and pro-angiogenic character of RCC. Utilizing the pharmacogenetic profile of the patient may optimize TKI selection. Currently, the drug arsenal for mRCC is growing with new available TKIs or TKIs in development, and new promising immune-based therapies for RCC treatment are on their way, such as anti-programmed death-ligand 1 (PD-L1).[82] Recent findings showed that in some patients, the efficacy of anti-angiogenic therapy might improve by PD-L1 blockade. However, as Liu *et al.* mention: 'The top priority and challenge is the selection of patients who are likely to respond to anti-PD-L1 therapy'.[81]

Within 5 years from now, we expect that some of the presented biomarkers here will be validated in replication cohorts and prospective validation studies with a longitudinal observation in time and a systematic data collection. These can be designed as noninterventional because blood samples (DNA) can be collected during routine monitoring visits of the patients. In retrospect, it will be investigated whether the determined genotypes of the patients have been a prediction for the outcome of TKI treatment. Two large prospective, nonrandomized clinical trials on precision medicine in oncology have already been set up as presented at the 2015 American Society of Clinical Oncology (ASCO) annual meeting: ASCO's Targeted Agent and Profiling Utilization Registry (TAPUR) study and the National Cancer Institute (NCI) Molecular Analysis for Therapy Choice (NCI-MATCH).[83,84] In these trials, more than 20 different targeted therapies will be investigated including sunitinib, and these drugs will be made freely available. For the NCI-MATCH study, a number of 3000 patients is anticipated. Patients are treated with a drug based on the molecular profile of the tumor, and important clinical outcomes (e.g. overall response rate and PFS) will be recorded to see how patients can benefit from these drugs.[83,84]

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SUPPLEMENTARY MATERIAL

Supplementary document 1: Systematic review protocol

1. TITLE

Systematic review: a decade of pharmacogenetic research on TKIs in metastatic renal cell carcinoma

2. REGISTRATION

PROSPERO registration number: CRD42015016509

3. AUTHORS

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Contributions: MD and JS drafted the manuscript. Jan Schoones developed the search strategy. All authors read, provided feedback and approved the final manuscript

4. AMENDMENTS

In the event of protocol amendments, the date of each amendment will be accompanied by a description of the change and the rationale

5. SUPPORT

MD is supported by the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 259939.

6. RATIONALE

INTRODUCTION

In the last ten years a number of targeted agents for treatment of solid tumors have become available. Sunitinib, a tyrosine kinase inhibitor (TKI) was the first targeted drug for mRCC and is on the market since 2006. While pazopanib, axitinib and sorafenib have since then become available, sunitinib is still the first-choice treatment for metastatic renal cell carcinoma (mRCC).

Patients respond very differently to sunitinib with sometimes severe toxicities that need dose reductions and/or a poor response with regard to tumor shrinkage. Therefore, we are not yet able to predict the individual treatment outcome on sunitinib.

Previous studies identified Single Nucleotide Polymorphisms (SNPs) in genes encoding enzymes or transporters related to pharmacokinetics and pharmacodynamics of TKIs which have been associated with toxicity and efficacy of sunitinib. These genetic biomarkers are potential predictors for TKI treatment outcome in mRCC patients. Ideally, pharmacogenetic testing of genetic biomarkers prior to start of therapy, could result in giving the individual patient the most effective TKI with the least possible side effects. However, findings on these genetic biomarkers have not been validated yet. This means clinical implementation of pharmacogenetic testing to guide sunitinib dosing or other TKIs is not yet possible.[1-14]

We perform a new systematic review up to January 2015 in order to have a more complete overview of all pharmacogenetic studies on sunitinib and other TKIs in mRCC to date and we only focus on genetic predictive biomarkers.

7. OBJECTIVES

The objective of this review is to systematically review the research findings on pharmacogenetics of sunitinib in mRCC to date and to evaluate the use of genetic biomarkers for sunitinib treatment outcome. Therefore, we try to answer the following questions:

What are the germline genetic variants that associate with sunitinib and other targeted agents (pazopanib, axitinib, sorafenib) and their efficacy and toxicity in mRCC patients?

What is the value and quality of the included studies in our review?

Based on the available research findings, what has been the result with regard to clinical implementation of predictive biomarkers for sunitinib treatment outcome up till now?

What should be the focus of future research in this field?

Supplementary document 1: Continued

8. ELIGIBILITY CRITERIA

Studies will be selected according to the criteria outlined below:
We will include studies with patients diagnosed with (metastatic) renal cell carcinoma (mRCC) receiving a targeted therapy (sunitinib, axitinib, sorafenib, pazopanib or other). In addition, we included studies that test the association of SNPs, any other genetic variants or gene/protein expression with the treatment outcome of these targeted therapies. Therefore, information that has to be available in the studies are DNA samples, genotyping data together with outcome data which are toxicity and efficacy data.
References were eligible when genetic biomarkers (i.e. SNPs, genetic variants, gene/protein expression) were investigated in relation to TKI treatment and mRCC. Excluded from our selection were review articles, preclinical studies (animal models and in vitro studies), phase I or II clinical trials and papers discussing pathology, prognosis, tumor biology or somatic mutations (e.g. Von Hippel-Lindau mutations). Accordingly, articles were excluded from our review if non-treatment related biomarkers were studied or if no TKI response was assessed, because these generally consider prognostic biomarkers.
Our literature search was done for articles that appeared between January 2004 and January 2015. Only English language papers will be included and conference abstracts are excluded from our search.
Excluded from our selection were review articles, preclinical studies (animal models and in vitro studies), phase I or II clinical trials and papers discussing pathology, prognosis or tumor biology, somatic mutations (e.g. VHL).
We have made sure that all authors of the review team agreed with the eligibility criteria prior to the selection process.

9. INFORMATION SOURCES

Search for references in the following databases:
PubMed
Embase (OVID version)
Web of Science
COCHRANE
CENTRAL

10. SEARCH STRATEGY

Together with a trained librarian, we will perform a search in PubMed, Embase (OVID version), Web of Science, COCHRANE Library and CENTRAL. The search consists of the combination of three subjects:
Renal cell carcinoma
Tyrosine kinase inhibitors (e.g. sunitinib, sorafenib)
Pharmacogenetics
The query will be applied in all databases taking into account the terminological and technical differences between these databases. Various synonyms and related terms for all subjects were used. Results will be limited to articles in the English language, and from the year 2004 onwards. Meeting abstracts are being excluded. See **Supplementary document 2**: search strategy

11. STUDY RECORDS

11a. DATA MANAGEMENT

Results from our literature search will be uploaded to EndNote X6 and references will be de-duplicated with every new insertion of search results from one of the databases. The selection of articles will be made within EndNote in order to easily check which articles are excluded. An overview of our selection including the data collection will be processed in an excel file and eventually presented in tables in the manuscript.

11b. SELECTION PROCESS

First of all, papers will be selected by title. Titles will be judged on the topic of renal cell carcinoma, tyrosine kinase inhibitors and pharmacogenetics. The first two authors of the review (MD and JS) will screen the titles from the first original search independently from each other. Disagreement on the selection by title will be resolved through discussion with the last author on the review (HJG).
Secondly, a selection will be made by abstract with the included articles from the first selection by title. Abstracts will be judged on the topic of renal cell carcinoma, tyrosine kinase inhibitors and pharmacogenetics. The first two authors of the review (MD and JS) will screen the abstracts from papers included in the first selection step independently from each other. Disagreement on the selection by title will be resolved through discussion with the last author on the review (HJG).
Thirdly, the final aim is to get a total of roughly 80 references. Full-text will be judged on the topic of renal cell carcinoma, tyrosine kinase inhibitors and pharmacogenetics. The first two authors of the review (MD and JS) will screen the full texts papers included in the second selection step independently from each other. Disagreement on the selection by title will be resolved through discussion with the last author on the review (HJG).
For every selection step, the reason for exclusion of the paper will be recorded. An overview of our selection criteria and selection process will be saved in an excel file.

Supplementary document 1: Continued

11c. DATA COLLECTION PROCESS

During the selection process, we will use excel-based forms to make a selection of articles. With the final selection of articles we will again use the excel file to extract relevant data to include in our review results.

12. DATA ITEMS

For the selection by title, abstract and full text of articles, we will make a selection based on the eligibility criteria mentioned in item 8. In addition, the excel file contains the reasons for exclusion.
The last separate worksheet is used for data extraction from the final selection of articles. Extracted data were: first author, year of publication, demographics (median age, number of patients, male percentage, ethnicity), sample material and origin (i.e. germline DNA from blood, serum or other), methods, tumor type, drug information (i.e. drug name, treatment line, dose), evaluated biomarkers, tested endpoints and statistical approach. The exact extraction data can be found in the excel file in the last worksheet.

13. OUTCOMES AND PRIORITISATION

End points to be tested for association with genetic variants will be toxicity and efficacy endpoints:
Progression-free Survival (PFS)
Overall Survival (OS)
Any type of toxicity (e.g. thrombocytopenia, leukopenia and many more) with an explanation on clinically relevant toxicities on severity grades.
Response to treatment (RECIST criteria; PD, SD, PR, CR)
Dose reductions
Any other endpoint related to toxicity or efficacy of the TKI treatment

14. RISK OF BIAS INDIVIDUAL STUDIES

Clinical validity (the robustness of the statistical association between a pharmacogenetic variant and outcome of drug therapy) and clinical utility (a test's health-care value) of the included articles was evaluated in a descriptive manner.[17,18]

15. DATA SYNTHESIS

15a. CRITERIA FOR SYNTHESIS STUDY DATA

Because of the diversity in study populations, ethnicities, outcomes, number of SNPs tested and differences in statistical approach in the studies, we will not perform a meta-analysis.

15b. PLANNED SUMMARY MEASURES

N/A

15c. ADDITIONAL ANALYSES

N/A

15d. PLANNED TYPE OF SUMMARY

We will visualize the study quality by using the NOS star system as described in item 14 and present the difference between studies in a more descriptive/qualitative way.

16. META BIAS(ES)

Meta bias was evaluated in a descriptive manner.

17. CONFIDENCE IN CUMULATIVE EVIDENCE

Clinical validity (the robustness of the statistical association between a pharmacogenetic variant and outcome of drug therapy) and clinical utility (a test's health-care value) of the included articles was evaluated in a descriptive manner.[17,18]

Supplementary document 2: Search strategy

Research question:
pharmacogenetics of sunitinib in metastatic renal cell carcinoma

Search strategy
Together with a trained librarian, we performed a search in PubMed, Embase (OVID version), Web of Science, COCHRANE Library and CENTRAL.
The search consisted of the combination of three subjects:
Renal cell carcinoma
Tyrosine kinase inhibitors (e.g. sunitinib, sorafenib)
Pharmacogenetics

Search results
This search resulted in a total of 1511 references. After removing duplicates automatically, a total of 973 references were obtained d.d. 14-01-2015, originating from:
PubMed: 680 [RefID 1-680]
Embase: 662, of which 256 unique [RefID 681-936]
Web of Science: 129, of which 35 unique [RefID 937-971]
COCHRANE: 20, of which 2 unique [RefID 972-973]
CENTRAL: 20, of which 0 unique
A final amount of **968 unique references** were obtained after removing the following 5 extra duplicates manually:
Bielecka, 2014
Dabora, 2011
Joosten, 2015
Pecuchet, 2013
Wu, 2012

Supplementary document 2: Continued

Database	Search Strategy	Number of references	Number of unique references
PubMed	("Carcinoma, Renal Cell"[Mesh] OR "renal cell carcinoma"[all fields] OR "renal tumor"[all fields] OR "renal tumour"[all fields] OR "renal tumors"[all fields] OR "renal tumours"[all fields] OR "RCC"[all fields] OR "mRCC"[all fields] OR "kidney cell carcinoma"[all fields] OR "kidney tumor"[all fields] OR "kidney tumour"[all fields] OR "kidney tumors"[all fields] OR "kidney tumours"[all fields] OR "renal cell adenocarcinoma"[all fields] OR "kidney cell adenocarcinoma"[all fields] OR "Grawitz"[all fields]) AND ("Genetic Variation"[Mesh] OR "genetic"[all fields] OR "genetics"[all fields] OR "genomics"[all fields] OR "genomic"[all fields] OR "genome"[all fields] OR "mutation"[all fields] OR "mutations"[all fields] OR "allelic"[all fields] OR "Allele"[all fields] OR "alleles"[all fields] OR "DNA"[all fields] OR "gene"[all fields] OR "genes"[all fields] OR "chromosome"[all fields] OR "chromosomes"[all fields] OR "germ-line"[all fields] OR "germline"[all fields] OR "INDEL"[all fields] OR "insertion"[all fields] OR "deletion"[all fields] OR "inversion"[all fields] OR "polymorphism"[all fields] OR "polymorphisms"[all fields] OR "polymorfism"[all fields] OR "polymorfisms"[all fields] OR "Genomic Structural Variation"[all fields] OR "Genomic Structural Variations"[all fields] OR "DNA Copy Number Variations"[all fields] OR "DNA Copy Number Variation"[all fields] OR "SNP"[all fields] OR "SNPs"[all fields] OR "nucleotide"[all fields] OR "nucleotides"[all fields] OR "Genetics"[Mesh] OR "pharmacogenetic"[all fields] OR "pharmacogenetics"[all fields] OR "hapmap"[all fields] OR "Pharmacogenetics"[Mesh] OR toxicogenetic*[all fields] OR "predictive marker"[all fields] OR "predictive markers"[all fields] OR "predictive biomarkers"[all fields] OR "predictive biomarker"[all fields] OR "Genetic Markers"[Mesh] OR "Genetic Phenomena"[Mesh] OR "GWAS"[all fields] OR "Genome-Wide Association Study"[Mesh] OR "Genome-Wide Association"[all fields] OR "Genetic Association Studies"[Mesh]) AND ("sunitinib"[Supplementary Concept] OR sunitinib*[all fields] OR "Sutent"[all fields] OR "SU 11248"[all fields] OR "SU11248"[all fields] OR "SU-11248"[all fields] OR "Protein-Tyrosine Kinases/antagonists and inhibitors"[Mesh] OR "tyrosine kinase inhibitor"[all fields] OR "Tyrosine kinase inhibitors"[all fields] OR "TKI"[all fields] OR "SU011248"[all fields] OR "SU-011248"[all fields] OR "SU 011248"[all fields] OR "Receptors, Vascular Endothelial Growth Factor"[Mesh] OR "vascular endothelial growth factor receptor"[all fields] OR "VEGF"[all fields] OR "VEGFR"[all fields] OR "VEGF-R1"[all fields] OR "VEGF-R2"[all fields] OR "VEGF-R3"[all fields] OR "VEGFR1"[all fields] OR "VEGFR2"[all fields] OR"VEGFR3"[all fields] OR "PDGF"[all fields] OR "PDGFR"[all fields] OR "sorafenib"[Supplementary Concept] OR "sorafenib"[All fields] OR "nexavar"[All fields] OR "BAY 545-9085"[All fields] OR "BAY-545-9085"[All fields] OR "BAY 43-9006"[All fields] OR "BAY-43-9006"[All fields] OR "BAY 439006"[All fields] OR "BAY43-9006"[All fields] OR "BAY439006"[All fields] OR "pazopanib"[Supplementary Concept] OR "votrient"[All fields] OR "GW 786034B"[All fields] OR "GW786034B"[All fields] OR "GW-786034B"[All fields] OR "GW 786034"[All fields] OR "GW786034"[All fields] OR "GW-786034"[All fields] OR "axitinib"[Supplementary Concept] OR "inlyta"[All fields] OR "AG 013736"[All fields] OR "AG013736"[All fields] OR "AG-013736"[All fields] OR "Angiogenesis Inhibitors"[Mesh] OR "angiogenesis inhibitors"[All fields] OR "angiogenesis inhibitor"[All fields] OR "anti angiogenic"[All fields] OR "anti-angiogenic"[All fields] OR "antiangiogenic"[All fields] OR "anti angiogenesis"[All fields] OR "anti-angiogenesis"[All fields] OR "antiangiogenesis"[All fields]) AND english[la] AND ("2004/01/01"[PDAT] : "3000/12/31"[PDAT])	680	680

Supplementary document 2: Continued

Database	Search Strategy	Number of references	Number of unique references
Embase (OVID-version)	("Kidney Carcinoma"/ OR "renal cell carcinoma".ti,ab OR "renal tumor".ti,ab OR "renal tumour".ti,ab OR "renal tumors".ti,ab OR "renal tumours".ti,ab OR "RCC".ti,ab OR "mRCC".ti,ab OR "kidney cell carcinoma".ti,ab OR "kidney tumor".ti,ab OR "kidney tumour".ti,ab OR "kidney tumors".ti,ab OR "kidney tumours".ti,ab OR "renal cell adenocarcinoma".ti,ab OR "kidney cell adenocarcinoma".ti,ab OR "Grawitz".ti,ab) AND (*"Genetic Variability"/ OR "genetic".ti,ab OR "genetics".ti,ab OR "genomics".ti,ab OR "genomic".ti,ab OR "genome".ti,ab OR "mutation".ti,ab OR "mutations".ti,ab OR "allelic".ti,ab OR "Allele".ti,ab OR "alleles".ti,ab OR "DNA".ti,ab OR "gene".ti,ab OR "genes".ti,ab OR "chromosome".ti,ab OR "chromosomes".ti,ab OR "germ-line".ti,ab OR "germline".ti,ab OR "INDEL".ti,ab OR "insertion".ti,ab OR "deletion".ti,ab OR "inversion".ti,ab OR exp *genetic polymorphism/ OR "polymorphism".ti,ab OR "polymorphisms".ti,ab OR "polymorfism".ti,ab OR "polymorfisms".ti,ab OR "Genomic Structural Variation".ti,ab OR "Genomic Structural Variations".ti,ab OR *"Copy Number Variation"/ OR "DNA Copy Number Variations".ti,ab OR "DNA Copy Number Variation".ti,ab OR "SNP".ti,ab OR "SNPs".ti,ab OR "nucleotide".ti,ab OR "nucleotides".ti,ab OR exp *"Genetics"/ OR "pharmacogenetic".ti,ab OR "pharmacogenetics".ti,ab OR "hapmap".ti,ab OR exp *"Pharmacogenetics"/ OR toxicogenetic*.ti,ab OR "predictive marker".ti,ab OR "predictive markers".ti,ab OR "predictive biomarkers".ti,ab OR "predictive biomarker".ti,ab OR exp *"Genetic Marker"/ OR "GWAS".ti,ab OR "Genome-Wide Association".ti,ab OR exp *"Genetic Association"/) AND (*"sunitinib"/ OR sunitinib*.ti,ab OR "Sutent".ti,ab OR "SU 11248".ti,ab OR "SU11248".ti,ab OR "SU-11248".ti,ab OR exp *"protein tyrosine kinase inhibitor"/ OR "tyrosine kinase inhibitor".ti,ab OR "Tyrosine kinase inhibitors".ti,ab OR "TKI".ti,ab OR "SU011248".ti,ab OR "SU-011248".ti,ab OR "SU 011248".ti,ab OR *vasculotropin receptor/ OR "vascular endothelial growth factor receptor".ti,ab OR "VEGF".ti,ab OR "VEGFR".ti,ab OR "VEGF-R1".ti,ab OR "VEGF-R2".ti,ab OR "VEGF-R3".ti,ab OR "VEGFR1".ti,ab OR "VEGFR2".ti,ab OR "VEGFR3".ti,ab OR "PDGF".ti,ab OR "PDGFR".ti,ab OR *"sorafenib"/ OR "sorafenib".ti,ab OR "nexavar".ti,ab OR "BAY 545-9085".ti,ab OR "BAY-545-9085".ti,ab OR "BAY 43-9006".ti,ab OR "BAY-43-9006".ti,ab OR "BAY 439006".ti,ab OR "BAY43-9006".ti,ab OR "BAY439006".ti,ab OR *"pazopanib"/ OR "votrient".ti,ab OR "GW 786034B".ti,ab OR "GW786034B".ti,ab OR "GW-786034B".ti,ab OR "GW 786034".ti,ab OR "GW786034".ti,ab OR "GW-786034".ti,ab OR *"axitinib"/ OR "inlyta".ti,ab OR "AG 013736".ti,ab OR "AG013736".ti,ab OR "AG-013736".ti,ab OR exp *"Angiogenesis Inhibitor"/ OR "angiogenesis inhibitors".ti,ab OR "angiogenesis inhibitor".ti,ab OR "anti angiogenic".ti,ab OR "anti-angiogenic".ti,ab OR "antiangiogenic".ti,ab OR "anti angiogenesis".ti,ab OR "anti-angiogenesis".ti,ab OR "antiangiogenesis".ti,ab) NOT conference abstract.pt AND english.la AND (2004 OR 2005 OR 2006 OR 2007 OR 2008 OR 2009 OR 201*).yr	662	256

Supplementary document 2: Continued

Database	Search Strategy	Number of references	Number of unique references
Web of Science	(TI=("Kidney Carcinoma" OR "renal cell carcinoma" OR "renal tumor" OR "renal tumour" OR "renal tumors" OR "renal tumours" OR "RCC" OR "mRCC" OR "kidney cell carcinoma" OR "kidney tumor" OR "kidney tumour" OR "kidney tumors" OR "kidney tumours" OR "renal cell adenocarcinoma" OR "kidney cell adenocarcinoma" OR "Grawitz") AND TS=("Genetic Variability" OR "genetic" OR "genetics" OR "genomics" OR "genomic" OR "genome" OR "mutation" OR "mutations" OR "allelic" OR "Allele" OR "alleles" OR "DNA" OR "gene" OR "genes" OR "chromosome" OR "chromosomes" OR "germ-line" OR "germline" OR "INDEL" OR "insertion" OR "deletion" OR "inversion" OR genetic polymorphism OR "polymorphism" OR "polymorphisms" OR "polymorfism" OR "polymorfisms" OR "Genomic Structural Variation" OR "Genomic Structural Variations" OR "Copy Number Variation" OR "DNA Copy Number Variations" OR "DNA Copy Number Variation" OR "SNP" OR "SNPs" OR "nucleotide" OR "nucleotides" OR "Genetics" OR "pharmacogenetic" OR "pharmacogenetics" OR "hapmap" OR "Pharmacogenetics" OR toxicogenetic* OR "predictive marker" OR "predictive markers" OR "predictive biomarkers" OR "predictive biomarker" OR "Genetic Marker" OR "GWAS" OR "Genome-Wide Association" OR "Genetic Association") AND TI=("sunitinib" OR sunitinib* OR "Sutent" OR "SU 11248" OR "SU11248" OR "SU-11248" OR "protein tyrosine kinase inhibitor" OR "tyrosine kinase inhibitor" OR "Tyrosine kinase inhibitors" OR "TKI" OR "SU011248" OR "SU-011248" OR "SU 011248" OR vasculotropin receptor OR "vascular endothelial growth factor receptor" OR "VEGF" OR "VEGFR" OR "VEGF-R1" OR "VEGF-R2" OR "VEGF-R3" OR "VEGFR1" OR "VEGFR2" OR "VEGFR3" OR "PDGF" OR "PDGFR" OR "sorafenib" OR "sorafenib" OR "nexavar" OR "BAY 545-9085" OR "BAY-545-9085" OR "BAY 43-9006" OR "BAY-43-9006" OR "BAY 439006" OR "BAY43-9006" OR "BAY439006" OR "pazopanib" OR "votrient" OR "GW 786034B" OR "GW786034B" OR "GW-786034B" OR "GW 786034" OR "GW786034" OR "GW-786034" OR "axitinib" OR "inlyta" OR "AG 013736" OR "AG013736" OR "AG-013736" OR "Angiogenesis Inhibitor" OR "angiogenesis inhibitors" OR "angiogenesis inhibitor" OR "anti angiogenic" OR "anti-angiogenic" OR "antiangiogenic" OR "anti angiogenesis" OR "anti-angiogenesis" OR "antiangiogenesis")) AND la=english AND py=(2004 OR 2005 OR 2006 OR 2007 OR 2008 OR 2009 OR 2010 OR 2011 OR 2012 OR 2013 OR 2014 OR 2015) NOT meeting abstracts	129	35
COCHRANE Library	(("Kidney Carcinoma" OR "renal cell carcinoma" OR "renal tumor" OR "renal tumour" OR "renal tumors" OR "renal tumours" OR "RCC" OR "mRCC" OR "kidney cell carcinoma" OR "kidney tumor" OR "kidney tumour" OR "kidney tumors" OR "kidney tumours" OR "renal cell adenocarcinoma" OR "kidney cell adenocarcinoma" OR "Grawitz") AND ("Genetic Variability" OR "genetic" OR "genetics" OR "genomics" OR "genomic" OR "genome" OR "mutation" OR "mutations" OR "allelic" OR "Allele" OR "alleles" OR "DNA" OR "gene" OR "genes" OR "chromosome" OR "chromosomes" OR "germ-line" OR "germline" OR "INDEL" OR "insertion" OR "deletion" OR "inversion" OR genetic polymorphism OR "polymorphism" OR "polymorphisms" OR "polymorfism" OR "polymorfisms" OR "Genomic Structural Variation" OR "Genomic Structural Variations" OR "Copy Number Variation" OR "DNA Copy Number Variations" OR "DNA Copy Number Variation" OR "SNP" OR "SNPs" OR "nucleotide" OR "nucleotides" OR "Genetics" OR "pharmacogenetic" OR "pharmacogenetics" OR "hapmap" OR "Pharmacogenetics" OR toxicogenetic* OR "predictive marker" OR "predictive markers" OR "predictive biomarkers" OR "predictive biomarker" OR "Genetic Marker" OR "GWAS" OR "Genome-Wide Association" OR "Genetic Association") AND ("sunitinib" OR sunitinib* OR "Sutent" OR "SU 11248" OR "SU11248" OR "SU-11248" OR "protein tyrosine kinase inhibitor" OR "tyrosine kinase inhibitor" OR "Tyrosine kinase inhibitors" OR "TKI" OR "SU011248" OR "SU-011248" OR "SU 011248" OR vasculotropin receptor OR "vascular endothelial growth factor receptor" OR "VEGF" OR "VEGFR" OR "VEGF-R1" OR "VEGF-R2" OR "VEGF-R3" OR "VEGFR1" OR "VEGFR2" OR "VEGFR3" OR "PDGF" OR "PDGFR" OR "sorafenib" OR "sorafenib" OR "nexavar" OR "BAY 545-9085" OR "BAY-545-9085" OR "BAY 43-9006" OR "BAY-43-9006" OR "BAY 439006" OR "BAY43-9006" OR "BAY439006" OR "pazopanib" OR "votrient" OR "GW 786034B" OR "GW786034B" OR "GW-786034B" OR "GW 786034" OR "GW786034" OR "GW-786034" OR "axitinib" OR "inlyta" OR "AG 013736" OR "AG013736" OR "AG-013736" OR "Angiogenesis Inhibitor" OR "angiogenesis inhibitors" OR "angiogenesis inhibitor" OR "anti angiogenic" OR "anti-angiogenic" OR "antiangiogenic" OR "anti angiogenesis" OR "anti-angiogenesis" OR "antiangiogenesis"))	20	2

Supplementary document 2: Continued

Database	Search Strategy	Number of references	Number of unique references
CENTRAL	(("Kidney Carcinoma" OR "renal cell carcinoma" OR "renal tumor" OR "renal tumour" OR "renal tumors" OR "renal tumours" OR "RCC" OR "mRCC" OR "kidney cell carcinoma" OR "kidney tumor" OR "kidney tumour" OR "kidney tumors" OR "kidney tumours" OR "renal cell adenocarcinoma" OR "kidney cell adenocarcinoma" OR "Grawitz") AND ("Genetic Variability" OR "genetic" OR "genetics" OR "genomics" OR "genomic" OR "genome" OR "mutation" OR "mutations" OR "allelic" OR "Allele" OR "alleles" OR "DNA" OR "gene" OR "genes" OR "chromosome" OR "chromosomes" OR "germ-line" OR "germline" OR "INDEL" OR "insertion" OR "deletion" OR "inversion" OR genetic polymorphism OR "polymorphism" OR "polymorphisms" OR "polymorfism" OR "polymorfisms" OR "Genomic Structural Variation" OR "Genomic Structural Variations" OR "Copy Number Variation" OR "DNA Copy Number Variations" OR "DNA Copy Number Variation" OR "SNP" OR "SNPs" OR "nucleotide" OR "nucleotides" OR "Genetics" OR "pharmacogenetic" OR "pharmacogenetics" OR "hapmap" OR "Pharmacogenetics" OR toxicogenetic* OR "predictive marker" OR "predictive markers" OR "predictive biomarkers" OR "predictive biomarker" OR "Genetic Marker" OR "GWAS" OR "Genome-Wide Association" OR "Genetic Association") AND ("sunitinib" OR sunitinib* OR "Sutent" OR "SU 11248" OR "SU11248" OR "SU-11248" OR "protein tyrosine kinase inhibitor" OR "tyrosine kinase inhibitor" OR "Tyrosine kinase inhibitors" OR "TKI" OR "SU011248" OR "SU-011248" OR "SU 011248" OR vasculotropin receptor OR "vascular endothelial growth factor receptor" OR "VEGF" OR "VEGFR" OR "VEGF-R1" OR "VEGF-R2" OR "VEGF-R3" OR "VEGFR1" OR "VEGFR2" OR "VEGFR3" OR "PDGF" OR "PDGFR" OR "sorafenib" OR "sorafenib" OR "nexavar" OR "BAY 545-9085" OR "BAY-545-9085" OR "BAY 43-9006" OR "BAY-43-9006" OR "BAY 439006" OR "BAY43-9006" OR "BAY439006" OR "pazopanib" OR "votrient" OR "GW 786034B" OR "GW786034B" OR "GW-786034B" OR "GW 786034" OR "GW786034" OR "GW-786034" OR "axitinib" OR "inlyta" OR "AG 013736" OR "AG013736" OR "AG-013736" OR "Angiogenesis Inhibitor" OR "angiogenesis inhibitors" OR "angiogenesis inhibitor" OR "anti angiogenic" OR "anti-angiogenic" OR "antiangiogenic" OR "anti angiogenesis" OR "anti-angiogenesis" OR "antiangiogenesis"))	20	0
Total			**973**

Supplementary document 3: Reported non-SNP biomarker associations ($P \leq 0.05$) with TKI treatment outcome in mRCC from 2009 to 2015.

First Author, year	TKI	No. of patients	ethnicity	Tumour type	Associated biomarkers	Study endpoint	Effect size	95%CI	p-value
Choueiri *et al.*, 2009 [45]	SUN	1	probably CAU (US)	CC on first diagnosis, then Xp11 translocation RCC	Xp11.2 translocation histological subtype (*TFE3* gene)	Response	N/A		
Beuselinck *et al.*, 2015 [46]	SUN	121	probably CAU (Belgium)	CC	ccRCC tumor molecular subtypes ccrcc1/ccrcc4 vs. ccrcc2/ccrcc3	Response			*P*=0.005
						PFS	HR:0.42	0.21-0.86	*P*=0.017
						OS	HR:0.38	0.19-0.76	*P*=0.0064
Paule *et al.*, 2010 [47]	SUN	23	probably CAU (France)	CC	VEGF (isoforms 121, 165, and 189),	Response	39%		*P*=0.04
					VEGF121/ VEGF165	OS	HR:5.8	1.4-24.5	*P*=0.02
Gruenwald *et al.*, 2010 [48]	SUN	26	probably CAU (Germany)	80.8% CC	Level of circulating endothelial cells (CEC)	PFS	Increase from baseline 40 ± 41 CEC/ml to 111 ± 61 at day 28		*P*=0.0109
D' Alterio *et al.*, 2012 [49]	SUN	62	probably CAU (Italy)	Mostly CC	CXCR4 expression	Objective response	-		*P*=0.0262
					CXCR4 expression + Fuhrman grading				*P*=0.0005
					CXCR4 expression	PFS	HR:2.04	1.08-3.84	*P*=0.0271
Garcia-Donas *et al.*, 2013 [50]	SUN	67	mostly European	CC	HIF2A protein expression	Objective response	HR:0.11	0.017-0.75	*P*=0.024
					PDGFRB protein expression		HR:0.04	0.002-0.68	*P*=0.026
					VEGFR3 protein expression	PFS	HR:0.403	0.20-0.82	*P*=0.012
					VEGFA protein expression	OS	HR:4.29	1.43-12.8	*P*=0.0092
					HIF2A protein expression		HR:0.39	0.15-0.99	*P*=0.048
					EGLN3 expression		HR:2.89	1.04-8.05	*P*=0.042

Supplementary document 3: Continued

First Author, year	TKI	No. of patients	ethnicity	Tumour type	Associated biomarkers	Study endpoint	Effect size	95%CI	p-value
Minardi *et al.*, 2013 [51]	SUN	41	probably European	CC	Endothelial cell VEGF expression VEGF staining (H-score)	distant metastasis free survival (DMFS)	-		*P*=0.031
									P=0.042
					tumour cell VEGF expression	OS			*P*=0.0145
					VEGF staining (H-score)				*P*=0.004
					Endothelial cell VEGF expression				*P*=0.016
Sato *et al.*, 2013 [52]	SUN	50	JAP	82% CC	EMMPRIN expression	Pathological T stage			*P*<0.0001
						Clinical M stage			*P*=0.0003
						AJCC stage			*P*=0.0001
						Fuhrman grade			*P*<0.0001
						OS			*P*=0.0345
						PFS			*P*=0.0066
						MVA	R^2=0.4801		
Motzer *et al.*, 2014 [33]	SUN	292	88% CAU	mRCC	HIF-1α	PFS	HR:1.55	1.03-2.34	*P*=0.0341
					baseline angiopoietin-2	best overall response (CR/ PR vs. SD/PD) at baseline	-		*P*=0.0197
					baseline matrix metalloproteinase-2				*P*=0.0327
del Puerto-Nevado *et al.*, 2014 [53]	SUN	23	probably CAU (Spain)	CC	protein expression: phosphorylated KDR (pKDR-Y1775)	PFS	HR:5.35	1.49–19.13	*P*=0.01
						OS	HR:5.13	1.25–21.05	*P*=0.02
Gambini *et al.*, 2014 [54]	SUN	1	probably CAU (Italy)	CC	neuroendocrine markers + pancreatic metastases	response	N/A		
Mikami *et al.*, 2014 [55]	SUN	25	probably JAP	CC	CD44 expression	Time to treatment failure	-		*P*=0.008
						OS			*P*=0.007
					TNF-a/CD44 expression	PFS			*P*=0.029

Supplementary document 3: Continued

First Author, year	TKI	No. of patients	ethnicity	Tumour type	Associated biomarkers	Study endpoint	Effect size	95%CI	p-value
Dornbusch *et al.*, 2013 [56]	SUN	42	probably CAU (Germany)	CC	Immuno-expression of CA9	Response after 6 months	-		*P*=0.034
					Immuno-expression of CD31				*P*=0.049
					Immuno-expression of HIF-1α	Response after 9 months			*P*=0.021
					Immuno-expression of VEGFR1				*P*=0.038
					Immuno-expression of PDGFRα				*P*=0.012
					Immuno-expression of PDGFRβ				*P*=0.041
					Immuno-expression of Ki67				*P*=0.004
					Immuno-expression of HIF-1α	PFS	HR:0.456	0.227-0.916	*P*=0.027
					Immuno-expression of CA9	OS	HR:0.271	0.110-0.669	*P*=0.005
					Immuno-expression of PDGFRα		HR:3.375	1.414-8.056	*P*=0.006
Gamez-Pozo *et al.*, 2012 [57]	SUN	38	probably CAU	CC: N=27, papillary: N=4, other: N=7	miRNA predictive model	OS	HR:0.463	0.219-0.979	*P*=0.044
						PFS	HR:0.385	0.161-0.963	*P*=0.032
Berkers *et al.*, 2013 [58]	SUN	20	probably CAU (Belgium)	CC	microRNA-141	PFS	Fold:-4.03	-	*P*=0.0098
Prior *et al.*, 2014 [59]	SUN	20 N=14 (sunitinib sensitive), N=6 (resistant)	probably CAU (Spain)	CC	microRNA-942	TTP	-		*P*=0.0030
					microRNA-628-5p				*P*=0.0270
					microRNA-133a				*P*=0.0153
					microRNA-484				*P*=0.0001
					microRNA-942	OS			*P*=0.0009
					microRNA-628-5p				*P*=0.0161
					microRNA-133a				*P*=0.0092
					microRNA-484				*P*=0.0036
					miR-942	sunitinib resistance			*P*=0.0074
Choueiri *et al.*, 2013 [60]	PAZ	225 (biomarker subset: N=116)	probably CAU	CC	1) VHL gene 2) HIF1a en HIF2a immunohistochemistry 3) HIF1a transcriptional signature		No significant associations		

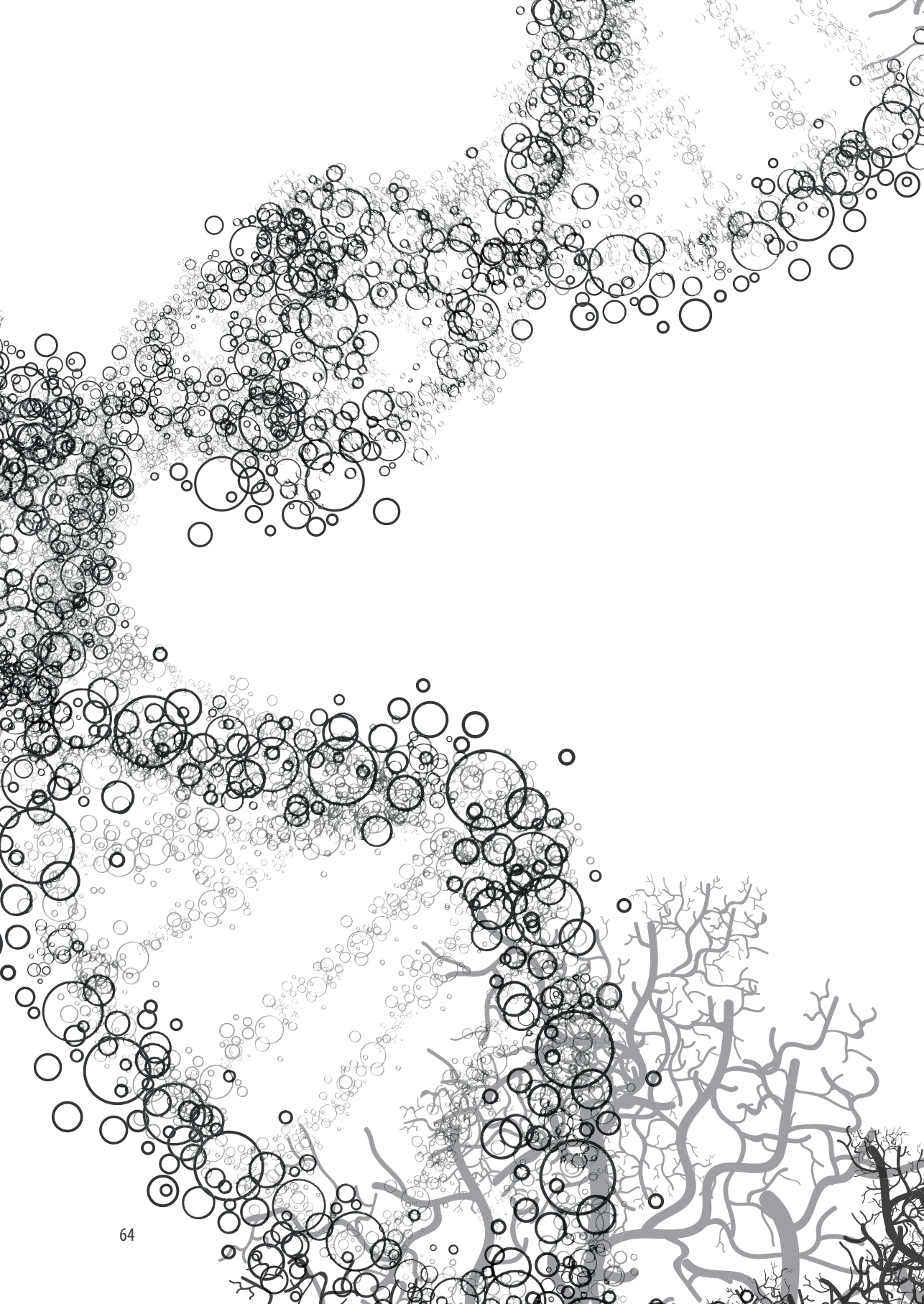
Supplementary document 3: Continued

First Author, year	TKI	No. of patients	ethnicity	Tumour type	Associated biomarkers	Study endpoint	Effect size	95%CI	p-value
Pena *et al.*, 2010 [61]	SOR (N=451, placebo (N=452)	903	probably CAU (USA)	Mostly CC	VEGF plasma protein levels week 3 and week 12	Sorafenib use	-		*P*<0.0001
					sVEGFR2 plasma protein levels week 3 and week 12				*P*<0.0001
					TIMP-1 plasma protein levels week 3				*P*=0.002
					TIMP-1 plasma protein levels week 12				*P*=0.006
Feng *et al.*, 2013 [62]	SOR	28 (18 mRCC vs 10 healthy)	probably CHI	CC	Plasma cell free DNA levels	Response week 8	-		*P*=0.019
						Response week 12			*P*=0.007
						Response week 16			*P*=0.004
						Response week 24			*P*<0.001
						Survival week 12			*P*=0.047
						Survival week 16			*P*=0.018
						Survival week 24			*P*=0.018
Aziz *et al.*, 2014 [63]	SOR	96	probably CAU (USA)	RCC (subtype not specified)	Microvessel area (MVA) in tumor samples	response	-	-	*P*=0.005 (after Bonferroni correction)
Zheng *et al.*, 2015 [64]	SOR	10	probably CHI	RCC (subtype not specified)	Micro RNA-30a (miR-30a)	Sorafenib resistance	-	-	*P*<0.05
Doberstein *et al.*, 2011 [65]	rapamycin, SUN and cisplatin	282	probably CAU (Germany)	CC=81.6% (n=230)	expression of L1 cell adhesion molecule (L1-CAM)	survival	RR:1.8		*P*=0.014
						sunitinib resistance	-	-	*P*<0.01

Supplementary document 3: Continued

First Author, year	TKI	No. of patients	ethnicity	Tumour type	Associated biomarkers	Study endpoint	Effect size	95%CI	p-value
Furuya *et al.*, 2011 [66]	SOR ± IFNα	66	JAP	CC	IFNAR2 mRNA levels	Response	-		*P*<0.0001
						OS			*P*<0.05
Sharpe *et al.*, 2013 [67]	SUN or PAZ	85	N/A, but probably all CAU (UK or Belgium)	CC	vessel density (CD31)	Use of TKI			*P*<0.05
					phospho-S6K expression				*P*<0.05
					PDL-1 expression				*P*<0.05
					FOXP3 expression				*P*<0.05
					increase in cytoplasmic FGF-2				*P*<0.05
					MET receptor expression in vessels				*P*<0.05
					Fuhrman tumor grade				*P*<0.05
					Ki-67				*P*<0.05
					levels of Ki-67	PD			*P*<0.05
					levels of CD31				*P*<0.05
Yamada *et al.*, 2014 [68]	SUN, SOR, AXI	13	probably JAP	CC: N=11, papillary: N=2	Plasma granulocyte macrophage colony-stimulating factor (GM-CSF) level	Response (PR vs SD or PD)	-	-	*P*=0.012
Tran *et al.*, 2012 [69]	PAZ, placebo	344		mRCC (subtype not specified)	IL-6	PFS	HR:0.55	0.28-0.71	*P*=0.009
Peters *et al.*, 2014 [70]	SUN, SOR, AXI, BEV	18	probably CAU (Hannover)	CC=88.9%	CST6 methylation status	PFS	HR:4.1	1.3-12.6	*P*=0.009
							HR:4.1	13.0-13.4	*P*=0.011
					LAD1 methylation status	OS	HR:6.4	1.6-26.0	*P*=0.004
							HR:2.9	1.0-8.6	*P*=0.043
Dubrowinskaja *et al.*, 2014 [71]	SUN, SOR, BEV, AXI	18	-	CC: N=16, papillary: N=1, chromophobe: N=1	NEFH methylation	OS	-	-	*P*=0.028
Choudhury *et al.*, 2015 [72]	SUN, PAZ	48	Asian	CC	Prognostic classification based on multigene assay: *CXCL5*, *EFNA5*, *EMCN*, *LAMB3*, *PLG*, *PRAME*, *RARRES1*, *SLC6A19*	Radiologic response	-	-	*P*=5.96 x10^{-4}
						OS	HR:3.58	1.162–11.03	*P*=0.0186

Abbreviations: ABCB1=ATP binding cassette member B1; ABCG2=ATP binding cassette member G2; sunitinib=SUN; pazopanib=PAZ; sorafenib=SOR; axitinib=AXI; bevacizumab=BEV; foretinib=FOR; valatinib=VAL; CAU=Caucasian; CHI=Chinese; JAP=Japanese; CC=clear cell renal cell carcinoma; GIST=gastrointestinal stromal tumor; PFS=progression-free survival; OS=overall survival; AST= aspartate aminotransferase; ALT= alanine aminotransferase



CHAPTER 3

Association analysis of genetic polymorphisms in genes related to sunitinib pharmacokinetics, specifically clearance of sunitinib and SU12662

Diekstra MH, Klümpen HJ, Lolkema MPJK, Yu H, Kloth JSL, Gelderblom H, van Schaik RHN, Gurney H, Swen JJ, Huitema ADR, Steeghs N, Mathijssen RHJ.

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ABSTRACT

Interpatient variability in the pharmacokinetics (PK) of sunitinib is high. Single nucleotide polymorphisms (SNPs) in PK candidate genes have been associated with the efficacy and toxicity of sunitinib, but whether these SNPs truly affect the PK of sunitinib remains to be elucidated. This multicenter study involving 114 patients investigated whether these SNPs and haplotypes in genes encoding metabolizing enzymes or efflux transporters are associated with the clearance of sunitinib and its active metabolite SU12662. SNPs were tested as covariates in a population PK model. From univariate analysis, we found that the SNPs in *CYP3A4*, *CYP3A5*, and *ABCB1* were associated with the clearance of both sunitinib and SU12662. In multivariate analysis, *CYP3A4*22* was found to be eliminated last with an effect size of –22.5% on clearance. Observed effect sizes are below the interindividual variability in clearance and are therefore too limited to directly guide individual dosing of sunitinib.

INTRODUCTION

Sunitinib is an orally administered tyrosine kinase inhibitor that is approved as a systemic therapy for metastatic renal cell carcinoma, gastrointestinal stromal tumors after failure on treatment with imatinib, and neuroendocrine tumors of the pancreas.[1-6] Sunitinib is a multitargeted tyrosine kinase inhibitor that inhibits vascular endothelial growth factor receptors type 1, 2, and 3; platelet-derived growth factor receptor-α and -β; Fms-related tyrosine kinase 3; colony stimulating factor-1 receptor; the cytokine receptor Kit; and the proto-oncogene tyrosine-protein kinase receptor Ret.[1-6]

Cytochrome P450 (CYP)3A4 metabolizes sunitinib to its active *N*-desethyl metabolite, SU12662, and subsequently into SU14335 and other inactive metabolites (**Figure 1**).[1,4,5,7-9] The involvement of CYP3A5, CYP1A1, and/or CYP1A2 in the metabolism of sunitinib has also been suggested, but no studies have yet confirmed this.[1] CYP3A4 expression is regulated by the pregnane X receptor and the constitutive androstane receptor, which are encoded by the *NR1I2* and *NR1I3* genes, respectively.[7,9] Sunitinib is a substrate of the efflux transporters ATP-binding cassette transporter P-glycoprotein and the breast cancer resistance protein encoded by the *ABCB1* and *ABCG2* genes, respectively.[8] No genetic polymorphisms affecting the distribution and excretion of sunitinib and SU12662 have been reported. Both sunitinib and SU12662 contribute to the total amount of active drug in plasma. When administered at the approved doses, sunitinib and SU12662 exhibit linear pharmacokinetics (PK).[4,10] Yet interpatient variability in the PK of sunitinib and SU12662 is high, and a large variability in efficacy and toxicity of sunitinib has been reported.[2-4,10] As a result, 32% of patients with renal cell carcinoma who were treated with sunitinib required a dose reduction for adverse events, including hypertension, fatigue, and hand-foot syndrome.[1,2,10,11]

Exploratory studies have found that single nucleotide polymorphisms (SNPs) in candidate genes in the PK pathway of sunitinib are associated with the efficacy and toxicity of sunitinib. Van Erp *et al.*[1] reported that the presence of a copy of the TT allele in the *ABCG2* haplotype (rs2231142 and rs55930652) was associated with occurrence of any toxicity higher than grade 2 and that the presence of a copy of the TTT alleles in the *ABCB1* haplotype (rs1128503, rs2032582, and rs1045642) was associated with an increased prevalence of hand-foot syndrome. The G allele in *CYP1A1* (rs1048943) showed an increased risk for both mucosal inflammation and leukopenia.[1] The absence of a CAG copy in the *NR1I3* haplotype (rs2307424, rs2307418, and rs4073054) was associated with an increased risk for leukopenia. In a subsequent analysis, van der Veldt *et al.*[3] reported an association between *CYP3A5*1* (presence of an A allele on rs776746) and an increased progression-free survival (PFS). The *CYP3A5*1* allele results in expression of the CYP3A5 enzyme, which is hypothesized to convert sunitinib to the active metabolite SU12662.

SU12662 has a longer half-life than sunitinib (80-110 vs. 40-60 h). This increased conversion into SU12662 could explain the observed increased occurrence of toxicities for *CYP3A5*1* carriers.[2] To maximize the potential for identifying novel associations, these studies did not correct for multiple testing, increasing the risk for inflation of type 1 errors and necessitating replication studies.[1-3]

Recently, several novel SNPs affecting CYP3A4 activity have been identified. The *CYP3A4*22* SNP results in decreased CYP3A4 activity and is associated with decreased levels of clearance (CL) for several drugs as well as increased levels of paclitaxel-induced neurotoxicity.[12-14] During treatment with midazolam or tacrolimus, an increased CYP3A activity was observed for *POR*28* (T allele carriers for rs1057868C/T) in the gene encoding P450 oxidoreductase (*POR*).[12,13,15-19]

The reported associations between SNPs in candidate genes in the PK pathway of sunitinib and sunitinib-related outcomes may present an interesting opportunity to guide individualization of sunitinib treatment. However, replication of exploratory findings in an independent cohort is necessary, and it remains to be elucidated if these SNPs truly affect the PK of sunitinib and SU12662. Therefore, the aim of this study was to investigate, using CL as the PK parameter of interest, whether polymorphisms in candidate genes involved in the metabolism and absorption of sunitinib result in altered PK of sunitinib and its active metabolite, SU12662.

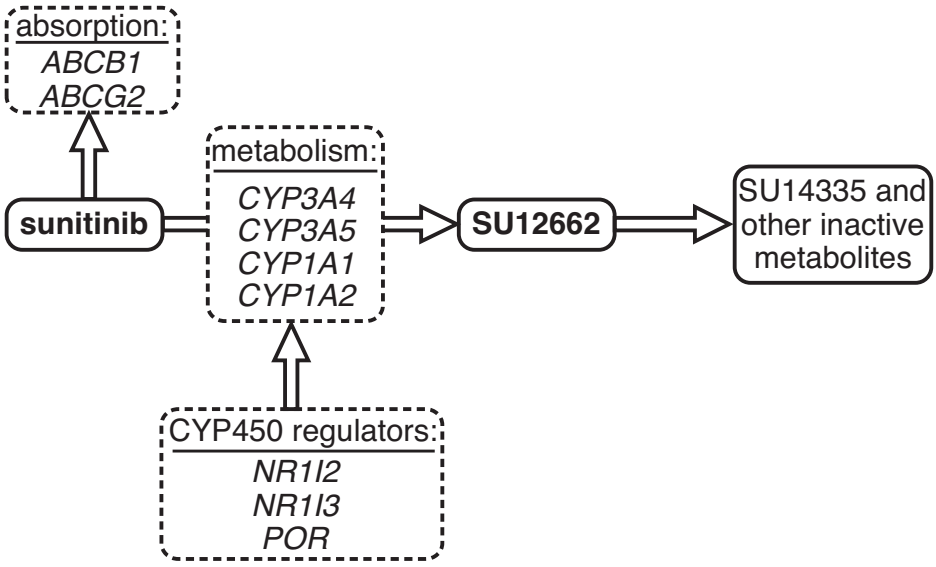


Figure 1 Pharmacokinetic pathway of sunitinib and its active metabolite, SU12662.

RESULTS

Population characteristics

Data from patients treated with monotherapy sunitinib were pooled from five prospective PK studies conducted at seven medical centers in the Netherlands and Australia (see **Supplementary Table S1** online).[20-24] Both PK data and pharmacogenetics (PG) data were available for a total of 114 of the 122 patients. The majority (60.5%) of subjects were diagnosed with metastatic renal cell carcinoma (**Table 1**).

Table 1 Patient characteristics

Demographic	**noted as *N* (%) or *N* (range)**
Gender	
Male	75 (65.8%)
Female	39 (34.2%)
Age (years)	58.7 (range 27-81)
Ethnicity	
Caucasian	109 (95.6%)
Asian	3 (2.6%)
Middle-eastern	1 (0.9%)
Hispanic	1 (0.9%)
Tumor type	
(m)RCC	69 (60.5%)
(p)NET	14 (12.3%)
GIST	8 (7.0%)
other solid tumor type	23 (20.2%)
Sunitinib, daily dose (mg)	
25	5 (4.4%)
37.5	60 (52.6%)
50	48 (42.1%)
62.5	1 (0.9%)

ECOG, Eastern Cooperative Oncology Group; GIST, gastrointestinal stromal tumor; (m)RCC, metastatic or nonmetastatic renal cell carcinoma; (p)NET, neuroendocrine tumors of the pancreas or elsewhere.

Effect of SNPs on CL of sunitinib and SU12662

Parameter estimates of the base model are presented in **Table 2**. Association analysis was performed following a stepwise procedure that comprises genotype inclusion, univariate testing for potential association (forward inclusion), and, finally, elimination of the genotypes of least significance (backward elimination). The correlation results of SNPs and CL are listed in **Table 3**. The genotypes rs1048943 GG, rs35599367 TT, rs776746 AA, rs2231142 AA, and rs2307418 AA were excluded from the analysis due to a frequency <5% (**Table 3**).

The remaining 37 genotypes and 3 haplotypes were individually tested by inclusion in a base model (without any covariates). Genotypes CC and CT of SNP rs35599367 in *CYP3A4*, GG of SNP rs776746 in *CYP3A5*, TT of SNP rs2032582 in *ABCB1*, and CC of SNP rs1128503 in *ABCB1* showed a potential association ($P<0.05$) with CL of sunitinib and were included in the full model (**Table 3**). In the univariate analysis, a 29.0% increase in CL of sunitinib ($P=0.01$) was observed for the CC genotype of SNP rs35599367 (*CYP3A4*) as compared with the CT and TT genotypes. By contrast, genotype CT of SNP rs35599367 (*CYP3A4*) showed a 21.3% decrease in sunitinib CL ($P=0.02$) as compared with the CC and TT genotypes. *CYP3A5*3* (genotype GG of SNP rs776746) showed a decrease in sunitinib CL of 18.4% ($P=0.01$). The AG genotype of rs776746 in *CYP3A5* (*CYP3A5*1/*3*) revealed potential association with CL of SU12662 with an effect size of 28.0% ($P=0.04$). None of the haplotypes showed an association with sunitinib or SU12662 CL (**Table 3**). **Figure 2a** shows an overview of the empirical Bayes estimates of CL for sunitinib and SU12662 plotted for the different tested genotypes in *CYP1A1*, *CYP3A4*, *CYP3A5*, *ABCB1*, *ABCG2*, *NR1I2*, *NR1I3*, and *POR*.

In the multivariate backward-elimination step, none of the tested SNPs reached the significance threshold of $P<0.0005$ (**Table 3**). SNP rs35599367 was the last SNP that was eliminated from the full model. In the presence of at least one T allele (*CYP3A4*22*) of rs35599367, a decrease of 22.5% in CL of sunitinib was observed ($P<0.01$; **Table 3**). The interindividual variability in CL of sunitinib decreased with 1.3% (percentage of coefficient variation (%CV)) after inclusion of this genotype as a covariate in the model (**Table 2**).

Table 2 Parameter estimates of the base model for sunitinib and SU12662

Parameter	Estimate (RSE)	IIV, $_{\%CV}$ (RSE)	IIV, $_{variance}$
Sunitinib			
CL_p ($l \cdot h^{-1}$)	38.8 (3.4%)	32.2% (10.9%)	0.104
Ka,p, h^{-1}	0.195	-	-
Vc,p, L	877 (16.9%)	64.7% (18.5%)	0.419
Q_p ($l \cdot h^{-1}$)	254 (12.5%)	-	-
$V_{p,p}$ (l)	1670 (11%)	49.1% (12.8%)	0.241
Proportional error	0.0287 (20.7%)	-	-
Additive error (nmol/l)	0.50	-	-
SU12662			
CL_m ($l \cdot h^{-1}$)	79.6 (4.2%)	42.9% (6.5%)	0.184
$V_{c,m}$ (l)	309 (19.5%)	-	-
Proportional error	0.0332 (22%)	-	-
Additive error (nmol/l)	0.54	-	-

%CV, percentage of coefficient variation; CLm, clearance of the active metabolite SU12662; CLp, clearance of the parent drug sunitinib; IIV, interindividual variability; Ka,p, absorption rate constant; *Q*p, intercompartment clearance; RSE, relative standard error; Vc,p, volume distribution of central compartment of the parent drug sunitinib; Vc,m, volume distribution of central compartment of the active metabolite SU12662; Vp,p, volume distribution of peripheral compartment of the parent drug sunitinib. From ref. 22.

Table 3 Results from association analyses of SNPs on sunitinib and SU12662 clearance

Gene(s)	rs Number	Genotype	N[a]	%	*P* value[b] (CL_p)	Effect size (%)[c] (CL_p)	*P* value[d] (CL_p)	*P* value[b] (CL_m)	Effect size (%)[c] (CL_m)	*P* value[d] (CL_m)
CYP1A1	rs1048943	AA (wt)	104	92.0	0.83	-2.5		0.34	-13.5	
		AG	8	7.1	0.86	-2.3		0.84	+3.0	
		GG	1	0.9	-	-		-	-	
CYP3A4	rs35599367	CC (wt)	99	88.4	0.01	+29.0	0.01	0.42	+11.0	
		CT	11	9.8	0.02	-21.3	0.84	0.58	-7.6	
		TT	2	1.8	-	-		-	-	
CYP3A5	rs776746	GG (wt)	92	82.9	0.01	-18.4	0.06	0.07	-17.7	
		AG	17	15.3	0.05	+19.0		0.04	+28.0	0.04
		AA	2	1.8	-	-		-	-	
ABCB1	rs1128503	CC (wt)	37	32.5	0.048	-4.8		0.45	-6.5	
		TC	52	45.6	0.35	-5.9		0.92	-0.8	
		TT	25	21.9	0.05	+17.0	0.66	0.33	+10.0	
ABCB1	rs2032582	GG (wt)	40	36.0	0.42	-5.3		0.46	-6.2	
		GT	41	36.9	0.23	-7.7		0.46	-6.3	
		TT	30	27.0	0.02	+18.0	0.02	0.20	+13.0	
ABCB1	rs1045642	TT (wt)	37	32.5	0.30	+7.0		0.94	+1.0	
		TC	51	44.7	0.69	+3.0		0.80	+2.0	
		CC	26	22.8	0.11	-11.5		0.71	-3.7	
ABCG2	rs2231142	CC (wt)	83	75.5	0.10	+13.0		0.86	+2.0	
		AC	24	21.8	0.14	-10.9		0.91	-1.2	
		AA	3	2.7	-	-		-	-	
ABCG2	rs55930652	CC (wt)	52	46.0	0.74	+2.0		0.58	-4.6	
		CT	52	46.0	0.28	-6.7		0.82	+2.0	
		TT	9	8.0	0.32	+13.0		0.32	+17.0	
ABCG2	rs2622604	CC (wt)	56	50.9	0.45	-4.8		0.17	-10.9	
		CT	47	42.7	0.84	-1.3		0.39	+8.0	
		TT	7	6.4	0.16	+21.0		0.08	+36.0	
NR1I2	rs3814055	CC (wt)	40	35.1	0.68	-2.7		0.77	+3.0	
		CT	56	49.1	0.19	+9.0		0.59	+5.0	
		TT	18	15.8	0.21	-10.5		0.26	-12.0	
NR1I3	rs2307424	CC (wt)	43	38.1	0.53	+4.0		0.57	-4.8	
		TC	55	48.7	0.30	-6.5		0.69	-3.3	
		TT	15	13.3	0.36	+9.0		0.07	+25.0	
NR1I3	rs2307418	AA (wt)	82	72.6	0.43	+6.0		0.56	-5.3	
		CA	29	25.7	0.25	-8.1		0.84	+2.0	
		CC	2	1.8	-	-		-	-	
NR1I3	rs4073054	TT (wt)	49	43.8	0.69	+3.0		0.14	+13.0	
		TG	47	42.0	0.50	-4.4		0.71	-3.1	
		GG	16	14.3	0.97	-0.2		0.40	-9.7	
*POR*28*	rs1057868	CC (wt)	53	46.9	0.87	-1.0		0.56	+5.0	
		CT	52	46.0	0.37	-5.6		1.00	-0.1	
		TT	8	7.1	0.10	+23.0		0.47	-11.0	
ABCB1	rs1128503	TTT - TTT	21	19.4	0.65	+3.0		0.90	+1.0	
	rs2032582	TTT - other	44	40.7						
	rs1045642	Other - other	43	39.8						

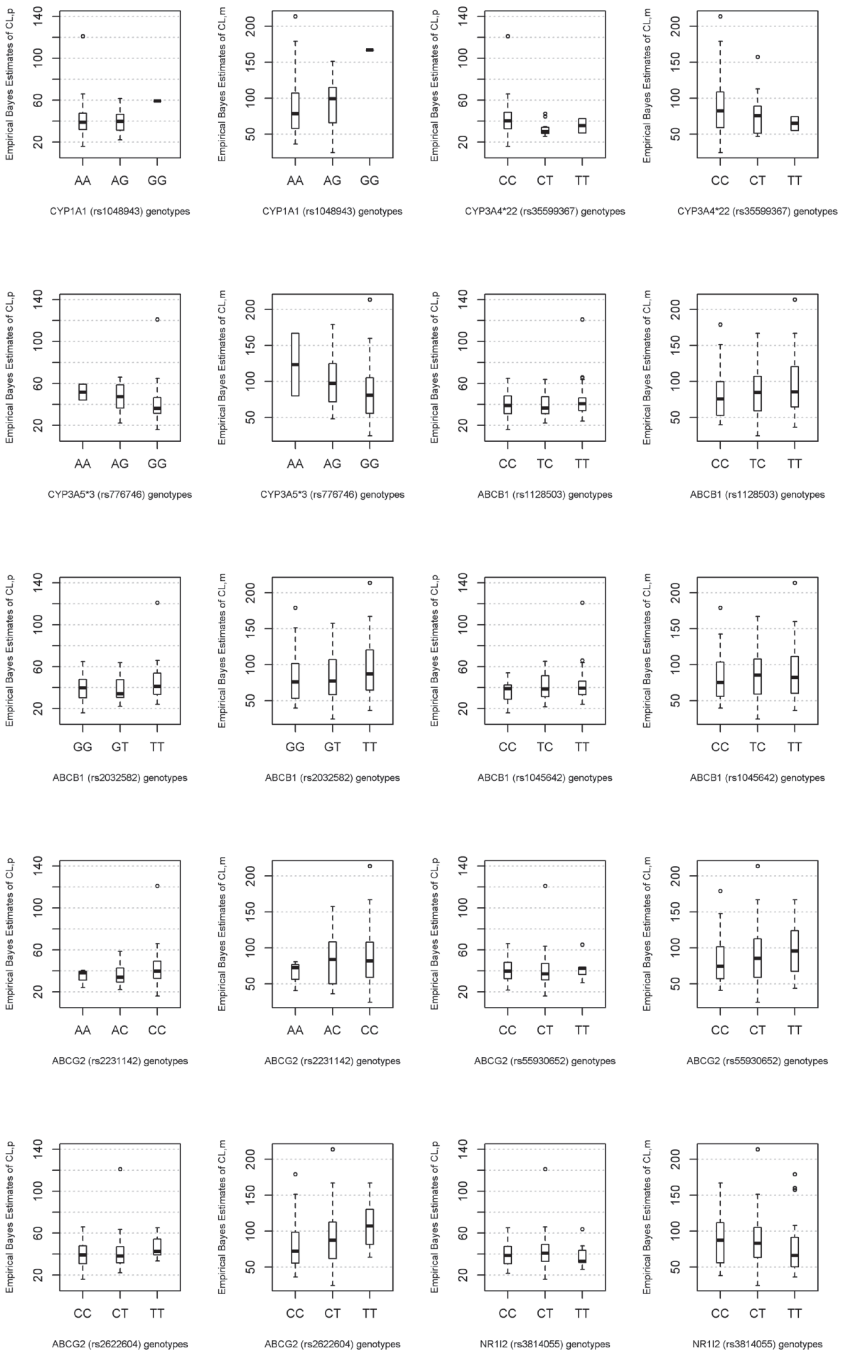
Table 3 Continued

Gene(s)	rs Number	Genotype	*N*[a]	%	*P* value[b] (CL_p)	Effect size (%)[c] (CL_p)	*P* value[d] (CL_p)	*P* value[b] (CL_m)	Effect size (%)[c] (CL_m)	*P* value[d] (CL_m)
ABCG2	rs55930652 rs2622604	TT - TT	7	6.4	0.71	+2.0		0.09	+15.0	
		TT - other	45	40.9						
		Other - other	58	52.7						
NR1I3	rs2307424 rs2307418	CAT - CAT	11	9.9	0.20	-7.9		0.34	-7.8	
		CAT - other	38	34.2						
		Other - other	62	55.9						
		CAG - CAG	16	14.4	0.49	-4.4		0.34	-7.7	
		CAG - other	47	42.3						
		Other - other	48	43.2						
CYP3A4	rs35599367	CT or TT vs. CC				-22.5	0.01		-9.9	
CYP3A5	rs776746	AG or AA vs. GG				+22.5	0.06		+21.5	
Genotypes	**rs Number**	**CYP3A genotype-predicted phenotype**	***N*[a]**	**%**	***P* value[b] (CL_p)**	**effect size (%)[e] (CL_p)**		***P* value[b] (CL_m)**	**Effect size (%)[e] (CL_m)**	
CYP3A4 CT or TT *and CYP3A5* GG	rs35599367 and rs776746	PM	12	11.0	0.01	-22.9		0.45	-9.7	
CYP3A4 CT or TT *and CYP3A5* AG or AA	rs35599367 and rs776746	IM_1	1	0.9	-	-		-	-	
CYP3A4 CC *and CYP3A5* GG	rs35599367 and rs776746	IM_2	78	71.6	1.00	0.0		0.31	-8.7	
CYP3A4 CC *and CYP3A5* AG or AA	rs35599367 and rs776746	EM	18	16.5	0.02	+22.0		0.01	+35.0	

CLm, clearance of SU12662; CLp, clearance of sunitinib; EM, extensive metabolizer; IM1, intermediate metabolizer type 1; IM2, intermediate metabolizer type 2; PM, poor metabolizer; SNP, single nucleotide polymorphism; wt, wild type.

[a]Number of subjects. Numbers differ per SNP, haplotype, or phenotype because not all subjects were successfully genotyped for all SNPs. [b]*P* value for the influence of genotype or haplotype on clearance from the univariate analysis (forward inclusion). [c]Effect size of the genotype or haplotype on the clearance with presence of the genotype as compared with the clearance with absence of the genotype or haplotype. [d]*P* value for the influence of genotype or haplotype on clearance from the multivariate analysis (backward elimination). [e]Effect size of the phenotype on the clearance with presence of the phenotype as compared with the clearance with the presence of the IM1 + IM2 phenotypes.

A


Empirical Bayes Estimates of CL_p
Empirical Bayes Estimates of CL_m
20
60
100
140
50
100
150
200
AA AG GG
CC CT TT
CC TC TT
GG GT TT
AA AC CC
CYP1A1 (rs1048943) genotypes
CYP3A4*22 (rs35599367) genotypes
CYP3A5*3 (rs776746) genotypes
ABCB1 (rs1128503) genotypes
ABCB1 (rs2032582) genotypes
ABCB1 (rs1045642) genotypes
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NR1I2 (rs3814055) genotypes

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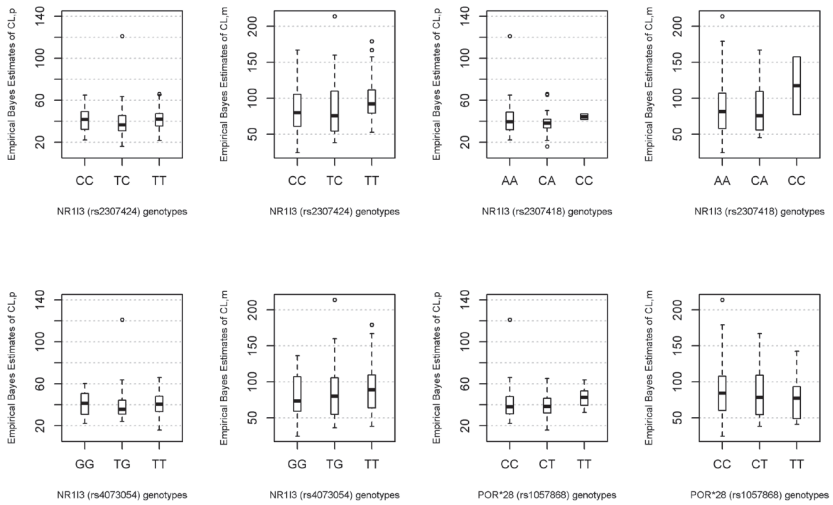

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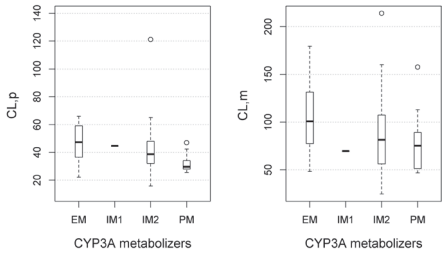

Figure 2 Clearance plotted against SNP genotypes. (a) Empirical Bayes estimates of clearance of sunitinib (CLp) and the active metabolite SU12662 (CLm) for base model stratified by different genotypes of single nucleotide polymorphisms or haplotypes in *CYP1A1*, *CYP3A4*, *CYP3A5*, *ABCB1*, *ABCG2*, *NR1I2*, *NR1I3*, and *POR*. The quartiles construct the upper and lower ranges of the boxes; the whiskers display the highest and lowest value within the 1.5 interquartile ranges; and the outliers are 1.5 interquartile ranges above the upper quartile or 1.5 interquartile ranges below the lower quartile. (b) Empirical Bayes estimates of clearance of sunitinib (CLp) and the active metabolite SU12662 (CLm) for the base model stratified by CYP3A phenotypes.

CYP3A genotype-predicted phenotype analysis

Because of a great similarity in CYP3A substrate specificity of CYP3A4 and CYP3A5, the *CYP3A4* and *CYP3A5* genotypes were combined as a *CYP3A* genotype-predicted phenotype (CYP3A phenotype).[17] In a *post hoc* analysis, *CYP3A* genotype-predicted phenotypes (poor metabolizers (PMs), intermediate metabolizers (IMs), or extensive metabolizers (EMs)) were tested as covariates in our population PK model. The possible combinations, together with the frequencies of *CYP3A4* and *CYP3A5* genotypes, were linked to the corresponding metabolizer status (CYP3A phenotype) and are listed in **Table 3**. The aberrant CYP3A phenotypes (PMs and EMs) were compared with the most frequent CYP3A-predicted phenotype (IM type 1 with CT or TT genotype of *CYP3A4* and AG or AA of *CYP3A5* and IM type 2 with wild-type CC genotype of *CYP3A4* and GG of *CYP3A5*). The observed effect sizes of these IMs were used as a reference point with respect to the observed effect sizes of PMs or EMs. For CYP3A PMs, a decrease of 22.9% in the CL of sunitinib was observed as compared with that of CYP3A IMs (P=0.01). For SU12662, a decrease in CL of 9.7% was observed for the group of CYP3A PMs as compared with CYP3A IMs (P=0.45), as shown in **Table 3**. For CYP3A EMs, a 22.0% increase in the CL of sunitinib was observed as compared with that of CYP3A IMs (P=0.02). For SU12662, a 35.0% increase in the CL of sunitinib was observed for the group of CYP3A PMs as compared with CYP3A IMs (P=0.01; **Table 3**). **Figure 2b** shows the empirical Bayes estimates of CL for sunitinib and SU12662 plotted for the different CYP3A phenotypes.

DISCUSSION

To our knowledge, this is the first PG study on the PK of sunitinib. Previous exploratory studies have directly related SNPs in candidate genes in the PK pathway of sunitinib to efficacy and toxicity, without involvement of plasma concentrations. Interestingly, none of the SNPs in candidate genes in the PK pathway of sunitinib that have been previously associated with outcomes of sunitinib treatment appear to be significantly associated with the CL of sunitinib and SU12662 in our population of 114 patients treated with sunitinib. Although not significant, *CYP3A4*22* carriers showed a 22.5% decrease in the CL of sunitinib. By contrast, *CYP3A5*1* carriers showed a 22.5% increase in the CL of sunitinib. When analyzed by CYP3A phenotype, PMs (defined as the concomitant presence of *CYP3A5*3* GG and *CYP3A4*22*) showed a 22.9% decrease in the CL of sunitinib and a 9.7% decrease in the CL of SU12662 as compared with those of CYP3A IMs, and CYP3A EMs (defined as the concomitant presence of *CYP3A5*3* AG or AA and *CYP3A4* CC) showed a 22.0% increase in the CL of sunitinib and a 35% increase in the CL of SU12662 as compared with those of CYP3A IMs. Unfortunately, our data do not allow exact replication of previously reported

exploratory analyses because no toxicity or efficacy data were available for the current data set. In addition, SNPs related to the pharmacodynamic pathway were not included in this study.[25,26] Furthermore, the small sample size of 114 patients limits the number of SNPs that could be investigated. Therefore, we selected only those SNPs and haplotypes that were significantly associated ($P<0.05$) with toxicity, PFS, or overall survival as reported in published exploratory PG studies on sunitinib. The minor allele frequency for the tested SNPs was >0.1, with the exception of the *CYP1A1* SNP rs1048943 (0.044), the *CYP3A4* SNP rs35599367 (0.067), and the *CYP3A5* SNP rs776746 (0.095).

The SNPs in *CYP3A4* (rs35599367) and *ABCB1* (rs2032582) deviated from Hardy-Weinberg equilibrium. However, the observed deviations were small, with $P=0.02$ for *CYP3A4* (rs35599367) and $P=0.01$ for *ABCB1* rs2032582. In addition, allele frequencies are highly similar to the frequencies reported in the HapMap-CEU The National Center for Biotechnology Information (NCBI) database. In this study, *ABCB1* rs2032582 showed an allele frequency of 45.5%, as compared with 46.9% reported in the NCBI database. For *CYP3A4*22*, an allele frequency of 6.7% was found, as compared with 2.5% reported in the NCBI database.

The observed decrease of 22.5% in the CL of sunitinib resulting from *CYP3A4*22* has a similar order of magnitude as reported for the effect of *CYP3A4*22* and the CYP3A phenotype on CL of tacrolimus, midazolam, erythromycin, and erlotinib.[12,13,27,28] The effect sizes of 22.5% (*CYP3A4*22*) and 22.9% (CYP3A PMs) observed in this study are not large enough to guide individual sunitinib dosing in clinical practice because these effect sizes are smaller than the interindividual variabilities of 32.2 and 42.9% in CL of sunitinib and SU12662, respectively. Houk *et al.*[4] reported an even larger interindividual variability of 37.9 and 52.2% in the CL of sunitinib and SU12662, respectively. In practice, the actual effect sizes of the studied SNPs on CL might be lower due to the effect on PK variability of environmental and other factors, such as smoking, alcohol use, body mass index, hormonal status, age, race, gender, and coadministration of other drugs.[4,29,30]

The analysis of CYP3A genotype-predicted phenotypes in relation to the CL of sunitinib has not been performed previously. Earlier, it was observed that carriers of the *CYP3A5*1* allele have a prolonged PFS and increased toxicity.[2,3] It was hypothesized that this might result from higher levels of SU12662, which has a longer half-life than sunitinib, resulting in increased exposure. Our data partly support this hypothesis by showing that carriers of a *CYP3A5*1* allele indeed have a 22.5% increased CL of sunitinib ($P=0.05$) as compared with GG carriers, and thus increased formation of SU12662. However, the theory regarding increased exposure to SU12662 is refuted because our data indicate that carriers of a *CYP3A5*1* allele, in addition to having a higher CL of sunitinib, have a 21.5% ($P=0.04$) higher CL of SU12662, resulting in a faster elimination and reduced exposure. Effect sizes of the PM CYP3A phenotype (22.9%) and *CYP3A4*22* (22.5%) on CL of sunitinib are comparable and

differ only by 0.4 percentage points. This can be explained by the low *CYP3A5*1* frequency in our study population, who are mainly Caucasians.

To date, studies looking for PG biomarkers for sunitinib-related outcomes have revealed a small set of potential markers. The available studies used the candidate-gene approach in retrospectively collected cohorts and thus are limited by our current knowledge of the PK and pharmacodynamics of sunitinib. Our results indicate that the currently available PG biomarkers cannot be used directly to guide individual dosing of sunitinib because they account for a limited part of the observed interindividual variability in CL of sunitinib and SU12662. Combinations of PG and environmental markers that predict the CL of this drug should be studied further. In addition, several other approaches to individualize sunitinib treatment remain to be explored. Therapeutic drug monitoring appears to be a promising method to guide sunitinib dosing that warrants further research.[31,32] In addition, it would be quite interesting to explore other PG biomarkers by using an unbiased approach—for example, by conducting a genome-wide association study in a well-defined cohort of prospectively collected data.[33]

In conclusion, of the 14 SNPs that are in the suggested PK pathway for sunitinib and that have been previously related to sunitinib outcome, none significantly influences the CL of sunitinib or SU12662 based on the *P* value <0.0005. The observed effect sizes are too small to directly guide individual dosing regimens of sunitinib.

METHODS

Study population

PK data were available from participating medical centers, including the Leiden University Medical Center, Academic Medical Center, University Medical Center Utrecht, Slotervaart Hospital, Erasmus MC Cancer Institute, Netherlands Cancer Institute-Antoni van Leeuwenhoek, Canberra Hospital, and the Westmead Hospital in Sydney.[20-24] Patients were eligible if PK sampling of sunitinib was performed and blood for PG analysis was available. Patients were excluded because of missing dose levels (N=2), concomitant use of mitotane (a strong CYP3A4 inducer; N=2), and genotype call rates <80% (N=3). All patients received sunitinib orally, administered in doses ranging from 25.0 to 62.5 mg once daily in a continuous dosing regimen or an intermittent schedule (4 weeks on treatment followed by 2 weeks off treatment). Patients were diagnosed with metastatic or nonmetastatic renal cell carcinoma (N=69), neuroendocrine tumors of the pancreas (N=14), gastrointestinal stromal tumor (N=8), or another solid tumor type (N=23) (**Table 1**). The local medical ethical boards of the participating medical centers approved all studies, and informed consent was obtained from all patients.

PK data

Steady-state plasma concentrations of sunitinib and SU12662 were measured before start of sunitinib administration and at one or more (fixed or random) time points after dosing (see **Supplementary Table S1** online).[20-24]

SNP and haplotype selection

SNPs related to the sunitinib PK pathway were selected from seven published exploratory PG studies on sunitinib.[1-3,12,15,18,19] SNPs and haplotypes significantly associated ($P<0.05$) with toxicity response, PFS, or overall survival were included in the current study. Selected SNPs and haplotypes are listed in **Supplementary Table S2a,b** online.

Genotyping

Germ-line DNA was isolated from 400 µl whole blood, using the Maxwell 16 system (Promega, Leiden, The Netherlands), according to the manufacturer's protocol. Prior to analysis, DNA concentrations were measured using the Nanodrop ND-1000 spectrophotometer (Isogen Life Science, IJsselsteijn, The Netherlands). Genotyping was performed with TaqMan probes (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) using the LightCycler480 (LC480) Real-Time PCR Instrument (Roche Applied Science, Almere, The Netherlands).

The mean genotype call rate was 98.4%, with a lowest observed call rate of 96% and a highest observed call rate of 100%. As a quality control, 5% of the samples were genotyped in duplicate for all assays, and no inconsistencies were observed. Patients with a genotype call rate of <80% were excluded from analysis. Minor allele frequencies of all 14 SNPs were calculated and compared with reported minor allele frequencies for European populations (http://www.ncbi.nlm.nih.gov/snp/). All SNPs were in Hardy-Weinberg equilibrium ($P>0.05$), except for rs35599367 in *CYP3A4* ($P=0.024$) and rs2032582 in *ABCB1* ($P=0.007$) (see **Supplementary Table S3** online). The maximum likelihood estimates of haplotype probabilities were calculated using PLINK software, version 1.07.[34] Haplotype probabilities with a likelihood ≥95% were included in statistical analysis. Haplotype likelihoods could be calculated for a mean percentage of patients of 97.3% (range: 96.3-98.2%).

Statistical analysis

Plasma concentration-time data and PG data were analyzed using the nonlinear mixed effect modeling software, NONMEM (version 7.1; ICON, Ellicott City, MD). The first-order conditional estimation method with interaction was used for the parameter estimation, and Piraña was used as the modeling environment. R (version 2.13.0; http:// cran.r-project.org) was used for handling of data and results.

A two-compartment model for sunitinib and one-compartment model for SU12662 were structured as schematically shown in **Figure 3**.[22] The base PK model was designated based on objective function value (ΔOFV >3.84; $P<0.05$), successful minimization, successful covariance estimation, and goodness-of-fit plots. The goodness-of-fit plots are presented in the Clearsun study.[22] The visual predictive checks are also described in the Clearsun study and shown in **Supplementary Figure S1** online.[22] The bioavailability (*F*) was unknown and therefore the parameter estimates of the population PK model were noted as apparent terms proportional to *F*, except for the absorption rate constant of sunitinib (*K*a,p). For CL, this means the parameter estimate is noted as CL/*F*. For SU12662, the PK parameters in the population PK model were estimated relative to *F* and fm, which is the fraction of sunitinib metabolized to SU12662. The *K*a,p was set at 0.195/h based on the results of a population PK metaanalysis of sunitinib and SU12662.4,22 An exponential error term was used to define the interindividual variability of all PK parameters. This exponential error term was quantified as both %CV and variance (calculated as (%CV/100)).[2] A combined error model with additive error terms fixed to the lower limit of quantification (**Table 2**) was used to model the residual error.[4,22]

Once the base model was defined, genotypes and haplotypes were tested as covariates on both CL parameters in a stepwise manner. Genotype frequencies of SNPs or haplotypes that were smaller than 5% were considered as missing values and ignored in analysis. Other covariates, i.e. gender, age, and tumor type, were not included in the building of the model, because the main research question of the current study was to identify potential relationships between the genotypes of relevant drug-metabolizing enzymes and the CL of sunitinib and SU12662. Furthermore, considering the relatively small sample size and the large variability in the PK of sunitinib, inclusion of other covariates is unlikely to have substantial effects on the outcome. The different PK studies in which the included patients previously participated were regarded as "PK study covariates." In the current analysis, these "PK study covariates" were considered during model building but were not included in the model. Instead, all goodness-of-fit diagnostics stratified on "PK study covariate" were evaluated in order to evaluate the influence of "PK study covariate" on PK parameters. No relevant differences between the different studies were identified.

First, a univariate forward-inclusion step was conducted. Covariates (genotypes or haplotypes) were screened by inclusion in a base model univariately using a power model, where $CL_{typical\ value}=\theta_1\times(\theta_2)^{pg1}$, where pg is the genotype or haplotype of interest whereby the presence of the certain genotypes or haplotypes in the patients was scored 1 and the absence was scored 0. θ_1 is the population CL estimate and θ_2 is the covariate effect size estimate. The threshold of this step was set at $P<0.05$ (likelihood ratio test, ΔOFV >3.84; degrees of freedom =1). In the next step, all potentially related covariates were included in a full model.

Subsequently, in the backward-elimination step, covariates were removed one at a time from the full model, and significance was again assessed using the likelihood ratio test. The likelihood ratio test itself may have anticonservative properties, and to account for multiple testing, we applied a threshold *P* value of 0.0005 (ΔOFV >12.12; degrees of freedom =1) in this step of the analysis. A separate analysis of combinations of the *CYP3A5*3* and *CYP3A4*22* genotypes (referred to as the CYP3A phenotype) was performed in order to evaluate correlations between each metabolizer group (PM, IM, or EM) and CLs. According to the literature, the presence of the T allele (CT or TT) in SNP *CYP3A*22* or the GG genotype in SNP *CYP3A5*3* results in decreased potency of metabolic enzymes.[17] The subjects were grouped separately according to their genotypes, as presented in **Table 3**.

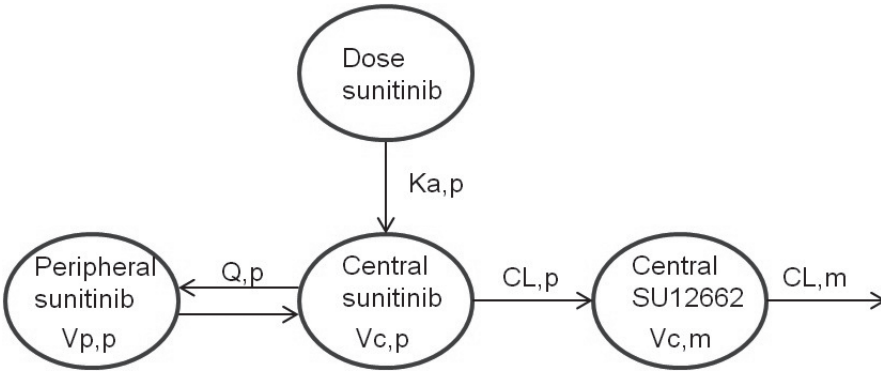

Figure 3 Pharmacokinetic model for sunitinib and SU12662. CLm, apparent clearance of SU12662; CLp, apparent clearance of sunitinib; Ka,p, absorption rate constant of sunitinib; *Q*p, apparent intercompartmental flow of sunitinib; Vc,p, apparent central volume of distribution of sunitinib; Vc,m, apparent volume of distribution of SU12662; Vp,p, apparent peripheral volume of distribution of sunitinib.[22]

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SUPPLEMENTARY MATERIAL

Supplementary table S1 Time points of blood sampling for included PK studies

Study acronym[REF]	Clinical trial registration number	Time points
NIB[20]	-	0 and one random sampling time point after start sunitinib
M10PKS[21]	NCT01286896	0 and 24 hour(s)
Clearsun[22]	NCT01098903	0, 4, 8, and 24 hour(s)
Chrono[23]	NTR3526	0, 1, 2, 4, 6, 8, 12 and 24 hour(s)
Phenotyping study[24]	NCT01743300	0, 10, 30, 40 minutes and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 hour(s)

Supplementary Table S2a 14 selected SNPs related to sunitinib pharmacokinetics

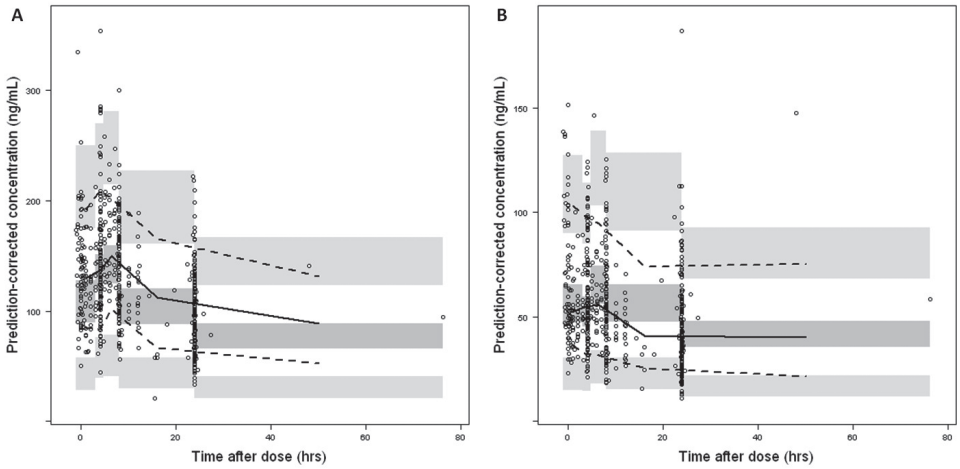
gene	rs number	Variant allele	P value	Associated effect	references
CYP1A1	rs1048943	G	0.029	increased risk for leukopenia and mucosal inflammation	van Erp NP *et al.*, J Clin Oncol, 2009[1]
*CYP3A4*22*	rs35599367	T	0.018	decreased CYP3A4 activity; 33% lower dose requirements for carriers of the T allele than for CC patients. The calculated dose-adjusted C0 was lower for CC than for CT/TT patients.	Elens L *et al.*, Clin Chem, 2011[12]
*CYP3A5*3*	rs776746	A	0.022 0.032	dose reductions due to toxicity [1] improved PFS [3]	Garcia-Donas J *et al.*, Lancet Oncol, 2011[2] van der Veldt AA *et al.*, Clin Cancer Res, 2011[3] van Erp NP *et al.*, J Clin Oncol, 2009[1] de Jonge H *et al.*, Pharmacogenomics, 2011[15]
ABCB1	rs1128503	T	0.09	less hypertension	
ABCB1	rs2032582	T	0.17 0.089 0.055	presence of T-allele: protective for hypertension worse PFS worse OS	
ABCB1	rs1045642	C	0.22	less hypertension (*P*=0.22)	
ABCG2	rs2231142	A	0.088	PFS	Garcia-Donas J *et al.*, Lancet Oncol, 2011[2] van Erp NP *et al.*, J Clin Oncol, 2009[1]
ABCG2	rs55930652	T	0.016	copy of TT: any toxicity higher than grade 2	
ABCG2	rs2622604	T			
NR1I2	rs3814055	T	0.03	response reduction	van Erp NP *et al.*, J Clin Oncol, 2009[1] Xu CF *et al.*, J Clin Oncol, 2011[18]
NR1I3	rs2307424	T	0.017 and 0.041	*P*=0.017 for haploblock; absence of CAT *P*=0.041 for haploblock; absence of CAG	van Erp NP *et al.*, J Clin Oncol, 2009[1] van der Veldt AA *et al.*, Clin Cancer Res, 2011[3]
NR1I3	rs2307418	C			
NR1I3	rs4073054	G			
*POR*28*	rs1057868	T	0.04	increase in CYP3A activity	de Jonge H *et al.*, Pharmacogenomics, 2011[15] Oneda B *et al.*, Pharmacogenet Genomics, 2009[19]

Supplementary Table S2b selected haplotypes related to sunitinib pharmacokinetics

gene	rs number	SNP	*P* value	Genotype and predicted functional change	references
ABCB1	1045642	C/T	0.035 (TTT) 0.033 (TCG copy)	presence of **TTT**: increased prevalence of hand-foot-syndrome **TCG copy:** improved PFS	van Erp NP *et al.*, J Clin Oncol, 2009[1] van der Veldt AA *et al.*, Clin Cancer Res, 2011[3]
	1128503	C/T			
	2032582	G/T			
ABCG2	rs55930652	C/T	0.016	copy of **TT**: any toxicity higher than grade 2	van Erp NP *et al.*, J Clin Oncol, 2009[1]
	2622604	C/T			
NR1I3	2307424	C/T	0.017 0.041	Absence of **CAG copy:** leukopenia absence of **CAT copy:** improved PFS	van Erp NP *et al.*, J Clin Oncol, 2009[1] van der Veldt AA *et al.*, Clin Cancer Res, 2011[3]
	2307418	A/C			
	4073054	T/G			

SNPs rs1128503, rs2032582 and rs1045642 in the *ABCB1* gene and rs2231142 in *ABCG2* show an association of $P > 0.05$, but were integrated in haplotypes having an association outcome of $P < 0.05$ and therefore included in analysis.

PFS = progression free survival.

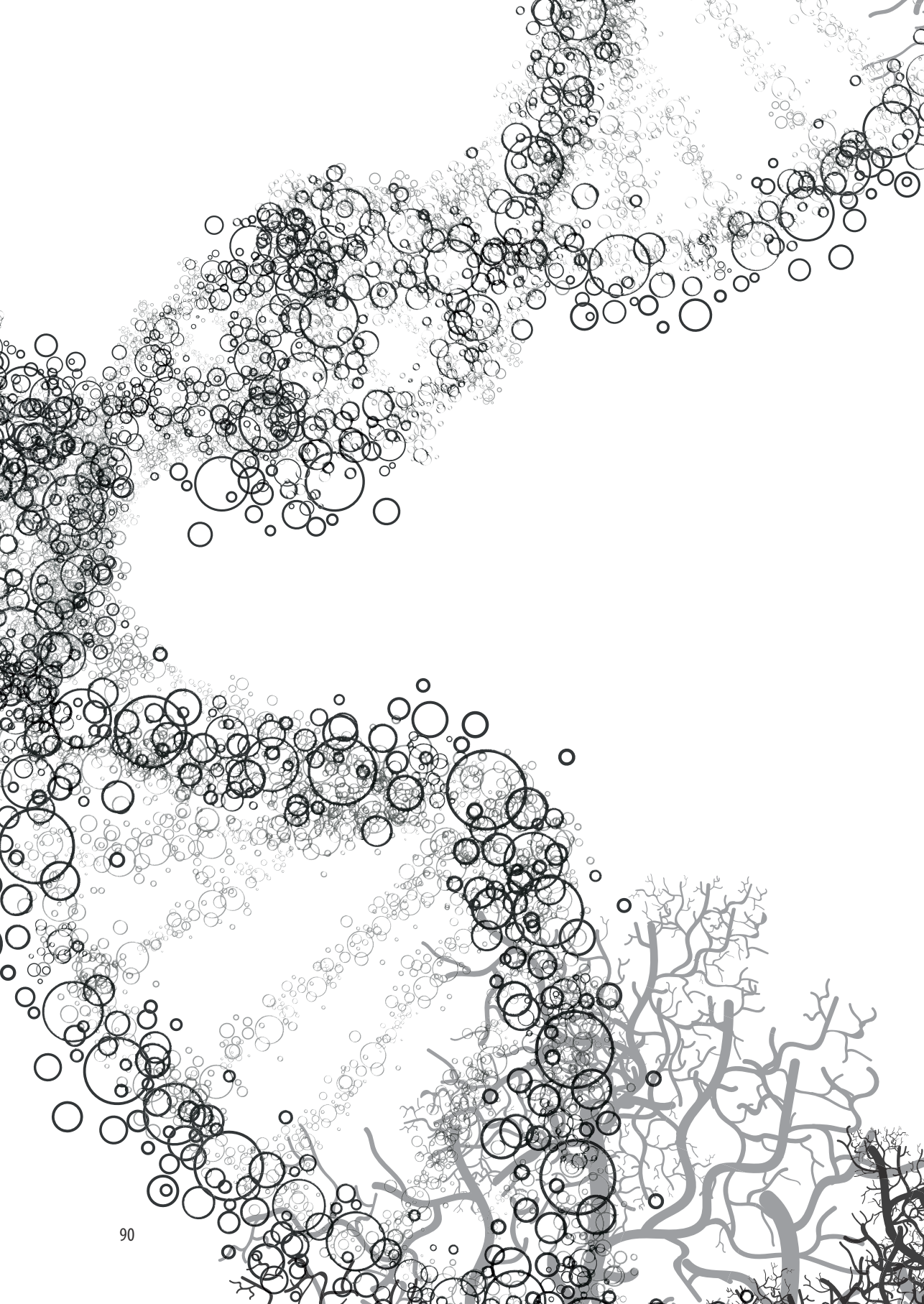


Supplementary Figure S1 Prediction-corrected visual predictive check of sunitinib (A) and SU12662 (B) concentration. Solid lines and dark grey areas represent the median observed values and predicted 90% confidence intervals (CIs). Dashed lines and light grey areas represent the 10% and 90% percentile s of the observed values and 90% CIs of the model predicted percentiles.[22]

Supplementary Table S3 SNPs incorporated in haplotypes associated with functional changes

gene	rs number	genotype	N[1]	%	MAF (%)	HWE (*P*-value[1])
CYP1A1	rs1048943	AA (wt)	104	92.0	G=4.4%	0.08
		AG	8	7.1		
		GG	1	0.9		
CYP3A4	rs35599367	CC (wt)	99	88.4	T=6.7%	0.02
		CT	11	9.8		
		TT	2	1.8		
CYP3A5	rs776746	GG (wt)	92	82.9	A=9.5%	0.27
		AG	17	15.3		
		AA	2	1.8		
ABCB1	rs1128503	CC (wt)	37	32.5	T=44.7%	0.41
		TC	52	45.6		
		TT	25	21.9		
ABCB1	rs2032582	GG (wt)	40	36.0	T=45.5%	0.01
		GT	41	36.9		
		TT	30	27.0		
ABCB1	rs1045642	TT (wt)	37	32.5	C=45.2%	0.30
		TC	51	44.7		
		CC	26	22.8		
ABCG2	rs2231142	CC (wt)	83	75.5	A=13.6%	0.44
		AC	24	21.8		
		AA	3	2.7		
ABCG2	rs55930652	CC (wt)	52	46.0	T=31.0%	0.42
		CT	52	46.0		
		TT	9	8.0		
ABCG2	rs2622604	CC (wt)	56	50.9	T=27.7%	0.49
		CT	47	42.7		
		TT	7	6.4		
NR1I2	rs3814055	CC (wt)	40	35.1	T=40.4%	0.82
		CT	56	49.1		
		TT	18	15.8		
NR1I3	rs2307424	CC (wt)	43	38.1	T=37.6%	0.69
		TC	55	48.7		
		TT	15	13.3		
NR1I3	rs2307418	AA (wt)	82	72.6	C=14.6%	0.75
		CA	29	25.7		
		CC	2	1.8		
NR1I3	rs4073054	TT (wt)	49	43.8	G=35.3%	0.39
		TG	47	42.0		
		GG	16	14.3		
*POR*28*	rs1057868	CC (wt)	53	46.9	T=30.1%	0.32
		CT	52	46.0		
		TT	8	7.1		

[1]*P*-value for the test for HWE. HWE: Hardy Weinberg Equilibrium.



CHAPTER 4

CYP3A5 and *ABCB1* polymorphisms as predictors for sunitinib outcome in metastatic renal cell carcinoma

Diekstra MH, Swen JJ, Boven E, Castellano D, Gelderblom H, Mathijssen RHJ, Rodríguez-Antona C, García-Donas J, Rini BI, Guchelaar H-J.

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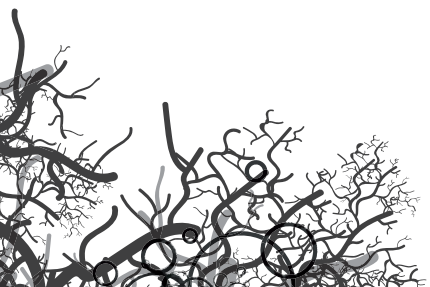

ABSTRACT

Background In our exploratory studies, we associated single nucleotide polymorphisms (SNPs) in candidate genes with the efficacy and toxicities of sunitinib in metastatic renal cell carcinoma (mRCC).

Objective To see whether previously reported associations of SNPs with sunitinib-induced toxicities and efficacy in mRCC can be confirmed in a large cohort of patients.

Design, setting, and participants The mRCC patients treated with sunitinib and a DNA sample available were pooled from three exploratory studies conducted in the United States, Spain, and the Netherlands. A total of 22 SNPs and 6 haplotypes in 10 candidate genes related to the pharmacokinetics and pharmacodynamics of sunitinib were selected for association testing.

Outcome measurements and statistical analysis SNPs and haplotypes were tested for associations with toxicity, dose reductions, progression-free survival (PFS), overall survival (OS), and best objective response.

Results and limitations A total of 333 patients were included. We confirmed 2 of the 22 previously reported SNP associations. The presence of *CYP3A5*1* was associated with dose reductions (odds ratio:2.0; 95% confidence interval [CI], 1.0-4.0, P=0.039). The presence of CGT in the *ABCB1* haplotype was associated with an increased PFS (hazard ratio: 1.9; 95% CI, 1.3-2.6; P<0.001) and remained significant after Bonferroni correction. These associations are consistent with prior observations.

Conclusions The confirmation of previously reported associations between polymorphisms in *CYP3A5* and *ABCB1* with sunitinib toxicity and efficacy, respectively, indicates that genotyping of these genetic variants will be useful for guiding sunitinib treatment. A prospective validation study is needed to confirm our findings on *ABCB1* and *CYP3A5* genetic polymorphisms.

Patient summary We confirmed that variants in genes involved in processing sunitinib through the body have an effect on sunitinib treatment outcome. These findings confirm the potential of testing for these genetic variants to improve individual patient care for patients with metastatic renal cell carcinoma treated with sunitinib.

INTRODUCTION

Sunitinib, a multitargeted tyrosine kinase inhibitor (TKI), is used as first- or second-line treatment of metastatic renal cell carcinoma (mRCC).[1-6] The drug is characterised by a wide interindividual variability in both pharmacokinetics and pharmacodynamics that are influenced by comedication, age, gender, and environmental factors. Increased sunitinib exposure results in an increase in time to tumour progression and overall survival (OS) accompanied by an increased risk of mild to moderate toxicities.[7] Dose reductions are needed in 32% of mRCC patients and may result in suboptimal therapeutic sunitinib levels.[8,9] Sunitinib-induced hypertension is associated with an improved clinical outcome.[10]

Exploratory analyses have reported statistically significant associations between single nucleotide polymorphisms (SNPs) in genes related to the pharmacokinetics and pharmacodynamics of sunitinib and the toxicity and efficacy of sunitinib in mRCC.[9,11-15] SNPs in *CYP1A1*, *ABCB1*, *ABCG2*, *VEGFR2* (synonym for *KDR*), *FLT3*, and *NR1I3* have been associated with an increased risk of leukopenia, mucosal inflammation, hand-foot syndrome (HFS), or the occurrence of any grade 3 or 4 toxicity.[9] The A allele in *CYP3A5* rs776746 (*CYP3A5*1*) has been associated with the need for more dose reductions of sunitinib due to toxicity.[12] Kim *et al.* reported that SNPs in *VEGF* and *VEGFR2* were associated with the prevalence and duration of sunitinib-induced hypertension.[14] Polymorphisms in *eNOS* (synonym for *NOS3*) and *VEGFA* were associated with grade 3 hypertension during the first cycle of sunitinib treatment.[15] For efficacy, SNPs in *VEGFR3* (synonym for *FLT4*) were associated with reduced progression-free survival (PFS)[12], and genetic polymorphisms in *NR1I3*, *ABCB1*, and *CYP3A5* were associated with an increased PFS.[13]

The SNP genotypes and haplotypes just listed are potential predictive biomarkers for sunitinib treatment outcome in mRCC patients and may enable individualised treatment. However, the exploratory studies applied fairly liberal statistical thresholds and had relatively small patient cohorts of 63-255 patients[9,12-15], increasing the risk for false-positive findings. Moreover, the selected SNP panels vary among studies. The objective of the present study is to see whether previously reported associations of SNPs with sunitinib-induced toxicities and efficacy in mRCC can be confirmed in a large cohort of patients.

METHODS

Study population

The mRCC patients treated with sunitinib were merged from three exploratory studies that were described in detail.[9,12-15] In brief, patients diagnosed with mRCC receiving 50.0 mg, 37.5 mg, or 25.0 mg sunitinib for at least 4 wk in a 4-wk-on/2-wk-off schedule or continuous dosing regimen were enrolled between 2004 and 2010 from the centres participating in the Dutch SUTOX consortium (five medical centres in the Netherlands), Spanish Oncology Genitourinary Group (SOGUG) medical centres (15 Spanish participating hospitals), and the Cleveland Clinic Foundation (CCF) Taussig Cancer Institute in the United States (**Figure 1**).[9,12-15] Patients with a non-clear cell histologic subtype of RCC were excluded. From each patient a blood sample had to be available. SUTOX samples were anonymised by a third party, according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org). The medical ethical review boards of all participating centres approved the study.[9,12-15] Patients gave written informed consent.

Single nucleotide polymorphism selection and genotyping

Germline DNA was isolated from whole blood, serum, plasma, or peripheral blood mononuclear cell samples. Isolated DNA was genotyped for 22 SNPs and 6 haplotypes in genes *CYP1A1*, *CYP3A5*, *ABCB1*, *ABCG2*, *NR1I3*, *VEGFA*, *eNOS*, *VEGFR2*, *VEGFR3*, and *FLT3* (**Supplementary Table 1**).[9,12-15] Samples with a call rate <80% were excluded from the analysis. All SNPs achieved a SNP call rate >95%. Minor deviations from the Hardy-Weinberg equilibrium were observed for SNPs rs776746 in *CYP3A5* (P=0.01) and rs1045642 in *ABCB1* (P=0.041). A 98% concordance in SNP genotypes was observed between the Leiden University Medical Centre and the Spanish National Cancer Research Centre samples. A more detailed description of methods for SNP selection, genotyping, and quality control are described in **Supplement 1**.[9,12-17]

Study design

Toxicity and efficacy data for the present study were obtained by a retrospective review of the medical record of each patient and scored according to the protocol of the current study. Toxicity was assessed at baseline, week 4, and week 6 of each treatment cycle for the first four cycles. Toxicities were scored according to the National Cancer Institute-Common Terminology Criteria for Adverse Events v.3.0 or v.4.0. Toxicities of grade ≥3 were considered clinically relevant. Tested toxicities were thrombocytopenia, leukopenia, mucosal inflammation, HFS, hypertension, and any toxicity grade >2 in agreement with exploratory studies.[9,10,12,14,15] Data on dose reductions were also documented for the first four cycles of sunitinib treatment.

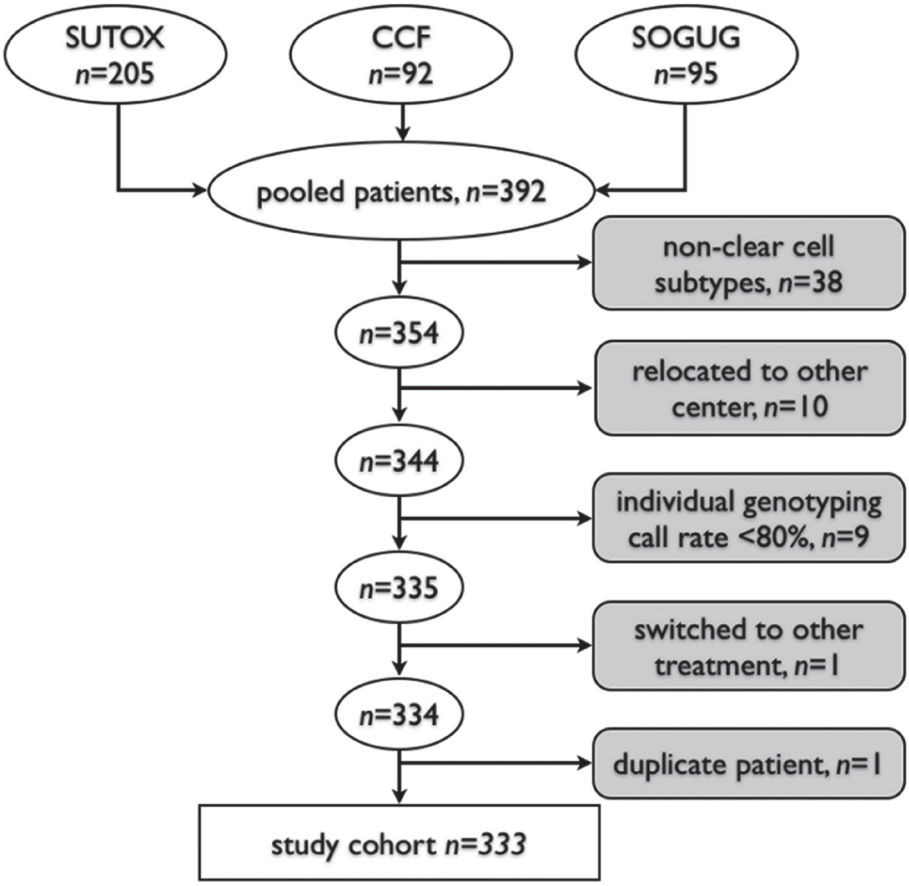

Figure 1 Patient flowchart on included patients. Fifty-nine patients had to be excluded from association analyses because of non-clear cell subtypes (N=38), relocation to another medical centre during follow-up (N=10), individual genotyping call rates <80% (N=9), double patient (N=1), or a change to another treatment than sunitinib directly after enrolment (N=1). A total of 333 sunitinib-treated metastatic renal cell carcinoma patients were available for analysis of toxicity and survival in the present study. CCF=Cleveland Clinic Foundation; SOGUG=Spanish Oncology Genitourinary Group; SUTOX=Dutch SUTOX consortium.

The primary outcome measure for efficacy analysis was PFS, defined as the time in months between the first day of sunitinib treatment and the date of progressive disease (PD) according to Response Evaluation Criteria in Solid Tumours (RECIST) v.1.0. If a patient had not progressed, PFS was censored at the time of the last follow-up. In addition, associations between SNPs and OS were tested. OS was defined as the time in months between the first day of sunitinib treatment and the date of death or the date at which patients were last known to be alive.

Statistical analysis

For toxicity analysis, baseline corrected toxicity scores were calculated by subtracting baseline values from the maximum recorded score in four cycles of treatment, and each toxicity end point was dichotomised as grade 0-2 versus grade 3-4. The end-point dose reduction was dichotomised as any dose reduction within cycle 1-4 or no dose reduction. Best response was dichotomised as PD versus other response (ie, stable disease, partial response, or complete response). SNPs and haplotypes, together with clinical covariates, were tested for associations with toxicities and dose reductions using an independent samples *t* test, chi-square test, or a Mann-Whitney *U* test depending on the type of data. Clinical variables and SNPs or haplotypes with $P \leq 0.1$ were included as covariates in the multivariate analysis. All SNPs and haplotypes were tested separately with adjustment for covariates using binary logistic regression analysis on the best fitting genetic model based on genotype distributions (additive, dominant, recessive, or general model). Association analysis on best response was performed using the covariates gender, Heng risk group, and study centre because these were $P \leq 0.1$ in the univariate analysis.

For survival analysis, SNPs and haplotypes were tested univariately using Kaplan-Meier survival analysis with the log-rank test. SNPs and haplotypes with $P \leq 0.1$ were included in the multivariate analysis. Dose reduction and prior treatment were tested for association with PFS and OS but not included in the multivariate analysis because of observed *P* values >0.1. Patients were assigned a prognostic risk group (favourable, intermediate, or poor) according to the Heng risk criteria.[18,19] SNPs and haplotypes together with Heng risk group and study centre were included as covariates in the Cox regression analysis. In addition, combined genotypes CC and GG in *VEGFA* rs3025039 and *VEGFR2* rs2305948 were tested for an association with OS.[14]

For multivariate analyses, associations with $P \leq 0.05$ were considered significant. Statistical analyses were performed using SPSS Statistics for Windows, v.20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Population characteristics

Data from 333 mRCC patients were available (**Figure 1**). **Table 1** presents the patient characteristics. Most of the patients had no toxicities at baseline (96-99% for the tested toxicities). Baseline grade 1-3 toxicities were observed for 0.3-3.9% of patients, and no baseline grade 4 toxicities were observed (**Table 2**). Dose reductions within cycles 1-4 of sunitinib were observed for 35% of the study population. For the 115 subjects who received a dose reduction, the reason for dose reduction was unknown for 54 subjects (47%) because of unavailable data. The remaining 61 patients (53%) received a dose reduction because of toxicity of grade 1 or 2 (N=18), grade 3 (N=39), or grade 4 (N=4). The distribution of response according to RECIST within cycles 1-4 is presented in **Table 1**. Overall, median follow-up times for PFS and OS analysis were 43 and 49 mo, respectively. Median PFS and OS of patients were 16 and 26 mo, respectively.

Table 1: Characteristics of metastatic clear cell renal cell carcinoma patients treated with sunitinib (N=333)

Characteristic	Value (quartiles: 25 and 75 percentiles)	%
Gender		
male	228	69
female	105	31
Age at start sunitinib (years)	61 (55, 69)	
BSA	1.97 (1.82, 2.13)	
Prior nephrectomy		
yes	281	84
no	48	14
unknown	4	1
WHO performance status		
0	146	44
1	156	47
2	23	7
3	1	0
unknown	7	2
Ethnicity		
Caucasian	321	96
Black	5	2
Asian	3	1
Latin-American	1	0
Arabian	3	1

Table 1: Continued

Characteristic	Value (quartiles: 25 and 75 percentiles)	%
Number of metastatic sites		
1	91	27
2	133	40
≥3	104	31
unknown	5	2
Metastatic sites		
Lung	228	70
Liver	62	19
Bone	95	29
Lymph nodes	152	46
Brain	19	6
kidney	77	23
Heng risk group*		
Good (0 risk factors)	67	20
Intermediate (1-2 risk factors)	176	53
Poor (3-6 risk factors)	90	27
Prior systemic antitumor treatment		
Yes	82	25
No	251	75
Duration of first prior treatment episode (months)**	6 (3, 11.5)	
Sunitinib, daily dose (mg) in first 4 cycles		
50 mg	315	95
37.5 mg	14	4
25 mg	4	1
Best response to sunitinib		
Progressive Disease	43	13
Stable Disease	129	39
Partial Response	130	39
Complete Response	11	3
unknown	20	6
Dose reduction after cycle 1, 2 or 3		
yes		
men	68	
women	47	
total	115	35
no		
men	153	
women	54	
total	207	62
unknown	11	3

Table 1: Continued

Characteristic	Value (quartiles: 25 and 75 percentiles)	%
Baseline chemistry and hematology		
SBP (mmHg)	138.5 (125.25, 150)	
DBP (mmHg)	80 (71, 88)	
LDH (units/L)	237 (170, 337)	
creatinine (µmol/L)	106.08 (88.4, 123.2)	
bilirubin (µmol/L)	6.84 (5.13, 9.00)	
albumin (g/L)	42 (38, 44.35)	
calcium (mmol/L)	2.38 (2.32, 2.48)	
AST (units/L)	20 (16, 27)	
ALT (units/L)	18 (13, 27)	
hemoglobin (mmol/L)	8 (7.3, 8.94)	
leukocytes ($x10^9$)	7.60 (6.46, 9.49)	
neutrophils ($x10^9$)	4.90 (3.83, 6.53)	
thrombocytes ($x10^9$)	264 (210, 353)	
MCV (fL)	88 (83, 91)	

ALT=alanine transaminase; AST=aspartate transaminase; BSA=body surface area; DBP=diastolic blood pressure; LDH=lactate dehydrogenase; MCV=mean corpuscular volume; SBP=systolic blood pressure; WHO=World Health Organisation. Values are presented as median unless otherwise indicated.

*Patients are grouped according to their Heng risk group based on the six Heng risk scores: deteriorated WHO performance status (≥2), low haemoglobin (lower than the lower limit of normal), high calcium (>2.5 mmol/l), time from initial diagnosis to treatment with sunitinib (<1 yr), neutrophil count (higher than the upper limit of normal), and thrombocytes (higher than the upper limit of normal).[18,19]

**Duration of first prior treatment episode refers to the number of months a patient has received any systemic anticancer treatment prior to start with sunitinib. In a case in which a patient had received more systemic antitumour treatments, only the duration of the first treatment was recorded.

Table 2: Distribution of delta toxicities within 4 cycles of sunitinib treatment

Type of toxicity	Toxicity grade	Number of patients (N)	Percentage of patients (%)
Thrombocytopenia	none	129	39
	grade 1	134	40
	grade 2	43	13
	grade 3	23	7
	grade 4	4	1
Leukopenia	none	169	51
	grade 1	95	28
	grade 2	60	18
	grade 3	9	3
Mucosal inflammation	none	144	43
	grade 1	107	32
	grade 2	67	20
	grade 3	15	5
Hand-foot-syndrome*	none	199	60
	grade 1	62	19
	grade 2	51	15
	grade 3	21	6
Any toxicity >grade 2	none	41	12
	grade 1	87	26
	grade 2	121	36
	grade 3	75	23
	grade 4	9	3
Hypertension grades	none	209	63
	grade 1	34	10
	grade 2	48	14
	grade 3	42	13

Baseline corrected toxicity scores were calculated by subtracting baseline values from the maximum recorded score in 4 cycles of treatment. In case the grade of toxicity was not recorded in the medical record of the patient, it was assumed that no toxicity had occurred (grade 0).
*For hand-foot syndrome, grade 3 is the highest possible grade according to the Common Terminology Criteria for Adverse Events v.4.0.

Single nucleotide polymorphism association results

All tested associations are presented in **Table 3** that include associations not previously reported. Two of the exploratory associations between individual SNPs and toxicities or efficacy were confirmed in the present study (**Table 4**). *CYP3A5*1* was associated with dose reductions (odds ratio [OR]:2.0; 95% confidence interval [CI], 1.0-4.0; *P*=0.039). PFS was improved in the case of the presence of CGT in the *ABCB1* haplotype (hazard ratio:1.9; 95% CI, 1.3-2.6; *P*<0.001), and the *P* value remained significant after Bonferroni correction (**Table 4**; **Figure 2**). Two of our current findings showed the same direction of effect and a similar effect size as observed in earlier exploratory studies but failed to meet the threshold for statistical significance in the present study. Presence of the G allele in *VEGFA* rs1570360 was associated with an increased risk for hypertension (OR:1.9; 95% CI, 0.8-4.5; *P*=0.17), and the T allele of *FLT3* rs1933437 was associated with an increased risk for leukopenia (OR:3.6; 95% CI, 0.8-15.6; *P*=0.088) (**Table 4**).

Table 3: Univariate and multivariate analyses of SNPs, haplotypes and other covariates on end-points of toxicity, dose reductions, response and survival during sunitinib treatment in mRCC.

Tested outcome	SNP or patient characteristic	Univariate analyses (*P*-value)	Multivariate analyses		
			P-value	OR/ HR	CI 95%
Thrombocytopenia	Age on start sunitinib	0.02	0.051	1.05	1.00-1.10
	Gender	0.05	0.6	1.28	0.48-3.38
	BSA	0.09	0.02	0.07	0.01-0.59
	SOGUG vs other centers		0.08	4.13	0.84-20.3
	CCF vs other centers		0.1	0.47	0.19-1.17
	rs1048943 in *CYP1A1*	0.07	0.1	3.04	0.72-12.7
	rs307826 in *VEGFR3*	0.07	0.2	1.76	0.69-4.44
Leukopenia	Age on start sunitinib	0.08	0.2	1.06	0.98-1.14
	BSA	0.04	>0.9	0.91	0.04-21.1
	rs2307418 in *NR1I3*	There are no patients with grade 3 or 4 leukopenia and the CA or CC genotype.			
	rs3025039 in *VEGFA*	0.07	**0.024**	5.42	1.25-23.5
	rs1933437 in *FLT3*	0.06	0.088	0.28	0.06-1.21
mucosal inflammation	Creatinine at baseline	0.03	0.3	1.01	0.99-1.03
	MCV at baseline	0.07	0.3	0.94	0.85-1.05
	SOGUG vs other centers		0.06	0.21	0.04-1.09
	CCF vs other centers	There are no patients from CCF having grade 3 or 4 mucosal inflammation.			
	rs1128503 in *ABCB1*	0.09	**0.028**	0.19	0.04-0.83
	rs2032582 in *ABCB1*	0.04	**0.048**	0.22	0.05-0.98
	rs2307418 in *NR1I3*	0.002	**0.013**	8.09	1.55-42.3
	rs1570360 in *VEGFA*	0.07	0.1	3.66	0.71-18.8

Table 3: Continued

Tested outcome	SNP or patient characteristic	Univariate analyses (*P*-value)	Multivariate analyses		
			P-value	OR/ HR	CI 95%
hand-foot syndrome	Age on start sunitinib	0.02	0.095	0.95	0.90-1.01
	Albumin at baseline	0.002	0.072	1.15	0.99-1.35
	Hb at baseline	0.09	0.5	1.20	0.72-2.02
	Leukocytes at baseline	0.014	0.2	0.83	0.64-1.08
	LDH at baseline	0.06	0.4	1.00	0.99-1.00
	rs2305948 in *VEGFR2*	0.03	**0.032**	2.84	1.09-7.38
	rs1933437 in *FLT3*	0.05	**0.037**	5.33	1.10-25.79
Tox > grade 2	Age on start sunitinib	0.03	0.077	1.03	1.00-1.07
	Gender	0.00	0.3	0.70	0.36-1.36
	Bilirubin at baseline	0.04	0.6	0.98	0.91-1.05
	Albumin at baseline	0.05	0.6	1.02	0.94-1.11
	Hb at baseline	0.00	0.015	0.65	0.46-0.92
	MCV at baseline	0.004	0.4	0.98	0.92-1.03
	rs1048943 in *CYP1A1*	0.06	**0.043**	3.65	1.04-12.8
	rs1045642 in *ABCB1*	0.04	0.4	1.43	0.65-3.15
	rs2307424 in *NR1I3*	0.02	**0.006**	0.46	0.27-0.80
	rs2010963 in *VEGFA*	0.08	0.3	1.28	0.81-2.04
	rs1570360 in *VEGFA*	0.05	0.093	0.65	0.39-1.08
	rs1933437 in *FLT3*	0.098	**0.037**	3.36	1.08-10.5
	haplotype *ABCB1* (rs1128503, rs2032582, rs1045642). Other-other vs Other-CGT + CGT-CGT	0.005	0.069	2.04	0.95-4.40
	haplotype *NR1I3* (rs2307424, rs2307418, rs4073054). Other-other (0) vs Other-CAT + CAT-CAT (1)	0.06	**0.045**	0.60	0.36-0.99
hypertension grades	BSA	0.011	0.1	3.92	0.71-21.6
	Albumin at baseline	0.001	0.001	1.30	1.12-1.51
	ALT at baseline	0.005	0.01	1.03	1.01-1.05
	Hb at baseline	0.08	0.2	0.77	0.50-1.18
	Leukocytes at baseline	0.04	0.4	0.95	0.84-1.08
	MCV at baseline	0.09	0.2	1.05	0.97-1.13
	SOGUG vs other centers		0.02	14.0	1.61-122.4
	rs776746 in *CYP3A5*	0.02	**0.009**	4.70	1.47-15.0
	rs2231142 in *ABCG2*	0.05	**0.040**	0.03	0.001-0.85
	rs55930652 in *ABCG2*	0.04	0.09	0.45	0.18-1.13
	rs2622604 in *ABCG2*	0.06	0.17	0.51	0.20-1.34
	rs1570360 in *VEGFA*	0.007	0.09	6.56	0.74-57.90
	rs3025039 in *VEGFA*	0.02	0.3	1.58	0.62-4.03
	haplotype *ABCG2* (rs55930652, rs2622604). Other-other vs TT-other+TT-TT	0.05	0.11	0.47	0.18-1.20

Table 3: Continued

Tested outcome	SNP or patient characteristic	Univariate analyses (*P*-value)	Multivariate analyses		
			P-value	OR/ HR	CI 95%
Dose reduction after cycle 1,2 or 3	Gender	0.006	0.13	1.59	0.88-2.87
	Hb at baseline	0.07	0.009	0.74	0.59-0.93
	SBP at baseline	0.095	0.13	0.99	0.97-1.00
	CYP3A5 rs776746	0.006	**0.039**	2.04	1.04-4.00
	ABCG2 rs2231142	0.07	**0.022**	0.36	0.15-0.86
	VEGFA rs699947	0.06	0.051	0.53	0.28-1.00
	VEGFA rs1570360	0.07	0.18	2.26	0.69-7.44
	NOS3 rs2070744	0.098	**0.014**	0.36	0.16-0.81
	haplotype *VEGFA* Other-other vs other-ACG + ACG-ACG	0.07	0.076	0.56	0.29-1.06
Response (PD vs other)	Gender	0.04	0.12	0.58	0.29-1.16
	Heng favorable risk (0)	0.00	0.004		
	Heng intermediate risk (1-2)		0.004	9.39	2.09-42.2
	Heng poor risk (3-6)		0.023	2.26	1.12-4.55
	CCF vs other centers		0.053	0.34	0.12-1.01
	rs55930652 in *ABCG2*	0.02	**0.045**	0.61	0.38-0.99
	rs2622604 in *ABCG2*	0.02	0.067	0.62	0.37-1.04
	rs833061 in *VEGFA*	0.087	0.086	0.44	0.17-1.12
	rs1933437 in *FLT3*	0.01	**0.008**	0.51	0.32-0.84
	haplotype *ABCB1 CGT* Other-other vs Other-CGT + CGT-CGT	0.077	**0.044**	2.83	1.03-7.79
	haplotype *ABCG2* Other-other > other-TT > TT-TT	0.03	0.069	0.62	0.37-1.04
	haplotype *NR1I3 CAT* Other-other(1) vs Other-CAT+ CAT-CAT (0)	0.01	**0.008**	3.79	1.41-10.2
PFS	Heng favorable risk (0)	0.00	<0.001		
	Heng intermediate risk (1-2)		<0.001	0.43	0.29-0.63
	Heng poor risk (3-6)		<0.001	0.53	0.40-0.72
	SOGUG vs other centers	0.04	0.3	0.84	0.62-1.14
	CCF vs other centers	0.002	0.016	1.52	1.08-2.13
	rs1128503 in *ABCB1*	0.02	**0.016**	1.42	1.07-1.90
	rs2032582 in *ABCB1*	0.05	**0.032**	1.36	1.03-1.81
	rs2622604 in *ABCG2*	0.04	0.082	1.26	0.97-1.65
	rs2070744 in *NOS3*	0.06	0.12	0.81	0.61-1.06
	rs1870377 in *VEGFR2*	0.04	**0.058**	0.59	0.35-1.02
	haplotype *ABCB1* (rs1128503, rs2032582, rs1045642). Other-other vs Other-CGT + CGT-CGT	0.002	**<0.001**	0.54	0.38-0.75
	haplotype *ABCG2* (rs55930652, rs2622604). Other-other vs Other-TT + TT-TT	0.042	0.074	1.28	0.98-1.67

Table 3: Continued

Tested outcome	SNP or patient characteristic	Univariate analyses (*P*-value)	Multivariate analyses		
			P-value	OR/ HR	CI 95%
OS	Heng favorable risk (0)	0.00	0.00		
	Heng intermediate risk (1-2)		0.00	0.32	0.21-0.49
	Heng poor risk (3-6)		<0.001	0.53	0.39-0.71
	SUTOX vs other centers	0.00	0.064	0.73	0.52-1.02
	CCF vs other centers	0.00	0.009	1.82	1.16-2.86
	rs1128503 in *ABCB1*	0.05	**0.021**	0.66	0.46-0.94
	rs2622604 in *ABCG2*	0.086	0.229	1.19	0.90-1.56
	rs1570360 in *VEGFA*	0.04	0.063	0.75	0.55-1.02
	rs1870377 in *VEGFR2*	0.089	**0.024**	0.50	0.28-0.92
	haplotype *ABCB1* (rs1128503, rs2032582, rs1045642). recessive: TTT-TTT vs Other-other + Other-TTT	0.06	**0.021**	0.63	0.43-0.93
	haplotype *ABCB1* (rs1128503, rs2032582, rs1045642). dominant: Other-other vs Other-CGT + CGT-CGT	0.05	**0.007**	0.63	0.45-0.88
	haplotype *ABCG2* (rs55930652, rs2622604). Other-other vs Other-TT + TT-TT	0.086	0.18	1.21	0.92-1.60

ALT=alanine transaminase; BSA=body surface area; CCF=Cleveland Clinic Foundation; CI=confidence interval; Hb=haemoglobin; HR=hazard ratio; LDH=lactate dehydrogenase; MCV=mean corpuscular volume; OR=odds ratio; OS=overall survival; PD=progressive disease; PFS=progression-free survival; SBP=systolic blood pressure; SNP=single nucleotide polymorphism; SOGUG=Spanish Oncology Genitourinary Group; SUTOX=Dutch SUTOX consortium. This table includes only SNPs, haplotypes, and other covariates with $P \leq 0.1$ in univariate analysis. Multivariate results with $P \leq 0.05$ are indicated in bold. Multivariate results are reported for a single base model including all patient characteristics as covariates without inclusion of SNPs. SNP results are presented for the singular SNP added to the base model of patient characteristics.

Table 4: **SNP association results from multivariate analysis.** This table presents an overview of single nucleotide polymorphisms (SNPs) and haplotypes in candidate genes related to the pharmacokinetics and pharmacodynamics of sunitinib tested for association with toxicity and efficacy of sunitinib in mRCC. Significant associated SNPs (P<0.05) from exploratory studies[9,12-15] are compared with the association results from the present study.

gene	rs number	Predicted functional change	Original association in exploratory studies				Results from present study		
			P-value	OR/HR	95% CI	ref	*P*-value	OR/HR	95% CI
CYP3A5	rs776746	A allele (*CYP3A5*1*) associated with dose reductions	0.0014	3.75	1.67-8.41	11	0.039	2.04	1.04-4.00
ABCB1	haplotype *ABCB1* (rs1128503, rs2032582, rs1045642)	CGT copy associated with increased PFS	0.033	1.92	1.05-3.45	12	<0.001	1.85	1.32-2.56
VEGFA	rs1570360	Increased risk for hypertension	0.035	2.04	1.05-3.96	11	0.17	1.86	0.76-4.52
FLT3	rs1933437	T allele associated with increased risk for leukopenia	0.008	2.78	1.30-5.88	9	0.088	3.57	0.83-15.6

CI=confidence interval; HR=hazard ratio; OR=odds ratio; OS=overall survival; PFS=progression-free survival. This table presents an overview of single nucleotide polymorphisms (SNPs) and haplotypes in candidate genes related to the pharmacokinetics and pharmacodynamics of sunitinib tested for association with toxicity and efficacy of sunitinib in metastatic renal cell carcinoma. Significantly associated SNPs (P<0.05) from exploratory studies[9,12-15] are compared with the association results from the present study.

DISCUSSION

Two of 22 previously reported pharmacogenetic biomarkers for efficacy or toxicity of sunitinib showed identical associations in a large cohort of 333 mRCC patients. *CYP3A5*1* is associated with dose reductions, and the presence of CGT in the *ABCB1* haplotype is associated with an improved PFS.[12,13] Compared with the exploratory studies, similar effect sizes were observed for the PFS increase in *ABCB1* CGT carriers, and a smaller effect size was observed for the chance of dose reductions in case of *CYP3A5*1* in the present study. Compared with the exploratory studies, smaller CIs were observed in the present study, and the association between *ABCB1* and improved PFS remains significant after

Bonferroni correction. These results indicate that genetic polymorphisms in the *CYP3A5* or the *ABCB1* gene play an important role in predicting the need for dose reductions and the length of PFS in individuals treated with sunitinib. Our observation that SNPs in *ABCB1* CGT are associated with sunitinib efficacy is supported by a recent study reporting that patients carrying the wild-type and heterozygote genotypes of rs2032582 and rs1128503 in *ABCB1* showed a decreased clearance of sunitinib and its active metabolite SU12662.[20] Another reinforcement of current findings in *ABCB1* was presented by Beuselinck *et al*., who reported an increased time-to-dose reduction for TT genotypes in rs2032582 and rs1128503.[21] For the same SNPs in *ABCB1*, Garcia-Donas *et al.* reported a protective effect for the T allele on hypertension.[12] These results could be explained by a dose-plasma concentration-effect relationship of sunitinib influenced by *ABCB1* genetic polymorphisms.[22] The studied variant genotypes in *ABCB1* result in an increased function of the ABCB1 efflux transporter leading to a decreased absorption or higher clearance of sunitinib and consequently a lower exposure to sunitinib, less hypertension, and a decrease in PFS in the absence of CGT or for TT variant homozygotes.[12,20,21] The *CYP3A5*1* allele results in CYP3A5 expression, and it was hypothesised by van der Veldt *et al.* that this may result in increased conversion from sunitinib to SU12662. This metabolite has a longer half-life than sunitinib resulting in increased exposure, and it could be responsible for any toxicity for which dose reduction is needed.[13,20] Our observation that *CYP3A5*1* is associated with more dose reductions agrees with this hypothesis and is supported by a recent study that reported a 22.5% increase in the clearance of sunitinib for *CYP3A5*1* carriers.[20] In contrast, Beuselinck *et al*. did not find any relation between *CYP3A5*1* and time-to-dose reduction.[21] This difference could be due to the time-to-event approach that is not the same end point as dose reductions or to the smaller number of subjects (96 patients) in their study.

The present study did not find any of the original exploratory association findings of SNPs in genes related to the pharmacodynamic pathway of sunitinib.[9,12-15] Scartozzi *et al*. reported significant associations of SNPs rs833061, rs699947, rs2010963, and rs6877011 in *VEGF* and *VEGFR-3* (*FLT4*) genes with PFS and OS on sunitinib.[23] Beuselinck *et al*. reported associations of the T allele in *VEGFR-3* rs307821 with a decrease in PFS and OS ($P<0.05$) compared with the GG genotype.[24] However, the studies of Scartozzi *et al*. and Beuselinck *et al*. only analysed 84 and 88 patients, respectively, which is rather limited for pharmacogenetic studies, and both studies emphasise the need for prospective validation of these findings in a larger patient cohort.[23,24] Moreover, the findings of Scartozzi *et al*. were based on genotyping results of tumour tissue instead of germline DNA, and expression of the *VEGF* gene is known to vary between tumour and normal tissue.[23] This could explain the lack of associations for SNPs in *VEGF* and *VEGFR3* in this large cohort of patients (N=333).

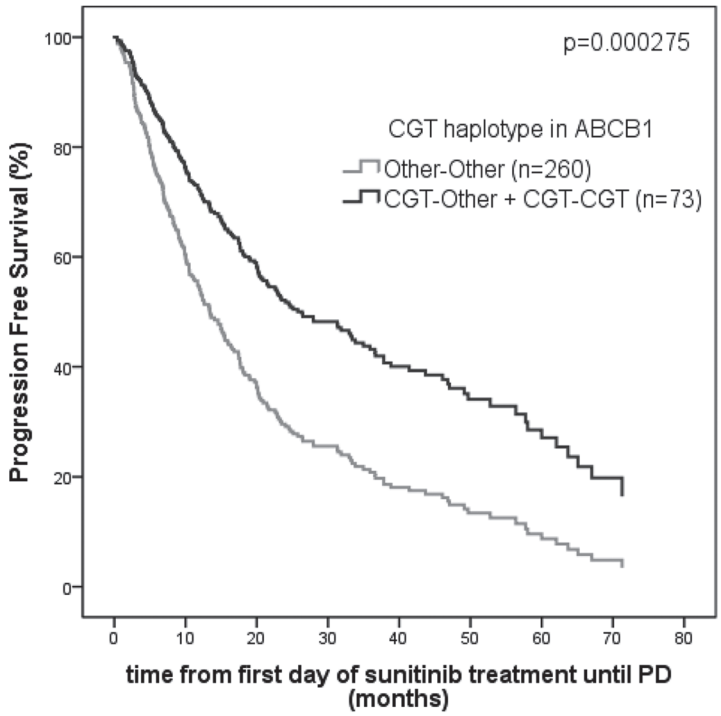

Figure 2 Multivariate Cox regression analysis for association of genetic polymorphisms in the *ABCB1* haplotype (rs1128503, rs2032582, and rs1045642) with progression-free survival in months, corrected for Heng risk group and study centre. PD=progressive disease.

Strengths of the current study are the large sample size for a pharmacogenomics study, the interrogation of significant SNPs from exploratory studies, and a technical validation on genotyping methods. In our analysis, toxicities were assessed during four cycles of sunitinib treatment, whereas most exploratory approaches only concerned one or two cycles.[9,13,15] The application of an extended time frame holds a small risk for bias of the number of sunitinib-induced toxicities by non-treatment-related effects such as withdrawal of patients due to early disease progression.

Survival analyses were adjusted for dose reductions by inclusion of a dichotomous covariate and regardless of starting dose. While this approach is valid, adjustment for the actual cumulative sunitinib dose would be preferable. Unfortunately, due to the retrospective character of the present study, available data were too sparse to calculate the cumulative dose accurately. It is conceivable that especially patients who are carriers

of a particular SNP genotype received dose reductions (eg, *CYP3A5*1*), thereby reducing sunitinib exposure. In this way, the potential effects of SNPs on survival outcomes could have been missed.

To date, most exploratory studies applied the candidate-gene approach limiting interrogated SNP panels to our current understanding of the biologic mechanisms of sunitinib. Consequently, relevant SNPs influencing sunitinib toxicity or efficacy may not yet have been identified. In contrast, a genomewide association study (GWAS) is an unrestricted hypothesis-free approach to find unbiased associations of SNPs with sunitinib treatment outcome.[25] Recently, a GWAS of sunitinib and pazopanib efficacy and toxicity in mRCC was reported. This study included 355 sunitinib-treated patients, and potential loci in *LOXL2*, *ENTPD4*, *IL2RA*, *LRRC2*, *ANAPC4*, and *SLC34A2* were identified.[26,27] However, no associations reached genomewide significance, and larger sample sizes are needed. A European collaborative project (EuroTARGET) was set up with the same objective and might provide additional information.[28]

CONCLUSIONS

Multiple targeted treatments are currently available for the treatment of mRCC in addition to sunitinib. Pharmacogenetic testing for *CYP3A5* and *ABCB1* may help make better informed decisions regarding TKI selection for individual patients and limit toxicity while increasing efficacy. A prospective validation study is needed to confirm our findings on *ABCB1* and *CYP3A5* genetic polymorphisms.

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SUPPLEMENTARY MATERIAL

Supplement 1 - Methods

SNP selection and genotyping

Twenty-two SNPs and six haplotypes in 10 genes (*CYP1A1*, *CYP3A5*, *ABCB1*, *ABCG2*, *NR1I3*, *VEGFA*, *eNOS*, *VEGFR2*, *VEGFR3* and *FLT3*) related to pharmacokinetics or pharmacodynamics of sunitinib were selected for this study because of reported associations ($P<0.05$) with toxicity, PFS or OS in previous exploratory studies (Supplementary Table 1).[9,12-15]

Genotyping

Germline DNA was isolated from whole blood, serum, plasma or peripheral blood mononuclear cell (PBMC) samples. SUTOX and CCF samples were genotyped at Leiden University Medical Center (LUMC) using Taqman probes (*Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands)* on the LightCycler480 (LC480) Real-Time PCR Instrument (*Roche Applied Science, Almere, The Netherlands*). SOGUG samples were genotyped by the Spanish National Cancer Research Centre (CNIO) using a KASPar SNP genotyping system (Kbiosciences, Hoddesdon, UK) and the sequence Detection System 7900HT (Applied Biosystems, Foster City, CA, USA) for fluorescence detection and allele assignment. Haplotype analysis was performed using PLINK software, version 1.07. Haplotype maximum likelihood estimates ≥95% were included.[16]

Quality control

For CCF and LUMC samples, 5% of the samples were genotyped in duplicate and no inconsistencies were observed. At CNIO, two samples representing each the wild-type homozygote, heterozygote and variant homozygote groups were confirmed by sequencing obtaining concordant results. Samples with a call rate <80% were excluded from the analysis. All SNPs achieved a SNP call rate >95%. Minor deviations from the Hardy-Weinberg equilibrium (HWE) were observed for SNPs rs776746 in *CYP3A5* ($P=0.014$) and rs1045642 in *ABCB1* ($P=0.041$). These 2 SNPs were included in the analysis, since only minor deviations from HWE were observed and Minor Allele Frequencies (MAFs) were similar to those previously described (7.5% versus 3.6% for rs776746 and 51.2% versus 42.9% for rs1045642, for observed MAFs and NCBI Hap Map CEU MAFs respectively).[17]
To exclude inter assay variation between LUMC and CNIO, A selection of 132 anonymized SOGUG DNA samples were re-genotyped in Leiden. A 98% concordance in SNP genotypes was observed between the LUMC and CNIO samples.

Statistical analysis

SNPs and haplotypes, together with age, gender, Body Surface Area (BSA), WHO performance status, study centre (SUTOX, CCF or SOGUG) and baseline creatinine, total bilirubin, albumin, aspartate transaminase (AST), alanine transaminase (ALT), hemoglobin, leukocytes, thrombocytes, Mean Corpuscular Volume (MCV), calcium and lactate dehydrogenase (LDH) were tested for associations with toxicities and dose reductions using an independent samples t-test, χ^2 test or a Mann-Whitney *U* test depending on the type of data. Clinical variables and SNPs or haplotypes with $P \leq 0.1$ were included as covariates in multivariate analysis. For related variables (e.g. the transaminases AST and ALT) with $P \leq 0.1$, the most significant variable was retained as covariate in multivariate analysis.

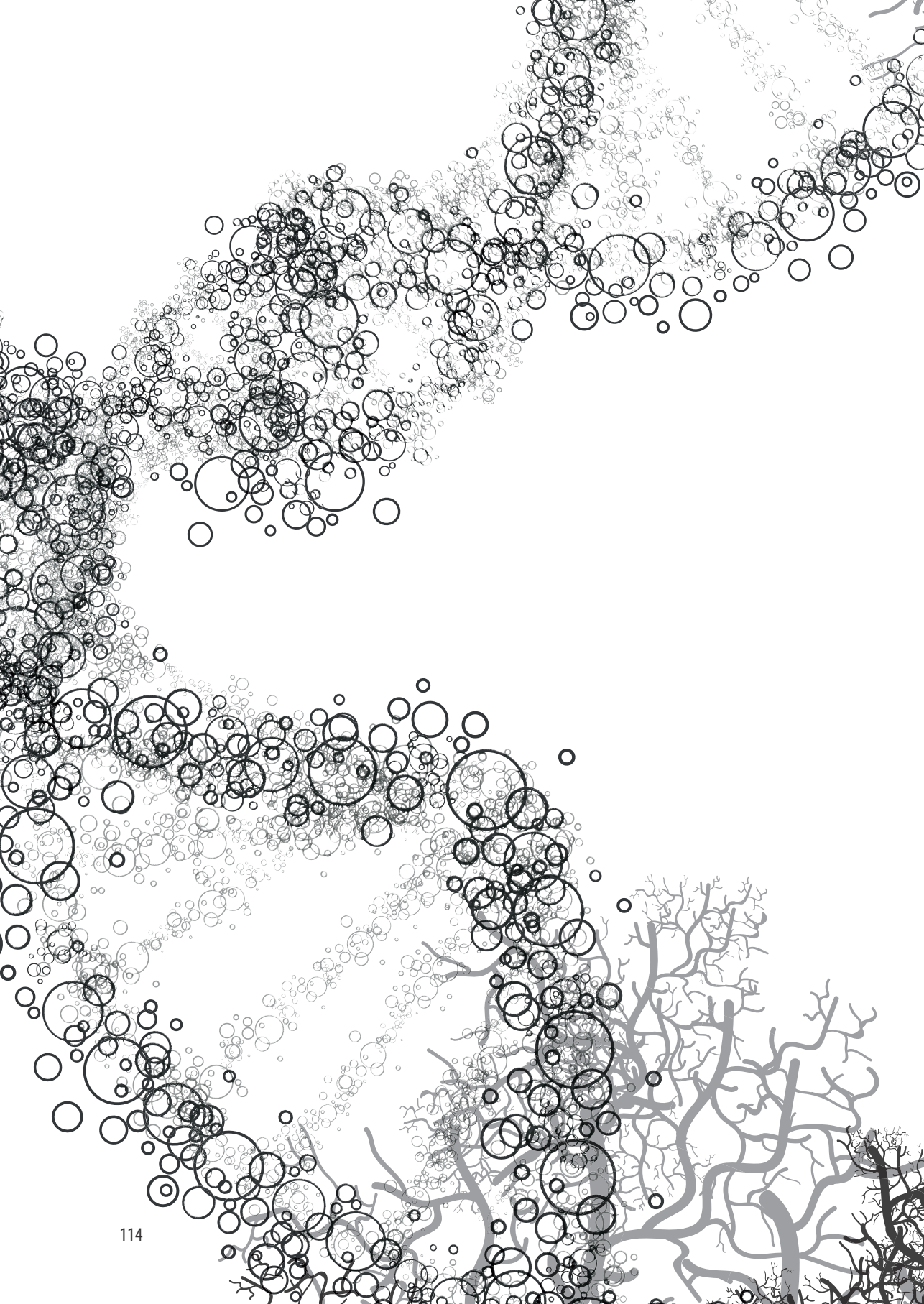
Supplementary Table 1 – Overview of single nucleotide polymorphisms and haplotypes in candidate genes related to the pharmacokinetics and pharmacodynamics of sunitinib that were significantly associated ($P<0.05$) with toxicity and efficacy of sunitinib in metastatic renal cell carcinoma in exploratory studies.[9,12-15]

gene	rs number	*P*-value	OR/HR	95% CI	Previously found associations	Reference
CYP1A1	rs1048943	0.029	6.24	1.20-32.42	G allele associated with increased risk for **leukopenia**	9
		0.021	4.03	1.24-13.09	G allele associated with increased risk for **mucosal inflammation**	
CYP3A5	rs776746	0.032	0.27	0.08-0.89	A allele (*CYP3A5*1*) associated with an increased **PFS**	13
		0.0014	3.75	1.67-8.41	A allele (*CYP3A5*1*) associated with **dose reductions** due to toxicity	12
ABCB1	rs1128503	0.011	0.41	0.20-0.81	T allele is protective for **hypertension**	12
	rs2032582	0.014	0.42	0.21-0.84	T allele is protective for **hypertension**	
	haplotype *ABCB1* (rs1128503, rs2032582, rs1045642)	0.035	0.39	0.16-0.94	TTT copy associated with increased occurrence of **hand-foot syndrome**	9
		0.033	0.52	0.29-0.95	CGT copy associated with increased **PFS**	13
ABCG2	rs2231142	0.042	0.11	0.01-0.92	Protective for **hand-foot-syndrome**	12
	haplotype *ABCG2* (rs55930652, rs2622604)	0.016	0.38	0.17-0.83	TT copy associated with **any toxicity higher than grade 2**	9
NR1I3	haplotype *NR1I3* (rs2307424, rs2307418, rs4073054)	0.041	1.74	1.02-2.96	absence of CAG copy associated with an increased risk for **leukopenia**	9
		0.017	1.76	1.11-2.79	absence of CAT copy associated with an increased **PFS**	13

Supplementary Table 1 – Continued

gene	rs number	*P*-value	OR/HR	95% CI	Previously found associations	Reference
VEGFA	rs699947	0.0074	2.43	1.27-4.66	Increased risk for **hypertension**	12
	rs833061	0.03	N/A	N/A	Increased prevalence of **hypertension**	14
	rs2010963	0.03 and 0.01	13.62	3.71-50.04	Increased prevalence + duration of **hypertension**; GG genotype (rs2010963) associated with sunitinib-induced **hypertension** compared to CC	
	rs1570360	0.035	2.04	1.05-3.96	Increased risk for **hypertension**	12
	rs3025039	0.03	3.18	1.12-8.99	concomitant presence of CC in *VEGF* (rs3025039) and GG in *VEGFR2* (rs2305948) associated with decreased **OS (poor prognosis)**	14
	haplotype *VEGFA* (rs699947, rs833061, rs2010963)	0.031	0.59	0.34-1.03	ACG haplotype associated with the **incidence of grade 3 hypertension**	15
NOS3 (=eNOS)	rs2070744	0.045	2.62	1.08-6.35	C allele associated with increased risk for **grade 3 hypertension** during 1st cycle of sunitinib	15
KDR (=VEGFR2)	rs2305948	0.046	2.39	1.02-5.60	presence of the T allele associated with **any toxicity > grade 2**	9
		0.03	3.18	1.12-8.99	concomitant presence of CC in *VEGF* (rs3025039) and GG in *VEGFR2* (rs2305948) associated with decreased **OS**	14
	rs1870377	0.0058	2.62	1.32-5.20	Increased risk for **hypertension**	12
FLT-4 (=VEGFR3)	rs307826	0.00049	3.57	1.75-7.30	Associated with reduced **PFS**	12
	rs307821	0.00085	3.31	1.64-6.68	Associated with reduced **PFS**	12
FLT3	rs1933437	0.008	0.36	0.17-0.77	T allele was associated with an increased risk for **leukopenia**	9

Abbreviations: OR = Odds Ratio; HR = Hazard Ratio; CI = Confidence Interval, OS = Overall Survival, PFS = Progression Free Survival



CHAPTER 5

Sunitinib-induced hypertension in *CYP3A4* rs4646437 A-allele carriers with metastatic renal cell carcinoma

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ABSTRACT

The single nucleotide polymorphism (SNP) rs4646437G>A in *CYP3A4* was suggested to be related to sunitinib toxicity. Our objective was to perform an in-depth investigation of the association between this SNP and sunitinib toxicity and efficacy using a large cohort of metastatic renal cell carcinoma (mRCC) patients. We collected DNA and clinical information of mRCC patients treated with sunitinib. SNP rs4646437 in *CYP3A4* was tested for associations with toxicity using logistic regression. Cox regression modeling was used for association analysis of rs4646437 with progression-free survival (PFS) and overall survival (OS). In a total of 287 patients, the A-allele of *CYP3A4* rs4646437 was associated with an increased risk for hypertension (odds ratio=2.4, 95% confidence interval: 1.1-5.2, *P*=0.021) and showed no significant association with PFS or OS. In conclusion, hypertension is more likely to occur in A-allele carriers of the *CYP3A4* rs4646437 variant in our cohort of mRCC patients treated with sunitinib.

INTRODUCTION

Sunitinib, a tyrosine kinase inhibitor, is widely prescribed for treatment of metastatic renal cell carcinoma (mRCC).[1] The large inter-individual variability in sunitinib response makes it difficult to predict treatment outcome in individual patients. Sunitinib-induced adverse events lead to dose reductions or treatment discontinuation with subsequent loss of treatment efficacy. This large variability can be explained not only by patient characteristics (for example drug-drug interactions, treatment adherence and environmental factors) but also by the genetic profile of patients. Studies using the candidate gene approach have identified single nucleotide polymorphisms (SNPs) to be associated with the efficacy and risk of toxicity on sunitinib treatment.[1-5]

Sunitinib is metabolized by CYP3A4 to its active metabolite SU12662. Urun *et al*.[6] have observed that A-allele carriers of *CYP3A4* rs4646437G>A showed a lower risk of grades 3-4 toxicity (odds ratio (OR)=0.27, 95% confidence interval (CI): 0.08-0.88, *P*=0.03) compared with wild-type (WT) GG in a cohort of 159 European mRCC patients treated with sunitinib. In a small number of reports on other types of drugs that are metabolized by CYP3A4, the variant A-allele of rs4646437 was associated with either an increased or decreased activity of CYP3A4.[7-9] He *et al*.[7] tested 22 SNPs in genes encoding CYP3A4, CYP3A5 and CYP2C19 for associations with voriconazole pharmacokinetics (PK) in a sample of 158 patients. The presence of A-allele of rs4646437 was associated with high plasma voriconazole concentrations of >4mg l^{-1} (OR=2.8, 95% CI: 1.09-7.38, *P*=0.03). The authors note that, although rs4646437 is located in the intron (99767460G>A), it could alter the splicing of primary transcripts or gene expression of *CYP3A4*.[7] In another cohort of 240 Chinese renal transplant recipients receiving tacrolimus, Li *et al*.[8] assessed 17 SNPs in *CYP3A5*, *CYP3A4*, *COMT*, *IL-10* and *POR* for association with tacrolimus PK. For rs4646437, a significant association was observed with dose-adjusted tacrolimus blood concentration (C_0/D) showing lower ratios for the variant A-allele carriers.[8] Crettol *et al*.[9] studied 73 patients receiving cyclosporine and reported that A-allele carriers of rs4646437 needed a higher dose of cyclosporine.

A potential explanation for the conflicting results of rs4646437 variant on CYP3A4 expression and metabolism may be related to gender. Schirmer *et al*.[10] reported that rs4646437 is a sex-dependent genetic marker with a higher CYP3A4 expression and activity for female A-allele carriers compared with male A-allele carriers. Therefore, it was suggested to take gender into account in the genetic analyses of *CYP3A4*.[10] The aim of the current study was to establish whether there is a relationship between rs4646437 in *CYP3A4* and sunitinib treatment outcome in a large cohort of clear cell mRCC patients.

MATERIALS AND METHODS

Study population

This exploratory *post-hoc* analysis is one of the largest pharmacogenetics studies in mRCC patients. In this large observational study, DNA samples together with clinical information were collected from previous pharmacogenetics studies on sunitinib-treated clear cell mRCC patients enrolled between 2004 and 2010 (**Figure 1**). The SUTOX consortium is a Dutch working group focusing on genotype-related sunitinib-induced toxicity and comprises five medical centers in the Netherlands. The Spanish Oncology Genitourinary Group (SOGUG) consists of 15 Spanish medical centers, and CCF is the Taussig Cancer Institute of Cleveland Clinic Foundation in the United States.[1-5,11] SUTOX samples were anonymized by a third party, according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org). The Medical Ethical review boards of all participating centers approved the study. Patients provided their written informed consent for participation.[2,11]

Genotyping methods

Germline DNA was isolated from EDTA-whole blood, peripheral blood mononuclear cell samples, serum or plasma. Samples from serum or plasma were preamplified in order to have a higher DNA concentration. DNA samples from SUTOX and CCF patients were genotyped at Leiden University Medical Center using Taqman probes (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) on the LightCycler480 (LC480) (Roche Applied Science, Almere, The Netherlands) and on ViiA 7500 (Applied Biosystems) Real-Time PCR Instruments. The latter was used for the analysis of preamplified samples for a better fluorescence chart review. DNA samples from SOGUG were genotyped by the Spanish National Cancer Research Centre using a KASPar SNP genotyping system (Kbiosciences, Hoddesdon, UK) and the sequence Detection System 7900HT (Applied Biosystems, Foster City, CA, USA) for fluorescence detection and allele assignment. In all, 5% of the samples were genotyped in duplicate, and no inconsistencies were observed.[11]

Study design

Sunitinib toxicity was evaluated during four 6-week treatment cycles and scored at baseline, week 4 and week 6 of each cycle according to the National Cancer Institute-Common Terminology Criteria for Adverse Events (NCI-CTCAE) v3.0 or v4.0.[11] Tested toxicity outcomes were thrombocytopenia, leukopenia, mucosal inflammation, hand-foot syndrome, hypertension and any toxicity >grade 2. Toxicities ≥grade 3 were considered clinically relevant. Tested toxicity outcomes were adjusted for toxicity grades observed at baseline.[11] Baseline corrected toxicity scores were calculated by subtracting baseline

values from the maximum recorded score in four cycles of treatment. Corrected toxicity scores were divided into grades 0-2 and grades 3-4.[11] Patient characteristics considered relevant for suffering any kind of adverse event were age at the start of sunitinib, gender and study center.

For survival analysis, progression-free survival (PFS) was defined as the time in months between the first day of sunitinib and the date of progressive disease (PD) according to the Response Evaluation Criteria in Solid Tumours version 1.0. If a patient had not progressed, PFS was censored at the time of the last follow-up or date of death. Overall survival (OS) was defined as the time in months between the first day of sunitinib and the date of death or the date at which patients were last known to be alive (last follow-up date). Variables considered to affect PFS or OS were age at the start of sunitinib, gender, study center and prognostic risk group according to Heng criteria.[11-12]

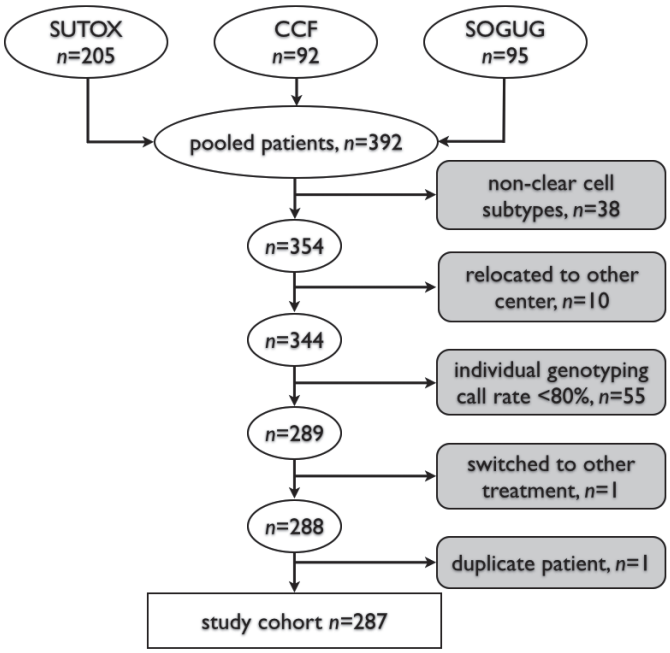

Figure 1 Patient flowchart on included patients. A total of 105 patients had to be excluded from association analyses because of non-clear cell subtypes (N=38), relocation to another medical center during follow-up (N=10), individual genotyping call rates <80% (N=55), double patient (N=1) or a change to another treatment than sunitinib directly after enrolment (N=1). A total of 287 sunitinib-treated metastatic renal cell carcinoma patients were available for analysis of toxicity and survival in the present study. CCF, cleveland clinic foundation; SOGUG, Spanish oncology genitourinary group; SUTOX, dutch SUTOX consortium.[11]

Statistical analysis

Genotyping results were dichotomized in a dominant model with WT (GG genotype) vs A-allele carriers (AG and AA genotype), analogous to the work of Urun *et al*.[6] Distribution of genotypes was tested for deviation from Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test. Distribution of the continuous variable 'age at the start of sunitinib' followed a normal distribution according to the Shapiro-Wilk test.

SNP rs4646437 (WT vs A-allele carriers) was tested univariately against toxicity end points using a χ^2 test. For multivariate analysis, SNP rs4646437 was tested against toxicity end points dichotomized as grades 0-2 and grades 3-4 using binary logistic regression ($P<0.05$), including the covariates age at the start of sunitinib, gender and study center.[11]

For PFS and OS analysis, age, gender, study center and Heng prognostic risk group[12] together with *CYP3A4* rs4646437 genotype were included in the multivariate Cox regression survival analysis. All results with a *P*-value <0.05 from multivariate analyses were considered significant.

In addition, the interaction between the SNP rs4646437 genotypes and gender was tested univariately for association with all of the toxicity outcomes and with PFS and OS.[10-11] This additional analysis was performed to see whether the interaction term of SNP × gender needed to be included as covariate in multivariate analysis. Interaction between gender and the rs4646437 genotype on PFS and OS was also tested using Cox regression survival analysis.

All tests were two sided and carried out using SPSS Statistical Package for Windows (version 20.0; IBM, Armonk, NY, USA).

RESULTS

Of the initial 391 patients, 287 clear cell mRCC patients met the inclusion criteria of which 68% were males (**Figure 1**). Baseline patient characteristics are summarized in **Table 1**. Genotype frequencies were 228 carriers of WT GG, 52 carriers of heterozygous AG and 7 carriers of the AA genotype. This is consistent with Hardy-Weinberg equilibrium ($\chi^2=3.46$, $P=0.063$). Obtained minor allele frequency was 6.97%.

Most patients had no toxicities at baseline (95.5-100% for the tested toxicities). Baseline grades 1-3 toxicities were observed for 0-4.5% of patients, and no baseline grade 4 toxicities were observed. Dose reductions were needed for 33% of patients within cycles 1-4 of sunitinib. Any toxicity >grade 2 was observed in 25% of patients (**Table 2**). Observed grades 3-4 adverse events within four cycles of sunitinib were thrombocytopenia (7.7%), leukopenia (2.8%), mucosal inflammation (3.1%), hand-foot syndrome (5.2%) and hypertension (13.6%). The interaction between SNP genotype

and gender was tested against all toxicity outcomes, but no significant interaction was found in univariate analysis (*P*=0.27). As a result, gender was included as an independent covariate instead of a combined 'gender × SNP' covariate. Genotype distributions for males and females are presented in **Table 1**. **Table 3** shows the results of association analyses. For A-allele carriers of CYP3A4 rs4646437, we observed an increased risk for hypertension (OR=2.4, 95% CI: 1.1-5.2, *P*=0.021). For other toxicity end points, no significant associations were observed.

The median PFS was 17 months with a median follow-up period of 45 months for PFS analysis. The median follow-up period was 49 months for OS and the median OS was 29 months. The interaction between gender and genotype was tested in PFS and OS analysis, but no significant interaction was found (*P*=0.23 and *P*=0.76 respectively). There was no significant difference in median PFS or median OS between CYP3A4 rs4646437 WT and A-allele carrier patients both uncorrected and corrected for age, gender, study center and Heng prognostic risk group in Cox regression survival analysis (**Table 3**).

Table 1 Baseline characteristics of clear cell metastatic renal cell carcinoma patients treated with sunitinib (N=287)

Feature	Value (range)	%
Gender:		
Male	195	68
Female	92	32
Median age at start of sunitinib	61 (34-87)	
Mean BSA	1.97 (1.2-2.7)	
Prior nephrectomy		
yes	242	84.3
no	41	14.3
unknown	4	1.4
WHO performance status		
0	120	42
1	142	50
2	17	6
3	1	0
unknown	7	2
Ethnicity		
Caucasian	277	97
Black	4	1
Asian	2	1
Arab	3	1
Latin American	1	0

Table 1 Continued

Feature	Value (range)	%
Gender genotype distributions		
Female AA carriers	2	1
Female AG carriers	17	6
Female GG carriers	73	25
Male AA carriers	5	2
Male AG carriers	35	12
Male GG carriers	155	54
HENG prognostic risk group[a]		
Good (0 risk factors)	61	21
Intermediate (1-2 risk factors)	157	55
Poor (3-6 risk factors)	69	24
Prior systemic antitumor treatment		
Yes	52	18
No	235	82
Sunitinib daily dose (mg)		
Only 50 mg	269	94
Only 37.5 mg	14	5
Only 25 mg	4	1
Dose reduction after cycle 1,2 or 3		
No	180	63
Yes	95	33
Unknown	12	4
Best response to sunitinib		
Complete response	9	3
Partial response	120	42
Stable disease	104	36
Progressive disease	37	13
Unknown	17	6
Median SBP (mmHg)	140 (95-205)	
Median DBP (mmHg)	80 (29-110)	
Median LDH (U/L)	241 (110-1190)	
Median creatinine (µmol/L)	106.1 (1.0-247.5)	
Median bilirubin (µmol/L)	6.84 (0.5-46.2)	
Median albumin (g/L)	42 (4.7-51.0)	
Median AST (U/L)	19 (8-107)	
Median ALT (U/L)	17 (4-119)	
Median haemoglobin (mmol/L)	8.1(4.7-14.2)	
Median leukocytes ($x10^9$)	7.6 (1.6-50.0)	
Median neutrophils ($x10^9$)	4.8 (0.7-26.4)	
Median thrombocytes ($x10^9$)	254 (92-1492)	
Median MCV (fL)	88 (68-110)	
Median calcium (mmol/L)	2.38 (1.92-2.93)	

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BSA, body surface area; DBP, diastolic blood pressure; LDH, lactate dehydrogenase; MCV, mean corpuscular volume;

SBP, systolic blood pressure; WHO, world health organization (performance status). [a]Patients are grouped according to their Heng risk group based on the six Heng risk scores: detoriated WHO performance status (≥2), low hemogblobin (<lower limit of normal), high calcium (>2.5 mmol l^{-1}), time from initial diagnosis to treatment with sunitinib (<1 year), neutrophil count (greater than upper limit of normal) and thrombocytes (greater than upper limit of normal).[11]

Table 2. Distribution of baseline corrected toxicity scores within four cycles of sunitinib t reatment

Type of toxicity	Toxicity grade	Number of patients (N)	Percentage of patients (%)
Thrombocytopenia	none	104	36
	grade 1	122	43
	grade 2	39	14
	grade 3	18	6
	grade 4	4	1
Leukopenia	none	151	53
	grade 1	78	27
	grade 2	50	17
	grade 3	8	3
Mucosal inflammation	none	126	44
	grade 1	93	32
	grade 2	59	21
	grade 3	9	3
Hand-foot-syndrome[a]	none	173	60
	grade 1	57	20
	grade 2	42	15
	grade 3	15	5
Any toxicity >grade 2	none	39	14
	grade 1	76	26
	grade 2	101	35
	grade 3	62	22
	grade 4	9	3
Hypertension	none	173	60
	grade 1	29	10
	grade 2	45	16
	grade 3	39	14

In case the grade of toxicity was not recorded in the medical record of the patient, it was assumed that no toxicity had occurred (grade 0).

[a]For hand-foot syndrome grade 3 is the highest possible grade according to CTCAE version 4.0.

Table 3. Association analysis results of *CYP3A4* rs4646437 vs toxicity endpoints and survival endpoints

Toxicity endpoint	*P*-value	OR	95% CI	
			Lower	Upper
Thrombocytopenia grade 0-2 vs3-4				
WT vs A carriers uncorrected	0.18	0.31	0.08	1.61
WT vs A carriers	0.21	0.38	0.08	1.74
Leukopenia grade 0-2 vs 3-4				
WT vs A carriers uncorrected	0.57	0.54	0.07	4.5
WT vs A carriers	0.78	0.73	0.08	6.4
Mucositis grade 0-2 vs 3-4				
WT vs A carriers uncorrected	0.90	1.11	0.22	5.48
WT vs A carriers	0.72	1.37	0.25	7.5
Hand-foot syndrome grade 0-2 vs 3-4[a]				
WT vs A carriers uncorrected	0.07	2.76	0.94	8.08
WT vs A carriers	0.14	2.33	0.77	7.09
Hypertension grade 0-2 vs 3-4				
WT vs A carriers uncorrected	0.013	2.51	1.21	5.22
WT vs A carriers	**0.021**	2.43	1.14	5.18
Any toxicity >grade 2				
WT vs A carriers uncorrected	0.59	0.83	0.42	1.64
WT vs A carrier	0.89	0.95	0.46	1.96
Survival endpoint	*P*-value	*HR*	*95% CI*	
			Lower	*Upper*
PFS				
WT vs A carriers uncorrected	0.38	0.86	0.61	1.20
WT vs A carriers	0.14	0.77	0.55	1.09
OS				
WT vs A carriers uncorrected	0.92	1.02	0.71	1.47
WT vs A carriers	0.45	0.86	0.59	1.26

Abbreviations: CI, confidence interval; HR, hazard ratio; OR, odds ratio; OS, overall survival; PFS, progression-free survival; WT, wild type. In case the grade of toxicity was not recorded in the medical record of the patient, it was assumed that no toxicity had occurred (grade 0). Toxicity end points are corrected for age, gender and study center. Survival end points are corrected for age, gender, Heng prognostic risk group and study center. [a]For hand-foot syndrome, grade 3 is the highest possible grade according to CTCAE version 4.0. Values in bold represent *P*-values <0.05 corrected for clinical covariates.

DISCUSSION

In this study, we observed a significant association between the presence of A-allele in *CYP3A4* rs4646437 and an increased risk for hypertension compared with WT carriers in sunitinib-treated mRCC patients. Hence, *CYP3A4* rs4646437 is a potential predictive biomarker for hypertension in sunitinib-treated mRCC patients, but this needs confirmation.

This is the first time that SNP *CYP3A4* rs4646437 is examined in a large cohort of mRCC patients. Grades 3-4 hypertension was observed in 14% of patients both in this study and in the study of Urun *et al*.[6] Our findings do not confirm the data of Urun *et al*.,[6] who observed no association with hypertension and who reported a reduced risk of grades 3-4 toxicities for AG carriers of *CYP3A4* rs4646437 (OR=0.27). In contrast to earlier reports, we did not demonstrate significant results for the interaction between genotype and gender.[10]

Our studied time period of four treatment cycles in which toxicity data were collected is comparable to Urun *et al*.[6] who reported a median treatment duration of 7.7 months. In both studies, Caucasians were included of whom the majority were male.[6] The difference in our findings with those of the group of Urun *et al*.[6] could partly be explained by our large sample size (287 vs 159 patients), a higher number of A carriers (20.6% vs 11.2%) and a lower rate of adverse events >grade 2 (24.7% vs 52%). In addition, our fixed covariates in multivariate testing ($P<0.1$) differ from the included clinical covariates by Urun *et al*.[6] using a significance threshold of $P<0.2$. The time frame of our study is long including 7 years, which could introduce a bias as clinical experience with sunitinib may have caused dose reduction before the occurrence of >grade 2 adverse events. A time-to-event analysis is preferred for toxicity analysis as many patients will not complete four cycles of sunitinib treatment, and patients may develop toxicities earlier or later in this time frame. However, it was not possible to collect these data accurately because of the retrospective character of this study.

The expression of CYP3A4 in the liver is twofold higher in women than in men that can be explained by sex-dependent gene expression differences.[10] However, as genders are not equally present in this study (only one-third is female) and merely 19 subjects (7%) were female A carriers, it is not possible to perform robust statistics for interaction testing between gender and SNP genotype.

Based on our results in this large cohort, it remains difficult to explain whether A-allele carriers of *CYP3A4* rs4646437 either have an increased or decreased function of CYP3A4. One could reason that an increased function would lead to conversion of sunitinib to the metabolite SU12662 with a similar activity and a longer half-life compared with the parent compound. On the other hand, a decreased function of CYP3A4 could lead to an

increase in total drug exposure all the same. In both hypotheses, A-allele carriers will have an increased exposure to the drug with a stronger inhibition of vascular endothelial growth factor receptor 2 (VEGFR-2) and therefore a higher risk of hypertension.[2-4,13]

A number of PK/pharmacodynamic analyses demonstrate that the increased plasma drug exposure of sunitinib (and SU12662) indeed results in hypertension or other types of toxicity. In a large meta-analysis of Houk *et al*.[14] on mainly Caucasian patients, it was shown that a higher exposure to sunitinib and SU12662 (total drug AUC) resulted in improved clinical outcomes and an increased risk of adverse events: fatigue, diastolic blood pressure elevation, or reduction of the absolute neutrophil count. Also, in a PK analysis of Mizuno *et al*.[15] on 19 Japanese RCC patients, the systemic exposure to sunitinib and its active metabolite was associated with the incidence of thrombocytopenia (≥grade 2) and hypertension (≥grade 2). Nagata *et al*.[16] studied six Japanese RCC patients of whom four received a starting dose of 50 mg and developed grade 2 or 3 thrombocytopenia. It was concluded that the total trough level of sunitinib should be <100 ng ml $^{-1}$ in order to avoid severe thrombocytopenia and the starting dose could be reduced to 37.5 or 25 mg in most cases. In another PK analysis, Noda *et al*.[17] investigated 21 RCC patients on the association of total sunitinib concentration with toxicities and clinical outcome. Patients with a total sunitinib concentration ≥100 ng ml $^{-1}$ showed a higher incidence of ≥grade 3 thrombocytopenia, anorexia or fatigue.[17] Recently, Teo *et al*.[18] investigated the effect of SNPs in *CYP3A5* and *ABCB1* on both PK and clinical outcome (that is, toxicity and response) in a Chinese population of 31 patients. The variant CC genotype of rs1045642 in *ABCB1* was associated with a higher exposure to sunitinib, an increased risk for rash and mucositis and progression of the disease as compared with T-allele carriers (P<0.05). No association with sunitinib exposure, response or toxicities was seen for rs776746 in *CYP3A5*.[18] In contrast, our research group investigated a considerably larger group of Caucasian patients and reported that rs776746 in *CYP3A5* was associated with hypertension (OR=4.70, 95% CI: 1.47-15.0, P=0.009) and with the need for dose reductions.[11] Another SNP in *CYP3A4* (*CYP3A4*22*) was tested in a set of 114 patients and associated with a 22.5% decrease in clearance of sunitinib, which explains the higher exposure to the drug.[19] However, this pharmacogenetic-PK analysis did not investigate the occurrence of toxicities and the SNP is different from that in the present study. In putting together these results, we should be aware that patients of Asian ethnicity have been reported to have a higher incidence of severe sunitinib-induced toxicities compared with Caucasians.[11,14-18]

Remarkable in our findings is that only an association was observed of SNP rs4646437 with sunitinib-induced hypertension, whereas no associations have been found with any of the other tested toxicities on sunitinib. Although mechanisms are currently not fully unraveled, it is thought that sunitinib-induced hypertension is caused by inhibition of

VEGFR-2 leading to a reduced amount of nitric oxide and thus vasoconstriction. SNPs in *VEGFA* and *eNOS* are reported to be related to sunitinib-induced hypertension.[4,11,13] Probably there are more factors involved that influence the development of hypertension, which can be related to both PK and pharmacodynamics. The current study and other recent findings of our group suggest that SNPs in *CYP3A4* and *CYP3A5* could have an essential role in hypertension development.[11]

Furthermore, hypertension can be considered as a different sort of toxicity as it is related to improved clinical outcomes on sunitinib.[4,20] Yet, no improved survival outcomes were seen for the variant A-allele carriers of rs4646437. Promising biomarkers for sunitinib efficacy have been identified in *ABCB1*. Corresponding results were observed for SNPs in *ABCB1*: the presence of WT CGT haplotype in rs1128503, rs2032582 and rs1045642 resulted in an improved clinical outcome (PFS, OS and response), the CC WT in rs1128503 showed a decrease in sunitinib clearance, and the TT variant in rs1128503 required less dose reductions.[11,19,21] In contrast, the variant CC genotype of rs1045642 showed an increased exposure to sunitinib in Japanese patients.[18] To predict hypertension and subsequent effects on survival, we may be searching for one biomarker that consists of multiple SNPs related to the PK and pharmacodynamics of sunitinib.

In conclusion, hypertension is more likely to occur in A-allele carriers of the *CYP3A4* rs4646437 variant. Confirmation of this finding in another cohort is needed to potentially use this SNP as a predictive biomarker for hypertension on sunitinib treatment.

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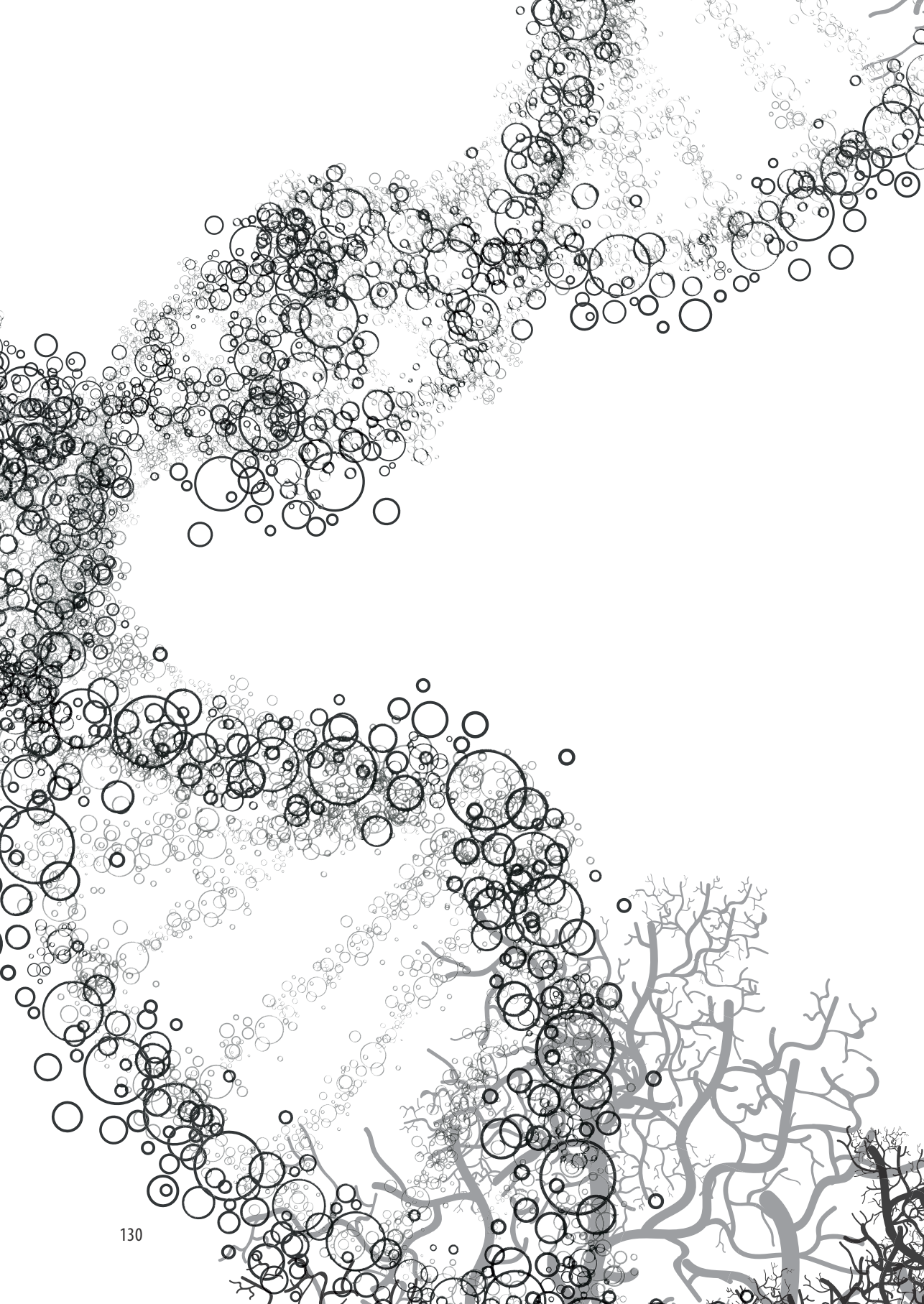

CHAPTER 6

Association of single nucleotide polymorphisms in *IL8* and *IL13* with sunitinib-induced toxicity in patients with metastatic renal cell carcinoma

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**Meta H. M. Diekstra and Xiaoyan Liu contributed equally to this work.*

ABSTRACT

Purpose Earlier, the association of single nucleotide polymorphisms (SNPs) with toxicity and efficacy of sunitinib has been explored in patients with metastatic renal cell carcinoma (mRCC). Recently, additional SNPs have been suggested as potential biomarkers. We investigated these novel SNPs for association with sunitinib treatment outcome in mRCC patients.

Methods In this exploratory study, we selected SNPs in genes *CYP3A4*, *NR1I2*, *POR*, *IL8*, *IL13*, *IL4-R*, *HIF1A* and *MET* that might possibly be associated with sunitinib treatment outcome. Each SNP was tested for association with progression-free survival (PFS) and overall survival (OS) by Cox-regression analysis and for clinical response and toxicity using logistic regression.

Results We included 374 patients for toxicity analyses, of which 38 patients with non-clear cell renal cell cancer were excluded from efficacy analyses. The risk for hypertension was increased in the presence of the T allele in *IL8* rs1126647 (OR=1.69, 95% CI=1.07-2.67, *P*=0.024). The T allele in *IL13* rs1800925 was associated with an increase in the risk of leukopenia (OR=6.76, 95% CI=1.35-33.9, *P*=0.020) and increased prevalence of any toxicity >grade 2 (OR=1.75, 95% CI=1.06-2.88, *P*=0.028). No significant associations were found with PFS, OS or clinical response.

Conclusions We show that polymorphisms in *IL8* rs1126647 and *IL13* rs1800925 are associated with sunitinib-induced toxicities. Validation in an independent cohort is required.

INTRODUCTION

Several targeted therapies have been approved for the treatment of metastatic renal cell carcinoma (mRCC). Of these, the tyrosine kinase inhibitor (TKI) sunitinib is widely used as first-line treatment option.[1-4] The response to sunitinib varies largely among patients. Only 35% of mRCC patients benefit from sunitinib, and about 30% of patients need dose reductions due to adverse events of which grades vary among patients.[1,5,6] To optimize treatment efficacy and to minimize the risk of adverse events of higher grades, it would be helpful to predict the individual treatment outcome at the initiation of therapy. Unfortunately, no biomarkers are yet available to fulfil this need.

Several studies used a candidate gene approach to select single nucleotide polymorphisms (SNPs) in genes involved in the pharmacokinetics and pharmacodynamics of sunitinib. SNPs and haplotypes in *CYP1A1*, *CYP3A5*, *ABCB1*, *ABCG2*, *NR1I3*, *VEGFA*, *NOS3* (=*eNOS*), *FLT1* (=*VEGFR1*), *KDR* (=*VEGFR2*), *FLT-4* (=*VEGFR3*) and *FLT3* have been described to have an association with either toxicity or efficacy of sunitinib ($P<0.05$) in mRCC patients.[5-11] The associations of *CYP3A5* and *ABCB1* with dose reductions and efficacy, respectively, have been confirmed recently.[12] Not all clinical outcomes can be explained by these potential biomarkers, which make identification of other markers possibly associated with clinical outcome an attractive prospect.

Novel SNPs in *CYP3A4*, *NR1I2*, *POR*, *IL8*, *IL4-R*, *IL13*, *HIF1A* and *MET* have been reported in patients with RCC, either prognostic or associated with treatment outcome, and might play a role in sunitinib treatment outcome (**Table 1**).[13-20] In some studies on these novel SNPs, the TKI pazopanib was given to patients with mRCC. Sunitinib and pazopanib have similar efficacy and both are used as first-line treatment options.[2] Further, both drugs have similarities in their metabolic pathways and affected targets, because of which SNPs associated with pazopanib outcome might also be meaningful for the sunitinib treatment outcome, i.e. toxicity or efficacy.[21] The T allele of SNP rs35599367 in *CYP3A4* (*CYP3A4*22*) was associated with decreased clearance of sunitinib.[13-15] The T allele of SNP rs3814055 in *NR1I2* was associated with a reduction in response to pazopanib and inferior progression-free survival (PFS) from sunitinib and pazopanib in univariate analysis.[6,16] The TT genotype of SNP rs1057858 in the P450 oxidoreductase gene (*POR*28*) was associated with higher CYP3A activity.[17,18] Variant alleles of rs4073 and rs1126647 in *IL8* were associated with an inferior PFS on pazopanib treatment.[16] SNPs rs1800925 and rs20541 in *IL13* and rs180510 in *IL4-R* are likely to influence tumour immune response and carcinogenesis.[19] The AG genotype of rs11549467 in *HIF1A* compared to wild-type GG was associated with a decreased PFS and a reduced response rate on pazopanib treatment.[16] The A-allele of SNP rs11762213 in *MET* was associated with an increased risk of recurrence or death in RCC patients.[20]

In this exploratory study, we evaluated the polymorphisms in the above-mentioned genes for possible associations with toxicity or efficacy of sunitinib in a large cohort of mRCC patients.

Table 1 Polymorphisms in candidate genes in the current study. Genetic polymorphisms were included if in previous exploratory studies associations were reported with a *P*-value <0.05

Gene	SNP	Reported results	References
CYP3A4	rs35599367C>T	*CYP3A4*22* associated with decreased CYP3A4 expression and decreased clearance of sunitinib.	Elens *et al*. 2011, 2013 [13,14] Diekstra *et al*. 2014 [15]
NR1I2	rs3814055C>T	T allele is associated with reduction of response to pazopanib and inferior PFS on sunitinib and pazopanib	van der Veldt *et al*. 2011 [6] Xu *et al*. 2011 [16]
*POR*28*	rs1057868C>T	POR*28 is associated with higher in vivo CYP3A activity.	de Jonge *et al*. 2011 [17] Oneda *et al* 2009 [18]
IL8	rs4073T>A	AA shows inferior PFS of pazopanib compared to wild type TT.	Xu *et al*. 2011 [16]
	rs1126647A>T	TT shows inferior PFS of pazopanib compared to wild type AA.	Xu *et al*. 2011 [16]
	Correlated SNPs rs4073T>A and rs1126647A>T	Inferior PFS for variant genotypes (r4073 AA + rs1126647 TT) compared to wild-types (rs4073 TT + rs1126647 AA).	Xu *et al*. 2011 [16]
HIF1A	rs11549467G>A	AG genotype is associated with inferior PFS and reduced response rate of pazopanib compared to wild type GG.	Xu *et al*. 2011 [16]
IL-4R	rs1805010T>C	CT/TT genotype is associated with decreased risk of RCC compared to CC genotype.	Chu *et al*. 2012 [19]
IL13	rs1800925C>T	TT genotype is associated with decreased risk of RCC.	Chu *et al*. 2012 [19]
	rs20541C>T		
MET	rs11762213G>A	A-allele is associated with an increased risk of recurrence or death	Schutz *et al.* 2013 [20]

SNP single nucleotide polymorphism, PFS progression-free survival, OS overall survival

METHODS

Study population

Patient data were collected from three exploratory studies (SUTOX, SOGUG and CCF) between the years 2004 and 2010 (**Supplementary document 1**).[12] SUTOX samples were anonymized by a third party, according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org). The study was conducted in accordance with the Declaration of Helsinki and approved by the medical ethics review board of all participating groups. Patients provided their written informed consent for participation.[12]

Study endpoints

PFS, defined as the time in months between the first day of sunitinib treatment and the date of progressive disease (PD) according to Response Evaluation Criteria in Solid Tumours (RECIST) v.1.0 or v1.1, was used as the primary endpoint to assess efficacy. Another endpoint was overall survival (OS), which was measured from the first day of sunitinib treatment until death or time of last follow-up.

We classified objective clinical response into three categories: (i) partial and complete response, (ii) stable disease and (iii) progressive disease (according to RECIST).

Specific sunitinib-related adverse events, i.e. thrombocytopenia, leukopenia, mucosal inflammation, hand-foot syndrome, hypertension and any toxicity >grade 2, were collected for this study (**Supplementary document 1**).[12]

Statistical analysis

For univariate analysis, a log-rank test was used for the association of each SNP with PFS and OS, and a chi-square test for clinical response and toxicity. SNPs with a *P* value <0.1 were included in the multivariate model. Based on previous results, we included well-established covariates age, gender and Heng prognostic risk group in the multivariate model for correction of PFS and OS.[22] In addition, the CGT haplotype of *ABCB1* was also used as covariate in multivariate Cox model because of the previously confirmed significant association with PFS.[6,12] For multivariate analysis of clinical response, we also included age, gender and Heng prognostic risk group as covariates. For multivariate analysis on toxicity endpoints, no biomarker was widely validated, so we corrected for age and gender. Because this association study used data from three study groups (SUTOX, SOGUG and CCF), study group was also tested as a covariate for all endpoints. It was not needed to correct for previous treatment in efficacy analyses, because this was already justified by using study group as a covariate.

Reported results from the multivariate analysis with a *P* value <0.05 were considered clinically significant. All tests were two-sided and carried out by SPSS Statistical Package for Windows (version 20.0 Armonk, NY: IBM Corp).

RESULTS

Patient characteristics

A total of 374 patients with mRCC treated with sunitinib were included for association analyses on toxicity endpoints. For efficacy analyses, only clear cell subtypes (N=336) were included (**Figure 1**). Patient characteristics are presented in **Table 2**. Median age of patients was 61 years, and most were men of Caucasian ethnicity. The majority of patients

had undergone nephrectomy. For the clear cell subjects, the good or intermediate Heng prognostic risk group consisted of 73% of the patients. Forty-five percent (N=143) of patients showed a partial response (PR) or complete response (CR) to sunitinib.

95.7 to 99.5% of patients had no toxicities at baseline. Within four cycles of sunitinib treatment, any grade of thrombocytopenia was observed in 61% of patients, mucosal inflammation in 59%, leukopenia in 49%, hand-foot syndrome in 41% and hypertension in 38%. Twenty-six percent of patients developed any toxicity >grade 2 (**Supplementary Table S1**).

For clear cell subtypes, median follow-up times for PFS and OS analysis were 43 and 49 months, respectively. Median PFS and OS of patients were 16 and 26 months, respectively.

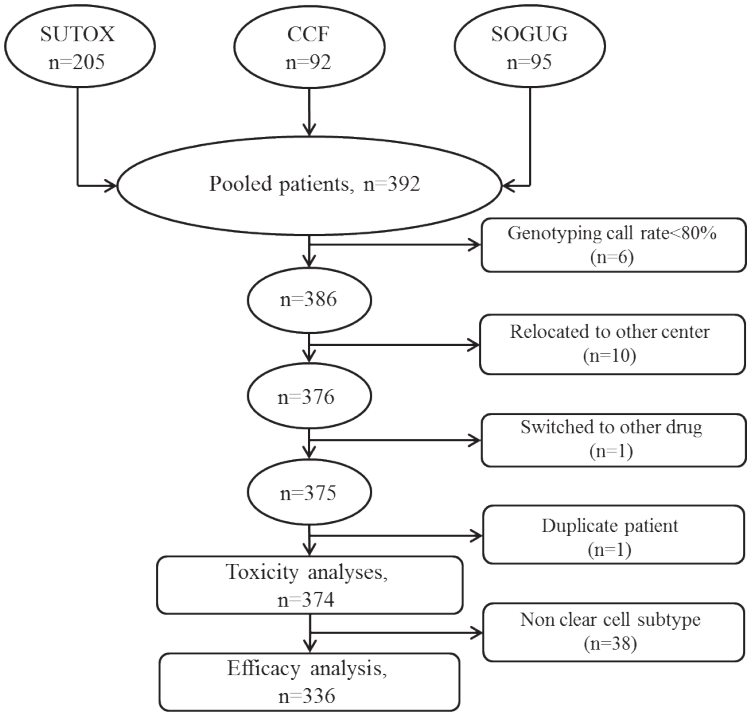

Figure 1 Patient flowchart on included patients. Fifty-six patients had to be excluded from association analyses because of individual genotyping call rates <80% (N=6), relocation to another medical center during follow-up (N=10), double patient (N=1), a change to another treatment than sunitinib directly after enrolment (N=1) or nonclear cell subtypes (N=38). A total of 374 sunitinib-treated clear cell mRCC patients were available for analysis of toxicity in the present study. For efficacy analysis, 336 sunitinib-treated clear cell mRCC patients were available.[12]

Table 2 Patient Characteristics

Characteristic	Efficacy cohort (N=336)	Toxicity cohort (N=374)
Age		
Median	61	61
Percentiles (25th, 75th)	55, 69	54, 68
BSA		
Median	1.98	1.96
Percentiles (25th, 75th)	1.82, 2.13	1.82, 2.12
Race		
White	324(96%)	358(96%)
Asian	3(0.89%)	3(0.80%)
Black	5(1.5%)	8(2.1%)
Arabian	3(0.89%)	4(1.0%)
Latin American	1(0.30%)	1(0.27%)
Gender		
Male	229(68%)	258(69%)
Female	107(32%)	116(31%)
Clinical response		
PR+CR	143(43%)	149(40%)
SD	130(39%)	149(40%)
PD	43(13%)	55(15%)
nknown	20(6.0%)	21(5.6%)
Heng prognostic risk group[a]		
Good (0 risk factor)	64(19%)	68(18%)
Intermediate (1-2 risk factors)	182(54%)	197(53%)
Poor (3-6 risk factors)	90(27%)	108(29%)
Metastatic sites		
1	91(27%)	105(28%)
2	137(41%)	149(40%)
>=3	104(31%)	116(31%)
Prior nephrectomy		
No	49(15%)	63(17%)
Yes	283(85%)	307(82%)
Prior treatment		
No	254(76%)	276(74%)
Yes	82(24%)	98(26%)

BSA body surface area, CR complete response, PR partial response, SD stable disease, PD progressive disease

[a]Patients are classified according to the six Heng risk criteria: poor WHO performance status (≥2), low haemoglobin (<lower limit of normal), high calcium (>upper limit of normal) and time from initial diagnosis to treatment with sunitinib (<1 year), neutrophil count (>upper limit of normal) and thrombocytes (>upper limit of normal)

Genotyping

IL8 rs4073 was excluded from statistical analysis because the call rate was less than 95%. The SNP genotype call rate for the remaining SNPs ranged from 97.6 to 99.7%. All SNPs were in Hardy-Weinberg equilibrium (HWE) (P>0.05 and χ^2<3.84). The allele frequencies of genotyped polymorphisms were similar as reported in the National Center for Biotechnology Information (NCBI) SNP database.[23] Only three patients with the *HIF1A* rs11549467 heterozygous genotype were detected. As a consequence, the effects of this SNP could not be analysed.

Genetic association analysis

No significant associations between SNPs and PFS, OS or objective response to sunitinib were observed in this study. Although not significant, A-allele carriers of *MET* rs11762213 had a better PFS compared to wild-type GG (HR=0.63, 95% CI=0.38-1.05, P=0.076), adjusted for age, gender, Heng prognostic risk group, study group and CGT haplotype in *ABCB1* (**Table 3**). In multivariate toxicity analysis, the T allele of *IL8* rs1126647 was associated with an increased risk of hypertension compared to wild-type AA (OR=1.69, 95% CI=1.07-2.67, P=0.024) after adjustment by age, gender and study group. Presence of the *IL13* rs1800925 T allele was associated with an increased risk of leukopenia (OR=6.76, 95% CI=1.35-33.9, P=0.020) and also with development of any toxicity >grade 2 (OR=1.75, 95% CI=1.06-2.88, P=0.028) (**Table 3**).

DISCUSSION

In the present study, we analysed eight candidate SNPs in seven genes for potential association with response and/or toxicity of sunitinib in a large cohort of mRCC patients. To our knowledge, we report for the first time that genetic polymorphisms in interleukin genes *IL8* and *IL13* are associated with the occurrence of clinically relevant adverse events from sunitinib. Patients carrying a T allele of *IL8* rs1126647 had an increased risk of hypertension compared to wild-type AA (OR=1.69). T carriers of *IL13* rs1800925 had an increased risk of leukopenia (OR=6.76) and an increased risk of any toxicity >grade 2 (OR=1.75).

Hypertension is a frequent toxicity caused by TKIs. The potential mechanism is suggested to be related to inhibition of VEGFR-2 and decrease of nitric oxide, resulting in vasoconstriction and elevated blood pressure.[8,24] It has been recognized that IL8, possibly by upregulating VEGF levels through NFkappaB[25,26], can play a role in stimulating VEGFR-2 transactivation. In our data, presence of the variant allele in *IL8* rs1126647A>T is significantly associated with hypertension. *IL8* rs1126647A>T is in linkage with *IL8*

Table 3. Univariate and multivariate analyses for SNPs associated with sunitinib treatment outcome

Treatment outcome	Number of patients	Univariate analyses			Multivariate analyses		
		HR or OR	95%CI	*P* value	HR or OR	95%CI	*P* value
PFS							
MET rs11762213 G>A		0.58	0.36-0.96	0.032	0.63	0.38-1.05	0.076[a]
GG versus	302						
AG+AA	33						
Hypertension							
IL8 rs1126647 A>T		1.70	1.08-2.68	0.022	1.69	1.07-2.67	0.024[b]
AA versus	128						
AT versus	174						
TT	61						
Thrombocytopenia							
IL13 rs20541 C>T		1.97	0.90-4.35	0.092	1,87	0,82-4,23	0,136[a]
CC versus	235						
CT+TT	117						
Leukopenie							
IL13 rs1800925 C>T		6.13	1.25-30.0	0.025	6.76	1.35-33.9	0.020[b]
CC versus	232						
CT+TT	137						
Any toxicity>grade 2							
IL13 rs1800925 C>T		1.91	1.15-3.15	0.012	1.75	1.06-2.88	0.028[b]
CC versus	234						
CT+TT	137						

HR hazard ratio, OR odds ratio, CI confidence interval, PFS progression-free survival

[a]Adjusted by age, gender, Heng prognostic risk group, study center and ABCB1 haplotype

[b]Adjusted by age, gender and study center

rs4073T>A (r^2=0.78).[16] It was shown that a haplotype of *IL8*, including the variant allele of rs4073T>A and rs1126647A>T, was associated with increased IL8 expression.[27] In contrast, Amaya *et al*.[28] have reported that the variant allele of rs4073 was associated with a lower production of IL8. It remains to be elucidated whether particular SNPs in *IL8* have prognostic or even predictive significance in metastatic renal cell carcinoma.

The occurrence of hypertension is a well-known predictor for increased survival in metastatic renal cell carcinoma treated with a TKI.[29] In addition to an increased occurrence of hypertension, patients with the variant allele in *IL8* rs1126647A>T showed a trend towards improved PFS/OS (22 vs 14 months for median PFS and 40 vs 25 months for median OS), although the differences between groups did not reach statistical significance. One possible reason for the lack of statistical significance in the

above association may be the ~2 fold larger (N=544) sample size of the study from Rini *et al*.[29] Xu *et al*.[16] described an inferior PFS of pazopanib for carriers of the variant allele of *IL8* rs1126647A>T. In a follow-up study, Xu *et al*.[30] reported that the variant allele of *IL8* rs1126647A>T was significantly associated with worse OS in mRCC patients treated with pazopanib or sunitinib, but could not confirm the initial association with PFS. Unfortunately, Xu *et al*. do not report data on associations between *IL8* rs1126647 and hypertension. In addition, many factors can have an impact on OS, including baseline conditions and previous and posterior treatments. Therefore, it is not possible to directly compare the association of *IL8* rs1126647 and hypertension/survival between the two datasets and further study in an independent cohort is required.

We observed an increased risk of leukopenia in carriers of the variant allele of *IL13* rs1800925C>T. Leukopenia is a common haematologic adverse event in sunitinib treatment of which the mechanism remains uncertain. It was reported that VEGF and its receptors (VEGFR) are essential for development of aberrant haematopoiesis, including leukopenia.[31] Shen *et al*.[32] have reported that VEGFR-2 can be upregulated by IL13. The T allele of *IL13* rs1800925 has been found to be associated with increased IL13 protein function.[33]

Little is known about the effects of SNPs in *IL8* and *IL13*, but it is likely that these SNPs will either increase or decrease the protein expression of IL8 and IL13 proteins.[24-26, 29,31-34] It also remains unclear whether the function of these interleukins will affect TKI treatment outcome and if and how these interleukin proteins would influence the VEGF(R) pathway directly or indirectly.[25-28, 30-36] Xu *et al*.[30] speculate that patients with the *IL8* variants having a high IL8 expression may have more aggressive tumors, and therefore a reduced survival. However, it is difficult to hypothesize because we noticed conflicting results: Hacking *et al*.[27] reported increased expression for *IL8* variants, while a more recent paper of Amaya *et al*.[28] reported the opposite effect on IL8 expression. If variant alleles in *IL8* and *IL13* SNPs alter protein expression and have an effect on VEGF-R2, a stronger inhibition of VEGF-R2 in the concomitant presence of sunitinib would explain our results on a higher risk for any toxicity, including hypertension and leukopenia.

HIF1A protein regulates the transcription of a large number of genes that respond to hypoxia, among which is angiogenesis. The A-allele frequency of *HIF1A* rs11549467 in our cohort was 0.45%, which is lower than that (A=3.0%) reported by Xu *et al*.[16], but corresponds with the result (A=0.6%) from Beuselinck *et al*.[10] The difference in A-allele frequency is potentially caused by the subject selection. Of note, the subjects recruited here and in the study of Beuselinck typically have Western European ancestry, whereas the patients in Xu's study are mostly from Eastern Europe.[10,16]

This study was conducted in a relatively large cohort, which decreases the chance for false-positive findings. In addition to Xu *et al*.[30], we have tested on toxicity as well

as efficacy endpoints, which is essential for clinical interpretation. SNPs could influence the drug exposure to the TKI and consequently have an effect on both adverse events, PFS and OS. Because this study only presents explorative findings without external validation, and our results are based on mainly Caucasian subjects, extrapolation to the entire mRCC population is difficult.

To capture adverse events that occur later on in treatment, all toxicity outcomes were recorded and evaluated up to four cycles of sunitinib treatment. In our cohort, hypertension and fatigue have a large contribution in the endpoint any toxicity >grade 2. Because it is difficult to evaluate fatigue objectively, we did not test fatigue as separate toxicity endpoint. Since our study represents data collected from 2004 to 2010, it is likely that clinical practice (i.e. toxicity management) has evolved in the course of time. In earlier years with limited experience on sunitinib treatment, dose reductions were applied in the case of grade 3 or 4 toxicities. Nowadays, physicians anticipate on the development of severe adverse events by already reducing the dose on the occurrence of grade 2 toxicity. However, we did not observe a difference in dose reductions between earlier years and later years (data not shown). Further, our dataset lacks sufficiently detailed information on the reasons and time points of dose reductions.

Our SNP selection was based on reported associations with toxicity and efficacy outcomes on treatment and the risk of developing RCC. SNPs are often categorized as being either predictive or prognostic biomarkers. However, this distinction may not have to be as rigorous as we currently assume. In fact, prediction and prognosis may be strongly correlated. Antitumor treatment can have a different effect in patients with a more aggressive tumor type that is possibly caused by an underlying SNP. For example, high IL8 protein levels in RCC are considered to have a poor prognosis.[35] However, elevated IL8 levels investigated in preclinical models resulted in resistance to sunitinib, which would be considered predictive.[36] Functional studies on immunological and angiogenic factors and a genome-wide approach can help us in understanding the mechanisms on the predictive or prognostic character of the SNPs.

In conclusion, this study suggests a relationship between interleukin genes *IL8* and *IL13* and the development of sunitinib-induced adverse events. Further validation in an independent cohort is warranted to confirm our findings. In addition, we consider functional studies on IL8 and IL13, with respect to regulation of VEGF(R) genes and sVEGF(R) plasma levels, to be crucial for our understanding of the mechanisms involved in sunitinib exposure and occurrence of adverse events.

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SUPPLEMENTARY MATERIAL

Supplementary document 1 – Additional methods

Study Population

The SUTOX consortium includes five medical centers in the Netherlands, the Spanish Oncology Genitourinary Group (SOGUG) consists of fifteen Spanish medical centers, and CCF represents the Taussig Cancer Institute of Cleveland Clinic Foundation in the United States. Patient characteristics with details on treatment, efficacy and toxicity were retrieved from the individual records. Sunitinib was administered orally either as first or second line treatment at a starting dose of 50mg, 37.5mg or 25mg daily, generally given as a 4-weeks-on and 2-weeks-off treatment cycle or as a continuous dosing regimen. Dose modification or dose delay was done for higher grades of adverse events. Eligible patients for the current study received sunitinib for at least one cycle of 6 weeks, and had a blood sample available for DNA extraction.

Genotyping

Germline DNA was extracted from whole blood, serum, plasma or peripheral blood mononuclear cell samples. Based on previous studies, we selected ten polymorphisms in genes *CYP3A4*, *NR1I2*, *POR*, *IL8*, *HIF1A*, *IL13*, *IL4-R* and *MET* (**Table 1**), which might be associated with sunitinib treatment.[6,13-20] Full genotyping methods are described in our previous study.[12] SUTOX and CCF samples were genotyped at Leiden University Medical Center (LUMC) using Taqman probes (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) on the LightCycler480 (LC480) Real-Time PCR Instrument (Roche Applied Science, Almere, The Netherlands). SOGUG samples were genotyped by the Spanish National Cancer Research Centre (CNIO) using a KASPar SNP genotyping system (Kbiosciences, Hoddesdon, UK) and the sequence Detection System 7900HT (Applied Biosystems, Foster City, CA, USA) for fluorescence detection and allele assignment.

To exclude inter assay variation between Leiden University Medical Center (LUMC) and Spanish National Cancer Research Centre (CNIO), a selection of 132 anonymized SOGUG DNA samples were re-genotyped in Leiden. A 98% concordance in SNP genotypes was observed between the LUMC and CNIO samples.[12]

Study Endpoints

Patients with unknown response status were excluded from response analysis. Cumulative toxicity for the first four treatment cycles was corrected by baseline conditions, and was used as dichotomous groups, grade 0-2 and grade 3-4.[12]

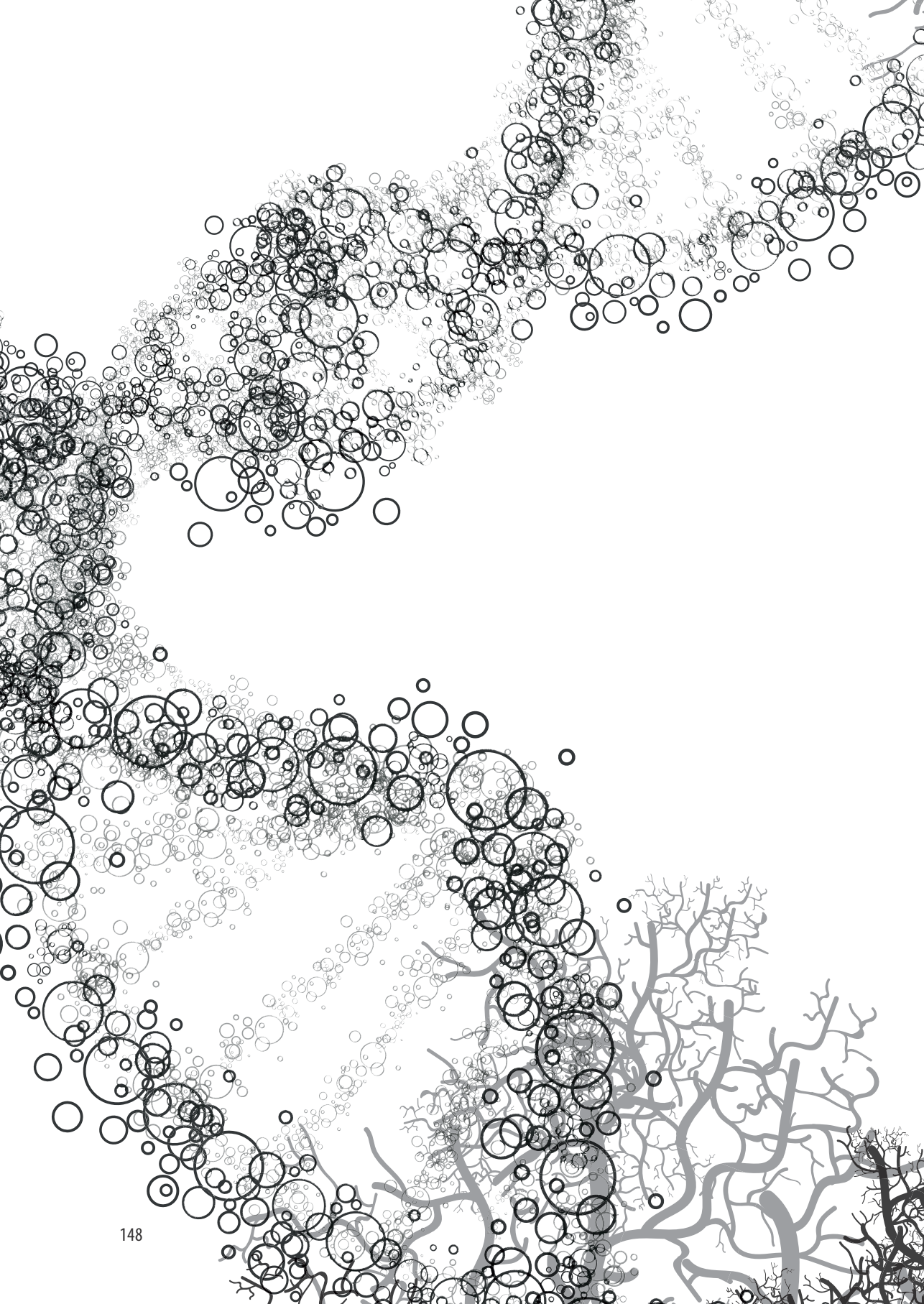
Adverse events were evaluated at baseline, after 4 weeks and 6 weeks in each cycle using the National Cancer Institute Common Terminology Criteria for Adverse Events v.3.0 or v.4.0.[12]

Statistical analysis

We fitted dominant and additive genetic models in this study, depending on the distribution of genotypes of each SNP (common homozygous, heterozygous and variant homozygous).

Supplementary Table S1. Distribution of most common adverse events within four cycles of sunitinib treatment

Toxicity by grade	No. of patients (N)	Percentage of patients (%)
Fatigue		
0	118	32
1	117	31
2	100	27
3	37	9.9
4	2	0.50
Thrombocytopenia		
0	145	39
1	148	40
2	49	13
3	28	7.5
4	4	1.1
Mucosal inflammation		
0	154	41
1	123	33
2	75	20
3	22	5.9
Leukopenia		
0	189	51
1	106	28
2	67	18
3	12	3.2
Hand-foot-syndrome		
0	222	59
1	69	18
2	57	15
3	26	7.0
Hypertension		
0	231	62
1	37	10
2	55	15
3	51	14
Diarrhoea		
0	227	61
1	90	24
2	44	12
3	13	3.5
Any toxicity >grade 2		
0, 1, 2	227	74
3, 4	97	26



CHAPTER 7

Evaluation of *KDR* rs34231037 as predictor of sunitinib efficacy in patients with metastatic renal cell carcinoma.

Apellániz-Ruiz M, Diekstra MH, Roldán JM, Boven E, Castellano D, Gelderblom H, Mathijssen RHJ, Swen JJ, Böhringer S, García-Donas J, Rini BI, Guchelaar H-J, Rodríguez-Antona C.

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ABSTRACT

Objective The identification of biomarkers able to predict clinical benefit from vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors is urgently needed. Recently, Maitland and colleagues described an association between *KDR*-rs34231037 and soluble VEGFR2 levels as well as pazopanib pharmacodynamics. We investigated in a well-characterized series of metastatic clear cell renal cell carcinoma patients whether rs34231037 could influence sunitinib response.

Methods Clinical data and DNA were available from an international series of 276 patients. *KDR*-rs34231037 association with sunitinib response, clinical benefit, and progression-free survival was analyzed using logistic and Cox regression analyses.

Results We found that G-allele carriers were over-represented among patients with clinical benefit during sunitinib treatment compared with those refractory to the treatment (odds ratio:3.78, 95%CI:1.02-14.06; *P*=0.047; multivariable analysis).

Conclusion In conclusion, rs34231037 variant carriers seemed to have better sunitinib response than wild-type patients. Moreover, the association with tumor size reduction suggests that this single nucleotide polymorphism might also identify patients with successful tumor downsizing under anti-VEGFR therapy.

INTRODUCTION

Renal cell carcinoma (RCC) accounts for 2-3% of all malignancies in adults. In most RCC, an aberrant activation of the vascular endothelial growth factor (VEGF) pathway is present because of inactivation of the Von Hippel–Lindau tumor-suppressor gene. This event leads to increased tumor vascularization that sustains tumor growth and metastasis. The development of drugs targeting VEGF or their receptors (VEGFR) has resulted in effective treatment options for patients with metastatic renal cell carcinoma (mRCC).[1] Sorafenib, sunitinib, pazopanib, axitinib, and cabozantinib are angiogenic inhibitors approved for the treatment of mRCC. These small-molecule tyrosine kinase inhibitors (TKIs) show high affinity for key proteins mediating tumor angiogenesis: VEGFR1, VEGFR2, VEGFR3, and platelet-derived growth factor receptor, among other targets. However, despite their effectiveness in RCC, a large interindividual variability has been observed in terms of drug response and toxicity.[2] Consequently, the identification of markers predictive of the response to these TKIs is urgently needed.

Antiangiogenic TKIs exert their effect on blood vessels rather than directly on tumor cells, suggesting that the patients' genomic profile could influence drug response. In fact, we and others have found single nucleotide polymorphisms (SNPs) associated with TKI therapy response.[3-8] Recently, Maitland *et al*.[9], through a Genome Wide Association Study, identified and validated an SNP in *KDR* (rs34231037; p.C482R) as the top hit associated with variations of soluble VEGFR2 (sVEGFR2) in blood. In addition, analysis of sVEGFR2 in RCC patients treated with pazopanib showed an association between the minor allele of this SNP and lower sVEGFR2 levels both at baseline and after 4 weeks of pazopanib treatment. sVEGFR2 is derived from an alternative splicing in the *KDR* gene.[10] In patients in whom sVEGFR2 was measured at baseline and during TKI treatment, a greater decrease in sVEGFR2 has been associated with increased response to the treatment.[11,12]

This suggests that rs34231037 might be predictive of anti-VEGFR2 drugs' outcome. However, to date, the association of rs34231037 with sunitinib response and progression-free survival (PFS) in patients remains unexplored. Here, we investigated the effect of *KDR*-rs34231037 on patient outcome in a large series of metastatic clear cell RCC patients treated with sunitinib.[5]

METHODS

Patients and sunitinib outcome

Patients' samples were collected by the Dutch SUTOX consortium, the Spanish Oncology Genitourinary Group (SOGUG), and the Taussig Cancer Institute of Cleveland Clinic

Foundation (CCF) as described previously.[6,13-16] In brief, from the original series of 333 clear cell RCC patients previously described[5], 42 samples were lost because of lack of DNA, 12 were eliminated because patients received previous anti-VEGF treatment, and three samples failed genotyping. Thus, 276 patients receiving sunitinib as first anti-VEGF therapy were analyzed for drug response and PFS. The study was approved by the corresponding ethical review boards.

Genotyping

Germline DNA was used to genotype rs34231037 (p.C482R). SUTOX and CCF samples were genotyped using a Taqman probe and SOGUG samples using the KASP genotyping chemistry (LGC, Teddington, UK). Genotype frequencies met Hardy–Weinberg equilibrium.

Statistical analysis

To investigate rs34231037 association with sunitinib response, patients were divided into groups according to their best response to this drug: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Logistic regression analysis was used to perform comparisons between CR/PR vs. SD/PD and between CR/PR vs. PD. In a subsequent analysis, we compared patients with clinical benefit defined as those with CR, PR, or SD longer than 1 year versus those who had PD or SD shorter than 1 year. Kaplan-Meier and Cox regression analyses were used to analyze PFS. In multivariate analyses, we adjusted for Heng prognostic group[17], age, sex, and study center.

RESULTS

We analyzed a series of 276 clear cell RCC patients receiving sunitinib as first anti-VEGF therapy. The majority of patients were men (68%), White (96%), had nephrectomy (84%), had received no previous antitumor treatment (86%), and the median age at the start of sunitinib was 61 years. The median PFS during sunitinib treatment was 16.0 months (95% CI=13.3-18.8) and 9, 116, 99, and 35 patients showed a CR, PR, SD, and PD, respectively.

We analyzed the association of rs34231037 with sunitinib response using univariate and multivariable analyses. We found a trend toward a better response among G-allele carriers compared with wild-type patients (multivariable analysis, odds ratio (OR):2.58; 95% CI: 0.95–6.99; P=0.062 for CR, PR or SD vs. PD and OR: 3.17; 95% CI:0.62–16.18; P= 0.165 for CR or PR vs. PD **Table 1**). Analyzing each subcohort of patients separately, we found the strongest association in the Spanish subcohort (**Figure 1** and **Supplementary**

Table 1, **Supplemental digital content 1**, http://links.lww.com/FPC/B209). When studying sunitinib clinical benefit, differences became statistically significant, with the G-allele carriers having better sunitinib outcome (OR:3.78; 95% CI: 1.02–14.06; P=0.047; **Figure 1** and **Table 1**).

In terms of PFS, time to progression of rs34231037 G-allele carriers was similar to that of wild-type individuals when considering the full series of patients (15.4 and 16.0 months, respectively; **Figure 1g** and **Table 1**). However, in the Spanish subcohort, rs34231037 G-allele carriers showed a longer PFS than wild-type patients (17.9 compared with 12.3 months; **Figure 1h**), although the difference was not statistically significant.

Table 1 Association of rs34231037 with response to sunitinib and progression-free survival

Clinical variables	Number of patients	Univariate		Multivariate[a]	
		OR/HR[b] (95% CI)	*P* value	OR/HR (95%CI)	*P* value
Response[c]					
CR, PR vs SD, PD	259	2.11 (0.81-5.46)	0.126	2.58 (0.95-6.99)	**0.062**
CR, PR vs PD	160	1.92 (0.41-8.92)	0.408	3.17 (0.62-16.18)	0.165
Clinical benefit[c]					
CR, PR, SD >1 years vs PD, SD <1 year	246	**2.93 (0.83-10.30)**	**0.094**	**3.78 (1.02-14.06)**	**0.047**
PFS	276	1.05 (0.61-1.82)	0.852	1.23 (0.7-2.15)	0.466

CR, complete response; HR, hazard ratio; OR, odds ratio; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; SOGUG, Spanish Oncology Genitourinary Group.

[a]Multivariate analyses included as covariates Heng prognostic risk group, age, sex, and study center.

[b]The data correspond to the effect of the wild-type A-allele of rs34231037, where OR/HR >1.0 indicates an increased risk of poor response/shorter PFS.

[c]Logistic regression comparing the groups of patients indicated.

Bold values indicate significant level *P*<0.1.

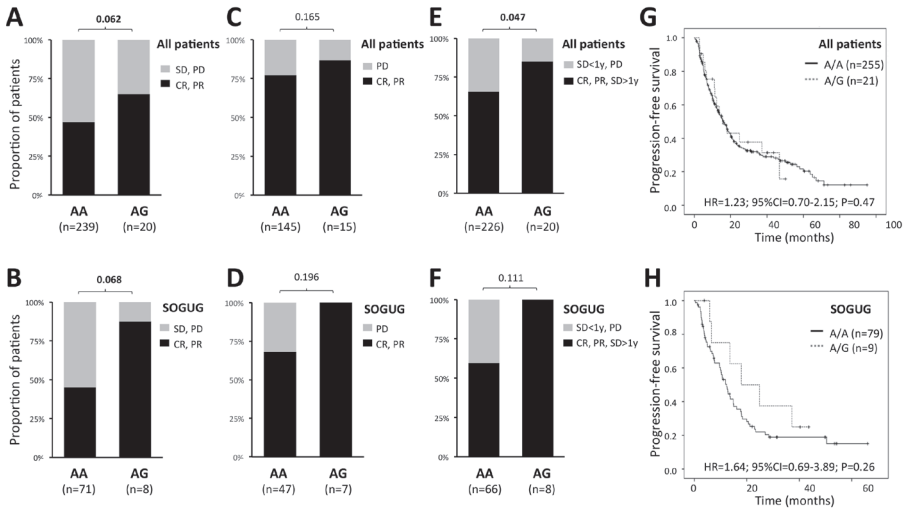

Figure 1 Effect of *KDR* rs34231037 on sunitinib response and PFS in metastatic ccRCC patients. Sunitinib response (responder defined as those with CR or PR) in the full series of patients (a) and in SOGUG series (b). Sunitinib response (responders defined as those with CR, PR, or SD longer than 1 year) in the full series of patients (c) and in SOGUG series (d). Sunitinib clinical benefit in the full series of patients (e) and in SOGUG series (f). PFS in the full series of patients (g) and in SOGUG series (h) according to the rs34231037 genotype. The P values correspond to multivariate analyses. ccRCC, clear cell renal cell carcinoma; CI, confidence interval; CR, complete response; HR, hazard ratio; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; SOGUG, Spanish Oncology Genitourinary Group; Time, time from the start of sunitinib treatment until PD (months).

DISCUSSION

Recently, Maitland *et al*.[9] identified rs34231037 as the most relevant SNP associated with sVEGFR2 baseline levels as well as with the extent of decline in sVEGFR2 upon pazopanib treatment. Our results suggest that rs34231037 variant carriers might have a better response to sunitinib treatment (**Figure 1**).

The relatively low MAF of rs34231037 in the White population results in a relatively small number of patients carrying the variant allele (21 out of 276) and decreased the statistical power of the analysis. Thus, the weak association found in this study indicates that *KDR* rs34231037 does not have a strong effect, although variant carriers seem to respond better to sunitinib. The association with tumor size reduction suggests that

this SNP might also be relevant to identify patients in whom tumor downsizing with TKI therapy before surgery would be successful.

We observed variability in the results among the series. In the SOGUG group, all patients received sunitinib as first line, whereas 20 and 22% of SUTOX and CCF patients received previous antitumor treatments. There were also differences in prognosis, sunitinib response, and PFS among the series. 11, 23, and 36% of SOGUG, SUTOX, and CCF patients had a poor prognosis; 19, 14, and 6% of patients had PD as the best response; and the median PFS was 13, 15, and 35 months in these series, respectively. This suggests that differences in the basal characteristics of the patients could be relevant for the effect of the SNP.

The sVEGFR2 soluble protein has been shown to be a physiologic inhibitor of developmental and reparative lymphangiogenesis in mice.[10] As carriers of the *KDR* rs34231037 G-allele have lower sVEGFR2 levels[9], the improved sensitivity to anti-VEGFR drugs might be explained by an intrinsic increased tumor lymphangiogenesis; however, this remains to be investigated.[10] How rs34231037 leads to decreased expression of sVEGFR2 is unknown. As sVEGFR2 derives from an alternative splicing of the *KDR* gene, one possibility would be that rs34231037, located in the immunoglobulin-like domain V of the VEGFR2, might be altering the splicing site signals in exon 11. Alternatively, it could lead to decreased stability of the mRNA or protein product. In addition, rs34231037 has been described as a risk factor for infantile hemangioma and when present in full-length VEGFR2 protein, it has been shown to alter the angiogenesis, decreasing the formation of complexes with β1 integrin and decreased VEGFR1 expression.[18]

In *VEGFR2* immunoglobulin-like domain V, there is one high-frequency missense polymorphism (rs1870377, p.Q472H) for which we described a trend toward shorter PFS and OS and an increased risk of hypertension upon sunitinib treatment in mRCC[5,6], and others have described higher levels of VEGF in sera.[19] In addition, in the immunoglobulin-like domain V of *VEGFR3*, a common missense variant (rs307826; p.T494A) has been associated with sunitinib response and PFS in RCC patients[6,7] and with altered VEGFR3 protein expression in tumors.[20] These results, together with the fact that the extracellular domain V of VEGFRs is essential for dimer formation and activation[21], suggest that variants altering this domain may influence the effect of anti-VEGFR TKIs.

The limitations of our study include that patients were treated with sunitinib, but in the study by Maitland *et al*.[9], patients were treated with pazopanib. Although both sunitinib and pazopanib inhibit VEGFRs, the binding affinity is not identical.[22] Thus, we can only speculate that rs34231037 would exert a similar effect on pazopanib-treated and sunitinib-treated patients. In addition, although the effect of rs34231037 on sVEFGR2 was large (explaining 23% of the variance[9]), the effect of the SNP on tumor response to TKI seems smaller. Because of the lack of serum samples in this study, measurement of sVEGFR2 levels during sunitinib treatment was not possible.

In conclusion, in this study, in a large and well-characterized series of sunitinib-treated patients, we report a potential moderate effect for *KDR*-rs34231037 on sunitinib response. Although Maitland and colleagues proposed that rs34231037 influenced pazopanib treatment outcome, this remains to be determined. Large series of patients treated with different anti-VEGFR TKIs and with sVEGFR2 pharmacodynamics and tumor response data are needed to validate these results and establish the clinical relevance of rs34231037 as a marker for antiangiogenic response.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1 Results of rs34231037 for response to sunitinib and PFS in the different study centers.

Clinical variable	Series of patients	Number of patients	Univariate		Multivariate[a]	
			OR/HR[b] (95%CI)	*P* value	OR/HR (95%CI)	*P* value
Response CR, PR vs SD, PD	All series	259	2.11 (0.81-5.46)	0.126	**2.58 (0.95-6.99)**	**0.062**
	SUTOX	116	2.09 (0.53-8.26)	0.291	2.50 (0.61-10.29)	0.204
	SOGUG	79	**8.53 (1.00-73.01)**	**0.050**	**7.74 (0.86-69.56)**	**0.068**
	CCF	64	0.26 (0.02-3.10)	0.286	0.33 (0.03-4.25)	0.398
Response CR, PR vs PD	All series	160	1.92 (0.41-8.92)	0.408	3.17 (0.62-16.18)	0.165
	SUTOX	61	0.88(0.15-5.03)	0.881	1.28 (0.19-8.50)	0.799
	SOGUG	54	1.22 (1.05-1.41)	0.171[f]	1.22 (1.05-1.41)	0.196[g]
	CCF	45	NC[e]	1.000	NC[e]	1.000
Clinical benefit[d] CR, PR, SD>1y vs PD, SD<1y	All series	246	**2.93 (0.83-10.30)**	**0.094**	**3.78 (1.02-14.06)**	**0.047**
	SUTOX	110	2.11 (0.42-10.69)	0.367	2.51 (0.47-13.50)	0.282
	SOGUG	74	**1.20 (1.06-1.36)**	**0.044[f]**	NC[e]	0.111[g]
	CCF	62	0.57 (0.05-6.70)	0.652	0.83 (0.04-17.88)	0.908
PFS	All series	276	1.05 (0.61-1.82)	0.852	1.23 (0.7-2.150)	0.466
	SUTOX	124	1.01 (0.44-2.32)	0.988	0.89 (0.38-2.08)	0.803
	SOGUG	88	1.59 (0.68-3.70)	0.281	1.64 (0.69-3.89)	0.261
	CCF	64	0.57 (0.14-2.42)	0.45	0.61 (0.14-2.65)	0.512

[a] Multivariate analyses included as covariates Heng prognostic risk group, age, gender and study center.

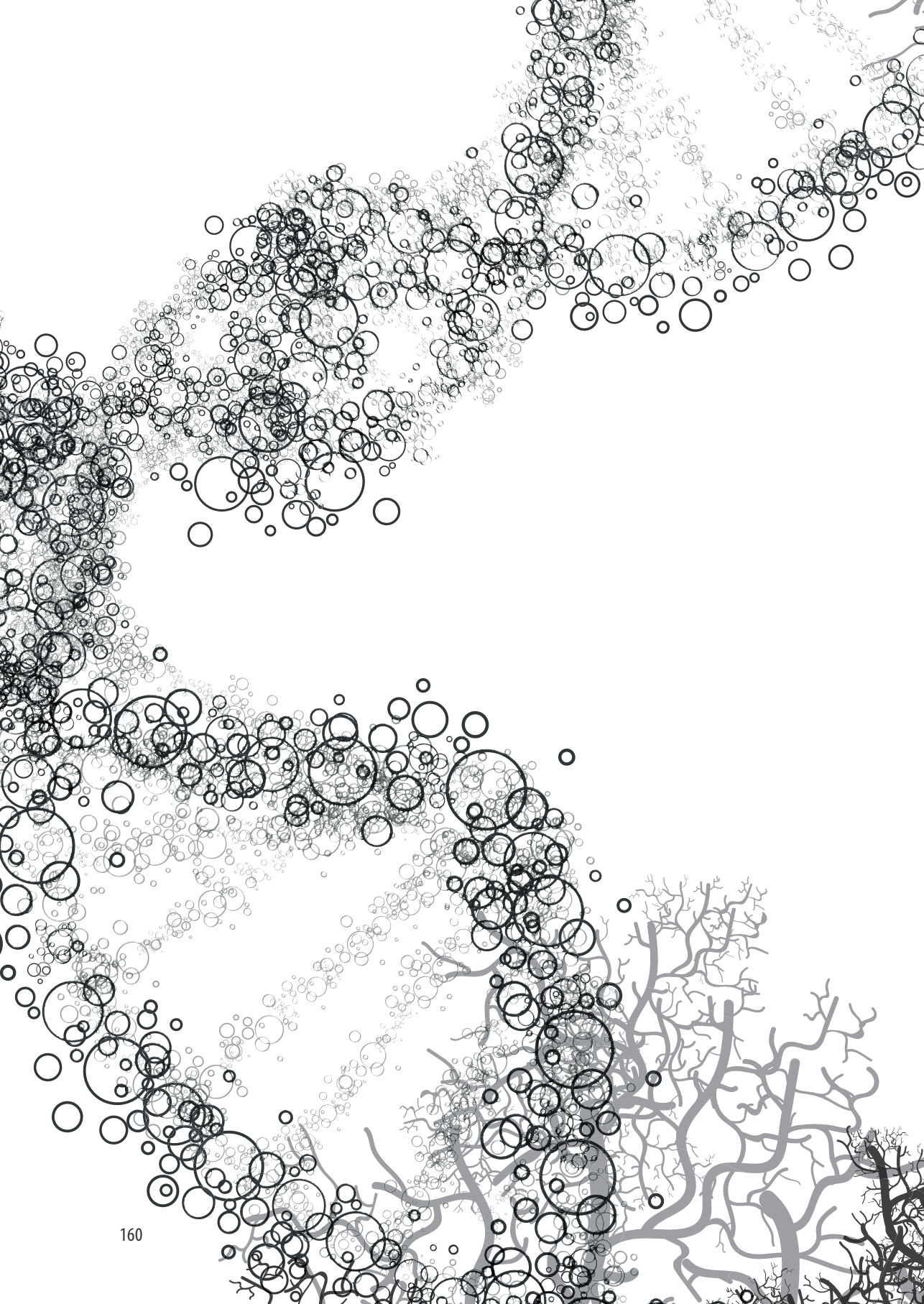
[b] The data corresponds to the effect of the wild type A-allele of rs34231037 where OR/HR>1.0 indicates increased risk of poor response/ shorter PFS.

[d] Logistic regression comparing patients with CR, PR or SD >1 year versus patients with SD <1 year/ PD upon sunitinib treatment.

[e] The associated risk could not be calculated (NC, not calculated).

[f] Since all variant carriers belonged to one group, regression analysis results were not conclusive and Fisher exact test was used.

[g] Since all variant carriers belonged to one group Mantel-Haenszel test was used.



CHAPTER 8

Population modelling integrating pharmacokinetics, pharmacodynamics, pharmacogenetics and clinical outcome in sunitinib-treated cancer patients

Diekstra MH*, Fritsch A*, Kanefendt F*, Swen JJ, Moes DJAR, Sörgel F, Kinzig M, Stelzer C, Schindele D, Gauler T, Hauser S, Houtsma D, Roessler M, Moritz B, Mross K, Bergmann L, Oosterwijk E, Kiemeney B, Guchelaar H-J, Jaehde U.

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ABSTRACT

The tyrosine kinase inhibitor sunitinib is used as first-line therapy in patients with metastasized renal cell carcinoma (mRCC), given in fixed-dose regimens despite its high variability in pharmacokinetics. Inter-individual variability of drug exposure may be responsible for differences in response. Therefore, dosing strategies based on pharmacokinetic and pharmacodynamics (PK/PD) models may be useful to optimize treatment. Plasma concentrations of sunitinib, its active metabolite SU12662 and the soluble VEGF receptors sVEGFR-2 and sVEGFR-3 were measured in 26 mRCC patients within the EuroTARGET project and 21 metastasized colorectal cancer (mCRC) patients from the C-II-005 study. Based on these observations, PK/PD models with potential influence of genetic predictors were developed and linked to time-to-event (TTE) models. Baseline sVEGFR-2 levels were associated with clinical outcome in mRCC patients, whereas active drug pharmacokinetics seemed to be more predictive in mCRC patients. The models provide the basis of PK/PD-guided strategies for the individualization of anti-angiogenic therapies.

INTRODUCTION

Sunitinib is a multi-target tyrosine kinase inhibitor (TKI), which is successfully used in the treatment of metastasized renal cell carcinoma (mRCC), gastrointestinal stromal tumors (GIST) and other solid tumor types. Sunitinib inhibits the vascular endothelial growth factor receptors (VEGFR-1, 2 and 3), the platelet-derived growth factor receptor (PDGFR) α and β, among other tyrosine kinases.[1,2] CYP3A4 converts sunitinib into its active *N*-desethyl metabolite (SU12662) and subsequently into inactive metabolites. The elimination half-life of sunitinib is 40-60 hours and 80-110 hours for SU12662. An increased exposure to sunitinib is associated with improved survival but also with an increased risk for adverse events.[3,4]

The individual response to sunitinib is highly variable: some patients experience severe toxicity and need dose reductions or even cessation of therapy whereas others show no response at all when using the same dose. Biomarker testing prior to start or during therapy may help provide the individual patient with the most effective treatment and the lowest possible risk of adverse effects. Whereas several potential biomarkers have been identified, they are not applied in clinical routine yet. However, sunitinib meets the requirements for therapeutic drug monitoring (TDM) enabling dose adjustment based on measured plasma drug concentrations.[3-5]

Soluble VEGFR-3 (sVEGFR-3) was observed to be a potential predictive biomarker for overall survival (OS) on sunitinib treatment in a study on 303 patients diagnosed with GIST.[6] Furthermore, VEGF-A and VEGFR-3 protein expression were associated with OS and progression-free survival (PFS), respectively, in 67 sunitinib-treated mRCC patients.[7] Likewise, levels of VEGF, sVEGFR-2, and sVEGFR-3 were associated with objective response in 63 mRCC patients.[8] With regard to genetic predictors, previous studies associated Single Nucleotide Polymorphisms (SNPs) in genes encoding metabolizing enzymes or transporters related to pharmacokinetics (PK) and pharmacodynamics (PD) of sunitinib with efficacy and toxicity.[9-22]

In order to find predictive biomarkers for the clinical outcome of sunitinib, a better understanding of the relationships between sunitinib exposure, the pharmacological response, and the clinical outcomes is vital. This is part of the objectives of the European collaborative project EuroTARGET.[23] Several pharmacokinetic models for sunitinib have previously been published. Here, we used a nonlinear mixed-effects PK model for analyzing data of both mRCC and metastasized colorectal cancer (mCRC) patients in a pooled data set.[23-26] This model was linked to pharmacodynamic models for sVEGFR-2 and sVEGFR-3 previously developed by our group.[27] The aim of our study was the development of pharmacokinetic models, linking sunitinib plasma concentrations to pharmacodynamic response and clinical outcome in a model-based time-to-event analysis including the identification of potential genetic predictors.

METHODS

Patient population

For the underlying PK/PD analysis, data were used from two pharmacokinetic studies which focused on sunitinib treatment in mRCC and mCRC patients.[23,25] Both studies were designed as prospective, open label, single arm, multicenter, non-randomized studies and performed in accordance with the Declaration of Helsinki. Patients gave written informed consent to give venous blood for PK/PD analsysis and genotyping taken in course of routine blood draw, and allowed the study sites to document clinical data.

The C-IV-001 study (EudraCT-No.: 2012-001415-23) was a phase IV PK/PD sub-study of the non-interventional EuroTARGET project.[23] Patients with mRCC were recruited in 9 medical centers in Germany and The Netherlands. Sunitinib doses ranged from 37.5 to 50 mg daily in the 4 weeks on/2 weeks off scheme. A patient was eligible for this study with a minimum age of 18 years, a diagnosed mRCC and a first-line treatment with sunitinib. Within the EuroTARGET project progression-free survival (PFS) was evaluated as primary endpoint.[23]

The C-II-005 study (EudraCT-No: 2008-00151537) was performed to investigate the beneficial effect of suntinib as add-on to biweekly FOLFIRI (folinate, fluorouracil, and irinotecan) in patients with mCRC and liver metastases.[25] Patients received a daily dose of 37.5 mg sunitinib on a 4 weeks on/2 weeks off treatment schedule. Primary endpoints were the reduction of tumor vessel permeability and blood flow determined by imaging techniques. Time to progression (TTP) was defined as a secondary endpoint. In case of toxicity sunitinib therapy was interrupted or continued after dose reduction to 25 mg per day until the symptoms disappeared.[25]

Data collection and sampling

Clinical information was collected, especially demographic characteristics, concomitant medication, clinical response to the treatment, and toxicity. Serial blood samples were drawn, immediately centrifuged (1000 g, 4 °C, 15 min) and stored at -80 °C. In the C-IV-001 study, up to 12 plasma samples were collected within three cycles during routine check-ups. Except for a mandatory baseline sample before treatment start, each centre was free to develop a schedule according to their specific clinical routine. In the C-II-005 study, plasma samples were collected within two cycles at baseline, day two of each cycle and afterwards approximately every two weeks, always before sunitinib intake.

Plasma concentrations of sunitinib and SU12662 were determined using high-performance liquid chromatography coupled with mass spectrometry (LC–MS/MS, MDS SCIEX API 5000 triple quadrupole mass spectrometer; Applied Biosystems/MDS SCIEX, Thornhill, Ontario, Canada). Between-run precision and accuracy ranged from 1.6 to 6.1% and 0.2 to 9.1% for sunitinib and from 1.1 to 5.3% and -0.1 to 6.2% for SU12662,

respectively.[28] sVEGFR-2 concentrations were determined by commercially available ELISA kits (R&D Systems, Minneapolis, MN). The soluble VEGFR-3 was measured using a validated immunoassay.[26] Within-laboratory precision and accuracy of all assays were within the acceptance criteria of the EMA[29] with 2.2 to 4.3% and 6.2 to 14.3% for sVEGFR-2, and 0.4 to 14.7% and -3.8 to +16.2% for sVEGFR-3, respectively. Quality control (QC) samples were analyzed in all assays and runs to determine run acceptance.

SNP selection and genotyping

The selection of SNPs was based on previously reported SNP associations ($P<0.05$) with sunitinib treatment outcome with regard to efficacy and toxicity. Herein, we have focused on SNPs that were very likely to have an effect on VEGF or VEGF receptors, or SNPs that have a high biomarker potential because of confirmatory findings in large cohorts. 13 SNPs were selected located in *CYP3A5*, *ABCB1*, *VEGF-A*, *VEGFR-2*, *VEGFR-3*, and *IL-8*.[9-22] Details are provided in **Supplementary Material 1**.

Germline DNA was isolated from whole blood samples taken at baseline (before treatment initiation), using the Chemagic™ Blood kit (PerkinElmer), and genotyping was performed using the LightCycler® 480 Real-Time PCR Instrument (Roche Applied Science, Almere, The Netherlands).

Pharmacokinetic/Pharmacodynamic modelling

Data from all patients were analyzed together using the first-order conditional estimation method with interaction implemented in NONMEM (version 7.3).[30] The PK/PD models were built in a sequential manner. The structure of the models is shown in **Figure 1**.

Pharmacokinetic model

The pharmacokinetic model was partially based on a semi-physiological model published by Yu et al.[24] This model features one-compartment model for sunitinib and a biphasic distribution for SU12662. Pre-systemic formation of SU12662 is handled via a hypothetical enzyme compartment incorporated into the central compartment of sunitinib. Central compartment and enzyme compartment are connected by an inter-compartmental clearance which was fixed to the liver blood flow. The addition of a peripheral compartment for sunitinib was tested since other published models featured this structure and the underlying data indicated a similar distribution as the active metabolite.[3,4,27] Inter-individual variability (IIV) was initially included for all parameters and removed, if their exclusion did not significantly worsen the model fit ($P<0.05$). A proportional, additive and a combined (additive + proportional) error model were tested for parent drug and metabolite separately to describe the residual unexplained variability (RUV).

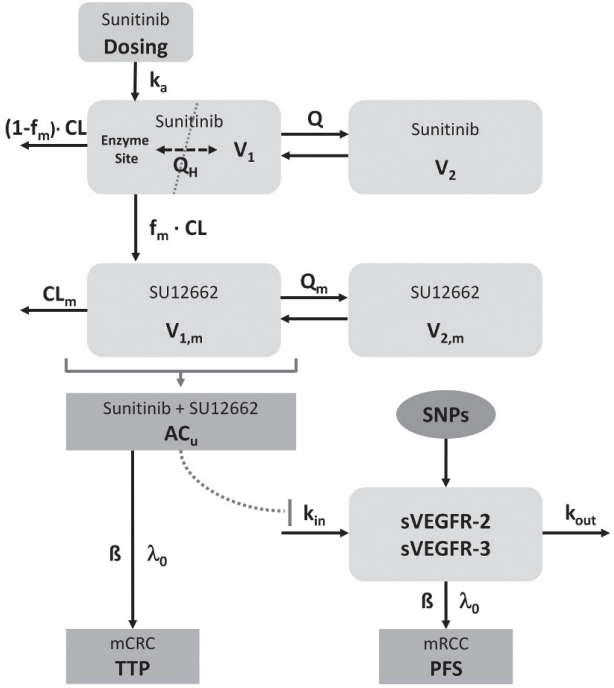

Figure 1 Model structure

Pharmacodynamic models

The concentration-time profiles of sVEGFR-2 and sVEGFR-3 were described using models developed previously by our group for healthy volunteers.[27] The concentration-effect relationship was described by a simple hyperbolic function (INH, **Eq. 1**) using the unbound concentration of the total active drug including SU12662 (AC_u) with a dissociation constant (k_d) fixed to 4 ng/ml obtained in vitro in a tyrosine kinase phosphorylation assay.[27,31] Unbound concentrations were computed by assuming a protein binding of 95% for sunitinib and 90% for SU12662.[32] Decreasing concentrations of the soluble receptors were described by an indirect response model with zero-order production (k_{in}) and first-order elimination (k_{out}). The inhibitory drug effect on k_{in} was included using an inverse-linear model with α as the intrinsic activity (**Eq. 2**):

$$INH = \frac{AC_u}{k_d + AC_u} \qquad (1)$$

$$\frac{dsVEGFR}{dt} = k_{in} \cdot \left[\frac{1}{1+\alpha \cdot INH}\right] - k_{out} \cdot sVEGFR \quad (2)$$

As for the PK model IIV was initially included for all model parameters and removed in case the model did not significantly worsen after exclusion.

Covariate analysis

Covariates were tested on the final models using the automated covariate search provided in PsN (Pearl speaks NONMEM, version 4.4.0).[33] In the forward inclusion step a potential covariate was significant when the OFV decreased by at least 3.84 (1 DF, *P*=0.05) and was kept in the model after the exclusion in the backwards step showed an OFV increase of at least 5.99 (1 DF, *P*=0.01). Covariates tested in the included the demographic parameters, tumor type and the preselected SNPs (see **Supplementary Material 1**).

Model qualification

Nested models were compared using the likelihood ratio test (LRT). Goodness-of-fit plots showing population (PRED) and individual predicted (IPRED) versus observed concentrations (OBS) and conditional weighted residuals (CWRES) versus IPRED or time were used to evaluate the models visually. For the final models precision of all model parameters were given as 90% confidence interval calculated by the non-parametric bootstrap approach (N = 1,000). Prediction-corrected visual predictive checks (pcVPCs) were generated for the patients using 1,000 simulations.[34] Both procedures, bootstrap and pcVPCs, were performed using PsN.[33]

Sensitivity analysis

The effect of fixed parameters on the model predictions was tested by varying the respective parameters between +50 and -50% in 10% steps of the base value derived from the literature. As time of drug intake or sampling time was missing in some patients, administration time was set to 8:00 a.m., assuming that an intake in the morning is the most likely scenario. A similar approach was used for missing sampling times. Here, 12:00 p.m. was chosen, since most check-ups across study centers were scheduled around mid-day. Moreover, the influence of dosing time on parameter estimates was tested randomly varying the time of drug intake between -3 and +3 hours of the documented or imputed value.

The deviation from the original estimate was quantified by calculating the relative prediction error (RPE) and the root squared mean prediction error (RSME,%), which are defined as (**Eq. 3,4**)

$$RPE\ (\%) = \left(\frac{\theta_{new} - \theta_{base}}{\theta_{new}}\right) \quad (3)$$

$$RSME\ (\%) = \sqrt{(RPE)^2} \quad (4)$$

where θ_{base} denotes for the parameter estimate of the original model and θ_{new} for the new estimate under changed conditions.

Outcome modelling

Outcome analysis was performed separately for mCRC and mRCC patients using time-to-event (TTE) models based on a proportional hazard model allowing the analysis of continuous and time-dependent covariates. Different TTE distributions were tested using NONMEM.[34] While a constant hazard is usually a viable assumption in cancer patients due to the short survival and progression times, models with time-dependent hazards were also tested for comparison (**Eq. 5,6,7**):

Constant hazard $$h(t) = \lambda_0 \cdot e^{\beta} \quad (5)$$

Time-dependent hazard (Gompertz) $$h(t) = \lambda_0 \cdot e^{\beta \cdot t} \quad (6)$$

Time-dependent hazard (Weibull) $$h(t) = \lambda_0 \cdot e^{\beta \cdot \ln(t)} \quad (7)$$

Dichotomous covariates were divided by their characteristic values while continuous covariates were grouped. Statistical significance of difference between groups was determined using the log-rank test.

In addition, the Kaplan-Meier analysis as classical non-parametric method was used to determine the median progression-free survival (PFS) in mRCC and the time-to-progression (TTP) in mCRC patients.[36,37] Kaplan-Meier analysis and Cox regression were performed using the *survival* package in R.[36]

RESULTS

Patients

Clinical characteristics of the included patients are presented in **Table 1**. 27 mRCC patients treated with sunitinib were recruited of which one patient was excluded from the analysis due to lack of pharmacokinetic data. 28 mCRC patients were recruited of which seven patients were excluded because of missing drug administration (N=5), missing data (N=1), or uncertainty in the documentation of sunitinib intake (N=1). Thus, 26 mRCC and 21 mCRC patients treated with sunitinib were included into the combined PK/PD analysis.

Outcome analysis was performed for each tumor entity separately with regard to the different endpoints of each study using data of 24 mRCC and 21 mCRC patients. Two of the 26 mRCC patients were excluded from the outcome analysis as both received sunitinib as second-line therapy.

Moreover, 25 mRCC and 14 mCRC patients could be genotyped on the 13 selected SNPs. Here, we observed SNP call rates of 94% to 100% and 10 of 13 SNPs were in Hardy-Weinberg equilibrium (HWE) with *P* values >0.05. Only the SNPs *ABCB1* rs1045642, and *VEGFA* rs699947 and rs2010963 were not in HWE with *P*=0.009, *P*=0.002, and *P*=0.03 respectively.

Table 1 Patient characteristics (median and range)

	mRCC patients (N=26)	mCRC patients (N=21)
Age (years)	64 (43-75)	61 (33-85)
Gender (m/f)	25/1	12/9
Weight (kg)	83 (65-106)	73 (57-106)
Height (cm)	180 (155-186)	172 (149-184)
BMI	25.7 (22.5-34.5)	26.0 (13.3-39.3)

Pharmacokinetic model

A PK model previously published by Yu et al. was adapted as basis for the structural model.[24] To allow comparison of the estimated parameters, volume and clearance (CL) parameters were, as in the reference model, allometrically scaled to a standard weight of 70 kg. Values for liver blood flow (Q_H) and the fraction metabolized (f_m) were fixed to their respective literature values as in the original model.[24] In contrast to the original model, a peripheral compartment for sunitinib was introduced, which improved the model significantly (difference in Objective Function Value (dOFV)=-123.98, *P*<0.0001). However, the volume of the peripheral compartment (V_2) could not be estimated with enough precision, hence the value was fixed to 588 L which was previously reported by Houk et al.[3,4] Compared to the base model this still improved the model fit significantly (dOFV=-112.37, *P*<0.0001). The model fit significantly worsened without inter-individual variability (IIV) for sunitinib CL (dOFV=90.58, *P*<0.0001), central volume of distribution (V_1) of sunitinib (dOFV=41.97, *P*<0.0001), f_m (dOFV=134.67, *P*<0.0001) as well as V_1 of SU12662 (dOFV=18.47, *P*<0.0001). Therefore, IIV was kept in the final model for these parameters. The estimation of covariances improved the model further (dOFV=-20.34, *P*<0.005).

The sensitivity analysis did not reveal major effects on parameter estimates when the fixed parameters Q_H and V_2 (sunitinib) were varied between +50% and -50%. However,

variation of f_m resulted in a high variation (RSME of up to 50%) for clearance, inter-compartmental clearance as well as the central and peripheral volume of distribution of SU12662 which could be expect by their definition. Randomly varying dosing time between +3 and -3 hours relative to the reported or imputed value had primarily an effect on the absorption rate constant (k_a). RSME was relatively high with 36.9% for this parameter. As expected the residual error for sunitinib was also highly affected with an RSME of 25.2%.

Forward inclusion and backward elimination of potential covariates did not reveal any significant effects on the tested model parameters. In addition, no statistical significant differences of pharmacokinetic parameters between both tumor entities were found, confirming that the underlying model can be used across different tumor types. A complete list of covariates tested is provided in **Supplementary Material 1.** Final parameter estimates are shown in **Table 2**. Visual predictive checks indicated that central tendency and variability of both active compounds could be described adequately with the underlying model (**Figure 2A,B**).

Pharmacokinetic/Pharmacodynamic models

The inverse-linear model previously developed for healthy volunteers was also applicable to describe the concentration-time profile of both soluble VEGF receptors in mRCC and mCRC patients after sunitinib therapy.[27] The shape of the concentration-time curves of both soluble receptors was comparable and their response was highly correlated in mRCC (r2=0.594, $P<0.0001$) and also mCRC patients (r2=0.635, $P<0.0001$). However, the covariate analysis performed on both models revealed pharmacodynamic differences between the tumor entities. Addition of a proportional covariate effect of "tumor type" on the intrinsic activity α of sunitinib on sVEGFR-2 levels improved the model significantly (dOFV=-7.45, $P=0.006$). It was shown that intrinsic activity (α) was 32.8% lower in patients with mCRC compared to mRCC.

Intrinsic activity was also influenced by *VEGFR-3* rs6877011 genotype (1=CG/GG; 0=CC). Presence of the G-allele (CG and GG genotypes) showed a decreased α compared to the wildtype CC (2.31 vs 1.00 in case of mRCC patients and 1.55 vs 0.65 for mCRC patients). A decreased intrinsic activity was also observed for patients with presence of a T-allele in *ABCB1* rs2032582 (2.31 vs 1.59 in case of mRCC patients and 1.55 vs 1.07 for mCRC patients).

Final parameter estimates of both models are shown in **Table 3.** Visual predictive checks indicated that central tendency and variability of both proteins could be described adequately with the underlying models (**Figure 2C,D**).

Table 2 Population parameter estimates of the final PK model compared to the model of Yu et al.[24]

		Our study				Yu et al.[24]	
Parameter	**Unit**	**Estimate (RSE, %)**	**IIV% (RSE, %)**	**Bootstrap mean**	**Bootstrap 90% CI**	**Estimate (RSE, %)**	**IIV% (RSE, %)**
Suntinib (parent drug)							
k_a	1/h	0.133 (34.6)	-	0.149	0.01-0.25	0.34 (10.8)	-
CL	L/h	33.9 (6.0)	30.3 (29.0)	33.92	30.76-37.53	35.7 (5.7)	33.9 (12.0)
V_1	L	1820 (6.6)	25.3 (30.3)	1812.1	1607.8-1812.2	1360 (6.0)	32.4(10.6)
V_2	L	588*	-	588*	-	-	-
Q	L/h	0.371 (18.9)	-	0.373	0.263-0.494	-	-
Q_H	L/h	80*	-	80*	-	80*	-
Residual error (proportional)	-	-0.367 (14.1)	-	-0.361	-0.450- -0.283	0.06 (13.5)	
SU12662 (metabolite)							
CL_m	L/h	16.5 (5.4)	-	16.5	15.0-17.9	17.1 (7.4)	42.1 (7.0)
$V_{1,m}$	L	730 (14.1)	42.9 (54.8)	713.6	545.9-872.9	635 (13.1)	57.9 (8.8)
$V_{2,m}$	L	592 (13.2)	-	604.9	481.0-737.4	388 (14.9)	-
Q_m	L/h	2.75 (24.6)	-	2.90	1.96-4.27	20.1 (32.6)	-
f_m	-	0.21*	34.6 (20.5)	0.21*	-	0.21*	-
Residual error (proportional)	-	-0.281 (10.8)	-	-0.276	-0.326- -0.229	0.03 (14.1)	-
Correlations							
ρ (CL, V_1)	-	-	-	-	-	-	-
ρ (CL, CL_m)	-	-	-	-	-	0.53	-
ρ (CL, $V_{1,m}$)	-	-0.0607 (48.3)	-	-0.0685	-0.127- -0.019	-	-
ρ (V_1, $V_{1,m}$)	-	0.0481 (51.8)	-	0.0534	0.0091-0.0996	0.48	-
ρ (CL_m, $V_{1,m}$)	-		-	-	-	0.45	-
ρ (CL, f_m)	-	-0.0425 (40.8)	-	-0.0392	-0.0671- -0.0130	-	-
ρ (V_1, f_m)		-	-	-	-	-	-
ρ (f_m, $V_{1,m}$)		-	-	-	-	-	-

*Parameter fixed to literature value CI, confidence interval, CL, clearance of sunitinib, CLm, clearance of the metabolite SU12262, fm, fraction metabolized to SU12662, IIV, inter-individual variability, ka, absorption rate constant, prop, proportional error model, Q, inter-compartmental clearance of sunitinib, QH, liver blood flow, Qm, inter-compartmental clearance of the metabolite SU12662, ρ, correlation coefficient, RSE, relative standard error, V1, volume of the central compartment of sunitinib, V1,m, volume of the central compartment of the metabolite SU12662, V2, volume of the peripheral compartment of sunitinib, V2,m volume of the peripheral compartment of the metabolite SU12662

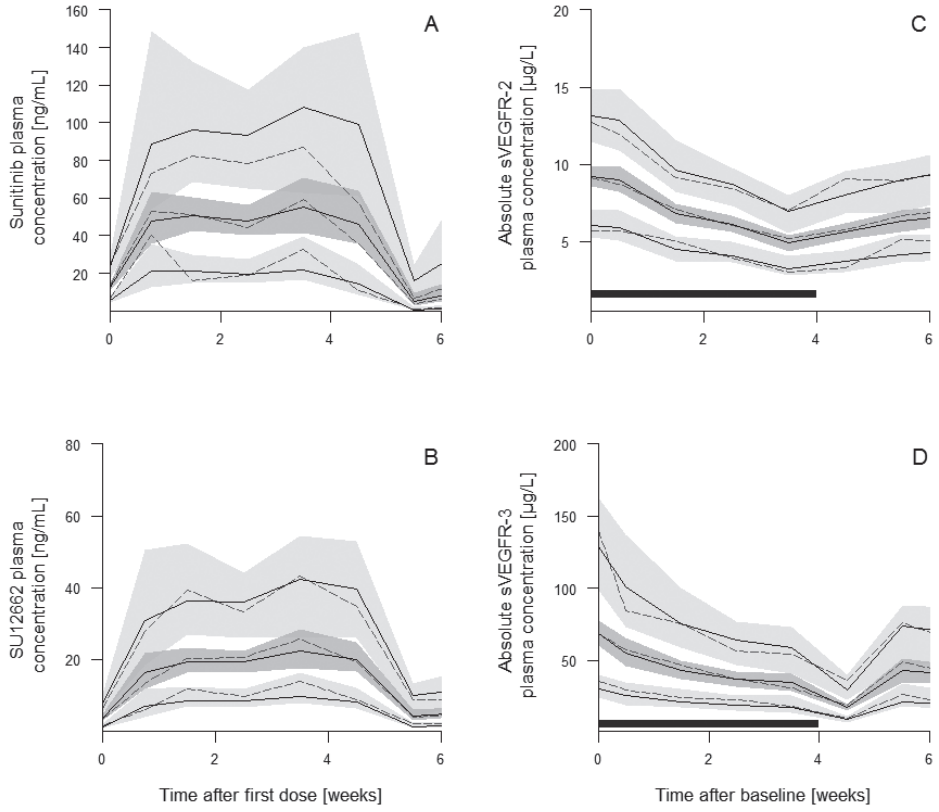

Figure 2 Prediction-corrected visual predictive checks of **A)** the final PK model of sunitinib, **B)** the final PK model of SU12662, **C)** the sVEGFR-2 model, and **D)** the sVEGFR-3 model for one treatment cycle. Solid lines indicate the estimated mean as well as the 90% prediction interval. Dashed lines show the respective observed mean and interval. Shaded grey areas represent the 90% confidence band of the predictions. The dark grey rectangle indicates the time on treatment.

Table 3 Population parameter estimates of the final PD models (sVEGFR-2, sVEGFR-3)

Parameter	Unit	Estimate (RSE, %)	IIV (RSE, %)	Bootstrap mean	Bootstrap CI (90%)
sVEGFR-2					
Baseline	µg/L	9.0 (2.9)	19.9 (21.4)	9.0	8.6-9.5
α	-	2.31 (8.8)	-	2.31	1.98-2.64
k_{out}	1/h	0.0043 (7.6)	-	0.0043	0.0038-0.0049
k_d	µg/mL	4*	-	4*	-
Residual Error	-	0.124 (6.8)	-	0.122	0.108-0.136
Tumor type on α (proportional)	-	-0.328 (24.6)	-	-0.322	-0.440- -0.186
***VEGFR*-3 rs6877011 on α (proportional)**	-	-0.565 (25.4)	-	-0.557	-0.787- -0.319
***ABCB1* rs2032582 on α (proportional)**	-	-0.311 (37.9)	-	-0.307	-0.497- -0.117
sVEGFR-3					
Baseline value	µg/L	63.5 (5.9)	42.6 (24.4)	63.7	57.3-69.8
α	-	1.74 (9.8)	54.3 (43.5)	1.76	1.49-2.05
k_{out}	1/h	0.0053 (7.2)	-	0.0054	0.0047-0.0060
k_d	µg/mL	4*	-	4*	-
Residual Error	-	0.15 (6.9)	-	0.15	0.13-0.17
Tumor type on baseline value (proportional)	-	-0.642 (6.5)	-	-0.640	-0.703- -0.569
Correlations					
P (baseline value, α)		0.124	-	0.123	0.045-0.209

*Parameter fixed to literature value

α, intrinsic activity, CI, confidence interval, IIV, inter-individual variability, k_d, dissociation constant, k_{out}, elimination rate constant, ρ, correlation coefficient, RSE, relative standard error

Outcome model for mRCC patients

Median PFS for mRCC patients was calculated with 6.9 months (N=24). The PFS could be described by a parametric time-to-event model assuming exponentially distributed data with a baseline hazard function λ_0 of 0.0252 weeks^{-1} (90% CI: 0.0168-0.0336). The inclusion of the measured and estimated sVEGFR-2 baseline value led to a decrease of the OFV by 4.14 or 4.67 (P<0.05), respectively. However, the dichotomized covariate, dividing patients into two groups with baseline values above and below the population median of 8.8 µg/L, had a stronger effect with a decrease of the OFV by -6.40 (P<0.025). β was estimated with 1.45 corresponding to a hazard ratio (HR) of 4.26 (with β defined as the natural logarithm of the HR). Inclusion of the active, unbound sunitinib/SU12662 concentrations resulted in an estimated β of -0.14 mL/ng indicating that a higher plasma level reduces the hazard and hence the probability of progression during treatment. However, the effect was not statistically significant (dOFV=-1.1, P=0.29). Likewise, plasma concentrations of sVEGFR-2 and sVEGFR-3 over time were not statistically significant

predictors of PFS either (dOFV, -3.7 and -0.99, respectively). Besides absolute plasma levels of both proteins, also the relative decrease with respect to individual baseline values predicted by the PK/PD models was tested as potential covariate. However, no significant improvement of the model fit could be observed either (dOFV=-0.31 and -0.98).

Best prediction of PFS in mRCC patients was achieved by a hazard function h(t) including the dichotomized baseline value of sVEGFR-2 which was independent of the developed PK/PD models (**Eq. 8**):

$$h(t)=\lambda_0 \cdot e^{\beta \cdot sVEGFR\text{-}2\ baseline\ (dichotom.)} \quad (8)$$

The observed Kaplan-Meier curve describing the progression-free survival function of the mRCC patients was within the predicted 90% prediction interval (PI) of 1,000 simulations and could sufficiently be described by the time-to-event model except for later time points as a result of censored data (**Figure 3A**). Final parameter estimates are shown in **Table 4**.

These findings were confirmed in a multivariate Cox regression analysis. The only covariates exhibiting a significant influence were the dichotomized baseline values of both soluble proteins (data not shown).

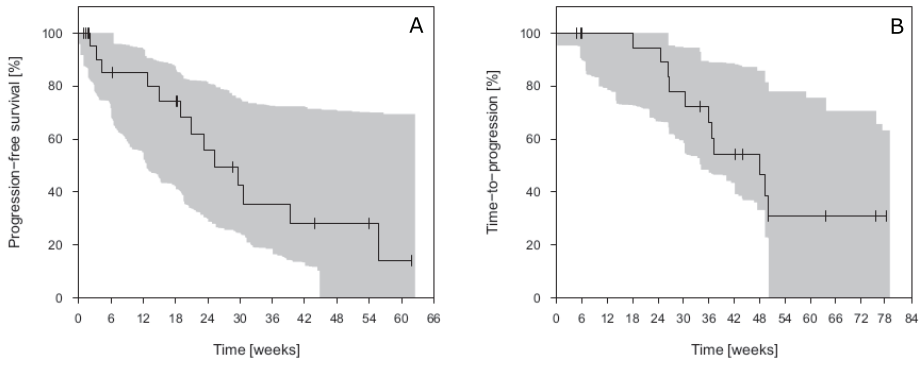

Figure 3 Prediction-corrected visual predictive checks of **A)** the final survival model for mRCC patients, and **B)** the final survival model for mCRC patients. Shaded grey areas represent the 90% prediction interval.

Table 4 Population parameter estimates of the final time-to-event models

Parameter	Covariate	Unit	Estimate (RSE,%)	Bootstrap mean	Bootstrap median	Bootstrap 90% CI
mRCC patients						
λ_0		weeks^{-1}	0.0118 (46.3)	0.0121	0.0117	0.0038-0.0220
β	sVEGFR-2 baseline value*	-	1.45 (43.3)	1.57	1.49	0.71-2.68
HR		-	4.26	4.81	4.44	2.03-14.59
mCRC patients						
λ_0		weeks^{-1}	0.0234	0.0256	0.0241	0.0120-0.0447
β	AC_u	ml/ng	-0.758	-0.919	-0.836	-0.366- -1.736
HR		-	0.47	0.40	0.43	0.18-0.69

*dichotomized above (1) and below (0) population median ACU, unbound active concentration (sunitinib + SU12662), ß, regression coefficient, CI, confidence interval, HR, Hazard Ratio, λ_0, baseline hazard, ρ, correlation coefficient, RSE, relative standard error

Outcome model for mCRC patients

Median time to progression (TTP) for mCRC patients was 8.4 months (N=21). Analogous to the mRCC patients, the TTP could be described by a parametric time-to-event model assuming exponentially distributed data. The baseline hazard function λ_0 was estimated with 0.0234 weeks^{-1} (90% CI:0.012-0.042 weeks^{-1}). The inclusion of the current concentration of the unbound, active drug (ACu) reduced the OFV by 6.07 (P<0.05). β was estimated to be -0.758 mL/ng corresponding to a HR of 0.47. None of the other variables describing the individual PK or biomarker response were identified to be predictive for TTP. Therefore, TTP in mCRC patients was best predicted by the pharmacokinetics of sunitinib and SU12262 with an appropriate hazard function h(t) dependent on the current $AC_u(t)$ (**Eq. 9**):

$$h(t)=\lambda_0 \cdot e^{\beta \cdot AC_u(t)} \quad (9)$$

The observed Kaplan-Meier curve describing the PFS function of the mCRC patients was within the predicted 90% prediction interval of 1,000 simulations and could sufficiently be described by the time-to-event model. However, TTP was difficult to predict for the time from one year onwards due to censored data (**Figure 3B**). Final parameter estimates are shown in **Table 4**. A multivariate Cox regression analysis confirmed these results exhibiting the AUC at steady-state (AUC_{ss}) of the unbound, active drug in combination with age as positive predictive covariates (data not shown).

DISCUSSION

In this study we successfully integrated distinct models for sunitinib in a modeling framework including PK, PD, pharmacogenetic and outcome data. The developed models adequately describe plasma concentration-time profiles of sunitinib, its active metabolite SU12662, sVEGFR-2 and sVEGFR-3 as well as clinical outcome in both tumor types. Similar models (but without pharmaogenetics) were published in patients with GIST[6] and recently hepatocellular carcinoma[38] but there is no model with integrated outcome data yet published for the tumor entities investigated here.

Covariate analysis on the PK parameters did not reveal any significant findings. The significant increase of sunitinib clearance in patients with *ABCB1* rs2032582 TT (18%) found in previous studies could not be confirmed.[17] Presumably this is due to the small and, with two tumor entities, relatively heterogeneous cohort. Biomarker response of sVEGFR-2 and -3 was highly associated in each tumor entity which suggested a comparable predictive value of both soluble receptors. As previously reported decreasing plasma concentrations were observed for both receptors after sunitinib administration with a subsequent increase after stop of treatment.[8,27,39] Independent of tumor entity and dosing scheme, baseline levels are not fully recovered after a two weeks off-phase.

A difference in sVEGFR-2 response to sunitinib between mRCC and mCRC patients could be identified. However, decrease of sVEGFR-2 plasma levels relative to the individual baseline did not have a significant impact on PFS or TTP in both studies, hence the clinical relevance of this effect might be negligible. Observed baseline values of sVEGFR-3 were in the same magnitude previously reported by Motzer et al. ranging between 22.3 and 129.2 µg/L for mRCC patients. However, they were significantly higher compared to mCRC patients.[40] This finding might indicate a higher expression of this protein in mRCC patients. However, data regarding the baseline values of sVEGFR-3 in mCRC patients is sparse, since the first- and second-line treatment usually does not involve tyrosine kinase inhibitors targeting sVEGFR.[41]

In this study, we found that the presence of the variant G-allele in SNP rs6877011 in *VEGFR-3* was associated with a 56.5% decrease in intrinsic activity on sVEGFR-2 compared to the wild-type CC. The same *VEGFR-3* SNP was associated with a decreased PFS in an earlier study.[12] Maitland et al. associated variant G-allele carriers of *VEGFR-2* (*KDR*) rs34231037 with sVEGFR-2 baseline levels and a decline in sVEGFR-2 in response to treatment with pazopanib.[42] We have recently found that rs34231037 variant G-allele carriers have a tendency towards a better response to sunitinib.[43] VEGFR-1, 2, and 3 have similar binding domains.[44] A SNP in any of the genes encoding these VEGF receptors could result in a conformation change and prevent or stimulate binding of the drug ligand to VEGF receptors, and change the ability of sunitinib to decrease sVEGFR-2 and sVEGFR-3. It is remarkable that the SNP effect of G-allele carriers of

rs6877011 in *VEGFR-3* was not found on the intrinsic activity of sunitinib on sVEGFR-3 but on VEGFR-2. Possibly, a lower activity of sunitinib on sVEGFR-3 could also affect sVEGFR-2. The conformation change may have more impact on VEGFR-2 binding affinity than VEGFR-3.

In both patient groups we succeeded in linking clinical outcome data to either pharmacodynamics (mRCC) or pharmacokinetics (mCRC). In mRCC patients, baseline levels of sVEGFR-3 and sVEGFR-2 as well as the decrease in sVEGFR-2 plasma levels over the treatment duration were previously reported to be related to clinical outcome.[7,45] These findings could be further confirmed by this study. Whereas the effect of sVEGFR-2 decrease over time was not significant in the time-to-event analysis, patients with a substantially higher baseline value of sVEGFR-2 showed a significantly worse PFS with an estimated Hazard Ratio (HR) of 4.26. The baseline value of sVEGFR-3 had a lower influence on PFS: for patients with a sVEGFR-3 baseline above the population median of the HR was 2.38 without statistical significance (P=0.2). An effect of similar magnitude (HR: 2.4, 95% CI: 1.13-5.11) was reported by Harmon et al. for the same covariate.[46] In contrast, in mCRC patients, the time-to-event model showed an effect of the pharmacokinetics on TTP with precise parameter estimates. Higher exposure to sunitinib and SU12662 included as active drug concentration over time was associated with a longer TTP. Similarly, a meta-analysis with 443 cancer patients including advanced GIST, mRCC and other solid tumors suggested that an increased AUCss is associated with a longer TTP and a longer OS.[4]

It is not surprising that plasma concentrations of proteins related to the VEGF pathway seem to be more predictive for clinical outcome in mRCC patients as most renal cell carcinoma cells overexpress VEGF due to mutations in the von-Hippel Lindau gene.[47] Furthermore, sunitinib showed no additional effects in patients with colorectal cancer[25], which is consistent with our findings that sVEGFR-2 and -3 levels were not correlated to outcome. The lower intrinsic activity of sunitinib on sVEGFR-2 baseline levels and the overall lower plasma concentrations of sVEGFR-3 may also underline the lower dependency of colorectal carcinomas on angiogenesis, especially via VEGF signaling.

In conclusion, a semi-mechanistic PK model for sunitinib could be successfully linked to pharmacodynamic models for sVEGFR-2 and sVEGFR-3 including various genotypes. Whereas we could show that sunitinib pharmacokinetics does not differ between the two tumor entities, we found differences in pharmacodynamic response with respect to the decrease of sVEGFR-2 and sVEGFR-3 plasma concentrations during therapy. Furthermore, sVEGFR-2 baseline levels seemed to be more predictive for clinical outcome in mRCC patients in contrast to mCRC patients where active drug pharmacokinetics showed the highest impact. Nevertheless, our study provides the basis of PK/PD-guided individualization strategies for the optimization of anti-angiogenic therapies and underlines that it is quite unlikely to identify a general, tumor entity-independent biomarker for sunitinib therapy response.

STUDY HIGHLIGHTS

What is the current knowledge on the topic? There is a high inter-individual variability in response to sunitinib. Hence, predictive biomarkers are needed in order to maximize efficacy and minimize toxicity.

What question did this study address? The objective of this study was the development of pharmacokinetic models, linking sunitinib plasma concentrations to pharmacodynamic response and clinical outcome including the identification of potential genetic predictors for metastasized renal cell cancer (mRCC) and metastasized colorectal cancer (mCRC) patients.

What this study adds to our knowledge? The developed PK/PD models adequately describe plasma concentration-time profiles of sunitinib, SU12662, sVEGFR-2, sVEGFR-3, and clinical outcome showing the strength of an integrated modelling approach. Clinical response in mRCC patients is best predicted by baseline sVEGFR-2 levels, whereas in mCRC patients active drug pharmacokinetics is more predictive.

How might this change drug discovery, development, and/or therapeutics? The PK/PD models presented in this study provide a better understanding of the relationship between sunitinib exposure, pharmacological response, and clinical outcome, and are hence an important step towards finding predictive biomarkers for clinical outcome of sunitinib.

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SUPPLEMENTARY MATERIAL

Supplementary Material 1: List of covariates tested with the respective models

Pharmacokinetic model	
Gender	-
Age	-
Tumor entity (mRCC or mCRC)	-
ABCB1 rs1128503	Additive and general model (if applicable)
ABCB1 rs2032582	Additive and general model (if applicable)
ABCB1 rs1045642	Additive and general model (if applicable)
VEGFA rs699947	Additive and general model (if applicable)
VEGFA rs833061	Additive and general model (if applicable)
VEGFA rs2010963	Additive and general model (if applicable)
IL8 rs1126647	Additive and general model (if applicable)
HAP_VEGFA	Additive and general model (if applicable)
Pharmacodynamic models	
Gender	-
Age	-
Weight	-
BSA	-
Tumor entity (mRCC or mCRC)	-
ABCB1 rs1128503	Additive and general model (if applicable)
ABCB1 rs2032582	Additive and general model (if applicable)
ABCB1 rs1045642	Additive and general model (if applicable)
VEGFA rs699947	Additive and general model (if applicable)
VEGFA rs833061	Additive and general model (if applicable)
VEGFA rs2010963	Additive and general model (if applicable)
IL8 rs1126647	Additive and general model (if applicable)
HAP_VEGFA	Additive and general model (if applicable)
FLT-4 (=VEGFR3) rs6877011	Additive and general model (if applicable)
FLT-4 (=VEGFR3) rs307826	Additive and general model (if applicable)
FLT-4 (=VEGFR3) rs307821	Additive and general model (if applicable)
Time-to-event models	
Active concentration of sunitinib and SU12662	-
AUC of active substance	-
Total concentration of sunitinib and SU12662	-
AUC of total substance	-
Absolute concentration of sVEGFR-2	-
Relative concentration of sVEGFR-2	-
AUC of sVEGFR-2	-
Absolute concentration of sVEGFR-3	-
Relative concentration of sVEGFR-3	-
AUC of sVEGFR-3	-
Systolic blood pressure	-

Supplementary Material 1: Continued

Time-to-event models	
Diastolic blood pressure	-
Rel. change in syst. Blood pressure	-
Rel. change in syst. Blood pressure	-

Supplementary Material 2: NONMEM Codes

Pharmacokinetic model

```
$SUBROUTINE ADVAN6 TOL=4

$MODEL
NCOMP =5
COMP = (DEPOT, DEFDOSE)
COMP = (OBSLIV)
COMP = (CENTRALM)
COMP = (PERIM)
COMP = (PERISUN)

$PK

WT = WEIGHT
IF(WEIGHT.EQ.-99.AND.SEX.EQ.1) WT = 83  ; ET Population mean - male
IF(WEIGHT.EQ.-99.AND.SEX.EQ.0) WT = 75  ; ET Population mean - female
ASCL   = (WT/70)**0.75
ASV    = WT/70

KA        = THETA(1)
V2        = THETA(2)*ASV * EXP(ETA(1))
QH        = THETA(3)*ASCL
CLP       = THETA(4)*ASCL* EXP(ETA(3))
CLM       = THETA(5)*ASCL
V3        = THETA(6)*ASV * EXP(ETA(2))
Q34       = THETA(7)*ASCL
V4        = THETA(8)*ASV
FM        = THETA(9)     * EXP(ETA(4))
```

```
Q25     = THETA(10)*ASCL
V5      = THETA(11)*ASV
```

```
K34 = Q34/V3
K43 = Q34/V4
K25 = Q25/V2
K52 = Q25/V5
```

```
S2 = V2
S3 = V3
```

```
$DES
CLIV = (KA*A(1) + QH/V2*A(2))/(QH+CLP)
```

```
DADT(1) = -KA*A(1)
DADT(2) = QH*CLIV-QH/V2*A(2) - K25*A(2) + K52*A(5)
DADT(3) = FM*CLP*CLIV-CLM/V3*A(3)-K34*A(3) + K43*A(4)
DADT(4) = K34*A(3)-K43*A(4)
DADT(5) = K25*A(2) - K52*A(5)
```

```
$ERROR
TY = LOG(F)
IF(F.LT.0.001) TY = 0.001
IPRED = TY
```

```
IF (CMT.EQ.2) THEN  ; DV log-transformed
W = SQRT(THETA(12)**2)
Y = IPRED+W*EPS(1)
ENDIF
```

```
IF (CMT.EQ.3) THEN  ; DV log-transformed
W = SQRT(THETA(13)**2)
Y = IPRED+W*EPS(2)
ENDIF
```

```
IRES = DV-IPRED
DEL = 0
IF(W.EQ.0) DEL = 0.0001
IWRES = IRES/(W+DEL)
```

```
$THETA
(0, 0.133)                  ;1 = KA
(0, 1820)                   ; 2 = V2
(80) FIX            ; 3 = QH
(0, 33.9)            ;4 = CLP
(0, 16.5)           ;5 = CLM
(0, 730)            ;6 = V3
(0, 2.75)           ;7 = Q34
(0, 592)            ;8 = V4
(0.21) FIX                  ;9 = FM
(0, 0.371)                  ;10 = Q25
(588) FIX                   ;11 = V5
(0,0.367)                   ;12 = Prop. Error Suni
(0,0.281)                   ;13 = Prop. Error Metab
```

```
$OMEGA BLOCK(4)
 0.0621             ;1 IIV V2
 0.0473 0.169               ;2 IIV V3
 0 -0.0613 0.088    ;3 IIV CLP
 0 0 -0.0421 0.113          ;4 IIV FM
```

```
$SIGMA
 1 FIX  ;
 1 FIX  ;
```

```
$ESTIMATION SIG=2 SIGL=4 PRINT=1 METHOD=1 INTER MAXEVAL=9999 NOABORT
```

```
$COVARIANCE PRINT=E
```

sVEGFR-2 PK/PD model

```
$SUBROUTINE ADVAN6 TOL=4
```

```
$MODEL
NCOMP=6
COMP    =(DEPOT,DEFDOSE)
COMP    =(OBSLIV)
COMP    =(CENTRALM)
```

```
COMP    =(PERIM)
COMP    =(PERISUN)
COMP    =(VEGFR2)
```

```
$PK
```

```
; Covariate relationships
```

```
;;; ALFAABCR2-DEFINITION START
IF(ABCR2.EQ.1) ALFAABCR2 = 1  ; Most common
IF(ABCR2.EQ.-99) ALFAABCR2 = 1  ; Missing data
IF(ABCR2.EQ.0) ALFAABCR2 = ( 1 + THETA(8))
;;; ALFAABCR2-DEFINITION END
```

```
;;; ALFASTUDY-DEFINITION START
IF(STUDY.EQ.1) ALFASTUDY = 1  ; Most common
IF(STUDY.EQ.2) ALFASTUDY = ( 1 + THETA(7))
;;; ALFASTUDY-DEFINITION END
```

```
;;; ALFAFLT1-DEFINITION START
IF(FLT1.EQ.0) ALFAFLT1 = 1  ; Most common
IF(FLT1.EQ.-99) ALFAFLT1 = 1  ; Missing data
IF(FLT1.EQ.1) ALFAFLT1 = ( 1 + THETA(6))
;;; ALFAFLT1-DEFINITION END
```

```
;;; ALFA-RELATION START
ALFACOV=ALFAFLT1*ALFASTUDY*ALFAABCR2
;;; ALFA-RELATION END
```

```
;PHARMACODYNAMICS
```

```
TVBLV2 = THETA(1)             ; sVEGFR-2 Baseline
BLV2   = TVBLV2*EXP(ETA(1))
```

```
TVALFA = THETA(2)             ; Intrinsic activity
```

```
ALFA   = TVALFA*ALFVACOV*EXP(ETA(2))
```

```
TVKOUT = THETA(3)          ; Kout
KOUT   = TVKOUT*EXP(ETA(3))
```

```
A_0(6)=BLV2
```

```
KIN = BLV2*KOUT
```

```
TVKD  = THETA(4)          ; Dissociation constant
KD    = TVKD*EXP(ETA(4))
```

```
;PHARMACOKINETICS
```

```
KA        = 0.133
V2        = V2X
QH        = QHX
CLP       = CLPX
CLM       = CLMX
V3        = V3X
Q34       = Q34X
V4        = V4X
FM        = FMX
Q25       = Q25X
V5        = V5X
```

```
K34 = Q34/V3
K43 = Q34/V4
K25 = Q25/V2
K52 = Q25/V5
```

```
S2 = V2
S3 = V3
```

```
$DES
```

```
CLIV = (KA*A(1) + QH/V2*A(2))/(QH+CLP)
```

```
DADT(1) = -KA*A(1)
DADT(2) = QH*CLIV-QH/V2*A(2) - K25*A(2) + K52*A(5)
```

```
DADT(3) = FM*CLP*CLIV-CLM/V3*A(3)-K34*A(3) + K43*A(4)
DADT(4) = K34*A(3)-K43*A(4)
DADT(5) = K25*A(2) - K52*A(5)
```

```
;TOTAL DRUG PLASMA CONCENTRATION
CONC = A(3)/V3+A(2)/V2
```

```
;FREE DRUG CONCENTRATION; 95% PB sunitinib, 90% PB SU12662
FC= A(3)/V3*(1-0.90)+A(2)/V2*(1-0.95)
```

```
;VEGFR2
 ;KINASE BINDING
  IF(FC.LE.0) THEN
   BND=0
  ELSE
   BND=FC/(KD+FC)
  ENDIF
```

```
 ;INHIBITORY SIGNAL
 INH = BND
```

```
 ;SIGNAL FUNCTION
 SF=1/(1+ALFA*INH)
```

```
;RESPONSE COMPARTMENT ABSOLUTE SVEGFR-2 LEVELS
```

```
 DADT(6)= KIN*SF-KOUT*A(6)
```

```
$ERROR
```

```
 IPRE   = A(6)
 RBL    = IPRE/BLV2
 IRES   = DV-IPRE
 W      = THETA(5)*IPRE
 Y      = IPRE+EPS(1)*W
 DVRL   = DV/BLV2
```

```
DEL          = 0
IF(W.EQ.0) DEL = 1
IWRE         = IRES/(W+DEL)
```

```
$THETA
(0, 9030)                    ; 1 TVBLV2
(0, 2.31)            ; 2 TValfa
(0, 0.00428)                 ; 3 THKOUT
(4) FIX                      ; 4 THKD
 (0, 0.124)                  ; 5 CV
(-1, -0.565,5)               ; ALFAFLT11
(-1, -0.328,5) ;              ALFASTUDY1
(-1, -0.311,5)               ; ALFAABCR21
```

```
$OMEGA 0.0388   ; 1 ETABLVR2
$OMEGA 0 FIX                 ; 2 ETAalfa
$OMEGA 0 FIX                 ; 3 ETAKOUT
$OMEGA 0 FIX                 ; 4 ETAKD
```

```
$SIGMA 1 FIX
```

```
$ESTIMATION SIG=2 PRINT=1 METHOD=1 INTER MAXEVAL=9999 NOABORT
```

```
$COVARIANCE PRINT=E
```

sVEGFR-3 PK/PD model

```
$SUBROUTINE ADVAN6 TOL=4
```

```
$MODEL
```

```
COMP=6
COMP    =(DEPOT,DEFDOSE)
COMP    =(OBSLIV)
COMP    =(CENTRALM)
COMP    =(PERIM)
COMP    =(PERISUN)
COMP    =(VEGFR3)
```

```
$PK

; Covariate relationships

;;; BLV3STUDY-DEFINITION START
IF(STUDY.EQ.1) BLV3STUDY = 1  ; Most common
IF(STUDY.EQ.2) BLV3STUDY = ( 1 + THETA(6))
;;; BLV3STUDY-DEFINITION END

;;; BLV3-RELATION START
BLV3COV=BLV3STUDY
;;; BLV3-RELATION END

;PHARMACODYNAMICS
 ;System specific

 TVBLV3 = THETA(1)
BLV3   = TVBLV3*BLV3COV*EXP(ETA(1))

 TVALFA = THETA(2)
 ALFA   = TVALFA*EXP(ETA(2))

 TVKOUT = THETA(3)
 KOUT   = TVKOUT

 A_0(6)=BLV3

KIN = BLV3*KOUT

TVKD  = THETA(4);
 KD    = TVKD

;PHARMACOKINETICS

K12        = 0.133
V2         = V2X
QH          = QHX
CLP         = CLPX
```

```
CLM      = CLMX
V3       = V3X
Q34      = Q34X
V4       = V4X
FM       = FMX
Q25      = Q25X
V5       = V5X
```

```
K34 = Q34/V3
K43 = Q34/V4
K25 = Q25/V2
K52 = Q25/V5
```

```
S2 = V2
S3 = V3
```

```
$DES
```

```
CLIV = (K12*A(1) + QH/V2*A(2))/(QH+CLP)
```

```
DADT(1) = -K12*A(1)
DADT(2) = QH*CLIV-QH/V2*A(2) - K25*A(2) + K52*A(5)
DADT(3) = FM*CLP*CLIV-CLM/V3*A(3)-K34*A(3) + K43*A(4)
DADT(4) = K34*A(3)-K43*A(4)
DADT(5) = K25*A(2) - K52*A(5)
```

```
;TOTAL DRUG PLASMA CONCENTRATION
CONC = A(3)/V3+A(2)/V2
```

```
;FREE DRUG CONCENTRATION; 95% PB sunitinib, 90% PB SU12662
FC= A(3)/V3*(1-0.90)+A(2)/V2*(1-0.95)
```

```
;VEGFR-3
 ;KINASE BINDING
  IF(FC.LE.0) THEN
    BND=0
  ELSE
    BND=FC/(KD+FC)      ;
  ENDIF
```

```
;INHIBITORY SIGNAL
INH = BND
```

```
;Signal Function
SF=1/(1+ALFA*INH)
```

```
;RESPONSE COMPARTMENT ABSOLUTE SVEGFR-3 LEVELS
```

```
 DADT(6)= KIN*SF-KOUT*A(6)
```

```
$ERROR
```

```
 IPRE     = A(6)
 RBL      = IPRE/BLV3
 IRES     = DV-IPRE
 W        = THETA(5)*IPRE
 Y        = IPRE+EPS(1)*W
 DVRL   = DV/BLV3
```

```
  DEL         = 0
 IF(W.EQ.0) DEL = 1
 IWRE        = IRES/(W+DEL)
```

```
$THETA
(0, 63500)            ; 1 TVBLV3
(0, 1.74)  ; 2 TValfa
(0, 0.0054)           ; 3 TVKOUT
(4) FIX               ; 4 TVKD
(0, 0.15)  ; 5 CV
( -0.642)  ;6 BLV3TUMOR
```

```
$OMEGA  BLOCK(2)
 0.167                ; 1 ETABLVR3
 0.124 0.258          ;  2 ETAalfa
```

```
$SIGMA 1 FIX
```

```
$ESTIMATION SIG=2 PRINT=1 METHOD=1 INTER MAXEVAL=9999 NOABORT
```

```
$COVARIANCE PRINT=E
```

Time-to-Event model (mRCC)

```
$SUBROUTINE ADVAN6 TOL=4
```

```
$MODEL
NCOMP   = 1
COMP    = (CUMHAZ)
```

```
$PK
```

```
 TVBLHAZ          = THETA(1)
  BLHAZ = TVBLHAZ*EXP(ETA(1))
```

```
 TVBETA           = THETA(2)
  BETA            = TVBETA*EXP(ETA(2))
```

```
PMN = 8814.3    ; measured pop. median
IF(VG2B.GT.PMN)THEN
VGB = 1
ELSE
VGB = 0
ENDIF
```

```
$DES
```

```
; Hazard Model
DADT(1) = BLHAZ*EXP(BETA*VGB)
```

```
$ERROR
```

```
 CHZ  = A(1)
```

```
 SUR   = EXP(-CHZ
HAZNOW = BLHAZ*EXP(BETA*VGB)
```

```
 IF(DV.EQ.1) THEN
```

```
  Y = HAZNOW*SUR                    ; prob density function of event
ELSE
  Y = SUR                           ; censored data
ENDIF
```

```
$THETA
(0, 0.00007)        ; 1 TV_BLHAZ      - BASELINE HAZARD
(1.45)              ; 2 TV_BETA       - FACTOR
```

```
$OMEGA 0 FIX     ; 1 ETA_HAZ
$OMEGA 0 FIX     ; 2 ETA_BETA
```

```
$ESTIMATION SIG=2 SIGL=6 MAXEVAL=9999 METHOD=COND LAPLACE LIKE PRINT=1
$COVARIANCE PRINT=E
```

Time-to-Event model (mCRC)

```
$SUBROUTINE ADVAN6 TOL=4
```

```
$MODEL
NCOMP=6
COMP = (DEPOT, DEFDOSE)
COMP = (OBSLIV)
COMP = (CENTRALM)
COMP = (PERIM)
COMP = (PERISUN)
COMP = (CUMHAZ)
```

```
$PK
```

```
TVBLHAZ          = THETA(1)
 BLHAZ = TVBLHAZ*EXP(ETA(1))
```

```
TVBETA           = THETA(2)
 BETA            = TVBETA*EXP(ETA(2))
```

```
;PHARMACOKINETICS
```

```
K12   = 0.133
V2    = V2X
QH    = QHX
CLP   = CLPX
CLM   = CLMX
V3    = V3X
Q34   = Q34X
V4    = V4X
FM    = FMX
Q25   = Q25X
V5    = V5X
```

```
K34 = Q34/V3
K43 = Q34/V4
K25 = Q25/V2
K52 = Q25/V5
```

```
S2 = V2
S3 = V3
```

```
$DES
```

```
CLIV = (K12*A(1) + QH/V2*A(2))/(QH+CLP)
```

```
DADT(1) = -K12*A(1)
DADT(2) = QH*CLIV-QH/V2*A(2) - K25*A(2) + K52*A(5)
DADT(3) = FM*CLP*CLIV-CLM/V3*A(3)-K34*A(3) + K43*A(4)
DADT(4) = K34*A(3)-K43*A(4)
DADT(5) = K25*A(2) - K52*A(5)
;FREE DRUG CONCENTRATION; 95% PB sunitinib, 90% PB SU12662
FC= A(3)/V3*(1-0.90)+A(2)/V2*(1-0.95)
```

```
; Hazard Model
DADT(6) = BLHAZ*EXP(BETA*FC)
```

```
$ERROR
```

```
 FREE  = A(3)/V3*(1-0.90)+A(2)/V2*(1-0.95)
```

```
CHZ   = A(6)
```

```
SUR    = EXP(-CHZ)
HAZNOW = BLHAZ*EXP(BETA*FREE)
```

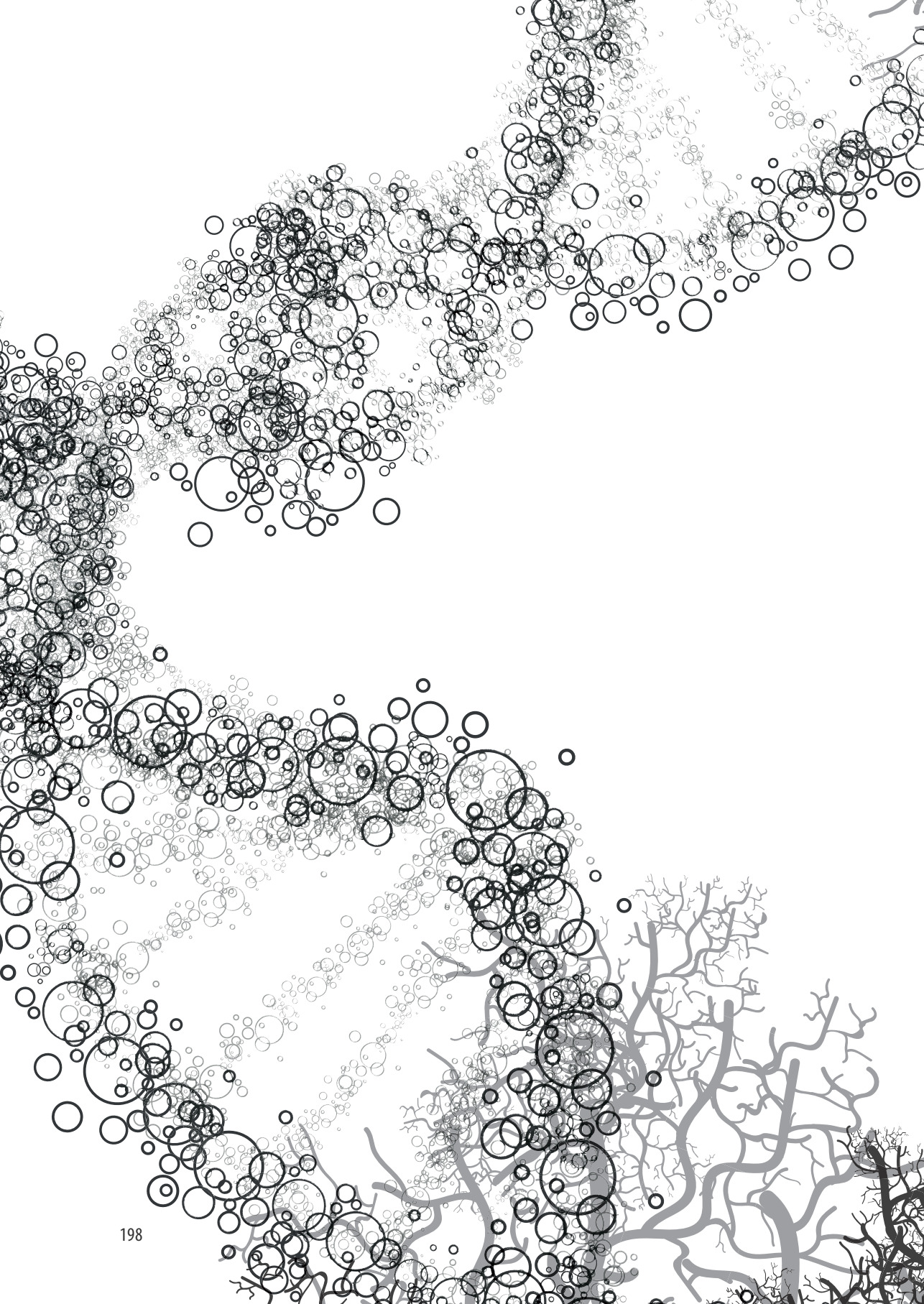
```
IF(DV.EQ.1) THEN
  Y = HAZNOW*SUR
ELSE
  Y = SUR
ENDIF
```

```
$THETA
(0, 0.00014)          ; 1 TV_BLHAZ        - BASELINE HAZARD
(-0.758)  ; 2 TV_BETA           - FACTOR
```

```
$OMEGA 0 FIX  ; 1 ETA_HAZ
$OMEGA 0 FIX  ; 2 ETA_BETA
```

```
$ESTIMATION SIG=2 SIGL=6 MAXEVAL=9999 METHOD=COND LAPLACE LIKE PRINT=1
```

```
$COVARIANCE PRINT=E
```



CHAPTER 9

Genetic biomarkers associated with efficacy of sunitinib treatment in patients with metastatic renal cell carcinoma: preliminary results from the EuroTARGET Genome-Wide Association Study.

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*Authors contributed equally to this work

COLLABORATORS

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Manuscript in preparation

Please note that this manuscript contains confidential information, since these preliminary results have not yet been published. The results presented here are not under consideration for publication and have not been made publically available.

ABSTRACT

Background Sunitinib is successfully used in metastatic renal cell carcinoma (mRCC), although the individual response is highly variable. In candidate gene studies, single nucleotide polymorphisms (SNPs) have been associated with sunitinib efficacy or toxicity. Genetic variants in *CYP3A5* and *ABCB1* show the most promising results for clinical use. A hypothesis-free genome-wide association study (GWAS) is not limited to our current understanding of pharmacological pathways and offers the potential to provide novel understanding on biological mechanisms underlying sunitinib action.

Methods We conducted a GWAS on sunitinib efficacy in 550 mRCC patients from centers in the Netherlands, Spain, Germany, Iceland, Romania, and the United Kindgdom that take part in the European consortium on "TArgeted therapy in Renal cell cancer: GEnetic and Tumor related biomarkers for response and toxicity" (EuroTARGET). After SNP imputation using the 1000Genomes reference panel, 6,540,327 SNPs were tested for association with progression-free survival (PFS) using Cox regression analyses.

Results Here, we identified 9 genetic variants on chromosome 21 that showed genome-wide significance ($P<5\times10^{-8}$) on association with sunitinib PFS. These variant alleles were predictive for an inferior PFS ($P<4\times10^{-8}$) with hazard ratios ranging from HR=2.31 (95%CI:2.71-10.06) to HR=2.44 (95%CI:2.66-11.44).

Conclusions This GWAS showed that SNPs located on chromosome 21 may well play a role in sunitinib PFS in mRCC patients. It seems likely that these SNP are involved in mechanisms related to drug resistance and consequently influence sunitinib efficacy. These findings will be tested for validation in an independent cohort of mRCC patients to assess the predictive value of these SNPs for clinical practice.

INTRODUCTION

Sunitinib, a multi-targeted tyrosine kinase inhibitor (TKI), is the first-choice treatment for patients with metastatic renal cell carcinoma (mRCC). There is a wide inter-individual variability in the response to sunitinib with regard to side-effects and efficacy. Currently, it is not possible to predict the individual treatment outcome to sunitinib, which results in suboptimal treatment for a considerable number of patients. At least one-third of patients require a dose reduction and up to 20% shows no clinical response to sunitinib. Biomarkers that enable prediction of an individual's response to sunitinib are imperative.[1-2]

In previous studies, the candidate gene approach was used to test Single Nucleotide Polymorphisms (SNPs) in genes related to the pharmacokinetics (PK) and pharmacodynamics of sunitinib. SNPs in *CYP1A1*, *CYP3A5*, *CYP3A4*, *NR1I2*, *NR1I3*, *ABCB1*, *ABCG2*, *VEGF-A*, *VEGF-R1*, *VEGFR2*, *VEGF-R3*, *FGF-R2*, *FLT3*, *eNOS*, *UGT1A1* and *IL8* were significantly associated with toxicities, dose reductions, clearance, drug exposure, best objective response, progression-free survival (PFS) or overall survival (OS).[3-19] A subsequent validation study confirmed that genetic polymorphisms in *CYP3A5* (rs776746) and *ABCB1* (rs1128503, rs2032582, rs1045642) are associated with 2-fold increases in dose reductions and in PFS respectively.[20] Hence, germline genetic variants are considered to be major contributors to the observed differences in response to sunitinib. However, sample sizes in these exploratory studies were limited (up to 350 subjects) and studies used inconsistent endpoint definitions.[3-20]

Only a decade ago, the Genome-Wide Association Study (GWAS) was first used to test about 1 million common genetic variants across the genome for association with drug response or adverse events. These first GWASs included commonly used drugs such as simvastatin used in cardiovascular disease, the anticoagulant warfarin, and flucloxacilin used in infectious disease. Yet, few GWAS results are available on SNPs associated with response to anticancer agents. Accordingly, the current GWAS is expected to be more informative than previous candidate gene studies since the hypothesis-free approach used in a GWAS could provide novel understanding on biological mechanisms underlying sunitinib action. However, a GWAS in cancer pharmacogenetics remains challenging because the need of a large sample size, the use of a stringent significance threshold to correct for multiple testing ($P<5.0*10^{-8}$), and validation in an independent cohort. Moreover, the drug response to anticancer therapy is determined by both germline genetic variants as well as somatic mutations.[21]

First GWAS results on response and toxicity in 1099 mRCC patients treated with pazopanib (N=744) or sunitinib (N=355) have been presented at the American Society of Clinical Oncology (ASCO) annual meeting 2014, but definitive results still remain to

be published.[22,23] Common intronic variants in *LOXL2* and *ENTPD4* were associated with the combined efficacy endpoint of PFS, OS and best response (BR) (P=1.7x10^{-8}). SNPs in *IL2RA, LRRC2* were associated with efficacy endpoints (P≤5x10^{-7}) at a threshold below genome-wide significance. A common variant intergenic between *ANAPC4* and *SLC34A2* was associated with HFS (P=4.6x10^{-8}) and, in agreement with earlier findings of Motzer et al., SNPs in *UGT1A1* were associated with bilirubin elevation in pazopanib treated patients (P=2.9x10^{-17}).[15,22] These findings have however not been validated in independent series.[22]

We established the "TArgeted therapy in Renal cell cancer: GEnetic and Tumor related biomarkers for response and toxicity" (EuroTARGET) consortium to look for sunitinib biomarkers.[24] Here we report the results of a GWAS to identify germline genetic variants that associate with sunitinib efficacy in a discovery cohort of mRCC patients.

METHODS

Patients

The EuroTARGET project consists of a prospectively collected set of patients that involves the collection of new blood samples and tissue material, and a set of 'historical patients' from earlier studies using previously stored samples. The current study describes findings from both patient cohorts. A more detailed description of the EuroTARGET cohort can be found in the article of van der Zanden et al.[25]

Inclusion criteria for our study were a pathologically confirmed diagnosis of mRCC, the use of sunitinib as a first TKI, availability of germline DNA and recorded clinical data on efficacy and toxicity endpoints. Patients were recruited at participating centers in The Netherlands, the United Kingdom, Iceland, Germany, Romania and Spain. Patient enrolment occurred from 2005 until 1 July 2015.[25,26] Patients were included only if genotyping data were available, the start date of sunitinib and a date after the start date were available, and if potential follow-up after start sunitinib until study end is more than 24 weeks. Only patients were included for whom no TKI or TKI-like systemic anti-tumor treatment was given prior to sunitinib (**Supplementary Table 1**). For efficacy analyses, only patients with a clear cell histological subtype or unknown histology were included.[25]

For the prospective studies, two ten ml blood samples were collected. One sample was stored in a central biobank in The Netherlands, and the other was used for genotyping (at deCODE Genetics in Reykjavik, Iceland). All subjects and samples were coded. The EuroTARGET study has been approved by the local research ethics committees of all participating centers and all patients gave their written informed consent. Retrospective samples were sent for genotyping to deCODE Genetics in Reykjavik, Iceland. For subjects from the Dutch SUTOX consortium, DNA samples were anonymized by a third party,

according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org).[3,6]

Clinical data collection

The clinical database that was used for the preliminary analyses as reported in this manuscript was created on 5 September 2016. Medical data with regard to treatment response, toxicities, and survival information were obtained by a retrospective review of the medical record of each patient and systematically documented in the EuroTARGET web-based electronic case record form (eCRF) (**Supplements 2a,b**). Response and date of response assessments were registered according to the Response Evaluation Criteria in Solid Tumors RECIST version 1.0 and 1.1.[24,25] Herein, the response as indicated by the physician in the medical record is leading and in case this was not recorded the response as recorded in the radiology report was used.[25]

Genotyping and quality control (QC)

Sample processing and genotyping procedures were conducted at deCODE genetics (Reykjavik, Iceland). Germline DNA was isolated from whole blood samples using the Chemagic Blood kit (PerkinElmer) and used for SNP genotyping on the Illumina Human OmniExpress BeadChips 12v1-1, 24v1-0, and 24v1-1 (Illumina, San Diego, CA, USA).[27] Quality control (QC) checks were performed using software R version 3.2.3[28], and PLINK software, version 1.07.[29] Individuals were excluded from analyses based on an individual genotype call rate <97%, gender mismatch between reported and estimated sex based on genotypes of the X-chromosome (using PLINK), or excess of heterozygous genotypes as measured by the inbreeding coefficient. An inbreeding statistic of F>0.1 was judged to be outlying and individuals were removed from the analysis. Genetic markers were excluded based on a SNP call rate <97%, minor allele frequency (MAF) <1%, and a *P*-value $\leq 10^{-7}$ for the Hardy-Weinberg equilibrium (HWE) goodness-of-fit test. After exclusion of individuals and markers in these marginal QCs, the remaining set was used for integrative QC assessment. In order to evaluate the possibility of population stratification or outliers, multidimensional scaling (MDS) analysis was performed in PLINK. In addition, pairwise identity by state (IBS) statistics were calculated to assess duplicates. Both MDS and IBS were computed using PLINK.[29] Individuals that were identified as outliers based on IBS clustering or the MDS analysis were excluded from the association analyses. SNP imputation was performed using the programs *shapeit* and *impute2* with default parameters in which the reference panel was 1000 genomes build version 3 with total, 'cosmopolitan', set of individuals.[30-33]

Association analysis

SNP genotypes were tested for association with efficacy outcomes. The primary efficacy endpoint was PFS and the secondary outcome was OS. PFS was defined as the time in months between the first day of sunitinib treatment and the date of progressive disease (PD) according to RECIST version 1.0 and 1.1. If no progression of the disease was observed during sunitinib treatment, PFS was censored at the time of the last follow-up or death. OS was defined as the time in months between the first day of sunitinib treatment and the date of death or the date at which patients were last known to be alive. SNPs and the efficacy covariates age, gender, country, and Heng prognostic risk group (favorable, intermediate or poor) were tested for association with PFS and OS using Cox-regression analysis under an additive genetic model.[34] In addition to the primary and secondary efficacy endpoints, an 'extreme analysis' was performed on patients with progressive disease versus patients having no progression within 3, 6, and 9 months respectively.

Statistical analyses were performed in R statistics version 2.3.2. Base packages *stats*, *survival*, and *MASS* were used to evaluate logistic, Cox, and ordinal regressions, respectively. R-package *mice* was used to impute missing values for Heng variables (WHO performance status, hemoglobin, neutrophil count, thrombocytes, calcium, and time from diagnosis until start of sunitinib) using 100 imputations. For the initial GWAS analysis Heng prognostic risk group was imputed to be the most likely category of each individual based on the 100 imputations. The 100 SNPs with lowest *P*-values were re-evaluated using a full multiple imputation procedure.

Associations with a *P*-value $\leq 5\times10^{-8}$ were considered genome-wide significant. Associations with a *P*-value between 5×10^{-8} and $\leq 5\times10^{-6}$ were considered suggestive. Both significant and suggestive SNPs will be re-tested in a validation cohort of mRCC patients treated with sunitinib. Post association QC was performed by visual inspection of Quantile-Quantile (QQ) plots of *P*-values of association tests and computation of the inflation factor given as: $\lambda=(\text{median}(T_1, \ldots, T_n)/0.675)^2$, where $T_1, \ldots, T_n$ are square roots of χ^2 quantiles.

RESULTS

Patient and genetic markers

For GWAS purposes, clinical data were available for a total of 713 sunitinib treated patients. 85 patients were removed from the analysis because their sample contained too little DNA. The remaining 628 patients entered the quality check procedure prior to association analyses. The flowchart on patient inclusion is shown in **Figure 1**.[25] The observed individual genotype call rates varied between 99.2 to 100% and therefore meets

the quality criteria. Based on further quality control steps, 24 patients were excluded from analysis. Genders were specified with a sample mix-up rate of 0.2% which means that 2 patients were removed prior to analyses. Results from the 7-dimensional MDS analyses showed two main clusters of patients derived from the principal component analysis with a cluster containing subjects from Spanish centers (SOGUG) and another cluster containing subjects from the other countries (Germany, Iceland, Netherlands, United Kingdom). The Romanian patients were in between these clusters (**Supplementary Figure 3**). In addition, MDS showed that 20 individuals were recognized as outliers and were therefore removed from analyses (**Supplementary Figure 3**). For the current analyses we have adjusted for country. However, based on the clusters of patients we observed from MDS analysis, we may include the principal component analysis (PCA) derived ancestry as a covariate in future analyses. The mean inbreeding coefficient was F=0.01 (95%CI: -0.02, 0.04). Pairwise IBS scores are plotted in **Supplementary Figure 4** and 2 patients were excluded because of an inbreeding coefficient of F>0.1. For efficacy analysis, 54 patients with a non-clear cell histological subtype of RCC were excluded resulting in a total of 550 patients included in our analyses (**Figure 1**). Patient characteristics for efficacy analyses are shown in **Table 1**. In brief, complete information on efficacy data and genotypes were available for 550 patients diagnosed with mRCC of which the majority received sunitinib with a starting dose of 50.0 mg (N=482, 87.6%) in a 4-weeks-on/2-weeks-off schedule and the remaining 68 patients (12.4%) started with 37.5 mg, 25 mg, 12.5 mg or an unknown dose (**Table 1**). Patients were enrolled between 15 August 2005 (first start date sunitinib) and 1 July 2015 (fixed censoring date).

At start of the analysis, a set of 680,729 genetic markers was available. After subjecting these markers to QC, 1,405 of the 680,729 SNPs (0.2%) were excluded because of a deviation from HWE (N=11), or a MAF <0.01 (N=1,394). All SNP call rates were >97%, and therefore no markers were excluded because of missing genotypes. This resulted in a number of 679,324 genetic markers that meet the quality criteria for statistical analyses (**Supplementary Table 5**). This number of genetic markers was supplemented using SNP imputation procedures with the 1000Genomes dataset as a reference panel and filtered at a MAF of >5%. This resulted in a total of 6,540,327 SNPs available for genetic association analyses.

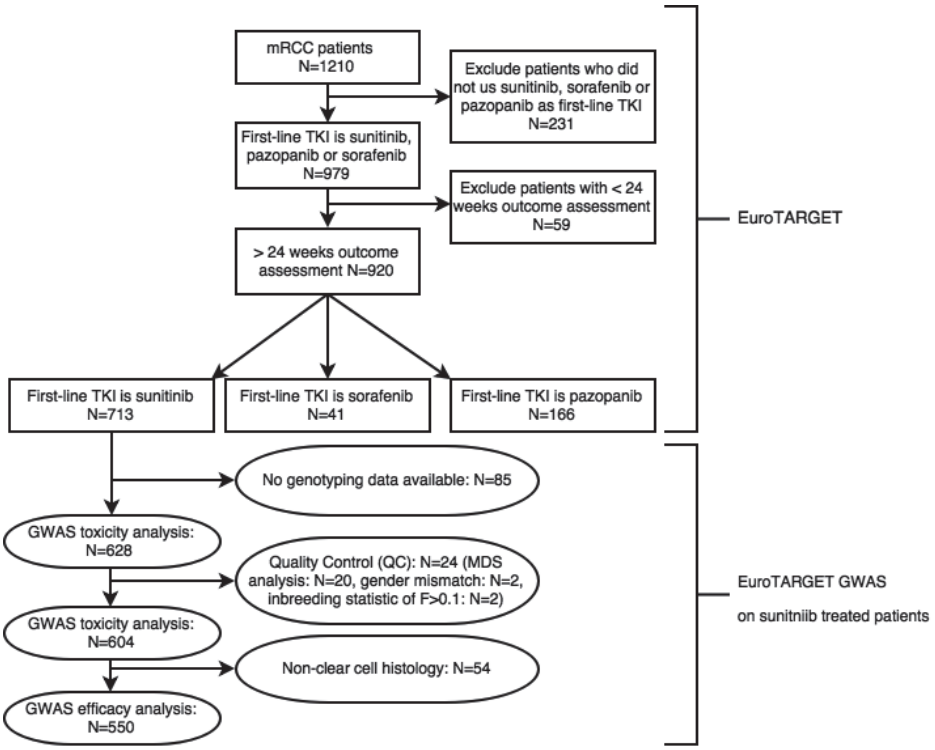

Figure 1 In total, 1210 mRCC patients were included in EuroTARGET, of which 748 were collected prospectively and 462 were available as historical (prospective) series at the start of EuroTARGET.[25] Of the 1210 patients, we selected the 979 patients (81%) who received sunitinib, sorafenib, or pazopanib as first TKI (remainder of patients did for example have no treatment or were treated with an mTOR inhibitor or other TKI). We did not exclude patients who used cytokine therapy before the TKI. To allow for informative analyses, we only focus on the subset of 920 patients for whom outcome could be assessed for at least 6 months (24 weeks). Patient with missing genotypes (N=85) were excluded for GWAS purposes. 24 patients were excluded after Quality Control (QC) checks. For efficacy analyses, 54 non-clear cell patients were excluded resulting in 550 patients available for testing efficacy endpoints.[25]

Table 1 Patient characteristics for patients diagnosed with metastatic renal cell carcinoma and treated with sunitinib, included for efficacy analyses (N=550)

Characteristic	Value (range)	%
Gender		
male	405	74
female	145	26
Median age at start sunitinib (years)	63 (33 – 87)	
Country		
Netherlands	281	51
Spain (SOGUG)	168	30.5
Germany (CESAR)	39	7
Iceland (Landspitali University Hospital)	27	5
United Kingdom (Addenbrooke's Cambridge)	20	3.5
Romania (University of Medicine and Farmacy Carol Davila)	15	3
Prior nephrectomy before start sunitinib		
yes	201	36.5
no	349	63.5
WHO performance status		
0	206	37.5
1	206	37.5
2	25	4.5
3	2	0.3
4	1	0.2
unknown	110	20
Treatment line sunitinib		
1	512	93
2	32	6
3	5	0.9
4	1	0.1
*Heng prognostic prisk group**		
Good (0 risk factors)	80	14.5
Intermediate (1-2 risk factors)	298	54.2
Poor (3-6 risk factors)	172	31.3
Prior drugs (excluding TKI or TKI-like antitumor treatment)		
Yes	38	7
No	512	93
Duration of prior drug treatment (months)	1 to 23	
Sunitinib starting dose (daily dose in mg)		
50	482	87.6
37.5	46	8.4
25	20	3.6
12.5	2	0.4
Treatment duration of sunitinib	10 months (2 days - 113 months)	

Table 1 Continued

Characteristic	Value (range)	%
Dose reduction		
yes	223	41
no	310	56
unknown	17	3
Dose reduction within 12 weeks (equal to 2 cycles) of treatment		
yes	111	20
no	416	76
unknown	23	4
Dose reduction within 24 weeks (equal to 4 cycles) of treatment		
yes	145	26
no	382	70
unknown	23	4

Values are presented as median unless otherwise indicated. Abbreviations: WHO = World Health Organization.

*The Heng prognostic risk group was based on six risk scores: WHO performance status (≥1), low hemoglobin (< lower limit of normal (LLN); for males LLN = 8.1 mmol/L or 13 g/dL, for females LLN = 7.1 mmol/L or 11.5 g/dL), high calcium (>2.5 mmol/L) and time from initial diagnosis to treatment with sunitinib (< 1 year), neutrophil count (> upper limit of normal (ULN)) and thrombocytes (> ULN).

Duration of prior drug treatment refers to the number of months that a patient has received an antitumor treatment prior to start with sunitinib that is not a TKI or TKI-like drug as mentioned in **Supplementary Table 1**, because these patients have already been excluded from analyses.

Genetic association analyses

A total of 6,540,327 SNPs were tested for associations with PFS and OS of sunitinib in 550 patients. Median follow-up times of PFS and OS were 9.9 months (range: 3 days – 104.5 months) and 17.8 months (range: 9 days – 104.5 months), and median PFS and OS of sunitinib treated patients were 10.5 months (range: 9 – 12 months) and 28.2 months (range: 24 – 33 months) respectively. Included covariates for PFS and OS analyses are gender, age at start of sunitinib treatment, Heng prognostic risk group, and country.

In the preliminary analysis presented in this manuscript we only report the results of genetic association analysis on the primary outcome PFS. Results of the most significant SNPs associated with sunitinib PFS are presented in **Table 2** and in the Manhattan plot in **Figure 2**. A number of 9 genome-wide significantly associated SNPs ($P<5\times10^{-8}$) with PFS of sunitinib are located on chromosome 21 with *P*-values between $P=1.11\times10^{-8}$ and $P=4.04\times10^{-8}$ and a hazard ratio of 2.3 to 2.4. Four of these SNPs were on the Illumina Human OmniExpress BeadChips. The other SNPs were imputed. Observed MAFs for the 9 associated SNPs in our study cohort were 0.061 to 0.067. For the most significantly

associated variant, median PFS for non-carriers (wild-types, N=528) was 12.7 months (95%CI: 11.1-15.6) versus 5.6 months (95%CI: 4.4-12.3) for variant carriers (N=72).

The 50 most significant SNPs are presented in **Supplementary Table 6**, of which the 30 most significant SNPs (including the 9 genome-wide associated SNPs) are located on chromosome 21. Furthermore, 11 SNPs located on chromosome 5 are suggestive for association with PFS ($P<7.1\times10^{-7}$).

The QQ plots of the observed *P*-values for association tests on PFS are shown in **Figure 3**, with a calculated inflation factor of 1.08.

Table 2 9 GWAS significant SNPs associated with PFS

SNP	Certainty of SNP imputation	Chromosome	MAF	*P* value	HR (95%CI)
1	0.93	21	0.065	1.1146E-08	2.42 (1.79-3.28)
2	0.98	21	0.063	1.3381E-08	2.44 (1.79-3.31)
3	0.99	21	0.063	1.7633E-08	2.41 (1.77-3.27)
4	0.97	21	0.064	2.5884E-08	2.35 (1.74-3.17)
5	1.00	21	0.067	2.7326E-08	2.32 (1.72-3.12)
6	1.00	21	0.067	2.9537E-08	2.31 (1.72-3.11)
7	0.98	21	0.064	3.0601E-08	2.32 (1.72-3.13)
8	1.00	21	0.067	3.1244E-08	2.31 (1.72-3.11)
9	1.00	21	0.061	4.0368E-08	2.34 (1.73-3.16)

Abbreviations: MAF=Minor Allele Frequency, HR=Hazard Ratio, CI=Confidence Interval.

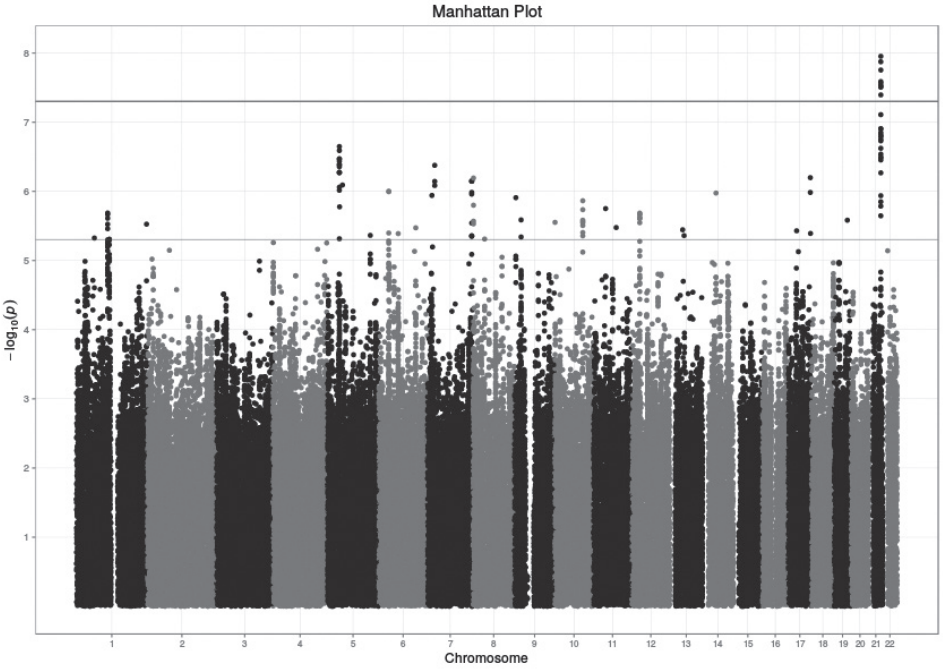

Figure 2 Manhattan plot for progression-free survival (PFS) analysis, including the covariates sex, age at start sunitinib, Heng prognostic risk group and country. Significance level (red horizontal line in bold) is set to $P=5\times10^{-8}$ for genome-wide significance and *P*-values between 5×10^{-8} and $\leq5\times10^{-6}$ (between the two red horizontal lines) were considered suggestive for association. Four actual measured non-imputed SNPs together with five added imputed SNPs were genome-wide significantly associated with PFS (*P*-values between $P=1.11\times10^{-8}$ and $P=4.04\times10^{-8}$). Many more SNPs were suggestive for an association with PFS with *P*-values between 5×10^{-8} and $\leq5\times10^{-6}$. The most significant SNPs for a suggestive association with PFS were located on chromosome 21 and 5 with the most significant association for an unimputed SNP located on chromosome 5.

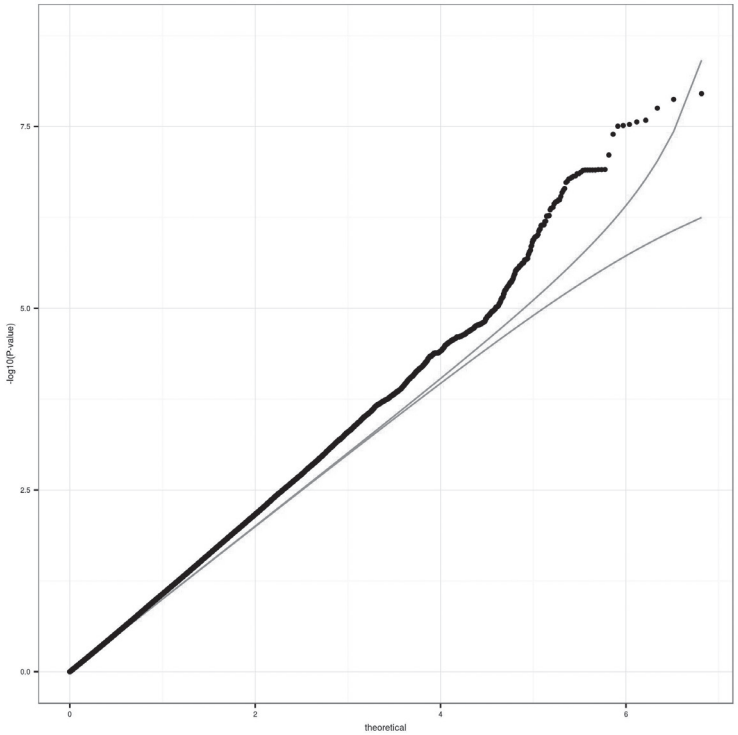

Figure 3 Quantile-Quantile (QQ)-plot. This QQ-plot shows the extent to which the observed distribution of the test statistic follows the theoretical null distribution. An inflation factor of λ=1.08 was observed.

DISCUSSION

This GWAS identified genetic markers that are potentially useful to predict PFS on sunitinib treatment as was tested using 6,540,327 SNPs in 550 mRCC patients. Variant alleles of 9 SNPs located on chromosome 21 were significantly associated with an inferior PFS ($P<5\times10^{-8}$) with a hazard rate of 2.42 (95%CI: 1.79-3.28) for the most significantly associated SNP. In addition, 21 SNPs on chromosome 21 show a suggestive association with PFS. It seems probable that these SNPs influence sunitinib efficacy, because these genes are possibly related to drug resistance or cancer cell survival. These markers and the other most significant findings need to be tested in a validation cohort of sunitinib treated mRCC patients to assess the predictive value of these SNPs for clinical practice.

None of the SNP associations reported in earlier candidate gene studies have been replicated in this GWAS. Neither have we observed any of the associations of SNPs in

LOXL2, *ENTPD4*, *IL2RA*, and *LRRC2* with efficacy of sunitinib or pazopanib reported in the GWAS by Motzer et al.[22]

This GWAS represents the largest pharmacogenetic association study of sunitinib in mRCC with a sample size of 550 patients. The use of a structured eCRF ensures consistency in data collection by all participating centers and a uniform data export. Adjustment of PFS outcomes might be somewhat distorted because of the need for imputation of Heng variables. Yet, by using a multiple imputations procedure, we observed an uncertain Heng risk group for only 17% of patients. A strength in this research is the imputation of genetic markers using the 1000Genomes dataset[30-33], which increased the number of SNPs to about 6 million SNPs being more informative. Our current findings in a discovery cohort need to be reassessed in a validation cohort. Moreover, this study only assessed the efficacy endpoint PFS while toxicity is an important reason to discontinue sunitinib treatment or reduce the dose. Toxicity data are available in eCRFs for the retrospective cohort and a prospective patient cohort has been set up including toxicity and efficacy data. We will perform follow-up studies including toxicity analyses and reassessment of the current findings, including a meta-analysis to compare the associations in sunitinib or pazopanib treated patients, and its role in different ethnicities (Asians versus Caucasians).

In conclusion, we identified SNPs on chromosome 21 as potential predictors for sunitinib efficacy. These findings will be re-tested for validation in an independent cohort.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1 List of TKIs or TKI-like antitumor treatments. Patients who received any of these treatments prior to start with sunitinib were excluded from analyses.

pazopanib + everolimus alternately (ROPETAR study)
sorafenib + capecitabine
axitinib
sunitinib + everolimus (SUNRISES study)
Tivozanib (KRN951)
AZD2171 (cediranib)
Sugen = SU014813 (sunitinib phase I study)
bevacizumab + pazopanib (VDF111687 study)
sorafenib + IL-2
AZD2171 (cediranib) + gevitinib
cabozantinib
cediranib and saractanib/placebo
AURA (=pha-739358) Hierna Bay 57-9352 (phase 1 study)
axitinib + everolimus
vandetanib + selumetinib
Dovitinib (TKI 258)
Everolimus + Cyclofosfamide
Pazopanib + docetaxel

Eligibility criteria

Eligibility criteria

In case a certain date is unknown, please use the following guidelines:
- In case the month and year are known: 01-mm-yyyy
- In case only the year is known: 01-01-yyyy
- In case the total date is unknown: uk

Metastasis	O Yes O No (In case of "No" a patient can NOT be enrolled in EuroTARGET)
Date of diagnosis of metastasis	__ __ -- __ __ -- __ __ __ __
Institute of diagnosis	…… (For the Netherlands: according to NKR codes for hospitals)
Date, the informed consent has been signed by the patient	__ __ -- __ __ -- __ __ __ __

General information at diagnosis

Height

Patient's height	..... (in cm)

Bilateral renal cell carcinoma

A patient has bilateral renal cell carcinoma, in case a second tumor in the other kidney was detected within three months. If the patient has bilateral renal cell carcinoma, it is not necessary to fill in the information for both individual tumors.

Bilateral renal cell carcinoma	O Yes O No O Unknown
In case of bilateral renal cell carcinoma: Synchronous occurrence?	O Synchronous (fill in the form using the first tumor) O Non-synchronous (fill in the form using the tumor with the worst prognosis) O Unknown

Tumor assessment

Multiple dates should be registered in this form.
In case a certain date is unknown, please use the following guidelines:
- In case the month and year are known: 01-mm-yyyy
- In case only the year is known: 01-01-yyyy
- In case the total date is unknown: uk

This information should be registered only once for each patient at time of recruitment.
In most cases metastasis will be diagnosed together with the primary tumor.
In case metastasis was diagnosed later, please fill in the information below on the PRIMARY TUMOR.

Date of initial diagnosis of primary tumor	__ __ -- __ __ -- __ __ __ __
Date of surgery / biopsy for primary tumor	__ __ -- __ __ -- __ __ __ __
Tumor sample on which the pathology diagnosis was based comes from	O Primary tumor O Metastasis O No pathology diagnosis
Pathology laboratorium of diagnosis	..... (For the Netherlands: according to NKR codes for hospitals)
Pathology identification number of specimen	.....
Type of renal cell cancer	O Clear renal cell carcinoma O Chromophobe renal cell carcinoma O Collecting duct carcinoma O Papillary renal cell carcinoma O Non-clear cell renal cell carcinoma O Unknown
Papillary type	O Papillary type I RCC O Papillary type II RCC O Unknown)
Please specify non-clear cell renal cell carcinoma	.....

Presence of sarcomatoid elements	O Yes O No O Unknown
Fuhrman grade	O Grade I O Grade II O Grade III O Grade IV O Unknown
Presence of tumor necrosis	O Yes O No O Unknown

cTNM classification

According to TNM staging system 7 (2009), based on clinical information. For instructions, please see header row.

TNM staging system - clinical
Indicate the TNM stage according to the TNM classification 7 (2009) from clinical information. In case a previous edition of the TNM staging system was used, re-evaluate the TNM stage according to this new edition (2009).

Summary of the changes in the TNM classification 7 (2009)
T2 has been subdivided in:
- T2a - Tumor more than 7 cm but not more than 10 cm
- T2b - Tumor more than 10 cm, limited to the kidney

T3 and T4 got changes concerning the text:
- T3a - Invasion into the adrenal gland is removed from this stage (now T4)
- T4 - Tumor invades beyond Gerota fascia (including contiguous extension into ipsilateral adrenal gland)

cT- Primary Tumor	O TX O T0 O T1 O T1a O T1b	O T2 O T2a O T2b	O T3 O T3a O T3b O T3c O T4
cN- Regional Lymph Nodes	O NX O N0 O N1 O N2		
cM- Distant	O M0 O M1		

pTNM classification

According to TNM staging system 7 (2009), based on pathology. For instructions, please see the header row.

TNM staging system - pathology
Indicate the TNM stage according to the TNM classification 7 (2009) from the pathology report. In case a previous edition of the TNM staging system was used, re-evaluate the TNM stage according to this new edition (2009). In case a patient did not get surgery the pTNM cannot be assessed, since no pathological grading took place. In this case, indicate the TNM classification based on clinical information.

Summary of the changes in the TNM classification 7 (2009)
T2 has been subdivided in:
- T2a - Tumor more than 7 cm but not more than 10 cm
- T2b - Tumor more than 10 cm, limited to the kidney

T3 and T4 got changes concerning the text:
- T3a - Invasion into the adrenal gland is removed from this stage (now T4)
- T4 - Tumor invades beyond Gerota fascia (including contiguous extension into ipsilateral adrenal gland)

pT- Primary Tumor	O TX	O T2	O T3
	O T0	O T2a	O T3a
	O T1	O T2b	O T3b
	O T1a		O T3c
	O T1b		O T4
pN- Regional Lymph Nodes	O NX		
	O N0		
	O N1		
	O N2		
pM- Distant	O M0		
	O M1		
	O pTNM classification not done		

Co-morbidities at start treatment of metastasis

If co-morbidities exist at the time of start of treatment of metastasis, these co-morbidities should be registered here. A co-morbidity is NOT related to the cancer-specific treatment, and NOT related to the disease itself.

Other malignancy excl. non-melanoma skin cancer and carcinoma in situ of the cervix uteri	O No O Yes, please specify ..... O Unknown
Diabetes mellitus	O No O Yes O Unknown
Hypertension	O No O Yes O Unknown
in case of “yes”, currently treated with medication?	O No O Yes O Unknown
Cardiovascular disease e.g. Intermittent claudication, bifurcation prosthesis, percutaneous transluminal angioplasty (PTA), tube prosthesis,	O No O Yes, please specify ..... O Unknown
abdominal aortic aneurysm, aortic aneurysm, generalized atherosclerosis, peripheral vascular disease, embolectomy, post myocardial infarction, myocardial infarction, angina pectoris, coronary artery bypass graft (CABG), coronary heart disease, percutaneous transluminal coronary angioplasty (PTCA), heart failure, cardiomyopathy, cerebrovascular accident (CVA) in history (not a transient ischemic attack (TIA), cerebral artery disease, cerebral hemorrhage, post carotid endarterectomy, heartvalve prosthesis (aortic valve prosthesis, mitral valve prosthesis), valvular heart disease (mitral, tricuspid, aortic or pulmonary valve stenosis or regurgitation), post heart transplantation, thrombosis, pulmonary embolism, pelvic vein thrombosis, arrhythmias, atrial fibrillation, ventricular fibrillation, AV-block, artificial pacemaker, cardioversion. Although DVT actually falls in this category, we would like to have a separate registration of DVT.	
Deep vein thrombosis Although DVT actually falls in category "cardiovascular diseases", we would like to have a separate registration of DVT.	O No O Yes, most recent year of diagnosis __ __ -- __ __ -- __ __ __ __ O Unknown
Other clotting disorder e.g. haemophilia	O No O Yes, please specify ..... O Unknown

Lung disease e.g. CARA, emphysema, chronic bronchitis, chronic obstructive pulmonary disease, obstructive	O No O Yes, please specify ..... O Unknown
pulmonary disease, bronchial hyperreactivity, pulmonary fibrosis, post-lung transplantation.	
Gastro intestinal e.g. peptic ulcer, gastric ulcer, duodenal ulcer, (reflux) esophagitis, post gastrectomy, total or partial (B1, B2-	O No O Yes, please specify ..... O Unknown
resection), inflammatory bowel disease (Morbis Crohn, CU (Colitis Ulcerosa) (no familial adenomatosis polyposis), liver disease (e.g. cirrhosis, hepatitis A, B, C, non-A and non-B), post liver transplantation, diverticulitis.	
Urinary tract and genital organs e.g.chron. glomerulonephritis (CGN), chron. pyelonephritis (CPN), chronic renal failure, nephrotic syndrome, post	O No O Yes, please specify ..... O Unknown
kidney transplantation, dialysis (haemodialysis, CIPD, peritoneal dialysis).	
Muscles, joints and connective tissue e.g. connective tissue diseases (e.g., Sarcoidosis = M. Besnier Boeck, Wegener, periarteriitis nodosa (PAN),	O No O Yes, please specify ..... O Unknown
Systemic lupus erythematosus (SLE), rheumatoid arthritis (RA)	
Central and peripheral NS e.g. dementia, Alzheimer's disease, dementia syndrome, hemiplegic, hemiparesis, quadriplegic, paraplegic,	O No O Yes, please specify ..... O Unknown
psychiatric disorders (this refers to severe chronic psychiatric problems, e.g. hospitalized in a psychiatric hospital or ward, severe depression, psychosis or schizophrenia), Parkinson's disease.	
Metabolic disease e.g. haemophilia, hypo-/hyperthyreoidia	O No O Yes, please specify ..... O Unknown
Infectious disease Only chronic infectious diseases, e.g. HIV-positive, AIDS, tuberculosis. No acute infectious diseases, e.g. pneumonia.	O No O Yes, please specify ..... O Unknown
Congenital disorder e.g. Down syndrome, clubfoot, facial clefts, 18q syndrome, sickle cell disease	O No O Yes, please specify ..... O Unknown
Other disorder	O No O Yes, please specify ..... O Unknown

Available patient material

Blood sample

In case a certain date is unknown, please use the following guidelines:
- In case the month and year are known: 01-mm-yyyy
- In case only the year is known: 01-01-yyyy
- In case the total date is unknown: uk

Blood sample	O Yes date of venepuncture __ __ -- __ __ -- __ __ __ __ O No

Frozen tumor sample

Frozen tumor sample available for EuroTARGET	O Yes O No
Available tumor material	O Primary tumor O Metastasis O Primary tumor and metastasis

Paraffin-embedded tumor sample

Paraffin-embedded tumor sample available for EuroTARGET	O Yes O No
Available tumor material	O Primary tumor O Metastasis O Primary tumor and metastasis

Treatment line

Treatment of metastasis

Information collected during treatment regimes for metastasis.
When another drug is administered a new treatment line starts.
Please use one form per treatment line!
"1st line treatment of metastasis" should always be filled in. 2nd, 3rd, 4th and 5th treatment lines should only be filled in in case of (multiple) medication change(s).

Multiple dates should be registered in this form. In case a certain date is unknown, please use the following guidelines:
- In case the month and year are known: 01-mm-yyyy
- In case only the year is known: 01-01-yyyy
- In case the total date is unknown: uk

Treatment line no	O 1 or palliative care or best supportive care O 2 O 3 O 4 O 5

Clinical data at start of treatment of metastasis

In which hospital is this treatment line administered	…… (For the Netherlands: according to NKR codes for hospitals)
Weight of the patient	…… (in kg)
WHO performance status Register the WHO performance status. Copy exactly what is written in the medical record. Do not interpret the performance status yourself. When the WHO performance status cannot be found, but the Karnofsky score is available, the WHO performance status can be derived from the Karnofsky score.	O 0. Fully active, able to carry on all predisease activities without restrictions O 1. Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature O 2. Ambulatory and capable of all self care but unable to carry out any \ work activities. Up and about more than 50 percent of waking hours O 3. Capable of only limited self-care, confined to bed or chair for 50 percent or more of waking hours O 4. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair O Unknown

WHO	*Karnofsky score*	*Description*
0	100	Normal no complaints, no evidence of disease.
0	90	Able to carry on normal activity; minor signs or symptoms of disease.
1	80	Normal activity with effort; some signs or symptoms of disease.
1	70	Cares for self; unable to carry on normal activity or to do active work.
2	60	Requires occasional assistance, but is able to care for most of his personal needs.
2	50	Requires considerable assistance and frequent medical care.
3	40	Disabled; requires special care and assistance.
3	30	Severely disabled; hospital admission is indicated although death not imminent.
4	20	Very sick; hospital admission necessary; active supportive treatment necessary.
4	10	Moribund; fatal processes progressing rapidly.
4	0	Dead

Number of locations with metastases	
Number of locations with metastases Indicate the number of locations (number of sites) with metastases at the start of this treatment line. Also include lymph node metastases. Regional and distant lymph node metastases count as one site.	O 1 O 2 O 3 O 4 O > 4

Location of metastases	
Location of metastases	O Lung O Lymph nodes (regional and/or distant) O Liver O Bone O Brain O Adrenal gland O Other, please specify .....

Lab values at start of treatment of metastasis

Enter the lab values at the start of this treatment line. Values must not be older than 3 months before start treatment. When necessary, the characters < or > can be used in the fields below.

Hemoglobin	.....	O mmol/L O g/dl	(calculated to 1 decimal place)
Leukocytes	.....	x 10^9/L (=10^3/µl)	(calculated to 1 decimal place)
Percentage of neutrophils	.....	%	(calculated to 1 decimal place)
Number of neutrophils	.....	x 10^9/L	(calculated to 2 decimal places)
Trombocytes (platelets)	.....	x 10^9/L (=10^3/µl)	
Creatinine	.....	O µmol/L O mg/dl	(calculated to 1 decimal place)
Albumin	.....	g/L	
Calcium	.....	O mmol/L	(calculated to 2 decimal places)
Total bilirubin	.....	O µmol/L O mg/dl	(calculated to 1 decimal place)
Alkaline phosphatase	.....	U/L	
Aspartate aminotransferase (SGOT/ AST/ ASAT)	.....	U/L	
Alanine transaminase (SGPT/GUID/ALT/ALAT)	.....	U/L	
LDH	.....	units/L	

Drug treatment	
Which drug(s) is/are being used	O None, palliative or best supportive care O Interferon-alpha (IFN-a) O Interferon-alpha (IFN-a) + vinblastine O Interleukin 2 O Sunitinib O Sorafenib O Bevacizumab + Interferon-alpha (IFN-a) O Temsirolimus O Everolimus O Pazopanib O Other, please specify ..... O Unknown

Dosing schemes

Please specify dose, frequency etc. in detail below.

A treatment line can consist of different cycles. This is the case for the treatment with Sunitinib. Usually Sunitinib is given during four weeks (weeks on), followed by a two-week rest-period (weeks off). This six-week period is equal to one cycle.

One dosing scheme is a (number of) treatment cycle(s). In case the treatment cycle has been changed (e.g. in case of dose reduction, change in frequency etc.), please fill in the new treatment cycle in dosing scheme 2, 3, etc. You can add extra schemes by clicking on the + button.

A treatment line can also consist of a treatment given during a certain number of weeks/ months. No cycles are involved. This is the case for the treatment with Interferon-alpha, Sorafenib, Everolimus, Pazopanib, Bevacizumab (+ interferon alpha) and Temsirolimus. Use "n/a" in the "weeks on", "weeks off", "total amount per cycle' and "no of cycles with this scheme" fields.

A treatment may consist of a combination of drugs (e.g. Bevacizumab + Interferon alpha). If this is the case, use two schemes for registering this treatment.

In case a new treatment line has started (change of medication), use a new therapy form ("2nd, 3rd, 4th, 5th line treatment of metastasis") for registering this line.

1.	Start Date 1	__ __ -- __ __ -- __ __ __ __				
	Dose 1	.....	O mg O million IU O ml O mmol O other, please specify ..... O unknown	(calculated to 2 decimal places)		
	Frequency 1	O 0.5 x O 1 x O 2 x O 3 x O unknown	per 1	O day O 3 days O week O month O unknown		
	Weeks on treatment 1	.....	Weeks off treatment 1	.....	In case no cycles are involved (eg., interferon alpha) use **n/a.**	
	Total amount per cycle 1	.....	No of cycles with this scheme 1	.....	Please use the unit chosen above. In case no cycles are involved (eg., interferon alpha) use **n/a.**	
	Stop date 1	__ __ -- __ __ -- __ __ __ __	Reason for stop/change 1	O toxicity grade 1 or 2 O toxicity grade 3 or 4 O progression / relapse O condition of patient O wishes of patiënt O patient has died O other, please specify ..... O unknown		

2.	Start Date 2	__ __ -- __ __ -- __ __ __ __				
	Dose 2	.....	O mg O million IU O ml O mmol O other, please specify ..... O unknown	(calculated to 2 decimal places)		
	Frequency 2	O 0.5 x O 1 x O 2 x O 3 x O unknown	per 2	O day O 3 days O week O month O unknown		
	Weeks on treatment 2		Weeks off treatment 2	.....	In case no cycles are involved (eg., interferon alpha) use **n/a.**	
	Total amount per cycle 2	.....	No of cycles with this scheme 2	.....	Please use the unit chosen above. In case no cycles are involved (eg., interferon alpha) use **n/a.**	
	Stop date 2	__ __ -- __ __ -- __ __ __ __	Reason for stop/change 2	O toxicity grade 1 or 2 O toxicity grade 3 or 4 O progression / relapse O condition of patient O wishes of patiënt O patient has died O other, please specify ..... O unknown		

3.	Start Date 3	__ __ -- __ __ – __ __ __ __			
	Dose 3	.....	O mg O million IU O ml O mmol O other, please specify ..... O unknown	(calculated to 2 decimal places)	
	Frequency 3	O 0.5 x O 1 x O 2 x O 3 x O unknown	per 3	O day O 3 days O week O month O unknown	
	Weeks on treatment 3		Weeks off treatment 3	.....	In case no cycles are involved (eg., interferon alpha) use **n/a.**
	Total amount per cycle 3	.....	No of cycles with this scheme 3	.....	Please use the unit chosen above. In case no cycles are involved (eg., interferon alpha) use **n/a.**
	Stop date 3	__ __ -- __ __ – __ __ __ __	Reason for stop/change 3	O toxicity grade 1 or 2 O toxicity grade 3 or 4 O progression / relapse O condition of patient O wishes of patiënt O patient has died O other, please specify ..... O unknown	

4.	Start Date 4	__ __ -- __ __ – __ __ __ __			
	Dose 4	.....	O mg O million IU O ml O mmol O other, please specify ..... O unknown	(calculated to 2 decimal places)	
	Frequency 4	O 0.5 x O 1 x O 2 x O 3 x O unknown	per 4	O day O 3 days O week O month O unknown	
	Weeks on treatment 4		Weeks off treatment 4	.....	In case no cycles are involved (eg., interferon alpha) use **n/a.**
	Total amount per cycle 4	.....	No of cycles with this scheme 4	.....	Please use the unit chosen above. In case no cycles are involved (eg., interferon alpha) use **n/a.**
	Stop date 4	__ __ -- __ __ – __ __ __ __	Reason for stop/change 4	O toxicity grade 1 or 2 O toxicity grade 3 or 4 O progression / relapse O condition of patient O wishes of patiënt O patient has died O other, please specify ..... O unknown	

(Concomitant) non drug treatment - Surgery	
	O No surgery
Date of surgery	__ __ -- __ __ -- __ __ __ __
Surgery type	O Open nephrectomy O Laparoscopic nephrectomy O Open partial nephrectomy O Laparoscopic partial nephrectomy O Adrenalectomy O Lymfadenectomy O Thrombectomy O Embolisation O Radio Frequency Ablation (RFA) O Cryosurgery O Metastasectomy O Nephrectomy + metastasectomy (in one session) O Partial nephrectomy + metastasectomy (in one session) O Nephrectomy + other (in one session) O Partial nephrectomy + other (in one session) O Other, please specify ..... O Unknown

(Concomitant) non drug treatment - Radiotherapy	
	O No radiotherapy
Start date	__ __ -- __ __ -- __ __ __ __
Stop date	__ __ -- __ __ -- __ __ __ __
Total dose	..... Gy.
Site of radiotherapy	.....
Subjective (symptomatic) response to radiotherapy	O No O Yes O Unknown

Toxicities

The items in this table must be updated on an ongoing basis.

Please collect only adverse events which are (likely) CAUSALLY related to the treatment.
All grade 3, 4 and 5 toxicities (NOT grade 1 and 2) developed until one month after the last treatment should be reported. Register per treatment line each toxicity only once, with the highest CTC grade reached. If a toxicity existed before the diagnosis of renal cell carcinoma was made, but the CTC grade has increased after diagnosis, this toxicity must also be reported. In this case the starting date is the day that the toxicity worsens.

If the CTC grade was not specified by the physician in the medical record of the patient, specify the grade of each specific toxicity yourself according to CTCAE version 4.0. The CTC grade refers to the severity of the toxicity. For each specific toxicity, the CTCAE guidelines give a unique clinical description which corresponds to the degree of toxicity. You can find the CTCAE at the following page: http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_8.5x11.pdf. Always consult these guidelines to find the specific CTC grade for the toxicity to be reported.

The following symptoms are not required to report as toxicity:
- Symptoms that can be linked to (progress/relapse of) renal cell carcinoma.
- Symptoms that already existed in the same degree of seriousness at the time of diagnosis.
- Symptoms that can be regarded as symptoms of a previously reported other toxicity (e.g. swallowing difficulties due to mucositis).

Fever must be registered. Please also report whether fever is accompanied by neutropenia or not.
Note: If fever occurs as a result of a (allergic) reaction to the medication, the so-called "drug fever", this fever should also be reported as a toxicity.

	Toxicity If not specified by the physician, consult terminology used in the PDF file mentioned above	**CTC grade** If not specified by the physician, consult the guidelines in the PDF file mentioned above to find the specific CTC grade for the specific toxicity	**Determined by**	**Treatment to which the toxicity is related***	**Start date of toxicity**	**End date of toxicity**	**Is toxicity treated?**
		Grade 3: Serious toxicity: influences general daily life activities, hospitalisation sometimes required **Grade 4**: Life-threatening toxicity: always requires hospitalization **Grade 5**: Death due to toxicity	Indicate whether the CTC grade was specified by the **physician** in the medical record of the patient, or whether the grade was interpreted by the data manager from the medical record (**DM**)	Report the treatment to which the toxicity is (very likely) related.	Note the date the toxicity has started.	Note the date the toxicity has ended.	Is the toxicity treated?
1.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
2.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
3.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
4.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
5.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes

	Toxicity If not specified by the physician, consult terminology used in the PDF file mentioned above	**CTC grade** If not specified by the physician, consult the guidelines in the PDF file mentioned above to find the specific CTC grade for the specific toxicity	**Determined by**	**Treatment to which the toxicity is related***	**Start date of toxicity**	**End date of toxicity**	**Is toxicity treated?**
6.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
7.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
8.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
9.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
10.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
11.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
12.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
13.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
14.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
15.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
16.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
17.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
18.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
19.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
20.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
21.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
22.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
23.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
24.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
25.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes

	Toxicity If not specified by the physician, consult terminology used in the PDF file mentioned above	**CTC grade** If not specified by the physician, consult the guidelines in the PDF file mentioned above to find the specific CTC grade for the specific toxicity	**Determined by**	**Treatment to which the toxicity is related***	**Start date of toxicity**	**End date of toxicity**	**Is toxicity treated?**
26.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
27.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
28.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
29.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
30.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes
31.	.....	.....	O physician O DM	.....	__-__-____	__-__-____	O no O yes

*** Related to:**

01 = Interferon-alpha (IFN-a)
02 = IFN-a + vinblastin
03 = Interleukin 2
04 = Sunitinib
05 = Sorafenib
06 = Bevacizumab + IFN-a
07 = Temsirolimus
08 = Everolimus
09 = Pazopanib
10 = Radiotherapy
11 = Surgery
88 = other, please specify below
99 = unknown

Related to, option 88, specification:

.....

.....

.....

Response (also in case of non treatment)

Register the response as mentioned in the RADIOLOGY report; as a substitute use the response as indicated by the physician in the medical record.
Information on response is required, and therefore, this part should always be filled out. Details of tumor assessments (in the part 'tumor assessment': 'date tumor assessment', 'target lesions' and 'non-target lesions' below) should be registered if available.
More information on RECIST version 1.1: www.eortc.be/recist/

	Date of response assessment	Treatment line	Response*	In case of PR or SD: Decrease according to RECIST	Date of relapse or progression	Further anti cancer treatment	Reason no further treatment**
	Date on which the response has been established, within 3 months of treatment. This is the date that the physician recorded the response in the medical record. If this is not listed, then record the date of the investigations that are required for the determination of response. Please note the last date; therefore, the date on which all of the required results were available.	1 = 1st line 2 = 2nd line 3 = 3rd line 4 = 4th line 5 = 5th line	0 = no (drug) treatment 1 = CR complete remission 2 = PR partial remission 3 = SD stable disease 4 = PD progressive disease 9 = unknown		Date on which a possible relapse or progress occurs, mostly based on radiology. If no relapse or progress has occured at end of follow-up, fill in 00-00-0000 (not applicable).	Indicate whether the patient receives active treatment as a result of relapse or progression of disease (or when a previous treatment was ineffective). 0= **no** (in case of best supportive care).	If a patient does not receive active treatment (anymore), register the reason why. Multiple reasons are possible. If the field code 88 is filled in, a specification must be given in the available field.
1.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
2.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
3.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
4.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
5.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
6.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
7.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
8.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
9.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
10.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....
11.	__-__-____	.....	.....	.....%	__-__-____	O no O yes	.....

	Date of response assessment	Treatment line	Response*	In case of PR or SD: Decrease according to RECIST	Date of relapse or progression	Further anti cancer treatment	Reason no further treatment**
12.	_ _-_ _-_ _ _ _	.....	.....	.....%	_ _-_ _-_ _ _ _	O no O yes	.....
13.	_ _-_ _-_ _ _ _	.....	.....	.....%	_ _-_ _-_ _ _ _	O no O yes	.....
14.	_ _-_ _-_ _ _ _	.....	.....	.....%	_ _-_ _-_ _ _ _	O no O yes	.....
15.	_ _-_ _-_ _ _ _	.....	.....	.....%	_ _-_ _-_ _ _ _	O no O yes	.....
16.	_ _-_ _-_ _ _ _	.....	.....	.....%	_ _-_ _-_ _ _ _	O no O yes	.....

*** Response**
0 = No (drug) treatment, adjuvant treatment or palliative treatment whereby the patient has died before the first evaluation time, therefore not applicable
1 = Complete remission (CR): Disappearance of all (target and non-target) lesions
2 = Partial remission (PR): At least 30% decrease in longest diameter (LD) of single tumor or sum of LDs of multiple masses (specify the decrease according to RECIST version 1.1)
3 = Stable disease (SD): Neither PR nor PD criteria met (specify the decrease (if any) according to RECIST version 1.1)
4 = Progressive disease (PD): Greater than 20% increase in LD of single tumor or the sum of LDs in multiple masses, or appearance of new lesions
9 = unknown (also when treatment was not completed)

**** Reason no further treatment**
00 = new treatment starts, so n.a.
01 = no further treatment possibilities
02 = wait-and-see policy
03 = refusal patient / family
04 = co morbidity
05 = functional status
06 = age
07 = short life expectancy
08 = patient has died
88 = other, please specify below
99 = unknown

Reason no further treatment, option 88, specification:

.....

.....

.....

Tumor assessment (also in case of non treatment)

Details on 'tumor assessment', in the parts 'date tumor assessment', 'target lesions' and 'non-target lesions' should be filled out, if available. So please fill out the dates on which tumor assessments were performed in this treatment line and provide detailed information on target and non-target lesions.
Click on the + buttons to add a new assessment.

Date tumor assessment

		Date
1.	Baseline assessment	__ __ -- __ __ -- __ __ __ __
2.	Tumor assessment 2	__ __ -- __ __ -- __ __ __ __
3.	Tumor assessment 3	__ __ -- __ __ -- __ __ __ __
4.	Tumor assessment 4	__ __ -- __ __ -- __ __ __ __
5.	Tumor assessment 5	__ __ -- __ __ -- __ __ __ __
6.	Tumor assessment 6	__ __ -- __ __ -- __ __ __ __
7.	Tumor assessment 7	__ __ -- __ __ -- __ __ __ __
8.	Tumor assessment 8	__ __ -- __ __ -- __ __ __ __
9.	Tumor assessment 9	__ __ -- __ __ -- __ __ __ __
10.	Tumor assessment 10	__ __ -- __ __ -- __ __ __ __
11.	Tumor assessment 11	__ __ -- __ __ -- __ __ __ __
12.	Tumor assessment 12	__ __ -- __ __ -- __ __ __ __
13.	Tumor assessment 13	__ __ -- __ __ -- __ __ __ __
14.	Tumor assessment 14	__ __ -- __ __ -- __ __ __ __
15.	Tumor assessment 15	__ __ -- __ __ -- __ __ __ __
16.	Tumor assessment 16	__ __ -- __ __ -- __ __ __ __

Target Lesions

All measurable lesions up to a maximum of 5 lesions (2 per organ) representative of all involved organs should be identified as target lesions and be recorded and measured at baseline. These 5 lesions should be selected on the basis of their size (lesions with the longest diameter) and their suitability for accurate repetitive measurements (either by imaging techniques or clinically). A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as reference to further characterize the objective tumor response of the measurable dimension of the disease. If there are >5 measurable lesions, those not selected as target lesions will be considered together with non-measurable disease as non-target lesions.

Kidney-ipsilateral = side of primary tumor
For lymph nodes: Take shortest diameter

	Location /type	Target lesion 1	Target lesion 2	Target lesion 3	Target lesion 4	Target lesion 5	Sum of lesion diameters	Overall Response
1	Location /type ipsi = ipsilateral con = contra-lateral adrenal g = adrenal gland	Okidney ipsi Okidney con Olung Olymphnode Oliver Obrain Oadrenal g Oother* On.a.	Okidney ipsi Okidney con Olung Olymphnode Oliver Obrain Oadrenal g Oother* On.a.	Okidney ipsi Okidney con Olung Olymphnode Oliver Obrain Oadrenal g Oother* On.a.	Okidney ipsi Okidney con Olung Olymphnode Oliver Obrain Oadrenal g Oother* On.a.	Okidney ipsi Okidney con Olung Olymphnode Oliver Obrain Oadrenal g Oother* On.a.		CR (complete remission) PR (partial remission) SD (stable disease) PD (pro-gressive disease) U (unknown)
2	Baseline assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
3	Tumor 2 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
4	Tumor 3 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
5	Tumor 4 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
6	Tumor 5 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
7	Tumor 6 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
8	Tumor 7 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
9	Tumor 8 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
10	Tumor 9 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
11	Tumor 10 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD

12	Tumor 11 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
13	Tumor 12 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
14	Tumor 13 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
15	Tumor 14 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
16	Tumor 15 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
17	Tumor 16 assessment	..... mm	..... mm	..... mm	..... mm	..... mm	..... mm	O CR O PD O PR O U O SD
*Target lesion other		.....	.....	.....	.....	.....		

Non-Target Lesions

All non-measurable lesions (or sites of disease) plus any measurable lesions over and above the 5 listed as target lesions. Measurements are not required but these lesions should be noted at baseline and should be followed as "equal", "increase" or "decrease".

	Location /type	Non-target lesion 1	Non-target lesion 2	Non-target lesion 3	Non-target lesion 4	Non-target lesion 5
1	Location /type ipsi = ipsilateral contr = contra-lateral adrenal gl = adrenal gland	O kidney ipsi O kidney contr O lung O lymphnodes O liver O bone O brain O adrenal gl O other* O n.a.	O kidney ipsi O kidney contr O lung O lymphnodes O liver O bone O brain O adrenal gl O other* O n.a.	O kidney ipsi O kidney contr O lung O lymphnodes O liver O bone O brain O adrenal gl O other* O n.a.	O kidney ipsi O kidney contr O lung O lymphnodes O liver O bone O brain O adrenal gl O other* O n.a.	O kidney ipsi O kidney contr O lung O lymphnodes O liver O bone O brain O adrenal gl O other* O n.a.
2	Baseline assessment	O no O yes	O no O yes	O no O yes	O no O yes	O no O yes
3	Tumor 2 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
4	Tumor 3 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
5	Tumor 4 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
6	Tumor 5 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
7	Tumor 6 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
8	Tumor 7 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
9	Tumor 8 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
10	Tumor 9 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
11	Tumor 10 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
12	Tumor 11 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease

	Location /type	Non-target lesion 1	Non-target lesion 2	Non-target lesion 3	Non-target lesion 4	Non-target lesion 5
13	Tumor 12 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
14	Tumor 13 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
15	Tumor 14 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
16	Tumor 15 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
17	Tumor 16 assessment	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease	O equal O increase O decrease
	*Target lesion other	.....	.....	.....	.....	.....

New Lesions	
New lesion during this treatment line?	O no O yes
Date new lesion	__ __ -- __ __ -- __ __ __ __

Final information on patient

Final information on patient

This form should be filled in when a patient has died or is lost to follow up

At which date has the final information (toxicities, treatment, etc.) been collected	__ __ -- __ __ -- __ __ __ __
Reason for last registration	O Lost to follow up (e.g., referral to another hospital, registration impossible) O Patient has died O Unknown
In case the patient is lost to follow up, last date patient was known to be alive	__ __ -- __ __ -- __ __ __ __
In case the patient has died, date of death	__ __ -- __ __ -- __ __ __ __
In case the patient has died, cause of death	O As a result of the malignancy O Due to toxicity (specify in “toxicities and response”) O Other, please specify below O Unknown
Other cause of death, option 8	…… …… ……
Remarks	…… …… ……

Supplement 2a EuroTARGET web-based electronic case record form (eCRF)

Version 9 August 2012

Web-based Case Record Forms

EuroTARGET portal

Clinical baseline and follow-up data of EuroTARGET patients will be collected in web-based case record forms (CRFs). These web-based CRFs can be filled out via the user-friendly EuroTARGET portal: **https://eurotarget.glean.nl**

The portal can be entered with your personal account. Please change your password the first time you login. A password has to:

- Be at least 10 characters
- Of which at least 2 numbers
- And at least 2 special characters
- And at least 6 letters, of which at least 1 should be capitalized

You will only have access to patients of your own institute.

Please ignore the Dutch Announcements! Announcements will be communicated to you via e-mail.

Please "**log out**" after you are finished. Just closing the application may give problems the next time you are logging in.

EuroTARGET study number

Once a new patient has been added a EuroTARGET study number will be generated automatically. Please use this EuroTARGET study number on blood and tumor samples.

CRFs

After adding a new patient the following CRFs will become available for this patient:

- **Eligibility criteria:**
  This information should be registered once for each patient at time of recruitment.
- **General information at diagnosis:**
  Information on "tumor assessment" and "co-morbidities at start treatment of metastasis". This information should be registered once for each patient at time of recruitment.
- **Available patient material:**
  Please register whether "blood sample" and "tumor sample" are available for EuroTARGET.
- **Treatment line:**
  Information collected during treatment regimes for metastasis. When another drug is administered a new treatment line starts. Please use one form per treatment line. "1st line treatment of metastasis" should always be filled out. "2nd, 3rd, 4th and 5th line treatment of metastasis" forms are exact copies of "1st line treatment of metastasis" and should only be filled out in case of (multiple) medication change(s). Information

Supplement 2b User Manual for the EuroTARGET web-based electronic case record form (eCRF)

Version 9 August 2012

on "toxicities" and "response" must be updated on an ongoing basis (at least once every 6 months), preferably after each check-up.
In the table "response (also in case of non treatment)", the column 'In case of PR or SD: Decrease according to RECIST' does NOT need to be filled out. In addition, the parts "Tumor assessment (also in case of non treatment)", "Date tumor assessment", "Target lesions", and "Non-target lesions" do NOT need to be filled out.

- **Questionnaire data:**
  Please insert data from the patient questionnaire. Information on "smoking habits", "family history", "medication", all prior to study entry. In case NO questionnaire is available (e.g., in case of retrospective data), just leave the status of the CRF at "unstarted".

- **Final information on patient:**
  This information should be registered when a patient has died or is lost to follow up.

CRFs – finished

CRFs that are not started yet will have the status "unstarted". After entering and saving data the status of the CRF will become "unfinished". You can save a partially filled out CRF and complete it later.

The CRF "treatment line" needs to be updated during follow up of the patient. Follow up ends when a patient dies, is lost to follow up, or at the end of the study. When follow-up is not complete yet, leave the status of the CRF at "unfinished".

After all fields have been completed please put the status of the CRF on "finished" (at right top corner of the CRF). The system will then check whether all fields are filled in correctly and "sentdate" will be generated automatically.

Export function

The homepage (begin screen) of the EuroTARGET portal has the option "Export eCRF". To export data of your patients, click this button, leave all export format options in the default settings, and click "export". This will generate a folder with 2 Excel files per CRF; one with the data, and one with information about the variables. To export not only the CRFs, but also the Patient Data, you will need to check that option in the export format options.

Test account

You can take a look at the CRFs of a test patient by logging in on the portal with the test account:

Username: et_testphys
Password: Testphys11!!

Please do NOT change the password and do NOT enter real patients for this test institute!

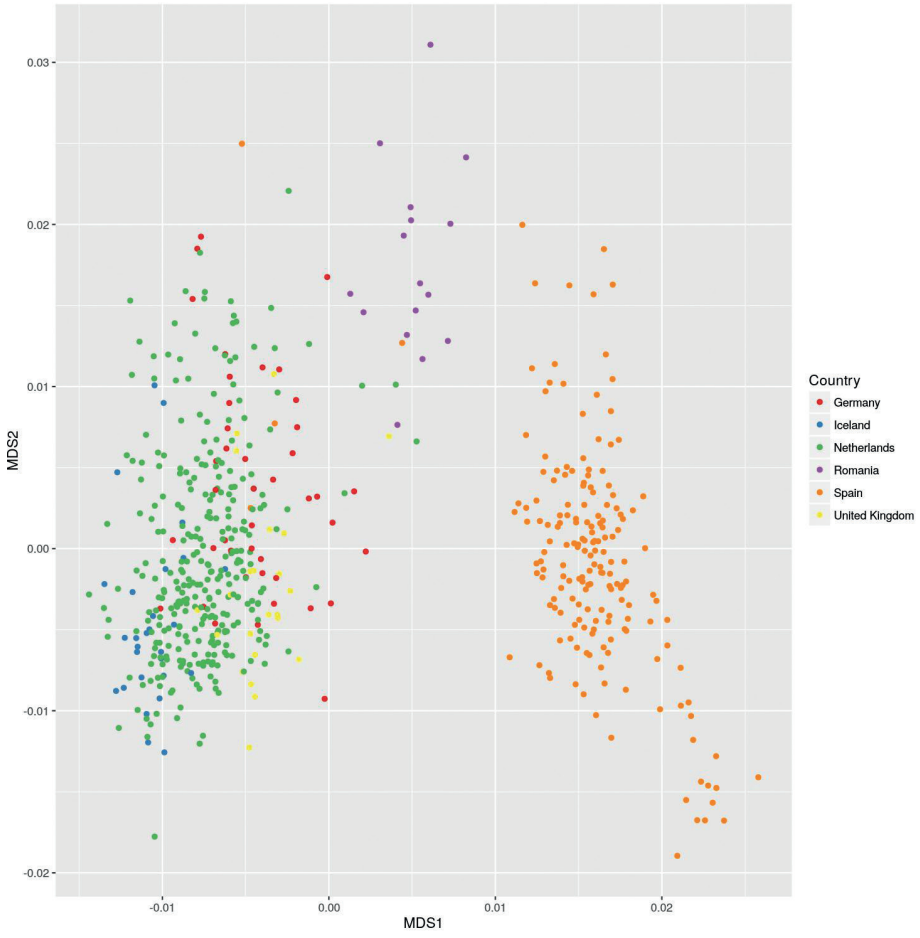
Please "**log out**" after you are finished. Just closing the application may give problems the next time you or another test account user are logging in.

Questions

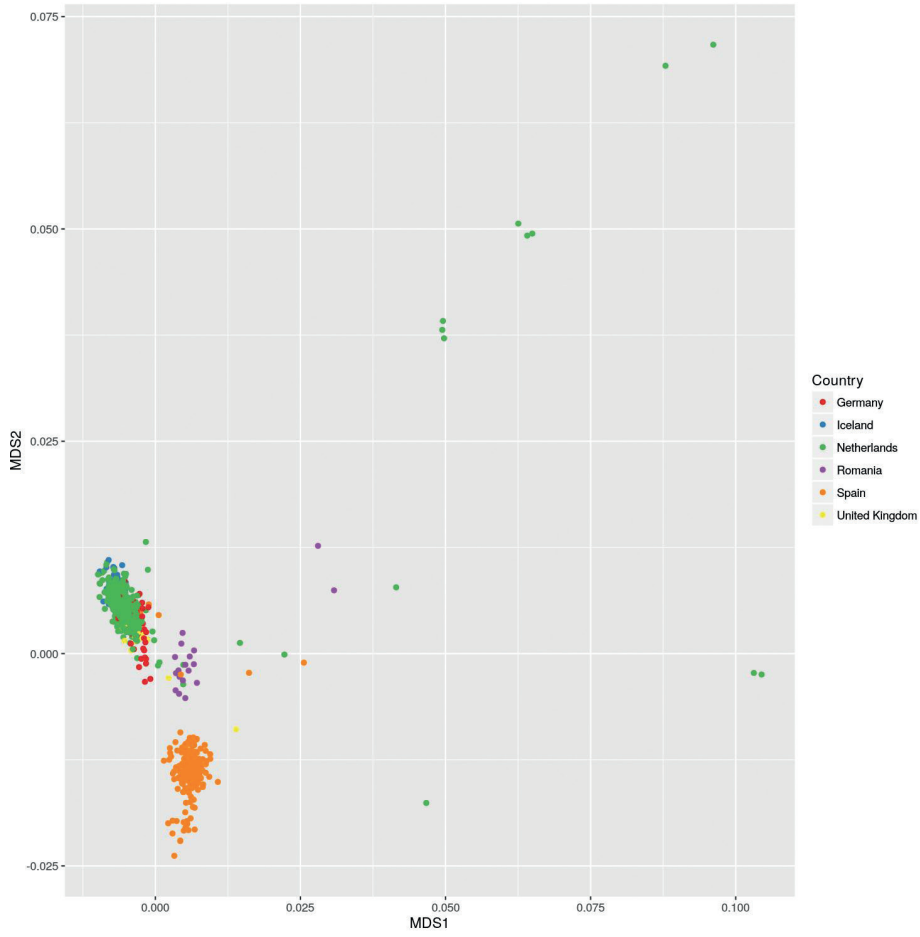
For questions please contact Dr Loes van der Zanden at Loes.vanderZanden@radboudumc.nl

Supplementary Figure 3 Multi-dimensional scaling (MDS). The sample was subjected to a MDS analysis in order to detect possible population stratification. MDS tries to find a low-dimensional map in which distances between original samples are optimally preserved. The distances between individuals is based on IBS similarity. 7 dimensions were used for the MDS map. The following plots plot set adjacent coordinates against each other. Plot **A** is after exclusion of 20 outlying individuals and plot **B** is before exclusion of these outliers.

A. After exclusion of 20 individuals



B. Including the 20 outlying individuals



Supplementary Figure 4 Pairwise IBS values form the basis for MDS and the IBD/IBS clustering. These pairwise IBS scores are plotted in the following figures. Each point represents a pair of individuals. Frequencies of IBS0, IBS1 and IBS2 genotype pairs are used for the coordinates. Pairwise plots, as well as a 3D-representation are plotted.

A. IBS0 vs. IBS1

B. IBS1 vs. IBS2

Supplementary Table 5 Summary of exclusions of markers for QC steps. The second part of the table contains pairwise overlap between QC steps.

	Ne	perc	Ncum	missing	HWE	MAF
Missing	0	0.0%	0	0	0	0
HWE	11	0.0%	11	0	11	0
MAF	1394	0.2 %	1405	0	0	1394

Abbreviations: *Ne*: number of exclusions; *perc*: percentage of exclusions from total sample after accounting for external exclusions; *Ncum*: cumulative number of excluded markers.

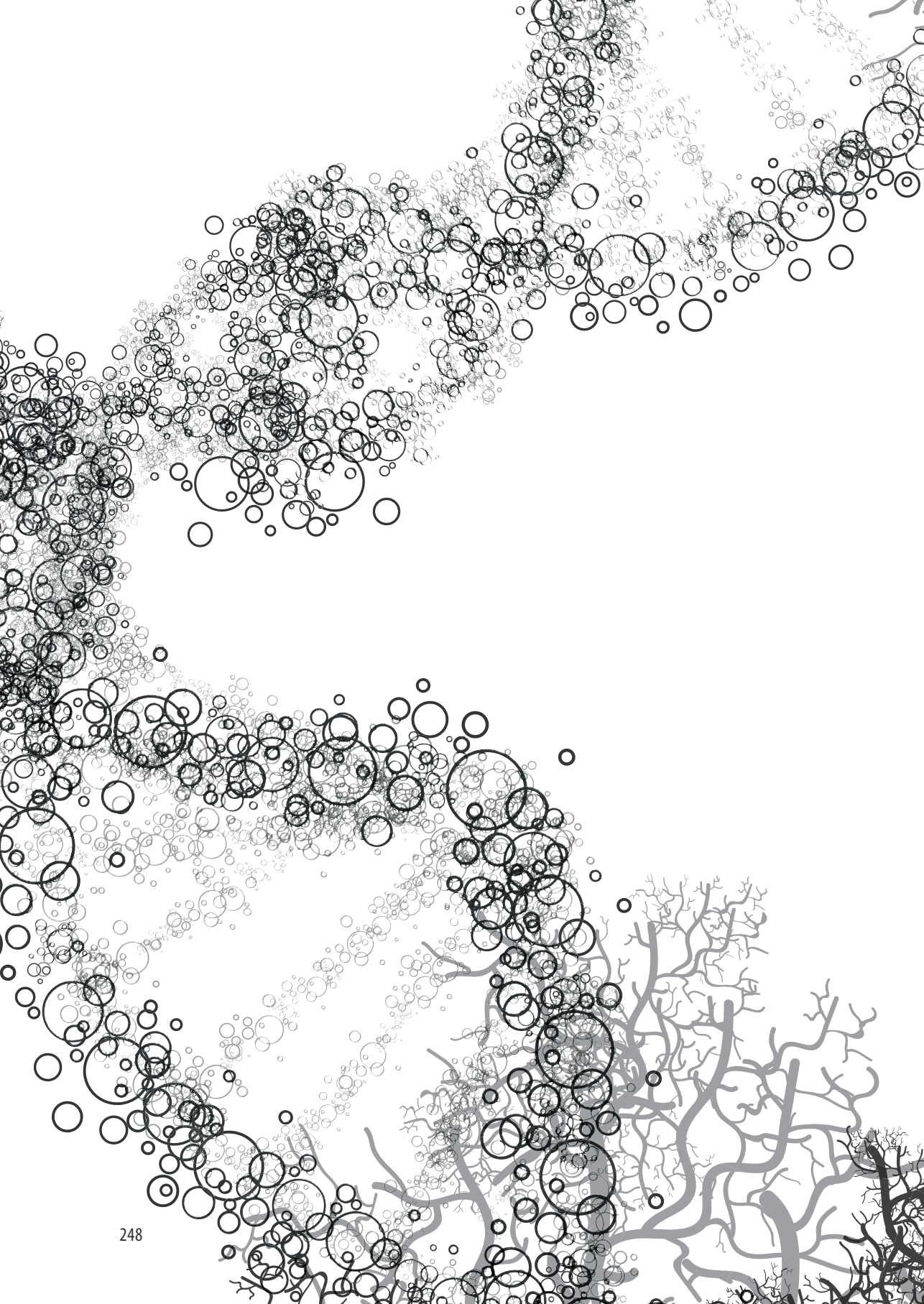
Supplementary Table 6: 50 most significant SNPs associated with PFS

SNP	Certainty of SNP imputation	Chromosome	MAF	P.value	HR	95%CI lower	95%CI upper
1	0.932	21	0.065	1.11458E-08	2.4236	1.7888	3.2839
2	0.977	21	0.063	1.33813E-08	2.4373	1.7924	3.3144
3	0.985	21	0.063	1.76333E-08	2.4080	1.7737	3.2692
4	0.971	21	0.064	2.58842E-08	2.3450	1.7372	3.1657
5	0.996	21	0.067	2.73259E-08	2.3212	1.7248	3.1238
6	1	21	0.067	2.95367E-08	2.3110	1.7186	3.1074
7	0.981	21	0.064	3.06015E-08	2.3224	1.7236	3.1293
8	0.998	21	0.067	3.12443E-08	2.3089	1.7167	3.1052
9	1	21	0.061	4.03684E-08	2.3369	1.7259	3.1642
10	0.921	21	0.051	7.78666E-08	2.5141	1.7960	3.5193
11	0.997	21	0.065	1.23328E-07	2.2416	1.6620	3.0232
12	0.998	21	0.065	1.23846E-07	2.2412	1.6618	3.0227
13	0.999	21	0.065	1.23891E-07	2.2423	1.6623	3.0247
14	1	21	0.065	1.25295E-07	2.2402	1.6611	3.0212
15	1	21	0.065	1.25295E-07	2.2402	1.6611	3.0212
16	1	21	0.065	1.25295E-07	2.2402	1.6611	3.0212
17	1	21	0.065	1.25295E-07	2.2402	1.6611	3.0212
18	1	21	0.065	1.25295E-07	2.2402	1.6611	3.0212
19	0.998	21	0.065	1.26663E-07	2.2419	1.6617	3.0246
20	0.997	21	0.065	1.33591E-07	2.2393	1.6596	3.0215
21	0.994	21	0.065	1.40066E-07	2.2394	1.6588	3.0232
22	0.994	21	0.065	1.41103E-07	2.2392	1.6586	3.0231
23	0.991	21	0.065	1.51718E-07	2.2385	1.6570	3.0240
24	0.982	21	0.063	1.52545E-07	2.3295	1.6988	3.1944
25	0.989	21	0.065	1.57844E-07	2.2388	1.6564	3.0259
26	0.988	21	0.065	1.62846E-07	2.2385	1.6557	3.0263
27	0.987	21	0.065	1.65752E-07	2.2382	1.6553	3.0263
28	0.929	21	0.054	1.77905E-07	2.3854	1.7212	3.3059
29	0.982	21	0.065	1.86134E-07	2.2366	1.6525	3.0271

Supplementary Table 6: Continued

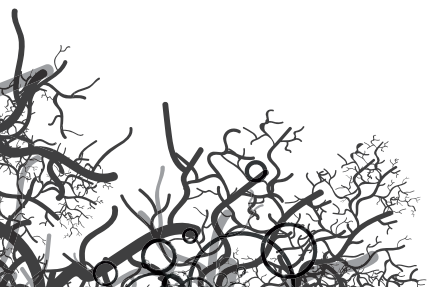
SNP	Certainty of SNP imputation	Chromosome	MAF	P.value	HR	95%CI lower	95%CI upper
30	1	5	0.561	2.24949E-07	0.6630	0.5675	0.7746
31	0.972	21	0.064	2.38494E-07	2.2324	1.6461	3.0276
32	0.996	5	0.559	2.56816E-07	0.6634	0.5675	0.7754
33	0.964	21	0.068	2.89726E-07	2.1975	1.6266	2.9687
34	0.955	21	0.069	3.21523E-07	2.1929	1.6227	2.9635
35	0.962	21	0.07	3.23447E-07	2.1571	1.6062	2.8971
36	0.999	5	0.56	3.38451E-07	0.6676	0.5715	0.7797
37	0.999	5	0.561	3.4011E-07	0.6676	0.5716	0.7798
38	0.91	21	0.051	3.51767E-07	2.4179	1.7214	3.3962
39	0.998	5	0.563	3.66796E-07	0.6675	0.5712	0.7800
40	0.999	5	0.56	4.07111E-07	0.6710	0.5751	0.7830
41	0.999	5	0.56	4.15443E-07	0.6713	0.5753	0.7833
42	0.895	7	0.077	4.2054E-07	2.1205	1.5848	2.8372
43	0.98	5	0.552	4.42056E-07	0.6665	0.5694	0.7801
44	0.983	5	0.554	5.30518E-07	0.6745	0.5782	0.7867
45	0.992	5	0.561	5.36346E-07	0.6734	0.5769	0.7860
46	0.992	5	0.561	5.3644E-07	0.6734	0.5769	0.7860
47	0.934	21	0.05	5.40247E-07	2.4091	1.7081	3.3978
48	0.944	17	0.602	6.34366E-07	1.5235	1.2909	1.7980
49	0.887	8	0.752	6.41902E-07	0.6241	0.5184	0.7514
50	0.99	7	0.059	7.11247E-07	2.1435	1.5857	2.8974

Abbreviations: MAF=Minor Allele Frequency, HR=Hazard Ratio, CI=Confidence Interval.



CHAPTER 10

GENERAL DISCUSSION AND FUTURE PERSPECTIVES



PHARMACOGENETICS AS A KEY TO INDIVIDUALIZE MRCC TREATMENT

Sunitinib treatment requires a personalized approach, since patients can respond very differently to this drug. Pharmacogenetics may improve our ability to provide a tailored therapy by studying how genetic variations could influence drug response. Accordingly, the objective of this thesis was to find genetic markers that can predict toxicity and efficacy of sunitinib particularly in patients with metastatic renal cell carcinoma (mRCC) (**Chapter 1**). In a population PK model, we observed that SNPs located in *CYP3A4*, *CYP3A5*, and *ABCB1* (genes involved in metabolism and drug absorption) are potentially associated with the clearance of sunitinib and its active metabolite. In analogy to this, we confirmed SNP associations from previous candidate gene studies for SNPs in *CYP3A5* and *ABCB1* that predict the need for dose reductions and improvement of PFS on sunitinib. Sunitinib-induced toxicity is possibly related to SNPs in *CYP3A4* and *CYP3A5*, and in the interleukin genes *IL8* and *IL13*. *VEGFR-2* (*KDR*) rs34231037 G-allele variant carriers were potentially associated with a favourable response to sunitinib: complete response (CR) or partial response (PR). Added to these findings, a GWAS learned us that SNPs in chromosome 21 are involved in sunitinib efficacy, probably by influencing drug resistance mechanisms.

Since sunitinib entered the market in 2006, many pharmacogenetic studies on sunitinib have been performed. An overview of observed associations between SNPs and treatment outcome on sunitinib or another TKI was presented in a systematic review in **Chapter 2**.[1] Overall, associations with toxicity or efficacy outcomes on sunitinib were observed for SNPs in the PK related genes *CYP1A1*, *CYP3A5*, *NR1I2*, *NR1I3*, *ABCB1* and *ABCG2* and in pharmacodynamic related genes *VEGFR-2*, *FLT3*, *eNOS*, *IL8*, *VEGF-A*, *VEGFR-1*, *VEGFR-3* and *FGF-R2*.[1] The research described in this thesis builds upon findings from candidate gene studies which are characterized by testing a selection of SNPs based on plausible involvement in PK or pharmacodynamics of the drug of interest. We performed new candidate gene studies selecting previously associated SNPs with the aim to confirm earlier findings. Additionally, we used a hypothesis free approach of a GWAS to test SNPs, independently of possible relationships with PK or pharmacodynamics, for association with sunitinib treatment outcome. Here, we discuss the results of these studies.

CANDIDATE GENE STUDIES

SNPs involved in sunitinib pharmacokinetics

The actual involvement of candidate SNPs in PK of sunitinib has been verified to rationalize further testing of the effect of SNPs on treatment outcome (**Chapter 3**).[2] In a set of 114 patients treated with sunitinib, a selection of 14 SNPs located in PK genes were tested as covariates in a population PK model to see if these would improve the model fit. Although not significant according to $P<0.0005$, SNPs in *CYP3A4* and *CYP3A5* encoding the crucial metabolizing CYP enzymes and SNPs in *ABCB1* encoding the drug efflux transporter showed a change in clearance of sunitinib and SU12662 ($P<0.05$).[2] *CYP3A4*22* (CT or TT genotypes) showed a 22.5% decrease in clearance, while *CYP3A5*1* (AG or AA genotypes) increased sunitinib clearance with 22.5%. In agreement, analyses of concomitant presence of *CYP3A4*22* and *CYP3A5*3* SNPs (poor metabolizers) showed a decrease in clearance of 22.9% for sunitinib, and for extensive metabolizers (*CYP3A4* CC and *CYP3A5*1*) we observed a 35% increase of SU12662 clearance. The effect sizes are below the interindividual variability of 32.2% for sunitinib and 42.9% for SU12662, and therefore dose changes based on *CYP3A4* or *CYP3A5* genotypes is not yet possible. For *ABCB1*, CC of rs1128503 potentially decreased CL of sunitinib with 4.8% compared to TC and TT, and TT of SNP rs2032582 showed a potential 18% increase of sunitinib CL compared to GT and GG ($P<0.05$).

Regarding SNPs in *CYP3A4* and *CYP3A5*, the effects were explained by a lower CYP3A4 expression for *CYP3A4*22* and higher CYP3A5 expression for *CYP3A5*1*. Yet, presence of the active metabolite of sunitinib with similar activity, makes effect interpretation ambivalent. Since *CYP3A5*1* was already associated with toxicity and improved PFS, it was reasoned that this is the result of increased CYP enzyme expression and increased SU12662 formation with a longer half-life than sunitinib, resulting in increased exposure.[3-5] Yet, one could also argue in the opposite direction; *CYP3A5*1* carriers also showed to have an increased SU12662 clearance reducing exposure which contradicts the abovementioned reasoning.[2] Also, a decreased CYP3A4 function would result in a decrease in clearance and an increased exposure to the total active drug (sunitinib + SU12662). In both cases, a person will have increased exposure to the drug.[2,6] Earlier, van der Veldt et al. reported an improved PFS for the CGT haplotype (rs1128503,rs2032582, rs1045642) of *ABCB1* (HR:0.522, 95%CI:0.287–0.950, $P=0.033$).[4] Our findings show a potential decrease in sunitinib clearance for CC in rs1128503 and no change in CL for wild-type GG in rs2032582, but a potential decrease for the variant TT genotype of rs2032582. This is in line with the reported decrease in clearance of sunitinib and its metabolite for wild-type and heterozygotes of rs1128503 and rs2032582 by Beuselinck et al.[7,8] GG carriers of rs2032582 in *ABCB1* associated with hypertension ≥ grade 2, hand-

foot syndrome (HFS), and > grade 3 toxicity, with similar MAFs for Europeans (G=0.53) and Asians (G=0.45).[9]

The above mentioned findings are even more meaningful since earlier associations of *CYP3A5*1* with the need for dose reductions and of the *ABCB1* haplotype with PFS were confirmed in a large combined cohort of 333 mRCC patients (**Chapter 4**).[4,5,8] In this study 22 SNPs in genes related to pharmacokinetics and pharmacodynamics were selected that have previously been associated with sunitinib treatment outcome. This study aimed to confirm earlier associations. Remarkably, 2 SNPs in pharmacokinetic genes have been confirmed, while no SNPs related to pharmacodynamics were found. In sum, all results described above indicate that genetic polymorphisms in *CYP3A5* and *ABCB1* play an important role in predicting the treatment outcome of sunitinib. The reported decrease in clearance of sunitinib and its active metabolite for G-allele carriers of rs2032582, clustered in the *ABCB1* haplotype, would explain the increased exposure to sunitinib. A decreased function of the ABCB1 drug efflux transporter for the CGT haplotype in *ABCB1*, could result in increased absorption of the drug and consequently a higher sunitinib exposure with an improved PFS. Vice versa, Beuselinck et al. reported an increased time-to-dose reduction for variant TT carriers of rs1128503 and rs2032582, which can be explained by a lower sunitinib exposure and thus less toxicity.[7,8]

Sunitinib-induced toxicities

However, not all clinical outcomes can be explained by the potential biomarkers in *CYP3A5* and *ABCB1*. Therefore, many studies aimed to identify other markers possibly associated with clinical outcome of sunitinib.[6,10,11] Urun et al. observed that variant A-allele carriers of *CYP3A4* rs4646437 showed a lower risk of grades 3 to 4 toxicity (OR:0.27, 95%CI:0.08–0.88, *P*=0.03) compared with wild-type (WT) GG in 159 mRCC patients treated with sunitinib.[6,10,11] In **Chapter 5**, we retested this SNP in *CYP3A4* on sunitinib toxicity and efficacy outcomes and observed that the variant A-allele was associated with hypertension in our cohort of 287 mRCC patients.[6] Furthermore, in a cohort of 336 mRCC patients we tested eight earlier associated SNPs, and showed that variant T-allele carriers of *IL8* rs1126647 associated with an increased risk of hypertension and variant T-allele carriers of *IL13* rs1800925 with an increased risk of leukopenia and any toxicity > grade 2 (**Chapter 6**).[11]

CYP3A4 has gender-specific gene expression and consequently a two-fold higher CYP3A4 expression in the liver was seen for females compared to males. Since only 19 females (7%) were female A-allele carriers, it was not possible to perform a robust test for interaction between SNP genotype and gender.[12] Hypertension is considered as an on-target effect of sunitinib, because sunitinib inhibits VEGF or VEGFR-2, resulting in a reduced production of nitric oxide and therefore vasoconstriction.[6,13,14] A greater

exposure to the total drug (sunitinib + active metabolite) results in increased risk for adverse events.[15] Unlike *CYP3A4*22* and *CYP3A5*1*, it remains unclear whether the variant allele carriers of rs4646437 have an increased or decreased CYP enzyme activity. Moreover, like discussed before on *CYP3A4*22* and *CYP3A5*1*, the effect of SNPs in CYP enzymes on drug exposure can be explained in different ways. Since we observed an association with hypertension, we hypothesized that A-allele carriers of rs4646437 have an increased exposure to the drug with a stronger VEGFR-2 inhibition. This could be due to an increased CYP3A4 function with more active metabolite formation, or due to a decreased CYP3A4 function with a higher total drug exposure.[6]

For *CYP3A4* rs4646437 A-allele carriers and *IL8* rs1126647 T-allele carriers we did not find improved PFS and/or OS outcomes, which would be expected according to the findings of Rini et al. and Xu et al.[13,16] Rini et al. observed improved survival outcomes in patients with sunitinib-induced hypertension and Xu et al. observed a worse OS for variant T-allele carriers of *IL8* rs1126647.[11,13,16] However, our findings agree with a later study by Goldstein et al. who observed no changes in survival in case of hypertension or blood pressure elevation above baseline on sunitinib.[14] Alike, it is unclear whether genetic variants in *IL8* or *IL13* would increase or decrease protein expression and whether this influences the VEGF(R)-pathway and sunitinib treatment outcome.[11,17-24] Based on the results in this thesis, we hypothesize that genetic variants in *IL8* or *IL13* cause altered protein expression of IL8 or IL13 and will affect VEGFR-2 inhibition which determines toxicity of sunitinib.[11,17-24]

The role of VEGF receptors in sunitinib efficacy

In 2014, Maitland et al. associated variant G-allele carriers of *VEGFR-2* (*KDR*) rs34231037 with sVEGFR-2 baseline levels and a decline in sVEGFR-2 in response to treatment with pazopanib.[25] In **Chapter 7**, we aimed to see if this finding is in line with outcomes of 276 sunitinib treated patients.[26] Our results suggest that rs34231037 variant G-allele carriers have a better response to sunitinib (OR=3.78; 95%CI=1.02-14.06; *P*=0.047). Since VEGFR-2 inhibits lymphangiogenesis, a stronger inhibition of VEGFR-2 with lower sVEGFR-2 levels will result in a higher lymphangiogenesis activity.[27] Here, we assume that these more aggressive tumors may respond better to VEGFR-inhibitors. Variant G-allele carriers of *KDR* rs34231037 could have a higher sensitivity to VEGFR-inhibitors, resulting in increased efficacy. For rs34231037 we did not find an association with PFS or OS for this SNP. SNP rs1870377 in *VEGFR-2*, has been previously shown to have a shorter PFS and OS and an increased risk for hypertension.[5,8]

Besides VEGFR-2, sunitinib also acts on VEGFR-3. SNP rs307826 in *VEGFR-3* was associated with an improved PFS and better response on sunitinib, accentuated with the observation that VEGFR-3 expression is related to PFS.[5,8,28] In **Chapter 8**, the effect

of SNPs on sVEGFR-2 and sVEGFR-3 is further studied in a nonlinear mixed-effects pharmacokinetics/pharmacodynamics model using blood sampling data of mRCC and mCRC patients treated with sunitinib. Compared with the earlier population PK model as described in **Chapter 3**, this model also contains soluble levels of the VEGF receptors (sVEGFR-2 and sVEGFR-3), clinical outcomes, and SNPs involved in pharmacodynamics (**Chapter 8**).[29] Presence of the variant G-allele in SNP rs6877011 in *VEGFR-3* was associated with a 56% decrease in intrinsic activity of sunitinib on sVEGFR-2 compared to wild-type CC (α=2.13 vs α=0.91). In earlier studies, the same *VEGFR-3* SNP was associated with a decreased PFS.[30] VEGFR-1, 2, and 3 have similar binding domains.[31] A SNP in any of the genes encoding these VEGF-receptors could result in a conformation change and change the binding ability of a drug ligand to VEGFR-receptors. This would affect the ability of sunitinib to decrease sVEGFR-2 and sVEGFR-3 and influence sunitinib efficacy overall. This explains our findings on *VEGFR-2* and *VEGFR-3* SNPs associated with response and PFS. However, it is remarkable that this SNP effect was not found for intrinsic activity of sVEGFR-3. A lower activity would also affect VEGFR-3. The conformation change may have more impact on VEGFR-2 binding affinity than VEGFR-3. sVEGFR-3 levels were associated with OS, and response on sunitinib and VEGFR-3 protein expression was associated with PFS and OS.[28,32,33] SNPs rs307821 and rs307826 are associated with PFS and/or OS of sunitinib.[34]

GWAS - MOVING BEYOND CANDIDATE GENES

Since 2007, the Genome-Wide Association Study (GWAS) concept has entered the world of pharmacogenetics. Other than the candidate gene studies, a GWAS may identify novel SNPs across the entire genome in genes that are directly or indirectly related to the pharmacokinetics and pharmacodynamics of a drug. This already revealed new understanding on biological mechanisms of other drugs.[35,36] In **Chapter 9**, we conducted a GWAS in which we tested 6,540,327 SNPs (obtained after imputation using the 1000Genomes reference panel) in 550 mRCC patients treated with sunitinib. In contrast with our expectations, we have not confirmed any of the earlier associated SNPs from candidate gene studies. Variant alleles of 9 SNPs (with MAFs of 0.061 to 0.067) on chromosome 21 were significantly associated with an inferior PFS ($P<5\times10^{-8}$) with 5.6 months PFS for variant carriers versus 12.7 months for wild-types (HRs: 2.3 to 2.4). It seems probable that these SNPs influence sunitinib efficacy.

POPULATION DIFFERENCES IN GENETIC ASSOCIATION ANALYSES

Population sizes

The studies presented in this thesis have relatively large sample sizes in the field of pharmacogenetics research in mRCC patients, ranging from 114 to 333 for the candidate gene studies and up to 600 patients for the discovery GWAS. For a pharmacogenetics GWAS to identify a SNP with a high effect, i.e. an OR of 2.0, a sample size of 300 patients is required. Significant SNPs with small effects (an OR of 1.2) can only be identified in a sample size of 5,000 patients.[35,36] The observed HR in our GWAS was 2.42 for the most significantly associated SNP. However, these calculations are based on an allele frequency of 0.25. The statistical power of our GWAS might be too low, since the observed Minor Allele Frequencies (MAFs) was C=0.07 for the most significant associated SNP located on chromosome 21. Similarly, rs34231037 in *VEGFR-2* has a very low MAF of G=0.038 in 276 Caucasian mRCC patients. This SNP came close to association with sunitinib response as mentioned in **Chapter 7**. For this SNP, an estimated effect of OR=2.0 tested in 300 patients would have a statistical power of merely 42% to identify a significant association with response (50% responders vs. 50% non-responders). In general, SNPs with an observed MAF ≥5% are considered to give robust statistical association analyses and will depend on sample size but also ethnicity.

Ethnicity

To extend the findings from this thesis globally is rather difficult, since we mainly investigated Caucasian populations while SNP genotype frequencies can vary greatly amongst different ethnicities which may result in different effects or effect sizes. A large inter-ethnic variability exists in the frequency of SNPs in genes involved in PK of sunitinib and many other drugs, such as CYP enzymes (*CYP3A4*, *CYP3A5*) and drug efflux transporters (*ABCB1*, *ABCG2*).[37] For *CYP3A5* rs776746, the NCBI database reports a MAF of 3.6% (HapMap-CEU) and 5.7% (1000Genomes) in European populations. In this thesis we reported higher MAFs of 9.5% (**Chapter 3**) and 7.5% (**Chapter 4**) for rs776746 in *CYP3A5*, which can be explained by the admixture of ethnicities other than European. For Asians, the MAF of rs776746 was 29% (1000Genomes) and for Africans 82%. A considerably higher incidence of adverse events of sunitinib was seen for Japanese patients compared to western populations. SNPs could explain these differences.[9] *ABCG2* rs2231142 A-allele carriers have a higher allele frequency in Japanese and Chinese populations (A=0.31 and A=0.29) compared to non-Asians (A=0.05 to 0.12).[38] Thrombocytopenia is the most occurring adverse event in Japanese RCC patients treated with sunitinib and can be explained by variant A-allele carriers of *ABCG2* rs2231142, which also showed severe

toxicity including hand-foot syndrome and increased drug exposure, i.e. a higher Area Under the Curve (AUC).[38-41] Furthermore, compared to white patients, African Americans diagnosed with clear cell RCC had less frequent VHL mutations and a decreased up-regulation of HIF-associated genes. This would mean that African Americans have a decreased response to TKIs which possibly contributes to the worse survival rates observed for African Americans.[42] Hence, adjustment for ethnicity differences in a grouped analysis should be taken into account in future pharmacogenetic association studies. We already incorporated this aspect in follow-up to our discovery GWAS (**Chapter 9**) where we aim to validate the most significantly associated SNPs with sunitinib treatment outcome in a European cohort and separately in an Asian cohort (N=219) to allow comparison of both ethnicities.[38]

OUTCOME DIFFERENCES IN GENETIC ASSOCIATION ANALYSES: PRACTICE MAKES PERFECT

To obtain reliable SNP associations within and across studies, it is essential to have similar and well-characterised phenotype definitions. Especially in pharmacogenetics studies in cancer this can be rather challenging, since sample sizes are relatively small to begin with. Furthermore, the drug of interest is usually part of a treatment combination and drug type and dosages can frequently change depending on the patient's condition. These factors each can significantly affect treatment outcomes. Moreover, more and more targeted therapies keep entering the market and application of these drugs and drug schedules changes rapidly.[36]

Survival differences

An interesting observation was the difference in survival between centers in the Netherlands, Spain and Cleveland Clinic in the United States (US) (**Chapter 4**). A significantly better PFS (P=0.016) and OS (P=0.009) was observed for patients treated in the Cleveland clinic compared to the other centers. Greater survival gains in the US are best explained by a higher spending of the US on cancer care compared to European countries. This allows the US to use the newest technologies on screening and diagnostic tools so that cancers are detected in an early phase. Furthermore, new drugs are often marketed faster in the US faster than in European countries. In our studies, this would not explain survival differences on sunitinib specifically since all patients had similar access to sunitinib treatment. However, new and more effective treatments as a second-line option started after sunitinib could influence overall survival.[43] Differences in surgical practice in the US compared to Europe may also affect survival outcomes. Cytoreductive

nephrectomy (CN) prior to first-line treatment with sunitinib may be beneficial for overall survival with 12.7 months OS compared to 8.0 months OS for sunitnib only.[44] These findings were underlined by a study on 15,390 patients on targeted therapy between 2006 and 2013 of whom 5,374 (35%) underwent CN. Here, OS was 17.1 months for patients who received CN and targeted therapy and 7.7 months for targeted therapy alone.[45] However, these findings need to be confirmed by the large Clinical Trial to Assess the Importance of Nephrectomy (CARMENA; NCT00930033) of which the primary results are expected in 2019. In this trial, mRCC patients are randomized to receive sunitinib alone or receive nephrectomy followed by sunitinib.[44,45] Besides country differences, survival can vary among several studies because of altered methodological approaches on survival analyses. Adjustment for prognostic factors for instance have a large influence on survival. In previous pharmacogenetics association analyses, the Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria were included, namely deteriorated WHO performance status (≥2), low haemoglobin (lower than the lower limit of normal), high calcium (>2.5 mmol/l), time from initial diagnosis to treatment with sunitinib (<1 yr), and serum lactate dehydrogenase (LDH) greater than 1.5 times the upper limit of normal (ULN). In our studies however, we adjusted survival outcomes using the prognostic index based on the Heng criteria, which validates the MSKCC criteria except for LDH levels and complemented with neutrophil count (higher than the upper limit of normal), and thrombocytes (higher than the upper limit of normal).[46,47] Moreover, most of these pharmacogenetic studies in mRCC deal with retrospective data and may need data imputation on missing MSKCC criteria or Heng variables. The method and outcomes for data imputation may be different among studies. Another example of methodological differences in survival analysis was described in the paper of Goldstein et al. in which outcomes were compared to a study of Rini et al. In the study of Rini et al. patients were excluded from analyses only if the event status of PFS or OS was "no", while Goldstein et al. also excluded patients if sunitinib treatment was discontinued for any reason which is considered to give a better assessment of suntinib-specific survival.[14] Recent evidence shows that a high body mass index (BMI) of ≥25 kg/m^2 should be included as covariate in survival analysis of mRCC patient as observed in the large cohort of 1,975 patients from the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) and in an external validation cohort of 4,657 patients.[48] Because of the lack of evidence in time of our analyses, we have not included BMI as covariate.

Earlier dose reductions and alternative dosing schedules: a change for the better

Another change in treatment outcome could be seen in toxicity grades. During the first years in which physicians prescribed sunitinib, patients who experienced severe adverse

events of grade 3 and 4 were not uncommon. In the course of time when physicians gained experience with sunitinib, severe adverse events were avoided at all costs. If possible physicians already intervened in case of grade 1 to 2 adverse events by reducing the dose (**Chapter 5** + **6**). Therefore, it is likely that a lower rate of > grade 2 events was reported in later studies. Another change in practice that reduces adverse events is the change of dosing schedule from 4 weeks on and 2 weeks off treatment (4/2) to 2 weeks on and 1 week off (2/1). In a multicenter analysis of 249 patients, it was shown that a switch from a 4/2 schedule to a 2/1 schedule resulted in an improved safety profile with a reduction of ≥grade 3 adverse events from 46% to 8%.[49] Altogether, this may have resulted in an underestimation of severe toxicity cases. Yet, it is very difficult to take into account this changing practice because of the lack of a fixed point in time. Moreover, sunitinib was introduced earlier in the US resulting in an extended experience in sunitinib use.

An omission in our toxicity analyses is that we have not performed a time-to-event analysis since heavy toxicities can occur early in treatment or later on for other patients or toxicity types. In addition, we have not tested for SNP associations with fatigue and quality of life (QoL) as endpoints. Fatigue is one of the most important complaints of patients treated with sunitinib and is experienced by about 50% of the patients. Yet, this type of toxicity is not well documented in the medical records and it cannot be clearly measured because of its subjectivity and fatigue is a state of general weakness which would apply to most patients with metastatic disease. But then again especially fatigue affects QoL of patients, which was not included as an endpoint. Most new studies that appear include QoL as an endpoint.[50]

Drug resistance, tumor heterogeneity, and circulating tumor DNA

Besides the large variability in response to sunitinib, another clinical challenge to overcome is the development of resistance to sunitinib which usually occurs within a year of treatment. It was shown that tumor angiogenesis was not merely regulated by VEGF signalling. Tumor cells have the ability to induce angiogenis despite of VEGF-inhibition.[51] This escape mechanisms from inhibited angiogenesis, enables the tumor to expand the tumor vasculature using already available blood vessels (vessel co-option), or the tumor itself can create blood vessels (vasculogenic mimicry).[52,53]

The exact mechanisms of how resistance to VEGFR-inhibitors arises is unclear, but it seems that DNA intratumor heterogeneity (ITH) is an important factor because these genetically different tumor cells cause adaptation and progression of the tumor.[54-56] In general, two main types of resistance can be distinguished referred to as "intrinsic" (patient related) or "acquired" (treatment related) resistance. Patients with intrinsic resistance will have no response to sunitinib at any time during treatment. Acquired

resistance to sunitinib develops while on treatment, for which two possible explanations have been hypothesized. The first hypothesis involves only one single clone that is sunitinib-resistant and that will prevail which results in a reduction of the ITH while being treated. The second hypothesis on acquired resistance would indicate that at first the ITH increases and then numerous resistant clones can develop. According to the findings of Stewart et al., sunitinib treatment could influence intratumoral heterogeneity.[55] This would support the second hypothesis of formation of multiple resistant clones because sunitinib treatment was associated with an increase in ITH. This study indicates that sunitinib may directly target the tumor and effectuate changes that result in a more genetically heterogeneous tumor that is more challenging to suppress using targeted therapy.[54,55] In our studies, we have analyzed germline DNA which excludes ITH to confound the predictive ability of SNPs on sunitinib outcome. Yet, it seems that sunitinib itself targets the tumor and induces genetic changes. To prevent resistance to VEGF-targeted therapies, new therapies should aim at inhibition of all proangiogenic factors, and not only VEGF stimulated angiogenesis.[51,57]

If the tumor genetically changes, treatment efficacy may reduce because the treatment is only partly effective on a specific type of tumor cells. Liquid biopsy of circulating tumor cells (CTC) or circulating tumor DNA (ctDNA) could provide an option to asses the current genetic status of the tumor without having to perform invasive tissue biopsies.[58,59] Different CTC profiles (epithelial, mesenchymal, stem cell-like or mixed-cell characteristics) were observed in mRCC patients at different time points on treatment with sunitinib, pazopanib or temsirolimus. These profiles were associated with PFS or protein expression levels which could indicate variability in treatment outcome.[59] Liquid biopsy of ctDNA could make it possible to see what genetic changes in the tumor are associated with sunitinib response.[58]

Change of therapy - Will sunitinib be surpassed by the new game changers in mRCC treatment?

Sunitinib has long been the drug of choice for mRCC and is therefore the drug of interest since the start of this thesis. Meanwhile, we are spoiled with choice on new TKIs that have entered the market after sunitinib such as pazopanib and axitinib but many more targeted therapies that are in development. Pazopanib showed a similar efficacy compared to sunitinib and patients preferred pazopanib over sunitinib because of a higher quality of life and a more favorable toxicity profile. This was presented in the PISCES study (a randomised, double-blind, cross-over study of pazopanib versus sunitinib, N=169) and the COMPARZ trial (a randomised open-label, phase 3 trial of 557 pazopanib treated patients and 553 patients on sunitinib).[60,61] Currently, both sunitinib and pazopanib are first choice of treatment in mRCC and it has been confirmed in a

large group of patient from the International mRCC Database Consortium (IMDC) using sunitinib (N=6519) or pazopanib (N=919) that both drugs have similar efficacy as a first-line treatment in mRCC.[62] Yet, using the COMPARZ data, it was shown that pazopanib has a higher quality-adjusted time without symptoms or toxicity (Q-TWIST), mainly because the duration of toxicity was 31 days longer for sunitinib compared to pazopanib.[63] In contrast to sunitinib, pazopanib treatment requires no stop weeks. Therefore, response measurements during sunitinib stop weeks cannot be compared to pazopanib, because tumors can grow during this 'drug holiday'. QoL is probably poorly rated at the end of sunitinib treatment since toxicity is then considerably high. To obtain a reliable overall view, QoL should also be assessed after the 2 weeks of rest, which is most likely better valued. The newer TKI axitinib is approved for second-line treatment of mRCC. The differences between individuals regarding PK, PD or response have not yet been explained. No SNP associations for axitinib were reported so far, but a first study in 2016 reported on the association between SNPs in *ABCG2* (rs2231142) and *ABCB1* (rs1128503, rs2032582, rs1045642) with a higher dose-adjusted AUC of axitinib at steady state but not with any adverse events. Increased axitinib plasma exposure was associated with adverse events and dose reductions.[64] We cannot justify comparison of genetic association results on the evaluation of different TKIs. TKIs have many similarities in inhibition of VEGFR-1,-2, and -3, and other tyrosine kinases but there are differences in binding affinity and conversion to (active or inactive) metabolites.[31] However, it would be interesting to study a possible class effect of SNPs related to TKI treatment outcome. In the follow-up of our GWAS study, we aim to compare SNP association results of sunitinib and pazopanib (**Chapter 9**).

TKIs are still standard of care in first line treatment of mRCC, but for second line treatment there are quite a few game changers in the field, namely lenvatinib, cabozantinib and nivolumab. These drugs show promising results as a second line therapy in mRCC after one or two prior VEGFR targeted TKIs. Lenvatinib is a TKI and inhibits VEGFR-1,-2,-3, FGFR1-4, PDGFR-β, RET and KIT proto-oncogenes. An improvement in PFS was seen for lenvatinib compared to everolimus.[65] The TKI cabozantinib targets the tyrosine kinases VEGFR2, MET, and AXL. The MET pathway is related to resistance to TKIs and poor prognosis. The METEOR study is a randomized phase 3 trial on 658 mRCC patients in which cabozantinib was compared to everolimus as a second-line therapy. Cabozantinib improves PFS, OS, and objective response compared to everolimus.[66,67]

The era of targeted therapy endures but it may mean a relegation of TKIs to make place for targeted immunotherapy. Immunotherapy has received a new momentum through the recent introduction of programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) checkpoint inhibitors. Atezolizumab is an anti-PD-L1 monoclonal immunoglobulin G1 antibody. In a phase I dose escalation and dose expansion trial it

was shown that atezolizumab had a long-term activity and safety in RCC patients, and is therefore considered as a promising agent to treat RCC patients.[68] Nivolumab is an anti-PD-1 human immunoglobulin G4 (IgG4) monoclonal antibody that was FDA approved for the treatment of melanoma, lung cancer and since October 2015 also for mRCC. From March 2016 nivolumab is reimbursed for mRCC in the Netherlands. Nivolumab binds to the PD-1 receptor located on cytotoxic T-lymfocytes (T cells). In this way it prevents binding of ligands PD-L1 and PD-L2 from the tumor cell which inhibits the blockade on the immune response mediated by the PD-1 pathway, and thus activates the anti-tumor response. In short, nivolumab releases the brakes on T-cells, activating tumor immune surveillance. The major benefit of immunotherapy compared to targeted therapy is the long-term survival that is observed in some of the patients. In the Checkmate 025 study nivolumab 3mg/kg intravenously every 2 weeks was compared with everolimus 10 mg orally once a day in 821 mRCC patients who were previously treated. OS was the primary outcome measure. It was shown that nivolumab had better OS (25.0 versus 19.6 months), better response rates, fewer adverse events, and an improved health-related quality of life.[69,70] The reason for a lack of difference in PFS between the drugs is not clear. Although progression occurred according to RECIST, patients can still have a durable response on nivolumab. In a randomized phase 2 trial, patients either received nivolumab treatment more than 6 weeks after the first progression occurred according to RECIST or discontinued nivolumab treatment before or at RECIST progression.[71] Patients who were treated beyond progression, had a tumor reduction, stable size of target lesions and a tolerable safety profile. Still, nivolumab shows a spectacular response for some patients, but no response for the majority of patients. About 80% of patients on nivolumab experience adverse events of which the most important were fatigue (33%), rash (10%), diarrhea (12%), and pneumonitis (4%).[72,73] Hence, similar to our research on sunitinib, a careful patient selection for nivolumab users is important. According to the Checkmate 025 study, patients cannot be selected based on PD-L1 expression as a marker for efficacy.[69] Moreover, PD-L1 expression in the tumor could be obscured by ITH. Combinations of available agents in mRCC are tested in ongoing studies with the ambition to achieve a synergistic anti-tumor effect.

FUTURE PERSPECTIVES AND NEW DEVELOPMENTS

10

Candidate gene studies have provided us several potential genetic biomarkers in pharmacogenes to guide individualized treatment of mRCC. And yet, in using the candidate gene approach we may have overlooked other genes containing essential variants. There will be many processes and pathways involved that determine how well

sunitinib works and specific tumor characteristics between patients and within a patient may be different and may change during treatment. It is clear that our search for reliable predictive markers is not yet over or, more positively stated, we are only two steps away from clinical implementation of precision medicine. First, we need to test all relevant genetic variants for association with sunitinib treatment outcome and secondly, these findings need to be prospectively validated. In the meantime, we should offer the best available options in terms of therapy (combinations) and patient monitoring.

The future: GWAS and Next Generation Sequencing (NGS)

The GWAS approach sheds new light on pharmacogenetic research and added new genetic variants on chromosome 21 that seem promising biomarkers to guide sunitinib treatment. We will perform follow-up studies to reassess these findings, and perform a meta-analysis to compare SNP associations in sunitinib or pazopanib treated patients. In addition, we will carry out a validation GWAS to see whether we could confirm any of the SNP associations from candidate gene studies and the first GWAS on sunitinib pharmacogenetics on 1099 mRCC patients treated with pazopanib (N=744) or sunitinib (N=355). It was shown that genetic variants in *LOXL2*, *ENTPD4, UGT1A1*, *ANAPC4* and *SLC34A2* were genome-wide significantly associated with efficacy or toxicity of sunitinib, and SNPs in *IL2RA, LRRC2* showed a suggestive association ($P \leq 5x10^{-7}$).[61,74]

Besides high expectations from GWASs that assess about 1 million common SNPs, next generation sequencing (NGS) is an encouraging prospect to select an effective therapy for mRCC. NGS can include the entire genome known as whole genome sequencing (WGS) or only the coding region known as whole exome sequencing (WES). Due to strong technological developments and the associated sharp drop in prices of NGS, precision medicine in oncology becomes a reality. Likewise, the 100,000 genomes project in the United Kingdom uses WGS with the aim to find genes which cause rare diseases and to identify genetic changes which occur in the tumour patients with cancer, to understand the mechanism of disease and develop therapies to personalise treatment.[76] In the Netherlands, the Center for Personalized Cancer Treatment (CPCT) aims to offer every patient a tailored treatment by analyzing DNA in the tumor.[75-77]

In addition, sequencing of rare genetic variants could be informative. One of the reasons for inconsistent results from pharmacogenetic association studies is the lack of a strong relation between genotype and phenotype, which may be caused by rare genetic variants. It was estimated that about 30 to 40% of functional variability in genes related to PK is governed by rare genetic variants (MAF <1%).[78] In a selection of 146 pharmacogenes, 12,152 genetic variants were observed of which 93% were rare variants. Individuals hold about 100 to 120 variants and rare variants are very geographically unique. These genes involved in drug PK have a high variability in genetics, such as *CYP3A4* and *CYP3A5*.

Because of these rare genetic variants, it is possible that an association between a SNP in *CYP3A5* was significant in one study and above the significance threshold in another study. Testing of rare genetic variants is not useful for treatment selection directly, but the understanding on mechanisms involved in sunitinib-induced adverse events can be very useful.[35,78] In a first report of a WES in mRCC patients, a somatic mutation in the *PBRM1* gene (encoding a component of the chromatin remodeling complex) was associated with a favorable response of sunitinib.[79]

Hurdles to overcome on clinical implementation of pharmacogenetic testing

The studies presented in this thesis provide potential biomarkers that can be used to predict sunitinib treatment response, and provided insight in genetic variants influencing PK and PD of sunitinib. However, these potentially useful markers have not yet been implemented into the clinic.

Who decides when it is clinical useful to genotype? Although not universally accepted, a pharmacogenetic test can be considered ready for clinical practice when it ensures analytical validity (the test is accurate and reliable), clinical validity (the SNP is associated with the outcome of interest) and clinical utility (it leads to improved patient outcome). The first two requirements are quite straightforward since these are technical properties, but there is no consensus on the definition of clinical utility. This absence of clearly defined requirements for the utility of pharmacogenetic tests is a major obstacle that prevents clinical implementation.[80-82]

The need for validation. While there is great controversy on requirements for clinical implementation of testing genetic biomarkers to guide treatment, (prospective) validation studies to confirm SNP associations are vital and provide clear evidence to translate findings to the clinic. In **Chapter 4** we confirmed earlier findings on SNPs in *CYP3A5*1* and *ABCB1* associated with sunitinib toxicity and efficacy.[4,5,8] This confirmation in a large cohort of 333 patients offers strong evidence for clinical utility. Nevertheless, this was strictly taken not an independent cohort of patients since patient cohorts from earlier candidate gene studies were pooled together and more or less the same investigators were involved. Other validation studies of Beuselinck et al. and Dornbusch et al. confirmed that variant AA and AC genotypes of rs9582036 in *VEGFR-1* are predictive for a better PFS and OS.[83,84] But although validated in completely independent cohorts, the sample size is small and these validation studies are based on retrospective data. This is a drawback for clinical implementation.

Predictive or prognostic value. To be sure about clinical validity on pharmacogenetic testing, the SNP should be confirmed to be predictive rather than prognostic. The distinction between predictive and prognostice SNPs is sometimes a grey area. A

prognostic SNP can be defined as a SNP that gives information on the disease outcome of the patient and the possible risk of metastases when untreated. A predictive SNP gives information on the effect of treatment on the tumor in terms of adverse events or survival, and is used to know which patients are likely to benefit from the drug.[85-87] To test whether a SNP is predictive for sunitinib treatment outcome, patients with the outcome of interest need to be compared to a group of patients without this outcome (e.g. severe versus mild adverse events or disease progression versus no progression). In some cases, the predictive and prognostic value of a SNP may be strongly correlated which is illustrated by findings reported in this thesis. SNP rs1126647 in *IL8* was shown to be a predictive marker for sunitinib-induced hypertension (**Chapter 6**).[85-87] At the same time, it is known that upregulated IL8 expression is a RCC tumor characteristic. This SNP in *IL8* may cause a higher IL8 expression which indicates a more aggressive and possibly drug resistant tumor type with a worse prognosis resulting in an ineffective treatment and a higher sensitivity to adverse events. In summary, to implement a pharmacogenetic marker the predictive value of a SNP is a strict requirement that has to be assured and well-founded notwithstanding any additional prognostic value.

Acceptance and interpretation of genotyping results. When in due course biomarkers are considered ready for the clinic, the Dutch Pharmacogenetics Working Group (DPWG) and the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines can inform physicians on how to interpret genotyping results to guide treatment.[88,89] CPIC provides an overview of the level of evidence for drug-gene interactions inlcuding the abovementioned criteria such as replication, sample size and statistical significance. The level of evidence is graded from A (the highest level of evidence) to D (lowest level of evidence with no prescribing recommendations). Still, the major hurdle is that healthcare professionals feel unprepared to apply pharmacogenetics into practice because they would like to see validation in prospective (pharmacogenetic) randomized studies. However, if multiple retrospective studies indicate the same relevance of a predictive pharmacogenetic marker and this marker receives a high CPIC score, this marker is ready for implementation. Pharmacists have an important role in making the use of pharmacogenetics part of routine care. Ubiquitous Pharmacogenomics (U-PGx) is a recently started research project of the Horizon 2020 programme in which major challenges and obstacles for implementation of PGx testing in patient care will be addressed.[81,85-91]

Therapeutic Drug Monitoring (TDM)

It is clear that we expect to find validated reliable genetic biomarkers to be implemented in clinical practice and improve mRCC treatment by knowing beforehand how an individual patient will respond to the drug. A priori pharmacogenetic testing could lead to effective

and efficient use of sunitinib in mRCC or any drug that will be used in the future because it has the great advantage that it gives information for a life-time. One could argue that it would even be unethical not to use genotypic information in treatment decisions and thereby ignore important information that could optimize an individual's treatment. But what do we do until pharmacogenetics of sunitinib in mRCC reaches the clinic? A useful patient management tool for dose individualization is TDM. TDM is a technique that uses measured drug concentrations/drug exposure in the blood of the patient to derive PK/PD parameters, and eventually adjust the dosage of the drug if needed. To justify TDM, the drug has to meet several criteria; the drug has a narrow therapeutic window, the dose cannot be optimized by clinical observation, there is a relation between the drug concentration and the effect, there is no clear relation between dose and effect, a high inter-individual variability in PK, and the intra-patient variability is less than the inter-patient variability. The relation between the drug concentration and effect has clearly been proven by Houk et al.[15,92] Sunitinib meets all requirements, except the lack of evidence on the intra-patient variability to be less than the inter-patient variability.[15] TDM has, in contrast to pharmacogenetic testing, no predictive ability but can only show after start of therapy to what concentration the patient is exposed which is a big disadvantage especially considering severe adverse events. Moreover, blood samples need to be taken at a fixed time point, which is subject to mistakes in interpretation, while for pharmacogenetics this is only needed once in a lifetime at any time. However, TDM is a very accurate method to determine drug plasma concentrations and thereby guide sunitinib dosing. It can prevent the onset of severe toxicities when applied timely after start of treatment. In addition, it is an appropriate method to know drug compliance which is not possible in pharmacogenetics. In our aim to ensure precision medicine, it is not a matter of either pharmacogenetics or TDM but these strategies should be used synergistically. Pharmacogenetic testing to predict the best drug and dose and TDM to adjust the dose based on measured concentrations.[93-95]

What exactly is our target? Angiogenesis, immunogenesis or drug resistance?

Instead of the former approach used in anticancer treatment that concentrates on the primary tumor location, oncologist are now more and more focused on molecular profiling of the tumor and to find a matching therapy. An important characteristic of clear cell RCC is a high VEGF expression and consequently a strong tendency for blood vessel formation (angiogenesis) that tumors need in order to grow and therefore many treatments were developed to inhibit angiogenesis with considerable success. Yet, immunotherapy is a relatively new and effective treatment option in mRCC and may be 'the future of cancer therapy' in strengthening the existing defense mechanisms against

cancer. A combination therapy or sequential therapy that targets the immunogenic and pro-angiogenic character of RCC and/or therapeutic resistance could be the perfect solution to treat mRCC. But how to optimally combine or sequence these drugs?[96] In melanoma, a combination of ipilimumab (an anti-CTLA4 antiboy) and nivolumab showed an improvement in PFS compared to ipilimumab alone but it also showed an increased risk for adverse events.[97] In mRCC, the combination of bevacizumab (a VEGF-inhibitor) and atezolizumab (a PD-1 checkpoint inhibitor) is promising. This treatment combination was related to changes in antitumor immune markers, namely antigen-specific T-cell migration.[98] Another interesting option is to use a computer algorithm to calculate the most ideal drug combination. This would be a really futuristic approach to find synergistic drug combinations with a high efficacy in mRCC and low drug doses to prevent side effects and drug resistance.[99]

Regardless of what the best drug combination or drug sequence may be, the main research question for the future will remain the same as in this thesis: "Can we use genetic information to predict an individual's response to therapy?" When we have sufficient scientific evidence, the use of pharmacogenetics should be a part of routine care with clear recommendations for physicians how to apply pharmacogenetic testing and how to interpret results. The newest technologies in cancer genetic research enable us to find reliable genetic markers, and most importantly to personalize the treatment of RCC patients and thereby improve patient outcomes.

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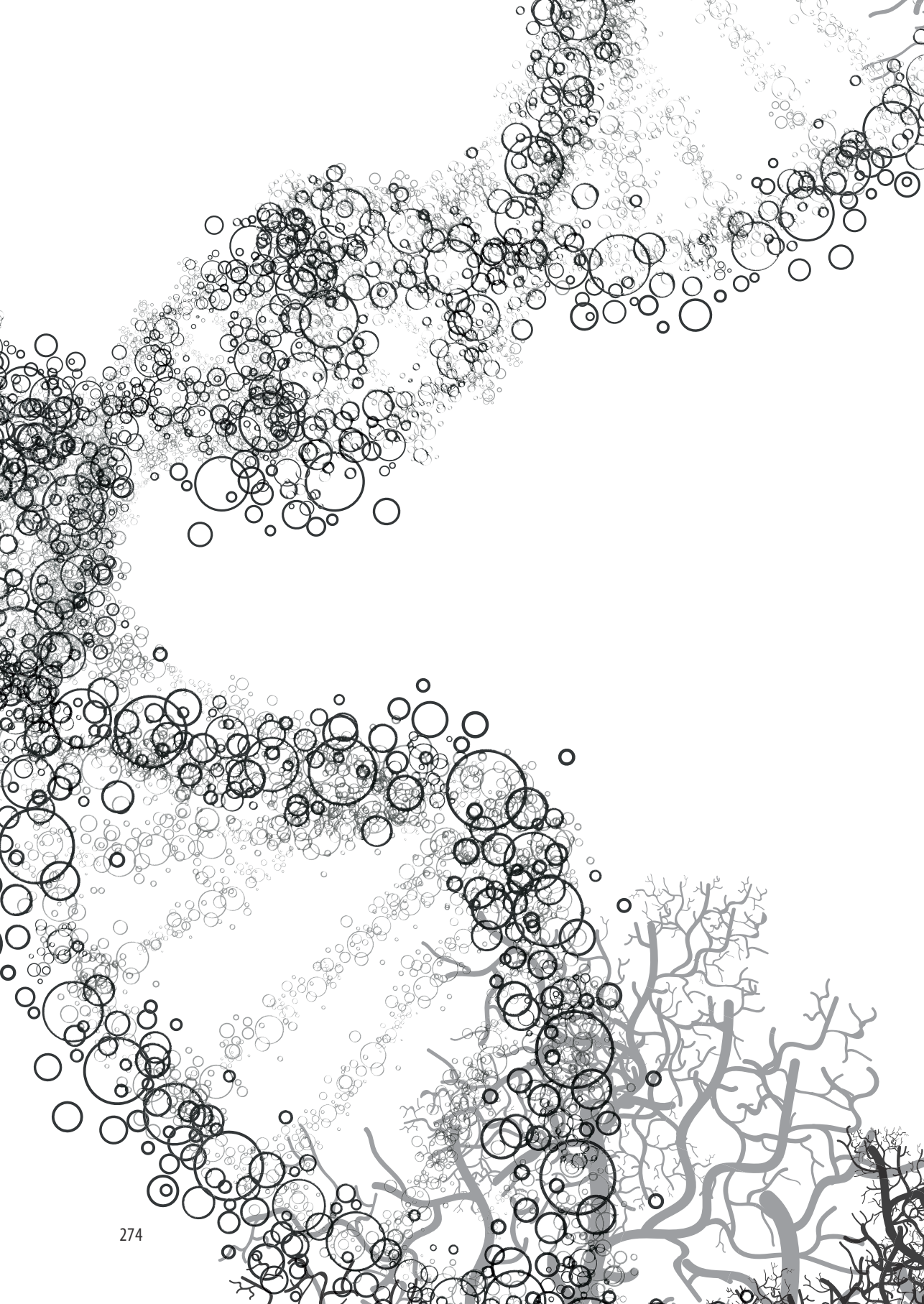
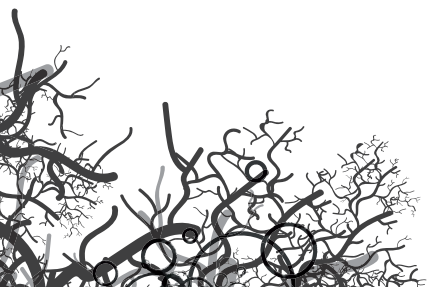


CHAPTER 11

Summary



Summary

Kidney cancer comprises about 2-3% of all malignancies in adults, of which >90% is renal cell carcinoma (RCC) and the incidence is increasing. There are about 2300 new cases a year in the Netherlands, and 350,000 people worldwide. Approximately 17% of RCCs are metastasized at first diagnosis. Preferred sites of metastasis are lung, liver, bone, brain, and only rarely adrenal glands or the contralateral kidney. With the arrival of targeted therapies about 10 years ago, many new successful treatment options against metastatic RCC (mRCC) became available. Especially the (multi)targeted tyrosine kinase inhibitors (TKIs) with an anti-angiogenic effect showed to be very successful drugs. In **Chapter 1** the treatment of mRCC is thoroughly described with a focus on sunitinib. Sunitinib is the TKI of first choice in mRCC, and is usually given as 50 mg in a 4 week on/2 week off schedule. Sunitinib is metabolized by cytochrome P450 (CYP)3A4 to its active metabolite SU12662. Both sunitinib and SU12662 embody the total amount of active drug in plasma. Patients show a high variability in pharmacokinetics (PK) of sunitinib and SU12662 and respond very differently to drugs with large differences in toxicity and efficacy. Most common side effects are fatigue, hand-foot syndrome (HFS), hypertension, mucositis, hair or skin discoloration and hypothyroidism. About 30% of RCC patients on sunitinib required a dose reduction for any of these adverse events. The great difficulty is that we cannot predict an individual's response to sunitinib, and thus are unable to give patients an optimally effective and safe treatment and to efficiently use the available drug arsenal without adjustments afterwards. Therefore, we need specific biomarkers. It is plausible that genetic variability in genes involved in the PK or pharmacodynamics (PD) of a drug are important determinants of a person's response to the drug with respect to adverse events and efficacy. The aim of all studies described in this thesis was to study the predictive ability of germline pharmacogenetics markers on toxicity and efficacy outcomes of sunitinib in mRCC patients.

Our study objective originated from earlier exploratory candidate gene studies in which significant associations were found of Single Nucleotide Polymorphisms (SNPs) with sunitinib treatment outcome. These studies were performed under the assumption that genetic variations have an effect on PK or pharmacodynamics and consequently on drug efficacy or toxicity. In **Chapter 2** we systematically review studies on SNPs (pharmacogenetics) but also gene expression or epigenetic biomarkers (pharmacogenomics) as predictors of TKI treatment outcome in mRCC containing studies from 2004 to 2015. The most promising SNPs in predicting adverse events, efficacy, clearance, or drug exposure on TKI treatment are related to genes encoding CYP450 enzymes, CYP450 regulators, or drug efflux transporters (PK) and genes encoding VEGF or VEGF receptors and interleukins (PD). Most findings on potential biomarkers have not been validated and therefore biomarker testing to guide choice of therapy and dose in mRCC is not yet feasible. Implementation of

pre-emptive pharmacogenetic testing will become likely when confirmed in independent cohorts preferably in prospective studies.

In chapter 3, we assessed whether it is correct to assume that SNPs in genes encoding metabolizing enzymes or efflux transporters influence the PK of sunitinib and SU12662. We tested a selection of 14 previously associated SNPs for association with clearance of sunitinib and its active metabolite in 114 patients with solid tumors. SNPs were included as covariates in a population PK model using non-linear mixed-effect modelling (NONMEM). SNPs in *CYP3A4*, *CYP3A5*, and *ABCB1* were potentially associated with clearance of both sunitinib and its active metabolite. The strongest evidence was observed for a SNP in *CYP3A4*, namely *CYP3A4*22*, that was found to be associated with a 22.5% decrease in clearance of sunitinib. However, all observed effect sizes were below the interindividual variability in clearance of sunitinib and SU12662 and are therefore too limited to directly guide individual dosing of sunitinib.

To investigate whether SNPs associated with toxicity and efficacy on sunitinib in earlier exploratory studies could be confirmed in a larger group, we included patients from previous pharmacogenetics analyses on sunitinib performed in the United States, Spain and the Netherlands and formed a pooled dataset (**Chapter 4**). Earlier associations of *CYP3A5*1* with the need for dose reductions and of the *ABCB1* haplotype with PFS were confirmed in this large combined cohort of 333 mRCC patients. This indicates that genetic polymorphisms in *CYP3A5* and *ABCB1* play an important role in predicting the treatment outcome of sunitinib.

However, not all clinical outcomes can be explained by the potential biomarkers in *CYP3A5* and *ABCB1*. Therefore, many other markers possibly associated with clinical outcome of sunitinib have been studied. Variant A-allele carriers of SNP rs4646437 in *CYP3A4* were reported to have a protective effect on sunitinib related grades 3 to 4 toxicity in a cohort of 159 mRCC patients. In **Chapter 5**, we retested this SNP in *CYP3A4* on sunitinib toxicity and efficacy outcomes and observed that the variant A-allele was associated with hypertension in a large cohort of 287 mRCC patients. It remains difficult to explain whether A-allele carriers of *CYP3A4* rs4646437 have an increased or decreased function of CYP3A4. Either way, we expect that this SNP will result in an increased exposure to sunitinib and/or the active metabolite and therefore cause hypertension. In **Chapter 6**, we tested a set of eight relatively new SNPs (reported from 2011 onwards) in *CYP3A4*, *NR1I2*, *POR*, *IL8*, *IL4-R*, *IL13*, *HIF1A* and *MET* that were earlier associated with sunitinib treatment outcome. Here, we observed that variant T-allele carriers of *IL8* rs1126647 associated with an increased risk of hypertension and variant T-allele carriers of *IL13* rs1800925 with an increased risk of leukopenia and any toxicity > grade 2. This suggests a relationship between interleukin genes *IL8* and *IL13* and the development of sunitinib-induced adverse events. Yet, further validation in an independent cohort is warranted to confirm these findings.

In **Chapter 7**, the earlier associated SNP rs34231037 in *VEGFR-2* (*KDR*) on pazopanib treatment outcome, was analyzed in our cohort of 276 mRCC patients treated with sunitinib. Variant G-allele carriers of this SNP showed to have a better response to sunitinib. Large series of patients treated with different anti-VEGFR TKIs together with sVEGFR2 measurements and tumor response data are needed to establish the role of rs34231037 as marker for antiangiogenic response.

In order to find a predictive biomarker, we aimed to have a better understanding of the relationships between sunitinib exposure, the pharmacological response, and clinical outcome. In **Chapter 8** a nonlinear mixed-effects population PK/PD model was built using NONMEM and by means of blood sampling data of sunitinib treated patients diagnosed with either mRCC or colorectal carcinoma (CRC) and a group of 12 healthy volunteers. Here, we evaluated the predictive performance of this model including PK parameters, blood pressure, soluble levels of the VEGF receptors (sVEGFR-2 and sVEGFR-3), and SNP genotypes. Presence of the variant G-allele in SNP rs6877011 in *VEGFR-3* was associated with a 56% decrease in intrinsic activity of sunitinib on sVEGFR-2. In earlier studies, the same *VEGFR-3* SNP was associated with a decreased PFS. The developed PK/PD models adequately describe plasma concentration-time profiles of sunitinib, SU12662, sVEGFR-2, sVEGFR-3 with potential influence of genetic predictors, and therefore provides the potential of PK/PD-guided individualization strategies for the optimization of anti-angiogenic therapies.

In **Chapter 9**, we conducted a Genome-Wide Association Study (GWAS) in which we tested 6,540,327 SNPs (obtained after imputation using the 1000Genomes reference panel) in 550 mRCC patients treated with sunitinib. Variant alleles of 9 SNPs located on chromosome 21 were significantly associated with an inferior PFS ($P<5\times10^{-8}$) compared to wild-types. These SNPs may influence sunitinib efficacy. However, the results reported in these thesis are only preliminary results which need further analyses including toxicity outcomes.

This thesis closes with a general discussion in **Chapter 10** on the potential biomarkers that have been revealed for prediction of either toxicity or efficacy of sunitinib in patients with mRCC. In addition, future perspectives in the field are presented. The most promising markers are related to important modulators of sunitinib pharmacokinetics (PK), namely SNPs in *ABCB1* (encoding the efflux transporters ATP-binding cassette) and *CYP3A5*. When we have sufficient scientific evidence, the use of pharmacogenetics should be a part of routine care with clear recommendations for physicians how to apply pharmacogenetic testing and how to interpret results. The newest technologies in cancer genetic research enable us to find reliable genetic markers, and most importantly to personalize the treatment of RCC patients and improve patient outcomes.

CHAPTER 11

Nederlandse samenvatting

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Nierkanker komt voor bij ongeveer 2 tot 3% van alle kwaadaardige ziekten bij volwassenen en daarvan wordt meer dan 90% aangeduid als niercelcarcinoom (RCC). De incidentie ervan neemt toe. Er zijn ongeveer 2300 nieuwe gevallen per jaar in Nederland en 350.000 mensen wereldwijd. Bij ongeveer 17% van de niercelcarcinoom patiënten is de ziekte gemetastaseerd bij de eerste diagnose. De voorkeursplaatsen voor metastasen zijn de longen, lever, botten, hersenen, en slechts zelden bijnieren of de contralaterale nier. Met de komst van doelgerichte therapieën circa 10 jaar geleden, zijn er veel nieuwe succesvolle behandelopties tegen gemetastaseerd niercelcarcinoom (mRCC) beschikbaar gekomen. Met name de (multi) doelgerichte tyrosinekinase remmers (TKIs) met een anti-angiogeen effect bleken zeer succesvolle geneesmiddelen. In **Hoofdstuk 1** wordt de behandeling van gemetastaseerd niercelcarcinoom grondig beschreven waarbij we ons specifiek richten op sunitinib. Sunitinib is de TKI van eerste keuze bij mRCC en wordt meestal gegeven in een dosering van 50 mg in een schema met 4 weken behandeling gevolgd door 2 weken rust. Sunitinib wordt gemetaboliseerd door cytochroom P450 (CYP)3A4 en daarbij omgezet naar de actieve metaboliet SU12662. Zowel sunitinib en SU12662 belichamen de totale hoeveelheid actieve stof in het plasma. Er is een grote variabiliteit tussen patiënten wat betreft de farmacokinetiek (PK) van sunitinib en SU12662 en patiënten reageren zeer verschillend op sunitinib met grote verschillen in bijwerkingen en effectiviteit. De meest voorkomende bijwerkingen zijn vermoeidheid, hand-voet syndroom, hypertensie, mucositis, verkleuring van de haren of de huid en hypothyreoïdie. Bij ongeveer 30% van de RCC patiënten die behandeld worden met sunitinib is een dosisreductie nodig naar aanleiding van (één van deze) bijwerkingen. Het grote probleem is dat we nog niet kunnen voorspellen hoe iemand op sunitinib zal reageren. Hierdoor zijn we niet in staat om patiënten een optimale behandeling te geven die zo effectief en veilig mogelijk is en waarbij we efficiënt gebruik kunnen maken van de beschikbare geneesmiddelen zonder dat aanpassingen achteraf nodig zijn. Hiervoor hebben we specifieke biomarkers nodig. Het is zeer aannemelijk dat genetische varianten, in genen die betrokken zijn bij de PK of farmacodynamiek (PD) van een geneesmiddel, in belangrijke mate bepalen hoe de individuele patiënt op sunitinib zal reageren met betrekking tot bijwerkingen en werkzaamheid. Het doel van alle studies die worden beschreven in dit proefschrift was om na te gaan of genetische varianten de behandeluitkomst van sunitinib met betrekking tot bijwerkingen en werkzaamheid kunnen voorspellen in patiënten met gemetastaseerd niercelcarcinom.

Het doel van de studies in dit proefschrift kwam voort uit eerdere exploratieve bevidingen waarin significante associaties werden gevonden van Single Nucleotide Polymorfismen (SNPs) met behandeluitkomsten van sunitinib. Deze studies werden uitgevoerd in de veronderstelling dat genetische variaties een effect hebben op de PK of

PD en daarmee op de werkzaamheid of bijwerkingen van het geneesmiddel. In **Hoofdstuk 2** herzien we op systematische wijze studies die gepubliceerd zijn tussen 2004 en 2015 waarin SNPs (farmacogenetica), maar ook genexpressie of epigenetische biomarkers (farmacogenomica) zijn gerapporteerd als voorspellers van behandeluitkomsten van TKIs in mRCC. De meest veelbelovende SNPs in het voorspellen van bijwerkingen, effectiviteit, klaring, of geneesmiddel blootstelling bij behandeling met een TKI werden gevonden in genen die coderen voor CYP450-enzymen, die CYP450 beïnvloeden, of in geneesmiddel transporters (PK) en genen die coderen voor VEGF of VEGF-receptoren en interleukinen (PD). De meeste bevindingen voor potentiële biomarkers zijn niet gevalideerd en daarom is het nog niet haalbaar om op basis van het testen van deze biomarkers de geschikte therapie en bijbehorende dosering te kiezen voor patiënten met niercelcarcinoom. Implementatie van farmacogenetische testen voorafgaand aan de behandelkeuze zal pas werkelijkheid worden wanneer deze SNP associaties worden bevestigd in een onafhankelijk patiënten cohort en bij voorkeur in prospectief opgezette studies.

In **Hoofdstuk 3** hebben we onderzocht of het een juiste veronderstelling is dat SNPs, in genen die coderen voor enzymen betrokken bij het metabolisme of efflux transporters, invloed hebben op de farmacokinetiek van sunitinib en SU12662. Daarvoor hebben we een selectie van 14 eerder geassocieerde SNPs opnieuw getest om te zien of deze SNPs ook geassocieerd zijn met de klaring van sunitinib en de actieve metaboliet bij 114 patiënten met solide tumoren. De SNPs werden meegenomen als covariaten in een populatie PK model met behulp van non-linear mixed-effect modelling (NONMEM). SNPs in *CYP3A4*, *CYP3A5* en *ABCB1* waren potentieel geassocieerd met de klaring van zowel sunitinib alsook de actieve metaboliet. Het sterkste bewijs werd gevonden voor SNP *CYP3A4*22*, welke werd geassocieerd met een 22,5% afname in klaring van sunitinib. Echter, alle effecten die werden waargenomen in deze studie waren kleiner dan de interindividuele variabiliteit in de klaring van sunitinib en SU12662 en zijn dus te beperkt om de individuele dosering van sunitinib rechtstreeks hierop aan te passen.

Om te onderzoeken of significant geassocieerde SNPs met toxiciteit en werkzaamheid op sunitinib uit eerdere exploratieve studies bevestigd konden worden in een grotere groep, hebben we patiënten geincludeerd van eerdere farmacogenetica analyses op sunitinib die zijn uitgevoerd in de Verenigde Staten, Spanje en Nederland. Op deze manier hebben we een samengevoegde dataset gecreëerd (**Hoofdstuk 4**). Hierbij vonden we dat de eerdere associaties van *CYP3A5*1* met dosisreducties en van het *ABCB1* haplotype met progressie-vrije overleving (PFS) werden bevestigd in dit grote gecombineerde cohort van 333 mRCC patiënten. Dit geeft aan dat genetische polymorfismen in *CYP3A5* en *ABCB1* een belangrijke rol spelen bij het voorspellen van de behandeluitkomst van sunitinib.

Echter, niet alle klinische uitkomsten kunnen verklaard worden door potentiële biomarkers in *CYP3A5* of *ABCB1*. Daarom zijn er veel andere markers bestudeerd die mogelijk verband houden met de klinische uitkomst van sunitinib. In eerdere studies werd aangetoond dat dragers van het variant A allel van SNP rs4646437 in *CYP3A4* een beschermend effect hadden voor het optreden van graad 3 of 4 bijwerkingen op sunitinib in een cohort van 159 patiënten gemetastaseerd niercelcarcinoom. In **Hoofdstuk 5** hebben we deze SNP in *CYP3A4* opnieuw getest voor associatie met bijwerking of effectiviteit van sunitinib. We zagen dat de dragers van het variant A-allel geassocieerd waren met het optreden van hypertensie in een groot cohort van 287 mRCC patiënten. Het is moeilijk te verklaren of de A-allel dragers van *CYP3A4* rs4646437 een toename of afname van de CYP3A4 functie hebben. In elk geval verwachten we dat deze SNP zal leiden tot een verhoogde blootstelling aan sunitinib en/of de actieve metaboliet en daardoor hypertensie veroorzaakt. In **Hoofdstuk 6**, hebben we een reeks van acht relatief nieuwe SNPs (gerapporteerd vanaf 2011) in *CYP3A4*, *NR1I2*, *POR*, *IL8*, *IL4-R*, *IL13*, *HIF1A* en *MET* getest die eerder al werden geassocieerd met sunitinib behandel uitkomsten. Dragers van het variant T allel van *IL8* rs1126647 waren geassocieerd met een verhoogd risico op hypertensie en dragers van het variant T allel van *IL13* rs1800925 met een verhoogd risico op leukopenie en bijwerkingen > graad 2. Dit suggereert een verband tussen de interleukine genen *IL8* en *IL13* en de ontwikkeling van bijwerkingen op sunitinib. Toch is validatie van deze bevindingen in een onafhankelijk cohort een vereiste om dit te bevestigen.

In **Hoofdstuk 7**, wordt de eerder geassocieerde SNP rs34231037 in *VEGFR-2* (*KDR*) met pazopanib behandel uitkomsten, geanalyseerd in een cohort van 276 mRCC patiënten die werden behandeld met sunitinib. Dragers van het variant G allel van deze SNP bleken een betere respons op sunitinib te hebben. Om de rol van rs34231037 als marker voor anti-angiogene respons vast te stellen, is een grotere groep patiënten nodig die behandeld is met verschillende TKIs en waarvan gegevens beschikbaar zijn over gemeten hoeveelheden sVEGFR2 en tumorrespons.

Om een voorspellende biomarker te vinden, richtten we ons op een beter begrip van de relatie tussen de blootstelling aan sunitinib, de farmacologische respons en de klinische uitkomst. In **Hoofdstuk 8** werd een populatie PK/PD model gebouwd met behulp van NONMEM en door middel van bloedmosters van sunitinib behandelde patiënten die gediagnosticeerd werden met ofwel mRCC of colorectaal carcinoom (CRC) en een groep van 12 gezonde vrijwilligers. Hier onderzochten we het voorspellende vermogen van dit model aangevuld met PK parameters, bloeddruk gegevens, de hoeveelheid VEGF-receptoren (sVEGFR-2 en sVEGFR-3) en SNP genotypes. De aanwezigheid van het variante G allel van SNP rs6877011 in *VEGFR-3* was geassocieerd met een afname van 56% intrinsieke activiteit van sunitinib op sVEGFR-2. In eerdere studies werd dezelfde SNP

in *VEGFR-3* geassocieerd met een verlaagde PFS. De PK/PD modellen zoals ontwikkeld in deze studie geven een functionele beschrijving van de plasma concentratie-tijd profielen van sunitinib, SU12662, sVEGFR-2 en sVEGFR-3 met de potentiële invloed van genetische voorspellers. Daarom is dit PK/PD model mogelijk geschikt voor het gericht behandelen op basis van PK/PD en het daarmee individualiseren van anti-angiogene geneesmiddelen.

In **Hoofdstuk 9**, voerden we een genoomwijde associatie studie (GWAS) uit op 550 mRCC patiënten behandeld met sunitinib waarin we 6.540.327 SNPs hebben getest (verkregen na imputatie vanuit de 1000Genomes referentie set). De variant allelen van 9 SNPs gelegen op chromosoom 21 waren significant geassocieerd met een verlaagde PFS ($P<5\times10^{-8}$) vergeleken met het wild-type. Deze SNPs zouden de effectiviteit van sunitinib mogelijk kunnen beïnvloeden. Echter, de resultaten die vermeld worden in dit proefschrift zijn slechts voorlopige resultaten waarvan verdere analyses nodig zijn waarin ook toxiciteit als eindpunt wordt getoetst.

Dit proefschrift wordt afgesloten met een algemene discussie in **Hoofdstuk 10** over potentiële biomarkers die zijn gevonden voor het voorspellen van bijwerkingen of effectiviteit van sunitinib bij patiënten met gemetastaseerd niercelcarcinoom. Bovendien worden toekomstverwachtingen in dit veld naar voren gebracht. De meest veelbelovende markers zijn gerelateerd aan de farmacokinetiek (PK) van sunitinib, namelijk SNPs in *ABCB1* (het gen dat codeert voor de ATP-bindende cassette efflux transporter) en SNPs in *CYP3A5*. Wanneer we voldoende wetenschappelijk bewijs hebben, zal het gebruik van farmacogenetica als standaard zorg moeten gelden. Hierbij dienen dan duidelijke aanbevelingen gegeven te worden aan artsen over hoe zij deze farmacogenetische testen kunnen toepassen en vervolgens kunnen interpreteren. De komst van nieuwe technologieën in genetisch onderzoek binnen de oncologie, stellen ons in staat om betrouwbare genetische markers te vinden. Dit is van groot belang, omdat we daarmee in de toekomst de behandeling van patiënten met niercelcarcinoom kunnen toespitsen op het individu en zo de behandeluitkomsten voor deze patiënten kunnen verbeteren.

CHAPTER 11

List of publications

List of publications
This thesis

Diekstra MH, Klümpen HJ, Lolkema MP, Yu H, Kloth JS, Gelderblom H, van Schaik RH, Gurney H, Swen JJ, Huitema AD, Steeghs N, Mathijssen RH. Association analysis of genetic polymorphisms in genes related to sunitinib pharmacokinetics, specifically clearance of sunitinib and SU12662. Clin Pharmacol Ther. 2014;96(1):81-89.

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Diekstra MH, Liu X, Swen JJ, Boven E, Castellano D, Gelderblom H, Mathijssen RHJ, Rodríguez-Antona C, García-Donas J, Rini BI, Guchelaar H-J. Association of single nucleotide polymorphisms in IL8 and IL13 with sunitinib-induced toxicity in patients with metastatic renal cell carcinoma. Eur J Clin Pharmacol., 2015;71(12):1477-1484.

Diekstra MH, Belaustegui A, Swen JJ, Boven E, Castellano D, Gelderblom H, Mathijssen RHJ, García-Donas J, Rodríguez-Antona C, Rini BI, Guchelaar H-J. Sunitinib-induced hypertension in CYP3A4 rs4646437 A-allele carriers with metastatic renal cell carcinoma. Pharmacogenomics J. 2017;17(1):42-46.

Diekstra MH, Swen JJ, Gelderblom H, Guchelaar H-J. A decade of pharmacogenomics research on tyrosine kinase inhibitors in metastatic renal cell cancer: a systematic review. Exp Rev of Mol Diagn. 2016;16(5):605-618.

Apellániz-Ruiz M, **Diekstra MH**, Roldán JM, Boven E, Castellano D, Gelderblom H, Mathijssen RHJ, Swen JJ, García-Donás J, Rini BI, Guchelaar H-J, Rodríguez-Antona C. Evaluation of KDR rs34231037 as predictor of sunitinib efficacy in patients with metastatic renal cell carcinoma. Pharmacogenet Genomics. 2017;27(6):227-231.

Diekstra MH, Fritsch A, Kanefendt F, Swen JJ, Moes DJAR, Soergel F, Kinzig M, Stelzer C, Schindele D, Gauler T, Hauser S, Houtsma D, Roessler M, Moritz B, Mross K, Bergmann L, Oosterwijk E, Kiemeney B, Guchelaar H-J, Jaehde U. Population modelling integrating pharmacokinetics, pharmacodynamics, pharmacogenetics and clinical outcome in sunitinib-treated cancer patients. CPT Pharmacometrics Syst Pharmacol. 2017, accepted for publication.

Other publications

Diekstra MH, Liu X, Swen JJ, Guchelaar H-J. What do we need to make genetic biomarker-guided treatment for renal cell carcinoma a reality? Pharmacogenomics. 2017;18(1):1-4.

Van der Zanden LF, Vermeulen SH, Óskarsdottir A, Maurits JSF, **Diekstra MH**, et al. Description of the EuroTARGET cohort: A European collaborative project on Targeted therapy in Renal cell cancer: Genetic and Tumour related biomarkers for response and toxicity. Urol Oncol. 2017 Apr 3. pii: S1078-1439(17)30109-6.

In preparation

Diekstra MH, Swen JJ, van der Zanden LF, Vermeulen SH, Boven E, Mathijssen RHJ, Oosterwijk E, Böhringer S, Kiemeney LALM, Guchelaar H-J. Genetic biomarkers associated with efficacy of sunitinib treatment in patients with metastatic renal cell carcinoma: preliminary results from the EuroTARGET Genome-Wide Association Study. Manuscript in preparation.

CHAPTER 11

Curriculum vitae

Curriculum vitae

Mathilde Hyacintha Maria Diekstra, given name Meta, was born on April 17th 1986 in Nijmegen, The Netherlands. She is the daughter of Frans Diekstra and Joke Hoefnagel and younger sister of Frank Diekstra. After Meta completed her secondary school (VWO) in 2004 at the Stedelijk Gymnasium Nijmegen, she studied pharmacy at the University of Groningen. During her studies, she completed her Bachelor project at the Department of Clinical Pharmacy at the University Medical Center in Groningen (UMCG) on the topic of ciprofloxacin use in children. In 2010, she performed her Master project at the School of Chemistry at the University of Leeds, United Kingdom where she investigated the role of ADP-catalysed phosphorylation and phosphate-dependent dephosphorylation of PPDK by PDRP in Escherichia coli. She followed her internships in SAL Apotheek Segbroek (The Hague), Canisius Wilhelmina Ziekenhuis (Nijmegen), and the St. Maartenskliniek (Nijmegen). In 2012, she graduated as a pharmacist and started her PhD project on pharmacogenetics of sunitinib in metastatic renal cell carcinoma at the department of Clinical Pharmacy & Toxicology of the Leiden University Medical Center (LUMC) under the supervision of prof. dr. Henk-Jan Guchelaar, prof. dr. Hans Gelderblom, and dr. Jesse Swen. This project was a part of the larger European collaborative project on "TArgeted therapy in Renal cell cancer: GEnetic and Tumour related biomarkers for response and toxicity" (EuroTARGET), funded by the European Commission under the Seventh Framework Programme (FP7). For one of her research projects on genetic polymorphisms associated with sunitinib treatment outcome she received the Presidential Trainee Award 2015 from the American Society for Clinical Pharmacology & Therapeutics (ASCPT) in New Orleans, US. In the summer of 2015, she worked on a Genome-Wide Association Study (GWAS) at the biopharmaceutical company deCODE genetics in Reykjavík, Iceland. During her PhD, she taught medical students on the topic of pharmacology and dermatics, supervised scientific internships, and was a board member of the LUMC Association for PhD candidates (LAP) in which she represented PhD candidates from the LUMC, organized several lectures and was president of the annual PhD Day in 2016. As a pharmacist in the LUMC, her responsibilities included the medication verification on aseptic preparation of parenteral products for children and oncolytic drugs. She believes that scientific research and patient care go hand in hand and she desires to combine both aspects in her future career. From February 2017, Meta has started her training to become a hospital pharmacist at Maastricht University Medical Center.

Linkedin profile: www.linkedin.com/in/metadiekstra

CHAPTER 11

Courses and training activities

COURSES AND TRAINING ACTIVITIES

Courses at Leiden University Medical Center (LUMC)

2014	Survival Analysis;
2014	Supervision of scientific internships;
2014	Workshop dealing with public databases and datasets in cancer research;
2013	Advanced genetic association analysis;
2013	Introduction Genetic epidemiology;
2013	Basic Methods and Reasoning in Biostatistics;
2012	Good Manufacturing Pratice (GMP) basic course;
2012	Design and interpretation of research involving human subjects;
2012	Basiscursus Regelgeving en Organisatie Klinisch onderzoekers (BROK);
2012	General overview of laboratory facilities.

Courses at Leiden University

2015	General course Spanish level 1, Academic Language Center;
2013	General course English level 6, Academic Language Center;
2013	Time management, self-management;
2013	Project management skills.

Symposia, meetings, and congresses

2013 - 2016	Oral presentation at annual EuroTARGET consortium meeting in Reykjavik (2013), Cambridge (2014), Bucharest (2015), Leiden (2015) and Madrid (2016);
2016	17th Golden Helix Pharmacogenomics Day (Erasmus UMC, Rotterdam);
2016	Werkgroep Immunotherapie Nederland voor Oncologie (WIN-O) Multidisciplinary symposium renal cell carcinoma (Ede);
2015	Oral presentation International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT) 2015 (Rotterdam);
2015	Poster presentation ASCPT 2015 annual meeting (New Orleans);
2014	Oral presentation FIGON Dutch Medicines Days 2014 (Ede);
2013	Poster presentation FIGON Dutch Medicines Days 2013 (Ede);
2013	Poster presentation ASCO annual meeting 2013 (Chicago);
2013	Training on targeted therapy, Nederlandse Vereniging voor Medische Oncologie (Amersfoort);
2012	NVVO Milestones day: targeted therapy (Rotterdam).

Memberships

American Society of Clinical Oncology (ASCO);
American Society for Clinical Pharmacology and Therapeutics (ASCPT);
Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie (KNMP);
LUMC Association for PhD candidates (LAP);
Nederlandse Vereniging van Ziekenhuisapothekers (NVZA);
Nederlandse Vereniging voor Klinische Farmacologie en Biofarmacie (NVKFB);
Vereniging van Apothekers in opleiding tot Ziekenhuisapotheker (VAZA).

CHAPTER 11

Dankwoord

DANKWOORD

Onderzoek doen is een weelde. Het was een zeer verrijkende ervaring. Een ervaring die er nooit was geweest zonder de bijdrage van vele mede geïnteresseerden die ik graag wil bedanken.

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Beste Nielka, Astrid en Karel, dank jullie voor het leggen van de basis van dit proefschrift. Het was een eer om op jullie werk te mogen voortbouwen.

Alle SUTOX collega's en co-auteurs, waaronder Alwin, Huixin, Ron (van Schaik), professor Boven, Ron (Mathijssen) en Neeltje, wil ik bedanken voor de interessante wetenschappelijke discussies, het delen van ervaringen uit de kliniek en het komen tot nieuwe inzichten. Jesus, Brian, Cristina, Maria and Ainhoa, thank you for a wonderful and fruitful collaboration and for asking me challenging questions that really moved us forward in our search for genetic biomarkers. Jesus and Cristina, thank you for receiving us in the beautiful and lively Madrid. Ainhoa, muchas gracias. It was great to have you over in Leiden at our department, to perform the genotyping together and to visit you in Bilbao. Bilbao es una ciudad hermosa, pero San Sebastián es muy agradable también.

All EuroTARGET partners, thank you for our international scientific adventure and working together on this project. Achim and Ulrich, thank you for a very enjoyable cooperation with a beautiful end result. Loes, Sita, Bart en Egbert, dank jullie voor alle overleggen om ervoor te zorgen dat het project op het goede spoor bleef en de vaart erin werd gehouden. Thorunn, Gisli, and Arna, thank you for your hospitality and ease in receiving me in Reykjavik. I enjoyed working with you on the scripts for our database and I had a memorable time in Iceland. Stefan, dank je voor al jouw hulp bij de statistische analyses en jouw belangrijke input in het EuroTARGET project.

Alle collega's uit de apotheek van het LUMC wil ik bedanken voor een plezierige samenwerking en in het bijzonder mijn kamergenoten Djoeke, Marloes, Lisanne, Marleen, Paul, Xiaoyan, Sofieke, Carin, Frank, en Cathelijne. Op de promovendikamer was het fijn om onze ervaringen, kleine frustraties en succesjes met elkaar te kunnen delen. Marion en Mieke, dank voor al jullie inspanningen en het proactief meedenken in de planning van overleggen en TC's. Tahar, Renée, Danielle en Rowena, het 'plaatjes draaien op de LC480' werd een echte sport en verliep gestroomlijnd na jullie goede instructies en het samen doornemen van de resultaten. Bedankt voor al jullie hulp en het overnemen van het genotyperen in de drukkere perioden.

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