Chapter 8

Summary, Discussion and Future perspectives
Von Hippel Lindau (VHL) disease is an autosomal dominant disorder with an estimated incidence of 1 in 36,000 to 85,000 newborns. The cause of this disease is a germline mutation in the VHL tumour suppressor gene leading to improperly functioning of the VHL protein. This loss-of-function of the VHL protein in VHL-disease causes via HIF1α increased downstream transcription of vascular endothelial growth factor (VEGF-A), and chemokine receptor 4 (CXCR4). As a result VHL disease patients develop vascularised tumours. Three VHL phenotypes are distinguished by their risk to develop pheochromocytoma and renal cell carcinoma: type 1 no pheochromocytoma; type 2, risk to develop all VHL-related manifestations and type 2a low risk and 2b high risk to develop renal cell carcinoma; type 2c, solitary pheochromocytoma. The penetrance rate of this disease is approaching 100% by 75 years of age. Because of the (yet) unpredictable clinical and biological behaviour of VHL-disease and the potential life threatening manifestations, intensive and lifelong surveillance of VHL-mutation carriers is recommended. In this thesis we aimed to elucidate clinical features and shed more light on the behaviour of VHL-disease. In addition, the role of CXCR4 in VHL-disease and VEGF-A in AML is studied.

Following an introduction and outline of the thesis in Chapter 1, Chapter 2 comprises a literature review on the role of chemokines and their receptors in cancer. Metastatic cancer is the result of several sequential steps and represents a highly organised, non-random and organ-selective process. A number of in vitro and in vivo models show that tumour cells use chemokine-mediated mechanisms during the metastasis process comparable to the regulation of leukocyte trafficking. Furthermore, chemokines modulate tumour behaviour such as the regulation of tumour-associated angiogenesis, activation of host tumour-specific immunological responses, and direct autocrine stimulation of tumour cell proliferation. We reviewed clinical trials with new drugs targeting chemokines or their receptors. Chapter 2 underscores the likely additional value of new drugs targeting chemokines and their receptors, especially the CXCR4/CXCL12 axis, for treatment of cancer patients.

In Chapter 3 the role of CXCR4 and CXCL12 in cancer cell-tumour microenvironment interaction is reviewed. The growing appreciation of the role of the microenvironment in driving the cancer cell biology has improved the understanding of cancer and accelerated the identification of new therapeutic targets. CXCR4 and CXCL12 are two key factors in the cross-talking between cancer cells and their microenvironment, what makes them promising targets for cancer therapy. In this review, we discussed the potential benefits of targeting CXCR4 with specific inhibitors to disrupt CXCR4-dependent tumour-stroma interactions for the sensitisation of cancer cells for conventional therapy.

In AML the CXCR4/CXCL12 axis is important for the apoptosis and mobilization of the leukemic cancer cells. In the AML blasts another downstream target of VHL protein, VEGF-A, correlates at high protein expression levels with poor relapse-free and overall survival. The biological significance of VEGF-A, known for its role in angiogenesis, depends on the content and ratio of the different VEGF-A isoforms. In Chapter 4 we determined the VEGF-A isoforms VEGF121,
VEGF145, VEGF148, VEGF165, VEGF183, and VEGF189 mRNA expression in 30 paediatric AML samples by quantitative RT-PCR. Thereafter the VEGF-A isoform expression levels were correlated to gender, age at diagnosis, white blood count, cytogenetic risk profile, and outcome. The size of the patient samples precluded measuring mRNA expression of all depicted VEGF-A isoforms in all patients. The VEGF-A isoforms VEGF121, VEGF165, and VEGF189 were expressed in all samples. The VEGF-A isoform VEGF183 was expressed in blasts of 19/22 patients. VEGF145 and VEGF148 were expressed in only 2 and 8 patients, respectively. None of the VEGF-A isoforms showed a correlation with clinico-pathological outcome or overall and progression free survival. A significant co-expression of VEGF-121, VEGF165, VEGF-183, and VEGF189 isoforms was apparent (mean rho = 0.716, p<0.0001). This means that measuring mRNA levels from a single VEGF-A isoform is representative for mRNA expression levels of all VEGF-A isoforms.

As previously described, mutations in the VHL-gene lead often to altered or repressed transcription of this gene. Promoter hypermethylation of a gene is a pathway for the repression of gene transcription. Therefore, hypermethylation of the VHL-gene promoter can have the same downstream consequences as mutations in the VHL-gene. To study the effect of a VHL-gene mutation or hypermethylation of the VHL promoter on downstream protein expression in haemangioblastoma, we compared in Chapter 5 VEGF-A, CXCR4 and CXCL12 protein expression levels in VHL-related haemangioblastomas to those in sporadic haemangioblastomas. In total 33 freshly frozen haemangioblastoma specimens, derived from 27 patients were collected, 16 of patients with VHL-disease and 17 from patients without VHL disease were collected. Immunohistochemical analyses were performed for H&E, VEGF-A, CXCR4 and CXCL12. From all tumour tissues DNA was isolated to analyse the VHL-gene mutation and methylation status. Areas of normal surrounding brain tissue, as defined by morphologic appearance, were apparent in 15 haemangioblastomas. We found that CXCR4, CXCL12, and VEGFA were higher expressed in haemangioblastoma tissue compared to normal surrounding tissue. In sporadic haemangioblastomas the mean percentage of CXCR4 positive cells per specimen (16%, SD 8.4) was higher than in VHL-related haemangioblastomas (8%, SD 4.4, p=0.002). This difference was not explained by a age at time of surgery or solid nodule and associated cyst size. CXCL12 was strongly expressed in 75% of sporadic haemangioblastomas and in 81% of VHL-related haemangioblastomas. VEGF-A was present in stromal haemangioblastoma cells and vascular endothelial cells of all VHL-related and sporadic haemangioblastomas. VHL-gene promoter hypermethylation was present in two sporadic haemangioblastomas and VHL-gene mutations in 57% of sporadic haemangioblastomas. The VHL-gene status showed no correlation with CXCR4 expression levels in the haemangioblastoma specimens.

In Chapter 6 the age of onset of the main VHL-related manifestations, including haemangioblastomas, in 82 VHL-mutation carriers is described. Moreover we aimed to define an organ specific age to start and interval in surveillance with a 5% detection probability to detect the first and subsequent VHL-related manifestations and to compare these calculations with current VHL-surveillance guidelines. We found that the calculated age to start surveillance was at birth for adrenal glands, 7 years for retina, 14 years for cerebellum, 15 years for spinal cord, 16 years for pancreas, and 18
years for kidneys. The calculated surveillance intervals were 4 years for adrenal glands, biennially for retina and pancreas, and annually for cerebellum, spinal cord and kidney. Compared to current VHL-guidelines, our calculated starting age of is 6 years later for retina, and 5 years earlier for adrenal gland. The calculated screening intervals were for retina 2x longer and for adrenal glands 4x longer than currently advised.

In Chapter 7 we studied haemangioblastoma progression in VHL-mutation carriers in the context of pregnancy. Using medical charts and imaging reports from 12 VHL-mutation carriers we assessed the progression of haemangioblastomas before, during, and after pregnancy and in 27 VHL-mutation carriers we analysed pregnancy outcome. The progression score of cerebellar haemangioblastomas was significantly changed between the single MRI prior to and the two after pregnancy (p= 0.049). Foetal mortality rate was 2% (n=1) caused by a pheochromocytoma in the mother. Maternal VHL-related complications occurred in 17% (n=8) of all pregnancies. In four patients a life-threatening situation emerged: hydrocephalus due to cerebellar haemangioblastoma (n=2) and pheochromocytoma (n=2). This suggests that pregnancy in VHL might induce cerebellar haemangioblastoma progression. Therefore we recommend during pregnancy to increase surveillance of VHL patients especially of cerebellar haemangioblastomas.

**DISCUSSION AND FUTURE PERSPECTIVES**

The need for surveillance in VHL is obvious as these patients can develop multiple benign lesions and malignant carcinomas throughout their lives but guidelines on the age to start and stop and intervals for screening are not evidence-based. Cancer-related surveillance guidelines in general are based on the detection of new lesions and the monitoring of growth of lesions, thereby supporting treatment decisions. International collaboration and surveillance guidelines are a first necessity to achieve optimal care of VHL-patients. This will enable to compose a large data base and thus comparison of long term follow-up data of all VHL-disease related manifestations from a large series of VHL patients. The differences of VHL-related manifestations like differences in growth pace and organ manifestations, will have to be further elucidated in order to identify risk factors that predict behaviour of the manifestations in the individual patient. Such large data base might also give more insight on the weight of risk factors. It would be of interest to study in such a large cohort genetic and epigenetic risk factors and the influence of other factors such as pregnancy.

Identifying the growth pace of a VHL-related manifestation in the individual patient might improve the decisions on timing of intervention that preferably are performed at times of high risk on symptoms in localized disease and low risk on complications. Previously, four studies two on respectively 19 and 160 haemangioblastoma patients and two on 64 and 16 renal cell carcinoma patients, analysed tumour growth (1-4). They showed stuttering growth of haemangioblastoma and continuous growth of renal cell carcinoma. We showed that the onset of VHL-related manifestations is according Poisson distribution models, representing the second hit theory. The first hit, mutation in VHL, is inherited and the acquired random second hit gives rise to VHL-related manifestations. Better understanding of the onset of these manifestations supports standardising guidelines. Ultimately, incorporation of standardised screening of VHL mutation carriers could lead
to potential better understanding of development and growth of all VHL-related manifestations. This insight will support the best timing and way of treatment.

In solid tumours RECIST criteria validated with a large warehouse of over 6000 patients are used to evaluate tumour response to treatment. However, the definition of growth of VHL-related manifestations is not validated or standardised. Using existing tools such as RECIST should enable the acquisition of criteria to assess VHL-related tumour growth. These data can be collected and form a warehouse. This would allow to determine the optimal criteria for tumour growth in VHL.

Currently there are ongoing trials in VHL patients with amongst others bevacizumab (anti-VEGFA), and the anti-FGFR tyrosine kinase inhibitor dovitinib therapy. The commonly used β-blocker propranolol showed promising results, significant regression of size in 94% of patients and diminished colour (p<.001), in a small study in 55 infants with haemangiomas (5). This finding deserves additional testing of propranolol effect on VHL-related manifestations. In a pilot study 15 VHL patients were treated with sunitinib (a multi tyrosine kinase inhibitor of VEGF-R, PDGF-R and c-KIT) and monitored for the effects on clear cell renal cell carcinoma (ccRCC), retinal angioma, haemangioblastoma, and pancreatic neuroendocrine tumour (pNET) (6). In total 22 haemangioblastoma, 7 retinal lesions, 18 ccRCC, and 5 pNET lesions were analysed. Stable disease occurred for the retinal lesions, pNETs and 20/22 haemangioblastomas while 2 haemangioblastoma lesions progressed. However, in 6 out of 18ccRCC lesions a partial response according to RECIST was observed. The FDA already approved sunitinib for treatment of ccRCC and pNET (7). This illustrated that sunitinib can delay progression of some of the VHL-related manifestations.

Insight in the microenvironment of VHL lesions might give access to other novel drug targets. Inactivation of VHL does not correlate with poor prognosis in ccRCC, but CXCR4 mRNA expression, one out of many of the HIF downstream target genes, does correlate with risk on metastasis in ccRCC (8). As HIF is degraded via VHL protein, measuring CXCR4 expression might be of importance in VHL patients to predict risk of metastasis of ccRCC. Immunohistochemical expression of CXCR4 can only be measured on resected ccRCC tissue. Recently CXCR4 tracers were developed for use in PET-imaging (9). Potentially these tracers might be used in VHL patient to analyse CXCR4 expression levels. As shown in this thesis CXCR4 expression is found in haemangioblastoma tissue and not in surrounding normal brain tissue. Others showed strong upregulation of CXCR4 in ccRCC tissue (10). Therefore, the CXCR4 expression can potentially be used to distinguish normal brain tissue from haemangioblastoma tissue and also ccRCC tissue from surrounding normal tissue. Moreover, by demarcating normal tissue from tumour tissue, CXCR4 receptor could serve as drug target.

VHL phenotypes differ in the type of manifestations that occur. These phenotypes are presumably existing because of the mutation specific effect on HIF inactivation. More recent research suggests that HIF2α might more important in the tumour formation process compared to HIF1α, especially for pheochromocytomas and paragangliomas (11, 12). More insight in the causative effect of specific VHL-mutations might identify either HIF1 or HIF2, as the most interesting target for therapy. High protein expression of HIF2α and PDGFRβ in ccRCC samples of patients treated with sunitinib was associated with better objective treatment response and stable disease as defined by RECIST. Moreover, high VEGFA protein expression in these tumours was associated
with shorter, and high HIF2α protein expression with longer overall survival time (13). Drug combinations including compounds targeting degradation of HIF might be further explored in VHL patients. Moreover, combining agents targeting VEGFA and CXCR4, both downstream of the VHL/HIF pathway, might be of interest in case of VHL-related manifestations. In AML high CXCR4 and VEGFA expression are predictors of poor prognosis (14, 15). Trials in AML patients and glioblastoma patients with anti-CXCR4 and anti-VEGFA agents to chemosensitise cancer cells and prevent angiogenesis respectively, are ongoing. The efficacy of this combination therapy in VHL patients deserves further testing. Additionally, also therapies inhibiting VEGFA and HIF1α might in VHL patients increase progression free intervals (16). Mutations in flanking genes associated with angiogenic, proliferative and migration pathways in VHL-related ccRCC and pancreatic tumours and single-nucleotide polymorphisms (SNPs) such as in MET in ccRCC are largely contributing to more aggressive tumour behaviour (17). Family members with the same germline mutation, can have different growth patterns of the various lesions. A recent study revealed that the VHL-HIF axis influenced the histone methylation and in this way upregulated CXCR4 levels (8). Therefore, genomic studies in VHL-disease should not only focus on the genetic mutations in a VHL patient but also on a patients’ gene methylation profile.

In addition other factors like pregnancy might alter the natural course of disease progression. We have shown that pregnancy seems to induce haemangioblastoma progression. However, conflicting results are found on the effect of pregnancy on haemangioblastoma progression as some suggest no effect (18). Progression during pregnancy could be explained by an increased level of plasma circulating VEGFA during pregnancy (19). CXCR4 is upregulated in pregnancy due to its role in the maternal/fetal interface and angiogenesis (20, 21). VEGFA and CXCR4 are known factors causing increased angiogenesis and tumour cell proliferation respectively. Also mechanical compression of the uterus and increased circulating volume potentially stimulates growth of VHL-disease related manifestations. More research is needed to clarify the consequences of pregnancy on VHL-related manifestations.

Altogether, insight in the best time to start with treatment intervention starts with incorporating international surveillance guidelines, followed by analysis of VHL-disease progression during life in large cohorts. The search for targeted therapies in VHL-patients adjusted to their risk profile can be encouraged by defining risk factors based on genetic and epigenetic profiles. Using the expertise and results found in other monogenetic diseases will contribute in this search for optimal treatment of VHL-patients.
REFERENCES


