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Pharmacogenetics of sunitinib in metastatic renal cell carcinoma

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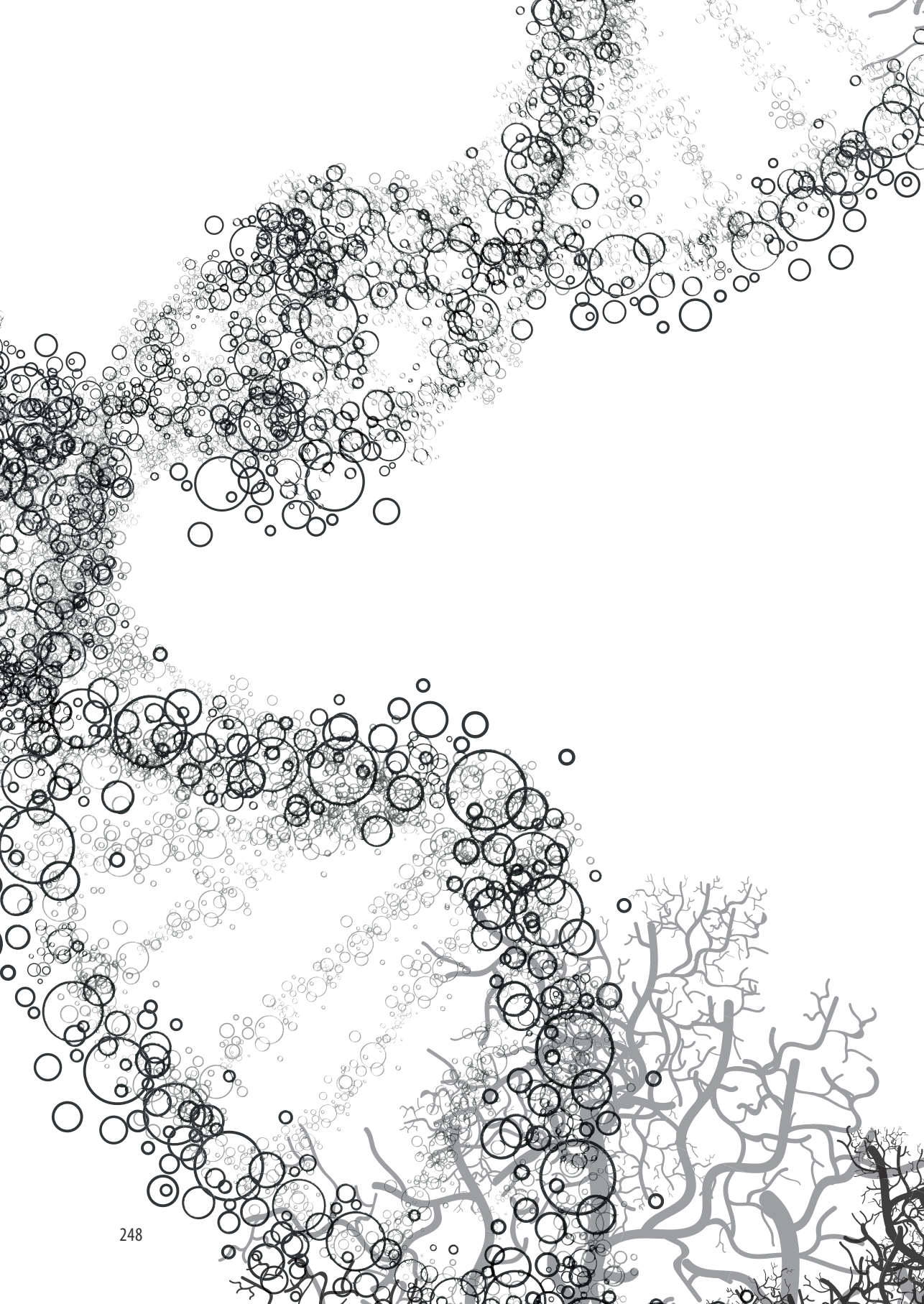


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CHAPTER 10

GENERAL DISCUSSION AND FUTURE PERSPECTIVES



PHARMACOGENETICS AS A KEY TO INDIVIDUALIZE MRCC TREATMENT

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

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

CANDIDATE GENE STUDIES

SNPs involved in sunitinib pharmacokinetics

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

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

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

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

Sunitinib-induced toxicities

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

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

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

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

The role of VEGF receptors in sunitinib efficacy

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

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

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

GWAS - MOVING BEYOND CANDIDATE GENES

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

POPULATION DIFFERENCES IN GENETIC ASSOCIATION ANALYSES

Population sizes

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

Ethnicity

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

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

OUTCOME DIFFERENCES IN GENETIC ASSOCIATION ANALYSES: PRACTICE MAKES PERFECT

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

Survival differences

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

nephrectomy (CN) prior to first-line treatment with sunitinib may be beneficial for overall survival with 12.7 months OS compared to 8.0 months OS for sunitinib only.⁴⁴ These findings were underlined by a study on 15,390 patients on targeted therapy between 2006 and 2013 of whom 5,374 (35%) underwent CN. Here, OS was 17.1 months for patients who received CN and targeted therapy and 7.7 months for targeted therapy alone.⁴⁵ However, these findings need to be confirmed by the large Clinical Trial to Assess the Importance of Nephrectomy (CARMENA; NCT00930033) of which the primary results are expected in 2019. In this trial, mRCC patients are randomized to receive sunitinib alone or receive nephrectomy followed by sunitinib.^{44,45} Besides country differences, survival can vary among several studies because of altered methodological approaches on survival analyses. Adjustment for prognostic factors for instance have a large influence on survival. In previous pharmacogenetics association analyses, the Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria were included, namely deteriorated WHO performance status (≥ 2), low haemoglobin (lower than the lower limit of normal), high calcium (>2.5 mmol/l), time from initial diagnosis to treatment with sunitinib (<1 yr), and serum lactate dehydrogenase (LDH) greater than 1.5 times the upper limit of normal (ULN). In our studies however, we adjusted survival outcomes using the prognostic index based on the Heng criteria, which validates the MSKCC criteria except for LDH levels and complemented with neutrophil count (higher than the upper limit of normal), and thrombocytes (higher than the upper limit of normal).^{46,47} Moreover, most of these pharmacogenetic studies in mRCC deal with retrospective data and may need data imputation on missing MSKCC criteria or Heng variables. The method and outcomes for data imputation may be different among studies. Another example of methodological differences in survival analysis was described in the paper of Goldstein et al. in which outcomes were compared to a study of Rini et al. In the study of Rini et al. patients were excluded from analyses only if the event status of PFS or OS was “no”, while Goldstein et al. also excluded patients if sunitinib treatment was discontinued for any reason which is considered to give a better assessment of sunitinib-specific survival.¹⁴ Recent evidence shows that a high body mass index (BMI) of ≥ 25 kg/m² should be included as covariate in survival analysis of mRCC patient as observed in the large cohort of 1,975 patients from the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) and in an external validation cohort of 4,657 patients.⁴⁸ Because of the lack of evidence in time of our analyses, we have not included BMI as covariate.

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

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

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

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

Drug resistance, tumor heterogeneity, and circulating tumor DNA

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

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

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

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

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

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

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

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

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

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

FUTURE PERSPECTIVES AND NEW DEVELOPMENTS

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

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

The future: GWAS and Next Generation Sequencing (NGS)

The GWAS approach sheds new light on pharmacogenetic research and added new genetic variants on chromosome 21 that seem promising biomarkers to guide sunitinib treatment. We will perform follow-up studies to reassess these findings, and perform a meta-analysis to compare SNP associations in sunitinib or pazopanib treated patients. In addition, we will carry out a validation GWAS to see whether we could confirm any of the SNP associations from candidate gene studies and the first GWAS on sunitinib pharmacogenetics on 1099 mRCC patients treated with pazopanib (N=744) or sunitinib (N=355). It was shown that genetic variants in *LOXL2*, *ENTPD4*, *UGT1A1*, *ANAPC4* and *SLC34A2* were genome-wide significantly associated with efficacy or toxicity of sunitinib, and SNPs in *IL2RA*, *LRR2* showed a suggestive association ($P \leq 5 \times 10^{-7}$).^{61,74}

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

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

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

Hurdles to overcome on clinical implementation of pharmacogenetic testing

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

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

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

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

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

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

Therapeutic Drug Monitoring (TDM)

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

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

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

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

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

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

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