Pharmacokinetics and Pharmacodynamics of Tyrosine Kinase Inhibitors

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Farmacokinetiek en farmacodynamiek van tyrosine kinase remmers

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Chapter 1

General introduction

CANCER

Cancer is the second most common cause of death worldwide after cardiovascular diseases, responsible for an estimated 8.4 million deaths in 2012 and its incidence is still increasing.[1] Through the introduction of many chemotherapeutic agents and hormone treatments in the last decades, treatment options for patients with advanced forms of cancer have improved. Mortality rates have decreased and in some cases cancer is now changing to become a chronic disease. More recent, increased insight in cancer cell biology has led to further improvement in anti-cancer treatment. Much attention has been focused on tyrosine kinases that comprise essential elements of cellular signalling cascades which control proliferation, cell survival and cell death. In cancer, tyrosine kinases can be found activated by mutations, thereby contributing to malignant transformation, tumour growth and metastasis.

TYROSINE KINASE INHIBITORS

Targeted therapies, especially tyrosine kinase inhibitors (TKIs), have largely contributed to the recent improvement in anti-cancer treatment. TKIs usually act by competing with adenosine 5'- triphosphate (ATP) for the intracellular ATP binding site of one or more tyrosine kinases.[2] ATP displacement by TKI binding results in inhibition of several processes which are necessary for tumour growth, such as angiogenesis, cell proliferation and cell migration (type I inhibitors).[3] More recently, TKIs have been developed which are non-ATP competitive inhibitors (type II and type III inhibitors). Currently, 17 TKIs are available for the treatment of a broad variety of cancer types, with approval by both the US Food and Drug Administration and European Medicines Agency (**Table 1**).[4]

TKIs are oral drugs which are usually administered on a daily base. In the year 2000, imatinib was the first TKI that became available on the market for the treatment of chronic myeloid leukaemia (CML). By blocking BCR-ABL, a fusion gene frequently mutated in patients with CML, imatinib is able to inhibit myeloid cell growth.[5, 6] In a later stage, imatinib was also found to be effective in the treatment of gastrointestinal stromal tumours (GIST), by inhibition of c-KIT and PDGFR-α.[7] c-KIT is mutated in over 90% of GISTs and PDGFR in most c-KIT negative GIST.[8-10] Since the introduction of imatinib, overall survival of patients with metastatic GIST has improved drastically. Earlier, only 15% of patients with advanced GIST were still alive 2 years after diagnosis. Nowadays, progression free survival on imatinib treatment is 2-2.5 years, and 10% of patients with advanced GIST has been treated with imatinib for more than 10 years without progression of the disease.[7, 11, 12]

The introduction of sunitinib, a multi-target tyrosine kinase inhibitor of c-KIT, PDGFR-α, PDGFR-β, VEGF receptors 1, 2 and 3, CSF-1R and FLT3,[12-14] as a second line treatment for imatinib resistant or intolerant GIST further increased progression free survival from 6 weeks to 27 weeks.[15] And since 2014, even a third-line TKI (regorafenib) is approved for GIST, further improving survival.[16] Besides the

indication as second line for GIST, sunitinib has proven efficacy in the treatment of advanced renal cell carcinoma and neuro-endocrine tumours of the pancreas.[17, 18] These are just some examples of how TKIs have improved anti-cancer therapies and have been implemented in today's anti-cancer treatment.

Table 1 Tyrosine kinase inhibitors

TKI	Inhibited kinases	Indication
Afatinib	EGFR	EGFR+ NSCLC[19]
Axitinib	VEGFR-1, VEGFR-2, VEGFR-3	Renal cell cancer[20]
Bosutinib	SRC, ABL	Ph+ CML[21]
Cabozantinib	MET, VEGFR-2, RET	Medullary thyroid carcinoma[22]
Crizotinib	ALK, HGFR, RON, ROS1	ALK+ NSCLC[23]
Dasatinib	BCR-ABL, c-KIT, PDGFR-β, SRC, EPHA-2	Ph+ CML, Ph+ ALL[24]
Erlotinib	EGFR	EGFR+ NSCLC, metastatic pancreas carcinoma[25]
Gefitinib	EGFR	EGFR+ NSCLC[26]
Imatinib	BCR-ABL, c-KIT, PDGFR-α, PDGFR-β, DDR-1, DDR-2, CSF-1R	GIST, CML, ALL, dermatofibrosarcoma protuberans[27]
Lapatinib	HER2, EGFR	HER2+ Breast cancer[28]
Nilotinib	BCR-ABL, PDGFR-α, PDGFR-β, c-KIT, CSF-1R, DDR	Ph+ CML[29]
Pazopanib	VEGFR-1, VEGFR-2, VEGFR-3, c-KIT, PDGFR-α, PDGFR-β, FGFR-1, FGFR-3, LTK, LCK, c-FMS	Renal cell cancer, soft tissue sarcoma[30]
Regorafenib	VEGFR-1, VEGFR-2, VEGFR-3, TIE2, c-KIT, RET, RAF-1, BRAF, PDGFR, FGFR	colorectal , GIST[31]
Sorafenib	VEGFR-2, VEGFR-3, PDGFR-β, C-RAF, B-RAF, FLT3, c-Kit	Renal cell cancer, HCC, non-medullary thyroid carcinoma[32]
Sunitinib	VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β, c-KIT, RET, FLT3, CSF-1R	Renal cell cancer, GIST, p-NET[33]
Vandetanib	EGFR, VEGFR-2, RET	Medullary thyroid carcinoma[34]
Vemurafenib	BRAF	Braf V600E+ melanoma[35]

PERSONALIZED MEDICINE

Although there is (at least) 30-35% inter-patient variability in both pharmacokinetics and pharmacodynamics for most TKIs, these drugs are still prescribed in a fixed dosing schedule. Side effects are common in TKI treatment and not occasionally patients require dose reductions or discontinuations due to adverse effects. Furthermore, not all patients respond similarly to TKI treatment. A small proportion of patients suffer from initial resistance to a drug, and regardless of which TKI is used for which indication, eventually all patients become drug resistant resulting in

tumour growth. These observations suggest that personalized medicine could further improve the treatment with TKIs.[36, 37]

Patient selection

There are several ways to improve and personalize treatment. A first step in personalized medicine is defining patients who are likely to have a favourable prognostic outcome of treatment in terms of little side effects and long survival. These patients will potentially be the best candidates for the treatment. Patients with a poor prognostic outcome may be prevented from side effects without positive treatment effects and may possibly receive another type of treatment, which in their specific case has a better prognosis. Patient stratification may be based on tumour characteristics, as in the example of patients with GIST where patients with a specific PDGFR-α mutation, D842V, have a poor response to imatinib treatment.[9, 38, 39] For these patients, there are no standard treatment options available currently, but this might change in the future.

Therapeutic drug monitoring

A second possibility to further personalize treatment is by defining which dose a specific patient should get. The large differences in the occurrence of side effects and survival to treatments suggests that TKIs have a narrow therapeutic window. The therapeutic window is the plasma concentration range in which the drug is effective, and does not result in too much (severe) side effects. It can be visualized in a figure with 2 boundaries. The lower boundary represents the lowest plasma concentration that is necessary for the drug to be effective, the upper boundary represents the plasma concentration above which severe side effects occur. In the ideal situation, the plasma concentration of a drug should be held within these boundaries, which are drug specific (**Figure 1**). In treatments with a narrow therapeutic window, therapeutic drug monitoring (TDM) may result in a more tailored treatment with more efficacy and less side effects. TDM is frequently applied in the treatment with anti-epileptics, antibiotics and in the treatment of patients with organ transplantation.[40-42] In anti-cancer therapy however, it is relatively new and not yet standard practise.[43, 44]

By applying TDM, patients start with a standard dose of the drug. At steady state, pharmacokinetics of the drug plasma drug concentrations are measured. If a patient's plasma concentration falls below the lower boundary of the therapeutic window, the daily drug dose may be increased to reach higher efficacy. In case the plasma concentration is too high, the drug dose may be decreased to protect the patient from side effects. If the plasma concentration lies within the upper and lower boundary and the patient does not suffer from severe side effects, no action is required with regard to the drug dose. A disadvantage of TDM is that the patient starts with a standard dose. Those patients with plasma levels above the upper limit at the standard starting dose may already suffer from severe side-effects shortly after start of treatment. This may be prevented by the use of predictive models.

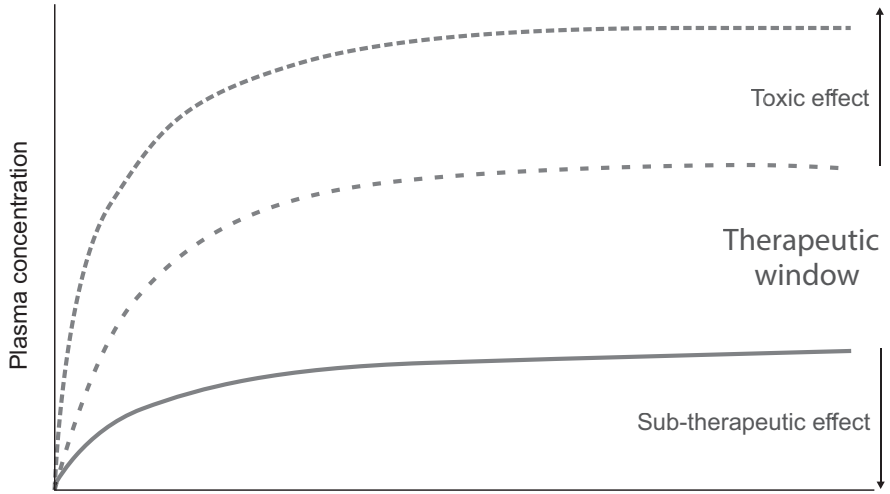

Figure 1 The therapeutic window of a drug

There are several factors which may influence the pharmacokinetics of a drug, such as patient characteristics (i.e. age, gender, BMI), genetic variability (i.e. single nucleotide polymorphisms [SNPs] in drug transporters and metabolizing enzymes), lifestyle habits (i.e. smoking, diet), organ function (i.e. kidney and liver function), illness related factors (i.e. ascites, weight loss) and the use of co-medication (**Figure 2**).[36, 45] Instead of, or besides using TDM, there are several other options to personalize the treatment of many drugs, including that of TKIs, by using predictors for either pharmacokinetics and/or pharmacodynamics.

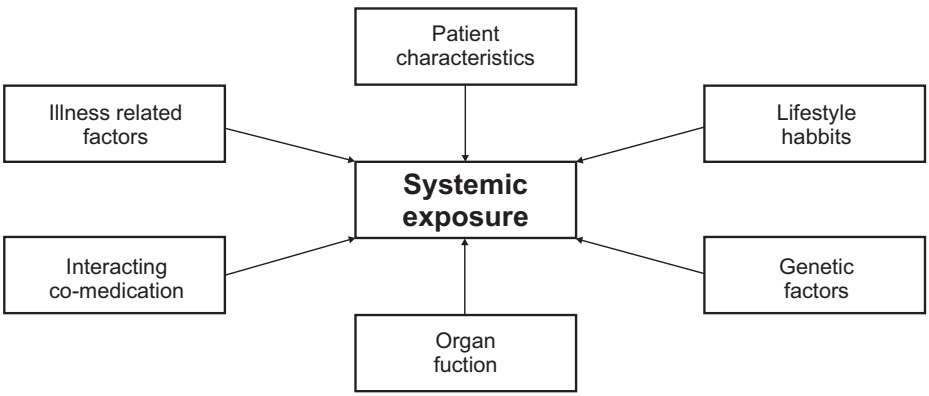

Figure 2 Factors influencing systemic drug exposure

Predictive tests and markers

Pharmacogenetics may be such a predictor. In pharmacogenetics, a certain SNP or combination of SNPs within the pharmacokinetic or pharmacodynamic pathway of the drug, may be used as a predictor of the outcome of treatment. The outcome may be survival rate,[4] the occurrence of side-effects,[46] or exposure to the drug.[47]

To further personalize treatment, phenotyping probes may also be applied. By using phenotyping probe testing, a compound with comparable pharmacokinetics as the drug of interest is administered to the patient. Afterwards, pharmacokinetic sampling of the mother compound and, if applicable, the (active) metabolite of the probe take place. As the probe and the drug of interest follow a similar pattern of uptake, metabolism and excretion, the ratio between the mother compound and the metabolite of the probe serve as a measure of the pharmacokinetic processes within that specific patient, regardless of possible factors that may influence the pharmacokinetics. Therefore, this may predict the pharmacokinetics of the drug of interest in the patient. In case of an accurate prediction, patients who show high levels of the phenotyping probe, will also show high levels of the drug of interest. This may particularly be useful in the treatment with drugs with severe side effects, as these can be avoided by starting at lower dose levels in patients who are prone to high drug levels. Phenotyping probes are usually drugs with little or no side effects (exogenous markers), but may also be a compound which is naturally present within the body (endogenous markers). In anticancer treatment, dextromethorphan, an ingredient of cough syrup and metabolized by both CYP2D6 and CYP3A, is such a phenotyping probe for tamoxifen, an anti-hormone drug that is frequently applied in the treatment of women with breast cancer.[48] An example of an endogenous marker, which may serve as a phenotyping probe, is the 4β-hydroxycholesterol/cholesterol ratio as a marker for CYP3A4 activity.[49]

Also, the occurrence of specific side effects may serve as marker for treatment efficacy. This was previously shown in patients with renal cell carcinoma. Patients who developed hypertension, caused by inhibition of the tyrosine kinase VEGFR, had a mean increase in overall survival of 7.2 months, compared to patients who did not develop hypertension.[50, 51] Another example is the occurrence of rash in the treatment of patients with lung cancer with erlotinib. Patients that do not develop a skin rash usually have a poor response to erlotinib.[52]

Although it is well known that patients treated with TKIs show broad inter-individual variations in pharmacokinetics, prospective studies investigating exposure-effect and exposure-toxicity relationships are limited.[53] In the treatment of sunitinib, a combined trough level of sunitinib and its active metabolite N-desethylsunitinib of at least 50 ng/mL is thought to be necessary for the drug to be effective in anti-cancer treatment. However, this trough level is based on preclinical and retrospective studies.[13, 54-56] So far, no prospective studies have been performed to evaluate whether

TDM of sunitinib, using the combined trough level of at least 50 ng/mL as a target value, results in longer progression free survival than standard dosing which is currently applied. This accounts for most studies on TKIs, and prospective randomized controlled trials in this field are warranted before TDM can be applied in clinical practice.[53]

The work described in this thesis includes research that focuses on a pharmacokinetic approach towards improved sunitinib treatment (chapter 2-4), and research that focuses on pharmacodynamic aspects of TKI treatment (chapter 5-8). In **chapter 2**, hepatobiliary clearance of technetium-99m-2-methoxy isobutyl isonitrile (^{99m}Tc-MIBI), as a phenotyping probe for the adenosine triphosphate binding cassette transporter ABCB1 and midazolam clearance test as a probe for cytochrome P450 3A (CYP3A) were correlated to sunitinib pharmacokinetics. Furthermore, sunitinib exposure was correlated to the occurrence of severe side effects due to sunitinib. Although it has been suggested that patients who are treated with sunitinib may benefit from TDM, it was not known whether TDM of sunitinib is safe. Therefore, a feasibility study was performed in patients treated with sunitinib in a phase I clinical trial (**chapter 3**). Sunitinib dose was elevated when total trough levels of sunitinib and its active metabolite SU12662 dropped below a pre-defined target level of 50 ng/mL, and sunitinib dose was decreased when patients suffered from unacceptable toxicity.

Circadian rhythms exist throughout the body. They originate in the suprachiasmatic nucleus (SCN) in the hypothalamus, which is stimulated by photo neurons in the eyes.[57] The pharmacokinetic processes absorption, distribution, metabolism and excretion are also thought to be subjected to circadian rhythms,[58-60] resulting in intra-individual variations in drug pharmacokinetics based on administration time. Such variations may also exist in the treatment with sunitinib. **Chapter 4** describes the results of preclinical and clinical studies of chronicity in sunitinib pharmacokinetics. Studies were performed in immortalized hepatic cells, mice and patients treated with sunitinib as standard of care.

As previously mentioned, another approach towards personalized treatment is by predicting treatment response and toxicity levels based the pharmacogenetic profile of the patient. In **chapter 5** and **chapter 6**, the efficacy of respectively imatinib and sunitinib treatment in patients with GIST was associated with SNPs within genes comprising the pharmacokinetic and pharmacodynamic pathway of the drug.

The last chapters of this thesis focus on the occurrence of specific side-effects in the treatment of TKIs.

QTc prolongation is a possibly dangerous side-effect of many drugs, which in some cases, such as rofecoxib (Vioxx), results in withdrawal of the drug from the market. It is usually caused by inhibition of the human ether-à-go-go- related gene (hERG) , within the cardiomyocyte.[61] Blocking of this potassium channel prevents potassium to rapidly flow out of the cell, resulting in prolonged repolarization which

is seen is on the surface electrocardiogram as a prolongation of the QTc interval. For many anti-cancer drugs, including TKIs, information on the effect of the drug on the QTc interval is lacking. In **chapter 7**, the prolongation of the QTc interval after start of TKI treatment was investigated in 363 patients treated with 8 different TKIs.

Several studies have described an increase in the mean corpuscular volume or erythrocytes after start of sunitinib or imatinib.[62-66] Since both sunitinib and imatinib are inhibitors of c-KIT, and no other cause of macrocytosis could be appointed, this has led to the hypothesis that the increase in MCV may be specific for c-KIT-inhibiting TKIs. In **chapter 8**, we describe which TKIs result in an increase of the MCV in a population of 550 patients from the Erasmus MC Cancer Institute. Furthermore, we show that macrocytosis can be used as a predictor of survival in renal cell cancer patients treated with sunitinib.

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Part I

Pharmacokinetic approach towards improved sunitinib treatment

Chapter 2

Predictive Value of CYP3A and ABCB1 Phenotyping Probes for the Pharmacokinetics of Sunitinib: The Clearsun Study

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ABSTRACT

Background and Objective

The wide inter-patient variability in drug exposure partly explains the toxicity and efficacy profile of sunitinib treatment. In this prospective study CYP3A and ABCB1 phenotypes were correlated to the pharmacokinetics of sunitinib and its active metabolite *N*-desethylsunitinib.

Methods

A correlation analysis was performed between sunitinib pharmacokinetics and 1'OH-midazolam/midazolam ratio and parameters derived from ^{99m}Tc-MIBI scans, respectively. A population pharmacokinetic model (NONMEM) was built, which included the phenotype tests as covariate.

Results

In 52 patients, the mean trough level of sunitinib plus metabolite increased from 21.4 ng/mL at day 1 of a cycle to 88.1 ng/mL in the 4th week of treatment. A trend for a correlation was observed between ^{99m}Tc-MIBI elimination constant and trough levels of *N*-desethylsunitinib, however, this was not significant. Correlations were found between 1'OH-midazolam/midazolam ratio and sunitinib clearance (P = 0.008) and day 1 *N*-desethylsunitinib trough concentrations (P = 0.005), respectively. Moreover, patients suffering from grade 3 toxicities had significant lower clearance of sunitinib than patients without grade 3 toxicities (34.4 L/h vs. 41.4 L/h; P = 0.025).

Conclusions

Phenotype tests for ABCB1 and CYP3A4 did not explain inter-individual variability of sunitinib exposure sufficiently. However, the correlation between sunitinib clearance and the occurrence of severe toxicity suggests a direct exposure-toxicity relationship.

INTRODUCTION

Sunitinib malate (Sutent®; Pfizer Pharmaceuticals Group, NY, USA) is an oral multi-targeted tyrosine kinase inhibitor with antitumor and anti-angiogenic activities. It is approved for first-line treatment of metastatic renal-cell cancer (mRCC), imatinib-resistant metastatic gastrointestinal stromal tumors (GIST) and pancreatic-neuroendocrine tumors (p-NET).[1-3]

Regular treatment schedules for mRCC and GIST consist of a flat-fixed daily dose of 50 mg (4 weeks on, 2 weeks off) and for p-NET a flat-fixed daily dose of 37.5 mg continuously. However, dose reductions due to toxicity are frequently necessary (almost one in every 3 patients).[1, 2, 4] Moreover, a small proportion of patients tolerate doses higher than 50mg daily without significant toxicity.[5] A plausible explanation for this wide range in tolerable dose is the wide inter-patient variability in sunitinib drug exposure. This variability may result from differences in activity of drug metabolizing enzymes and transporters. To determine the activity of sunitinib metabolism in individual patients, so-called phenotype tests may be applied. Phenotype tests consist of the administration of a "model" or probe drug metabolized by an individual specific enzyme or transporter. This means that an easily detectable and safe compound of which the pharmacokinetic fate highly correlates with that of the drug of interest is administered to the patient, and different pharmacokinetic parameters of the probe drug and its metabolites, or the determination of a ratio between the drug and its metabolite (metabolic ratio; MR), are determined.[6] By measuring this MR, the activity of an enzyme or transporter of interest may be estimated at a certain moment of time, which allows the definition of an individual metabolic profile. In other words, the pharmacokinetics of this compound (or 'probe'-drug) may act as a predictor for enzymatic or transporter function.[7]

Midazolam is such a probe-drug for cytochrome P450 3A (CYP3A) as it is exclusively metabolized by CYP3A. CYP3A activity has been successfully associated with the pharmacokinetics and pharmacodynamics of several anti-cancer drugs, including docetaxel[8] and irinotecan.[9] One of the possible drawbacks of phenotype tests is the occurrence of side effects from the probe drug. Microdoses of midazolam have, therefore, been validated [10]. The ratio between total 1'OH-midazolam and midazolam plasma concentration 30 minutes post-administration is now used as a predictor of CYP3A activity.[10, 11]

ABCB1 is an adenosine triphosphate binding cassette (ABC) transporter, involved in hepatic clearance of many classes of anticancer drugs, including sunitinib. Hepatobiliary clearance of technetium-99m (^{99m}Tc)-2-methoxy isobutyl isonitrile (MIBI) is markedly reduced in the presence of ABCB1 inhibitors in humans.[12, 13] Thus, it is likely that ABCB1 is the principle mediator of ^{99m}Tc-MIBI elimination. Hepatic scans provide a ^{99m}Tc-MIBI elimination constant (kH) and can be used as a probe of ABCB1 activity.[14] Hepatic ^{99m}Tc-MIBI clearance (kHxliver volume) has previously been studied as a predictor of vinorelbine clearance.[15]

As sunitinib is primarily metabolized by CYP3A (Figure 1), resulting in the formation of its active metabolite *N*-desethylsunitinib, midazolam is a putative probe-drug for sunitinib metabolism.[16] The hepatic elimination of sunitinib is primarily mediated by ABCB1 and ABCG2.[17] Therefore, hepatic ^{99m}Tc-

MIBI scan, as a probe for ABCB1, may also serve as a possible predictor of sunitinib pharmacokinetics. The primary aim of this study was to investigate whether these CYP3A and ABCB1 phenotype probes correlate with the clearance of sunitinib ('ClearSun'), and may therefore be used as a predictor of sunitinib exposure. This may ultimately be used in clinical practice to dose this drug on an individualized way, instead of the current flat-fixed dosing standard. As a secondary aim, we studied the association between drug exposure and sunitinib-induced toxicity.

METHODS

In four centres in Australia (Westmead Hospital, Sydney and Canberra Hospital, Garran) and the Netherlands (Erasmus MC Cancer Institute, Rotterdam and Academic Medical Centre, Amsterdam), patients with solid tumours treated with single agent sunitinib were asked to participate in this study between February 2009 and September 2011. Patients were eligible at any cycle during treatment, and all dose levels of sunitinib were allowed in the study. Additional eligibility criteria were age at least 18 years; Eastern Cooperative Oncology Group (ECOG) performance status of 2 or less; adequate hematopoietic, hepatic, and renal functions. Patients were excluded for any significant systemic disorders and pregnancy. All patients provided informed consent according to federal and institutional guidelines. The trial was conducted in accordance with current Good Clinical Practice and approved by the local ethical committee.

Study design

This was a prospective pharmacokinetic study of patients treated with sunitinib. Patients were asked to undergo three study tests at day 1 of a new treatment cycle and in the fourth week of continuous treatment. Study tests consisted of pharmacokinetic sampling of sunitinib concentrations, 1'OH-midazolam/midazolam MR assessment and ^{99m}Tc-MIBI liver scans at both time-points during the study. Demographic and clinical data of patients were reported on case record forms designed for data collection in this study. All plasma samples for pharmacokinetics on sunitinib and midazolam, and the performed ^{99m}Tc-MIBI lever scans 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).

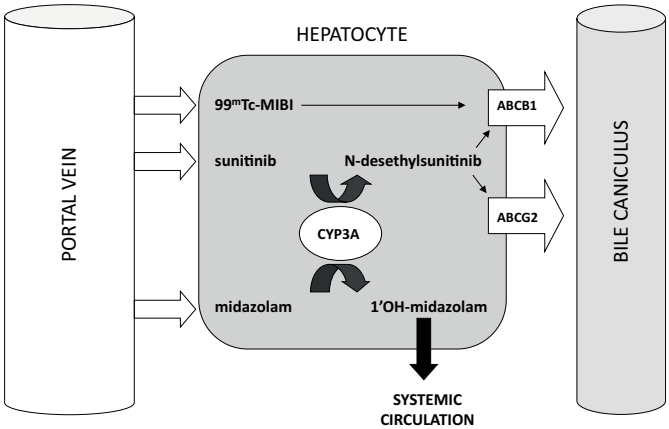

Figure 1 Schematic figure for sunitinib, midazolam and ^{99m}Tc-MIBI metabolism and/or transport. Abbreviations: ^{99m}Tc-MIBI, technetium-99m-2-methoxy isobutyl isonitrile; CYP3A, cytochrome P-450 enzyme 3A; ABCB1; ATP-binding cassette sub-family B member 1; ABCG2, ATP-binding cassette sub-family G member 2; min, minutes

Pharmacokinetic sampling of sunitinib

Pharmacokinetic blood withdrawal was performed on day 1 and at repeated continuous dosing of sunitinib treatment and stored at -80°C at the participating center. A limited blood sampling regimen was used with accurately timed blood samples taken pre-dose and at t=4h, t=8h and t=24h after sunitinib administration. All pharmacokinetic measurements of sunitinib were undertaken at the Laboratory of Translational Pharmacology of Erasmus MC Cancer Institute. Sunitinib and its active metabolite *N*-desethylsunitinib in human plasma were quantified using a validated UPLC-MS/MS method consisted of a Waters Acquity UPLC sample manager coupled to a triple quadruple mass spectrometer operating in the multiple reaction monitoring mode (MRM) with positive ion electrospray ionisation (Waters, Etten-Leur, The Netherlands). The multiple reaction monitoring transitions were set at 399→326, at 371→283 and at 409→326 for sunitinib, *N*-desethylsunitinib and the deuterated internal standard sunitinib-d10, respectively. Chromatographic separations were achieved on an Acquity UPLC® BEH C18 1.7 µm 2.1 x 50 mm column eluted at a flow-rate of 0.250 mL/min on a gradient of acetonitrile. The overall cycle time of the method was 4 minutes. The calibration curves were linear over the range of 0.200 to 50.0 ng/mL with the lower limit of quantification validated at 0.200 ng/mL for both sunitinib and *N*-desethylsunitinib. As sunitinib was found to be extremely sensitive to light causing the Z (*cis*)-isomer to rapidly convert to the E (*trans*)-isomer, the sample extraction and cleaning-up were performed under sodium light and in amber vials. The extraction of 100 µL of plasma involved a simple liquid-liquid extraction with *tert*-butyl methyl ether.[18]

Plasma concentrations were corrected for dose-adjustment, using the assumption that dose adjustment of sunitinib results in equally adjusted exposure to sunitinib and its active metabolite.

1'OH-midazolam/midazolam metabolic ratio assessment

A micro-dose of midazolam (75 µg) was administered orally before sunitinib administration as a measure of CYP3A activity as previously validated.[10] 1'OH-midazolam/midazolam MR was determined 30 minute post-administration in a single plasma sample using liquid chromatography tandem mass spectrometry as previously described.[19] Midazolam and 1-hydroxymidazolam were quantitated using a fully validated method with an API 4000 triple quadruple mass spectrometer (AB Sciex, Concord, ON, Canada) coupled to an Agilent series 1100 (Waldbronn, Germany) LC system. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with positive ion electrospray ionization. The MRM transitions were 326→291, 342→324 and 330→295 midazolam, 1-hydroxymidazolam and internal standard midazolam-d4 (IS), respectively. Chromatography was performed on a Phenomenex Kinetex C18 analytical column (50 mm × 2.1 mm, 2.6 µm; Torrance, CA, USA) using water and acetonitrile with 0.1% formic acid under gradient conditions. One ml of plasma was submitted to enzymatic hydrolysis using β-glucuronidase and a liquid-liquid extraction procedure was performed using hexane-chloroform as previously described.[10] The limits of quantification were 0.05 and 0.1 ng/ml for midazolam and 1-hydroxymidazolam, respectively.

All pharmacokinetic measurements on midazolam were performed by the department of Clinical Pharmacology & Toxicology of Geneva University Hospitals (Switzerland) using the method described by de Loor et al.[19]

Hepatic technetium labelled sestamibi (^{99m}Tc-MIBI) clearance

Within 2 days before the start of the treatment and after repeated daily dosing, hepatic elimination of ^{99m}Tc-MIBI was determined and interpreted as an indicator of ABCB1 (P-glycoprotein, MDR1) activity.[14] A correlation analysis was performed between ^{99m}Tc-MIBI kH and both sunitinib dose-adjusted trough levels and clearance. All hepatic ^{99m}Tc-MIBI scans were interpreted by a single person (M.W.) at Westmead Hospital, Sydney, Australia.

Definition of Toxicity

All adverse events were graded according to National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0 scores at the participating centres. Clinical symptoms and haematological abnormalities were considered drug induced, such as: fatigue, mucosal inflammation, hand-foot syndrome, thrombocytopenia, neutropenia and any other adverse events higher than grade 2. The considerations if an adverse event is related to sunitinib treatment were up to the investigators discretion.

Statistical Design and Data Analysis

To correlate phenotype tests (1'OH-midazolam/midazolam MR and hepatic ^{99m}Tc-MIBI scans) with sunitinib dose-adjusted trough levels, Spearman's correlation coefficient was calculated using SPSS version 17.0. The paired Student's *t*-test was used to compare sunitinib trough levels, 1'OH-midazolam/

midazolam MR assessments and ^{99m}Tc-MIBI kH at day 1 of a treatment cycle and after repeated daily dosing.

A population PK model was developed in order to identify the correlation between both phenotype tests and clearance of sunitinib and *N*-desethylsunitinib. Nonlinear mixed effects modeling (NONMEM version 7.1, ICON, Ellicot City, Maryland, USA with Piraña as modeling environment[20]) was used to develop an integrated base compartmental model for sunitinib (2-compartmental model) and *N*-desethylsunitinib (1-compartmental model) (Supplementary figure 1[20, 21]). This base pharmacokinetic (PK) model was selected according to change in objective function value (OFV, Δ OFV > 3.84, P < 0.05), successful minimization, successful covariance estimation, and goodness-of-fit plots. Parameter estimation was performed by first order conditional estimation method with interaction. Due to unknown bioavailability (F), all the parameter estimates (except for absorption rate constant of sunitinib ($k_{a,p}$)) were presented as apparent terms relative to F. For *N*-desethylsunitinib, parameters were estimated relative to F and f_m, the fraction sunitinib metabolized to *N*-desethylsunitinib. $K_{a,p}$ was fixed to 0.195 h^{-1} according to the result from an informative sunitinib modeling.[22] Inter-individual variability (IIV) in all parameters was described by an exponential error term and provided as both percentage of coefficient variation (%CV) and variance (calculated as $(\%CV/100)^2$). And the residual error was modeled using a combined error model with additive error terms fixed to lower limit of quantification (LLOQ). Results presented in Supplementary table 1.

Subsequently, the phenotype tests were tested as covariates on clearance parameters for both compounds in a univariate analysis. P-values were calculated by chi-squared distribution from Δ OFV. Goodness-of-fit was judged by various graphical and statistical measures.[23]

The independent *t*-test was used to test a possible correlation between both sunitinib clearance and dose-adjusted trough levels and the occurrence of toxicities, as well as for the correlation between both phenotype tests and the occurrence of toxicities.

A probability value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics

A total of 52 patients were enrolled in this study. Forty-two of all participating patients underwent a midazolam clearance test at start of a new cycle and in the fourth week of treatment (steady state). A total of 39 patients had ^{99m}Tc-MIBI scans of the liver at both time points and were evaluable for the analysis. Of the 52 patients enrolled in this study, 28 patients had undergone a dose reduction or temporary discontinuation of treatment due to toxicity. As sunitinib has linear pharmacokinetics, a dose-reduction could not have influenced the outcomes of this study.[24] Patient's demographics and disease characteristics are summarized in Table 1.

Table 1 Patient characteristics

Characteristic	Value
Total no. of patients	52
Age (years)	59 (29-81)
Sex	
Male	33 (64)
Female	19 (36)
Mean BSA m^2	2.01
Range	1.26-2.5
Ethnicity	
Caucasian	47 (90)
Asian	3 (6)
Hispanic	1 (2)
Other	1 (2)
Type of malignancy	
RCC	43 (83)
p-NET	7 (13)
GIST	2 (4)
ECOG performance status	
0	29 (56)
1	21 (40)
2	2 (4)
Duration of treatment (months)	9 (0-54)
Dose of sunitinib	
0 mg[a]	6 (12)
25 mg	6 (12)
37.5 mg	14 (26)
50 mg	26 (50)
Previous dose reduction	28 (54)

Values are expressed as mean (range) or n (%)

Abbreviation: N, number of patients; BSA, Body Surface Area; RCC, renal cell carcinoma; p-NET, pancreatic neuro-endocrine tumor; GIST, gastrointestinal stromal tumor; ECOG, Eastern Cooperative Oncology Group

[a] Patients who did not use sunitinib by the time of pharmacokinetics measurements were excluded from the analysis

Sunitinib pharmacokinetics and drug elimination phenotype

Mean combined trough levels of sunitinib and *N*-desethylsunitinib, adjusted for dose, were 21.4 ng/mL (range 10.5 - 38.3 ng/mL) at day 1 of a new course, and 88.1 ng/mL (range 22.3 - 189.7 ng/mL) in the 4th week of a treatment cycle (mean difference 66.7 ng/mL, $P < 0.005$; Table 2). In the population PK model, a mean clearance of 36.5 L/h for sunitinib and metabolic clearance of 91.3 L/h for the metabolite was estimated (Supplementary Table 1).

A good correlation between 1'OH-midazolam/midazolam MR on the 1st day and in the 4th week of a treatment cycle was found ($P = 0.003$), as well as between hepatic ^{99m}Tc-MIBI kH at day 1 and week 4 ($P < 0.001$, Supplementary Figure 2).

The 1'OH-midazolam/midazolam MR did not significantly correlate with combined trough levels of sunitinib and metabolite at steady state (n = 42, $P = 0.22$, Table 3). However, the ratio correlated with *N*-desethylsunitinib levels at 24 hours after first intake of sunitinib in the first course of treatment (n = 21, $P = 0.005$, Figure 2). There was no correlation between 1'OH-midazolam/midazolam MR and N-desethylsunitinib/sunitinib MR at steady state (n = 42, $P = 0.60$). The correlations between hepatic ^{99m}Tc-MIBI kH and *N*-desethylsunitinib trough levels during any course were substantial, although not significant (n = 39, $P = 0.067$).

At steady state, the 1'OH-midazolam/midazolam MR weakly correlated with the clearance of sunitinib (n = 42, $P = 0.0081$), but not with clearance of *N*-desethylsunitinib (n = 42, $P = 1$).

We found no significant correlation between hepatic ^{99m}Tc-MIBI kH and sunitinib clearance or *N*-desethylsunitinib clearance (n = 39, $P = 1$).

Table 2 Pharmacokinetics of sunitinib and N-desethylsunitinib, and CYP3A and ABCB1 phenotype tests during treatment with sunitinib.

	n	Day 1	n	Steady-state	Difference mean	*P*
Pharmacokinetic measurements[a]						
Sunitinib trough level (ng/mL)	51	16.4 ±5.4 (7.2-28.9)	46	57.9 ±22.5 (19.1-151.1)	41.6	<0.005
N-desethylsunitinib (ng/mL)	51	5.0 ±2.4 (1.5-13.0)	46	30.1 ±12.8 (3.2-57.8)	25.2	<0.005
Sum (ng/mL)	51	21.4 ±6.7 (10.5-38.2)	46	88.1 ±31.4 (22.3-189.7)	66.7	<0.005
Phenotype tests						
1'OH- midazolam/midazolam metabolic ratio	48	2.73 ±2.18 (0.3-9.9)	42	2.78 ±2.0 (0.2-7.9)	0.05	0.86
^{99m}Tc-MIBI scan[b]	48	1.23 ±0.66 (0.42-3.56)	42	1.19 ±0.53 (0.45-2.81)	0.05	0.55

Values are expressed as mean ±SD (range)

Abbreviations: ^{99m}Tc-MIBI, technetium-99m-2-methoxy isobutyl isonitrile

[a] all pharmacokinetic measurements were adjusted for dose

[b] described as distribution of elimination constant (kH) min^{-1} x 10^{-2}

Table 3 Correlation between pharmacokinetics and phenotype tests at steady state

Parameter	1'OH- midazolam/midazolam MR (R^2/Δ OFV, P-value)	^{99m}Tc-MIBI scan (R^2/P-value)
C_{trough} sunitinib[a]	0.06 (0.126)	0.01 (0.540)
C_{trough} N-desethylsunitinib[a]	0.01 (0.478)	0.09 (0.067)
Sum[a]	0.04 (0.217)	0.01 (0.520)
CL sunitinib	6.992 (0.0081)	0 (1)
CL N-desethylsunitinib	0 (1)	0 (1)

[a] trough levels at steady state treatment

Abbreviation: C-trough, trough concentration; CL, clearance; R^2, correlation coefficient; OFV, Objective Function Value; ^{99m}Tc-MIBI, technetium-99m-2-methoxy isobutyl isonitrile

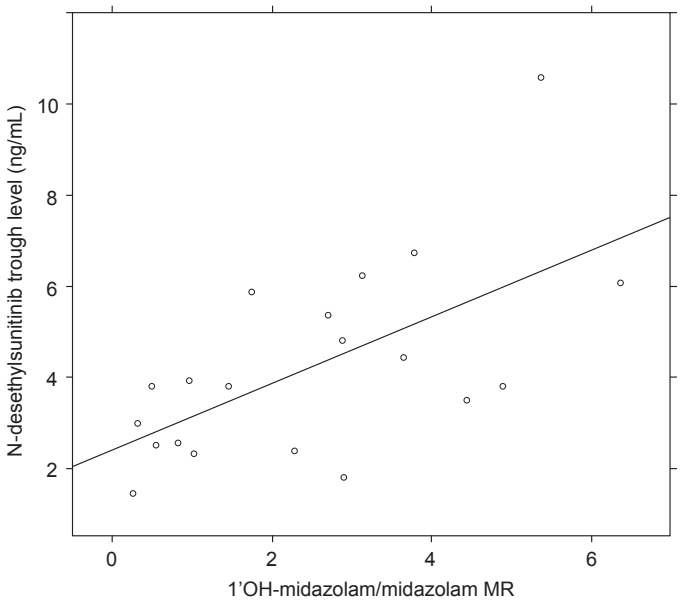

Figure 2 Correlation (R = 0.59, P = 0.005) between 1'OH-midazolam/midazolam MR (x-axis) and N-desethylsunitinib trough concentration after the first intake of sunitinib (y-axis). Each dot expresses an individual patient; the line expresses the correlation coefficient.
Abbreviation: MR, metabolic ratio

Toxicities

All patients experienced at least grade 1-2 toxicity from sunitinib treatment. Twenty-two (42%) of 52 patients suffered from grade 3 toxicities and 28 patients (54%) underwent a dose reduction at any time Hand-foot syndrome was the most frequently reported grade 3 toxicity, occurring in 6 patients (12%, Table 4). Interestingly, patients with any type of grade 3 toxicity had a significantly lower clearance of sunitinib than patients without grade 3 toxicities (34.4 L/h vs. 41.4 L/h; P = 0.025), suggesting an exposure-toxicity relationship. Combined trough levels of sunitinib and metabolite at steady state were positively correlated with the occurrence of fatigue (P = 0.007). There was no significant correlation between 1'OH-midazolam/midazolam MR and occurrence of severe toxicities (P = 0.23).
No toxicities were seen as a result of the use of the midazolam clearance tests or the ^{99m}Tc-MIBI-scans.

Table 4 Toxicity seen during treatment with sunitinib.

Adverse effect	Grade 1 – 2	Grade 3
NON-HEMATOLOGICAL		
Fatigue	42 (81)	4 (8)
Stomatitis[a]	33 (63)	2 (4)
Diarrhea	31 (60)	2 (4)
Nausea	21 (40)	4 (8)
Hand-foot syndrome	21 (40)	6 (12)
Hypertension	21 (40)	5 (10)
Anorexia	20 (39)	1 (2)
Taste alterations	21 (40)	
Skin rash	19 (37)	
Hypothyroidism	7 (14)	
Any	52 (100)	22 (42)
HEMATOLOGICAL		
Thrombocytopenia	25 (48)	
Leukopenia	25 (48)	

Values are expressed as n (%).

Abbreviation: n, number of patients

[a] Stomatitis including aphetic ulceration and mucositis of the mouth.

DISCUSSION

Considering that sunitinib is mainly metabolised by CYP3A4 to its active metabolite *N*-desethylsunitinib, and eliminated in the liver by ABCB1 (and ABCG2) transporters, probe drugs for CYP3A and ABCB1 activity were used in this study. Earlier publications suggested that midazolam clearance or MR, and hepatic ^{99m}Tc-MIBI elimination scans can be used as a predictor of CYP3A activity and ABCB1 activity, respectively.[10, 25, 26]

Although in the current study a significant correlation between 1'OH-midazolam/midazolam MR and the clearance of sunitinib was found, no correlation was seen between 1'OH-midazolam/midazolam MR and the active metabolite of sunitinib. This is somewhat surprising, as the conversion of sunitinib into *N*-desethylsunitinib is thought to be fully CYP3A-mediated.[16] The elimination of *N*-desethylsunitinib by other pathways or by efflux transport may have interfered, resulting in this negative outcome. Variable drug uptake may also have confounded an existing relationship. Furthermore, considering first order kinetics, non-continuous dosing of midazolam may have influenced the outcome. To predict sunitinib metabolism by use of an ABCB1 probe, we correlated the hepatic ^{99m}Tc-MIBI elimination

constant with the clearance of, and exposure to, sunitinib and its active metabolite. We found no significant correlations between hepatic clearance of ^{99m}Tc-MIBI and pharmacokinetic measurements of sunitinib. However, a substantial, although not significant, correlation was seen between ^{99m}Tc-MIBI kH and trough concentrations of *N*-desethylsunitinib. Sunitinib is eliminated by a number of efflux transporters other than ABCB1, such as ABCG2, and so ^{99m}Tc-MIBI elimination may be an incomplete probe for biliary elimination of this drug.[16] On the other hand, the stronger correlation between the ^{99m}Tc-MIBI and the sunitinib metabolite, suggests that ABCB1 is relatively more important than ABCG2 in the transport of *N*-desethylsunitinib.

A strong correlation was seen in hepatic ^{99m}Tc-MIBI kH between the first day and fourth week of a treatment cycle, indicating that the ABCB1 efflux transporter is not affected by sunitinib treatment over this time period. Also for 1'OH-midazolam/midazolam MR, a significant correlation was seen between the first day and fourth week of treatment, although the correlation coefficient was low. In addition, there was a large inter-individual variation in 1'OH-midazolam/midazolam MR, suggesting a substantial inter-individual variation in CYP3A activity.

Various drugs are well-known modulators of CYP3A4 activity, but no CYP3A4 inhibitors or inducers were started or stopped during the study period. Hypothetically, four weeks of sunitinib treatment may have affected CYP3A activity, leading to an altered 1'OH-midazolam/midazolam ratio. Other factors which may potentially change CYP3A activity, such as food intake, smoking and daily variation in drug intake may also have caused this variation.[27, 28]

All patients in our study experienced at least grade 1 toxicity and 42% of them had grade 3 toxicity. There was a significantly lower clearance of sunitinib in patients with grade 3 toxicity, as previously prescribed by Houk et al.[29] These data suggest a direct relationship between exposure to sunitinib and toxicity, and emphasize the need of a more tailored sunitinib treatment than current clinical practice. Thirty-three patients in our study suffered from any grade of diarrhea, as an indicator of mucosal inflammation. Inflammation may suppress drug metabolizing enzymes and efflux transporters and may hypothetically be the cause of increased levels of sunitinib.

CONCLUSION

Overall, we may conclude that although 1'OH-midazolam/midazolam MR may significantly correlate with measures of sunitinib pharmacokinetics, it does not predict variability in sunitinib exposure sufficiently to be useful in a clinical dosing-strategy. The same is true for the ABCB1 probe tested. However, this study provides important new information to unravel the clinical pharmacokinetics of sunitinib. Additional research remains necessary to provide more information on the variability in the pharmacokinetics of sunitinib. For example, possibly pharmacogenetic variation in genes coding for transporters or enzymes, like *CYP3A*22*, may explain pharmacokinetic variation for a large(r) part.[30] Furthermore, therapeutic drug monitoring can contribute to tailored sunitinib treatment,[30, 31] although this can only be applied after the start of treatment Ultimately, to prevent patients from

toxicity shortly after start of treatment, possible phenotyping probes should be used to determine the starting dose, and therapeutic drug monitoring to determine dose levels during treatment. Hopefully, all these efforts will provide tools to finally optimize the dosing of sunitinib, leading to a minimum of side-effects and a maximized anti-tumour effect.[31]

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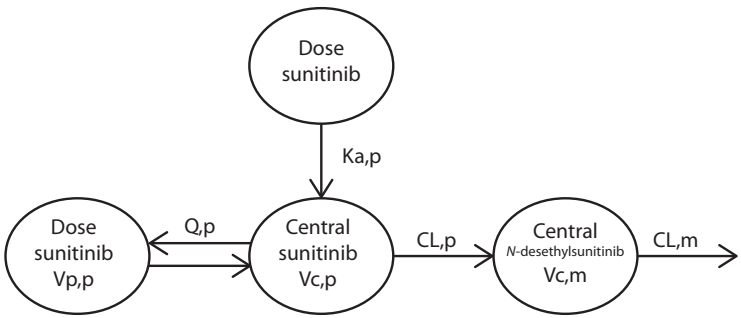
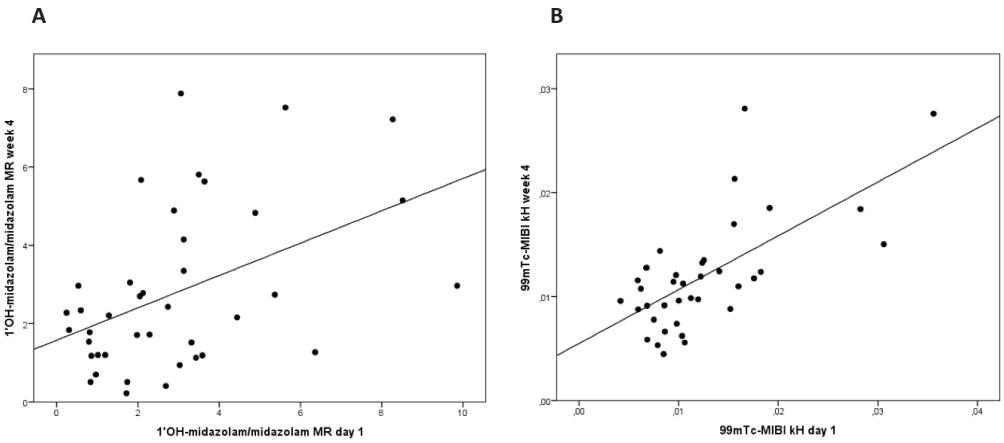
Supplementary table 1 Parameter estimated of the base model for sunitinib and *N*-desethylsunitinib

Parameter	Estimate (RSE)	IIV, $_{\%CV}$ (RSE)	IIV,$_{variance}$
Sunitinib			
CL,p, L·h^{-1}	36.5 (5.5%)	31.2% (10.9%)	0.0974
$k_{a,p}$, h^{-1}	0.195	-	-
Vc,p, L	616 (23.1%)	74.4% (19.5%)	0.553
Q,p, L·h^{-1}	281 (11.0 %)	-	-
Vp,p, L	1800 (9.1%)	41.2% (12.9%)	0.170
Proportional error	0.0459 (17.9%)	-	-
Additive error, ng/mL	0.2	-	-
***N*-desethylsunitinib**			
CL,m, L· h^{-1}	91.3 (6.8%)	43.6% (8.1%)	0.190
Vc,m, L	358 (18.3%)	-	-
Proportional error	0.0473 (20.2%)	-	-
Additive error, ng/mL	0.2	-	-



Supplementary figure 1 PK model for sunitinib and *N*-desethylsunitinib. $K_{a,p}$: absorption rate constant of sunitinib; Vc,p: apparent central volume distribution of sunitinib; Vp,p: apparent peripheral volume distribution of sunitinib; Q: apparent intercompartmental flow of sunitinib; CL,p: apparent clearance of sunitinib; Vc,m: apparent volume distribution of *N*-desethylsunitinib; CL,m: apparent clearance of *N*-desethylsunitinib.



Supplementary figure 2 Correlation in 1'OH-midazolam/midazolam MR (**A**) and hepatic ^{99m}Tc-MIBI kH (**B**) between the first day and fourth week of sunitinib treatment. Each dot expresses an individual patient; the line expresses the correlation coefficient.

Chapter 3

Pharmacokinetically-guided Sunitinib dosing: a feasibility study in patients with advanced solid tumours

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ABSTRACT

Background Plasma exposure of sunitinib shows large inter-individual variation. Therefore, a pharmacokinetic (PK) study was performed to determine safety and feasibility of sunitinib dosing based on PK levels.

Methods Patients were treated with sunitinib 37.5 mg once daily. At day 15 and 29 of treatment, plasma trough levels of sunitinib and N-desethyl sunitinib were measured. If the total trough level (TTL) was <50 ng/mL and the patient did not show any grade ≥3 toxicity, the daily sunitinib dose was increased by 12.5 mg. If the patient suffered from grade ≥3 toxicity, the sunitinib dose was lowered by 12.5 mg.

Results Twenty-nine out of 43 patients were evaluable for PK assessments. Grade ≥3 adverse events were experienced in seven patients (24%) at the starting dose and in nine patients (31%) after dose escalation. TTLs were below target in 15 patients (52%) at the starting dose. Of these, 5 patients (17%) reached target TTL after dose escalation without additional toxicity.

Conclusion In a third of the patients that were below target TTL at standard dose, the sunitinib dose could be increased without additional toxicities. This could be the basis for future studies and the implementation of a PK-guided dosing strategy in clinical practice.

INTRODUCTION

Sunitinib (Sutent®) has proven efficacy as single agent in several solid tumor types and is approved for use in advanced renal cell cancer (RCC), imatinib-resistant or -intolerant gastrointestinal stromal tumors (GISTs) and pancreatic neuroendocrine tumors (pNET).[1-3] Recent findings demonstrated a positive dose-efficacy relationship for sunitinib treatment.[4] As deduced from pharmacokinetic/pharmacodynamic preclinical data, target total plasma concentrations of sunitinib plus active metabolite (N-desethylsunitinib) are in the range of 50 to 100 ng/mL.[5-9] In line with these preclinical data, total trough levels (TTLs) below 50 ng/mL have been associated with decreased therapeutic efficacy in patients compared to patients with TTL above this level.[7] It is therefore hypothesized that for optimal sunitinib therapy a TTL above 50 ng/mL should be reached in each individual patient. However, sunitinib exposure shows considerable variation due to patient non-compliance (for example due to drug-related toxicity), drug interactions with co-medication, variability in oral drug availability and many other factors.[10] Despite this considerable inter-patient variability in systemic exposure, sunitinib is currently prescribed at a fixed dose. Given the narrow therapeutic index, the large inter-individual variability in systemic exposure, and the positive exposure-efficacy relationship, there is a strong rationale for pharmacokinetically (PK) guided dosing also known as therapeutic drug monitoring (TDM) of sunitinib.[4, 7, 11] Such an approach could contribute to a tailor made sunitinib treatment with improved therapeutic efficacy and decreased risk for toxicity.[12]

Thus far, no prospective clinical trials investigating the safety and efficacy of PK guided dosing for sunitinib therapy have been performed. Hence, the ultimate proof that reaching target trough concentrations increases treatment efficacy remains to be awaited. As a first step towards individualized PK based dosing, we investigated the safety and feasibility of PK-guided sunitinib dosing in a pilot study by measuring sunitinib trough levels. The main purpose of the study was to assess whether PK-guided dosing could be performed without causing additional toxicities. Establishing a feasible and safe PK-guided dosing strategy could provide a rationale for a large prospective clinical trial.

METHODS

Patient population

This multicenter prospective pilot trial (NCT01286896) was initiated in 2011 and was performed in three medical centers in The Netherlands. Eligible patients were patients with histologically or cytologically confirmed advanced tumors for which sunitinib was considered standard therapy or patients with advanced or metastatic tumors for whom no standard therapy was available.

Other inclusion criteria included age ≥ 18 years; an Eastern Cooperative Oncology Group (ECOG) performance status ≤ 1; measurable or evaluable disease according to Response Evaluation Criteria Solid Tumors (RECIST) 1.1 criteria; estimated life expectancy > 12 weeks; adequate hematologic, hepatic and renal function; no cardiac instability within the previous six months. Additionally, patients

should be able and willing to undergo blood sampling; and patients should be able to swallow oral medication.

The protocol was approved by local independent ethics committees, and the study was conducted in accordance with the Declaration of Helsinki. All patients received information regarding the purpose and conduct of this study and provided written informed consent.

Study design

Eligible patients started treatment at a dose level of 37.5 mg sunitinib once daily continuously. At day 15 of sunitinib treatment, TTLs of sunitinib plus N-desethylsunitinib were measured. If the TTL was <50 ng/mL and the patient did not experience any grade ≥ 3 toxicity (CTCAE 4.02), the daily sunitinib dose was increased by 12.5 mg at day 22. At day 29, seven days after the first dose adjustment, the second TTL was measured. If indicated, a second dose adjustment based on TTL and/or toxicity was performed at day 36, as described before. After 8 weeks a final TTL evaluation was performed. No further dose increments were allowed.

If the patient suffered from grade ≥ 3 toxicity or intolerable grade 2 toxicity despite supportive care at any moment during the study, the sunitinib treatment was interrupted until adequate recovery (CTC grade < 2) was achieved. Subsequently, sunitinib treatment was resumed at the next lowest dose level. Sunitinib dose levels allowed within this study were 12.5, 25, 37.5, 50 and 62.5 mg QD. Patients experiencing grade > 2 toxicity with sunitinib 12.5 mg once daily, discontinued the treatment and went off study. No dose escalations were allowed after a previous dose reduction for toxicity. Treatment was continued until progressive disease, until patient refusal or until adverse events which required discontinuation of therapy were observed.

Pharmacokinetic analyses

Samples for pharmacokinetics (PK) were collected at day 15 ± 1 day, day 29 ± 1 day and after 8 weeks (day 57 ± 1 day) of sunitinib treatment. EDTA blood samples were collected and, thereafter, directly sent to the laboratory by ordinary mail at ambient temperature. After receipt of the samples, within 36 h after blood collection, plasma was harvested and stored at -20°C until analysis.

Trough levels of sunitinib and N-desethylsunitinib in plasma were measured by LC-MS/MS as described before.[13] TTLs were determined by calculating the sum of sunitinib and N-desethylsunitinib plasma levels and were reported to the treating physician within one week after blood collection. Patients were evaluable for pharmacokinetic analyses if they had undergone all three PK blood samplings.

Safety assessments

Adverse events (AE), serious adverse events (SAE) and their relationship with study medication were assessed throughout the study. The incidence and severity of AEs were evaluated and graded using the National Cancer Institute Common Toxicity Criteria for Adverse Events version 4.02 (CTCAE 4.02). Patients who received at least one dose of the study treatment were included in the safety evaluation.

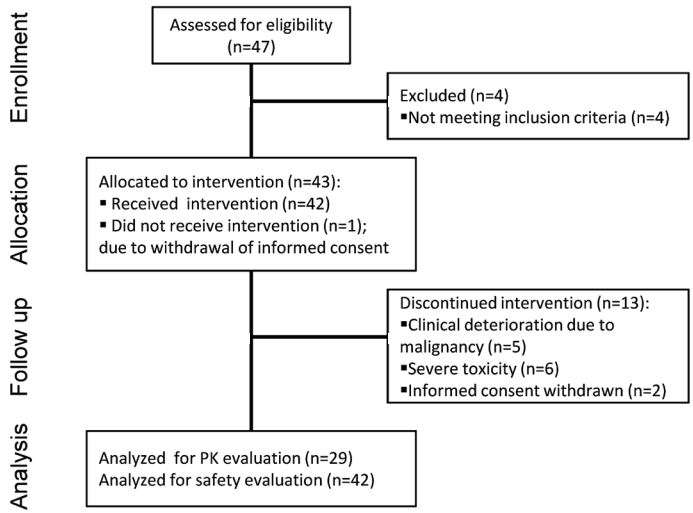

Figure 1 Patient flow diagram

Statistical analysis

The number of patients recruited was based on the number estimated to be required to evaluate at least 8 patients for toxicity after PK guided dose escalation. It was expected that 45% of patients would experience clinically relevant toxicity at the starting dose of 37.5 mg once daily.[14-18] In addition, it was expected that about 50% of the patients without toxicity (55%) would have TTL ≥ 50 ng/mL.[15, 16] In both occasions, patients were not eligible for dose escalation. Thus, to be able to evaluate at least 8 patients after dose escalation, it was necessary to include about four times as many patients (at least 30 patients).

Descriptive statistics were used to summarize the patient characteristics, toxicity data, response data and sunitinib TTLs.

Table 1 Baseline characteristics of all evaluable patients

Characteristic	N (percentage)
Gender	
Male	28 (67)
Female	14 (33)
Age (y)	61 (28 – 74)
Bodyweight (kg)	77 (44 – 108)
Ethnicity	
Caucasian	42 (100)
ECOG Performance status	
0	10 (24)
1	32 (76)
Primary tumour	
Neuroendocrine carcinoma	8 (19)
Colorectal carcinoma	8 (19)
Renal cell carcinoma	6 (14)
Adenocarcinoma of unknown primary (ACUP)	3 (7)
Uveal melanoma	3 (7)
Miscellaneous*	14 (33)
Clinical stage, pre-treatment	
Locally advanced	2 (5)
Metastatic	40 (95)
Prior treatment	
TKI therapy	5 (36)
Chemotherapy	31 (74)
1 regimen	12 (29)
2 regimens	3 (7)
≥3 regimens	17 (40)
Surgery	28 (67)
Radiotherapy	16 (38)

*Miscellaneous: pancreatic carcinoma (n=2), hepatocellular carcinoma (n=2), oesophageal carcinoma (n=2), prostate carcinoma, cervix carcinoma, head and neck carcinoma, mesothelioma, liposarcoma, ewing sarcoma, myo-epithelioma, osteosarcoma.

RESULTS

Patient population

From April 2011 until June 2012, 43 patients with a variety of advanced solid tumors were enrolled (18 patients at the Netherlands Cancer Institute - Antoni van Leeuwenhoek Amsterdam, 15 patients at the University Medical Center Utrecht and 10 patients at the Erasmus MC Cancer Institute Rotterdam). Forty-two patients received at least one dose of sunitinib and were evaluable for toxicity assessments. Twenty-nine patients completed all three pharmacokinetic blood samples and were therefore evaluable for pharmacokinetic assessments (see Figure 1 for the CONSORT diagram). In total, 14 out of 29 patients were evaluable for toxicity assessment after PK-guided dose escalation. At the time of the database lock (August 2012), four patients (9.5%) were still on sunitinib therapy. Demographical and clinical characteristics for all patients are provided in Table 1.

Target trough levels

After 14 days of sunitinib treatment, the median TTL was 49.5 ng/mL [IQR 41.8 – 64.0] (see Table 2). Considerable inter-patient variability of TTLs was observed at the starting dose with a coefficient of variation (CV%) of 32.1%. Moreover, 15 out of 29 patients (52%) did not reach the target TTL of 50 ng/mL at the starting dose of 37.5 mg per day. Therefore, dose escalations to 50 mg per day were indicated in 15 patients. However, in one patient it was not possible to increase the sunitinib dose due to hematological toxicity, and therefore dose escalation was performed in 14 patients. At the second PK evaluation (day 29), the median TTL was increased to 57.6 ng/mL [IQR 48.3 – 61.9] with an inter-patient variability of 35.2% for the entire population of 29 patients. The 14 patients that had undergone dose escalation after day 15 PK measurement (median TTL 42.0 ng/mL [IQR 36.3-47.3]) reached median TTL of 51.3 ng/mL [IQR 44.7-58.7] at day 29. In 2 patients dose escalation at day 15 resulted in reduced TTL at day 29. Moreover, 19 out of 29 patients (66%) reached the target TTL. Of the 10 patients below the target level, the sunitinib dose was increased to 62.5 mg per day in 3 patients and dose escalations were not possible due to toxicity in 7 patients. At the final PK evaluation (day 57), the median TTL was reduced to 51.8 ng/mL [40.3 – 63.7] with an inter-patient variability of 45.0% and 15 patients (52%) reached the target TTL. Six patients in group 2 showed a decrease in TTL throughout the study without dose adjustments. In 2 cases this resulted in TTL below the target TTL at day 57. In Figure 2, the measured TTLs of individual patients at day 15, day 29 and day 57 are presented.

Table 2 Therapy outcomes regarding reached total trough level (TTL), dose and target TTL, stratified by patient group

Outcome	**Group 1a**	**Group 1b**	**Group 2a**	**Group 2b**	**Total**
	TTL<50	TTL<50	TTL≥50	TTL≥50	
	No severe TOX	Severe TOX	No severe TOX	Severe TOX	
	(n=5)	(n=10)	(n=8)	(n=6)	(n=29)
TTL (ng/mL)					
(median [IQR])					
day 15	44.0 [41.8-48.8]	39.1 [30.1-43.0]	65.5 [56.8-67.9]	62.2 [56.0-64.7]	49.5 [41.8-64.0]
day 29	51.8 [45.6-61.5]	50.2 [43.3-56.3]	61.4 [58.7-79.3]	53.1 [40.1-65.8]	57.6 [48.3-61.9]
day 57	63.9 [56.2-78.3]	39.6 [31.1-48.2]	61.9 [55.0-69.6]	56.4 [37.5-53.6]	51.8 [40.3-63.7]
Dose (mg)					
(mean (SD))					
day 29	50.0 (-)	47.5 (7.9)	37.5 (-)	33.3 (6.5)	42.7 (7.8)
day 57	55.0 (6.8)	35.0 (5.3)	37.5 (-)	25.0 (-)	37.9 (9.7)
Pts on target TTL (number (%)					
day 15	0 (0)	0 (0)	8 (100)	6 (100)	14 (48)
day 29	3 (60)	5 (50)	8 (100)	3 (50)	19 (66)
day 57	5 (100)	1 (10)	6 (75)	3 (50)	15 (52)

IQR, interquartile range; SD, standard deviation; TTL, total trough level, TOX, toxicity.

Patients were stratified by TTL measured at day 15 and toxicity within 8 weeks of treatment. Patients with TTL<50 ng/mL at day 15 are in group 1. Patients with TTL≥50 ng/mL are in group 2. In addition, subgroups are formed by patients without severe toxicity within 8 weeks of treatment (group 1a and 2a) and patients suffering from severe toxicity within 8 weeks of treatment (group 1b and 2b).

PK guided dosing

Based on TTL reached at day 15, two patient groups were distinguished: Group 1 consisted of patients who did not reach target TTL and Group 2 of patients who reached target TTL. Based on toxicity in the first 8 weeks of treatment, these groups could be subdivided further into four patient subgroups with different results of the PK guided dosing strategy. The defined groups were as follows: Group 1a patients with TTL <50 ng/mL at day 15 and no relevant toxicity (n = 5; 17%), Group 1b patients with TTL < 50 ng/mL at day 15 with relevant toxicity (n = 10; 34%), Group 2a patients with TTL > 50 ng/mL at day 15 and no relevant toxicity (n = 8; 28%), Group 2b patients with TTL > 50 ng/mL at day 15 with relevant toxicity (n = 6; 21%). As shown in Table 2, the 5 patients (17%) who did not reach target TTL at day 15 and had PK-guided dose elevations without relevant toxicity, tolerated treatment with 47% higher mean daily dose compared to standard therapy. After 8 weeks of treatment the distribution of daily sunitinib doses in the study population was as follows: 2 patients using 62.5 mg, 4 patients using 50 mg, 15 patients using 37.5 mg, and 8 patients using 25 mg. Presumably without PK-guided dosing the result would have been 21 patients on 37.5 mg, 8 on 25 mg, and therefore a possible under-dosing in 6 of 29 (21%) of patients.

In Figure 3, an overview of all dose adjustments and TTLs is shown per individual patient within the 8 week study period.

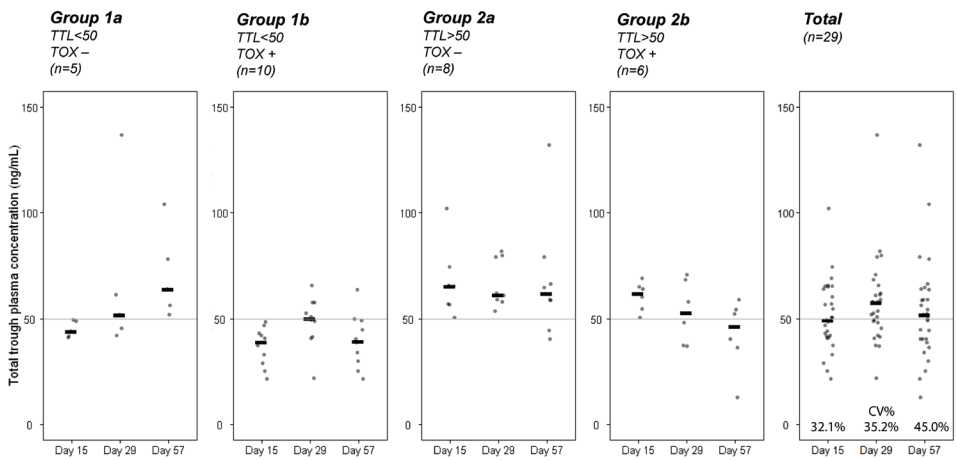

Figure 2 TTLs measured at day 15, day 29 and day 57 of sunitinib treatment of all patients who were evaluable for PK evaluation, stratified by patient group. The black bars represent the median TTL.

Treatment toxicity

The most frequently occurring treatment related adverse events are listed in Table 3. Grade ≥3 adverse events were observed in 29 patients (69%). The main grade ≥ 3 adverse events attributed to study treatment included hypertension (14%), fatigue (12%), anemia (12%), thrombocytopenia (12%) and hand-foot syndrome (HFS) (10%). Common grade 1 or 2 non-hematologic treatment-related toxicities were fatigue (60%), nausea (50%), dysgeusia (55%), oral mucositis (52%), diarrhea (40%), HFS (33%) and vomiting (29%).

Six patients discontinued sunitinib treatment (at the standard dose of 37.5 mg per day) due to adverse events before the final PK-evaluation at day 57; five of these discontinuations were considered treatment related and included fatal cardiac failure (n = 1, grade 5), fatigue, increased blood bilirubin, nausea (all n = 1, grade 3) and fatigue (n = 1, grade 2). Dose reductions of sunitinib due to treatment related adverse events during the PK evaluation period were performed in sixteen patients (Group 1b + 2b). Moreover, nine of these patients (56%, group 1b) who had an initial TTL guided dose increase did not tolerate this higher dose level, as shown in Figure 3. In addition, five patients discontinued sunitinib treatment due to toxicity after the PK evaluation period; two of these discontinuations were considered treatment related and included a combination of anemia and thrombocytopenia (n = 2, grade 3).

The main purpose of the study was to assess whether PK-guided dosing could be performed without causing additional toxicities. Therefore, the occurrence of toxicities in the patients who required dose escalations (Group 1) was compared with patients who did not need dose interventions based on PK and remained at the standard dose (Group 2). In all patient groups, the frequency of grade ≤ 2 toxicity was similar. TTLs above the target level at day 15 of therapy did not correlate to frequency of severe toxicity (grade ≥ 3). In Group 1, 10 out of 15 patients (67%) experienced severe toxicities and in Group 2 with TTL > 50 ng/mL, 6 out of 14 patients (43%) experienced severe toxicity.

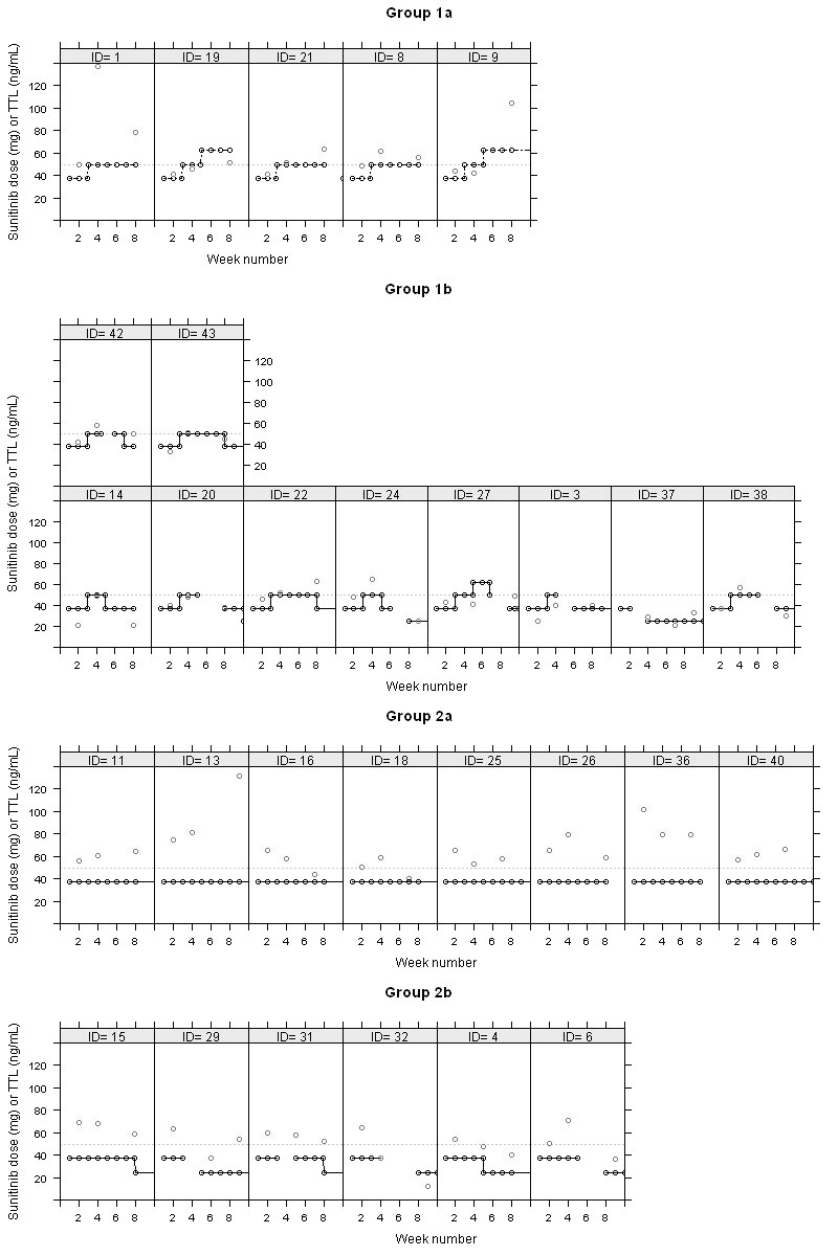

Figure 3 Course of sunitinib dose levels and TTLs during the first 8 weeks of treatment of all individual patients who were evaluable for PK evaluation, stratified by patient group. On the X-axis is the week number, on the Y-axis both sunitinib dose level and TTLs are presented. Grey dots represent TTLs. Black squares represent dose levels. The dotted line represents the target TTL (50 ng/mL).

Group 1a: patients with TTL<50 ng/mL at day 15 and without toxicity; Group 1b: patients with TTL<50 ng/mL at day 15 and with toxicity; Group 2a: patients with TTL>50 ng/mL at day 15 and without toxicity; Group 2b: patients with TTL> 50 ng/mL at day 15 and with toxicity.

DISCUSSION

In this pilot study, the safety and feasibility of PK guided sunitinib dosing was investigated. At the standard dose of 37.5 mg, 52% of patients did not reach target TTLs of sunitinib after 14 days of sunitinib treatment. Ultimately, 5 out of 29 patients (17%) had successful dose escalations with final doses of 50 mg (n = 3) and 62.5 mg (n = 2), leading to TTLs above the target without causing additional toxicities. This implies that PK guided sunitinib dose escalations rather than fixed doses can contribute to optimization of therapy in a part of the patients.

Similar to classical anticancer chemotherapy regimens, it is often reasoned that increasing the dose of an anticancer drug in patients who lack toxicity might increase the likelihood of treatment efficacy.[19-22] Fixed dosing may lead to under-dosing due to inadequate drug exposure in some patients. Dosing to toxicity might lead to overdosing and unnecessary side effects since in some patients adequate drug exposure will already be accomplished with a lower dose. This is complex since drug exposure, toxicity and efficacy generally do not show a linear relationship and it is not known whether toxicity is accompanied by adequate exposure. Therefore, therapeutic drug monitoring for the individualization of dosing of anticancer drugs with an considerable and unpredictable inter-patient variability in pharmacokinetics is gaining popularity. For example, PK-guided dosing has been mentioned for docetaxel leading to a decrease in the inter-patient variability of drug exposure.[23] However, to our knowledge, the current study is the first study in which PK guided dosing is applied to sunitinib treatment.

The sunitinib starting dose of 37.5 mg (continuously once-daily) was based on previously reported studies investigating a continuous dosing strategy for sunitinib.[14-16] Since no safety data were available regarding long term continuously daily dosing of high doses sunitinib, the highest dose level was maximized to 62.5 mg per day.[14-16, 24]

A limitation of this study is that target TTL have not been established in clinical studies, thus far. The association between sunitinib exposure and efficacy was based on the steady state area under the concentration-time curve (AUC).[4] Since AUC and trough level increase both proportionally with dose, these parameters should correlate with each other.[7, 25] Hence, the target plasma levels used in this study were deduced from preclinical studies.[5, 6, 8, 9] Furthermore, our study was performed in a small cohort of patients with a large variety of advanced solid tumors and without a control group. Therefore, it was not possible to investigate the relationship between plasma exposure and treatment efficacy.

In the previously reported studies, mean TTLs were approximately 40-65 ng/mL and inter-patient variability was high with a coefficient of variation (CV) of more than 30%.[14-16, 24] Our patient cohort showed comparable results after 14 days of treatment (before any dose change) with median TTL of 49.5 ng/mL and an inter-patient variability of 31.2%. When assuming that target TTLs are needed

for adequate treatment responses, this means that more than half of the patients were at sub-target levels (< 50 ng/mL) at the standard dose.

Based on the elimination half-life of sunitinib (±40h) and N-desethylsunitinib (±80h) it was expected that steady state concentrations would be reached within 14 days of treatment and that parent and metabolite accumulate 3-4 fold and 7-10 fold, respectively.[25] However, no significant drug accumulation was observed after three weeks of continuous sunitinib treatment in previous studies.[15, 26] Additionally, in case of a dose escalation from 37.5 mg to 50 mg (33% increment) more than 90% of the new steady state level would already be reached after two half-lives since the new steady state is close to the former level. Therefore, TTL measurement seven days after a dose escalation was expected to be adequate. However, in some patients TTLs still tended to increase after two weeks of sunitinib treatment even when the dose remained equal. A longer period before collection of the first TTL sample was considered, but this would postpone potential beneficial dose increments. In addition, it was observed that TTLs in 2 out of 8 patients decreased to below the target TTL without a dose reduction after 8 weeks of treatment. It is not known whether this is due to unexplained intra-patient variability, patient non-adherence (despite using diaries and pill counts) or whether sunitinib levels tend to decrease after long term treatment as was shown for imatinib[27] and sorafenib.[28, 29] Hence, further insights in TTLs and inter-patient pharmacokinetic variability during sunitinib treatment are warranted to allow rational design of future PK-guided dosing studies.[30]

The total occurrence of toxicity grade 3 or higher observed in this study was consistent with previously reported studies on continuous daily dosing regimens of sunitinib.[14-16, 24] Due to the small patient number this study was not powered to compare the occurrence of toxicities within the different patient subgroups.

Of all 16 patients who required dose reductions due to toxicity (Group 1b + 2b), 7 patients suffered from toxicities at the standard dose and would also have experienced these toxicities if they were treated without the PK-guided dosing strategy. The same goes for 1 patient in the study who died due to a probably treatment related adverse event (diffuse cardiac ischemia followed by cardiac failure) at the starting dose of 37.5 mg per day. However, as could be expected, patients did experience additional toxicities after PK-guided dose escalations (9 patients). These toxicities were manageable by dose reductions.

As suggested by previous data, it was expected that toxicity was correlated to sunitinib exposure[4] However, results indicated that the frequency of severe toxicities (grade ≥ 3) was not correlated to TTL at day 15, as toxicity levels at that time-point were comparable in the patients with TTL <50 ng/mL (Group 1) and TTL >50 ng/mL (Group 2). Probably toxicity is also correlated to cumulative sunitinib exposure instead of the initial steady state TTL, as was also reported for absolute neutrophil counts by Houk et al.[4]

In addition, the daily sunitinib doses after 8 weeks of treatment in our patient cohort also give insights in a possible added value of a PK-guided dosing strategy. Six patients (21%) were treated with sunitinib doses above the standard dose of 37.5 mg per day. These patients would probably be under-dosed in

a fixed dosing regimen.

There are several benefits of a PK guided dosing approach for sunitinib. First, one could hypothesize, based on our results and previous work by other groups, that if an effective TTL cannot be reached at a dose level with tolerable toxicity in these patients, TTL assessments could substantiate the choice to switch to another more effective therapy. Second, when assuming that TTLs > 50 ng/mL are needed for adequate treatment responses, 17% of patients (the 5 patients in Group 1a (TTL < 50 ng/mL and no toxicity)) benefit from PK-guided dose increases without additional toxicity. Third, compared to a toxicity based approach, additional toxicities might be avoided since adequate drug exposure is reached before toxicity occurs. However, efficacy of both approaches have not been studied or compared in great detail so far.

In the current oncology field we are more and more searching for personalized treatments options for a sometimes small number of patients. With little effort we can sometimes optimize the treatment options we already have. Individualizing dosing of sunitinib based on a simple measurement of plasma concentration is worthwhile to investigate further in order to utilize the scarce treatment options available for many tumor types in a most optimal way. Further research is therefore required to investigate the safety and therapeutic efficacy of PK guided dosing of sunitinib in order to reach a systemic exposure above the target TTL compared with that from a standard fixed dose.

Table 3 Toxicity data

Adverse event	Grades ≤ 2												Grade ≥ 3												Any grade	
	Discon-tinued		Group1				Group 2				Total		Discon-tinued		Group 1				Group 2				Total		Total	
	(n=13)		Group 1a (n=5)		Group 1b (n=10)		Group 2a n=(8)		Group 2b n=(6)		(n=42)		(n=13)		Group 1a (n=5)		Group 1b (n=10)		Group 2a (n=8)		Group 2b (n=6)		(n=42)		(n=42)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Treatment-related non-heamatological adverse events (ocurring in ≥10% of patients)																										
fatigue	7	54	4	80	6	60	5	63	3	50	25	60	2	15	0	0	2	20	0	0	1	17	5	12	30	71
nausea	6	46	3	60	4	40	4	50	4	67	21	50	2	15	0	0	0	0	1	13	0	0	3	7	24	57
dysgeusia	6	46	4	80	5	50	4	50	4	67	23	55	0	0	0	0	0	0	0	0	0	0	0	0	23	55
oral mucositis	3	23	2	40	8	80	5	63	4	67	22	52	0	0	0	0	0	0	0	0	1	17	1	2	23	55
diarrhea	3	23	3	60	3	30	4	50	4	67	17	40	0	0	1	20	1	10	1	13	0	0	3	7	20	48
hand-foot syndrome	2	15	3	60	5	50	2	25	2	33	14	33	0	0	0	0	3	30	1	13	0	0	4	10	18	43
vomiting	4	31	5	100	2	20	1	13	0	0	12	29	0	0	0	0	0	0	1	13	0	0	1	2	13	31
hypertension	1	8	1	20	0	0	3	38	0	0	5	12	2	15	2	40	0	0	1	13	1	17	6	14	11	26
anorexia	2	15	0	0	2	20	0	0	2	33	6	14	1	8	0	0	0	0	0	0	0	0	1	2	7	17
dry skin	2	15	2	40	2	20	0	0	2	33	8	19	0	0	0	0	0	0	0	0	0	0	0	0	8	19
rash	0	0	0	0	3	30	4	50	1	17	8	19	0	0	0	0	0	0	0	0	0	0	0	0	8	19
constipation	1	8	1	20	3	30	1	13	0	0	6	14	1	8	0	0	0	0	0	0	0	0	1	2	7	17
epistaxis	2	15	0	0	2	20	2	25	0	0	6	14	0	0	0	0	0	0	0	0	1	17	1	2	7	17
dyspnea	2	15	0	0	2	20	1	13	0	0	5	12	1	8	0	0	0	0	0	0	0	0	1	2	6	14
skin yellow discoloration	2	15	0	0	1	10	2	25	1	17	6	14	0	0	0	0	0	0	0	0	0	0	0	0	6	14
dyspepsia	0	0	2	40	1	10	1	13	1	17	5	12	0	0	0	0	0	0	0	0	0	0	0	0	5	12
hair depigmentation	0	0	2	40	1	10	2	25	0	0	5	12	0	0	0	0	0	0	0	0	0	0	0	0	5	12
oral pain	1	8	0	0	0	0	4	50	0	0	5	12	0	0	0	0	0	0	0	0	0	0	0	0	5	12

Adverse event	Grades ≤ 2												Grade ≥ 3										Any grade	
	Discon-tinued		Group1				Group 2				Total		Discon-tinued		Group 1				Group 2				Total		Total	
	(n=13)		Group 1a (n=5)		Group 1b (n=10)		Group 2a n=(8)		Group 2b n=(6)		(n=42)		(n=13)		Group 1a (n=5)		Group 1b (n=10)		Group 2a (n=8)		Group 2b (n=6)		(n=42)		(n=42)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
periorbital edema	2	15	0	0	2	20	0	0	1	17	5	12	0	0	0	0	0	0	0	0	0	0	0	0	5	12
periph. neuropathy	1	8	0	0	2	20	1	13	1	17	5	12	0	0	0	0	0	0	0	0	0	0	0	0	5	12
Laboratory abnormalities																										
Haematology																										
anemia	1	8	0	0	0	0	0	0	0	0	1	2	1	8	0	0	1	10	1	13	2	33	5	12	6	14
white blood cells	0	0	0	0	0	0	1	13	1	17	2	2	0	0	0	0	1	10	0	0	1	17	2	5	4	10
neutrophils	0	0	1	20	2	20	0	0	3	50	6	14	1	8	1	20	2	20	0	0	1	17	5	12	11	26
platelets	1	8	1	20	0	0	2	25	0	0	4	10	0	0	0	0	1	10	0	0	2	33	3	7	7	17
Clinical chemistry																										
ALAT	0	0	0	0	1	10	2	25	0	0	3	7	0	0	1	20	0	0	1	13	0	0	2	5	5	12
ASAT	0	0	0	0	0	0	1	13	0	0	1	2	0	0	1	20	1	10	1	13	0	0	3	7	4	10
creatinine increased	0	0	0	0	1	10	1	13	1	17	3	7	1	8	0	0	0	0	0	0	0	0	1	2	4	10
Cardial events																										
heart failure	0	0	0	0	0	0	0	0	0	0	0	0	1	8	1	20	0	0	0	0	0	0	2	5	2	5
acute coronairy syndrome	0	0	0	0	0	0	0	0	0	0	0	0	1	8	0	0	0	0	0	0	0	0	1	2	1	2
prolonged QT interval	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	13	0	0	1	2	1	2
myocardial infarction	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	13	0	0	1	2	1	2
Any toxicity	13	100	5	100	10	100	8	100	6	100		100	7	54	3	60	9	90	5	63	5	83	29	69		100

ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase. Group 1a: patients with TTL<50 ng/mL at day 15 and without toxicity; Group 1b: patients with TTL<50 ng/mL at day 15 and with toxicity; Group 2a: patients with TTL>50 ng/mL at day 15 and without toxicity; Group 2b: patients with TTL> 50 ng/mL at day 15 and with toxicity.

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Chapter 4

Relationship between Sunitinib Pharmacokinetics and Administration Time: Preclinical and Clinical Evidence

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ABSTRACT

Background and Objective Circadian rhythms may influence the pharmacokinetics of drugs. This study aimed to elucidate whether the pharmacokinetics of the orally administered drug sunitinib are subject to circadian variation.

Methods We performed studies in male FVB-mice aged 8-12 weeks, treated with single-dose sunitinib at 6 dosing times. Plasma and tissue samples were obtained for pharmacokinetic analysis and to monitor mRNA expression of metabolizing enzymes and drug transporters. A prospective randomized cross-over study was performed in which patients took sunitinib once daily at 3 courses at 8AM, 1PM and 6PM. Patients were blindly randomized in two groups, which determined the sequence of sunitinib dosing time. Primary endpoint in both studies was the difference in plasma area-under-the-concentration-time-curve (AUC) of sunitinib and its active metabolite SU12662 between dosing times.

Results Sunitinib and SU12662 plasma AUC in mice followed a ~12h-rhythm as a function of administration time ($p \leq 0.04$). The combined $AUC_{0\text{-}10h}$ was 14-27% higher when sunitinib was administered at 4AM and 4PM, than at 8AM and 8PM. Twenty-four hr-rhythms were seen in the mRNA levels of drug transporters and metabolizing enzymes. In 12 patients, sunitinib trough concentrations were higher when the drug was taken at 1PM or 6PM, than when taken at 8AM ($C_{trough1PM}$ 66.0ng/mL; $C_{trough\text{-}6PM}$ 58.9ng/mL; $C_{trough\text{-}8AM}$ 50.7ng/mL, $p=0.006$). The AUC was not significantly different between dosing times.

Conclusions This indicates that sunitinib pharmacokinetics follow a ~12h-rhythm in mice. In humans, morning dosing resulted in lower trough concentrations, probably resulting from differences in elimination. This can have implications for therapeutic drug monitoring.

KEYPOINTS

Sunitinib is known for its narrow therapeutic window and wide inter-patient variability in drug exposure, which in part may be explained by a within-patient variability in drug exposure, possibly due to daily variations in absorption, distribution, metabolism and excretion of sunitinib.

In this study, we showed that the area under the concentration-time curve of sunitinib and its active metabolite SU12662 follows a 12-hour rhythm in mice. Patients had lower trough concentrations of both sunitinib and SU12662 when the drug was administered in the morning, rather than intake at later moments of the day.

This is particularly interesting, since therapeutic drug monitoring (TDM) is currently being suggested as a step forward in the individualization of sunitinib treatment. In TDM, drug dose may be increased or decreased based on trough concentrations to aim for better survival. Therefore, it is crucial to take the administration time into account to prevent erroneous dose escalations or reductions.

INTRODUCTION

Sunitinib (Sutent®; Pfizer Pharmaceutical Group, New York, NY, U.S.A.) is a multi-targeted tyrosine kinase inhibitor that is registered for the treatment of advanced clear cell renal cell carcinoma (RCC), imatinib-resistant or intolerant gastrointestinal stromal tumors (GIST), and pancreatic neuro-endocrine tumors (pNET).[1-5] There is a broad inter-patient variability in plasma concentrations of sunitinib, which may be due to patient non-compliance, drug-drug interactions (CYP3A4-modulating drugs) and inter-patient variability in pharmacokinetics.[6] Besides inter-patient variability in drug exposure, there also may exist variability within a patient due to intra-patient variations in pharmacokinetic processes.

After oral intake, sunitinib is absorbed from the gastrointestinal tract, resulting in peak plasma concentrations 6-12 hours after administration.[1] Food has no significant effect on the exposure to sunitinib.[1, 7] In the liver, sunitinib is mainly metabolized by the cytochrome P450 enzyme 3A4 (CYP3A4) to its primary active metabolite SU12662, which is further converted by CYP3A4 into several inactive compounds. Both sunitinib and SU12662 are eliminated from the body via the bile in the feces through efflux transporters in the liver, namely the ATP-binding cassette sub-family B member 1 (ABCB1) and sub-family G member 2 (ABCG2).[8]

Enzymes and transporters involved in the pharmacokinetics of drugs, such as CYP3A4 and ABCB1, have time-dependent variations in expression which may have profound effect on the exposure to several drugs.[9, 10] These variations may change the efficacy and/or toxicity profile of drugs. This was previously shown in patients treated with tacrolimus, a drug frequently used to prevent organ rejection after transplantation, where morning administration resulted in higher AUC levels than evening administration.[11]

Two previous studies have compared the toxicity levels of patients treated with sunitinib in the morning with those of patients treated with sunitinib in the evening. No differences in toxicity levels between morning and evening dosing of sunitinib were found in either study. Although the authors

concluded that it is safe to take the drug either in the morning or at night, sunitinib pharmacokinetics were only examined during morning sunitinib administration and these studies were not designed to study the chrono-efficacy and chrono-tolerance of sunitinib exposure.[12, 13]

Several studies have shown an association between higher exposure to sunitinib and improved survival.[14, 15] In order to improve the efficacy of sunitinib treatment, we wanted to gain insight into possible time-dependent changes in sunitinib pharmacokinetics, for which we designed a translational study. The primary aim of this study was to examine whether administration time of sunitinib influences its pharmacokinetics. We studied the effect of administration time on pharmacokinetic parameters of sunitinib by measuring sunitinib and its active metabolite in plasma and organ tissue of mice as well as in plasma of cancer patients treated with monotherapy sunitinib. As a secondary aim, we studied the daily variation in the activity of sunitinib-metabolizing enzymes and transporters in murine hepatocytes and bowel tissue and through the midazolam clearance test in patients, which is a well-known marker of CYP3A activity.[16]

METHODS

Mice

Seven groups of 18 male FVB mice with the age of 8-12 weeks were used in this study. For logistic reasons, mice were housed under normal and reversed 12 hours light/12 hours dark cycles (lights on/off or off/on at 8 AM and 8 PM) for at least one week before start of the experiment. Mice were fed a standard diet *ad libitum*. Three hours before pharmaceutical treatment, mice were fasted until 1 hour after administration. Water was available *ad libitum* throughout the entire experiment. This study was approved by an independent Animal Ethical Committee under protocol number EMC139-12-19.

Study protocol

Sunitinib was dissolved in acidified water with a pH of maximum 6.0, at a concentration of 5 mg/mL. All mice were administered a fixed single dose of 1.06 mg, which was based on a dose of 42.4 mg/kg and mean weight of mice of 25 g. Every four hours, one group consisting of 18 mice was administered a single dose sunitinib through gavage, starting at 8AM.

At six different times after the administration of sunitinib (t = 1, 2, 3, 5, 10, 20 h), three mice with identical sunitinib dosing time were administered isoflurane as anesthetic followed by eye extraction for 1 mL blood withdrawal and euthanasia by cervical dislocation. Directly after euthanasia one liver lobe and the small bowel were removed. Blood from each single mouse was separately processed to plasma. Both plasma and tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

The seventh group of mice served as a control group and was administered solvent. Mice in this group were sacrificed with four hours intervals. Blood and organs of the mice in this group were collected

and stored under the same conditions as those of the treated mice. Quantitative real-time PCR was applied on tissue samples to reveal potential time-dependent changes in the circadian expression of murine orthologues of genes encoding drug transporters and metabolizing enzymes involved in sunitinib pharmacokinetics, using known core clock genes as a control (**see Supplementary material**).

Patients

A prospective randomized cross-over trial was performed in patients treated with sunitinib at the Department of Medical Oncology of the Erasmus MC-Cancer Institute, Rotterdam, the Netherlands. Patients were allowed to participate in the study at any time during sunitinib treatment, with both 4 weeks on/2 weeks off treatment and with continuous dosing regimen. Patients could participate in the study if (i) aged ≥ 18 years, (ii) having histologically or cytologically confirmed diagnosis of advanced renal cell carcinoma, GIST or pancreatic neuro-endocrine tumor for which treatment consisted of monotherapy sunitinib, (iii) using any stable dose of sunitinib at study entry (defined as at least 2 weeks continuous dosing without dose modifications), (iv) hematologic, renal and liver functions were adequate, and (v) written informed consent was given. Exclusion criteria consisted of (i) pregnancy or breastfeeding, (ii) medical unstable condition requiring treatment, (iii) symptomatic central nervous system metastases or history of psychiatric disorder that would prohibit the understanding and giving of informed consent, (iv) major surgery within 2 weeks before start of the protocol, (v) use of CYP3A4 inhibiting or inducing drugs, (vi) problematic blood withdrawal and (vii) a known allergy to sunitinib and/or midazolam.

Study protocol

Patients were randomized to start intake of sunitinib either at 8AM (group A) or 6PM (group B) in the next treatment course after inclusion. In the course thereafter a cross-over was performed to 6PM for group A and 8 AM for group B. Accordingly, the sequence of the time of dosing of sunitinib in group A was morning-evening and in group B evening-morning. During both courses, patients were hospitalized for 24 hours in the third or fourth week of the treatment cycle, when steady state plasma concentrations were achieved.[8] Blood samples for pharmacokinetic measurements were taken at time-points t = 0, 1, 2, 4, 6, 8, 12 and 24 hours after oral sunitinib intake.

At both hospitalization days, patients were subjected to a midazolam clearance test as a marker of hepatic CYP3A activity, 2 hours after administration of sunitinib. Midazolam (2.5 mg) was administered intravenously through slow infusion, and blood withdrawals for determining the pharmacokinetics of midazolam and its metabolite 1'OH-midazolam were taken at time-points t = 0.5, 2 and 6 hours after administration, as previously described by Lee et al.[17]

After amendment of the study protocol, based on the outcome of the animal experiments, a subset of patients was asked to participate in the study for a third treatment course in which sunitinib was taken at 1PM.

This study was approved by the local medical ethical board (MEC2012-138), and registered at the

Dutch trial registry (www.trialregister.nl, number NTR3526). According to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org) all samples for pharmacokinetic assessment on sunitinib and midazolam were coded and anonymized. The analytical methods for sunitinib and midazolam quantification are described in the **Supplementary material**. Parameters for sunitinib and SU12662 pharmacokinetics were combined AUC, trough concentrations and clearance of sunitinib.

Statistics

For a probability of 83% that the study would detect a treatment difference at a two-sided 0.05 significance level, and a 20% true mean difference in AUC and/or clearance between treatment times, 18 patients were required. This is based on the assumption that the within-patient standard deviation of the pharmacokinetic parameters is 0.2. Patients were considered eligible after completing pharmacokinetic blood withdrawal during two treatment courses. To compare parameters of sunitinib pharmacokinetics between morning, afternoon and evening dosing time of sunitinib, the Wilcoxon signed-rank test was used. CircWave Batch v5.0 with cosinor analysis was used for harmonic regression analysis of circadian oscillation using a 24-hour wave in the expression of genes, and a 12-hour wave in the pharmacokinetics of sunitinib, with forward linear harmonic regression using an *F*-test. User defined alpha was chosen at 0.05.

RESULTS

Sunitinib pharmacokinetics in mice

A total of 108 mice were treated with a single dose of sunitinib at six different time points over 24 hour. Cosinor analysis of the concentration-time curves from time zero to 10 hours ($AUC_{(0\text{-}10)}$) plot showed a clear 12-hour rhythm in the exposure to sunitinib and SU12662 ($p = 0.0342$, and $p = 0.0027$ respectively; **Figure 1A**) and the combined exposure ($p = 0.0174$) as a function of administration time. The combined AUC of sunitinib and SU12662 was 14-27% higher when the drug was administered at 4AM and 4PM, rather than at 8AM and 8PM.

Figure 1B-E shows the areas under the concentration-time curves of sunitinib and SU12662 for liver and 3 consecutive sections of the intestine, corresponding with duodenum, jejunum and ileum, respectively. As is shown, oscillations also occur in the accumulation of sunitinib and its metabolite in these tissues. However, the 12-hour rhythm was only statistically significant for SU12662 accumulation in the duodenum ($p = 0.0179$, **Figure 1C**) but not for sunitinib or SU12662 accumulation in other tissues. Sunitinib and SU12662 concentrations in both plasma and tissue samples showed a broad inter-mouse variability.

Quantitative real-time PCR on liver tissue samples taken from mice in various stages of their circadian rhythm revealed daily fluctuations with a 24 hour period in the activity of the clock genes *Bmal1*,

Cryptochrome 1 (*Cry1*), *Dbp*, *Period* 2 (*Per2*), and *Rev-erbα*. This was confirmed by the cosinor analysis ($p \leq 0.0214$), showing that the mice in normal and reversed light-dark cycle were properly entrained at the time of the experiment (**Supplementary Figure 1**).

In the liver, cosinor analysis showed circadian fluctuations in the mRNA levels of *Abcb1a*, *Abcb1b*, and *Cyp3a11* ($p \leq 0.047$), but not in the expression of *Abcg2* ($p = 0.254$, **Supplementary Figure 2A**). In duodenum and jejunum, the expression of *Abcb1a*, *Abcg2* an *Cyp3a11* followed a circadian rhythm ($p \leq 0.0162$, **Supplementary Figure 2B and 2C**). In the ileum, only a circadian variation in *Abcb1a* expression was seen ($p < 0.001$, **Supplementary Figure 2D**). This implicates circadian expression of the genes involved in the uptake and metabolism of sunitinib as a cause for daily variations in sunitinib pharmacokinetics. The peak activities, as judged by their mRNA expression levels for these genes were all at different times of the day.

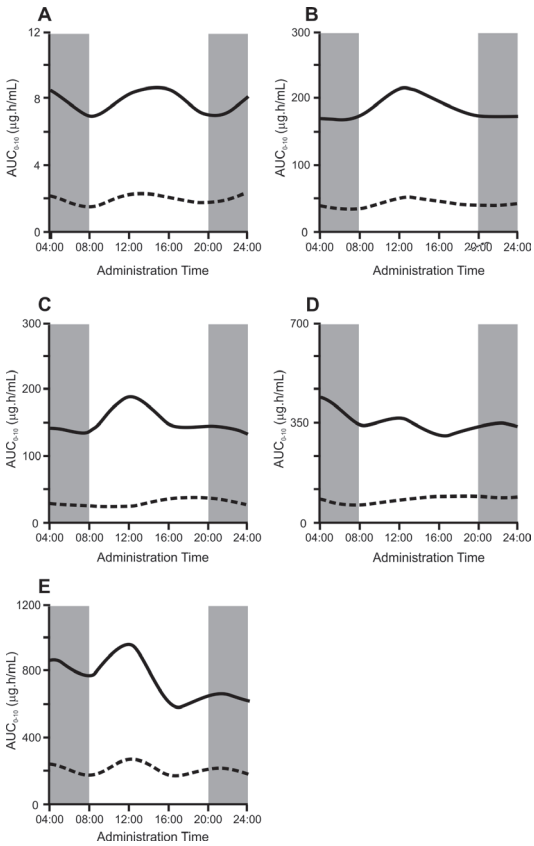

Figure 1

Daily variations in area under the concentration-time curve (AUC) for sunitinib (black) and SU12662 (dotted line) in plasma (A), liver (B), duodenum (C), jejunum (D) and ileum (E) of FVB mice treated with a single dose sunitinib. The X-axis indicates the time of sunitinib administration, the Y-axis represents the mean AUC corresponding with the 6 consecutive administration times. Gray areas indicate it was dark in the cages of the mice, white areas indicate it was light.

Patient demographics

Twenty-seven patients were included in this study, of whom 16 patients completed at least 2 courses with pharmacokinetic blood withdrawals (group A, n=7; group B, n=9). One patient was excluded from the study due to inability to withdraw blood, and one patient retracted informed consent before the end of the study protocol. Nine patients stopped sunitinib treatment during the study due to toxicity or progressive disease. A subgroup of twelve patients had pharmacokinetic measurements during 3 courses using sunitinib at three different times of the day each course. All patients used sunitinib as first line anti-cancer treatment.

Group A and group B were comparable in sex, age, number of courses within the study protocol and amount of dose reductions before and within the study protocol. From the 16 patients who completed at least 2 courses with pharmacokinetic blood withdrawals, three patients underwent dose reductions in between the study courses. Six patients had already undergone dose-reductions before the start of the study protocol. There were no dose escalations prior to, or during the study protocol in any of the patients. Demographic characteristics are presented in **Table 1**.

Table 1 Descriptives of 27 individuals who participated in the clinical study

Characteristic	**Value**	
Cohort	**A**	**B**
No of patients	13 (48.1)	14 (51.9)
No of courses within study		
0-1	6 (46.2)	5 (35.7)
2	7 (53.8)	9 (64.3)
3	5 (38.5)	7 (50.0)
Sex		
Male	10 (76.9)	12 (85.7)
Female	3 (23.1)	2 (14.3)
Age (years)	63.1 (10.9)	61.3 (7.4)
WHO-PS		
0-1	13 (100)	13 (92.9)
2	0 (0)	1 (7.1)
Tumour type		
RCC	13 (100)	12 (85.7)
p-NET	0 (0)	2 (14.3)
Dose-reductions during study	2 (22.2)	1 (11.1)
Duration of sunitinib treatment (months)	16.7 (26.8)	19.3 (22.2)

Abbreviations: no, number of patients; WHO-PS, World Health Organization Performance Score; RCC, renal cell carcinoma; p-NET, pancreatic neuro-endocrine tumor.

Values are expressed as n (%), except for age and duration of treatment, which are mean (SD)

Sunitinib pharmacokinetic parameters

Mean AUC_{τ} (AUC from time zero to end of dosing period) in 16 patients was 1599 ± 592 ng x h/mL, when sunitinib was administered at 8AM (AUC_{8AM}) and 1444 ± 536 ng x h/mL when sunitinib was administered at 6PM (AUC_{6PM}). In the 12 patients who underwent pharmacokinetic blood sampling during three courses, no difference was seen in AUC_{1PM} when compared to dosing times 8AM (p = 0.21) or 6PM (p = 0.24), as is shown in **Figure 2**. However, a relevant difference in combined trough concentrations was seen between morning and evening dosing (n=16, $C_{trough\text{-}8AM}$ 50.7 ± 17.6 ng/mL,

$C_{trough\text{-}6PM}$ 58.9 ± 21.6 ng/mL; p = 0.006). This was also seen when comparing morning dosing and dosing at noon (n = 12, $C_{trough\text{-}8AM}$ 56.0 ± 19.6 ng/mL; $C_{trough1PM}$ 66.0 ± 25.2 ng/mL; p = 0.003). The clearance of sunitinib was not significantly different between the three dosing times (CL_{8AM} 42.0 ± 14.7, CL_{1PM} 40.2 ± 13.5, CL_{6PM} 39.8 ± 9.8, p ≥ 0.5). Patients in group A had slightly higher AUC_{8AM} than patients in group B. For all other pharmacokinetic parameters, there were no significant differences between the both treatment groups (**Table 2**).

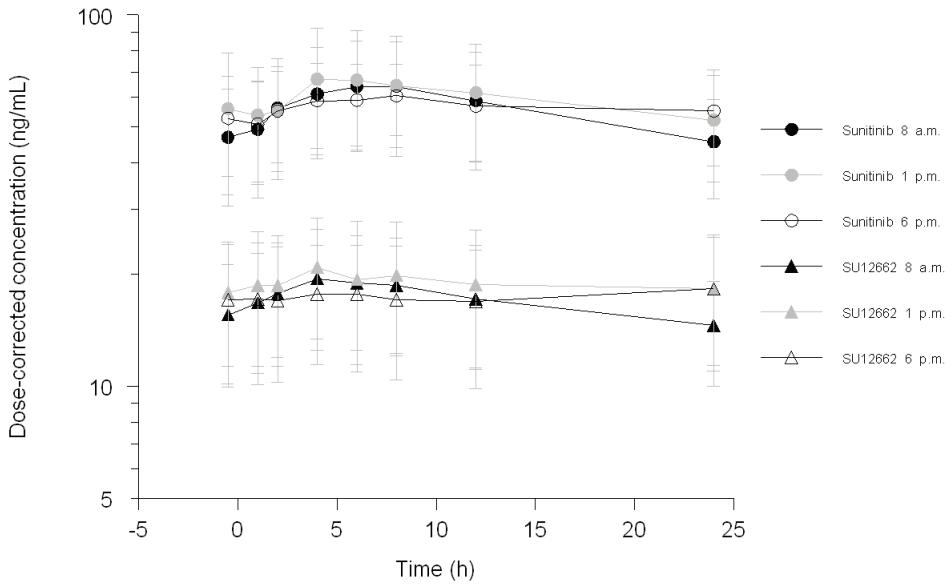

Figure 2 24-hour concentration curves of sunitinib and SU12662 for patients treated with sunitinib at 8AM, 1PM and 6PM.

Midazolam clearance test

Three out of 16 patients did not undergo midazolam clearance tests at both hospitalization days, as a patient's request. In the remaining 13 patients there was no significant difference in the ratio between midazolam and 1'OH-midazolam between the administration times 10AM and 8PM (mean difference in 1'OH-midazolam/midazolam ratio 0.0184, p = 0.256). Group A was not different from group B in outcome of the 1'OH-midazolam/midazolam ratio in the morning nor in the evening (**Table 2**).

Table 2 Pharmacokinetic parameters in group A and group B

Pharmacokinetic measurement	Group A Mean (sd)	Group B Mean (sd)	P-value
AUC_{8AM}	1832 (623)	1314 (426)	**0.048**
AUC_{1PM}	1965 (679)	1572 (665)	0.340
AUC_{6PM}	1711 (663)	1274 (377)	0.092
$C_{trough8AM}$	64.1 (22.6)	48.1 (16.4)	0.094
$C_{trough1PM}$	76.3 (26.2)	58.6 (23.6)	0.249
$C_{trough6PM}$	69.0 (27.8)	52.4 (15.4)	0.122
CL_{8AM}	35.1 (14.0)	45.5 (12.0)	0.145
CL_{1PM}	34.5 (16.5)	44.3 (25.4)	0.435
CL_{6PM}	36.0 (12.9)	42.7 (5.8)	0.235
1'OH-MD/MD_{10AM}	0.081 (0.014)	0.095 (0.027)	0.251
1'OH-MD/MD_{8PM}	0.087 (0.027)	0.121 (0.084)	0.311

Abbreviations AUC8AM, AUC1PM, AUC6PM, combined area under the concentration-time curve of sunitinib and SU12662; Ctrough8AM, Ctrough1PM, Ctrough6PM, combined trough concentration of sunitinib and SU12662; CL8AM, CL1PM, CL6PM, sunitinib clearance. Both measurements at sunitinib dosing time 8AM, 1PM and 6PM respectively; 1'OH-MD/MD, ratio between 1'OH-midazolam and midazolam at 10AM and 8PM

Units of AUC in ng·h/mL, Ctrough ng/mL, CL L/h.

DISCUSSION

In this translational study, sunitinib pharmacokinetics in mice showed daily variations depending on the time of administration. Sunitinib and SU12662 AUC follow a similar oscillation pattern as a function of administration time, with 14-27% higher combined plasma AUC when sunitinib was administered at 4AM or 4PM, rather than at 8AM or 8PM, which is in the middle of the active or inactive phase of the mouse. Although a recent study in rabbits revealed significant differences in the exposure to sunitinib between dosing at 8AM or 8PM, this was not seen in our study in mice.[18]

Both the sunitinib and the SU12662 AUC in plasma follow a similar pattern in an apparent 12-hour rhythm. The oscillation pattern is probably due to circadian rhythms in both Cyp3a11 enzyme activity and expression of the efflux transporters Abcb1a, Abcb1b and Abcg2. The 12-hour rhythm in sunitinib and SU12662 pharmacokinetics may be due to counteracting activities of these drug transporters and metabolizing enzyme, since we observed variation in mRNA expression with peak levels at different times of the day.

In the 16 patients treated with sunitinib, the plasma AUC was equal at 3 dosing times, which is in contrast to our pre-clinical results. Despite the equality in daily exposure to sunitinib, the trough concentrations of sunitinib were significantly lower when patients were administered sunitinib in the morning, than at noon or evening dosing. Trough concentrations were sampled just before the intake

of a new dose of sunitinib. In this phase of the pharmacokinetic process metabolism and elimination are the most important determinants of drug concentrations.

This study showed that the midazolam clearance test in patients was similar at 10AM and 8PM, suggesting similarity in hepatic CYP3A activity between these time-points. Since CYP3A4 has a key role in sunitinib metabolism,[8] this suggests that sunitinib metabolism was similar at morning and evening dosing. Based on the midazolam clearance test, it can be assumed that metabolism did not vary between these time-points. Therefore it is hypothesised that the difference in sunitinib trough concentrations may be attributed to daily changes in elimination.

The daily variation in sunitinib trough concentrations is relevant for future research. For sunitinib and other TKIs, a threshold concentration for efficacy is known.[15] Therapeutic drug monitoring (TDM) is currently being investigated as a potential improvement of sunitinib therapy, using trough concentrations of sunitinib and SU12662 as an indicator for daily exposure. In these studies, dose levels of sunitinib are increased if trough concentrations are below the threshold. A recently published study revealed that it is feasible to dose sunitinib based on trough concentrations, with threshold concentrations between 50-100 ng/mL.[19] Patients in our study had less fluctuations in daily sunitinib concentration when sunitinib was administered at noon or in the evening. Therefore, the chance of dropping below the threshold of 50 ng/mL, where sunitinib is thought to be ineffective, is smaller when the drug is administered at these times of the day, although the daily exposure is the same. Patients dosed in the morning may therefore potentially undergo erroneous dose escalations and suffer from more toxicity.[20]

Interestingly, the daily variation in sunitinib AUCs observed in mice was not confirmed in patients. The discrepancy between pre-clinical models and patients may be due to various reasons. First, the pharmacokinetics of sunitinib may be different between mice and humans due to species specificity in the orthologous of CYP-enzymes and drug transporters involved in the pharmacokinetics of sunitinib. Second, the mice in this study only received a single dose of sunitinib and therefore steady-state plasma concentrations were not reached at the time of pharmacokinetic sampling. This is in contrast with the patients in our study who were administered sunitinib for at least 2 weeks before pharmacokinetic sampling took place. A third explanation can be that while mice were kept under strict light/dark cycles resulting in synchronized behavior, the circadian rhythm in the patients in this study may be disturbed by life style or during the hospital stay for pharmacokinetic sampling. pharmacokinetic samples were taken both during day times and during night hours, which resulted in waking of the patient. Fourthly, the mice in this study were genetically homogeneous, while in the human population there is genetic heterogeneity.

Of note, there were some limitations in the pre-clinical study. From each mouse only one blood withdrawal for pharmacokinetic determination was possible. Therefore, the AUC is calculated from sunitinib concentrations that are measured in different mice, which showed a large inter-individual difference in sunitinib and SU12662 concentrations. These large variations in pharmacokinetics may have been due to several causes. For instance, sunitinib was administered through gavage. Possibly,

the solvent was partially left in the gavage tube and it was not noticed whether mice vomited after gavage. Also, mice ranged in age from 8-12 weeks and therefore may differ in weight. In addition, mice were fasted from 3 hours before gavage until 1 hour after oral gavage, to make sure the stomachs of all mice were empty at the time of sunitinib administration. However, this may have caused stress to the animals and may have affected the phase of peripheral circadian rhythms, although analysis of mRNA levels of the core clock genes excluded the latter possibility.

This study was not designed to detect differences in pharmacodynamics (efficacy of treatment and toxicity) between different dosing times of sunitinib. Patients were allowed to participate at any time during sunitinib treatment, and there was a broad variation in the on-treatment time between patients. Six out of 16 (38%) patients had previously undergone dose reductions due to severe toxicity, and at the time of participation in the study these patients were treated at a dose level with an acceptable toxicity profile. Therefore, differences in toxicity levels between dosing times were not an endpoint in this study. However, previous studies have reported that toxicities from sunitinib treatment are similar in morning and evening dosing.[12, 13]

CONCLUSION

Altogether, we conclude that daily variation in the pharmacokinetics of sunitinib exists, most likely resulting from differences in sunitinib elimination. Patient's plasma trough concentrations of sunitinib are higher when administered in the afternoon or evening, and during these dosing times more stable drug concentrations are achieved than when administered in the morning. It is therefore advised that sunitinib should be dosed in the afternoon or evening in daily clinical practice, and if TDM is implemented in clinical practice, the administration time of sunitinib should be taken into account.

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SUPPLEMENTARY MATERIAL

Pharmacokinetic measurements on sunitinib and midazolam

All pharmacokinetic assessments for sunitinib, SU12662, midazolam and 1'OH-midazolam were performed at the Laboratory of Translational Pharmacology of the department of Medical Oncology in the Erasmus MC-Cancer Institute. Tissue samples from mice were diluted in human plasma (1:4 w/v) into a 2-mL eppendorf tube. Hereafter a 5-mm stainless steel bead (Qiagen, Venlo, The Netherlands) was added and the samples were homogenized with a Tissuelyser (Qiagen, Venlo, The Netherlands) and processed for 4 minutes at 40 Hz. Beads were removed and homogenized samples were stored at -70^{o}C until analyses. Sunitinib and SU12662 concentrations in tissues and plasma were quantitated using a validated UPLC-MS/MS method as previously described by de Bruijn et al.

Midazolam and 1'OH-midazolam were quantitated using a fully validated method with a Waters 2795 Separation Module coupled to a Quatro micro API Mass spectrometer (Waters, Etten-Leur, The Netherlands). The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with positive ion electrospray ionization. The MRM transition was 326→244 for midazolam, 342→324 for 1'OH-midazolam and 301→255 for the internal standard temazepam (IS). Chromatography was performed on a Zorbax Eclipse XDB-C8 analytical column, 150 mm × 4.6 mm, 5 µm (Agilent, Amstelveen, The Netherlands) using water and acetonitrile with 0.1% formic acid under gradient conditions. The sample extraction and cleaning-up involved a simple liquid-liquid extraction by addition of 50 µL aliquots of 4% ammonium hydroxide, 100 µL of internal standard solution and 1 mL aliquots of N-butylchloride to 200 µL plasma. The limits of quantification were 0.400 for midazolam and 0.200 ng/ml for 1'OH-midazolam.

Individual pharmacokinetic parameters were estimated by non-compartimental anlaysis in the software WinNonlin. In the experiment with mice, mean concentrations of sunitinib and SU12662 at each time-point after administration of sunitinib were calculated from 3 sacrificed mice at that specific time-point, with minimum and maximum value. Mean AUC, minimum and maximum AUC were calculated using the corresponding sample values.

Semi-quantitive real time PCR

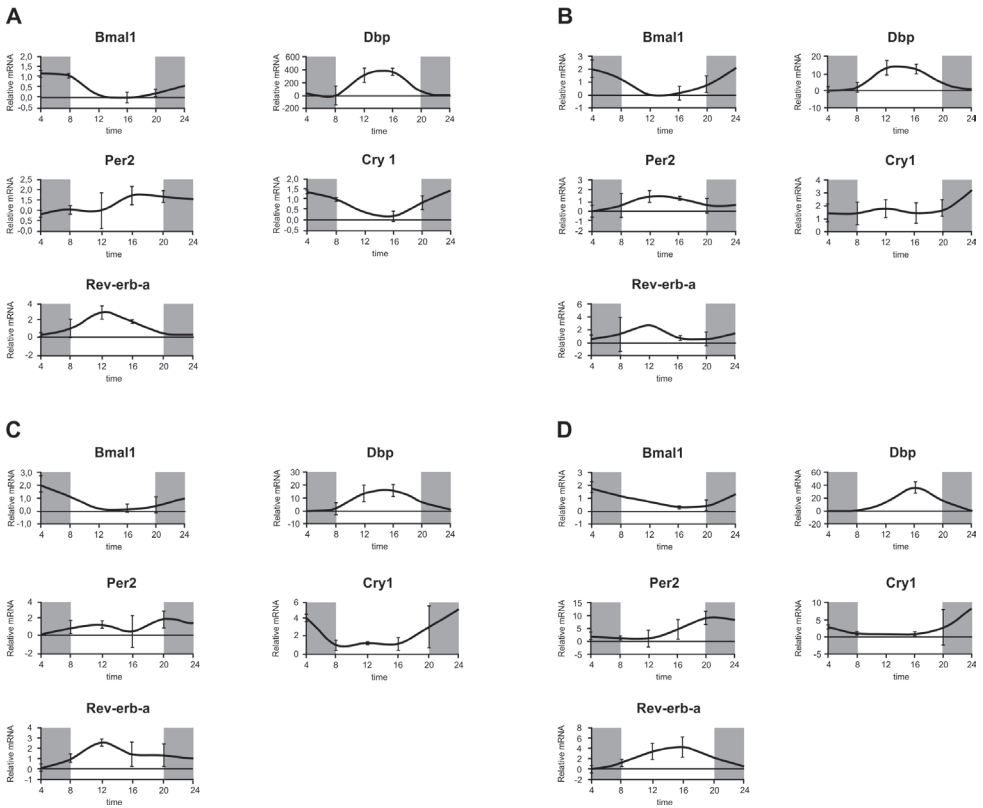
Total RNA was isolated from mice liver and intestine tissue using TriPure reagent (Roche Diagnostics) according to the manufacturer's protocol. RNA quality and concentrations were analyzed using the Nanodrop ND1000 (NanoDrop Technologies). One ug of RNA was used for cDNA preparation using iScript (Biorad) according to the manufacturer's protocol.

Semi-quantitative RT-qPCR was performed using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's protocol on a Biorad C1000 Touch Thermal Cycler using a standard 2-step amplification program with annealing/extension at 60^{0}C. Reactions for samples with housekeeping genes (B2M, Hprt and/or Gapdh) were always performed within the same plate as reactions for genes of interest. qPCR data represents the average of at least 2 housekeeping genes. The primers used for

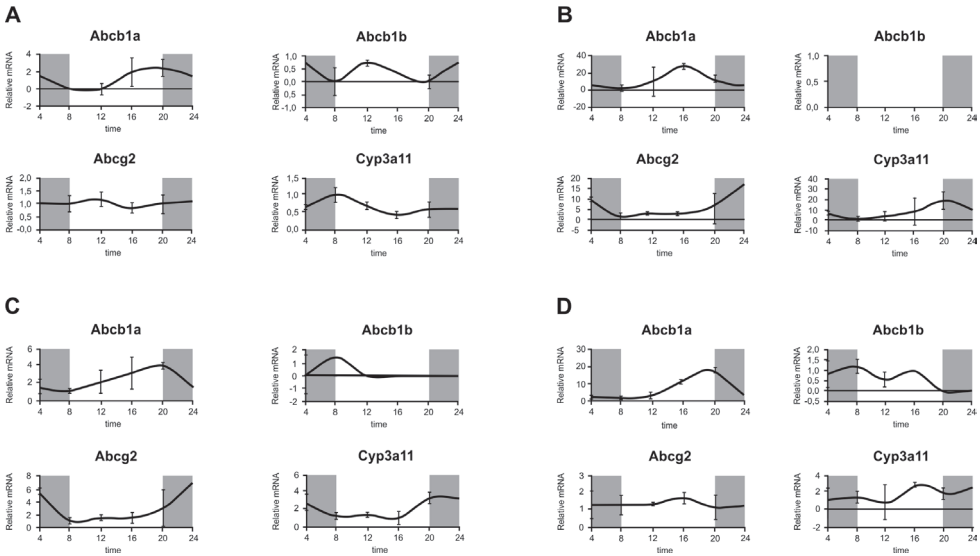
RT-qPCR are presented in **Supplementary table 1**. Relative gene expression was calculated using the comparative C(t) method and was normalized to relative expression at time 8AM (relative expression = 1).

Supplementary table 1 Primers used for RT-qPCR of mice liver and intestine tissue

Gene	Forward primer	Reverse primer
B2M	5'-CCGGCCTGTATCCAGAAA-3'	5'-ATTTCAATGTGAGGCGGGTGGAAC-3'
Hprt	5'-CGAAGTGTTGGATACAGGCC-3'	5'-GGCAACATCAACAGGACCTCC-3'
Gadph	5'-CAGAACATCATCCCTGCATCC-3'	5'-GTCATCATACTTGGCAGGTTTCTC-3'
Bmal1	5'-GCACTGCCACTGACTACCAAGA-3'	5'- TCCTGGACATTGCATTGCAT-3'
DBP	5'-ACCGTGGAGGTGCTAATGAC-3'	5'-CCTCTTGGCTGCTTCATTGTT-3'
Per2	5'-GGCTTCACCATGCCTGTTGT-3'	5'-GGAGTTATTTCGGAGGCAAGTGT-3'
Cry1	5'-CAGACTCTCGTCAGCAAGATG-3'	5'-CAAACGTGTAAGTGCCTCAGT-3'
Rev-erbα	5'- ACCTTACTGCTCAGTGCCTGGAAT-3'	5'- TGGACCTTGACACAAACTGGAGGT-3'
Cyp3a11	5'- ACCTGGGTGCTCCTAGCAAT-3'	5'- ACCATCAAACAACCCCCATGT-3'
Abcb1a	5'- CAGATACCATACAGAAATGCGA-3'	5'- CTCAATGATCCTGATGATGTGG-3'
Abcb1b	5'- GCATTACTAATCAAAGTGGACCC-3'	5'- ATCAAACCAGCCTATCTCCT-3'
Abcg2	5'- AAGTCTTCGTTGCTAGATGTC-3'	5'- GTCATCTTGAACCACATAACCT-3'



Supplementary figure 1 Relative mRNA expression of the genes involved in the regulation of circadian rhythms measured with 4-hours intervals in mouse liver (A), ileum (B), jejunum (C) and duodenum (D) of FVB mice by quantitative real-time PCR, normalized to relative expression at 8AM. Circadian rhythms were shown in the expression of *Bmal1*, *Per2*, *Cry1*, *Rev-erbα* and *Dbp.* Gray areas indicate it was dark in the cages of the mice, white areas indicate it was light.



Supplementary figure 2 Relative mRNA expression of *Cyp3a11, Abcb1a, Abcb1b and Abcg2* measured with 4-hours intervals in mouse liver (A), ileum (B), jejunum (C) and duodenum (D) from FVB mice. Data are relative to expression at time 8AM. Gray areas indicate it was dark in the cages of the mice, white areas indicate it was light.

Part 2

Pharmacodynamic aspects of treatment with tyrosine kinase inhibitors

Chapter 5

Genetic Polymorphisms as Predictive Biomarker of Survival in Patients with Gastro-Intestinal Stromal Tumours treated with Sunitinib

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Submitted

ABSTRACT

Background The purpose of this study was to identify single nucleotide polymorphisms (SNPs) that are associated with outcome to treatment with sunitinib in patients with advanced GIST.

Methods In this retrospective multicenter study 49 SNPS in genes encoding proteins involved in the pharmacokinetic and pharmacodynamic pathway of sunitinib, as well as clinical factors, were explored for their association with progression free survival (PFS) and overall survival (OS) in 127 patients with advanced GIST who have been treated with second line sunitinib.

Results PFS was significantly longer in carriers of the TT genotype in *POR* rs1056878 C/T (Hazard Ratio [HR] 4.310, 95% CI: 1.457-12.746, p = 0.008). The presence of the T-allele in *SLCO1B3* rs4149117 G/T (HR 2.024, 95% CI: 1.013-4.044, p = 0.046), the CCC-CCC alleles in *SLC22A5* haplotype (HR 2.603, 95% CI: 1.216-5.573, p = 0.014), and the GC-GC alleles in the *IL4R* haplotype (HR 7.131, 95% CI: 1.518-33.496, p = 0.013) were predictive for OS. When these factors were combined in a model, PFS and OS were significantly longer with an increasing number of favorable genetic alleles (HR 0.654, 95% CI 0.512-0.836, p = 0.001 and HR 0.359, 95% CI 0.156-0.826, p = 0.016 respectively).

Discussion This study shows that polymorphisms in genes encoding for proteins involved in the pharmacokinetic and pharmacodynamic pathway of sunitinib are associated with survival in GIST patients. If validated, this may help to identify patients that benefit more from treatment with sunitinib and therefore may be useful in therapeutic decision making.

INTRODUCTION

Since the introduction of imatinib as first line treatment for advanced gastrointestinal stromal tumors (GIST), progression free survival and overall survival of patients with this malignancy has dramatically improved. Unfortunately, eventually the vast majority of patients develop resistance to imatinib, mainly due to secondary mutations, while in others severe toxicity occurs, both resulting in the need to switch to second line treatment with sunitinib (Sutent; Pfizer Pharmaceuticals Group, New York, NY).[1] Sunitinib is a multi-targeted tyrosine kinase inhibitor.[2, 3] Its clinical value in the treatment of patients with metastatic GIST has been shown in a randomized trial showing a median time to tumor progression of 27.3 weeks for patients treated with sunitinib, versus 6.4 weeks for patients treated with placebo.[1] However, there is a large inter-individual difference in the efficacy of sunitinib in patients with GIST. This may in part be explained by the presence of specific mutations within the tumor[4] but another factor that may contribute to the variability in efficacy may be germline genetic variation. In patients treated with sunitinib for metastatic renal cell cancer, single nucleotide polymorphisms (SNPs) in genes related to the pharmacokinetic and pharmacodynamic pathways of sunitinib have been associated with outcome in terms of progression-free survival (PFS) and overall survival (OS).[5]

In patients with GIST, the role of germline genetic polymorphisms as biomarkers predicting outcome has never been investigated. To further personalize treatment in this group of patients, it is meaningful to get better insight into the factors predicting the efficacy of a drug before starting, especially when alternative treatment options exist such as in the case of advanced GIST. Therefore, we performed a multicenter association analysis to explore whether polymorphisms in candidate genes within the pharmacokinetic or pharmacodynamic pathway of sunitinib are associated with PFS and OS in patients with GIST.

METHODS

Study population and design

From a large multicenter Dutch cohort of 365 patients with GIST, those patients who have been treated with second line sunitinib were selected. Patients had started sunitinib treatment between March 2004 and June 2014 in the Erasmus MC Cancer Institute, Leiden University Medical Center, Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, or University Medical Center Groningen. Sunitinib could be administered in a 4 weeks on/2 weeks off treatment scheme, or in a continuous dosing regimen, with any dose of sunitinib. Patients who have had dose reductions or dose escalations were allowed to be included in this study.

Demographic data of patients was retrospectively collected in an electronic case record form, designed for this study. Collected patient characteristics were age, gender, self-declared ethnicity, Eastern

Cooperative Oncology Group (ECOG) WHO performance score, weight, length, tumor characteristics (*i.e.* histology, mutation status, mitotic index (per 50 HPF), site of origin tumor, previous surgery), prior therapy and therapy after sunitinib, and survival estimates. For PFS and OS, data collection took place until August 2014.

From each patient one sample of whole blood, serum or tumor surrounding tissue containing germline DNA was collected for DNA isolation. Samples could be either residuals or prospectively obtained samples in a study approved by the local medical ethical board. Samples were stored at -20°C or colder at the local hospital laboratory until further process. All samples were anonymized, according to the Codes for Proper use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org).

Genetic polymorphisms and haplotype estimation

Forty-nine SNPs in 23 genes involved in the pharmacokinetics and pharmacodynamics of sunitinib were selected for genotyping, based on literature (see **Table 1**). SNPs were selected from the genes *ABCB1*, *ABCC2*, *ABCG2*, *CYP1A1*, *CYP1A2*, *CYP3A4*, *NR1I2*, *NR1I3*, *POR*, *SLCO1B3*, *SLC22A1*, *SLC22A4* and *SLC22A5* within the pharmacokinetic pathway and the genes *FLT1, FLT3, IL-4R, IL-8, KDR, PDGFRA, RET* and *VEGFA* within the pharmacodynamic pathway.

DNA isolation and genotyping were performed at the department of Clinical Pharmacy and Toxicology, Leiden University Medical Center. DNA was isolated from serum or whole blood using Magna Pure compact (Roche, Almere, the Netherlands), or from tumor surrounding tissue using Maxwell (Promega, Leiden, the Netherlands). DNA isolated from serum or tissue was pre-amplified as described before.[6]

SNPs were determined using the QuantStudio 12K Real-Time PCR System (Life Technologies, Bleiswijk, the Netherlands), with custom designed arrays. Custom designed pyrosequencing assays were used to enhance the call-rate above 90%. The mean genotype call-rate was 98.6% with a lowest call-rate of 93.2% and highest call-rate of 100%. The allele frequencies of seven out of 49 SNPs were not in Hardy Weinberg equilibrium, but frequencies were comparable to the frequencies reported in the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) and all SNPs were therefore kept within the analysis.

SNPs within a gene were tested for linkage disequilibrium (LD) using Haploview (Broad Institute). Haplotypes were estimated for polymorphisms with an LD of more than 95%. The maximum likelihood estimates of haplotype probabilities were calculated using PLINK software, version 1.7 (http://pngu.mgh.harvard.edu/purcell/plink/). Haplotype probabilities with a likelihood ≥95% were included in the statistical analysis. Haplotypes were formed from SNPs in *NR1I3* (rs2307418, rs2307424, rs4073054), *PDGFRA1* (rs1800810, rs1800812, rs1800813), *PDGFRA2* (rs2228230, rs35597368), *IL8* (rs1126647, rs4073), *SLC22A5* (rs2631367, rs2631370, rs2631372), *VEGFA* (rs2010963, rs699947, rs833061), *IL4R* (rs1801275, rs1805015).

Table 1 Selected polymorphisms within the pharmacodynamic and pharmacokinetic pathway of sunitinib

Gene	Protein	SNP	Allele change
Pharmacodynamic genes			
IL4	*IL4*	rs224350[9]	C/T
IL4R		rs1801275[9]	A/G
		rs1805010[9]	A/G
		rs1805015[9]	T/C
IL8	*IL8*	rs4073[12]	A/T
		rs1126647[12]	A/T
IL13	*IL13*	rs1800925[9]	C/T
		rs20541[9]	G/A
FLT1	*FLT1*	rs7993418[13]	A/G
FLT3	*FLT3*	rs1933437[14]	T/C
FLT4	*VEGFR3*	rs6877011[15]	C/G
KDR	*VEGFR2*	rs1870377[16]	A/T
		rs2071559[14]	C/T
		rs2305948[16]	C/T
PDGFRA1	*PDGFRA1*	rs1800810[14]	C/G
		rs1800812[14]	G/T
		rs1800813[14]	A/G
PDGFRA2	*PDGFRA2*	rs2228230[17]	C/T
		rs35597368[14, 16]	C/T
RET	*RET*	rs1799939[14]	G/A
VEGFA	*VEGFA*	rs1570360[16]	G/A
		rs2010963[16, 18]	G/C
		rs25648[15]	C/T
		rs3025039[19]	C/T
		rs699947[16, 18, 19]	A/C
		rs833061[18, 19]	C/T

Pharmacokinetic genes			
ABCB1	*ABCB1*	rs1045642[20, 21]	C/T
		rs868755[8, 21]	G/T
		rs28656907[22]	C/T
ABCC2	*ABCC2*	rs717620[21]	C/T
ABCG2	*ABCG2*	rs2231137[8]	G/A
		rs2231142[8, 21]	C/A
CYP1A1	*CYP1A1*	rs1048943[14]	A/G
CYP1A2	*CYP1A2*	rs762551[14]	A/C
CYP3A4	*CYP3A4*	rs2740574[8]	A/G
NR1I2	*NR1I2*	rs3814055[14]	C/T
		rs1054191[14]	G/A
NR1I3	*NR1I3*	rs2307424[5, 14]	C/T
		rs2307418[5, 14]	A/C
		rs4073054[5, 14]	G/T
POR	*POR*	rs1057868[23]	C/T
SLC1B3	*OATP1B3*	rs4149117[8]	G/T
SLC22A1	*hOCT1*	rs628031[20, 21]	G/A
		rs683369[8, 21]	C/G
		rs6935207[20]	G/A
SLC22A4	*OCTN1*	rs1050152[8]	C/T
SLC22A5	*OCTN2*	rs2631367[8]	C/G
		rs2631370[8]	T/C
		rs2631372[8]	C/G

Statistics

PFS was defined as the time between the first day of sunitinib treatment, and the day of progressive disease (PD), or death due to PD, whatever came first. If PD had not occurred in a patient, or in those cases where a patient was lost to follow-up, the patient was censored at the day of last follow-up. OS was defined as the time between the first day of sunitinib treatment and the date of death. Patients who had not died or of whom it was unknown whether they had died were censored at the last day of follow up.

All SNPs and haplotypes were univariately tested against PFS and OS using the Kaplan-Meier method with the log-rank test. Patient characteristics were also univariately tested against PFS and OS, using either the Kaplan-Meier method with the log-rank test, or Cox regression analysis, based on the type of data. Variables and SNPs with a p-value ≤ 0.10 in the univariate analysis were selected for inclusion in a multivariate Cox-regression analysis, using PFS and OS as dependent variables. For SNPs, the best

fitted model (multiplicative, wildtype dominant or mutant dominant) was chosen to enter into the multivariate analysis, based on the univariate analyses. Missing data from baseline characteristics that were associated with PFS or OS in the univariate analysis, were randomly imputed before entering the variable in the multivariate regression model. Depending on the variable, 1-40% of data was imputed. Multivariate analysis were performed twice, with and without replacement of missing variables. If results were similar in size and direction of effect, replacement was considered legitimate.

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 17.0 (SPSS, Chicago, IL). Given the explorative nature of this study, all results from multivariate analysis with p-value ≤ 0.05 were considered statistically significant and no correction for multiple testing was performed.

RESULTS

Study population

The study population consisted of 127 patients with GIST treated with sunitinib, of whom 63% were men. The mean age at start of sunitinib was 61.2 ± 13.4 year. The stomach was the most frequent site of primary GIST location (38%). In fourteen patients (11%) a *c-KIT* exon 9 mutation was found, and 58 patients (46%) had a tumor with an exon 11 mutation in *c-KIT* in the primary tumor. Other mutations were found in *c-KIT* exon 13 (n = 2), exon 14 (n =1), exon 17 (n =2) or in *PDGFR* exon 18 (n = 7). In 43 patients (33.8%) the mutation in the primary tumor was unknown. Most patients (76%) received sunitinib in an intermittent dosing scheme, starting sunitinib with 50 mg a day (n = 91, 72%) during the first 4 weeks, continued by 2 weeks off-dosing.

At the time of analysis, 110 patients had stopped sunitinib treatment. In 87 patients (85%), this was because of PD and in all other cases because of severe toxicity. In the entire population, the median PFS was 7.6 months (interquartile range [IQR] 3.1-17.0 months) and the median OS was 18.3 months (IQR 9.7-29.3 months). The baseline characteristics of the study population are presented in **Table 2**.

Table 2 Baseline characteristics

Variable	N (%) or mean (sd)
Gender	
male	80 (63)
female	47 (37)
Age at start sunitinib (years)	61.2 (13.4)
Hospital	
LUMC	60 (47)
EMC	43 (34)
NKI	18 (14)
UMCG	6 (5)
Primary location tumor	
stomach	48 (38)
small bowel	36 (28)
colon	7 (5)
rectum	6 (5)
unknown	30 (24)
Histology of primary tumor	
spindle cell	70 (55)
epitheloid	12 (9)
mixed	21 (17)
unknown	24 (19)
Mutation	
exon 9	14 (11)
exon 11	58 (46)
other mutation or wild type	32 (25)
unknown	21 (16)
WHO PS at start sunitinib	
0-1	98 (77)
2-3	11 (9)
unknown	18 (14)
Type of sunitinib treatment	
intermittent	97 (76)
continuous	28 (22)

Variable	N (%) or mean (sd)
unknown	2 (2)
Dose of sunitinib at start treatment	
12.5 mg	1 (1)
25 mg	5 (4)
37.5 mg	28 (21)
50 mg	91 (72)
unknown	3 (2)
Reason to stop sunitinib	
PD	87 (69)
toxicity	23 (18)
continued treatment	17 (13)

Abbreviations: BSA; body surface area, LUMC; Leiden University Medical Center, EMC; Erasmus MC Cancer Institute, NKI; Netherlands Cancer Institute, UMCG; University Medical Center Groningen, WHO PS; World Health Organization performance score, PD; progressive disease

Pharmacogenetic biomarkers for PFS

In the univariate analysis, PFS was longer for patients with the presence of the T-allele in *KDR* rs1870377 T/A (p = 0.033), the presence of the G-allele in *IL13* rs20451 G/A (p = 0.025), the presence of the C-allele in *VEGFA* rs25648 T/C (p = 0.014), and in the absence of 2 GCT copies in the *VEGFA* haplotype (p = 0.042) in the pharmacodynamic genes. With respect to the pharmacokinetic SNPs that were tested, the presence of the homozygous TT-allele in *POR* rs1057868 C/T (p = 0.008), and the absence of two CCC-copies in the *SLC22A5* haplotype (p = 0.007) were univariately associated with prolonged PFS. From the baseline characteristics length (HR 1.028; 95% CI: 1.002-1.055, p = 0.032), mitotic index of the primary tumor (HR 1.006, 95% CI: 1.000-1.012, p = 0.042), age at start of sunitinib (HR 0.986; 95% CI: 0.972-0.999, p = 0.037) and the reason to stop imatinib (PD 13.7 months, other than PD 29.9 months; p = 0.01) were included in the multivariate analysis.

Only the homozygous TT genotype in *POR* rs1057868 C/T (HR 0.232, 95% CI: 0.078-0.686, p = 0.008) was associated with PFS in the multivariate Cox regression analysis (**Table 3**). A trend towards shorter PFS was seen for the presence of 2 copies of the CCC *SLC22A5* haplotype, compared to 1 or 0 copies (HR 2.358, 95% CI: 0.978-5.684, p = 0.056).

Table 3 Univariate and multivariate analysis of progression free survival in patients with GIST treated with sunitinib

		Univariate analysis*			Multivariate analysis		
Factors	**No.**	**Mean PFS (months)**	**95% CI**	**P-value**	**HR****	**95% CI**	**P-value**
Clinical factors							
Reason to stop imatinib				0.10			0.238
PD	102	13.7	11.3-16.1		1,565	0.744-3.929	
other	23	29.9	14.9-45.0		1		
Length (HR 1.028)	96		1.002-1.055	0.032	1,008	0.994-1.007	0.582
Mitotic index (HR 1.006)	76		1.000-1.012	0.042	1,001	0.994-1.007	0.804
Age at start sunitinib (HR 0.986)	125		0.972-0.999	0.037	0.990	0.974-1.007	0.240
Genetic factors pharmacodynamic pathway							
KDR rs1870377				0.033			0.423
TT & TA	114	17.9	13.6-22.2		0.696	0.286-1.691	
vs AA	9	8.1	1.9-14.2		1		
IL13 rs20541				0.025			0.756
GG & GA	113	18.0	13.7-22.3		0.870	0.362-2.090	
vs AA	11	8.0	4.8-11.3		1		
VEGFA rs25648				0.014			0.347
CC & CT	117	17.7	13.5-21.8		0.626	0.236-1.661	
vs TT	8	7.0	2.5-11.4		1		

		Univariate analysis*			Multivariate analysis		
Factors	**No.**	**Mean PFS (months)**	**95% CI**	**P-value**	**HR****	**95% CI**	**P-value**
VEGFA GCT-haplotype				0.042			0.081
GCT-GCT *vs*	1	3.0	3.0-3.0		6,488	0.793-53.060	
GCT-other & other-other	116	16.5	12.8-20.3		1		
Genetic factors pharmacokinetic pathway							
POR rs1057868				0.001			**0.008**
TT	9	46.5	17.6-75.4		0.232	0.078-0.686	
vs CC & CT	115	14.5	11.8-17.2		1		
SLC22A5 CCC-haplotype				0.007			0.056
CCC-CCC *vs*	15	7.7	4.3-11.1		2,358	0.987-5.684	
CCC-other & other-other	105	18.5	14.1-23.0		1		

*Only factors with P-value < 0.10 level are presented; these were selected for multivariate analysis **Hazard ratio. HR < 1 indicates that the factor is associated with improved PFS, HR > 1 indicated that the factor is associated with worse PFS.

Pharmacogenetic biomarkers for OS

In the univariate analysis two pharmacodynamic SNPs within *VEGFA* were predictive for longer OS (rs1570360 G/A, absence of the A allele; p = 0.005 and rs699947 C/A, presence of the C-allele; p = 0.036), as well as the presence of a CGG-copy in the *PDGFRA1* haplotype (p = 0.007) and the presence of the GC-other or other-other alleles in the *IL4R* haplotype (p = 0.008). Within the pharmacokinetic pathway, the presence of the C-allele in *ABCC2* rs717620 C/T (p = 0.006), as well as presence of the T-allele in *SLCO1B3* rs4149117 G/T (p = 0.054). Two haplotypes within the pharmacokinetic pathway were associated with longer OS: the absence of 2 CTT-copies in *NR1I3* (p < 0.0001) and the absence of 2 CCC-copies in *SLC22A5* (p = 0.001).

From the baseline characteristics that were univariately tested against OS, a better survival was seen in patients who stopped imatinib for another reason than PD (PD 25.8 months OS, other than PD 55.4 months OS, p = 0.001), the absence of liver metastasis at start of sunitinib (44.2 vs 27.4 months, p = 0.093), and the absence of metastases at the time of diagnosis (37.6 vs 25.8 months OS, p = 0.025). Multivariate Cox regression analysis showed *SLCO1B3* rs4149117 G/T, the absence of a T-allele (HR 2.024, 95% CI: 1.013-4.044, p = 0.046), the presence of 2 copies of the CCC *SLC22A5* haplotype (HR 2.603, 95% CI: 1.216-5.573, p = 0.014), and the presence of 2 copies of the GC *IL4R* haplotype (HR 7.131, 95% CI: 1.518-33.496, p = 0.013) as predictors for OS, as well as PD as a reason to stop imatinib (HR 3.025, 95% CI: 1.358-6.742, p = 0.007) and the presence of metastases at the time of the primary diagnosis GIST (HR 1.773, 95% CI: 1.044-3.012, p = 0.034). Data are presented in **Table 4**.

Favorable genetic profile

Polymorphisms and haplotypes that were significantly associated with OS (*SLCO1B3* rs4149117 G/T, the presence of the T-allele, the absence of a CCC-copy in the *SLC22A5* haplotype and the absence of a GC-copy in the *IL4R* haplotype) were combined in a favorable genetic profile for PFS and OS, using the number of favorable genetic factors.

The number of favorable genetic factors was significantly associated with longer survival (PFS 9.2 *vs* 15.6 *vs* 28.4 months for respectively one, two or three favorable genetic factors, p = 0.005). There was only 1 patient with no favorable genetic factors in our population. In a multivariate regression model including the clinical factors (reason to stop imatinib, length and mitotic index of the primary tumor), this was confirmed (HR 0.654, 95% CI 0.512-0.836, p = 0.001, **Figure 1A**).

OS was significantly longer with an increasing number of positive predicting genetic factors (mean OS 16.0 *vs* 31.5 *vs* 49.5 months for respectively one, two or three positive predictive genetic factors, p = 0.001). This was confirmed in a multivariate regression analysis, including the amount of favorable genetic factors and the clinical factors reason to stop imatinib, metastasis at primary diagnosis and liver metastasis at the start of sunitinib (HR 0.359, 95% CI 0.156-0.826, p = 0.016, **Figure 1B**).

Table 4 Univariate and multivariate analysis of overall survival in patients with GIST treated with sunitinib

		Univariate analysis			Multivariate analysis		
Factors	**No**	**Mean OS (months)**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Clinical factors							
Reason to stop imatinib				0.001			**0.007**
PD	102	25.8	21.8-29.8		3,025	1.358-6.742	
other	24	55.4	37.5-73.3		1		
Metastasis at time of diagnosis				0.025			**0.034**
no	66	37.6	28.8-46.4		1		
yes	59	25.8	19.5-32.2		1,773	1.044-3.012	
Liver metastasis at start sunitinib				0.093			0.127
no	37	44.2	28.1-30.3		1		
yes	86	27.4	23.2-31.6		0.660	0.315-1.155	
Genetic factors pharmacodynamic pathway							
VEGFA rs1570360				0.005			0.128
GG *vs*	66	38.9	29.6-48.2		0.654	0.378-1.130	
GA & AA	58	22.0	18.1-25.9		1		
VEGFA rs699947				0.036			0.390
CC & CA	94	35.8	28.6-43.0		0.755	0.398-1.433	
vs AA	28	21.6	17.6-25.5		1		

PDGFRA CGG-haplotype				0.007			0.066
CGG-CGG & CGG-other	120	33.1	27.1-39.1		0.189	0.085-0.418	
vs other-other	6	13.7	6.6-20.7		1		
IL4R GC-haplotype				0.008			**0.013**
GC-GC *vs*	4	8.2	2.0-14.5		7,131	1.518-33.496	
GC-other & other-other	117	32.8	26.7-38-8		1		
Genetic factors pharmacokinetic pathway							
ABCC2 rs717620				0.006			0.168
CC & CT	121	32.7	26.8-38-6		0.248	0.090-0.682	
vs TT	5	10.2	8.5-11.8		1		
SLCO1B3 rs4149117				0.054			**0.046**
GG *vs*	97	28.1	23.3-32.9		2,024	1.013-4.044	
GT & TT	23	47.9	28.5-67.2		1		
NR1I3 CTT-haplotype				<0.001			0.062
CTT-CTT *vs*	4	9.1	3.1-15.0		4,599	0.927-22.810	
CTT-other & other-other	122	33.0	27.0-38.9		1		
SLC22A5 CCC-haplotype				0.001			**0.014**
CCC-CCC *vs*	14	15.6	10.5-20.8 28.4-41.5		2,603	1.216-5.573	
CCC-other & other-other	107	34.9			1		

*Only factors with P-value < 0.10 level are presented; these were selected for multivariate analysis

**Hazard ratio. HR < 1 indicates that the factor is associated with improved OS, HR > 1 indicated that the factor is associated with worse OS

Abbreviations: OS; overall survival, 95% CI; 95% confidence interval, PD; progressive disease

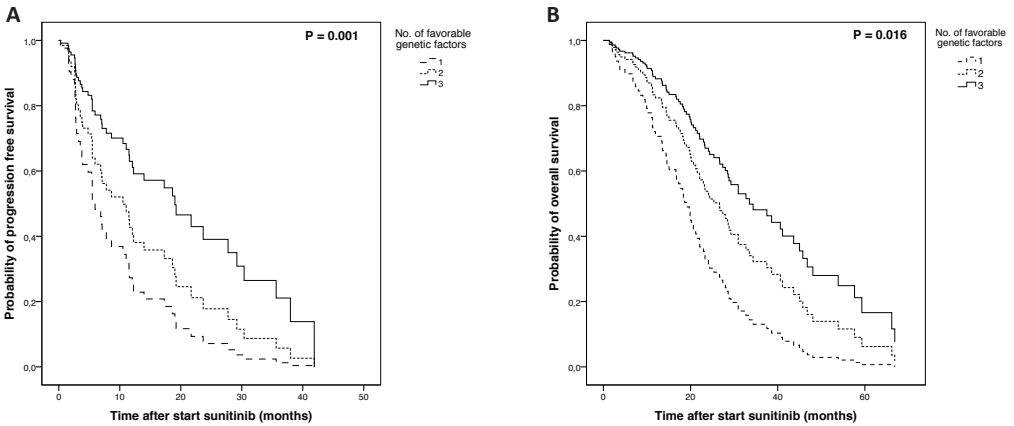

Figure 1. PFS (Figure 1A) and OS (Figure 1B) in patients with GIST treated with sunitinib being carriers of one, two or three favorable genetic variations

DISCUSSION

Patients with GIST treated with sunitinib have a large inter-patient difference in PFS and OS. This may in part be explained by various tumor cell-related factors such as secondary mutations and by some clinical factors.[4] However, genetic polymorphisms within the pharmacokinetic and pharmacodynamic pathways may add to this as they affect the exposure to and the efficacy of the drug, and thereby influence the outcome of treatment as well. In this explorative study we showed in a population of 129 patients with GIST, that polymorphisms in both the pharmacokinetic (*SLCO1B3*, *SLC22A5* and *POR*) and the pharmacodynamic (*IL4R*) pathway of sunitinib are associated with PFS and OS in patients with advanced GIST treated with sunitinib.

These findings indirectly suggest that survival to sunitinib in patients with GIST is subjected to exposure to sunitinib and its active metabolite. Sunitinib is metabolized by CYP3A4 and CYP3A5 into its active metabolite SU12662. This is converted to several inactive compounds by the same enzymes. The activity of cytochrome P450-enzymes is regulated by P450 oxydoreductase (POR). In our study, rs1056878, otherwise known as *POR*28*, was associated with prolonged PFS in sunitinib treated patients with GIST. Rs1056878 encodes for the amino acid variant A503V, and has been associated with lower activity of CYP1A2, CYP2D6, CYP3A5, but not of CYP3A4.[7] The finding that the polymorphic variant of rs1056878 is associated with better PFS suggests that carriers of this variant have a lower activity of metabolizing enzymes resulting in higher plasma concentrations. Sunitinib is a substrate of the ATP-binding cassette ABCB1 and ABCG2 efflux transporters, playing a role in both uptake and efflux of sunitinib. However, none of the SNPs in these genes were associated with survival in our analysis. The

precise role of members of the organic cation transporter novel (OCTN) family and the organic anion-transporting peptide (OATP) family in sunitinib absorption and elimination is unclear. However, SNPs in *SLC22A5*, which is the gene encoding for OCTN2, have been found to be associated with survival to imatinib in patients with GIST and CML.[8] Interestingly, we found the *SLC22A5* haplotype, consisting of rs2631367, rs2631370 and rs2631372 to be significantly associated with longer OS. Carriers of the two CCC-copies had significantly shorter OS than patients with other allelic combinations. This is consistent with the finding in imatinib treated patients with GIST.[8] Other member of the OCTN family that were tested in this study did not show a significant association with PFS or OS. In *SLCO1B3*, which encodes OATP1B3, rs4149117 was also associated with prolonged OS. Possibly, sunitinib is a substrate of these efflux transporters as well, but this needs to be elucidated.

The homozygous GC-copy in the *IL4R* haplotype consisting of rs1801275, rs1805015 (Ser478Pro and Gln551Arg) was significantly associated with longer OS. In a previous study, SNPs in *IL4R* have been associated with the development of renal cell carcinoma.[9] The finding that SNPs within *IL4R* are associated with OS in patients with GIST treated with sunitinib may be related to IL4R being involved in the tumor biology of GIST as well.

A limitation of this study is that no pharmacokinetics of sunitinib as an intermediate endpoint were measured in this group of patients. Therefore, it can only be assumed that the effects of the SNPs on survival is caused by differences in pharmacokinetics. In a recent pharmacogenetic-pharmacokinetic study, *CYP3A4*22* was found to have an effect size of > 20% on clearance.[10] However, this finding was not statistically significant.

Another limitation of this study is the sample size. Although this is the largest pharmacogenetic study in patients with GIST treated with sunitinib so far, the number of patients with specific genotypes is too small to draw conclusions from. Since this was an exploratory study, no formal correction for multiple testing was performed and results from the multivariate analyses with a p-value less than 0.05 were considered significant. Currently, the False Discovery Rate (FDR) is frequently used to control for reporting false positives in exploratory studies. Therefore, we calculated FDR values for each separate endpoint in a post-hoc analysis. FDR was below 10% for all SNPs with $p<0.05$ indicating a low likelihood of false positive findings.

In our current study, SNP that were found associated with prolonged PFS, were not associated with OS and *vice versa*. This is somewhat surprising, since PFS and OS can be expected to be related to each other. However, while PFS only includes the effects of sunitinib treatment, OS also embodies the effects of any subsequent lines of treatment. Patients in our study received sunitinib over a broad area of time. In the first years after the registration of sunitinib, no good third line of treatment was available, but patients were frequently offered other treatment in the context of clinical studies. Since

recently, regorafenib has been approved for third line treatment of GIST after failure of imatinib and sunitinib.[11] This may have caused a bias in the overall survival in our analysis. Still, we showed in a large group of patients that genetic polymorphisms can serve as a biomarker for overall survival.

Progressive disease as the reason to stop imatinib treatment was univariately associated with both worsened PFS and worsened OS. In the multivariate analysis this was only confirmed for OS, but not for PFS. The existence of metastases at the time of the primary diagnosis was also associated with worse OS. Possibly, the tumor has a more aggressive behavior when metastasis are present at first diagnosis and when the tumor has already progressed on imatinib, rather than the patient switched to sunitinib for other reasons, resulting in shorter OS.

Previously it has been described that primary mutations in *c-KIT* and *PDGFRA* may be predicting for the survival obtained by sunitinib in patients with GIST, this was not seen in our study. This may be explained by the fact that all patients were pre-treated with imatinib. It has been shown that during the treatment with imatinib, secondary mutations may arise, leading to imatinib-resistance.[4] Therefore, mutations that are found in the primary tumor may not be representative of the mutations within the tumor after treatment with imatinib.

Altogether we may conclude that polymorphisms in genes encoding for proteins related to the pharmacokinetic and pharmacodynamic pathways of sunitinib are associated with survival in patients with GIST treated with sunitinib. When validated in the future, this may be useful to predict which patient is going to respond to sunitinib therapy, and which patients may better respond to other treatment types.

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Chapter 6

Genetic Polymorphisms in Angiogenesis Related Genes are predictive for Survival of Patients with Advanced Gastrointestinal Stromal Tumors treated with Imatinib

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ABSTRACT

Background Imatinib is first line therapy for advanced gastrointestinal stromal tumors (GIST). Clinical response is high, but primary and secondary resistance are common. This study explores the effects of single nucleotide polymorphisms (SNPs) in genes related to the pharmacokinetics and pharmacodynamics of imatinib on its efficacy in patients with advanced GIST.

Methods In 255 advanced patients with GIST, a retrospective pharmacogenetic pathway analysis was performed using genotype data from 36 SNPs in 18 genes, as well as clinical factors, to investigate their effects on progression free survival (PFS) and overall survival (OS. Selected genetic and clinical factors were entered into a multivariate model.

Results In the multivariate model significant hazard ratios were found for PFS in patients with synchronous metastasis (HR 1.74, P=0.005), *KIT* exon 9 mutation (HR 1.94, p=0.040), and the SNPs *KDR* rs2305948 (TT genotype, HR 4.57, p=0.041) and *VEGFA* rs1570360 (*AA* genotype, HR 1.77, p=0.025). Significant hazard ratios for OS were found in patients with synchronous metastasis (HR 2.07, p=0.004), and the SNPs *KDR* rs1870377 (*AA* genotype, HR 2.33 ,p=0.017), *VEGFA* rs1570360 (*AA* genotype, HR 2.01, p=0.026) were found, whereas a T allele in *SLCO1B3* rs4149117 was associated with longer OS (HR 0.475, p=0.017).

Conclusion This exploratory pathway analysis of imatinib efficacy in advanced GIST patients shows that polymorphisms in *VEGFA*, *KDR* and *SLCO1B3* may be predictive for an effect on PFS and/or OS. If validated, these SNPs may serve as biomarkers to identify patients with an increased chance of progressive disease.

INTRODUCTION

Imatinib mesylate (Gleevec®, Glivec®, Novartis, Switzerland) is the first line therapy for chronic myeloid leukemia (CML) and advanced gastrointestinal stromal tumors (GIST).[1,2] It has revolutionized the treatment of these two malignancies in achieving marked survival benefit with limited toxicity.[3] In these two malignancies, clinical response to this oral tyrosine kinase inhibitor (TKI) is determined by etiologic mutation(s), as well as by germline genetic variations.[4,5] Single Nucleotide Polymorphisms (SNPs) are the most common genetic variations in germline. SNPs can have various outcomes, ranging from silent mutations to affecting gene expression and enzyme function. The pharmacokinetics and pharmacodynamics of imatinib may be changed in due to SNPs in genes encoding for enzymes and target protein regulating imatinib pharmacology.

GIST is a mesenchymal tumor of the digestive tract, often caused by gain-of-function mutations in the genes encoding for KIT or PDGFR-α or –β.[6,7] These mutations result in ligand independent autophosphorylation of these receptors leading to uncontrolled proliferation of the interstitial cells of Cajal, which serve as pacemaker cells in the gastrointestinal tract. However, in some GISTs no *KIT* or *PDGFR- α* or - *β* mutations are found. This group of so called 'wild type' GIST becomes increasingly rare as new mutations in other genes are found to be etiologically related to this malignancy.[8] *KIT* mutations are routinely screened in GIST to predict imatinib efficacy which is dependent on the location of the *KIT* mutation; presence of exon 11 mutations predict good response to imatinib, whereas mutations in exon 9 are predictive for limited objective response to imatinib in the standard dose.[4] Disease progression has also been associated with a variety of clinical factors, such as the location of the primary tumor.[9] It may also result from decreased imatinib concentrations; a mechanism called pharmacokinetic resistance.[10]

In CML treatment, complete cytogenetic response to imatinib has been associated with germline SNPs in genes encoding for enzymes which have a role in the metabolism of imatinib. In addition, polymorphisms in the genes encoding for the efflux transporter ABCG2 and for the influx transporter SLC22A1, have been associated with poor response and progression to advanced disease, respectively.[11] For patients with advanced GIST treated with imatinib, associations have been reported for SNPs in *SLC22A4* and *SLC22A5* and time to progression. The effects of the genes encoding for the influx transporters OCTN1 and OCTN2, respectively, was independent of mutational status and tumor size.[12] Since this single report, no studies have been published exploring the effects of SNPs on the efficacy of imatinib in patients with advanced GIST.

Therefore, this study investigates the effects of genetic variants on imatinib efficacy in patients with advanced GIST. A pharmacogenetic pathway analysis was performed including SNPs in genes encoding proteins and enzymes related to the pharmacokinetics and pharmacodynamics of imatinib.

METHODS

Patients

For this retrospective study GIST patients were included who have been treated in four Dutch referral centers (Erasmus MC Cancer Institute, Leiden University Medical Center, Antoni van Leeuwenhoek - Netherlands Cancer Institute and University Medical Center Groningen). All patients had a histology of proven GIST and documented advanced disease, being either relapsed or metastatic. Patients started imatinib therapy between November 2000 and May 2013. All patients had to be treated at least until the first evaluation of anti-tumor activity, with the exemption of patients with clinical progression before this moment, who were scored as such. DNA was obtained from residual blood samples that were collected for routine patient care or, in the Erasmus MC Cancer Institute, after specific informed consent was obtained (MEC 02.1002), and stored at -20°C or colder until genotyping. In the Antoni van Leeuwenhoek - Netherlands Cancer Institute only serum of these samples was stored. If a residual blood or serum sample was not available, DNA was obtained from residual formalin fixated paraffin embedded (FFPE) resection specimen. All samples were anonymized by a third party and the Code for Proper Secondary Use of Human Tissue was adhered to (www.federa.org/codes-conduct).[13]

SNP selection

SNPs in genes related to imatinib pharmacokinetics and pharmacodynamics were selected using a pathway approach. Metabolizing enzymes and potential targets of imatinib were selected and the literature was screened for SNPs in applicable genes. Using Haploview and HapMap data (release 28), SNPs in linkage were identified to efficiently select candidate SNPs. A minimum minor allele frequency of 0.1 was set. Additionally, the database of the National Institute of Environmental Health Sciences was used to select only the SNPs with an expected functional change. Finally, a total of 36 SNPs in 18 genes were selected (**Table 1)**.

Genotyping

DNA was isolated from blood, serum or FFPE samples using the MagnaPure Compact (Roche Diagnostics, Almere, the Netherlands) and stored at -20°C or colder. For optimal genotyping results, DNA isolated from serum and FFPE samples was pre-amplified.[14] A custom array was developed for the QuantStudio™ 12K Flex Real-time PCR system (Life Technologies, Bleiswijk, the Netherlands) and DNA was genotyped according to the manufacture's protocol. To achieve a satisfactory call rate for all SNPs, a number of SNPs were subsequently genotyped using commercially available realtime PCR genotyping assays (Life Technologies, Bleiswijk, the Netherlands) according to the manufacture's protocol or in house developed Pyrosequencing assays (Qiagen, Venlo, the Netherlands.

To explore haplotypes in the study population Haploview 4.2[15] and Plink 1.7[16] were used. SNPs in the same gene were tested and considered to be in a haplotype in case D' was at least 95%. Only patients with a ≥95% probability of the assigned allele were included in the analyses.

Table 1 selected SNPs in pharmacokinetics and pharmacodynamics of imatinib

Gene	Rs number	Chromosome	Allele change	Change type	Call rate	p value HWE	Study MAF
ABCG2	rs2231137	4	G/A	Splicing	93.7	0.016	0.061
ABCG2	rs2231142	4	C/A	Splicing	99.6	0.225	0.128
SLC22A5	rs2631367	5	C/G	TFBS	97.6	0.502	0.45
SLC22A5	rs2631370	5	T/C	TFBS	99.6	0.226	0.39
SLC22A5	rs2631372	5	C/G	TFBS	100	0.776	0.294
SLC22A1	rs628031	6	G/A	Splicing	98.8	0.378	0.421
SLC22A1	rs683369	6	C/G	Splicing	99.6	0.561	0.281
SLC22A1	rs6935207	6	G/A	TFBS	95.7	0.114	0.236
ABCB1	rs1045642	7	C/T	Splicing	99.6	0.316	0.498
ABCB1	rs868755	7	G/T	Splicing	99.6	0.546	0.433
ABCB1	rs28656907	7	C/T	TFBS	91	0.285	0.517
SLC22A4	rs1050152	5	C/T	Splicing	99.6	0.713	0.409
CYP3A4	rs2740574	7	A/G	TFBS	99.6	0.046	0.047
POR	rs1057868	7	C/T	nsSNP	99.6	0.468	0.323
ABCC2	rs717620	10	C/T	TFBS	99.2	0.346	0.186
CYP1A1	rs1048943	15	A/G	nsSNP	100	0	0.045
CYP1A2	rs762551	15	A/C	TFBS	99.2	0.867	0.304
SLCO1B3	rs4149117	12	G/T	Splicing	92.2	0.311	0.126
PDGFRA	rs1800810	4	C/G	TFBS	99.2	0.382	0.231
PDGFRA	rs1800812	4	G/T	TFBS	99.6	0.284	0.234
PDGFRA	rs1800813	4	A/G	TFBS	98.4	0.421	0.217
PDGFRA	rs2228230	4	C/T	Splicing	100	0.95	0.186
PDGFRA	rs35597368	4	C/T	Splicing	100	0.153	0.135
KDR	rs1870377	4	A/T	nsSNP	97.6	0.332	0.239
KDR	rs2071559	4	C/T	TFBS	99.2	0.489	0.457
KDR	rs2305948	4	C/T	nsSNP	100	0.935	0.086
VEGFA	rs1570360	6	G/A	TFBS	96.5	0.041	0.319
VEGFA	rs2010963	6	G/C	TFBS	98	0.284	0.336
VEGFA	rs25648	6	C/T	Splicing	100	0.104	0.188
VEGFA	rs3025039	6	C/T	miRNA	100	0.012	0.131
VEGFA	rs699947	6	A/C	TFBS	96.5	0.614	0.51
VEGFA	rs833061	6	C/T	TFBS	93.7	0.219	0.498
FLT4	rs6877011	5	C/G	miRNA	100	0.54	0.073
RET	rs1799939	10	G/A	Splicing	100	0.514	0.161
FLT3	rs1933437	13	T/C	Splicing	99.2	0.27	0.411
FLT1	rs7993418	13	A/G	Splicing	100	0.19	0.19

Splicing; Splicing modifying, TFBS; Transcription Factor Binding Site, nsSNP; Non-Synonymous SNP, miRNA; Micro RNA aleration, HWE; Hardy Weinberg Equilibrium, MAF; Minor Allele Frequency

Statistics

Clinical and demographic factors were collected from patient files, including ethnicity. Progression free survival (PFS) was defined as the time between the date of start of imatinib treatment and the date of progressive disease, according to either clear clinical progression or to RECIST 1.1 definition of progressive disease. If patients were still on treatment at the last date of follow-up, PFS was censored at that date. Overall survival (OS) was defined as the time between the date of start of imatinib treatment and death due to GIST. OS was censored at the last date of follow-up if a patient was alive at that time, or a day before death if a patient died due to an unrelated event or disease.

SNPs and haplotypes were univariately tested with Kaplan Meier analysis for an association with PFS and OS, using the general model. Clinical factors were tested univariately with either Kaplan Meier or Cox regression. If univariate analyses showed a trend for a difference in survival, with the threshold for the p value set a $P < 0.1$, these factors were entered into the multivariate Cox regression model. For genetic factors the most appropriate genetic model was selected (multiplicative, dominant or recessive). These models tested for associations for either PFS or OS with the respective variables found in the univariate analyses. Missing clinical factors were imputed using SPSS multiple imputation tool with the following factors entered: sex, age at diagnosis, length, weight, ethnicity (Caucasian or else), histology (spindle cell or else), primary tumor location (stomach or else), mutation (*KIT* exon 9, *KIT* exon 11, or rest group), metastasis at diagnosis, WHO performance score at start of imatinib treatment, liver metastasis at start of imatinib, and WHO performance score at start of sunitinib treatment, liver metastasis at start of sunitinib. Data at the start of sunitinib was used, as these are be correlated with liver metastasis and WHO performance at start of imatinib. The number of imputations was set at 100 and the results of the pooled analyses was reported. Factors in these analyses with $P < 0.05$ were deemed statistically. Due to the explorative nature of this study no correction for multiple testing was performed. SPSS version 20 (IBM Corp., Armonk, NY, United States) was used.

RESULTS

Study population

DNA was available for a total of 255 patients. The majority of the samples was isolated from residual blood (215 patients) and smaller numbers from serum (26 patients) and FFPE samples (14 patients). The baseline characteristics of the study population are depicted in **Table 2**, including the percentages of missing data per variable. The median PFS for the study population was 37.6 months (95% confidence interval (CI): 27.8 - 47.3 months) and the median OS 84.7 months (95% CI: 74.0 - 95.5 months). At the time of analysis, 132 patients (51.8%) had had progressive disease, 95 patients (37.3%) had died due to GIST and 10 patients had died due to other diseases, such as myocardial infarction or lung cancer. The median time of follow-up was 72 months (95% CI: 61.1 - 82.8 months), as calculated by the reversed Kaplan Meier estimator.

Baseline characteristics associated with PFS were length (p=0.003), ethnicity (p=0.027), histology (p=0.017), mutation status (p=0.040) and synchronous metastasis at the time of diagnosis (p=0.0001) (**Table 3)**. Overall survival was associated with length (p=0.004), primary location (p=0.085), histology (p=0.003), synchronous metastasis (p=0.0003) and the WHO performance score at start of imatinib (p=0.026) (**Table 4)**. Mutation status was not associated with OS (p=0.554, data not shown). These factors were included in the applicable Cox regression model.

Table 2: baseline characteristics of study population

		median or N (%)
Age at diagnosis (years)		59
Length (cm)		174
Weight (kg)		78
Sex	male	151 (59.2)
	female	104 (40.8)
Ethnicity	Caucasian	220 (86.3)
	other	20 (7.8)
	unknown	15 (5.9)
Primary location	stomach	94 (36.9)
	other	107 (42.0)
	small bowel	*75 (29.4)*
	rectum	*20 (7.8)*
	colon	*10 (4.0)*
	esophagus	*2 (0.8)*
	unknown	54 (21.1)
Histology	spindle cell	228 (60.8)
	epithelioid and mixed	52 (20.4)
	unknown	48 (18.8)
Mutation found	KIT exon 11	126 (49.4)
	KIT exon 9	22 (8.6)
	other	58 (22.8)
	unknown	49 (19.2)
Mitosis index	≤ 5 per 50 HPF	37 (14.5)
	>5 per 50 HPF	122 (47.8)
	unknown	96 (37.7)
Metastases at diagnosis	present	102 (40.0)
	not present	151 59.2)
	unknown	2 (0.8)
Liver metastasis	present	147 (57.6)
	not present	108 (42.4)
WHO PS	0-1	208 (81.6)
	2-Mar	13 (5.1)
	unknown	34 (13.3)

Pharmacogenetic factors associated with PFS

In the univariate analysis, SNPs related to the pharmacodynamics of imatinib showed (a trend for) shorter PFS for patients with *AA* genotype in *KDR* SNP rs1870377 (*TT+AT vs AA*, p=0.052) and for the *TT* genotype in *KDR* rs2305948 (*CC+CT vs TT*, p=0.019). The *AA* variant in *VEGFA* rs1570360 was also associated with shorter PFS (*GG+GA vs AA*, p=0.027) (**Table 3)**. All SNPs related to the pharmacokinetics of imatinib did not show an association with PFS in univariate tests.

Together with the selected clinical factors, these SNPs were entered into the multiple imputed Cox regression model. This model showed that presence of synchronous metastasis and a *KIT* exon 9 mutation are predictive for shorter PFS (HR 1.74, p=0.050 and HR 1.94, p=0.040, respectively). The *KDR* rs2305948 and *VEGFA* rs1570360 SNPs also showed a significant association for PFS, and in both cases the homozygote minor variant was associated with shorter PFS (HR 4.57, p=0.041 and HR 1.77, p=0.025, respectively).

Table 3 univariate and multivariate analyses of progression free survival (PFS) of GIST patients treated with imatinib

			Univariate Kaplan Meier analyses			Multivariate Cox regression analyses		
		N patients	median PFS	95% CI	*p* value	HR	95% CI	*p* value
Clinical factors								
Length	(HR= 1.031 per cm increase)			1.010 - 1.051	0.003	1.012 per cm increase	0.992 - 1.032	0.245
Ethnicity	Caucasian	220	31.3	23.3 - 39.3	0.027	1		0.201
	non-Caucasian	20	not reached	- *		0.548	0.215 - 1.377	
Histology	spindle cell	155	39.4	23.7 - 55.1	0.017	1		0.202
	epithelioid and mixed	52	24.8	17.8 - 31.8		1,012	0.992 - 1.032	
Synchronous metastasis	absent	151	60.9	16.9 -105.0	0.0001	1		0.005
	present	102	24.8	19.1 - 30.61		1,740	1.186 - 2.554	
Mutation	*KIT* exon 9	22	19.1	5.2 - 32.9	0.004	1,938	1.029 - 3.650	0.040
	KIT exon 11	126	43.8	31.4 - 56.2		1		
	other group	58	24.8	13.8 - 35.9		1,349	0.874 - 2.081	0.176
Genetic factors								
***KDR* rs1870377**	TT + AT	232	39.1	28.0 - 50.1	0.052	1		0.150
	vs AA	17	19.5	6.4 - 32.6		1,646	0.835 - 3.246	
***KDR* rs2305948**	CC + CT	253	37.7	28.5 - 46.9	0.019	1		0.041
	vs TT	2	6.6	- *		4,574	1.064 -19.664	
***VEGFA* rs1570360**	GG + GA	214	39.4	27.5 - 51.4	0.035	1		0.025
	vs AA	32	28.0	14.1 - 42.0		1,767	1.074 - 2.908	

In univariate analyses missing clinical factors were left missing; only univariate analyses shown with $p < 0.1$: in multivariate analyses missing clinical factors were imputed, the pooled analysis of 100 imputations is reported: HR= Hazard Ratio: HR > 1.0 indicates association with worse survival and vice versa: in the multivariate Cox regression, the effect of the mutation has *KIT* exon 11 compared to both *KIT* exon 9 and the other group (other mutations in *KIT*, *PDGFR* or 'wild-type'): 95% CI = 95% confidence interval: * = 95% CI not computed

Table 4 univariate and multivariate analyses of overall survival (OS) of GIST patients treated with imatinib

			Univariate Kaplan Meier analyses			Multivariate Cox regression analyses		
		N patients	median OS	95% CI	*p* value	HR	95% CI	*p* value
Clinical factors								
Length	(HR= 1.034 per cm increase)			1.011 - 1.057	0.004	1.015 per cm increase	0.991 - 1.039	0.223
Primary location	stomach	91	85.3	48.8 -121.8	0.085	1		0.245
	elsewhere	106	89.9	68.5 -111.2		0.734	0.436 - 1.236	
Histology	spindle cell	154	119.1	86.3 -151.8	0.003	1		0.221
	epithelioid and mixed	50	75.0	57.8 - 92.2		1.394	0.819 - 2.372	
Synchronous metastasis	absent	148	119.1	- *	0.00003	1		0.004
	present	101	63.1	41.5 - 84.7		2,075	1.261 - 3.416	
WHO performance	0-1	207	86.5	68.8 -104.2	0.026	1		0.243
score at start of imatinib	2-Mar	11	75.1	26.7 -123.4		1,792	0.673 - 4.772	
Genetic factors								
***KDR* rs1870377**	TT + AT	229	86.4	69.1 -103.7	0.037	1		0.017
	vs AA	16	67.3	16.0 -118.6		2,334	1.161 - 4.692	
***VEGFA* rs1570360**	GG + GA	212	86.6	57.1 -116.1	0.027	1		0.026
	vs AA	31	57.8	34.9 - 80.7		2,012	1.088 - 3.722	
***SLCO1B3* rs4149117**	GG vs	175	75.0	60.5 - 89.5	0.042	1		0.017
	GT + TT	56	98.9	- *		0.475	0.257 - 0.877	

In univariate analyses missing clinical factors were left missing; only univariate analyses shown with $p < 0.1$: in multivariate analyses missing clinical factors were imputed, the pooled analysis of 100 imputations is reported: HR= Hazard Ratio: HR > 1.0 indicates association with worse survival and vice versa: 95% CI = 95% confidence interval: * = 95% CI not computed

Pharmacogenetic factors associated with OS

In the univariate analysis, rs4149117 in *SLCO1B3* was associated with PFS. Patients with the *GG* genotype in *SLCO1B3* rs4149117 had longer PFS (*GG vs GT+TT*, p=0.042), see **Table 4**. The *AA* genotype in KDR rs1870377 and the *AA* genotype in *VEGFA* r1570360 were associated with shorter OS (p=0.037 and p=0.027, respectively).

When these SNPs were entered into the multiple Cox regression model together with the selected clinical factors, only synchronous metastasis was significantly associated was (HR 2.08, p=0.004). The *AA* genotype in *KDR* rs1870377 and in *VEGFA* rs1570360 were associated with a shorter OS as compared to patients with at least one wild-type allele (HR 2.3, p=0.017 and HR 2.01, p=0.026, respectively). The *GG* genotype variant in *SLOC1B3* showed a shorter OS compared to patients either *GT* or *TT* (HR 0.47, p=0.017).

(Un)favorable genetic profile

The study population was sorted into a group with a favorable genetic profile (195 patients) and a group with an unfavorable profile (46 patients), based on the multivariate analysis of PFS. For selection into the unfavorable profile, patients had to have either the *AA* genotype in *KDR* rs1870377, the *TT* genotype in *KDR rs2305948*, or the *AA* genotype in *VEGFA* rs1570360. Univariate Kaplan Meier curves of PFS and OS showed a significant difference for both groups. The median PFS for patients with the unfavorable profile was 18.8 months short of the PFS for the favorable profile (43.8 vs 25.0 months, p=0.004). The difference in median OS was 32.3 months (66.6 vs 98.9 months, p=0.008), as showed in **Figure 1**. Taking the selected clinical factors into account, this effect was consistent in the multiple imputed multivariate Cox regression model for PFS (HR 2.00, 95% CI 1.30 - 3.01, p=0.002) and for OS (HR 2.62, 95% CI 1.56 - 4.39, p=0.0003), as depicted in **Table 5**.

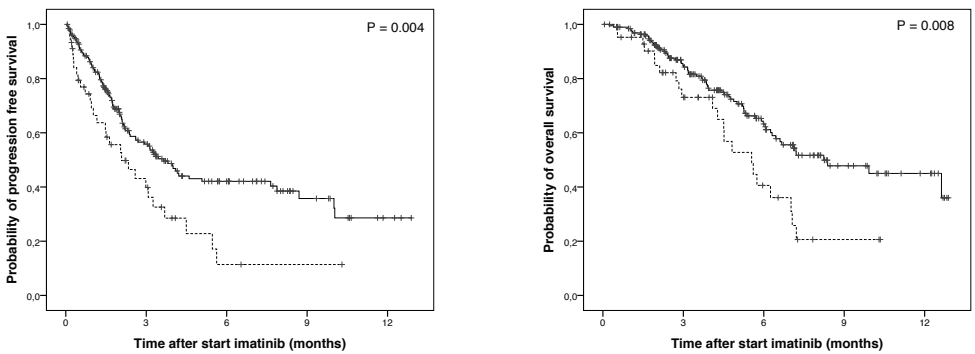

Figure 1 Progression free survival (PFS) and overall survival (OS) in months of GIST patients treated with imatinib according to (un)favorable genetic profile. Out of a total of 255 patients, 195 had a favorable profile and 46 had an unfavorable profile (the latter based on either *AA* genotype in *KDR* rs1870377, *TT* genotype in *KDR* rs2305948, or *AA* genotype in *VEGFA* rs1570360). For PFS, p=0.004, for OS, p=0.008

Table 5 multivariate analysis with genetic profile of progression free survival (PFS) and overall survival (OS) in GIST patients treated with imatinib

			PFS multivariate Cox regression analyses			**OS multivariate Cox regression analyses**		
		N patients	HR	95% CI	*p* value	HR	95% CI	p value
Length			1.013 per cm increase	0.994 - 1.033	0.180	1.021 per cm increase	0.997 - 1.045	0.090
Ethnicity	Caucasian	220	1		0.222	n/a		
	non-Caucasian	20	0.563	0.224 - 1.414				
Primary location	stomach	91	n/a			1		0.100
	elsewhere	106				0.662	0.404 - 1.082	
Histology	spindle cell	155	1		0.258	1		0.112
	epithelioid and mixed	52	1,295	0.828 - 2.025		1,516	0.908 - 2.531	
Synchronous metastasis	absent	151	1		0.005	1		0.005
	present	102	1,820	1.238 - 2.674		1,978	1.223 - 3.200	
Mutation	*KIT* exon 9	22	1,891	1.011 - 3.538	0.046			
	KIT exon 11	126	1			n/a		
	other group	58	1,303	0.874 - 2.008	0.230			
WHO ps	0-1	207	n/a			1		0.277
	2-Mar	11				1,741	0.641 - 4.732	
***KDR* rs1870377,**	other vs	195	1		0.002	1		0.0003
***KDR* rs2305948 and**	AA or TT or AA	46	2,004	1.302 - 3.085		2,616	1.557- 4.393	
***VEGFA* rs1570360**								

In multivariate analyses missing clinical factors were imputed, the pooled analysis of 100 imputations is reported; HR= Hazard Ratio: HR > 1.0 indicates association with worse survival and vice versa: the effect of the mutation has *KIT* exon 11 compared to both *KIT* exon 9 and the other group (other mutations in *KIT*, *PDGFR* or 'wild-type'): 95% CI= 95% confidence interval, n/a= not applicable

DISCUSSION

This exploratory pharmacogenetic study shows that SNPs in the genes encoding for VEGFA, KDR (also known as VEGFR2) and SLCO1B3 (also known as OATP1B3) are associated with PFS and OS in patients with advanced GIST treated with imatinib. To the best of our knowledge, this cohort of 255 patients is the largest patient group to be used for a pharmacogenetic study concerning imatinib treatment in GIST. The median follow-up of 72 months is long enough to qualify these data as mature. The SNP selection for this study was performed using predefined criteria, including a biological rationale based on imatinib pharmacology and expected functionality. Therefore, the SNPs found warrant validation in a prospective study.

So far, only one small pharmacogenetic study exploring the effects of SNPs in genes related to imatinib pharmacokinetics on its efficacy was performed in patients with advanced GIST. This Italian study investigated 31 SNPs in a population of 54 patients.[12] Three SNPs in the influx transporters *SLC22A4* (rs1050152) and *SLC22A5* (rs2631367 and rs2631372) were associated to time to progression, independent of mutational status, tumor size, age and sex. These SNPs were also tested in the present study, but the univariate tests with the general model were inconclusive.

Several SNPs in vascular endothelial growth factor A (*VEGFA*) were tested. VEGFA has many effects, most prominently being the activity in angiogenesis. In this study, the *AA* genotype in rs1570360 was a predictive marker for both PFS and OS. Other SNPs in *VEGFA* such as rs699947 have been associated to imatinib effect in CML patients, but none other of the tested SNPs showed a significant association in this study population.[5] In adjuvant GIST patients, SNPs in *VEGFA* have been tested for effect on relapse rate, but for none of those SNPs, which were also tested in this study, an association was observed.[17] The kinase insert domain receptor (KDR) is a tyrosine kinase receptor for VEGF. The *TT* genotype in rs2305948 had a profound effect on PFS, despite only two patients had this genotype. Larger studies will need to be performed to assess this SNP in this setting. The *AA* genotype in rs1870377 appears to have a negative effect on OS (and less so on PFS) in the present study population, but this was not seen in the study that also investigated SNPs in *KDR* in for an effect on GIST relapse rate.[17] In contrast, a study with CML patients reported better clinical outcome for patients with the *AA* genotype in rs1870377.[5] This contradiction may be caused by differences in tumor biology of solid vs hematological malignancies.

Patients with at least one T allele in rs4149117 in *SLCO1B3* had longer OS. The solute carrier organic anion transporter family member (SLCO) 1B3 is an influx transporter with imatinib as a substrate and mainly expressed in hepatocytes, and also in leucocytes.[18] Although this SNP did not show a trend when tested for PFS in the general model, the *GG* vs *GT+TT* model did show a trend (data not shown). A recent study performed with Brazilian CML patients reported that the frequency of patients with the *TT* genotype was higher in the responder group than in the non-responder group.[19] These results

are in line with a Japanese study, which found enhanced transporter function in patients with the *TT* genotype, as measured by higher intracellular imatinib levels.[20]

In order to perform multivariate analyses, missing baseline factors were imputed, assuming that these data were missing at random. In the analysis of PFS, the significant clinical and genetic factors in the imputed model had a similar size and direction of effect as in the non-imputed model. *KDR* rs1870377 was not significantly associated with PFS in the imputed model and it had a different direction of effect in the non-imputed model. For OS, the variables had similar size and direction of effect in both the imputed and non-imputed Cox regression model, thus legitimating the imputation of missing variables. The distribution of the mutational status and location of the primary tumor differed to clinical trials. This may be attributed to the specific selection biases affecting both clinical trials (with a generally fitter study population) and retrospective studies (where baseline data may be missing). To include GIST patients whose DNA was not available by means of residual blood or serum samples, pathology samples were used instead, if these could be obtained. Out of the 38 SNPs tested, 5 were not in Hardy-Weinberg equilibrium. These SNPs were retained in the analyses, as the MAF was did not differ substantially from the National Center Biotechnology Information database (www.ncbi.nlm.nih.gov/snp), and patient selection due to the retrospective nature of the study was considered the most plausible reason. The raw genotyping output indicated adequate assay quality and duplications gave the compatible results.

As previously reported, the effects of the oncogenic somatic mutation on imatinib efficacy were also found in this study. Tumors with a *KIT* exon 11 mutation were more sensitive to imatinib, especially compared to tumors with *KIT* exon 9 mutations.[4] Having GIST metastases was clearly associated with reduced survival. These metastases may be considered heterogeneous and some clones will progress despite imatinib activity in the majority of GIST lesions.[21] Other clinical factors were not associated with survival in this advanced patient group, even though factors such as the primary tumor site have been reported in other studies.[9] Remarkably, SNPs in pharmacokinetic genes encoding for ABCB1, ABCG2, SLC22A1, SL22A5 or CYP3A4, or haplotypes herein were not associated with a difference in survival, despite previous, sometimes conflicting, reports.[5,10-12,17,22-25]

This retrospective exploratory study investigated the effects of polymorphisms in genes related to the pharmacokinetics and pharmacodynamics of imatinib in the treatment of advanced GIST. One SNP in the pharmacokinetic pathway (rs4149117 in *SLCO1B3*) and two SNPs related to pharmacodynamics (rs1870377 in *KDR*, and rs1570360 in *VEGFA*) showed significantly different Hazard Ratios for PFS or OS. These polymorphisms, together with clinical factors such as tumor mutation and metastases, may identify patients who are most at risk of developing progressive disease before it can be expected in this population. If these findings are validated, they may proof useful in selecting patients whom may benefit from more frequent evaluation of antitumor efficacy.

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Chapter 7

Incidence and relevance of QTc-interval prolongation caused by Tyrosine Kinase Inhibitors

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ABSTRACT

Background Tyrosine kinase inhibitors (TKIs) are associated with prolongation of the QTc-interval on the ECG. QTc-interval prolongation increases the risk for life threatening arrhythmias. However, studies evaluating the effects of TKIs on QTc-intervals are limited and only consist of limited patient numbers.

Methods In this multicentre trial in four centres in the Netherlands and Italy we screened all patients who were treated with any TKIs. To evaluate the effects of TKIs on the QTc-interval we investigated ECGs prior to and during treatment with erlotinib, gefitinib, imatinib, lapatinib, pazopanib, sorafenib, sunitinib, or vemurafenib.

Results A total of 363 patients were eligible for the analyses. At baseline measurement, QTc-intervals were significantly longer in females than in males ($QTc_{females}$ = 404 ms *vs*. QTc_{males} = 399 ms, $P = 0.027$). A statistically significant increase was observed for the individual TKIs sunitinib, vemurafenib, sorafenib, imatinib, and erlotinib, after the start of treatment (median ΔQTc ranging from +7 to +24 ms, $P < 0.004$). The CTCAE grade for QTc-intervals significantly increased after start of treatment ($P = 0.0003$). Especially patients who are treated with vemurafenib are at increased risk of developing a QTc ≥ 470 ms, a threshold associated with an increased risk for arrhythmias.

Conclusion These observations show that most TKIs significantly increase the QTc-interval. Particularly in vemurafenib treated patients, the incidence of patients at risk for arrhythmias is increased. Therefore, especially in case of combined risk factors, frequent ECG controls in patients treated with TKIs are strongly recommended.

INTRODUCTION

Long QT syndrome (LQTS) is a myocardial repolarization disorder characterized by prolongation of the QT-interval on the surface electrocardiogram (ECG). The clinical presentation of LQTS consists of palpitations, syncope, seizures, and sudden cardiac death due to a characteristic arrhythmia known as *torsades de pointes* (TdP).[1-3]

Several factors, such as gender, age, electrolyte disturbances, cardiovascular diseases (CVD) and different types of drugs can affect the duration of the QT-interval.[4, 5] Due to the risk of fatal arrhythmias, the US Food and Drug Administration (FDA) now obliges to perform thorough studies to evaluate the potency of new drugs to induce QT-interval prolongation in preclinical and early phase I clinical trials in healthy individuals.[2] However, for new anticancer drugs these studies are usually not performed in healthy individuals because of their toxicity profile.[6, 7]

As with many drugs, tyrosine kinase inhibitors (TKIs) are reported to prolong the QT-interval.[8-16] In vitro studies demonstrated that lapatinib and imatinib interact with the phosphorylation of the cardiac hERG channel. This results in a reduction of the repolarizing current (IKr), which can lead to action potential prolongation and subsequent QT-interval prolongation.[11, 13]

In a small prospective clinical study to evaluate the cardiac safety of lapatinib in 21 patients, an overall QTc increase of 8.63 ms was seen.[9] In three prospective post-marketing studies in patients with solid tumours treated with sorafenib (*N* = 31), pazopanib (*N* = 48) and sunitinib (*N* = 24) a modest increase of 9.0 ms of QTc-interval with the use of sorafenib, 4.4 ms after start of pazopanib and 9.6 ms after start of sunitinib was seen.[8, 12, 16]

Still, these drugs have been approved by the FDA because they appear highly effective in situations where treatment options are limited. With an increasing number of TKIs on market, a relative long on-treatment time, and the application in the adjuvant setting, which is for example already standard for imatinib in patients with localised GIST with a high risk to relapse, and which is being explored for other TKIs in various tumour types, thorough QTc studies in this group of drugs is necessary to get more insight into their cardiac safety. In this multicentre study performed in four centres in the Netherlands and Italy, we describe the incidence and relevance of QTc-interval prolongation in patients with cancer treated with different types of TKIs.

METHODS

Study design

We undertook a retrospective study in patients with solid malignancies, who were treated with any type of TKI. Patients from four centres in the Netherlands (Erasmus MC Cancer Institute, Rotterdam, Leiden University Medical Centre, Leiden, and Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam) and Italy (Salvatore Maugeri Foundation, Pavia) were included.

This study was reviewed and approved by the Erasmus MC Medical Ethical Board (MEC 2013-148). All ECGs were obtained as standard clinical care. Demographical and clinical characteristics of the analysed cohort were collected using clinical record forms designed for this study.

Patients were considered eligible if they were aged ≥ 18 years, were treated for solid tumours with any type of TKI and if at least one ECG prior to start of TKI treatment and one ECG during treatment with TKI were available. Exclusion criteria were as follows: ECGs which do not match criteria for accurate QTc-interval measurements (intra-ventricular conduction delay and/or pace-maker driven rhythm), missing ECGs at baseline or during therapy, and patients with a time-lapse between baseline ECG and start of TKI treatment > 1 year. Patients who were subsequently treated with different TKIs were included once.

Definition of QTc prolongation

Since increases of the heart rate results in shortening of the QT-interval, a correction for heart rate was applied using the Bazett formula:[17]

$$QTc = QT/\sqrt{RR}$$

In this formula RR is the interval between two subsequent R-tops.

QTc was discretized according to the common terminology criteria for adverse events (CTCAE) guidelines version 4.03 (grade 0, QTc < 450; grade 1, QTc 450 – 479 ms; grade 2, QTc 480 – 499 ms; grade 3, QTc ≥ 500 ms; grade 4, QTc ≥ 500 ms with life-threatening signs or symptoms; grade 5, death).[18] Relevant CVD were defined as myocardial infarction and/or heart failure.

Outcome measures used in this study were: *i*) the quantitative difference in terms of QTc-interval between on therapy and baseline ECG measurements (ΔQTc, ms), *ii*) the transition from a condition of normal repolarization to a condition in which QTc is prolonged to an extent with high risk of arrhythmia as a consequence of the TKI therapy (*i.e.*, from QTc < 470 to QTc ≥ 470 ms),[3, 19] *iii*) clinically relevant ΔQTc (defined as ΔQTc ≥ 30ms, above which the risk for TdP is significantly increased)[20, 21] and *iv*) QTc-interval CTCAE grade increase during TKI therapy.[18]

Statistical analyses

The distribution of the quantitative variables analysed deviated significantly from normality (Shapiro-Wilk test $P < 0.05$), thus they were described by median (IQR). The presence of statistically significant differences in terms of QTc distributions before *vs.* during TKI therapy were tested by the Wilcoxon Signed Rank Test for paired samples. Differences in terms of quantitative variables distribution as function of categorical outcomes were tested by the Wilcoxon Rank-Sum test or by the Kruskal-Wallis test as appropriate. The McNemar test was applied to compare the distributions of categorical variables before *vs.* during TKI therapy. Differences in terms of categorical variables distributions between binary outcomes were evaluated by the Fisher's exact test. The impact of quantitative and categorical variables on the probability of binary outcomes was tested by stepwise logistic regression. Calcium and potassium levels, age, gender, co-medications and the presence of relevant cardiovascular

diseases and/or diabetes mellitus were tested as potentially informative variables in the analysis since they are known to influence the QTc interval and the risk of developing drug induced LQTS.[26] Both baseline and on therapy calcium and potassium levels were discretized into three levels (*hypo-*, *normo-* and *hyper-*) according to lab-specific normality ranges and analysed as categorical variables. Ethnicity, time-lapse between baseline ECG and start of TKI treatment, tumor type and recruitment center were considered potential confounders when evaluating multivariate models. The significance threshold was set to *P*-value < 0.05, all tests were two-tailed. The statistical software R version 3.0.1 was used for all statistical analyses (http://www.r-project.org/).

RESULTS

Patient characteristics

After applying inclusion and exclusion criteria, our study cohort consisted of 363 patients. The median age at start of treatment was 60 years (interquartile range [IQR] 51-67), and 59% of patients was male. Sunitinib was the most frequently used TKI in our study cohort, with a total of 110 treated patients. The median QTc-interval at baseline visit was 401 ms (IQR 388-415), where 346 patients (95.3%) had a normal QTc-interval (CTCAE grade 0), 14 (3.9%) had grade 1, 2 patients (0.6%) had grade 2, and 1 patient (0.3%) had a grade 3 QTc-interval. A total number of 37 patients (10.2%) had a known history of relevant CVD, while 34 patients (9.4%) used co-medication, that can lead to QTc-interval prolongation.[15] Patients' demographics and disease characteristics are presented in **Table 1**.

Variables modulating QTc-interval at baseline visit

At baseline measurements, QTc was slightly, but significantly longer in females than in males ($QTc_{females}$ = 404 ms [IQR 392-417] *vs*. QTc_{males} = 399 ms [IQR 385-414], *P* = 0.027), which is consistent with previous studies.[15] Patients treated with co-medication known to prolong the QTc-interval, such as anti-depressants, anti-epileptics and anti-emetics, had a statistically significant higher baseline QTc-interval than the patients who did not use such co-medication (409 ms [IQR 398-424] *vs*. 400 ms [IQR 387-414] respectively, *P* = 0.035). Consistent with previous studies, patients suffering from hypokalaemia had longer median QTc-intervals than patients with normokalaemia and hyperkalaemia (median QTc in hypokalaemic, normokalaemic and hyperkalaemic patients 416 ms [IQR 376-431], 401 ms [IQR 389-415] and 391 ms [IQR 381-408], *P* = 0.028, respectively). A more detailed report about baseline QTc-intervals according to the evaluated variables is found in **Supplementary Table 1**.

Table 1 Patient characteristics

Variable	N (%) or Median (IQR)
Gender	
Male	215 (59)
Female	148 (41)
Age (years)	60 (51-67)
QTc-interval baseline	401 (388-415)
QTc-interval therapy	415 (397-431)
Tumour type	
RCC	101 (27.8)
GIST	49 (13.5)
HCC	45 (12.4)
Lung cancer	27 (7.4)
Breast cancer	16 (4.4)
Melanoma	69 (19.0)
Other	56 (15.4)
Type of TKI	
Sunitinib	110 (30.3)
Vemurafenib	67 (18.5)
Sorafenib	52 (14.3)
Pazopanib	46 (12.7)
Imatinib	41 (11.3)
Erlotinib	21 (5.8)
Lapatinib	16 (4.4)
Gefitinib	10 (2.8)
WHO PS baseline	
0	155 (42.7)
1	195 (53.7)
2	12 (3.3)
3	1 (0.3)
CVD	37 (10.2)
QTc co-medications	34 (9.4)
Race	
Caucasian	349 (96.1)
Other	14 (3.5)
Site	
EMC	184 (50.7)
NKI	118 (32.5)
LUMC	54 (14.9)
SMF	7 (1.9)

Abbreviations: GIST, gastro-intestinal stromal tumour; RCC, renal cell cancer; HCC, hepatocellular carcinoma; TKI, tyrosine kinase inhibitor; WHO PS, world health organisation performance score; CVD, cardiovascular disease; EMC, Erasmus MC - Cancer Institute; NKI, Netherlands Cancer Institute – Antoni van Leeuwenhoek; LUMC, Leiden University Medical Centre; SMF, Salvatore Maugeri Foundation

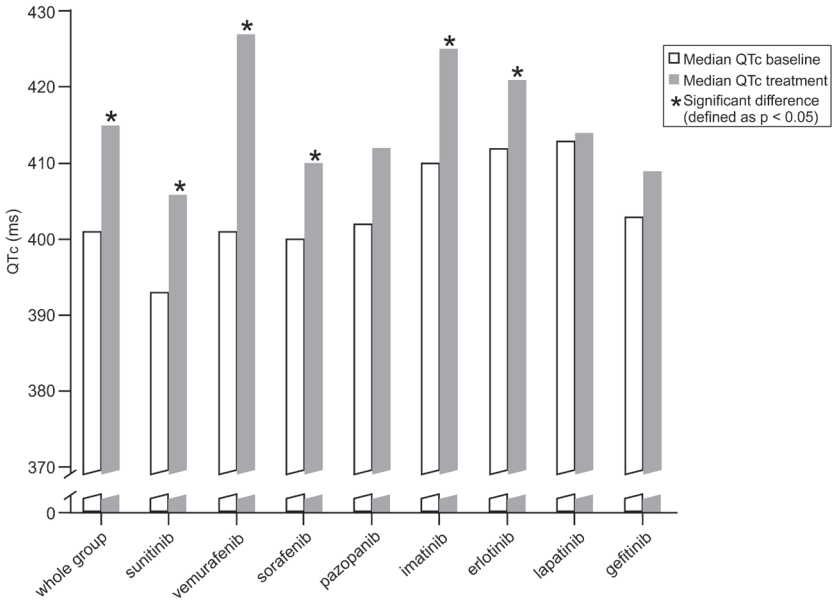

Figure 1: ΔQTc in the whole cohort and in specific TKIs.

White bars represent the median baseline QTc-interval, grey bars represent the median QTc-interval during treatment. At the y-axis, the QTc-interval is expressed in ms.

Quantitative variations of the QTc-interval

Start of any type of TKIs resulted in a statistically significant increase in QTc-interval, with a median ΔQTc of +11 ms ($P < 0.00001$). The distribution of ΔQTc was significantly different across TKIs ($P = 0.0001$). When analysing subgroups of patients treated with specific TKIs, patients treated with sunitinib ($N = 110$), vemurafenib ($N = 67$), sorafenib ($N = 52$), imatinib ($N = 41$) and erlotinib ($N = 21$) showed a statistically significant increase in QTc-interval after start of treatment (median ΔQTc ranging from +7 to +24 ms, $P < 0.004$; **Figure 1**). For lapatinib ($N = 16$) and pazopanib ($N = 46$) no statistically significant increase in QTc-interval after start of treatment was found, probably due to low patient numbers (**Table 2**).

Table 2 TKI-induced change in QTc-interval

		QTc-Interval (ms)		
		Median (IQR)		*P*-value
TKI	**n**	**Baseline**	**Therapy**	
Whole	363	401 (388-415)	415 (397-431)	**< 0.00001**
Sunitinib	110	393 (380-410)	406 (390-424)	**< 0.00001**
Vemurafenib	67	401 (394-417)	427 (415-442)	**< 0.00001**
Sorafenib	52	400 (386-412)	410 (394-425)	**0.0004**
Pazopanib	46	402 (390-411)	412 (395-431)	0.079
Imatinib	41	410 (396-424)	425 (410-439)	**0.002**
Erlotinib	21	412 (398-430)	421 (414-440)	**0.004**
Lapatinib	16	413 (405-423)	414 (397-428)	0.982
Gefitinib	10	403 (396-417)	409 (390-429)	0.919

Abbreviations: TKI, tyrosine kinase inhibitor; n, number of patients within each TKI group; ΔQTc, median difference between QTc-interval during TKI treatment and QTc-interval at baseline

Increase in CTCAE grade and prevalence of high-risk patients

A statistically significant increase in CTCAE grade for QTc-intervals was observed after start of TKI therapy in the whole cohort (*P* = 0.0003). In detail, 33 patients (9.1%) were characterized by a worsened CTCAE grade. Of these, 31 passed from grade = 0 to grade ≥ 1, while the remaining 2 individuals passed from grade = 1 to grade 2 or 3. Of the remaining patients, 321 (88.4%) did not have an increase or decrease in CTCAE grade after start of TKI treatment, while 9 patients (2.5%) had a reduced CTCAE grade for QTc-interval (**Table 3**).

Similarly, a statistically significant increase in the prevalence of high risk patients was observed after TKI therapy start ($QTc_{baseline}$ ≥ 470 ms = 1.7% *vs.* $QTc_{therapy}$ ≥ 470 ms = 5.8%, *P* = 0.005), with 20 individuals (5.5%) who transitioned from a low-risk to a high-risk condition. Moreover, five patients (1.4%) developed QTc ≥ 500 ms (CTCAE grade 3) after therapy start, and 76 patients (20.9%) experienced a clinically relevant QTc increase after TKI start. All five patients who developed QTc ≥ 500 ms after start of therapy had a ΔQTc ≥ 100 ms.

When focusing on specific TKI subgroups, we observed that individuals treated with vemurafenib (*N* = 67) were characterized by a statistically significant increase both in terms of CTCAE grade for QTc-intervals (*P* = 0.008) and in the probability of becoming high risk patients (*P* = 0.023), also showing the greatest probability of clinically relevant QTc increase (34.3%). No statistically significant variations in the evaluated outcomes were observed in the other TKI subgroups.

Table 3 Increase in CTCAE grade and prevalence of high-risk patients

TKI	N	ΔQTc ≥ 30 ms	Change in CTCAE Grade after start of therapy				QTc ≥ 470		
		N (%)	Increased N(%)	Unchanged N(%)	Reduced N(%)	*P*-value	Baseline N(%)	Therapy N(%)	*P*-value
Whole	363	76 (20.9)	33 (9.1)	321 (88.4)	9 (2.5)	**0.0003**	6 (1.7)	21 (5.8)	**0.005**
Sunitinib	110	22 (20.0)	4 (3.6)	104 (95.6)	2 (1.8)	0.746	1 (0.9)	3 (2.7)	0.617
Vemurafenib	67	23 (34.3)	9 (13.4)	58 (86.6)	0 (0)	**0.008**	1 (1.5)	8 (11.9)	**0.023**
Sorafenib	52	11 (21.2)	6 (11.6)	45 (86.6)	1 (1.9)	0.073	1 (1.9)	2 (3.9)	1
Pazopanib	46	6 (13.0)	3 (6.5)	41 (89.1)	2 (4.4)	0.410	1 (2.2)	2 (4.4)	1
Imatinib	41	8 (19.5)	5 (12.2)	34 (82.9)	2 (4.9)	0.430	1 (2.4)	1 (2.4)	1
Erlotinib	21	3 (14.3)	3 (14.3)	18 (85.7)	0 (0)	0.174	0 (0)	2 (9.5)	NA
Lapatinib	16	1 (6.3)	1 (6.3)	14 (87.5)	1 (6.3)	1	1 (6.3)	1 (6.3)	1
Gefitinib	10	2 (20.0)	2 (20.0)	7 (70.0)	1 (10.0)	0.423	0 (0)	2 (20.0)	NA

Abbreviations: TKI, tyrosine kinase inhibitor; N, number of patients; ΔQTc, difference between QTc-interval during TKI treatment and QTc-interval at baseline measurement; CTCAE, common terminology criteria for adverse events; NA, not applicable

Characterization of TKI-induced QTc variability

The median age of patients who did have a worsening of the CTCAE grade for QTc-interval was significantly higher than that of patients who did not (62 years [IQR 59-72] *vs.* 60 years [IQR 51-67] respectively, *P* = 0.023). These patients also more often suffered from hypokalaemia (20.7% *vs*. 3.1%, *P* = 0.0009; **Table 4**). Multivariate logistic regression confirmed that age and hypokalaemia were independent predictors of worsened CTCAE grade for QTc-interval (OR = 1.10, 95% CI = 1.05-1.16, *P* = 0.0002 and OR = 10.30, 95% CI = 2.22-4.64, *P* = 0.002).

Similarly, patients who did became at high risk for developing ventricular arrhythmia after start of TKI treatment were significantly older than patients who did not (66 years [IQR 60-76] and 60 years [IQR 51-66] respectively, *P* = 0.007) and were more frequently treated with QTc-prolonging co-medication (25% *vs*. 8.5%, *P* = 0.030). This was confirmed by multivariate logistic regression (OR = 1.10, 95% CI = 1.04-1.15, *P* = 0.0004 and OR = 4.38, 95% CI = 1.14-15.25, *P* = 0.023).

We did not identify variables that have a statistically significant impact on quantitative ΔQTc or on the probability of clinically relevant ΔQTc (**Supplementary Table 2**).

Table 4 Variables influencing CTCAE grade for QTc-interval and the probability to become a high risk patient

Variable		N	CTCAE Grade During TKI			Transition to High Risk (QTc ≥ 470 ms)		
			Increased (n = 33) median (IQR) or N (%)	Unchanged / Reduced (n = 330) median (IQR) or N (%)	*P*-value	Yes (n = 20) median (IQR) or N (%)	No (n = 343) median (IQR) or N (%)	*P*-value
Age (years)		363	62 (59-72)	60 (51-67)	**0.026**	66 (60-76)	60 (51-66)	**0.007**
Gender	Males	215	22 (66.7)	193 (58.5)	0.458	14 (70)	201 (58.6)	0.358
	Females	148	11 (33.3)	137 (41.5)		6 (30)	142 (41.4)	
Co-medication	Yes	34	6 (18.2)	28 (8.5)	0.107	5 (25)	29 (8.4)	**0.030**
	No	329	27 (81.8)	302 (91.5)		15 (75)	314 (91.6)	
CVD	Yes	37	5 (15.2)	32 (9.7)	0.360	4 (20)	33 (9.6)	0.134
	No	326	28 (84.9)	298 (90.3)		16 (80)	310 (90.4)	
DM	Yes	43	3 (9.1)	40 (12.1)	0.782	1 (5)	42 (12.2)	0.489
	No	320	30 (90.9)	290 (87.9)		19 (95)	301 (87.8)	
Ca^{2+}	Normo/Hyper	169	13 (61.9)	156 (71.6)	0.451	8 (80)	161 (70.3)	0.728
	Hypo	70	8 (38.1)	62 (28.4)		2 (20)	68 (29.7)	
K^{+}	Normo/Hyper	304	23 (79.3)	281 (96.9)	**0.0009**	16 (88.9)	288 (95.7)	0.204
	Hypo	15	6 (20.7)	9 (3.1)		2 (11.1)	13 (4.3)	

Abbreviations: CVD; cardiovascular disease; DM, diabetes mellitus; Ca^{2+}, calcium level at time of QTc measurement during treatment; K^{+}, potassium level at time of QTc measurement during treatment; N, number of analysed patients with non-missing values; CTCAE, common terminology criteria for adverse events; TKI, tyrosine kinase inhibitor; IQR, interquartile range; TKI, tyrosine kinase inhibitor

DISCUSSION

We found a significant increase in QTc-intervals after start of treatment with sunitinib, vemurafenib, sorafenib, imatinib and erlotinib. In most cases, the increase in QTc-interval is only modest and under normal conditions not clinically relevant. However, in 76 of the 363 patients the start of TKI treatment resulted in a clinically relevant increase of the QTc-interval. Still, only for vemurafenib treated patients and for the whole group of patients taken together, the incidence of high risk patients, defined as QTc ≥ 470 ms,[3] increased during treatment.

These findings suggest that although a substantial part of the treated patients have a clinically relevant increase in QTc-interval, this occurs mostly in patients with low QTc-interval at the start of treatment, and only in a small proportion of patients leads to a high risk of developing arrhythmias. Unfortunately it is not possible to differentiate which patient is at risk at the start of treatment.

Therefore, treating physicians should anticipate on this phenomenon by performing ECGs during treatment with TKI, and being aware of symptoms, such as palpitation, seizures and collapse, which may be the result of long QTc syndrome (LQTS). In those diseases where alternative treatment are available, such as in metastatic renal cell carcinoma where sunitinib and pazopanib have equivalent efficacy,[22] switch to a less cardio-toxic TKI may be possible in case of the occurrence of severe LQTS.

Furthermore, many patients use co-medication during TKI treatment. As drugs of a broad variety are known for drug induced QTc-interval prolongation, it is likely that patients use several drugs which can lead to QTc-interval prolongation and thereby intensifying the effect on the QTc-interval. This was shown in this study, where patients using such co-medication were more likely to become a high risk individual. In those cases, extra awareness may be necessary and switching towards drugs that are not likely to have an effect on QTc-interval should be considered.

This study has several limitations. This was a retrospective study in patients treated with cancer, and therefore in most cases ECGs were not made on predefined times before, during and after TKI therapy. Since fluctuations in QTc-interval are frequent and may be caused by many factors,[4, 5] this is a weakness of our study, and may have influenced outcome.[23] Furthermore, there may be a bias in patient selection since patients with cardiac events may be more likely to have had ECGs performed. Patients who died from arrhythmia may not have been included in analyses when no ECGs were available. Possible effects from electrolyte disorders on the QTc-interval may have been missed, because of missing data. However, we showed in a large group of patients treated with TKIs that there is an overall increase in QTc-interval after start of treatment, which may possibly be harmful for patients treated with these drugs. Future prospective studies could improve the current knowledge about TKI-induced QTc prolongation.

Overall, we may conclude that most TKIs tend to cause an increase in QTc-intervals. In some cases, this increase is clinically relevant, and therefore the QTc-interval should be verified in patients before starting TKI treatment and during therapy. Monitoring QTc-intervals during TKI treatment is particularly important in patients with a history of QTc-interval prolongation, in patients using co-medication which can prolong the QTc-interval, in patients with electrolyte disorders and in patients

with pre-existing CVD. Furthermore, during treatment with TKIs physicians should be aware of clinical symptoms, which may be attributed to QTc-interval prolongation.

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Supplementary Table 1 Impact of predictors and confounders on baseline QTc interval

Variable	N	Statistic rho or median QTc (IQR)	*P*-value
Age (years)	363	0.01	0.8532
Gender			**0.0266**
Male	215	399 (385-414)	
Female	148	404 (392-417)	
Co-medication			**0.0349**
No	329	400 (387-414)	
Yes	34	409 (398-424)	
CVD			0.0792
No	326	400 (388-415)	
Yes	37	408 (396-429)	
DM			0.628
No	320	401 (389-415)	
Yes	43	401 (384-415)	
Baseline Ca^{2+}			0.5699
Hypocalciemie	22	409 (385-418)	
Normocalciemie	193	400 (388-411)	
Hypercalciemie	10	391 (383-411)	
Baseline K^{+}			**0.0276**
Hypokaliemie	3	416 (376-431)	
Normokaliemie	255	401 (389-415)	
Hyperkaliemie	41	391 (381-408)	

Abbreviations: CVD, cardiovascular disease; DM, diabetes mellitus; Ca^{2+}, calcium; K^{+}, potassium; n, number of patients

Supplementary Table 2 Impact of predictors and confounders on outcome measures

Variable	N	ΔQTc		ΔQTc≥ 30 ms			Transition to high risk (QTc≥470ms)			CTCAE grading scale		
				Yes	No		Yes	No		Increased		
		Median (IQR)	*P*-value	Median (IQR) or N (%)	Median (IQR) or N (%)	*P*-value	Median (IQR) or N (%)	Median (IQR) or N (%)	*P*-value	Median (IQR) or N (%)	Unchanged/ decreased Median (IQR) or N (%)	*P*-value
Age (years)	363	0	0.9519	60 (53-70)	60 (51-66)	0.1263	66 (60-76)	60 (51-66)	0.0072	62 (59-72)	60 (51-67)	0.0263
Gender												
Male	215	12 (-3-28)	0.1774	47 (61.8)	168 (58.5)	0.6940	14 (70)	201 (58.6)	0.3578	22 (66.7)	193 (58.5)	0.4582
Female	148	10 (-4-24)		29 (38.2)	119 (41.5)		6 (30)	142 (41.4)		11 (33.3)	137 (41.5)	
Co-medication												
No	329	12 (-3-25)	0.7915	65 (85.5)	264 (92.0)	0.1181	15 (75)	314 (91.6)	0.0296	27 (81.8)	302 (91.5)	0.1066
Yes	34	9 (-3-34)		11 (14.5)	23 (8.0)		5 (25)	29 (8.4)		6 (18.2)	28 (8.5)	
CVD												
No	326	11 (-3-26)	0.7729	68 (89.5)	258 (89.9)	1	16 (80)	310 (90.4)	0.1343	28 (84.9)	298 (90.3)	0.3598
Yes	37	12 (-4-24)		8 (10.5)	29 (10.1)		4 (20)	33 (9.6)		5 (15.1)	32 (9.7)	
DM												
No	320	11 (-3-25)	0.7651	65 (85.5)	255 (88.9)	0.4275	19 (95)	301 (87.8)	0.4893	30 (90.9)	290 (87.9)	0.7816
Yes	43	11 (-1-30)		11(14.5)	32 (11.1)		1 (5)	42 (12.2)		3 (9.1)	40 (12.1)	
Ca^{2+}												
Normo/hyper	169	10 (-3-24)	0.9434	30 (71.4)	139 (70.6)	1	8 (80)	161 (70.3)	0.7277	13 (61.9)	156 (71.6)	0.4510
Hypo	70	14 (-5-23)		12 (28.6)	58 (29.4)		2 (20)	68 (29.7)		8 (38.1)	62 (28.4)	
K^+												
Normo/hyper	304	11 (-3-24)	0.2419	60 (92.3)	244 (96.1)	0.1992	16 (88.9)	288 (95.6)	0.2042	23 (79.3)	281 (96.9)	0.0009
Hypo	15	21 (3-42)		5 (7.7)	10 (3.9)		2 (11.1)	13 (4.3)		6 (20.7)	9 (3.1)	

Abbreviations: CVD, cardiovascular disease; DM, diabetes mellitus; Ca^{2+}, calcium; K^+, potassium; N, number of patients; IQR, interquartile range; ΔQTc, difference between QTc interval during treatment with a tyrosine kinase inhibitor and QTc interval at baseline measurement; CTCAE, common terminology criteria for adverse events

Chapter 8

Macrocytosis as a potential parameter associated with outcome in the treatment with Tyrosine Kinase Inhibitors

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Submitted

ABSTRACT

Introduction A rise in mean corpuscular volume (MCV) of the erythrocyte is frequently seen during the treatment with the tyrosine kinase inhibitors (TKIs) imatinib and sunitinib. We investigated whether macrocytosis also occurs as a class-effect in other TKIs and whether occurrence of macrocytosis is associated with outcome.

Methods In this retrospective study in 533 patients with solid tumours using imatinib, pazopanib, sorafenib, sunitinib or vemurafenib, we investigated levels of MCV prior to and during the treatment with these TKIs. Macrocytosis and MCV increases were correlated to progression free survival (PFS) and overall survival (OS) in specific tumour-treatment combinations.

Results Patients treated with imatinib, pazopanib or sunitinib had a significant increase in MCV levels starting 3 months after start of treatment and plateauing at 6 months ($p < 0.001$). Macrocytosis as well as a substantial increase in MCV (from baseline of +10fL (ΔMCV +10fL)), when included as a time-dependent covariate, were associated with improved OS in patients with renal cell cancer (RCC) treated with sunitinib ($p \leq 0.001$), but not in other cohorts. Also, when corrected for the time to onset of macrocytosis or time to ΔMCV +10fL, this correlation remained significant for OS (macrocytosis HR = 0.61, 95%CI 0.39-0.96, p = 0.031, ΔMCV +10fL HR = 0.58, 95%CI 0.37-0.90, p = 0.016).

Conclusions These observations show that imatinib, pazopanib and sunitinib induce an increase in MCV. The finding that occurrence of macrocytosis is associated with a good outcome to sunitinib in patients with RCC is worthwhile to explore further.

INTRODUCTION

Tyrosine kinase inhibitors (TKIs) are frequently used in anticancer treatment. They act by targeting the ATP-binding site of one or more tyrosine kinases, that are frequently constitutively activated in cancer cells.[1] Since the introduction of imatinib as targeted therapy in the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GISTs), several TKIs have been registered for the treatment of cancer patients.

In patients treated with imatinib or sunitinib the mean corpuscular volume (MCV) of the red blood cells increases.[2-8] In several cases this even resulted in macrocytosis (defined as MCV > 100 fL), which is usually only seen in patients with vitamin B12 or folic acid deficiency, alcoholism, hypothyroidism and myelodysplastic syndromes.[9] Since TKI-induced macrocytosis can occur in the absence of such conditions,[6] increase in MCV levels are considered a direct (side-)effect of the drug itself, as is seen with other drugs (*i.e.* hydroxyurea, methotrexate, zidovudine, azathioprine, capecitabine, cladribine and stavudine).[10-15] The increase in MCV levels in patients treated with imatinib or sunitinib may be caused by inhibition of the stem cell factor (c-KIT).[7] C-KIT is expressed on the surface of hematopoietic progenitor cells, where it regulates survival, proliferation and differentiation of the red blood cells by the activation of signal transduction molecules.[16] Interference with the c-KIT pathway in rodents resulted in macrocytic anemia.[17] Additionally, treatment with sorafenib, which is a weaker inhibitor of c-KIT than imatinib and sunitinib, did not result in macrocytosis.[7]

Whether macrocytosis is a clinically relevant side-effect of TKIs remains unknown. Furthermore, it is not known which TKIs, other than imatinib and sunitinib, give rise to an increase in the MCV. To study this, we set up an exploratory retrospective study in which we collected data from patients with solid tumors who have been treated with the TKIs imatinib, pazopanib, sorafenib, sunitinib or vemurafenib at the department of Medical Oncology of the Erasmus MC Cancer Institute, examined whether the use of these drugs was accompanied by a MCV rise, and if so, whether or not this was associated with outcome in terms of progression-free and overall survival.

PATIENTS AND METHODS

Study design

Patients who have had a solid tumor and have been treated with one of these 5 TKIs were included in this study if at least one baseline MCV level and one MCV level during treatment were available. Patients with subsequent use of TKIs were included as unique cases for each TKI if the time between treatments was at least one week and if a baseline MCV level was measured within this off-treatment period.

Data were retrospectively collected from the electronic patient record. Patient characteristics were collected as well as survival estimates and laboratory values. The variables considered to be relevant for the analysis were hematological parameters (hemoglobin [Hb], MCV, and red cell distribution width [RDW]). In addition to their baseline level, levels were collected during the first year of treatment or until date of stop if this occurred within a year after start of treatment. Due to the retrospective character of this study, no written informed consent was necessary from patients.

Statistical design and data analysis

One-way analysis of variance (ANOVA) test was used to discover any baseline differences between TKI treatments. For each TKI, and each individual patient, differences between baseline and corresponding on-treatment laboratory values were analyzed by using the paired-sample Student's *t*-test. To detect differences in the occurrence of baseline macrocytosis and on-treatment macrocytosis, the McNemar's test was used.

PFS was defined as the time between first day of treatment and the day of progressive disease (PD) or death from any cause, whichever came first. OS was defined as the time between the first day of treatment and the date of death from any cause. If progression or death had not occurred or if the patient was lost to follow-up, PFS and OS were censored at the date of last follow-up. Macrocytosis and a difference in MCV between baseline and on-treatment (ΔMCV) of more than +10 fL were included as a time-dependent covariate for PFS and OS in a univariate Cox regression analysis in the diverse specific indications for TKI treatment.

The statistical package software system version 21.0 (SPSS Inc, Chicago, IL) was used for all data analyses, except the time-dependent COX-regression analysis which was performed using STATA version 13.1 (StataCorp. 2013. Stata: Release 13. Statistical Software. College Station, TX: StataCorp LP). Results from statistical analyses with a two-sided p-value ≤ 0.05 were considered statistically significant in all analyses.

RESULTS

Study population and baseline variables

A total of 533 individual patients with a solid tumor for which the patient has been treated with imatinib, pazopanib, sorafenib, sunitinib, or vemurafenib were included in this study. There were 45 patients with subsequent use of 2 TKIs (usually imatinib followed by sunitinib in patients with GIST), and 2 patients with subsequent use of 3 TKIs, resulting in 582 cases with the use of a TKI. Sunitinib and imatinib were the most frequently used TKIs in the study population, with 213 and 182 cases respectively. Other TKIs were sorafenib (n=97), pazopanib (n=66) and vemurafenib (n=24). The median amount of MCV measurements in the entire population during the first year of treatment was 9 (IQR 5-13). The mean age at start of the TKI was 61 years, and 64% of patients was male (**Table 1**). The mean time of follow-up was 27.2 months.

At baseline visit, the mean MCV was 88.8 ± 6.6 fL, and there was no significant difference in baseline MCV among the TKIs used ($p = 0.29$). Twenty patients already had macrocytosis at baseline. None of these 20 patients was known with vitamin B12 or folic acid deficiency or with hypothyroidism, but 4 out of 20 patients used 3 or more alcohol units/day.

Changes in laboratory levels after start of treatment

The mean on-treatment MCV, calculated per patient from all on treatment MCV values, was significantly higher than baseline MCV for patients who have been treated with imatinib (mean ΔMCV = 4.47 fL, $p < 0.001$), pazopanib (mean ΔMCV = 2.13, $p < 0.001$) and sunitinib (mean ΔMCV = 4.78, $p < 0.001$, see **Table 2**). When MCV levels from the first year were plotted, it was seen that for imatinib, pazopanib and sunitinib, the MCV remained more or less stable within the first 3 months of treatment, and increased thereafter. For sorafenib and vemurafenib treated patients however, MCV significantly decreased within the first 3 months of treatment, but also showed a slight increase after 3 months of treatment (**Figure 1-A**). In **Figure 1-B** it is shown that RDW increases within the first months after start of treatment with imatinib, pazopanib and sunitinib, indicating an increased spread in the volume of erythrocytes. After 3 months the RDW decreases again. This was statistically significant for all TKIs.
The prevalence of macrocytosis was statistically significantly higher during the treatment with imatinib, pazopanib or sunitinib treatment, than at baseline ($p < 0.001$), but not for patients treated with sorafenib or vemurafenib. Hemoglobin levels significantly differed between baseline and on-treatment measurement for patients treated with imatinib, sorafenib and vemurafenib, but differences were small.

Table 1 Descriptive characteristics

Variable	N (%) or Mean (sd)
Gender	
Male	339 (63.6)
Female	194 (36.4)
Age	60.9 (11.2)
No of subsequent TKI treatments	
1	486 (91.2)
2	45 (8.4)
3	2 (0.4)
Type of TKI*	
Imatinib	182 (31.3)
Pazopanib	66 (11.3)
Sorafenib	97 (16.7)
Sunitinib	213 36.6)
Vemurafenib	24 (4.1)
Tumour type	
Colorectal	14 (2.6)
GIST	186 (34.9)
HCC	81 (15.2)
pNET	4 (0.8)
RCC	161 (30.2)
Sarcoma	6 (1.1)
Other	54 (10.1)
MCV baseline	88.8 (6.6)
MCV on-treatment	91.9 (6.6)
Reticulocytes baseline	61.0 (28.6)
Reticulocytes on-treatment	57.9 (23.6)
RDW baseline	14.7 (1.9)
RDW on-treatment	15.8 (1.9)

Abbreviations: No, number; TKI, tyrosine kinase inhibitor, GIST, gastrointestinal stromal tumour, HCC, hepatocellular carcinoma, pNET, neuro-endocrine tumour of the pancreas; RCC, renal cell carcinoma; MCV, mean corpuscular volume (of the erythrocyte); RDW, red cell distribution width. On-treatment values are expressed as the mean of all

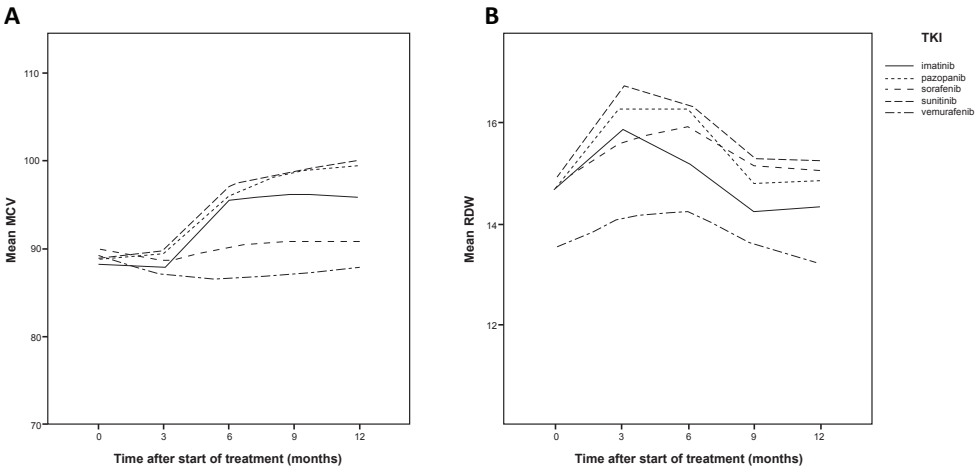

Figure 1 The course of MCV levels (Figure 1A) and RDW levels (Figure 1B) during the first 12 months of TKI treatment.

Table 2 Comparison between baseline and on-treatment levels

TKI	Variable	Baseline Mean (sd) or N (%)	On-treatment Mean (sd) or N (%)	P-value
Imatinib	Hb (mmol/L)	8.0 (1.16)	7.7 (0.85)	<0.01
	MCV (fL)	88.3 (6.41)	92.8 (5.36)	<0.01
	Macrocytosis (N (%))	5 (2.7)	45 (24.7)	<0.01
	RDW	14.6 (2.0)	15.0 (1.37)	<0.01
Pazopanib	Hb (mmol/L)	7.7 (1.09)	7.8 (1.20)	0.26
	MCV (fL)	88.7 (6.85)	90.9 (7.77)	<0.01
	Macrocytosis	3 (4.5)	20 (30.3)	<0.01
	RDW	14.7 (1.68)	16.2 (1.75)	<0.01
Sorafenib	Hb (mmol/L)	8.3 (1.04)	8.5 (1.07)	0.01
	MCV (fL)	90.1 (7.54)	89.0 (7.25)	<0.01
	Macrocytosis	5 (5.5)	9 (9.3)	0.29
	RDW	14.9 (1.94)	15.7 (2.12)	<0.01
Sunitinib	Hb (mmol/L)	7.6 (1.20)	7.7 (0.93)	0.62
	MCV (fL)	88.7 (6.44)	93.4 (6.41)	<0.01
	Macrocytosis	7 (3.3)	94 (44.1)	<0.01
	RDW	14.9 (2.02)	16.5 (1.87)	<0.01
Vemurafenib	Hb (mmol/L)	8.7 (1.09)	8.3 (1.17)	<0.01
	MCV (fL)	89.2 (4.35)	86.8 (4.89)	<0.01
	Macrocytosis	0 (0)	0 (0)	NA
	RDW	13.5 (0.90)	14.1 (0.88)	0.01

Survival analyses

Because in most patients MCV or ΔMCV > +10fL occurred somewhere after start of treatment, these parameters had to be included as time-dependent covariates in univariate Cox regression analyses for PFS and OS. In patients with RCC who were treated with sunitinib, MCV (hazard ratio (HR) = 0.61, 95% confidence interval (CI) 0.39-0.96, p = 0.031) as well as ΔMCV +10fL (HR = 0.58, 95% CI 0.37-0.90, p = 0.016) were associated with improved OS (**Figure 2**).

For other tumor-treatment combinations, there were no clear associations between macrocytosis or impact of MCV and ΔMCV +10fL with PFS or OS in univariate analyses.

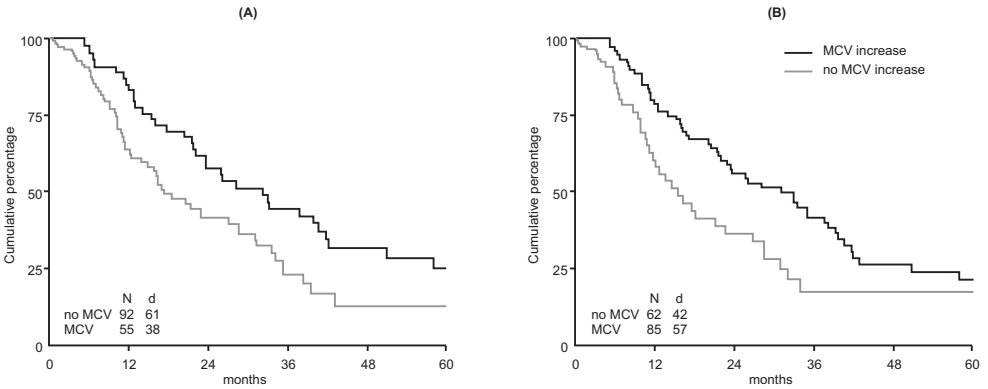

Figure 2 Influence of MCV according to original definition of macrocytoses (A), or defined as increase of ≥ 10 fL (B) on survival in patients with RCC treated with sunitinib. De black line represents patients with macrocytosis or MCV increase of ≥ 10 fL, the grey line represents patients without macrocytosis of MCV increase of ≥ 10 fL.

8

DISCUSSION

This study shows an increase in MCV levels in patients treated with imatinib, pazopanib or sunitinib. For sorafenib and vemurafenib, a rise in MCV is seen after a nadir MCV value in the first 6 months of treatment. In the subgroup of patients with advanced RCC treated with sunitinib, OS was clinically relevantly longer in patients with a rise in MCV of more than 10 fL or if a level above 100 fL (macrocytosis) was reached.

The increase in MCV levels in patients treated with imatinib and sunitinib is in line with previous research in small groups of patients.[2-8] To the best of our knowledge, in patients treated with pazopanib or vemurafenib, MCV levels had never been investigated before. We found an overall decrease in MCV levels in the first year of treatment with sorafenib and vemurafenib. Interestingly, this decrease occurs only in the first three months of treatment, after which in patients treated with sorafenib the MCV levels slowly rise. In the group of vemurafenib treated patients, none of the patients developed macrocytosis, nor an increase in MCV > 10fL. In patients treated with imatinib, pazopanib or sunitinib, the rise in MCV

levels generally occurs roughly after 3 months of treatment, without a decrease in the first 3 months.

Importantly, a patients needs to be exposed for a certain period of time to a TKI before a rise in MCV occurs. In the entire population, the time to onset of macrocytosis was 4.3 months. In tumor types such as melanoma and HCC, a substantial part of the population has already progressed at this stage, and as a result, may never have got the chance to develop macrocytosis or a substantial increase in the MCV levels.

Thus far it remains unknown how the effect of imatinib and sunitinib on the MCV occurs. The hypothesis was that binding of the TKI to c-KIT on the hematopoietic stem cell resulted in disturbed cell maturation and the release of larger erythrocytes into the blood stream.[7] Our study was not designed to detect any mechanism behind TKI-induced macrocytosis. Still, we found increases in MCV levels in imatinib, pazopanib and sunitinib, which are all inhibitors of c-KIT. Sorafenib is also an inhibitor of c-KIT, but sorafenib treated patients did not show a significant increase in MCV levels, possibly due to a drop in MCV during the first months of treatment. Most of the patients treated with sorafenib (83%) were treated for HCC, and the median time of treatment was 4.3 months. MCV levels significantly decreased in the first 3 months of treatment, and significantly rose thereafter. However, 3 months after initiation of sorafenib only 61% of patients was still being treated with sorafenib, and at 6 months this was 34%, which may have caused a bias in the analysis.

Side-effects of TKI-treatment, such as an increase in blood pressure in RCC patients treated with sunitinib and rash in patients treated with anti-EGFR function, are known for their prognostic abilities in these treatments.[18-20] One of the aims of our study was to investigate if macrocytosis/ MCV rise is associated with survival in specific subgroups of patients, which indeed appeared to be the case for overall survival in patients with RCC treated with sunitinib. Patients who developed macrocytosis had a significantly longer OS than patients with normocytosis or microcytosis. In the same group of patients, an increase in MCV levels from baseline to on-treatment of > 10 fL was associated with prolonged OS. Clearly, the correlations found have to be validated in a prospective setting. For other tumor-TKI combinations there was no significant difference in survival between patients with and without macrocytosis or substantial increase in MCV levels from baseline.

Since the effect of TKIs on the MCV is speculated to be driven by c-KIT, it was expected that the effects on survival would be seen in patients with GIST, since the anti-tumor effect of imatinib and sunitinib in GIST is driven by c-KIT. The anti-tumor effect of pazopanib and sunitinib in patients with RCC is caused by blocking of vascular endothelial growth factor (VEGF), and not by blocking c-KIT. These results suggest another, yet unknown, mechanism by which MCV is increased by TKIs.

Altogether we may conclude that a rise in MCV or even macrocytosis occurs in the treatment of several TKIs. In the treatment of patients with RCC with sunitinib, the occurrence of macrocytosis, or a substantial increase in MCV levels after start of treatment, could potentially serve as a positive prognostic factor, if validated prospectively.

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Chapter 9

Summary

For many drugs there is a broad inter-patient variability in the pharmacokinetic (PK) processes absorption, distribution, metabolism and excretion (ADME), which results in a certain amount of inter-individual variability in exposure to these drugs. Differences in the activity of ADME may be caused by several factors, such as the use of co-medication, genetic differences, lifestyle habits and probably many more (currently unknown) factors. The tyrosine kinase inhibitor sunitinib is such a drug.
Although there is an average 30-35% inter-patient variability in both pharmacokinetics and pharmacodynamics for sunitinib, it is (still) prescribed in a fixed dosing schedule. Severe side effects occur frequently in sunitinib treatment and not occasionally patients require dose reductions or discontinuations due to adverse effects. Furthermore, not all patients respond similarly to sunitinib treatment. These observations suggest that therapeutic drug monitoring (TDM) could add to further personalize sunitinib treatment. The work described in the first part of this thesis includes research that focuses on a pharmacokinetic approach towards improved sunitinib treatment (chapters 2 to 4).

Thesis part I

In **chapter 2**, hepatobiliary clearance of technetium-99m-2-methoxy isobutyl isonitrile (^{99m}Tc-MIBI), as a phenotyping probe for the adenosine triphosphate binding cassette transporter ABCB1 and midazolam clearance test as a probe for cytochrome P450 3A (CYP3A) were correlated to the pharmacokinetics of sunitinib and its active metabolite SU12662. A population pharmacokinetic model was built, which included the phenotype tests as covariate. In 52 patients, both phenotyping probes were insufficient to predict sunitinib pharmacokinetics strongly enough to be useful in clinical practice. Interestingly, we found that patients with low sunitinib clearance had significantly more frequent severe toxicities, suggesting an exposure-toxicity relationship for sunitinib. Such a relationship suggests that patients who are treated with sunitinib may benefit from TDM of sunitinib. Since it was unknown whether sunitinib dosing based on the pharmacokinetics was safe, a pharmacokinetic study was performed to determine the safety and feasibility of sunitinib dosing based on total trough levels of sunitinib and SU12662 (**chapter 3**). Twenty-nine patients with a broad variety of tumour types for whom no standard therapy was available were administered sunitinib 37.5 mg on a daily base. At 3 times during the study period of 8 weeks, PK sampling of sunitinib and SU12662 took place. The sunitinib dose was elevated when total trough levels of sunitinib and SU12662 dropped below a predefined target level of 50 ng/mL, and sunitinib dose was decreased when patients suffered unacceptable toxicity. In this study period of 8 weeks, it was possible to increase sunitinib dose levels in 5 patients (17%) without additional severe toxicity. Nine patients (31%), who underwent initial dose escalation, required a dose reduction due to toxicity. In the 15 patients who reached target TTL at the starting dose of 37.5 mg, 6 patients (21%) required dose reduction because of toxicity. This could be the basis for future studies and the implementation of a PK-guided dosing strategy in clinical practice.

There is increasing evidence that circadian rhythms in physiology may influence the PK of drugs. However, for TKIs, this has never been investigated before. **Chapter 4** describes the results of preclinical

and clinical studies where the effect of the time of administration of sunitinib on its PK has been investigated. A study was performed in mice, treated with sunitinib at 6 different times of the day. In parallel, a prospective randomized cross-over PK study was performed in which patients took sunitinib once daily at 3 courses at 8AM, 1PM and 6PM. Primary endpoint in both studies was the difference in plasma area under the curve (AUC) between dosing times.

Although a clear ~12-hour rhythm in sunitinib PK was observed in mice, leading to 30% more exposure to the compound when administered around noon than when administered in morning or evening, there was no significant difference in AUC between the three dosing times in patients. However, in patients, sunitinib trough levels were higher when the drug was taken at 1PM or 6PM, than when taken at 8AM resulting in better balanced concentrations of sunitinib during the day. This is interesting, since it could have implications for dosing based on therapeutic drug monitoring if a patient takes sunitinib in the morning, or in the afternoon or evening. Using TDM, a drug can be dosed based on its trough level. If in the case of sunitinib the trough levels are lower when the patient takes the drug in the morning than when taking the drug at noon or in the evening, without differences in overall daily exposure, the dose of sunitinib may falsely be increased in patients taking sunitinib in the morning.

Thesis part II

The second part of this thesis focusses on the pharmacodynamics (both survival and toxicity) of several TKIs. As already mentioned for sunitinib, there is a broad spread in how long patients respond to TKI treatment. To further personalize treatment in patients treated with TKIs, it is meaningful to get better insight into the factors predicting the efficacy of a drug before starting, especially when alternative treatment options exist such as in the case of advanced GIST. A possible approach towards personalized treatment is by predicting treatment response levels based on genetic polymorphisms (SNPs).

The purpose of the study described in **chapter 5** and **chapter 6** was to identify SNPs that can serve as predictive biomarkers for the treatment with respectively first line imatinib and second line sunitinib in patients with advanced GIST. Chapter 5 explores the effects of 36 SNPs in 18 genes related to the PK and PD of imatinib on its efficacy in 255 patients with advanced GIST treated with imatinib. In a multivariate model PFS was significantly shorter in patients synchronous metastasis at the time of the diagnosis GIST, patients with *KIT* exon 9 mutation, and patients with the TT-genotype in *KDR* rs2305948 and the AA genotype in *VEGFA* rs1570360. OS was also significantly shorter in patients with synchronous metastasis at the time of the diagnosis GIST, and further in patients with the AA-genotype in *KDR* rs1870377 or the AA-genotype in *VEGFA* rs1570360, whereas a T-allele in *SLCO1B3* rs4149117 was associated with longer OS.

In chapter 6, we retrospectively studied if 49 SNPS in genes within the pharmacokinetic and pharmacodynamic pathway of sunitinib were associated with PFS and OS in 127 patients with advanced GIST who had been treated with sunitinib. PFS was significantly shorter in carriers of the C-allele in *POR* rs1056878 C/T. The presence of the T-allele in *CYP1A2* rs4149117 G/T, the CCC-CCC alleles in the

SLC22A5 haplotype, and the GC-GC alleles in the *IL4R* haplotype were predictive for OS. When these factors were combined in a favorable genetic model, both PFS and OS were significantly longer with an increasing number of favorable genetic factors. If validated prospectively, this may identify patients that are likely to survive longer on sunitinib and therefore be a useful tool in therapeutic decision making. For now however, it is still too early to use SNPs in clinical practice of sunitinib treatment.

Almost all patients who are treated with a TKI are subject to side-effects of the given drug, varying from modest to severe side effects for which treatment needs to be discontinued or even stopped. The last two chapters of this thesis focus on the occurrence of side effects due to TKI treatment.
Tyrosine kinase inhibitors (TKIs) are associated with prolongation of the QTc-interval on the ECG. QTc-interval prolongation increases the risk for life threatening arrhythmias. However, studies evaluating the effects of TKIs on QTc-intervals are limited and only consist of limited patient numbers. In **chapter 7**, we investigated ECGs prior to and during treatment with erlotinib, gefitinib, imatinib, lapatinib, pazopanib, sorafenib, sunitinib, or vemurafenib. A significant increase in QTc interval was observed in patients treated with sunitinib, vemurafenib, sorafenib, imatinib and erlotinib. Especially patients who had been treated with vemurafenib were found to be at increased risk of developing a QTc ≥ 470 ms, a threshold associated with an increased risk for arrhythmias. These observations indicate that it is advisable to perform ECGs during treatment with these TKIs to prevent patients from arrhythmia.
Another side effect that has been observed in TKI treatment is the increase of the mean corpuscular volume (MCV) of the erythrocyte. This has previously been shown for patients treated with sunitinib and imatinib. For other TKIs, it has never been investigated before. **Chapter 8** describes a study in which we explored which TKIs can cause an increase in MCV or even macrocytosis (MCV >100 fL) and related this to clinical relevance. Therefore, we retrospectively studied >500 patients treated with imatinib, pazopanib, sorafenib, sunitinib or vemurafenib. We found a significant increase in MCV levels in patients treated with imatinib, pazopanib or sunitinib. Patients with renal cell cancer treated with sunitinib appeared to have a longer PFS if they developed macrocytosis than patients who did not develop macrocytosis. This may therefore serve as a potential parameter associated with outcome during treatment with TKIs.

Altogether we may conclude that treatment with TKIs is only ‘personalized’ in a limited way thus far. Many new TKIs are currently being developed and in the nearby future there will probably be several TKI-options available for a specific tumour type, as is already the case for renal cell cancer where both sunitinib and pazopanib are registered in the first line of treatment. Therefore, future research should focus on which patient could be treated best with which TKI. This can be achieved by investigating patient characteristics and tumour characteristics. Many current studies are focussing on tumour characteristics. Different signalling pathways may be found to drive tumour growth even in the same tumour types or in tumours that originate in the same tissue. By selectively inhibiting the pathway that is overexpressed in the tumour, regardless of the tumour type, future anti-cancer treatment may

be improved.

Otherwise, treatment with TKIs may be improved by TDM. The knowledge obtained from the studies described in this thesis focus on the pharmacokinetics of sunitinib, and may be a first step towards personalized sunitinib dosing. However, evidence is limited whether this improves survival. Some retrospective studies have been performed, showing an association between parameters of pharmacokinetics and efficacy and toxicity of treatment, but this has not yet been validated prospectively.

Many studies have been performed in which genetic polymorphisms are associated with survival or toxicity levels. However, this has not yet resulted in implementation of testing genetic polymorphisms in the clinical treatment setting to improve survival or decrease toxicity levels. Since the effects of most genetic polymorphisms on survival or toxicity is small it is of doubt whether this will change in the future. However, more research in the field of pharmacogenetics is required before final conclusions can be drawn. The same is true for other potential markers for efficacy and toxicity.

Appendix 1

Nederlandse samenvatting

Kanker is, na hart- en vaatziekten, wereldwijd de op een na meest voorkomende doodsoorzaak . In 2012 stierven ongeveer 8.4 miljoen mensen aan de gevolgen van kanker. Nog steeds neemt het aantal mensen dat kanker heeft toe. Behandeling met chemotherapie en hormonale therapie heeft in de afgelopen decennia de overleving van patiënten met kanker aanzienlijk verbeterd. In de meest recente jaren hebben de zogenaamde 'biologicals' de overleving van specifieke tumoren nog eens aanzienlijk doen verbeteren, en er wordt verwacht dat dit in de toekomst nog beter wordt. Tyrosine kinase remmers, afgekort TKIs, vallen onder de biologicals. Het zijn middelen die oraal worden ingenomen, meestal op dagelijkse basis.

In 2000 was imatinib de eerste TKI die op de markt kwam voor de behandeling van uitgezaaide (gemetastaseerde) gastro-intestinale stromacel tumoren (GIST). Deze tumoren reageren nauwelijks op de behandeling met conventionele chemotherapie en de overleving van patiënten met deze vorm van kanker was dan ook slecht, zo'n 6 maanden vanaf het moment waarop werd vastgesteld dat de ziekte was uitgezaaid. Nu kan de gemiddelde patiënt 2-2.5 jaar imatinib gebruiken zonder dat de tumor groeit. Indien de tumor tijdens behandeling groeit, is dit een teken dat de GIST niet meer reageert op imatinib. Verder behandelen met imatinib is dan niet meer zinvol. Er kan dan worden overgegaan op een vervolgbehandeling ('tweede lijn') met sunitinib. Sunitinib is een TKI die in 2006 werd geregistreerd voor 2 indicaties, namelijk uitgezaaide nierkanker en uitgezaaide GIST na eerdere behandeling met imatinib. Sinds enkele jaren is het middel ook geregistreerd voor neuro-endocriene tumoren van de alvleesklier, waardoor er nu drie indicaties zijn voor behandeling met sunitinib.
Naast imatinib en sunitinib zijn er momenteel nog 15 andere TKIs op de markt voor verschillende vormen van kanker, en dit aantal zal vermoedelijk aanzienlijk stijgen.

Zoals elk medicament, hebben ook TKIs belangrijke bijwerkingen. Welke bijwerkingen optreden en in welke ernst, is erg verschillend tussen patiënten. Nog steeds moet regelmatig de dosis van een TKI worden verlaagd en soms zelfs de behandeling definitief worden gestaakt vanwege bijwerkingen. Daarnaast zit er een groot verschil in hoe lang de groei van de tumor effectief wordt onderdrukt door de TKI. In het voorbeeld van imatinib, bij patiënten met uitgezaaide GIST, wordt ongeveer 10% van de patiënten langer dan 10 jaar effectief behandeld met imatinib, zonder dat de tumor groeit. Daar tegenover staat dat ongeveer 10% van de patiënten binnen 6 maanden na start van de behandeling al groei van de tumor heeft en het middel dus niet effectief (meer) is. Daartussen zit een grote groep patiënten die tussen de 6 maanden en 10 jaar baat heeft bij behandeling met imatinib.

Voor het verschil in zowel effectiviteit als in het optreden van bijwerkingen zijn verschillende mogelijke verklaringen. Hiertoe worden allereerst twee basisprincipes uit de medicijnleer uitgelegd, de zogenaamde 'farmacokinetiek' en de 'farmacodynamiek'.

Farmacokinetiek staat voor wat doet het lichaam met het medicijn. Dit omvat vier processen, namelijk absorptie, distributie, metabolisme en excretie, samen afgekort tot ADME. Nadat een tablet wordt geslikt moet het oplossen tot kleine partikels en vanuit het maagdarmkanaal worden opgenomen in de bloedbaan (*absorptie*). Via de bloedbaan wordt het geneesmiddel, al dan niet aan eiwit gebonden, verspreid door het lichaam (*distributie*). Onder andere in de lever wordt het medicijn door verschillende enzymen (dit zijn eiwitten) omgezet in andere stoffen (*metabolisme*). De nieuwe stoffen die hierdoor ontstaan worden afbraakproducten of metabolieten genoemd. Deze metabolieten worden in sommige gevallen ook weer omgezet in andere metabolieten. Gelijktijdig wordt een geneesmiddel en diens metaboliet(en) via de lever en/of de nieren uitgescheiden (*excretie*). De activiteit van deze vier processen tezamen bepalen hoeveel van het geneesmiddel in het lichaam aanwezig is; oftewel hoe hoog de blootstelling aan een bepaald medicijn in een bepaald persoon is. Dit laatste kan gemeten worden in bijvoorbeeld het bloed van de patiënt. Er is een grote variatie tussen mensen in de farmacokinetiek van geneesmiddelen. Dit wil zeggen: als meerdere mensen eenzelfde dosering van een bepaald medicijn innemen, dan zal er een variatie zitten in de gemeten medicijnspiegels. Dit wordt veroorzaakt door verschillen in de activiteit van deze vier processen.

Farmacodynamiek staat voor welk effect het medicijn heeft op het lichaam. Het betreft zowel gunstige als ongunstige effecten van een medicijn. In het geval van medicijnen tegen kanker zien we als gunstig (therapeutisch) effect dat een tumor niet verder groeit, of zelfs kleiner wordt. Ongunstige effecten (bijwerkingen) zijn onbedoelde neveneffecten van een medicijn. Voorbeelden zijn diarree, afwijkingen aan hart- en bloedvaten, hoge bloeddruk, lever- en nierfunctiestoornissen, schildklierafwijkingen, veranderingen van haar en huid, en nog veel meer.

In het eerste deel van dit proefschrift wordt een drietal studies besproken die tot doel hebben om de behandeling met sunitinib bij patiënten te verbeteren, gebaseerd op de farmacokinetiek van sunitinib. Patiënten die worden behandeld met sunitinib vertonen een grote spreiding in de blootstelling aan het middel in het bloed. Dit houdt in dat als 10 patiënten eenzelfde dosis sunitinib innemen, bijvoorbeeld 50mg, dat de ene patiënt dan bijvoorbeeld een spiegel van 20 ng/mL zal hebben en de ander een spiegel van 80 ng/mL. Mogelijk is het zo dat verschillen in het optreden van bijwerkingen en verschillen in hoe effectief het middel is om de groei van kanker af te remmen, hieraan gerelateerd is. Om de behandeling met sunitinib in patiënten met kanker in de toekomst te verbeteren hebben wij de volgende drie studies gedaan.

De eerste studie, weergegeven in **hoofdstuk 2** van dit proefschrift, is een studie waarbij we van 2 eenvoudig uit te voeren tests, de zogenaamde ‘midazolam klaringstest’ en de ‘sestamibi scan’ van de lever, hebben onderzocht of de uitkomst voorspellend is voor de medicijnspiegels van sunitinib. De midazolam klaringstest kan worden gebruikt als maat voor de activiteit van CYP3A4 in de lever. CYP3A4 is een enzym dat belangrijk is voor de omzetting van medicijnen in metabolieten. Het komt

onder andere voor in de lever en het maagdarmkanaal. De sestamibi scan van de lever is een maat voor de activiteit van het transporteiwit ABCB1. Dit eiwit zorgt er in de lever voor dat veel medicijnen, waaronder sunitinib, in de gal worden uitgescheiden. Zowel de midazolam klaringstest als de sestamibi scan van de lever bleken de medicijnspiegels van sunitinib onvoldoende te kunnen voorspellen om toegepast te kunnen worden in de klinische praktijk. Wel zagen we, dat patiënten met een lage klaring van sunitinib, en daarmee hogere medicijnspiegels, vaker ernstige bijwerkingen hadden dan patiënten met een hoge klaring en daarmee lagere medicijnspiegels. Dit suggereert dat er voor het medicijn sunitinib een relatie bestaat tussen de blootstelling en het optreden van toxiciteit.

Hieruit volgt de vraag of het beter zou zijn om sunitinib te doseren aan de hand van de medicijnspiegels, in plaats van een standaard dosis. Doseren op basis van de medicijnspiegels gebeurt al regelmatig bij de behandeling met onder andere antibiotica, en medicijnen ter voorkoming van afstoting na transplantaties. In **hoofdstuk 3** van dit proefschrift hebben we onderzocht, in patiënten die geen standaard behandelingsopties meer hebben voor hun kanker, of sunitinib gedoseerd kan worden op basis van de medicijnspiegels. Patiënten startten met 37.5 mg sunitinib per dag en 2 weken later werd voor de eerste keer een medicijnspiegel afgenomen. Indien deze spiegel onder de 50 ng/mL was, werd de dosis sunitinib opgehoogd met 12.5 mg naar 50 mg per dag. In de 4^{e} week van behandeling werd nogmaals een medicijnspiegel afgenomen. Indien deze nog steeds of juist nu onder de 50ng/mL was, werd de dosis wederom met 12.5 mg opgehoogd. Patiënten die ernstige bijwerkingen van de medicijnen ondervonden werden in dosis gereduceerd en nadien niet meer opgehoogd. Deze studie liet zien, dat het in 17% van de patiënten die behandeld werden met sunitinib, mogelijk is om de dosis aan de hand van de spiegels op te hogen tot boven de minimale waarde van 50 ng/mL, zonder dat dit gepaard ging met het optreden van ernstige bijwerkingen. We concludeerden dat het veilig is om sunitinib te doseren op basis van de spiegels. Toekomstige studies zullen moeten uitwijzen of dit ook daadwerkelijk resulteert in een verbetering van de behandeling met sunitinib (een betere effectiviteit en minder bijwerkingen).

Er is steeds meer bewijs dat circadiane ritmes (‘de klok’) de farmacokinetiek van medicijnen kunnen beïnvloeden. Voor sunitinib (of andere TKIs) was dit nooit eerder uitgezocht. In **hoofdstuk 4** van dit proefschrift hebben we onderzocht of de farmacokinetiek van sunitinib anders is wanneer het middel op een ander tijdstip gedurende de dag wordt ingenomen. Dit zou veroorzaakt kunnen worden door het dag-nachtritme waaraan ons lichaam onderhevig is. We hebben dit onderzocht in zowel muizen als in patiënten. Bij muizen zagen we dat wanneer sunitinib om 4 uur ’s middags of 4 uur ’s nachts werd toegediend, de blootstelling aan sunitinib 14-27% hoger lag dan wanneer sunitinib om 8 uur ’s ochtends of 8 uur ’s avonds werd toegediend. De patiënten in onze studie namen sunitinib in 3 achtereenvolgende kuren op 3 verschillende tijdstippen in; om 8 uur ’s ochtends, om 1 uur ’s middags en om 6 uur ’s avonds. De blootstelling gedurende de dag was bij patiënten niet verschillend bij verschillende tijdstippen van inname. Wel zagen we dat patiënten bij middag- of avond inname

van sunitinib hogere spiegels hadden vlak voor de inname van een nieuwe capsule (de zogenaamde 'dalspiegel'), en dat daarmee een meer stabiele blootstelling/concentratie gedurende de dag werd bereikt. Dit is relevant mocht sunitinib in de toekomst op basis van de dalspiegel worden gedoseerd (ook wel *therapeutic drug monitoring* of TDM genaamd). Immers, patiënten die sunitinib in de ochtend innemen kunnen een lagere dalspiegel hebben dan patiënten die sunitinib in de middag of avond innemen. Hypothetisch gezien zou bij deze patiënten de dosering kunnen worden verhoogd, terwijl de blootstelling gedurende niet lager is.

Het tweede deel van mijn promotieonderzoek gaat over de farmacodynamiek van verschillende TKIs, dus wat TKIs doen met het lichaam. Oftewel, treden er bijwerkingen op en is het middel effectief in de bestrijding tegen kanker?

De hoofdstukken 5 en 6 beschrijven een tweetal studies waarin we onderzochten of kleine afwijkingen in het menselijk DNA, 'single nucleotide polymorfismen' ofwel SNPs genaamd, een verschil in overleving kunnen verklaren bij patiënten met een uitgezaaide vorm van GIST. Dit werd onderzocht bij zowel gebruik van eerstelijns therapie met imatinib (**hoofdstuk 5**) en tweedelijns behandeling met sunitinib (**hoofdstuk 6**).

In **hoofdstuk 5** werden 36 SNPs in 18 genen die betrokken zijn bij de farmacokinetiek en farmacodynamiek van imatinib gerelateerd aan de progressie vrije overleving (PFS) en algehele overleving (OS) na start van imatinib behandeling in 255 patiënten met een GIST. PFS bleek significant korter in patiënten die ten tijde van het stellen van de diagnose al uitzaaiingen hadden, in patiënten met een zogenaamde *KIT exon 9 mutatie* en patiënten met het TT-genotype in de SNP *rs2305948 in KDR* of het AA-genotype in de SNP *rs1570360 in VEGFA*. OS was ook significant korter in patiënten met synchrone metastasen ten tijde van de diagnose, en verder in patiënten met het AA-genotype in *KDR rs1870377*, of het *AA-genotype in VEGFA rs1570360*. Patiënten die een T-allel hadden in *SLCO1B3 rs4149117* hadden juist een langere overleving.

In **hoofdstuk 6** werden 49 SNPs in genen betrokken bij de farmacokinetiek en farmacodynamiek van sunitinib geassocieerd met PFS en OS na start van sunitinib behandeling in 127 patiënten met een uitgezaaide vorm van GIST. PFS was korter in patiënten met een C-allel in de SNP *POR rs1056878*. De aanwezigheid van een T-allel in *CYP1A2 rs4149117*, een dubbel CCC-allel in *SLC22A5* en een dubbel GC-allel in *IL4R* waren voorspellend voor OS. Wanneer deze factoren werden gecombineerd in een genetisch model, bleken zowel PFS als OS significant langer naarmate het aantal gunstige genetische factoren in een patiënt groter was.

Wanneer de resultaten van deze beide studies ook zouden worden gevonden in een volgende studie, zou het mogelijk zijn om patiënten op voorhand te identificeren die een grotere kans hebben om te

reageren op de behandeling.

In **hoofdstuk 7** van dit proefschrift beschrijven we het optreden van een verlengde 'QTc-tijd' op het electrocardiogram, of ECG. Het ECG is een optelsom van positieve en negatieve stromen door de hartcellen, die optreden tijdens activiteit van het hart, ook wel depolarisatie en repolarisatie genoemd. De QTc-tijd is een onderdeel van het ECG, die de tijd aangeeft die het hart nodig heeft om in de ruststand te komen. Dit is de repolarisatiefase. Dit is belangrijk, omdat in geval van verlenging van de QTc-tijd een ernstige hartritmestoornis kan ontstaan, die zonder behandeling kan leiden tot de dood. Zo'n verlenging van de QTc-tijd is een frequent voorkomende bijwerkingen van medicijnen en kan soms zelfs reden zijn om medicijnen van de markt te halen. Van TKIs was tot voor kort weinig bekend over het effect op de QTc-tijd. In onze studie hebben we bij 363 patiënten die behandeld zijn met een TKI onderzocht of deze de QTc-tijd verlengd. Dit bleek het geval te zijn voor de TKIs sunitinib, vemurafenib, sorafenib, imatinib en erlotinib, en als we de hele groep patiënten als een geheel zagen. Tenslotte, in **hoofdstuk 8** van dit proefschrift wordt ingegaan op een ander verschijnsel wat optreedt na de start van behandeling met een TKI. Dit betreft het veranderen van de grootte van de rode bloedcellen, aangegeven met de term *mean corposcular volume* of MCV. Voor imatinib en sunitinib was eerder al eens beschreven in de literatuur dat het MCV toenam na de start van behandeling. In deze studie hebben wij dit eveneens aangetoond voor imatinib en sunitinib, en daarnaast voor het middel pazopanib. De klinische betekenis hiervan was altijd onduidelijk. Wij hebben aangetoond dat patiënten met gemetastaseerde nierkanker die behandeld werden met sunitinib en waarbij het MCV steeg tot boven de 100 fL tijdens de behandeling, een betere overleving hadden dan die patiënten waarbij het MCV in mindere mate of algeheel niet steeg tijdens de behandeling. Het stijgen van het MCV is hier dus een gunstig prognostisch teken voor de behandeling.

Al met al kunnen we concluderen dat de behandeling met TKIs tot dusver weinig op de individuele patiënt is afgestemd. Momenteel bevinden zich veel TKIs in de ontwikkelingsfase en in de nabije toekomst zullen er meerdere TKI-behandelingen beschikbaar zijn voor specifieke tumor types. Dit is inmiddels al het geval voor niercelkanker, waar zowel sunitinib als pazopanib geregistreerd zijn voor de eerstelijns behandeling. Daarom is het belangrijk dat er verder onderzoek gedaan wordt naar welke patiënt het beste met welk medicament behandeld kan worden. Dit kan bereikt worden door het onderzoeken van patiënten karakteristieken, maar ook door te focussen op tumor karakteristieken. Verschillende cascades van tumor activatie kunnen worden gevonden in een identiek tumor type of in tumoren die stammen uit hetzelfde soort weefsel. Door selectief een cascade van tumor activatie te blokkeren met TKIs, onafhankelijk van het tumortype, kan de behandeling van kanker in de toekomst aanzienlijk verbeterd worden.

Anderzijds kan de behandeling met TKIs verbeterd worden door de introductie van TDM. De kennis verkregen uit de studies in het eerste deel van dit proefschrift kunnen een eerste stap zijn in de richting van een "tailor made" behandeling met sunitinib. Echter, er is nog weinig bewijs of dit ook daadwerkelijk resulteert in een betere behandeling met sunitinib, en toekomstige studies zullen zich hierop moeten richten.

Veel studies zijn verricht waarbij SNPs werden geassocieerd met overleving en bijwerkingen van een bepaald middel. Tot op heden heeft dit er echter niet toe geleid dat dit ook is geïmplementeerd in de klinische praktijk om de overleving te verbeteren en bijwerkingen te minimaliseren. Aangezien de effecten van SNPs op overleving en bijwerkingen in het algemeen heel klein zijn, is het ook maar de vraag of dit in de toekomst zal veranderen. Hoe dan ook, meer onderzoek op het gebied van farmacogenetica is nodig, en datzelfde geldt voor andere potentiele markers voor effectiviteit en toxiciteit van TKIs.

Appendix 2

Curriculum Vitea

Jacqueline Saskia Leonore Kloth werd geboren op 3 februari 1985 in Dordrecht. In 2003 behaalde zij haar VWO diploma aan de Rijks Scholen Gemeenschap de Hoeksche Waard. Aansluitend startte zij haar studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In 2008 behaalde zij haar doctoraal examen. Van 2007-2009 doorliep zij haar coschappen in verschillende ziekenhuizen in de regio Rotterdam. Deze periode werd afgesloten met een oudste-coschap op de afdeling Interne Geneeskunde van het Maasstad ziekenhuis te Rotterdam.

Direct na het behalen van haar artsdiploma op 11 september 2009 was Jacqueline werkzaam als arts niet in opleiding tot specialist op de afdeling Interne Geneeskunde van het Maasstad ziekenhuis. Zij werkte op de afdelingen MDL, spoedeisende hulp, reumatologie en nefrologie.

In januari 2011 maakte Jacqueline de overstap naar de Daniel den Hoed kliniek. Hier was zij 10 maanden werkzaam als arts niet in opleiding tot specialist op de afdeling Interne Oncologie (afdelingshoofd prof. dr. J. Verweij), onder begeleiding van dr. A. van der Gaast. In die periode werd tevens begonnen aan de opzet van wetenschappelijk onderzoek naar een verbetering van de behandeling met tyrosine kinase remmers bij patiënten met kanker.

In november 2011 werd dit onderzoek gecontinueerd in het kader van een promotietraject onder supervisie van aanvankelijk prof. dr. J. Verweij en dr. R.H.J. Mathijssen. Bij de aanstelling van prof. dr. J. Verweij als decaan werd de supervisie overgedragen aan prof. dr. R.H.J. Mathijssen en dr. E.A.C. Wiemer (afdelingshoofd prof. dr. S. Sleijfer), wat resulteerde in dit proefschrift.
In december 2014 startte Jacqueline als arts in opleiding tot specialist op de afdeling Interne Geneeskunde van het Maasstad ziekenhuis (opleider dr. M.A. van den Dorpel), in het kader van haar opleiding tot reumatoloog (opleiders dr. R.J.E.M. Dolhain en dr. E. Barendregt).

Appendix 3

Publications

Kloth J.S.L., Klumpen H.J., Yu H., Eechoute K., Samer C.F., Kam B.L., Huitema A.D., Daali Y., Zwinderman A.H., Balakrishnar B., Bennink R.J., Wong M., Schellens J.H.M., Mathijssen R.H.J., Gurney H. Predictive value of CYP3A and ABCB1 phenotyping probes for the pharmacokinetics of sunitinib: the ClearSun study
Clin Pharmacokinet. 2014 Mar; 53(3):261-9

Schiavon G., Ruggiero A., Sleijfer S., Bekers D.J., van der Holt B., Kloth J.S.L., Krestin G.P., Schoffski P., Verweij J., Mathijssen R.H.J. Accuracy of actual tumor volume measurements with RECIST 1.1 versus Three-Dimentional CT criteria in GIST patients during imatinib treatment
Eur J Cancer. 2014 Mar;50(5):972-80

Diekstra M.H.M., Klumpen H.J., Lolkema M.P.J.K., Yu H., Kloth J.S.L., Gelderblom H., van Schaik R.H.N., Gurney H., Schwen J.J., Huitema A.D.R., Steeghs N., Mathijssen R.H.J. Association analysis of genetic polymorphisms in genes related to sunitinib pharmacokinetics with clearance of sunitinib and its primary metabolite (SU12662)
Clin Pharm and Ther. 2014 Jul;96(1):81-9

Lankheet N.A.G.*, Kloth J.S.L.*, Gaddellaa-van Hooijdonk C.G.M., Cirkel G.A., Mathijssen R.H.J., Lolkema M.P.J.K., Schellens J.H.M., Voest E.E., Sleijfer S., Beijnen J.H., Huitema A.D.R., Steeghs N. Individual PK-guided sunitinib dosing: a feasibility study in patients with advanced solid tumors
Br J Cancer. 2014 May 13; 110(10):2441-9

Kloth J.S.L., Binkhorst L., de Wit A.S., de Bruijn P., Lam M.H., Burger H., Machado I., Wiemer E.A.C., van der Horst G.T.J., Mathijssen R.H.J. Chronicity in sunitinib pharmacokinetics
Clin Pharmacokinet. 2015 Feb 3 [Epub ahead of print]

Kloth J.S.L.*, Pagani A.*, Verboom M.C., Malovini A., Napolitano C., Kruit W.H.J., Sleijfer S., Steeghs N., Zambelli A., Mathijssen R.H.J. Incidence and relevance of QTc-interval prolongation caused by tyrosine kinase inhibitors
Br J of Cancer. Accepted

Yu H., Steeghs N., Kloth J.S.L., de Wit D., van Hasselt J.G.C., van Erp N.P., Beijnen J.H., Schellens J.H.M., Mathijssen R.H.J., Huitema A.D.R. Integrated mechanism-based pharmacokinetic model for sunitinib and its active metabolite
Br J of Clin Pharmacokinet. 2014 Nov 12 [Epub ahead of print]

Binkhorst L., Kloth J.S.L., de Wit A.S., de Bruijn P., Lam M.H., Chaves-Machado I., Burger H., van Alphen R.J., Hamberg P., van Schaik R.H.N., Jager A., Koch B.C.P., Wiemer E.A.C., van Gelder T., van der Horst G.T.J., Mathijssen R.H.J. Circadian variation of tamoxifen pharmacokinetics
Submitted

Kloth J.S.L., Verboom M.C., Swen J.J., van Straaten T., Sleijfer S., Reyners A.K.L., Steeghs N., Gelderblom A.J., Guchelaar H.J., Mathijssen R.H.J. Genetic polymorphisms as predictive biomarker of survival in patients with gastro-intestinal stromal tumours treated with sunitinib
Submitted

Kloth J.S.L., Hamberg P, van der Holt B, Mendelaar P.C., Wiemer E.A.C., Kruit W.H.J., Sleijfer S. Mathijssen R.H.J. Macrocytosis as a potential parameter associated with outcome in the treatment with tyrosine kinase inhibitors
Submitted

Verboom M.C., Kloth J.S.L., van der Straaten T., Swen J.J., Sleijfer S., Reyners A.K.L., Mathijssen R.H.J., Guchelaar H.J., Steeghs N. and Gelderblom A.J. Genetic polymorphisms in angiogenesis related genes are predictive for survival of patients with advanced gastrintestinal stromal tumors treated with imatinib
Submitted

Appendix 4

PhD Portfolio

1. PhD training

	Year	Workload ECTS
General courses		
- Basic introduction course on SPSS, MolMed	2012	0.8
- BROK (Basiscurcus Regelgeving Klinisch Onderzoek) course, Erasmus MC	2012	1
- Integrity in research, Erasmus MC	2013	1
- Training OpenClinica, Erasmus MC	2013	0.4
- Biomedical English Writing and Presenting, MolMed	2014	3
Specific courses (e.g. Research school, Medical Training)		
- Genomics in Molecular Medicine, NIHES	2012	1
- Biostatistical Methods: Basic Principles, NIHES	2012	5.7
Seminars and workshops		
- Personalized Medicine research meeting Medical Oncology	2012	1
- Principles of Clinical Pharmacology, NIH course	2012	1
- Graphic style and plot vs table, Erasmus MC workshop	2012	0.2
- How to write a successful grant proposal, Erasmus MC workshop	2012	0.2
- Clinical Pharmacology, COIG workshop	2012	1
- Systematic literature search in Pubmed, Erasmus MC Workshop	2012	0.2
- Endnote, Erasmus MC Workshop	2012	0.2
- Systematic literature search in other databases, Erasmus MC Workshop	2012	0.2
- Genomic approaches to cancer, MolMed lecture	2013	0.1
- Photoshop & Illustrator CS5, MolMed workshop	2013	0.3
- Indesign CS5, MolMed workshop	2014	0.3
- Clinical Pharmacology meeting, Erasmus MC	2014	0.5
Presentations		
- Research Meeting Medical Oncology, oral presentation	2011	0.5
- ESMO Annual Meeting 2012, poster presentation	2012	1
- ESMO Annual Meeting 2013, poster presentation	2013	1
- ASCO Annual Meeting 2014, poster presentation	2014	1

(Inter)national conferences			
-	ESMO Annual Meeting 2012, Vienna, Austria	2012	1
-	NVKF&B Annual Meeting 2013, Utrecht	2013	0.3
-	ESMO Annual Meeting 2013, Amsterdam	2013	1
-	NVKF&B Annual Meeting 2014, Leiden	2014	0.3
-	ASCO Annual Meeting 2014, Chicago, Illinois, USA	2014	1
Other			
-	Research meeting Medical Oncology, Erasmus MC	2011 / 2013	0.4
-	IKNL networking days Vlissingen / Middelburg	2011 / 2013	1
-	PhD day Erasmus MC	2012- 2014	1
-	MOLMED research day, Erasmus MC	2012-2013	0.8
-	Clinical Oncology meeting, Erasmus MC	2011-2013	1.5
-	OMBO training	2011-2013	1
-	Rheumatology journal club, Cicero	2013-2014	0.5
2. Teaching			
Lecturing			
-	Personalized Medicine research meeting Medical Oncology	2011-2013	2
-	Clinical lessons to Oncology nurses, Erasmus MC	2012	0.2
Supervising Master's thesis			
-	Anna Pagani	2012-2013	1
-	Pauline Mendelaar	2014	1

Appendix 5

Dankwoord

De afgelopen 3.5 jaar heb ik me bezig gehouden met dit onderzoek. Het was een leerzame periode waarin ik zowel op professioneel als op persoonlijk gebied ben gegroeid. Dit proefschrift, waar ik ontzettend trots op ben, is het eindresultaat van mijn werk. Alleen was mij dit nooit gelukt. Vele anderen hebben op verschillende wijze bijgedragen aan de totstandkoming van dit proefschrift. Hen wil ik graag bedanken voor alle hulp en inzet.

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