" The role of the placental growth factor and prolyl hydroxylase containing domain protein 2 in the pathogenesis of hepatocellular carcinoma "

Femke Heindryckx

Promotor: prof. H. Van Vlierberghe
Co-Promotor: prof. I. Colle

Chapter I: Introduction

Chapter II: Aims

Chapter III: Results

Chapter IV: Discussion

Chapter V: Summary

Chapter VI: Curriculum vitae

Abbreviation list

[18] FMCH	Fluoromethylcholine
AASLD	American Association for the Study of Liver Diseases
Ang	Angiopoetin
bFGF	Basic fibroblast growth factor
CC	Cholangiocarcinoma
CD105	Endoglin
CK19	Cytokeratin 19
CLD	Chronic liver disease
CNV	Choroidal neovascularisation
DEN	Diethylnitrosamin
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
Egln1	Gene encoding for PHD2
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
FABP	Fatty acid binding protein
FGF	Fibroblast growth factor
FIH	Factor inhibiting hypoxia inducible factor
GMM	Genetically modified mouse models
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIF	Hypoxia inducible factor
HSC	Hepatic stellate cell
ICD	Intercapillary distances
mAb	Monoclonal antibody
MMP	Matrix methaloproteinase
mTOR	Mammalian target of Rapamycin
NASH	Non-alcoholic steatohepatitis
NO	Nitric oxide
PB	Phenobarbital
PDGF	Platelet derived growth factor
PEI	Percutaneous ethanol injection
Pgf	Gene encoding for the placental growth factor
PH	Partial hepatectomy
PHD	Prolyl hydroxylase containing domain protein
PlGF	Placental growth factor

PlGFKO	Knock out of the *Pgf*-gene
qPCR	Quantitative real time PCR
RFA	Radiofrequence ablation
SEM	Scannin electron microscopy
TACE	Transarterial chemo-embolisation
TAM	Tumour associated macrophages
TARE	Transarterial radio-embolisation
TNF	Tumour necrosis factor
US	Ultrasound
VCC	Vascular corrosion casts
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wild type
αPlGF	Antibody targeting the placental growth factor

Chapter I
Introduction

1 Hepatocellular carcinoma

1.1 Pathophysiology

Hepatocellular carcinoma (HCC) is a primary liver tumour that originates in a background of chronic liver diseases (CLD). Every year almost 500 000 new patients are diagnosed with HCC, making it the 5th most common and the 3rd most deadly cancer worldwide. The tumour is usually diagnosed at a late stage, limiting the therapeutic options, thus mortality is high with an average survival of 4 months after diagnosis (1). The most common causes of cirrhosis and HCC are chronic alcohol abuse, hepatitis B and C; and non-alcoholic steatohepatitis (NASH). Exposure to chemical carcinogens, such as aflatoxins, is a frequent origin of HCC in Asia and Africa. Other rare risk factors for HCC are alpha-1-antitrypsin deficiency, haemochromatosis and tyrosinemia.

Most of the cases of HCC are found in Southeast Asia and in sub-Saharan Africa. This reflects the prevalence of hepatitis B infection, which is the most common cause of this cancer worldwide. Recent data show, however, that the frequency of liver cancer in the Western countries is rising, primarily due to increasing obesity rates, and to chronic hepatitis C. Notably, HCC strikes almost 4 times as many men as woman, which is partially explained by the protective role of oestrogens and the HCC-stimulating effect of androgens (2).

Like any other cancer, HCC develops as a result of mutations in the cellular machinery that causes the cell to replicate at a higher rate and/or by escaping apoptosis. The preceding CLD results in a constant cycle of liver damage, followed by repair, resulting in the replacement of liver tissue by fibrosis, scar tissue and regenerative nodules. Unfortunately, the liver's repair mechanisms are not flawless, leading to mutations in the proto-oncogenic and tumour suppressor genes, causing uncontrollable cell growth. The molecular steps of hepatocarcinogenesis are being actively investigated. The *p53*-tumour suppressor gene – the most commonly mutated gene in human cancers – has been suggested to play an important role in HCC, since multiple mutations have been found on this gene (3). In carriers of the hepatitis B virus (HBV), *p53*

expression might be directly suppressed by the virus itself and various *p53* mutations have been described in HBV-related tumours (4,5). HBV-DNA integration and genetic instability are also frequently seen in the oncogenic progression (6). Also in chronic hepatitis C it is often observed that the viral genome integrates in the infected cells and can induce HCC, even in a non-cirrhotic liver (7).

Hepatic carcinogenesis is a multistep process: the presence of specific risk factors previously mentioned, promotes gene damage leading to a cascade of molecular and cellular deregulations that ultimately result in the malignant transformation of hepatocytes. While in healthy cells there is a balance between apoptosis and cell growth, malignant cells are characterised by uncontrolled growth and cell division. Whether these malignant cells will undergo local and distant spread is defined by determinants of the tumour cells such as proliferating activities and the ability of the tumour cells to respond to growth and survival signals derived from its microenvironment. One important factor that determines tumour growth and metastasis, is hypoxia and the subsequent angiogenic response, which we will discuss later on (8).

1.2   Diagnosis

HCC often arises and grows silently, making its discovery very challenging. The various clinical presentations generally relate closely to their underlying CLD, therefore patients present with non-specific signs and symptoms such as jaundice, hepatic encephalopathy, ascites and variceal bleeding. Patients whose tumours have been identified prior to the development of hepatic decompensation or other complications, are more likely to be better candidates for curative interventions. Therefore, high-risk patients are screened using ultrasonography (US) on a 6 to 12 month interval. When a small (<1cm) lesion is discovered, the standard procedure (as stated by the AASLD-guidelines 2011 (9)) is to repeat the US after 3 months to evaluate its stability. Routine surveillance of high-risk patients has made the discovery of asymptomatic HCC more common.
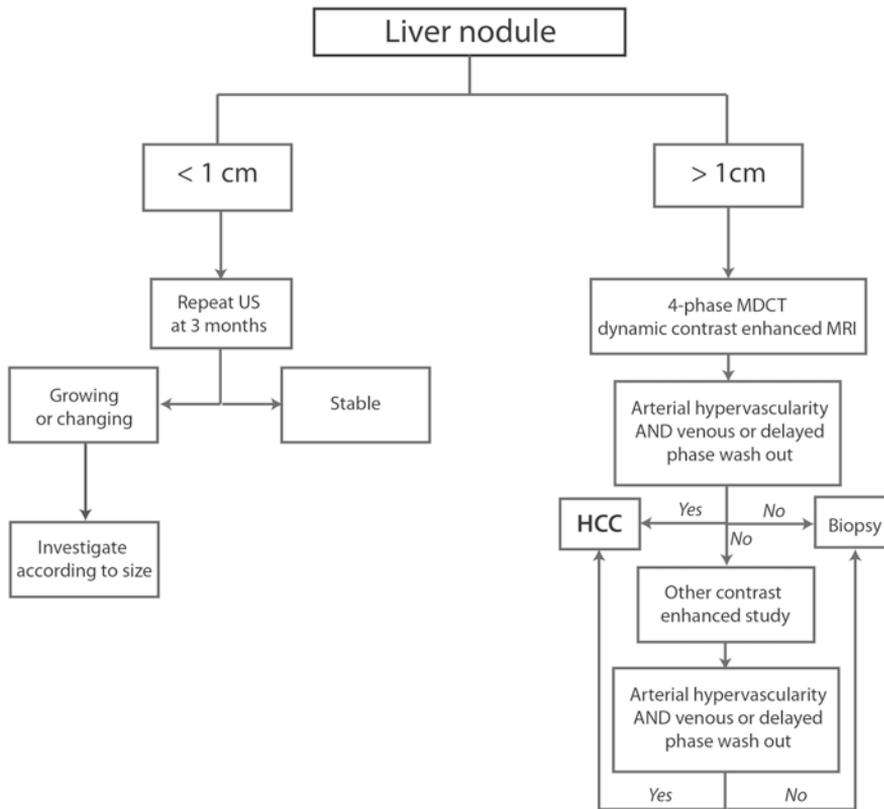
Fig1: *diagnostic algorithm for suspected HCC* (9).

The tests used to diagnose HCC include radiology, biopsy and α-fetoprotein-serology. Yet, the use of the latter is currently under discussion. Imaging plays a key role in the diagnosis of HCC, however, regardless of the immense advances in imaging technology, the characterisation of small tumours remains difficult, particularly in cirrhotic livers, characterised by a disrupted parenchymal architecture.

Contrast-enhanced CT plays a major role in the detection and characterisation of HCC in patients with chronic liver diseases. HCC lesions are usually hypervascularised and characterised by an increased arterial blood supply, therefore they are best assessed with a multiphase imaging technique, focussing on the arterial phase, such as a quadruple-phase CT-scan, consisting out of four phases (pre-contrast, arterial, portal-venous and delayed) (10). MRI

is superior to CT in providing better soft-tissue contrast, a more nuanced depiction of different tissue properties with a higher sensitivity, without ionizing radiation (11,12). In a dynamic contrast enhanced MRI repeated imaging is used to track the entrance of diffusible contrast agents in tissue over time. Both imaging techniques are used to detect and characterise liver lesions (>1cm), as different types of tumours enhance differently during each phase depending on whether they are hypervascular or hypovascular lesions. HCC-lesions show an increased arterial vascularisation and a venous or delayed phase wash out. If these characteristics are seen on a quadriple MDCT or a dynamic contrast enhanced MRI, the lesion can be diagnosed as HCC. If not, other techniques must be applied such as a biopsy or another contrast enhanced imaging technique (Figure 1).

Another fundamental technique for imaging of the liver is ultrasound. Its advantages are speed and simplicity. Its disadvantages, which include rather limited resolution for small liver lesions and limited specificity, can be overcome with the use of ultrasound contrast agents. These contrast agents open the possibility to identify HCC-nodules >2 cm in diameter, when enhancement of the lesion is seen during the arterial phase with washout during the portal phase (13).

Usually, the diagnosis of HCC depends on the measurement of the imaging techniques mentioned above. Still, a liver biopsy can be recommended in uncertain cases (14). A liver biopsy is a more invasive, however, more sensitive and specific technique. Histological samples can be obtained by needle core biopsy under CT-guidance. Nevertheless, the invasive nature of this technique implies certain risks, therefore a liver biopsy will not be performed under circumstances in which the diagnosis of HCC is positive after clinical, laboratory and radiographic evaluation.

1.3   Treatment

Treatment of HCC is often discussed in a multidisciplinary setting, depending on the tumour's stage (Figure 2).
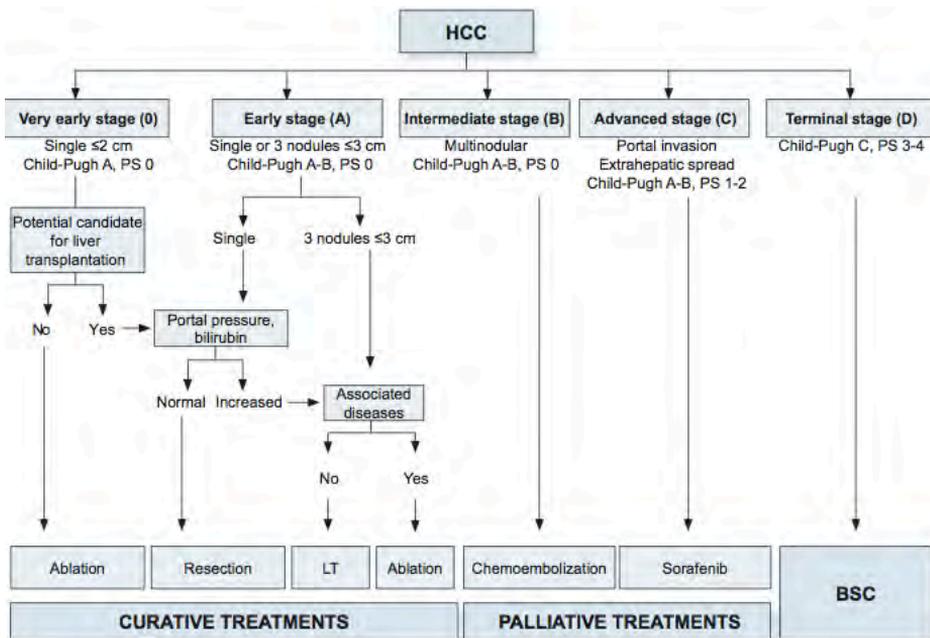
Fig 2: *Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy (15)(16,17), showing that very early and early stage HCC can be treated using surgical resection, liver transplantation or local ablation strategies. While patients with more advanced tumours are subjected to systemic treatment, such as the angiogenic inhibitor sorafenib. When the terminal stage of this tumour is reached, no treatment is feasible and best supportive care (BSC) is provided. This figure was obtained from (17).*

In the early stages of HCC surgical resection and liver transplantation are feasible curative therapeutic options, but the high level of recurrence limits the chances of survival. Because most HCCs occur in a cirrhotic liver, patients are in danger of decompensation or liver failure if an overaggressive resection is

14

attempted. Most surgeons will only perform a surgical resection if the patient has cirrhosis of Child-Pugh class A, no portal hypertension and a single definable tumour (18).

Another curative option for HCC is liver transplantation. Experience with transplantation has shown that tumour size is a very important variable in predicting recurrence. Therefore, the current guidelines for liver transplantation are based on the Milan-criteria, which support transplantation in the setting of one lesion ≤5 cm or up to three lesions ≤3 cm in diameter, which has an associated 5-year survival rate of >70% and tumour recurrence rate of <15% (19). Patients on the liver transplant waiting list are regularly screened, to detect if their HCC has not progressed beyond the Milan-criteria.

Local ablative strategies are the best treatment option for patients with small tumours who can not apply for surgical resection or liver transplantation (20). These local ablative modalities involve the destruction of tumour cells by altering the temperature (RFA, cryotherapy, microwave, laser) or injection of chemical substances (PEI, acetic acid or boiling saline). During precutaneous ethanol injection (PEI) ethanol is injected under ultrasound guidance in one or more sessions and may cause complete necrosis of the tumour (21). Yet, radiofrequency ablation (RFA), in which tumours are ablated using radiofrequency waves transmitted by an RFA-probe inside the tumour, is more frequently applied. For small HCC lesions RFA can be preferred over a surgical approach, since similar overall and disease-free survival is obtained but with less complications and a shorter hospital stay compared to surgical options (22).

Transarterial chemoembolization (TACE) is the recommended palliative treatment for patients with large or multifocal tumours (intermediate stage B). The liver is an ideal organ for chemoembolization, because of its dual blood supply from the hepatic artery and portal vein. During the progression of HCC, its blood supply becomes increasingly arterialized (>90% arterial), while the noncancerous liver parenchyma remains supplied largely by the portal vein (80% portal venous). This dual blood supply anatomy provides the rationale in support of arterial embolization as an effective therapeutic option to induce ischaemic tumour necrosis given that the hepatic artery almost exclusively supplies the HCC-tumours. In TACE, various chemotherapeutic agents

(doxorubicin, cisplatin or epirubicin) are delivered to the lesion prior to the arterial obstruction, to enhance the anti-tumoural effect (23). Transarterial radioembolisation (TARE) with intra-arterial injection of yttrium-90 microspheres is another form of hepatic arterial therapy, in which spheres are trapped in the tumour's capillary bed, where they deliver β-radiation to induce tumour necrosis (24).

The underlying liver disease often conceals the presence of liver tumours; therefore most tumours are discovered in an advanced stage. At this point no curative option is feasible, and systemic treatment is limited due to the high drug resistance of HCC. Recently, anti-angiogenic treatment has proved to be a valuable option for advanced HCC, which will be discussed later on.

2   Angiogenesis

2.1   Molecular factors in angiogenesis

A great number of factors contribute to the formation of new blood vessels. Among these angiogenic growth factors are wide-spectrum multifunctional mitogens (e.g. the fibroblast growth factors) and factors with distinct specificity for vascular endothelial cells (ECs) (e.g. the platelet-derived endothelial cell growth factor). Another group of factors induce angiogenesis indirectly (e.g. transforming growth factor-beta) by stimulating target cells to release angiogenic factors or by other mechanisms. The differential expression, release and activation of these factors might regulate angiogenesis under various physiological and pathological conditions. Due to the almost unlimited number of factors involved in angiogenesis it is not possible to discuss all of them. Hence, we will mainly focus on the role of prolyl hydroxylase containing domain proteins (PHD) and the placental growth factor (PlGF), since they are of uttermost importance in our research.

2.1.1   Hypoxia inducible factors and prolyl hydroxylase domain containing proteins

Hypoxia is the principal physiological stimulus that induces angiogenesis, providing a response by which cells are assured of adequate oxygenation. The response of cells to hypoxia is mediated by hypoxia-inducible factors (HIFs), HIF1-α, HIF2-α and HIF3-α, heterodimeric transcription factors consisting of an α- and β-subunit. Each of the 3 isoforms can heterodimerize with the β-subunit, thus forming an active transcriptional complex that is able to regulate hundreds of genes, including those involved in cell survival, metabolism and angiogenesis. However, HIF1-α and HIF2-α have distinct, non-redundant roles in angiogenesis. For instance, while HIF1-α is ubiquitously expressed, HIF2-α is only found in subset of tissues, including the liver and several tumours (25). Although HIF2-α is expressed in EC's, its exact function here remains elusive, since EC proliferation and migration is mainly regulated by HIF1-α. Additionally, HIF2-α deficient mice develop with severe organ abnormalities, however without apparent vascular defects (26,27). Yet, studies have suggested a role of

HIF2-α in the formation of abnormal blood vessels, mainly due to its capacity to induce the expression of eNOS (28,29).

Both subunits of HIF are constitutively expressed at the mRNA level; however, O_2 levels post-translationally regulate the α-subunit. In normoxia, the α-subunit is hydroxylated on specific, conserved proline residues by HIF-targeting prolyl hydroxylase domain-containing proteins (PHD1, PHD2, and PHD3) (30,31). Hydroxylated HIF binds the von Hippel–Lindau protein that is part of an E3-ubiquitin ligase. After ubiquitination, HIF1-α and HIF2-α molecules are rapidly degraded by the proteasome. In the event of hypoxia, PHDs are unable to hydroxylate HIF, thus preventing its degradation leading to heterodimerisation with HIF-β, followed by translocation to the nucleus, and binding to cis-regulatory hypoxia-response elements in target genes.

Four human HIF-α hydroxylases have been characterized so far (PHD1–PHD3 and factor-inhibiting-HIF or FIH1) and they belong to a family of 2-oxoglutarate-dependent, non-haem iron-binding dioxygenases. Notably, PHD2-inhibition by RNAi, but not inhibition of PHD1 or PHD3, is sufficient to upregulate HIF1-α in normoxia, indicating that the enzymes are not simply redundant and that PHD2 may be the main cellular oxygen sensor (32).

While the oxygen-dependent regulation of HIF has been well established, there is increasing evidence that HIF1-α can be regulated independent of oxygen levels. Growth promoting stimuli and oncogenic pathways play a pivotal role in this O_2-independent activation of HIF. A first group of O_2-independent regulators of HIF1-α are transition metal ions, such as cobalt and nickel. It is suggested that they substitute for the iron atom in the haem-group of oxygen-sensing proteins (33,34) or for the ferrous ion bound to PHD-molecules (35,36). The second kind of O_2-independent regulator is nitric oxide (NO). Indeed, hypoxia can stimulate NO-production through the induction of inducible NOS, the transcription of which is regulated by HIF-1. But independent of oxygen-levels, NO can block the stabilisation of HIF-1 and is able to repress its transcriptional activity (37,38). Another O_2-independent regulator is heat-shock protein 90 (HSP90), a molecular chaperone that induces proteosomal degradation of HIF1-α (39). Also, a variety of peptides, such as insulin (40,41) and insulin-like growth factors (42), interleukin-1 (41) and tumour necrosis

factor-α (TNFα) (43,44), angiotensin (45) and thrombin (46) are involved in the activation of HIF under normoxic conditions.

Under pathological conditions, strikingly in cancer, HIF becomes deregulated. The oxygen-independent activation of HIF plays an important role in the oncogenic progression, as the deregulation of growth factors and/or their respective receptors can result in a continuous activation of the HIF-pathway. Clinically, HIF is overexpressed in a variety of cancers, predicting poorer prognosis (47,48), resistance to radiotherapy and chemotherapy and increased patient mortality (49,50). Experimentally, HIF is required for the growth of solid tumours (51,52). HIFs regulate the expression of a variety of angiogenic factors, such as the vascular endothelial growth factor (VEGF), PlGF and angiopoetins (Ang) (53). This excess of pro-angiogenic factors, leads to an angiogenic response (Figure 3). Besides its importance for tumour-angiogenesis, HIF has also been associated with increased tumour aggressiveness and tumour cell heterogeneity (54). Intratumoural hypoxia has been linked with metastasis, and HIF has been identified to regulate several steps of the metastatic process, such as invasion through the basement membrane and extracellular matrix by up regulating proteases, increasing production of fibronectin to facilitate the establishment of an extracellular matrix; increased intravasation and regulation of genes that promote the pre-metastatic niche (55). The epithelial to mesenchymal transition (EMT) is the process in which epithelial cells lose their polarity and are converted towards a mesenchymal phenotype, is regarded as a critical event in the morphogenetic changes during metastasis. The essential features of EMT are the disruption of intercellular contacts and the enhancement of cell motility, thereby leading to the release of cells from the parent epithelial tissue. Indeed, the hypoxic microenvironment of cancer cells has been identified as an important factor in the induction of EMT. Loss *of E-cadherin* is a functional requirement of EMT and is induced by HIF, through upregulation of transcription repressors of *E-cadherin*.

Studies in PHD2-deficient mice indicate that PHD-inhibition may have interesting clinical implications (56). Specific knockouts of the *Egln1* gene (alias *Phd2*) in EC affect cell proliferation, whereas heterozygosity in normal mouse stroma plays a role in vessel maturation (56,57). Xenograft tumours grown in *Phd2*-heterozygotes are less hypoxic and have more functional vessels than those in control mice. ECs from these animals are more quiescent and exhibit

an altered transcriptional program dependent on HIF2-α. This transcriptional response directs ECs to form more organised vessels in tumours, leading to less hypoxia and thus a less aggressive, metastatic tumour phenotype. It has also been suggested that normalisation of tumour vessels can enhance tumour perfusion and potentially improve drug delivery (58). Therefore, PHD-inhibition could be effective in several aspects of cancer therapy. However, these studies have been conducted in xenograft models where PHD-activity was only impaired in the microenvironment, not in the tumour cells. This is an important distinction given the oncogenic role of HIFs. The reduction of PHD2 in a number of cancer cell lines suggests the possibility that PHD2 loss contributes to tumourigenesis and represents a pathway for HIF-activation in a number of tumours. Studies in cancer gene expression datasets revealed that *Phd2*-expression is significantly decreased in tumours, compared with normal tissue (59). Although this reduction of PHD2 impairs the degradation of HIF, studies have shown that PHD2 also has an additional HIF-independent regulatory function (60–62).

Thus, while the importance of HIF in angiogenesis and cancer has long been established, the exact role of PHD-molecules remains elusive. They have been proposed as both tumour suppressors and drivers of tumourigenesis, therefore their exact function in the progression of solid tumours forms an interesting field of research.
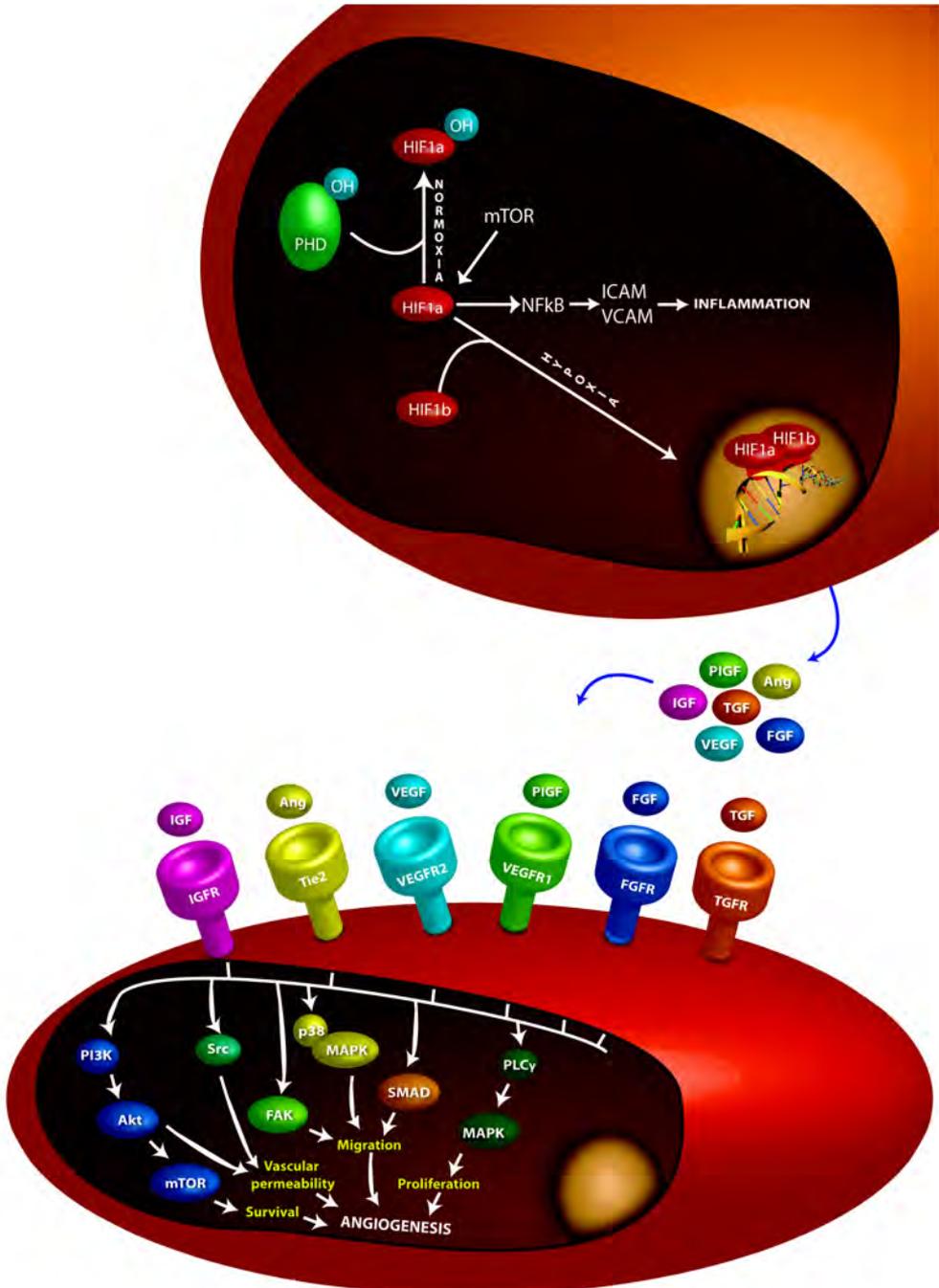
Fig.3: In the event of hypoxia, PHD2 is unable to hydroxylate HIF, thus an active transcriptional complex is formed, able to regulate hundreds of genes including several factors involved in angiogenesis.

2.1.2   Vascular endothelial growth factor

VEGF is most likely the best-known angiogenic factor and is of crucial importance for the vascular and lymphatic system. The VEGF-familiy consists out of 5 homologues, VEGF-A, -B, -C, -D and PlGF. All VEGFs bind with different affinities to their receptors, VEGF receptor-1 (VEGFR-1 or Flt-1), VEGFR-2 (or Flk) and VEGFR-3, of which only the first two are responsible for angiogenic signal transduction. VEGF-A is the key factor in the induction of angiogenesis and vasculogenesis, by binding to VEGFR-2 and increasing vascular permeability through a NO-dependent pathway (63,64). It causes vasodilatation by induction of endothelial nitric oxide synthase (eNOS) and increasing NO-production. VEGF-A promotes EC survival by inducing expression of anti-apoptotic proteins through activation of phosphatidylinositol-3-kinase (PI3K) pathways. Binding of VEGF-A to VEGFR-1 does not induce signalling transduction; it merely serves as a decoy receptor. Next to its established role in angiogenesis, evidence has emerged that VEGF-A also promotes a wide range of neuronal functions, both *in vitro* and *in vivo*, including neurogenesis, neuronal migration, neuronal survival and axon guidance (65,66). VEGF-A is mitogenic for astroglia and Schwann cells *in vitro* (67).

Furthermore, VEGF-A is known to promote leukemia cell survival through the activation of NF-κB and the activation of the MAPK/Erk-pathways (68). Also in HCC-cell lines it has been shown that VEGF regulates cell survival and proliferation via various signaling pathways depending on the cellular and environmental context (69).

VEGF-B is a ligand for VEGFR-1, but can also form heterodimers with VEGF-A (70). It is probably involved in the formation of coronary collaterals (71) and inflammatory angiogenesis (72), nevertheless, its precise function is unknown.

VEGF-C has high affinity for VEGFR-2 and VEGFR-3. It is primarily a lymphangiogenic growth factor that induces mitogenesis, migration and survival of ECs through activation of VEGFR-3. The angiogenic action of VEGF-C lies in its potential to bind VEGFR-2 (73–75). However, VEGF-C is also involved in the progression of several types of tumours, including non-small cell lung cancer, colorectal cancer and breast cancer (76–78). Recent studies have reported that VEGF-C induces tumour cell proliferation, invasion and

metastasis, partially through the VEGFR3-Src-p38MAPK-C/EBP-contactin-1 pathway however, other possible mechanisms of induction of cell locomotion by VEGF-C remain largely unknown (79,80).

VEGF-D is responsible for proliferation of ECs (through VEGFR-2) and also shows lymphangiogenic potential (through VEGFR-3) (81,82).

2.1.3   Placental growth factor

PlGF is a member of the VEGF-family of growth factors. The structure of PlGF exhibits remarkable similarities to the structure of VEGF-A, although PlGF and VEGF-A show only a 42% amino acid sequence identity, as well as significant functional differences (83). Human PlGF occurs in at least four isoforms, PlGF-1 (PlGF$_{131}$), PlGF-2 (PlGF$_{152}$), PlGF-3 (PlGF$_{203}$), and PlGF-4 (PlGF$_{224}$) due to alternative mRNA splicing of the *PGF*-transcripts. Apart from size, the PlGF-isoforms differ in terms of both their secretion properties and their binding affinities.
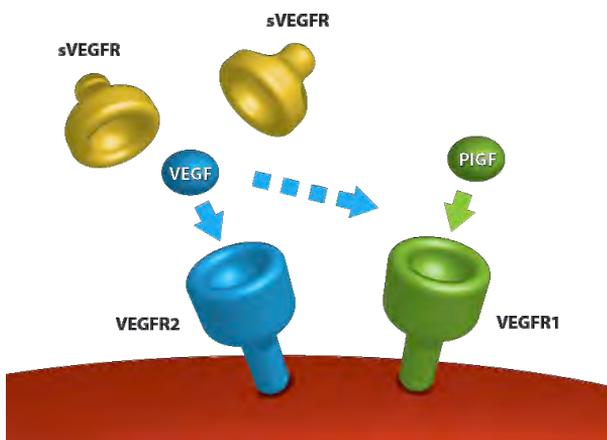
Fig 4: *VEGF receptors in PlGF-signalling. VEGF is able to bind on VEGFR2 and VEGFR1, yet only the former induces a signalisation cascade while the latter serves as a decoy receptor. However, VEGFR1 does serve as functional receptor for PlGF. Therefore, PlGF exerts is function independent from VEGF by inducing signalling reaction after binding with VEGFR1, and dependent from VEGF by preventing its binding to the decoy receptor.*

PlGF was originally discovered in the human placenta, where it has been proposed to control trophoblast growth and differentiation. Now, it is known that PlGF is expressed by a variety of cell types and organs, such as heart, lung, thyroid gland and skeletal muscle, but its expression remains low in healthy tissue. In the liver, PlGF-levels have been detected in hepatocytes, EC's, HSC and küppfer cells. It mainly has two mechanisms of action: one independent of VEGF and one dependent of VEGF. The independent action mechanism binding of PlGF to VEGFR-1 and induces auto-phosphorylation, thereby activating the VEGFR-1 signalling cascade (Figure 4). Nevertheless, only a weak phosphorylation takes place when binding to VEGFR1, hence the major angiogenic action might lay in its potential to bind to neuropilin-1 (84). VEGF-dependent activities of PlGF include displacement of VEGF from membrane-anchored or soluble VEGFR1, thereby liberating VEGF for stimulation of VEGFR2. Other VEGF-dependent actions of PlGF include the upregulation of VEGF, Fibroblast growth factor-2, platelet derived growth factor, matrix methaloproteinases (MMP) and other angiogenic factors (85,86); and an intermolecular cross-talk from VEGFR1 to VEGFR2, leading to enhanced activation of VEGFR2, thus increasing the response to VEGF (64).

PlGF induces a variety of biological effects. For instance, PlGF participates in the angiogenic process by stimulation of EC growth, migration and survival (87–90). Furthermore, it stimulates proliferation and regulates the contractile response of mesenchymal fibroblasts and mural cells. PlGF also reconstitutes haematopoiesis by recruiting VEGFR1 positive stem cells from the bone marrow, attracts inflammatory cells and recruits endothelial and other angio-competent bone marrow progenitors to the site of tumour and collateral vessel growth (89,91–93). In addition, PlGF is an important regulator of in the process of vessel stabilisation and maturation, by recruiting bone marrow cells, smooth muscle cells, ECs, pericytes and monocytes. This pleiotropic action of PlGF explains why PlGF stimulates revascularization of ischemic tissue, increases tumour vascularization and metastasis. Several studies have shown that PlGF is involved in numerous pathological conditions, such as fracture repair (94); atherosclerosis (95), coronary artery disease (96), wound healing in diabetes (97) and nervous degeneration (98). PlGF is also able to directly influence cellular motility, in breast cancer cells (99), lung carcinoma cells (100) and leukemia (101).

In addition, PlGF has been shown to be important for leukemia cell growth and survival, through auto- and paracrine pathways (102).

In contrast to the other members of the VEGF-family, PlGF is not essential for physiological angiogenesis during development and reproduction. *Pgf*-knock out mice are born without apparent vascular defects, resulting in viable and fertile mice that do not display any major abnormalities. A possible explanation for this is that PlGF is nearly undetectable in most organs in healthy conditions (103). However, in ischemic conditions such as myocardial infarction or following ligation of the hind limb artery, these mice show a reduced ability to respond to ischemic damage through angiogenesis and adaptive arteriogenesis. Hence, together with VEGFR-1, PlGF is an important mediator in regulating the angiogenic switch in pathological conditions [3, 22-23]. Several studies have shown that PlGF contributes to the angiogenic and inflammatory switch in disease, including tumour growth, ischemia, arthritis, ocular neovascularisation, atherosclerosis, obesity and portal hypertension (89,94,104). Therefore, PlGF has become an attractive drug candidate. A PlGF-inhibitor would be expected to selectively inhibit pathological angiogenesis, without affecting physiological vessel growth, possibly causing fewer adverse effects.

However, the role of PlGF in tumour angiogenesis is controversial. While several independent studies showed that PlGF enhances pathologic angiogenesis by initiating crosstalk between VEGFR-1 and VEGFR-2, other studies provided evidence that PlGF may also function as a negative regulator of tumour growth and angiogenesis. The mechanism of negative regulation of angiogenesis involves the formation of VEGF–PlGF heterodimers that do not display significantly angiogenic activity as compared to VEGF homodimers (105). They deplete the intracellular pool of VEGF, thereby reducing the formation of angiogenic VEGF–VEGF homodimers, lacking the ability to induce angiogenesis and failing to activate the VEGFR-2 mediated signalling pathways. Also, in a recent study conducted by Ferrara, PlGF neutralisation with antibodies did not inhibit tumour growth nor angiogenesis in 15 orthotopic tumour models (106).

Nevertheless, multiple studies did provide evidence for the potential use of anti-PlGf and anti-VEGFR-1 antibodies as anti-angiogenic agents in pre-clinical models of tumour growth. Fisher *et al.* developed a specific blocking monoclonal antibody against mouse PlGF (90), which was tested on mouse melanoma, pancreatic carcinoma, colon carcinoma, and lymphoma cell lines. All these tumours express PlGF, and it was demonstrated that the anti-PlGF antibody inhibited tumour growth. The anti-PlGF antibody also inhibited metastases of these tumours and enhanced the efficacy of the chemotherapeutic agents in melanoma and pancreatic tumour models. A recent study has demonstrated that downregulation of PlGF leads to decreased tumour growth and metastasis by promoting the anti-tumour immunity through polarizing tumour-associated macrophages (107).

Thus, while VEGF has been acknowledged as an indispensible factor in the angiogenic switch, both in physiologic as pathologic setting, the importance of PlGF has only recently been discovered. Although its almost exclusive involvement in pathological angiogenesis provides a unique potential for the development of new anti-angiogenic drugs, there have been contradictory findings on its role in tumour angiogenesis. Therefore, additional research is essential to investigate its potential function as a therapeutic target against cancer.

2.2 Mechanisms of angiogenesis

Several different mechanisms of angiogenesis have been described so far. The first type is the establishment of new vessels from a pre-existing vessel by **sprouting**. This type of neovascularisation has been greatly studied and can be defined in several stages. Vasodilatation is the first step in the formation of blood vessels. The influence of ang-2 and VEGF gives rise to the occurrence of fenestrations, which increases the vascular permeability allowing extravasation of plasma proteins (figure 5; 1-2) (108–110). These proteins serve as a scaffold for migrating ECs. Integrins are crucial in the migration of ECs to communicate between the scaffold proteins and the ECs, thus providing information about the location of the angiogenic site. Attracted by proangiogenic signals, ECs become motile and invasive, yet the basement membrane and the coat of mural cells prevent them from leaving their positions. Therefore, at the onset of sprouting, ECs must be liberated, a process requiring proteolytic breakdown of the basement membrane and detachment of mural cells, mediated by MMPs, allowing subsequent EC-migration and proliferation (figure 5; 2). Several angiogenic factors (VEGF, fibroblast growth factor (FGF), and epidermal growth factor (EGF)) induce EC proliferation and migration through the matrix (figure 5; 3). VE-cadherin and integrins coordinate the EC-binding while TNFα, FGF, and PDGF induce tube formation (figure 5; 4-5). Endothelial progenitors differentiate towards ECs and form a primitive vessel (figure 5; 5-6). The surrounding vessel layers, composed of pericytes (or smooth muscle cells (SMC) in large vessels), need to be recruited (a platelet derived growth factor (PDGF)- and VEGF mediated process) (figure 5; 6). Under the influence of TGF-β ECs will tighten up, pericytes are recruited and a new basement membrane and ECM is generated (figure 5; 7). Ang-1 is responsible for vessel stabilisation leading to durable and mature blood vessels (figure 5; 8).
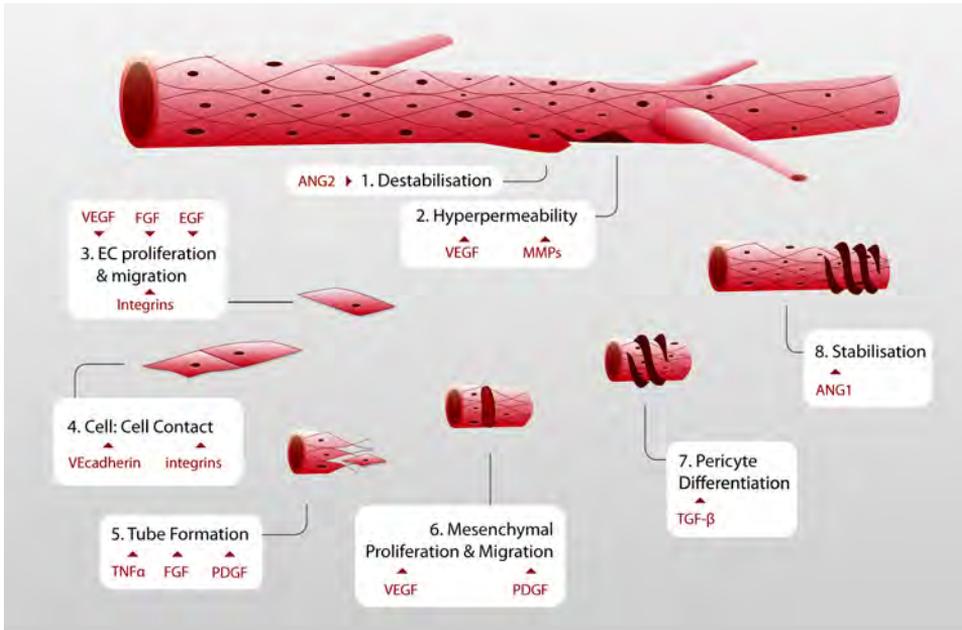
A second type of angiogenesis is the formation of a new vessel by **intussusception** of an existing vessel (111). An internal division forms within a pre-existing vessel, through the formation of transcapillary tissue pillars, resulting in two new vascular units. Stabilisation of the newly formed blood vessels occurs by invagination of surrounding pericytes and extracellular matrix.

A third type is called **vasculogenesis,** which is the term used for the *de novo* formation of new blood vessels and is thus not strictly speaking a form of angiogenesis since this term implies the formation of new blood vessels out of existing vessels. Endothelial precursor cells (angioblasts) migrate and differentiate in response to local stimuli (such as growth factors and the extracellular matrix) to form new blood vessels. These vascular trees are then pruned and extended through angiogenesis. Vasculogenesis occurs mostly during embryologic development of the circulatory system (112), but can also

occur in the adult organism from circulating endothelial progenitor cells (derivatives of stem cells) (113), especially in tumours (114,115).

A fourth type of blood vessel formation, **looping angiogenesis**, has recently been discovered (116,117). This is the non-angiogenic expansion of preexisting vessels, which occurs rapidly after tissue injury and is responsible for rapid occurrence of fully functional vessels in granulation tissue, shortly after wounding. Biomechanical forces, mediated by myofibroblastic contraction, induce vessel translocation by pulling on existing vessel loops into the granulation tissue, thereby providing fully functional vasculature. Similarly, tumours are known to incorporate the hosts existing vasculature, without inducing neo-angiogenesis, a process that is called "**vessel-cooption"** and in which angiopoetins play a pivotal role (118,119).

Another way of forming new vessels is **vascular mimicry**, a process that is described in the section "Angiogenesis in cancer".

2.3   Angiogenesis in cancer
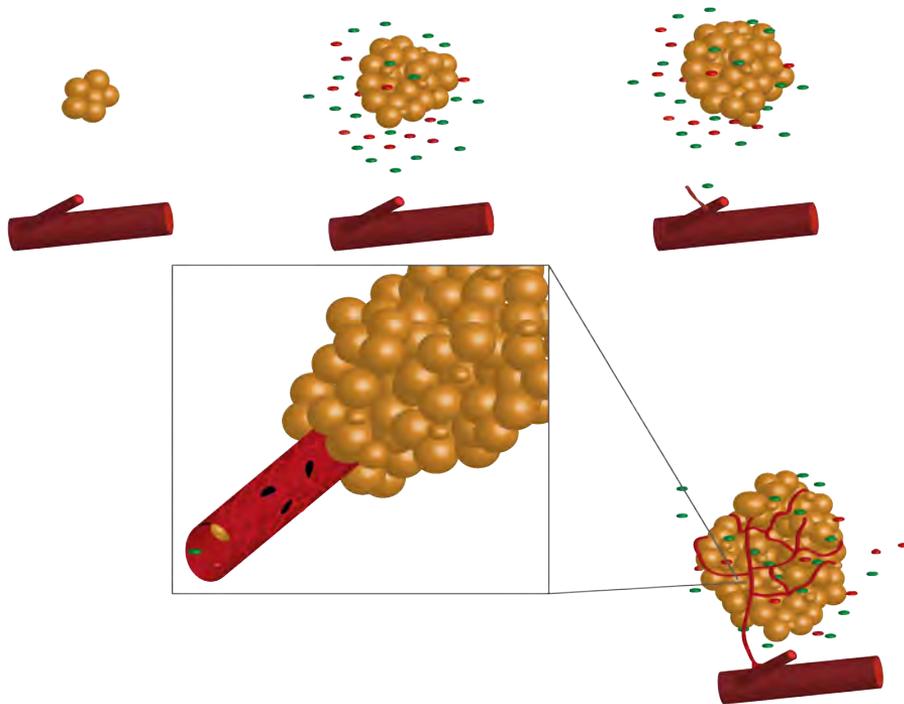
Fig 6: *Tumour angiogenesis. Tumours produce pro-angiogenic factors (green) but also factors that constrain the endogenous angiogenic-inhibitors (red), thus causing an angiogenic switch which stimulates the formation of new blood vessels and enhances the tumour's blood supply.*

Tumours consist out of rapidly growing and dividing cells and thus, have a high need for oxygen and nutrients. For solid tumours these needs can be fulfilled by passive diffusion up to the size of 1-2 mm^3 after which they become hypoxic and need additional blood supply, achieved by inducing an "angiogenic switch". At this point the net balance between positive and negative angiogenic regulators is disrupted in favour of pro-angiogenic pathways, leading to the formation of new vessels (figure 6). The angiogenic switch is not only triggered by metabolic stress (low O_2-levels, low pH or hypoglycaemia) (120), but also by mechanical stress (121), the inflammatory response (122,123) and genetic mutations. The collaboration between cancer cells and the tumour microenvironment leading to neovascularisation is a complex matter since pro- and anti-angiogenic factors can originate from cancer cells, ECs, stromal cells,

blood and the ECM. Their relative contribution changes throughout the tumour's progression.

Tumour vessels usually develop by sprouting or intussusception from pre-existing vessels, however, circulating endothelial precursor cells can also induce *de novo* formation of blood vessels (111,114,115). Because the temporal and spatial expression of angiogenic regulators in tumours is not as well coordinated as in physiological angiogenesis, tumour vessels are characterized by a limited functionality. They often lack functional perivascular cells, needed to protect vessels against oxygen or hormonal changes and providing them necessary vasoactive control to adapt to metabolic needs (124). Tumour vasculature is highly disorganised, vessels are dilated, with uneven diameters, and show excessive branching and shunts (125). Consequently, tumour blood flow is chaotic, leading to hypoxia and thus, increasing the pro-angiogenic stimulus, creating a vicious circle.

Morphologically, these tumour vessels are also abnormal: their walls have numerous fenestrae, widened inter-endothelial junctions and a discontinuous or absent basement membrane. This causes vessels to be leaky (126), which facilitates metastasis and enables the dispersion of macromolecules. Furthermore, the ECs are abnormal in shape, forming multilayers and bulging in the lumen. Often the vessel walls are not only aligned by ECs, but by a mosaic of ECs and cancer cells mimicking the former, a phenomena called "vascular mimicry". During this process, tumour cells can form highly structured vascular channels, independent of angiogenesis. These channels are composed of a basement membrane and can either by lined solely by tumour cells or by a mosaic of ECs and cancer cells.

Another process to enhance the tumour's blood supply independent from angiogenesis is vessel-cooption (127,128). In early stages of tumour progression, the tumour can co-opt host vessels, without inducing an angiogenic switch. Interestingly, these vessels regress after a period of time, under the influence of Ang2 which elicits mural cell detachment and EC apoptosis, possibly as a host defence mechanism inducing tumour cell-death around the regressed vessels (118). However, this is followed by a massive production of pro-angiogenic factors in the tumour, thus inducing the angiogenic switch.

The exact cause of these abnormalities in tumour vessels remains elusive. Indeed, the imbalanced production of angiogenic factors in tumours might cause the disorganised vessel formation. Especially VEGF is known to trigger the unusual vessels in experimental models and angiopoetins are important regulators of vessel (de)stabilisation. But less is known about the role of these factors in tumour vessel leakiness (129).

Thus, tumour cells outgrow their vascular supply and therefore induce angiogenesis by producing angiogenic factors and suppressing angiogenic inhibitors. This stimulates the formation of a typical abnormal, disorganized tumour vasculature, which is leaky and largely dysfunctional. Not only does this induce hypoxia, but it also stimulates metastasis.

2.4   Angiogenesis in chronic liver disease

The progression from fibrosis to cirrhosis and hepatocellular carcinoma, the end-point of CLDs, is distinguished by a prolonged inflammatory and fibrogenic process that leads to an abnormal angio-architecture distinctive for cirrhosis. Several mechanisms are responsible for the angiogenic switch during the pathogenesis of CLDs (figure 7). First, CLDs are characterised by chronic inflammation (130). Increased intrahepatic vascular resistance is primarily caused by anatomical changes, such as fibrotic scar tissue compressing portal and central venules. In addition, the formation of fibrotic septa, as well as sinusoidal capillarisation, can result in an increased resistance to blood flow and oxygen delivery. This results in hypoxia and the transcription of hypoxia-sensitive pro-angiogenic genes, usually modulated trough HIF. Also, an increased contribution of the hepatic artery to the sinusoidal blood flow leads to sensitisation of the hepatocytes to abnormal high oxygen conditions (131).

Fig 7: *Link between angiogenesis, inflammation and chronic liver diseases, portal hypertension, NASH and HCC, showing how inflammation and angiogenesis are involved in nearly all stages of liver disease, starting from the onset of fibrosis and the activation of hepatic progenitor cells, to the occurrence of HCC and metastasis.*

Hepatic stellate cell (HSC) motility and migration promote coverage of HSC around sinusoids, causing sinusoidal constriction and contributing to the hepatic resistance in liver cirrhosis. The characteristic fenestrated phenotype of the sinusoidal ECs is lost and an organised basement membrane is established, which leads to an impairment of oxygen diffusion even though the increased arterial flow provides a high supply of oxygenated blood (132). The deposition of collagen in the space of Disse accentuates the narrowing and distortion of the sinusoidal lumen, further restricting microvascular blood flow. This is aggravated by leukocytes, either mechanically trapped in the narrowed sinusoids or adhering to the endothelium, as a result of activation of a hepatic microvascular inflammatory response. The hypoxic liver tissue causes an up-regulation of VEGF, Ang and their receptors in HSCs, not only enhancing the

hypoxia-induced angiogenesis but also stimulating activation and migration of HSC (133–135). As previously stated, activated HSCs induce an inflammatory response and enhance angiogenesis (136,137). New vessels themselves can significantly contribute to the perpetuation of the inflammatory response by expressing chemokines and adhesion molecules promoting the recruitment of inflammatory cells. Furthermore, angiogenesis, early in the course of a CLD, may contribute to the transition from acute to chronic inflammation (138).

2.5 Angiogenesis in hepatocellular carcinoma

2.5.1 Hypoxia and angiogenesis in HCC

HCC is a primary malignancy that mostly emerges on a background of CLDs. It is one of the most vascular solid tumours in which angiogenesis plays an important role in its development, progression and metastasis (139). The hypervasculature found in CLDs facilitates the progression from small dysplastic nodules, through neoplastic lesions to large HCC tumours. Dysplastic nodules in the pre-malignant (cirrhotic) environment are responsible for the angiogenic switch and angiogenesis carries on throughout the process of tumour progression (140) (figure 6). The phenotypic switch to angiogenesis is linked to a disturbance in the equilibrium between positive and negative angiogenic regulators. Tumours secrete a number of angiogenic growth factors, such as VEGF, PDGF, PlGF and TGF-β1. Furthermore, expression of endogenous inhibitors, for instance TSP-1, endostatin and angiostatin, are down-regulated (141). This activates ECs and basement membranes to remodel existing vessels, and stimulates the release of endothelial progenitor stem cells from the bone marrow to form new vessels. HCC cells display rapid growth and are consequently in need for a high oxygen and nutrient supply. Hence, tumour cells induce the formation of new blood vessels to fulfil this aspiration. HCC lesions are characterised by arterial hypervascularity to provide the tumour with highly oxygenated blood (139). However, these new vessels are marked by a disorganised vasculature, consisting of leaky, hemorrhagic and tortuous vessels. The irregularity of tumour vessels results in chaotic blood flow with poorly oxygenated blood. Furthermore, these leaky blood vessels provide a

passage for tumour cells into the blood circulation and, hence, facilitate metastasis.

Hypoxia plays an essential role in promoting the invasive and metastatic behavior of HCC cells, mostly by exerting significant effects on cellular metabolism, with numerous downstream consequences. One of the first links discovered between O_2-levels and tumour behavior is that O_2-deprivation provides a selective advantage within tumours for the clonal expansion of cells that have acquired p53-mutations, thus avoiding apoptosis (142). HIFs also affect the *Myc*-pathway, which is an important regulator of proliferation and anabolic metabolism. Whereas HIF1-α opposes *Myc,* HIF2-α promotes *Myc*-activity (143,144). These observations describe crosstalk between responses to changes in O_2 availability and a key transcription factor regulating cell growth. Thus, hypoxia and subsequent HIF-signalling can influence the tumour cells proliferation. It has also been found that hypoxic tumours are more likely to metastasise in humans (120,145) and in rodent models, and it has been demonstrated that exposure of tumour cells to hypoxia *in vitro* can enhance their metastatic potential when they are reinjected in mice. Hypoxia-induced point mutations, deletions and gene amplifications may promote the development of metastatic disease by several mechanisms, including genomic instability, enhanced expression of pro-metastatic genes, growth and survival advantages of metastatic tumours cells and inactivation of metastatic-suppressor genes (146). Degradation of the ECM via specific proteases has also been suggested to play a key role in tumour metastasis. These proteases may also play a role in the release of growth factors from the ECM to assist growth of tumour cells at a new site, and indeed hypoxia is known to stimulate factors involved in this proteolytic breakdown, hence facilitating this metastatic process (147,148). Recently, an increasing amount of evidence demonstrates that alterations in microenvironmental O_2 levels and activation of hypoxic signaling through HIF are emerging as important triggers and modulators of the EMT, which is a key event in tumour progression and metastasis (149).

Hypoxia also influences the tumours metabolism. During tumoural hypoxia, there is metabolic shift away from oxidative phosphorylation in mitochondria towards glycolysis, a state also involved in the 'Warburg effect', during which more glucose is consumed and less ATP is produced. However, tumour cells do

not switch back to oxidative phosphorylation under aerobiosis. Interestingly, constitutive activation of HIF2-α results in the development of severe hepatic steatosis associated with impaired fatty acid beta-oxidation, decreased lipogenic gene expression, and increased lipid storage capacity (150). Thus, demonstrating that HIF2-α functions as an important regulator of hepatic lipid metabolism, which also has implications for HCC since these lesions often accumulate lipids and/or develop in a background of fatty liver disease.

The best-studied effect of hypoxia on tumour development and metastasis, is angiogenesis. VEGF is one of the uttermost important factors involved in the angiogenic switch of HCC. Already in an early phase of CLDs an upregulation of VEGF is observed (151–153), an increase that is tremendously enhanced during HCC progression. The degree of VEGF expression increases with tumour stage (154,155) and is associated with poor prognosis (156–158). Furthermore, VEGF levels have been correlated to vascular invasion (159,160), metastasis (161), recurrence (162), vascular density (163,164), differentiation (155) and tumour aggressiveness (165). Recently, the importance of PlGF in the pathogenesis of CLD has been established. PlGF levels are strongly up regulated in HCC and correlated with prognosis and recurrence after resection (166–168). However, less is known about the involvement of PlGF in the hepatocarcinogenesis, but *in vitro* experiments have shown that both PlGF and its receptors are expressed in hepatocytes, EC and HSC.

2.5.2   Inhibition of angiogenesis as systemic therapy for HCC

HCC is potentially curable by surgical resection and liver transplantation, as previously discussed. However, the majority of patients present with advanced-stage disease, which is most commonly accompanied by severe CLDs. Therefore, surgery is only feasible for a small fraction of patients. Moreover, systemic chemotherapy has very limited impact on advanced HCC, partly because HCC is a chemotherapy-resistant tumour (169,170). Furthermore, the underlying cirrhosis may lead to portal hypertension with hypersplenism, varices and gastrointestinal bleeding, hypoalbuminemia, hepatic encephalopathy, altered drug binding, distribution, and pharmacokinetics, limiting the selection and optimal dosing of most cytotoxic agents (171). Until recently, systemic therapy of advanced HCC provided marginal benefit. Since

the discovery of anti-angiogenic agents as potential inhibitors for tumour growth it has fundamentally landmarked a new era of anti-cancer therapy (table 1) (172). Anti-angiogenic agents are unique cancer-fighting targets. Their predominant effect is via inhibiting vessel formation and thus indirectly targeting tumour cell growth. By inhibiting angiogenesis, the tumour is withheld from its blood supply and therefore its growth is inhibited (173).

Significant progress on the treatment of advanced HCC has been made by the introduction of sorafenib. Sorafenib is a small molecular inhibitor that targets several tyrosine protein kinases in the Raf/MEK/ERK-pathway (anti-proliferative effect); and PDGF, VEGFR1 and VEGFR2 (anti-angiogenic effect). In vitro, sorafenib has shown to stop tumour growth and induce apoptosis of HCC cell lines, and thus inhibiting the growth of HCC in xenografts (174,175). A large phase III clinical trial (SHARP) was conducted in 602 patients with advanced HCC; resulting in a 31% decrease in the risk of death with a median survival for sorafenib of 10.6 months vs 7.9 months for placebo resulting in a significant increase of survival compared to placebo (176). The main adverse effects are diarrhoea, weight loss, hand–foot skin reaction, hair loss, anorexia, asthenia, fatigue, and voice changes. Sorafenib has become the standard of care for patients with advanced HCC and also for those progressing after loco-regional therapies. Therefore, the SHARP-study was a milestone in the management of advanced HCC offering hope for the more than 600 000 patients who die each year of HCC worldwide.

Other important inhibitors of the VEGF-pathway are bevacizumab and sunitinib. Bevacizumab is a recombinant, humanised monoclonal antibody (IgG1) that selectively binds and neutralises all isoforms of VEGF. In addition to its direct anti-angiogenic effects, bevacizumab may enhance chemotherapy administration by decreasing the elevated interstitial pressure in tumours. Reports from phase II trials showed promising results for bevacizumab monotherapy in advanced HCC (177). Combinations of bevacizumab with chemotherapy obtained responses of 10% - 20% with a median survival of 9-10 months (178). Despite the initial safety concerns for bevacizumab in 13 patients with liver cirrhosis and portal hypertension, the most common side effects were hypertension, bleeding, and proteinuria (177). Sunitinib is a small molecule inhibiting VEGFR1 and -2, PDGFR, c-Kit and Flt3. In a preclinical study, sunitinib suppresses tumour growth in HCC xenografts, by increasing apoptosis,

reducing microvessel density and inhibiting tumour cell proliferation (179). Although in a significant number of patients sunitinib led to tumour necrosis in two phase II trials, the results obtained in HCC-patients are rather disappointing given the treatment-related adverse events potentially leading to death due to gastrointestinal bleeding, encephalopathy and hepatorenal syndrome (180,181). The worse safety profile of this drug in HCC compared with other tumours points out the need of caution in the management of potent anti-angiogenic agents in cirrhotic patients. Other inhibitors of the VEGF-pathway, such as brivanib (182,183), foretinib (NCT00920192), vandetanib (184,185), vatalanib (186–188), cediranib, linifanib, and lenvatenib are currently being tested in several trials (189), as summarised in table 1.

Thalidomide is a glutamic acid derivative with immunomodulatory and anti-inflammatory qualities. It is mostly known for its teratogenicity when it was used as a sedative drug to treat pregnancy related nausea, but has become the subject of interest in oncology after its anti-angiogenic properties have been discovered in 1994. Still, the exact mechanism on how it exerts it anti-angiogenic potential, remains unclear. Studies have shown that thalidomide inhibits the production of NO, a factor that is involved in the early phases of angiogenesis (190). The anti-angiogenic effect of thalidomide may also be due to its ability to alter VEGF and bFGF production (191)(192). Several studies have shown that thalidomide is moderately tolerated and minimally effective in large HCC (193).

Another pathway that is currently under active investigation for potential anti-angiogenic drugs against HCC, is the mammalian target for rapamycine (mTOR) – pathway. mTOR is a serine-threeonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis and transcription; and its most known inhibitor is rapamycin. In *in vivo* mouse models, rapamycin displays strong inhibitory effects on tumour growth and angiogenesis, which are related to a reduced production of VEGF (194). Other mTOR targeting agents that are currently investigated in clinical trials are everolimus and temsirolimus, as shown in table 1.

Drugs (trade name)	Targets	Number of trials	Ref
Sorafenib (Nexavar®)	PDGF, VEGFR1 and VEGFR2, Raf/MEK/ERK	133 – Phases I, I-II , II, III & IV	(174,176,195,196)
Bevacizumab (Avastin®)	VEGF	20 – Phases I & II	(177,178,197–200)
Thalidomide (Thado®)	NO	19 – Phases I/II, II, II/III & III	(193,201–207)
Erlotinib (Tarceva®)	EGFR tyrosine kinase	14 – Phases I, I-II, II & III	(208–210)
Sunitinib (Sutent®)	VEGFR1,VEGFR2, PDGFR-a/b, c-KIT, FLT3 & RET kinases	12 - Phases I, II, II-III & III	(180,211,212)
Everolimus (Afinitab®)	mTOR	7 – Phases I, I-II, II & III	(213–215)
Brivanib	VEGFR & FGFR	6 – Phases I, II & III	(183)
Sirolimus (Rapamune®)	mTOR	5 - Phase I, II, II-III & III	(213,216–218)
AZD6244	MAP/ERK-kinase	3 – Phases I/II & II	(219,220)
Temsirolimus (Torisel®)	mTOR	3 – Phases I & II	(221)
BIBF1120 (Vargatef®)	VEGFR, PDGFR & FGFR	2 – Phase II	(222)
Cediranib (Recentin®)	VEGFR, PDGFR & c-KIT	2 - Phase II	(184,223–227)
Cetuximab (Erbitux®)	EGFR	2 - Phase II	(228–230)
Gefitinib (Iressa®)	EGFR	2 - Phase II	(231–233)
Lapatinib (Tyverb®)	EGFR & HER/NEU	2 – Phase II	(234)
Linifanib	VEGF & PDGF	2 – Phases II & III	(235)
Pazopanib	VEGFR, PDGFR & c-KIT	2 – Phase I	(236,237)
Pazopanib (Votrient®)	VEGFR, PDGFR & c-KIT	2 – Phase I	(238)
Ramucirumab	VEGFR2	2 – Phases II & III	(239)
Vandetanib (Caprelsa®)	VEGFR	2 – Phases I & II	(185)
AMG386	Ang	1 –Phase II	(240)
Foretinib	C-MET and VEGFR2	1 - Phase I	(241)
Lenvatinib	VEGFR, FGFR & SCFR	1 – Phase I/II	(242)
TSU-68	VEGFR, FGFR & PDGFR	1 – Phase I/II	(243)

Table 1: Overview of trials with anti-angiogenic agents as potential therapies against hepatocellular carcinoma, registered at www.clinicaltrials.gov (on 1st November '11) (244).

2.5.3   Limitations of anti-angiogenic therapy

The use of VEGF-pathway inhibitors to impair angiogenesis now represents a clinically validated treatment for a variety of solid tumours. Targeting of a single angiogenic protein, such as VEGF, might initially be effective, but ultimately leads to the failure due to escape mechanisms. Despite the benefit of disease stabilisation in patients and the increase in progression-free survival, tumours may eventually relapse and escape therapy with an absolute gain in overall survival of months rather than years (245). Escape from anti-angiogenic therapy is likely to involve multiple mechanisms (246).

The benefits of VEGF-targeted agents in the treatment of late-stage cancers can be temporary, resulting in eventual drug resistance, tumour growth and/or regrowth, and rapid vascular recovery when therapy is stopped. Ebos *et al.* and Pàez-Ribes *et al.* demonstrated that angiogenesis inhibition in mice can lead to opposing effects on tumour growth and metastasis depending on tumour stage and treatment duration (246–248). Anti-angiogenic therapy disturbs the tumour vasculature and in some cases (e.g. sunitinib) also disrupts pericyte coverage, which destabilises vessels, making them more leaky and immature, and hence facilitating intravasation of tumour cells and metastasis (249).

Anti-angiogenic therapy is expected to prune the tumour vasculature, thereby depriving the tumour cells from oxygen. The hypoxic conditions created by anti-angiogenic therapy, may select for more invasive tumour variants better adapted to survive and proliferate under reduced oxygen tension (56,145,250,251). Also, as mentioned previously, hypoxia is known to induce factors involved in the proteolytic breakdown of the ECM, induce EMT and activate pro-metastatic pathways, thus stimulating a more aggressive and pro-metastatic tumour phenotype (142,146–149,252).

In addition, anti-angiogenic therapies that inhibit a single target cause the upregulation of additional angiogenic factors, in a process called "angiogenic rescue" (253). Alternatively, these angiogenic escape mechanisms can also function independent from hypoxia, by inducing vascular mimicry, vessel cooption or switching the "mechanism" of angiogenesis. Blockage of angiogenesis also commonly leads to a switch from sprouting angiogenesis to intussusceptive angiogenesis (254). This might represent an adaptive response

to treatment with various anti-tumour and anti-angiogenic compounds. It serves to repair a damaged tumour vasculature in order to restore the tumour's oxygen supply and thus preventing the action of anti-angiogenic treatment.

Also, angiogenic inhibitors are known to inhibit vessels formed by ECs, but do not affect tumour cells that mimic the vasculature (255). Therefore, vascular mimicry could be used as an escape mechanism by tumour tissue, providing progressive growth without angiogenesis and could thereby contribute to the failure of anti-angiogenic therapy aimed to fully deprive tumours from their blood supply.

In addition, a recent study of Ebos *et al.* shows that pre-treatment of healthy mice with VEGF-inhibitors (prior to tumour cell implantation) promotes metastasis (247). A possible hypothesis is the creation of a "pre-metastatic niche". Administration of VEGF-inhibitors prunes quiescent vessels in healthy tissues, which will show rapid rebound growth after withdrawal of therapy (256) and such a well-vascularised niche may promote metastasis. Furthermore, VEGF-inhibitors are known to induce a state of chronic inflammation, which leads to the upregulation of several inflammatory cytokines that recruit angiogenic bone marrow-derived progenitors (212) and might promote the formation of a premetastatic niche (257).

3   Experimental mouse models for hepatocellular carcinoma research

3.1   Introduction

Hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to the malignant transformation of hepatocytes (258). The availability of a wide range of experimental mice models for HCC research, has given researchers the opportunity to assess tumour-host interactions, to perform drug-screening and to mimic the complex multistep process that leads to malignancy. When selecting a model for HCC research, one must first understand the limitations and advantages that the specific model possesses. HCC rarely occurs spontaneously in humans, in contrast to certain mouse strains, which have a high background incidence of spontaneous hepatic tumour occurrence (259). The same mouse strains are very susceptible to hepatocarcinogens. It also needs to be added that the spectacular results of potential anti-tumoural agents seen in mice, can seldom be repeated in human studies (260). In this introduction we will provide a concise overview of mouse models for HCC-research, thereby mainly focussing on diethylnitrosamine and providing a general outline of the principles of xenograft and transgenic mouse models. For a more complete summary, we would like to refer to our previously published review on mouse models for HCC, supplied as a supplement in this thesis (261).

3.2   Chemically induced mouse models

Several chemical reagents induce tumour formation when administrated in sufficient high doses and time span. There are two types of carcinogenic compounds: (1) genotoxic compounds which are characterised by their capacity to induce structural DNA changes and (2) promoting compounds lacking direct genotoxic capability, but enhance tumour formation after initiation by a hepatoxic compound (262). Treatment with a tumour-promoting agent facilitates the clonal expansion of the pre-neoplastic cells.

The advantage of chemically induced models is the similarity with the injury-fibrosis-malignancy cycle seen in humans. This makes them favourable models for HCC research, since their pathogenesis is more similar to the human situation. Different hepatocarcinogenic agents are metabolised through diverse metabolic pathways and extrapolation should be considered with caution when it is uncertain if the agents would be metabolised differently in humans. Age and dose of administration of hepatoxins might lead to different tumour phenotypes, which might influence experimental outcomes.

In the interest of space and convenience, we will only discuss the diethylnitrosamine (DEN) model. For a more detailed summary of chemically induced-mouse models, we would like to refer to our previously published review on mouse models for HCC (261).

DEN is often used as a carcinogenic reagent and the target organ in which DEN induces tumours is species specific. Mice mostly develop liver tumours, but also gastrointestinal (263), skin, respiratory (264,265) and hematopoietic tumours have been found (266,267). The carcinogenic capacity of DEN is situated in its capability of alkylating DNA-structures (figure 8). In the first step DEN is hydroxylated to α-hydroxylnitrosamine (268,269). This bioactivation step is oxygen and NADPH-dependent and is mediated by cytochrome P450 (cyp450), an enzyme that has its highest activity in the centrilobular hepatocytes. After cleavage of acetaldehyde, an electrophilic ethyldiazonium ion is formed. This ethyldiazonium ion causes DNA-damage by reacting with nucleophiles such as DNA-bases.
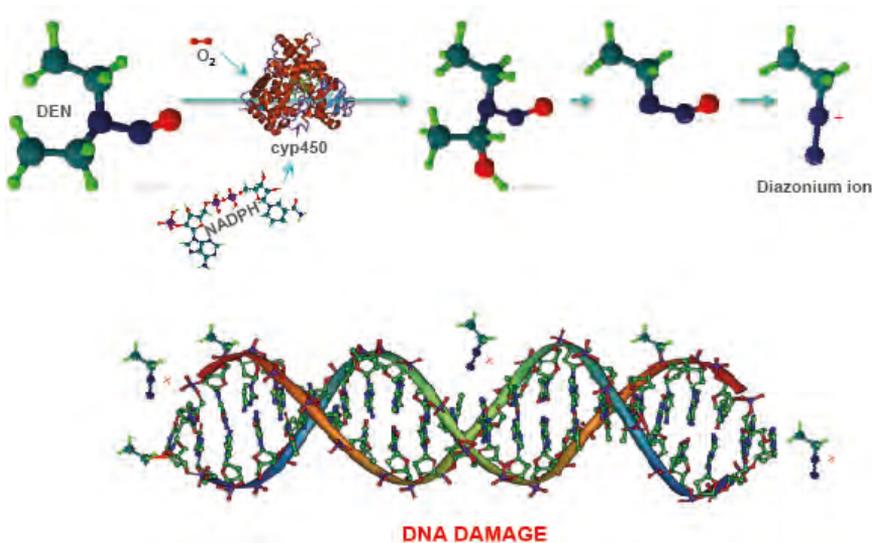
Fig. 8: the carcinogenic action of diethylnitrosamine (DEN) during which cyp450 hydroxylate DEN and cause the formation of the highly reactive diazonium ions, which can react with DNA-bases thus creating DNA-damage.

Furthermore, oxidative stress caused by DEN can contribute to the hepatocarcinogenesis (270,271). Reactive oxygen species (ROS) generated by the P450-dependent enzymatic system might induce oxidative stress due to the formation of hydrogen peroxide and superoxide anions. Production of ROS is known to cause DNA, protein and lipid damage; therefore oxidative stress has been known to play an important role in carcinogenesis (272).

One of the advantages of DEN, is that DEN-induced HCC has been shown to be molecularly similar to human HCC with poor prognosis. Genes involved in the regulation of cell growth and proliferations are known to be predictors for an unfavourable outcome of human HCC, and are highly expressed in DEN-induced HCC. For instance, the positive regulators of the cell cycle such as *Cdk4, Cdc25a, Cdc7 and Mapk3* are more highly expressed in DEN-induced HCC compared to other mouse models. And the expression of poor prognostic markers such as *Cd63, Rab3d, Afp and Tff3* is seen in DEN-induced HCC, similar to patients with HCC (273,274). In addition, mouse tumours induced by DEN harbour activating mutations in the *H-ras* proto-oncogene (268,269), correlated with metastasis and poor prognosis in human HCC (275,276).

Furthermore, *in vitro* studies have shown that DEN-induced hepatocellular carcinomas display characteristics, including the formation of Mallory bodies, that are similar to livers with alcohol induced HCC.

DEN works in a dose-dependent manner (277), a single low initiation dose does not lead to the formation of neoplasms, administration of a high dose induces HCC after a period of latency. Several DNA-repair mechanisms, such as non-homologous end joining, recombinational repair, base excision repair and many more, prevent the mutagenity of DNA-adducts when DEN is administrated in a single low dose (278).

The time needed after a single DEN-injection to develop HCC does not only depend on the administrated dose, but also on sex, age and strain of mice (277,279). The younger the mice, the faster HCC will occur due to the high hepatocyte proliferation rates found in juvenile animals (280). After a long term repetitive administration of DEN HCC develops in 100% of the male mice and in 30% of females. The gender-related difference in HCC incidence is due to the inhibiting effect of oestrogens and the stimulating effect of androgens on hepatocarcinogenesis (281). The methylation status of certain genes that contribute to the susceptibility to hepatocarcinogens might be one of the genetic factors that differ between tumour sensitive and tumour resistant strains (282,283). Tumour sensitive mice, such as C3H/HE-strain are characterized by a hypermethylation in promoter regions of tumour suppressor genes after hepatocarcinogen exposure, a high degree of methylation inhibits the DNA-transcription (284).

A dose dependent formation of carcinomas after a single injection (5,0 mg/kg – 90 mg/kg) of DEN in 15-day-old mice is observed after 45 to 104 weeks (285). When B6C3F1 mice are exposed to a single dose of 5,0 mg/kg DEN, it takes approximately 64 weeks to develop HCC (286). HCC occurs either within existing adenomas, which are visible after 24 weeks, or within the hepatocellular parenchyma. Metastasis to the lungs happens quite fast after the development of HCC (287). The time for tumour development differs between strains, for example C3H/HE (288,289) (60 mg/kg DEN, HCC after 45-75 weeks), and C57BL6/J (290) (5 mg/kg DEN, HCC after 40-70 weeks). Only 30 to 70% of the male mice develop HCC after a single injection with DEN, so the method is poorly reproducible. When mice are subjected to a short time weekly

administration of DEN it leads to a higher tumour incidence in a shorter time span. Six weeks of intraperitoneal injections (3 weeks with 75 mg/kg followed by 3 weeks of 100 mg/kg) lead to 100% tumour incidence after 52 weeks and the first tumours already occur after 30 weeks (291). Long term, weekly administration of DEN could enhance this process (292).

A two-stage model in which the initiation by a genotoxic compound is followed by a promotion phase is another strategy to induce HCC in mice. In this setup, DEN can be used as initiator and phenobarbital (PB) as a promoting agent. Several mechanisms might be responsible for the tumour promoting effect of PB. First, PB can increase the expression of cyp450 a 100-fold, leading to an enhanced effect of DEN (293). Second, the increased cyp450 activity induces oxidative stress (294,295). Third, PB can cause hypermethylation in promoter regions of tumour suppressor genes, therefore enhancing hepatocarcinogenesis (296,297). Fourth, PB also interferes with the intracellular communication and it might influence cell proliferation. Promotion with PB leads to tumours that exhibit mutations in the β-catenin proto-oncogene (298). Mutant nuclear *β-catenin* expression is associated with non-invasive tumours and a significantly higher 5-year survival rate in humans (299)

The effects of PB-promotion on DEN-initiated mice also vary considerably depending upon strain, sex and age. While the tumour promoting activity of PB is generally accepted a tumour inhibiting effect is seen when juvenile (15-days-old) $B6C3F_1$ mice are treated with DEN followed by a long-term (36 weeks) exposure to PB (300)(301). The inhibitory effect is not seen in female $B6C3F_1$-mice (282) or other mice strains such as Balb/c (298) and C3H (302), initiated with DEN in juvenile mice promoted with PB.

Timing of initiation with DEN is a critical determinant for the paradoxical effect of PB (300). DEN-initiation alone leads to the formation of lesions that express the *Bcl2* gene (*Bcl-2$^+$*), regardless of the moment of initiation. Bcl-2 family members can be functionally divided into anti-apoptotic and pro-apoptotic groups. The balance between these two groups may determine the fate of tumour cells. In HCC, this balance is often shifted towards the anti-apoptotic members, such as *Bcl*-2, leading to resistance to cell death and rapid proliferation. Although, human HCC is often characterised by a higher *BCL2* expression, no correlation has been found with tumour stage or prognosis.

When adult male B6C3F$_1$-mice are initiated with DEN between 6 and 10 weeks of age followed by exposure to PB in drinking water for 36 weeks, PB serves as a tumour-promoting agent. Ninety per cent of the tumours consist out of eosinophilic lesions that lack the *Bcl2* expression (*Bcl2*$^-$), indicating that PB offers a selective growth advantage for the latter. When juvenile male B6C3F$_1$-mice are initiated with DEN at 15 days of age and submitted to long-term PB exposure, PB serves as a tumour-inhibiting agent. Only 4% of the lesions are *Bcl2*$^-$, even after PB-promotion (which normally suppresses the proliferation of *Bcl2*$^+$ tumour cells and promotes the proliferation of *Bcl2*$^-$ cells). The decreased number of tumours is explained by the lack of *Bcl2*$^-$ lesions in juvenile mice and the growth-inhibiting effect of PB on the *Bcl2*$^+$ lesions. Juvenile livers are less differentiated because hepatocytes actively proliferate in juvenile mice (303). These qualitative differences between infant and adult hepatocytes might be the reason for the age-dependent initiation properties. Although PB-promotion stimulates the growth of *Bcl2*$^-$ lesions, it is not known if this significantly influences apoptosis, since PB-promoted Bcl2$^-$ lesions often overexpress the anti-apoptotic gene *Bcl-X$_L$*. Also, no significant differences are seen in the apoptotic index of PB-promoted and non-PB-promoted basophilic HCC-lesions (298,304).

There is also a gender-related difference in the response to PB-promotion. Female mice initiated with DEN at 15 days of age followed by long-term exposure to 0,05% PB show an increase of carcinomas compared to the DEN-only group, instead of the decrease that is seen in male mice (305).

When male BALB/c mice are initiated with DEN a long-term treatment of PB promotes the development of liver tumours, regardless of the age of DEN-administration. Genetic differences between strains of mice are likely to contribute to the different response to PB. Because PB induces an increase in methylation of GC-rich region genetic differences in factors contributing to the methylation status, might be one of the causes of the differential response to PB.

Another two-step hepatocarcinogenesis model is known as the Solt-Farber protocol (306). In this model initiation by a hepatocarcinogenic compound is followed by a partial hepatectomy (PH), which induces hepatic cell proliferation of the liver, leading to a fast expansion of the initiated cells. There is a fast

occurrence of altered hepatic foci and visible nodules after PH. Unfortunately, PH is a difficult procedure in mice associated with high mortality and has mostly been conducted in rats.

Thus, DEN is the best-known hepatic carcinogen, valued for giving occurrence to hepatic tumours that closely resemble human HCC. Several protocols are available, varying from single injections with a high dose to combinations with promoting agents and PHD, yet strain and gender specific characteristics might influence the outcome of these protocols. Furthermore, most experimental set-ups take almost 1 year to develop HCC, resulting in a time-consuming practice and tumours only rarely occur in a background of fibrosis.

3.3   Xenograft mouse models

In xenograft models, injecting human cancer cells from a lab culture in immune deficient mice creates the tumours. Athymic (nude) or severe combined immune deficient mice are often used as hosts (307). Tumour xenografts can be established either by direct implantation of biopsy material or by inoculation of human tumour cell lines. Several kinds of xenograft models can be distinguished. First, the ectopic xenograft model, in which human tumour cells are subcutaneously injected in mice. Second, the orthotopic model, where tumour cells are injected intrahepatically into mice. The orthotopic xenograft model is more suitable for extrapolation to humans and gives information about the metastatic spread of the tumour. The advantage of xenograft mouse models, is the short time span needed for the development of tumours and the fact that it is an efficient way to demonstrate proof-of-principle when enough cell lines are used. Tumour phenotypes can vary remarkably between lines, it is therefore important to use different cell lines when using the xenograft model. (308).

Human tumour progression is a complicated process during which the interaction of neoplastic cells and the surrounding tumour environment plays an important role. A micro-evolutionary process takes place in which advantaged tumour cells rapidly expand, a process that is altered when using cell cultures. Therefore, the resemblance between xenograft tumours and human tumours is rather poor (309,310). Due to the heterogeneity of cell lines,

multiple cell lines should always be used for drug screening, which often leads to different outcomes depending on the cell line's phenotype. Significant differences in tumour growth inhibition are often noted between cell lines, leading to discrepancy between studies. The lack of predictability of results obtained from xenograft mice models has convinced many researchers to use models in which tumours arise in a background that resembles the natural history of HCC.

An interesting setup consists of orthotopic implantations of HCC cells in fibrotic livers (311). Fibrosis is established by intraperitoneal injection of thioacetamide or subcutaneous injection of CCL_4 and oral alcohol intake. Tumours in fibrotic livers not only grow significantly larger and more rapidly than those in normal liver, they also have the capacity to metastasise and form satellite nodules. This model provides a useful tool for testing drug efficacy in orthotopic xenografts within the context of liver fibrosis, but there is also variable responsiveness to drugs between cell lines.

Metastatic HCC xenograft models are used for the research on the mechanism of metastasis and relapse. By orthotopic implantation of preserved metastatic tumour tissues of 30 surgical specimens, the first highly metastatic model of HCC in nude mice was developed (312). Orthotopic transplantation of this cell line (LCD-D20) leads to highly metastatic HCC that exhibits several characteristics of human tumour behaviour and the tumour tissue histologically resembles human HCC. Hepatitis B viral-DNA has been integrated in the cellular DNA of the LCI-D20 tumour cells. Metastasis to the lungs occurs in 100% of the mice 15 days after orthotopic inoculation. Genetically comparing these highly metastatic cell lines, to less metastatic cell lines, such as LCI-D35, leads to the identification of several genes involved in the invasive potential of tumour.

To conclude, xenograft models offer a fast solution for drug screening and are an easy way for proof-of-principle experiments. Xenograft models are often used for drug screening, nevertheless it must be noted that the spectacular results often seen in xenograft mouse models can seldom be repeated in cancer patients. It should also be noted that multiple cell lines have to be used in experiments because of the heterogenity between cell lines. In addition, ectopic implantation does not allow research about complex tumour-host

interactions, but orthotopic implantation makes it possible to investigate the neoplastic hepatocytes in their natural environment.

3.4   Transgenic mouse models

Genetically modified mouse models (GMM) are engineered to mimic pathophysiological and molecular features of HCC (313,314). It is a unique model for assessing the effects of oncogenes either alone or in combination with other oncogenes or carcinogenic agents. GMMs facilitate detailed investigation of carcinogenic pathways and allow the assessment of pathway cooperativity and dependency *in vivo* (315). Tissue-specific expression can be achieved by designing cDNA-constructs that contain promoter elements. Although the effect of the mutation is constitutive, its expression is limited by the use of tissue-specific promoters. An alternative to the constitutive tissue-specific expression is the induction of specific genes with molecules. This approach allows research toward the role of oncogenes in tumour maintenance and the influence of age on the carcinogenesis.

Due to the immense amount of transgenic mice, only a small selection of representative models for HCC-research will be discussed in this doctoral thesis.

3.4.1   Transgenic models expressing viral genes

The main cause of HCC worldwide is the hepatitis B virus (HBV). HBV is an enveloped hepatotropic DNA virus causing several liver diseases such as acute to chronic hepatitis, cirrhosis and HCC. During prolonged infection, viral DNA sequences integrate into the host genome, causing mutations, chromosomal instability and general genomic rearrangements. The genome of HBV is a circular molecule partially double-stranded DNA molecule and it is characterized by its four overlapping open reading frames (ORF) that encode for surface (S), core (C), polymerase (P) and X proteins (HBx). There is increasing evidence that the expression of viral genes, in particular genes encoding for HBx, might deregulate the control of cellular growth and viability and sensitize hepatocytes to exogenous and endogenous carcinogens (316). The oncogenic capacity of HBx is a result of the fact that some of the genes activated by HBx,

such as *Icam*-1, *c-myc* and *c-fos* are important for cell adhesion and proliferation (317,318).

Many aspects of HBV induced hepatocarcinogenesis have been analyzed by generating transgenic mice expressing complete fragments of the HBV genome, under control of either the HBV promoter, or constitutive (mouse albumin) or inducible (mouse metallothionein) liver-specific promoters. The first HBV-related transgenic mice model was produced in 1985 by two independent research groups (319,320).

Most of the HBV-related transgenic animals express the HBx-genes, which are associated with altered hepatocellular functions and HCC development (321–323). The livers of HBx transgenic mice exhibit megalocytosis, nuclear pleomorphism, hyperchromatism and a slightly increased nuclear/cytoplasmic ratio after approximately 15 weeks (324). The birthrate of the HBx-transgenic mice is a bit lower than that of other transgenic mice indicating an interaction with the prenatal development of mice (321,322). The progression to HCC takes approximately 52-104 weeks (324,325). Transgenic lineages with lower HBx copy number showed lower tumour incidence comparable with that of wild-type mice (326), while other experiments do not encounter any tumours in HBx transgenic mice (327,328). HBx-transgenic mice seem to be more sensitive for HCC-development after a single DEN-injection when compared to their non-transgenic counterparts (329).

Viral Hepatitis C (HCV) infection is an important viral risk factor for HCC (330). Chronic HCV can cause cirrhosis and increases the risk of HCC with approximately 1% to 3%. A number of HCV-proteins, such as core, NS3 and NS5A play an important role in the hepatocarcinogenesis (331–333). The proteins encoded by the HCV-genome, especially core, interrupt the intracellular signal transduction pathways. Numerous transgenic mouse models are made expressing different HCV proteins and differences occur in the development of tumours. When the complete viral protein is expressed, steatosis and HCC occurs in 15% of the mice after approximately 90-100 weeks (334). Other researchers did not encounter the tumour phenotype, indicating

that genetic background of mice and/or oncogene expression level plays an important role in the outcome of transgenic mice models (335).

The core protein interferes with the lipid metabolism by activating PPARα, leading to lipid accumulation in the hepatocytes (336). The expression of HCV core leads to progressive hepatic steatosis in several lines of constitutive transgenic mice, followed by HCC after 80-105 weeks in 32% of the male mice (337). Inducible expression of core-protein by tetracycline and Dox administration leads to a peak in steatosis after 2 months but no HCC occurs (338). An accelerated tumour progression occurs after DEN-injection, after merely 32 weeks HCC is found in 100% of the male mice. HCV proteins and chemical carcinogens such as DEN have a synergistic influence on HCC-development, because HCV proteins blocks apoptosis in the hepatocytes, therefore leading to an accelerated expansion of the neoplastic hepatocytes (339).

3.4.2 Transgenic mice over-expressing oncogenes

An oncogene is a protein-encoding gene that participates in the onset and development of cancer. Genetic alterations resulting in the activation or over-expression of oncogenes, increases the chance of tumour development.

The MYC protein is a transcription factor that activates the expression of several genes through binding on consensus sequences and recruiting histone acetyltransferases. When *myc* is mutated or over-expressed, it is associated with a variety of tumours. Transgenic mice over-expressing *c-myc* develop liver tumours after a long period of latency (65-90 weeks) (340). Five to 10% of the tumours show increased *β-catenin* expression.

β-catenin, a subunit of the cadherin-complex, plays an important role in the development and regeneration of the liver. β-catenin is one of the key downstream effectors of the *Wnt*-signaling pathway, a network of proteins that is known for its involvement in tumour development. β-catenin mutations are considered to be an early event in the hepatocarcinogenesis. Approximately 30% of the human hepatic tumours harbour activated *β-catenin* mutations. A *Wnt*-activating *β-catenin* mutation alone causes hepatomegaly, but to induce

hepatocarcinogenesis additional mutations or epigenetic changes are required (341). When mutations in both the *β-catenin* and *H-ras* genes are introduced by adenovirus-mediated Cre expression, early HCC is found in all the mice sacrificed 8 weeks after induction (342). High grade HCC is established after approximately 26 weeks.

3.4.3 Transgenic mice models over-expressing growth factors

During HCC an unregulated expression of hepatocyte mitogens occurs, leading to an uncontrolled expansion of the hepatocytes. Over-expression of growth factors that mediate hepatocyte growth induces HCC.

Transforming growth factor α (TGFα) is a potent hepatotrophic mitogen synthesized in hepatocytes during regeneration. Transgenic mice over-expressing human TGF-α under the inducible methallothionein 1 promotor develop liver tumours as well as an abnormal development of the pancreas and mammary glands (343). Zinc administrated through the drinking water enhances the tumour formation and after 40 to 70 weeks HCC occurs in 50% of the mice. Double transgenic mice carrying both *c-myc* (albumin promotor) and TGF-α (methallothionein promotor) leads to a tremendous acceleration of the neoplastic development compared to both single transgenic models (344). The faster occurrence of HCC in the double transgenic model, compared to the parental lines, suggests that the interaction of *c-myc* and TGF-α increases the malignant conversion by the selection and expansion of preneoplastic cells.

Epidermal growth factor (EGF) is a growth factor that plays an important role in the regulation of cell growth, proliferation and differentiation. Its over-expression is associated with hepatocellular carcinoma. Over-expression of the secreted form of EGF results in multiple highly malignant hepatic tumours after 24-36 weeks (345).

While most transgenic mouse models use tissue specific promoters to ensure liver-specific expression, there is one model that does not require liver-specific expression for the development of HCC. A transgenic mouse model over-expressing fibroblast growth factor 19 (*Fgf19*) in skeletal muscle leads to the occurrence of HCC in 50% of the mice after approximately 52 weeks (346). In

contrast to the other mice models, this model produces a higher HCC-incidence in female mice than male. All female mice tumours exhibit an increased *β-catenin* expression, which is not found in the male mice tumours. The exact mechanism of the *Fgf19*-induced hepatocarcinogenesis is still unclear, but might be a result of the increased metabolic rate which leads to an increase in ROS-production.

3.4.4   Other transgenic models

Alpha-1 antitrypsin (AAT) is a glycoprotein that is produced in the liver. Transgenic mice expressing a human form of transport-impaired AAT represent a good model for studying the effects of AAT-deficiency on the liver. AAT-deficiency is an autosomal recessive disorder in which a mutation causes the production of AAT that is unable to be transported. This leads to decreased AAT activity in serum and deposition of excessive AAT in the liver. Both heterozygous and homozygous individuals develop cirrhosis and HCC after 52-90 weeks (347).

Phosphatase and tensin homolog (*Pten*) is a tumour suppressor gene that regulates the serine-threonine kinase protein kinase B (PKB/akt) pathway. *Pten* deficiency induces cellular hyperproliferation, anti-apoptosis and oncogenesis (348). Liver-specific *Pten*-deficient mice develop hepatic steatosis, inflammation, fibrosis and tumours that are very similar to human NASH (349). Liver tumours are present in 66% of male and 30% of female *Pten*-deficient mice by 40–44 weeks of age.

The Simian vacuolating virus 40 (SV40) is a DNA-virus that has the potential to cause tumours. It suppresses the transcriptional properties of the tumour-suppressing p53-gene. When the SV40 T-antigen is brought to expression under a specific or inducible promotor such as albumin, α1-antitrypsin, serum amyloid P component or antithrombin III, liver tumours are found after a short period of latency (4 to 12 weeks) (350–354). Metastasis to the lungs can occur. Tumour progression is very rapid in these models and therefore differs radically from the development of human tumours which progress more gradually.

Sleeping Beauty (SB) is a genetically engineered insertional mutagenesis system. Its ability to rapidly induce cancer in SB-transgenic mice as well as the ease of identification of the mutated genes suggests important roles for SB in the discovery of novel cancer genes (355). The range of SB-related tumours extends from haematopoietic to solid cancers such as HCC. For the latter Rosa26-LsL-SB11; T2/Onc-low copy mice are crossed with Albumin-Cre mice and in order to increase susceptibility to HCC, mice can carry a dominant-negative Trp53 allele. Several male but no female mice develop HCC with lung metastases at approximately 15 months of age and liver nodules are observed in approximately half of these male mice sacrificed between 3 and 10 months of age (356).

To conclude, genetically modified mouse models are favourable to identify a range of discrete molecular and histological stages during the multistep process of hepatocarcinogenesis. GMMs harbouring multiple mutations allow the investigation of cooperation and dependency between oncogenes, growth factors and viral genes. Use of an inducible promoter offers significant advantages because the mutation can be switched on and off when needed. The biological insight gained from transgenic mice is invaluable. Nevertheless, a mutation in a gene does not always result in the expected phenotype and the phenotypic outcome can be influenced by many environmental and genetic factors. It should also be stated that most models do not demonstrate the advanced neoplastic lesions and that metastasis occurs only seldom. One should also consider the fact that natural tumours consist out of a heterogenic group of cells in which multiple mutations have occurred. The interpretation of results from studies using GMMs is not always as simple, especially when extrapolating to a human setting.

4   References

1.   Tseng PL, Wang JH, Tung HD, Hung CH, Kee KM, Chen CH, et al. Optimal treatment increased survival of hepatocellular carcinoma patients detected with community-based screening. Journal of gastroenterology and hepatology. 2010;25(8):1426–34.

2.   Yeh S-H, Chen P-J. Gender disparity of hepatocellular carcinoma: the roles of sex hormones. Oncology. 2010 Jul;78 Suppl 1:172–9.

3.   Chen S, Chen J, Ma G, Xi W, Xu Q, Xu W, et al. Clinical Therapeutic Effect and Biological Monitoring of p53 Gene in Advanced Hepatocellular Carcinoma. American journal of clinical oncology. 2011 Jan 6;

4.   Shapiro Ii, Shabrov AV, Karev VE, Leenman EE, Kvetnoi IM, Tsinzerling VA, et al. [Clinical and molecular correlation between chronic hepatitis and hepatocellular carcinoma induced by hepatitis B virus]. Arkhiv patologii. 2005;67(2):6–10.

5.   Vivekanandan P, Torbenson M, Ramakrishna B. Hepatitis B virus-associated hepatocellular carcinoma from India: role of viral genotype and mutations in CTNNB1 (beta-catenin) and TP53 genes. Journal of gastrointestinal cancer. 2011 Mar;42(1):20–5.

6.   Matsuzaki Y, Chiba T, Hadama T, Asaoka H, Doy M, Shoda J, et al. HBV genome integration and genetic instability in HBsAg-negative and anti-HCV-positive hepatocellular carcinoma in Japan. Cancer Letters. 1997 Oct;119(1):53–61.

7.   Jahan S, Khaliq S, Ijaz B, Ahmad W, Hassan S. Role of HCV Core gene of genotype 1a and 3a and host gene Cox-2 in HCV-induced pathogenesis. Virology journal. 2011 Jan;8:155.

8.   Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000 Sep 14;407(6801):249–57.

9.   Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology (Baltimore, Md.). 2011 Mar;53(3):1020–2.

10.   Schima W, Hammerstingl R, Catalano C, Marti-Bonmati L, Rummeny EJ, Montero FT, et al. Quadruple-phase MDCT of the liver in patients with suspected hepatocellular carcinoma: effect of contrast material flow rate. AJR. American journal of roentgenology. 2006 Jun;186(6):1571–9.

11.   Lee JM, Choi BI. Hepatocellular nodules in liver cirrhosis: MR evaluation. Abdominal imaging. 2011 Jun;36(3):282–9.

12. Sano K, Ichikawa T, Motosugi U, Sou H, Muhi AM, Matsuda M, et al. Imaging Study of Early Hepatocellular Carcinoma: Usefulness of Gadoxetic Acid-enhanced MR Imaging. Radiology. 2011 Oct 13;

13. Claudon M, Cosgrove D, Albrecht T, Bolondi L, Bosio M, Calliada F, et al. Guidelines and good clinical practice recommendations for contrast enhanced ultrasound (CEUS) - update 2008. Ultraschall in der Medizin (Stuttgart, Germany : 1980). 2008 Feb;29(1):28–44.

14. Borzio M, Borzio F, Macchi R, Croce AM, Bruno S, Ferrari A, et al. The evaluation of fine-needle procedures for the diagnosis of focal liver lesions in cirrhosis. Journal of hepatology. 1994 Jan;20(1):117–21.

15. Llovet JM, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Seminars in liver disease. 1999 Jan;19(3):329–38.

16. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012 Feb 17;379(9822):1245–55.

17. de Lope CR, Tremosini S, Forner A, Reig M, Bruix J. Management of HCC. Journal of hepatology. 2012 Jan;56 Suppl 1:S75–87.

18. Ueno S, Sakoda M, Kubo F, Hiwatashi K, Tateno T, Baba Y, et al. Surgical resection versus radiofrequency ablation for small hepatocellular carcinomas within the Milan criteria. Journal of hepato-biliary-pancreatic surgery. 2009 Jan;16(3):359–66.

19. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. The New England journal of medicine. 1996 Mar 14;334(11):693–9.

20. Calvisi DF, Ladu S, Gorden A, Farina M, Lee JS, Conner EA, et al. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. The Journal of clinical investigation. 2007;117(9):2713–22.

21. Uno T, Itami J, Shiina T, Toita T, Mikuriya S, Hatano K, et al. Radiation therapy in patients with unresectable hepatocellular carcinoma. Cancer chemotherapy and pharmacology. 1992 Jan;31 Suppl:S106–10.

22. Khan MR, Poon RTP, Ng KK, Chan AC, Yuen J, Tung H, et al. Comparison of percutaneous and surgical approaches for radiofrequency ablation of small and medium hepatocellular carcinoma. Archives of surgery (Chicago, Ill. : 1960). 2007 Dec 1;142(12):1136–43; discussion 1143.

23. Georgiades CS, Hong K, D'Angelo M, Geschwind J-FH. Safety and efficacy of transarterial chemoembolization in patients with unresectable hepatocellular carcinoma

and portal vein thrombosis. Journal of vascular and interventional radiology : JVIR. 2005 Dec;16(12):1653–9.

24. Kulik LM, Atassi B, van Holsbeeck L, Souman T, Lewandowski RJ, Mulcahy MF, et al. Yttrium-90 microspheres (TheraSphere) treatment of unresectable hepatocellular carcinoma: downstaging to resection, RFA and bridge to transplantation. Journal of surgical oncology. 2006 Dec 1;94(7):572–86.

25. Wiesener MS, Jürgensen JS, Rosenberger C, Scholze CK, Hörstrup JH, Warnecke C, et al. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003 Feb;17(2):271–3.

26. Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan L-J, et al. Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nature genetics. 2003 Dec;35(4):331–40.

27. Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, et al. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nature medicine. 2002;8(7):702–10.

28. Coulet F, Nadaud S, Agrapart M, Soubrier F. Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. The Journal of biological chemistry. 2003 Nov 21;278(47):46230–40.

29. Le Bras A, Lionneton F, Mattot V, Lelièvre E, Caetano B, Spruyt N, et al. HIF-2alpha specifically activates the VE-cadherin promoter independently of hypoxia and in synergy with Ets-1 through two essential ETS-binding sites. Oncogene. 2007 Nov 22;26(53):7480–9.

30. Ivan M, Haberberger T, Gervasi DC, Michelson KS, Günzler V, Kondo K, et al. Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. Proceedings of the National Academy of Sciences of the United States of America. 2002 Oct 15;99(21):13459–64.

31. Appelhoff RJ, Tian Y-M, Raval RR, Turley H, Harris AL, Pugh CW, et al. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. The Journal of biological chemistry. 2004 Sep 10;279(37):38458–65.

32. Berra E, Benizri E, Ginouvès A, Volmat V, Roux D, Pouysségur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. The EMBO journal. 2003 Aug 15;22(16):4082–90.

33. Shibayama N, Morimoto H, Kitagawa T. Properties of chemically modified Ni(II)-Fe(II) hybrid hemoglobins. Ni(II) protoporphyrin IX as a model for a permanent deoxy-heme. Journal of molecular biology. 1986 Nov 20;192(2):331–6.

34. Sinclair P, Gibbs AH, Sinclair JF, de Matteis F. Formation of cobalt protoporphyrin in the liver of rats. A mechanism for the inhibition of liver haem biosynthesis by inorganic cobalt. The Biochemical journal. 1979 Mar 15;178(3):529–38.

35. McNeill LA, Hewitson KS, Gleadle JM, Horsfall LE, Oldham NJ, Maxwell PH, et al. The use of dioxygen by HIF prolyl hydroxylase (PHD1). Bioorganic & medicinal chemistry letters. 2002 Jun 17;12(12):1547–50.

36. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001 Oct 5;107(1):43–54.

37. Sogawa K, Numayama-Tsuruta K, Ema M, Abe M, Abe H, Fujii-Kuriyama Y. Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. Proceedings of the National Academy of Sciences of the United States of America. 1998 Jun 23;95(13):7368–73.

38. Huang LE, Willmore WG, Gu J, Goldberg MA, Bunn HF. Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. The Journal of biological chemistry. 1999 Mar 26;274(13):9038–44.

39. Liu YV, Baek JH, Zhang H, Diez R, Cole RN, Semenza GL. RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. Molecular cell. 2007 Jan 26;25(2):207–17.

40. Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, Cohen B. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. The EMBO journal. 1998 Sep 1;17(17):5085–94.

41. Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Bürgel T. Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. FEBS letters. 2002 Feb 13;512(1-3):157–62.

42. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer research. 1999 Aug 15;59(16):3915–8.

43. Jung Y, Isaacs JS, Lee S, Trepel J, Liu Z-G, Neckers L. Hypoxia-inducible factor induction by tumour necrosis factor in normoxic cells requires receptor-interacting protein-dependent nuclear factor kappa B activation. The Biochemical journal. 2003 Mar 15;370(Pt 3):1011–7.

44.   Haddad JJ, Land SC. A non-hypoxic, ROS-sensitive pathway mediates TNF-alpha-dependent regulation of HIF-1alpha. FEBS letters. 2001 Sep 14;505(2):269–74.

45.   Sánchez-López E, López AF, Esteban V, Yagüe S, Egido J, Ruiz-Ortega M, et al. Angiotensin II regulates vascular endothelial growth factor via hypoxia-inducible factor-1alpha induction and redox mechanisms in the kidney. Antioxidants & redox signaling. 7(9-10):1275–84.

46.   Karna E, Szoka L, Palka J. Thrombin-dependent modulation of β(1)-integrin-mediated signaling up-regulates prolidase and HIF-1α through p-FAK in colorectal cancer cells. Molecular and cellular biochemistry. 2011 Oct 13;

47.   Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer research. 1999 Nov 15;59(22):5830–5.

48.   Semenza G. Signal transduction to hypoxia-inducible factor 1. Biochemical pharmacology. 2002 Sep;64(5-6):993–8.

49.   Unruh A, Ressel A, Mohamed HG, Johnson RS, Nadrowitz R, Richter E, et al. The hypoxia-inducible factor-1 alpha is a negative factor for tumor therapy. Oncogene. 2003 May 22;22(21):3213–20.

50.   Dewhirst MW, Cao Y, Moeller B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. Nat Rev Cancer. 2008;8(6):425–37.

51.   Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, et al. Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer research. 2000 Aug 1;60(15):4010–5.

52.   Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. The EMBO journal. 1998 Jun 1;17(11):3005–15.

53.   Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, et al. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature. 1998 Jul 30;394(6692):485–90.

54.   Axelson H, Fredlund E, Ovenberger M, Landberg G, Påhlman S. Hypoxia-induced dedifferentiation of tumor cells--a mechanism behind heterogeneity and aggressiveness of solid tumors. Seminars in cell & developmental biology. 16(4-5):554–63.

55.   Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010 Dec 15;16(24):5928–35.

56.   Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. Cell. 2009;136(5):839–51.

57.   Takeda K, Cowan A, Fong GH. Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system. Circulation. 2007;116(7):774–81.

58.   Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nature reviews. Drug discovery. 2011 Jun;10(6):417–27.

59.   Chan DA, Kawahara TLA, Sutphin PD, Chang HY, Chi J-T, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. Cancer cell. 2009 Jun 2;15(6):527–38.

60.   Lee KA, Lynd JD, O'Reilly S, Kiupel M, McCormick JJ, LaPres JJ. The biphasic role of the hypoxia-inducible factor prolyl-4-hydroxylase, PHD2, in modulating tumor-forming potential. Molecular cancer research : MCR. 2008;6(5):829–42.

61.   Su Y, Loos M, Giese N, Metzen E, Buchler MW, Friess H, et al. Prolyl hydroxylase-2 (PHD2) exerts tumor-suppressive activity in pancreatic cancer. Cancer. 2011;

62.   Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F, et al. Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(48):18154–9.

63.   Bootle-Wilbraham CA, Tazzyman S, Thompson WD, Stirk CM, Lewis CE. Fibrin fragment E stimulates the proliferation, migration and differentiation of human microvascular endothelial cells in vitro. Angiogenesis. 2001 Jan;4(4):269–75.

64.   Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, et al. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nature medicine. 2003;9(7):936–43.

65.   Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2002 Sep 3;99(18):11946–50.

66.   Antequera D, Portero A, Bolos M, Orive G, Hernández RMRA, Pedraz JL, et al. Encapsulated VEGF-secreting cells enhance proliferation of neuronal progenitors in the hippocampus of AβPP/Ps1 mice. Journal of Alzheimer's disease : JAD. 2012 Jan 1;29(1):187–200.

67.   Schwarz Q, Gu C, Fujisawa H, Sabelko K, Gertsenstein M, Nagy A, et al. Vascular endothelial growth factor controls neuronal migration and cooperates with Sema3A to

pattern distinct compartments of the facial nerve. Genes & development. 2004 Nov 15;18(22):2822–34.

68. Santos SCR, Dias S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. Blood. 2004 May 15;103(10):3883–9.

69. Zhang L, Wang J-N, Tang J-M, Kong X, Yang J-Y, Zheng F, et al. VEGF is essential for the growth and migration of human hepatocellular carcinoma cells. Molecular biology reports. 2012 May;39(5):5085–93.

70. Nash AD, Baca M, Wright C, Scotney PD. The biology of vascular endothelial growth factor-B (VEGF-B). Pulmonary pharmacology & therapeutics. 2006 Jan;19(1):61–9.

71. Bellomo D, Headrick JP, Silins GU, Paterson CA, Thomas PS, Gartside M, et al. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circulation research. 2000 Feb 4;86(2):E29–35.

72. Mould AW, Tonks ID, Cahill MM, Pettit AR, Thomas R, Hayward NK, et al. Vegfb gene knockout mice display reduced pathology and synovial angiogenesis in both antigen-induced and collagen-induced models of arthritis. Arthritis and rheumatism. 2003 Sep;48(9):2660–9.

73. Haiko P, Makinen T, Keskitalo S, Taipale J, Karkkainen MJ, Baldwin ME, et al. Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. Molecular and cellular biology. 2008 Aug;28(15):4843–50.

74. Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nature immunology. 2004 Jan;5(1):74–80.

75. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science (New York, N.Y.). 1997 May 30;276(5317):1423–5.

76. Yamashita T, Uramoto H, Onitsuka T, Ono K, Baba T, So T, et al. Association between lymphangiogenesis-/micrometastasis- and adhesion-related molecules in resected stage I NSCLC. Lung cancer (Amsterdam, Netherlands). 2010 Dec;70(3):320–8.

77. Miyazaki T, Okada N, Ishibashi K, Ogata K, Ohsawa T, Ishiguro T, et al. Clinical significance of plasma level of vascular endothelial growth factor-C in patients with colorectal cancer. Japanese journal of clinical oncology. 2008 Dec;38(12):839–43.

78. Arinaga M, Noguchi T, Takeno S, Chujo M, Miura T, Uchida Y. Clinical significance of vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 in patients with nonsmall cell lung carcinoma. Cancer. 2003 Jan 15;97(2):457–64.

79. Su J-L, Yang P-C, Shih J-Y, Yang C-Y, Wei L-H, Hsieh C-Y, et al. The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. Cancer cell. 2006 Mar;9(3):209–23.

80. Chen Y, Jiang L, She F, Tang N, Wang X, Li X, et al. Vascular endothelial growth factor-C promotes the growth and invasion of gallbladder cancer via an autocrine mechanism. Molecular and cellular biochemistry. 2010 Dec;345(1-2):77–89.

81. Bhardwaj S, Roy H, Heikura T, Ylä-Herttuala S. VEGF-A, VEGF-D and VEGF-D(DeltaNDeltaC) induced intimal hyperplasia in carotid arteries. European journal of clinical investigation. 2005 Nov;35(11):669–76.

82. Borg SA, Kerry KE, Royds JA, Battersby RD, Jones TH. Correlation of VEGF production with IL1 alpha and IL6 secretion by human pituitary adenoma cells. European journal of endocrinology / European Federation of Endocrine Societies. 2005 Feb;152(2):293–300.

83. Tjwa M, Luttun A, Autiero M, Carmeliet P. VEGF and PlGF: two pleiotropic growth factors with distinct roles in development and homeostasis. Cell and tissue research. 2003;314(1):5–14.

84. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell. 1998 Mar 20;92(6):735–45.

85. Marcellini M, De Luca N, Riccioni T, Ciucci A, Orecchia A, Lacal PM, et al. Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor. The American journal of pathology. 2006 Aug;169(2):643–54.

86. Roy H, Bhardwaj S, Babu M, Jauhiainen S, Herzig K-H, Bellu AR, et al. Adenovirus-mediated gene transfer of placental growth factor to perivascular tissue induces angiogenesis via upregulation of the expression of endogenous vascular endothelial growth factor-A. Human gene therapy. 2005 Dec;16(12):1422–8.

87. Adini A, Kornaga T, Firoozbakht F, Benjamin LE. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. Cancer research. 2002 May 15;62(10):2749–52.

88. Cai J, Ahmad S, Jiang WG, Huang J, Kontos CD, Boulton M, et al. Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. Diabetes. 2003 Dec;52(12):2959–68.

89.   Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nature medicine. 2001;7(5):575–83.

90.   Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell. 2007;131(3):463–75.

91.   Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, et al. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nature medicine. 2002 Aug;8(8):841–9.

92.   Kaplan PE, Granger CV, Pease WS, Arnett JA, Huba JC. Development of an academic productivity scale for departments of physical medicine and rehabilitation. Arch Phys Med Rehabil. 1997;78(9):938–41.

93.   Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2006;20(9):1495–7.

94.   Maes C, Coenegrachts L, Stockmans I, Daci E, Luttun A, Petryk A, et al. Placental growth factor mediates mesenchymal cell development, cartilage turnover, and bone remodeling during fracture repair. The Journal of clinical investigation. 2006 May;116(5):1230–42.

95.   Khurana R, Moons L, Shafi S, Luttun A, Collen D, Martin JF, et al. Placental growth factor promotes atherosclerotic intimal thickening and macrophage accumulation. Circulation. 2005 May 31;111(21):2828–36.

96.   Torry RJ, Tomanek RJ, Zheng W, Miller SJ, Labarrere CA, Torry DS. Hypoxia increases placenta growth factor expression in human myocardium and cultured neonatal rat cardiomyocytes. The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation. 2009 Feb;28(2):183–90.

97.   Cianfarani F, Zambruno G, Brogelli L, Sera F, Lacal PM, Pesce M, et al. Placenta growth factor in diabetic wound healing: altered expression and therapeutic potential. The American journal of pathology. 2006 Oct;169(4):1167–82.

98.   Chaballe L, Close P, Sempels M, Delstanche S, Fanielle J, Moons L, et al. Involvement of placental growth factor in Wallerian degeneration. Glia. 2011 Mar;59(3):379–96.

99.   Taylor AP, Leon E, Goldenberg DM. Placental growth factor (PlGF) enhances breast cancer cell motility by mobilising ERK1/2 phosphorylation and cytoskeletal rearrangement. British journal of cancer. 2010 Jun 29;103(1):82–9.

64

100. Chen J, Ye L, Zhang L, Jiang WG. Placenta growth factor, PLGF, influences the motility of lung cancer cells, the role of Rho associated kinase, Rock1. Journal of cellular biochemistry. 2008 Sep 1;105(1):313–20.

101. Casalou C, Fragoso R, Nunes JFM, Dias S. VEGF/PLGF induces leukemia cell migration via P38/ERK1/2 kinase pathway, resulting in Rho GTPases activation and caveolae formation. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K. 2007 Jul;21(7):1590–4.

102. Ikai T, Miwa H, Shikami M, Hiramatsu A, Tajima E, Yamamoto H, et al. Placenta growth factor stimulates the growth of Philadelphia chromosome positive acute lymphoblastic leukemia cells by both autocrine and paracrine pathways. European journal of haematology. 2005 Oct;75(4):273–9.

103. Marrony S, Bassilana F, Seuwen K, Keller H. Bone morphogenetic protein 2 induces placental growth factor in mesenchymal stem cells. Bone. 2003 Sep;33(3):426–33.

104. Van Steenkiste C, Geerts A, Vanheule E, Van Vlierberghe H, De Vos F, Olievier K, et al. Role of placental growth factor in mesenteric neoangiogenesis in a mouse model of portal hypertension. Gastroenterology. 2009;137(6):2112–6.

105. Xu L, Cochran DM, Tong RT, Winkler F, Kashiwagi S, Jain RK, et al. Placenta growth factor overexpression inhibits tumor growth, angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. Cancer research. 2006 Apr 15;66(8):3971–7.

106. Bais C, Wu X, Yao J, Yang S, Crawford Y, McCutcheon K, et al. PlGF blockade does not inhibit angiogenesis during primary tumor growth. Cell. 2010 Apr 2;141(1):166–77.

107. Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer cell. 2011 Jan 18;19(1):31–44.

108. Cao Y, Sonveaux P, Liu S, Zhao Y, Mi J, Clary BM, et al. Systemic overexpression of angiopoietin-2 promotes tumor microvessel regression and inhibits angiogenesis and tumor growth. Cancer Res. 2007;67(8):3835–44.

109. Jain RK, Carmeliet PF. Vessels of death or life. Scientific American. 2001;285(6):38–45.

110. Carmeliet P, Collen D. Molecular basis of angiogenesis. Role of VEGF and VE-cadherin. Annals of the New York Academy of Sciences. 2000;902:244–9.

111. Djonov V, Baum O, Burri PH. Vascular remodeling by intussusceptive angiogenesis. Cell and tissue research. 2003 Oct;314(1):107–17.

112. Krah K, Mironov V, Risau W, Flamme I. Induction of vasculogenesis in quail blastodisc-derived embryoid bodies. Developmental biology. 1994 Jul;164(1):123–32.

113. Li Calzi S, Neu MB, Shaw LC, Kielczewski JL, Moldovan NI, Grant MB. EPCs and pathological angiogenesis: when good cells go bad. Microvascular research. 2010 May;79(3):207–16.

114. Mund JA, Case J. The role of circulating endothelial progenitor cells in tumor angiogenesis. Current stem cell research & therapy. 2011 Jun 2;6(2):115–21.

115. Oh H-K, Ha J-M, O E, Lee BH, Lee SK, Shim B-S, et al. Tumor angiogenesis promoted by ex vivo differentiated endothelial progenitor cells is effectively inhibited by an angiogenesis inhibitor, TK1-2. Cancer research. 2007 May 15;67(10):4851–9.

116. Kilarski WW, Samolov B, Petersson L, Kvanta A, Gerwins P. Biomechanical regulation of blood vessel growth during tissue vascularization. Nature medicine. 2009 Jun;15(6):657–64.

117. Kilarski WW, Gerwins P. A new mechanism of blood vessel growth - hope for new treatment strategies. Discovery medicine. 2009 Jun;8(40):23–7.

118. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science (New York, N.Y.). 1999 Jun 18;284(5422):1994–8.

119. Döme B, Paku S, Somlai B, Tímár J. Vascularization of cutaneous melanoma involves vessel co-option and has clinical significance. The Journal of pathology. 2002 Jul;197(3):355–62.

120. Avni R, Cohen B, Neeman M. Hypoxic stress and cancer: imaging the axis of evil in tumor metastasis. NMR in biomedicine. 2011 Jul 17;24(6):569–81.

121. Qazi H, Shi Z-D, Tarbell JM. Fluid Shear Stress Regulates the Invasive Potential of Glioma Cells via Modulation of Migratory Activity and Matrix Metalloproteinase Expression. PloS one. 2011 Jan;6(5):e20348.

122. Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. The codependence of angiogenesis and chronic inflammation. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1997 May;11(6):457–65.

123. Imhof BA, Aurrand-Lions M. Angiogenesis and inflammation face off. Nat Med. 2006;12(2):171–2.

124. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. The Journal of clinical investigation. 1999 Jan;103(2):159–65.

125.   Oczeretko E, Juczewska M, Kasacka I. Fractal geometric analysis of lung cancer angiogenic patterns. Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society. 2001 Jan;39 Suppl 2:75–6.

126.   Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. The American journal of pathology. 2000 Apr;156(4):1363–80.

127.   Nagano N, Sasaki H, Aoyagi M, Hirakawa K. Invasion of experimental rat brain tumor: early morphological changes following microinjection of C6 glioma cells. Acta neuropathologica. 1993 Jan;86(2):117–25.

128.   Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene. 1999 Sep 20;18(38):5356–62.

129.   McDonald DM, Baluk P. Significance of Blood Vessel Leakiness in Cancer. Cancer Res. 2002;62(18):5381–5.

130.   Marra F. Chemokines in liver inflammation and fibrosis. Front Biosci. 2002;7:d1899–914.

131.   Le Couteur DG, Hickey H, Harvey PJ, Gready J, McLean AJ. Hepatic artery flow and propranolol metabolism in perfused cirrhotic rat liver. The Journal of pharmacology and experimental therapeutics. 1999 Jun;289(3):1553–8.

132.   DeLeve LD, Wang X, McCuskey MK, McCuskey RS. Rat liver endothelial cells isolated by anti-CD31 immunomagnetic separation lack fenestrae and sieve plates. American journal of physiology. Gastrointestinal and liver physiology. 2006 Dec;291(6):G1187–9.

133.   Novo E, Cannito S, Zamara E, Valfrè di Bonzo L, Caligiuri A, Cravanzola C, et al. Proangiogenic cytokines as hypoxia-dependent factors stimulating migration of human hepatic stellate cells. The American journal of pathology. 2007 Jun;170(6):1942–53.

134.   Yoshiji H, Kuriyama S, Fukui H. Blockade of renin-angiotensin system in antifibrotic therapy. Journal of gastroenterology and hepatology. 2007 Jun;22 Suppl 1:S93–5.

135.   Ankoma-Sey V, Wang Y, Dai Z. Hypoxic stimulation of vascular endothelial growth factor expression in activated rat hepatic stellate cells. Hepatology. 2000;31(1):141–8.

136.   Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. Best practice & research. Clinical gastroenterology. 2011 Apr;25(2):195–206.

137.   Eng FJ, Friedman SL. Transcriptional regulation in hepatic stellate cells. Seminars in liver disease. 2001 Aug;21(3):385–95.

138. Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. The codependence of angiogenesis and chronic inflammation. FASEB J. 1997;11(6):457–65.

139. Yamamoto T, Kaneda K, Hirohashi K, Kinoshita H, Sakurai M. Sinusoidal capillarization and arterial blood supply continuously proceed with the advance of the stages of hepatocarcinogenesis in the rat. Japanese journal of cancer research : Gann. 1996 May;87(5):442–50.

140. Kim KW, Bae SK, Lee OH, Bae MH, Lee MJ, Park BC. Insulin-like growth factor II induced by hypoxia may contribute to angiogenesis of human hepatocellular carcinoma. Cancer research. 1998 Jan 15;58(2):348–51.

141. Sugimachi K, Tanaka S, Terashi T, Taguchi K-ichi, Rikimaru T, Sugimachi K. The mechanisms of angiogenesis in hepatocellular carcinoma: angiogenic switch during tumor progression. Surgery. 2002 Jan;131(1 Suppl):S135–41.

142. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature. 1998 Mar 26;392(6674):405–8.

143. Keith B, Johnson RS, Simon MC. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. Nature reviews. Cancer. 2012 Jan;12(1):9–22.

144. Fer N, Melillo G. The HIF-1α-c-Myc pathway and tumorigenesis: evading the apoptotic gate-keeper. Cell cycle (Georgetown, Tex.). 2011 Oct 1;10(19):3228.

145. Höckel M, Vaupel P. Biological consequences of tumor hypoxia. Seminars in oncology. 2001 Apr;28(2 Suppl 8):36–41.

146. Rofstad EK. Microenvironment-induced cancer metastasis. International journal of radiation biology. 2000 May;76(5):589–605.

147. Graham CH, Forsdike J, Fitzgerald CJ, Macdonald-Goodfellow S. Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. International journal of cancer. Journal international du cancer. 1999 Feb 9;80(4):617–23.

148. Graham CH, Fitzpatrick TE, McCrae KR. Hypoxia stimulates urokinase receptor expression through a heme protein-dependent pathway. Blood. 1998 May 1;91(9):3300–7.

149. Azab AK, Hu J, Quang P, Azab F, Pitsillides C, Awwad R, et al. Hypoxia promotes dissemination of multiple myeloma through acquisition of endothelial to mesenchymal transition-like features. Blood. 2012 Mar 6;

150.   Rankin EB, Rha J, Selak MA, Unger TL, Keith B, Liu Q, et al. Hypoxia-inducible factor 2 regulates hepatic lipid metabolism. Molecular and cellular biology. 2009 Aug;29(16):4527–38.

151.   Marino G, Piazzese E, Gruttadauria S, Nicotra G, Guarnaccia M, Emmanuele G, et al. [Innovative use of the vascular endothelial growth factor in an experimental model of acute liver failure]. Il Giornale di chirurgia. 2004 Mar;25(3):61–4.

152.   Park YN, Kim YB, Yang KM, Park C. Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. Arch Pathol Lab Med. 2000;124(7):1061–5.

153.   Fiore M, Mancinelli R, Aloe L, Laviola G, Sornelli F, Vitali M, et al. Hepatocyte growth factor, vascular endothelial growth factor, glial cell-derived neurotrophic factor and nerve growth factor are differentially affected by early chronic ethanol or red wine intake. Toxicology letters. 2009 Aug 10;188(3):208–13.

154.   Ng IO, Poon RT, Lee JM, Fan ST, Ng M, Tso WK. Microvessel density, vascular endothelial growth factor and its receptors Flt-1 and Flk-1/KDR in hepatocellular carcinoma. American journal of clinical pathology. 2001 Dec;116(6):838–45.

155.   Gershtein ES, Dubova EA, Shchegolev AI, Kushlinskii NE. Vascular endothelial growth factor and its type 2 receptor in hepatocellular carcinoma. Bulletin of experimental biology and medicine. 2010 Nov;149(6):749–52.

156.   Ferrero S, Ragni N. Prognostic value of plasma and serum VEGF levels in patients with resectable hepatocellular carcinoma. Annals of surgical oncology. 2003 Nov;10(9):1123.

157.   Chao Y, Li C-P, Chau G-Y, Chen C-P, King K-L, Lui W-Y, et al. Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. Annals of surgical oncology. 2003 May;10(4):355–62.

158.   Wada H, Nagano H, Yamamoto H, Yang Y, Kondo M, Ota H, et al. Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1 alpha. Liver international : official journal of the International Association for the Study of the Liver. 2006 May;26(4):414–23.

159.   Yao D-F, Wu X-H, Zhu Y, Shi G-S, Dong Z-Z, Yao D-B, et al. Quantitative analysis of vascular endothelial growth factor, microvascular density and their clinicopathologic features in human hepatocellular carcinoma. Hepatobiliary & pancreatic diseases international : HBPD INT. 2005 May;4(2):220–6.

160.   Huang G-W, Yang L-Y, Lu W-Q. Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on

neovascularization and survival. World journal of gastroenterology : WJG. 2005 Mar 21;11(11):1705–8.

161. Zhang D-hong, Zhu W-guo, Yue S. [Expression and clinical significance of VEGF in tissue of primary hepatocellular carcinoma]. Xi bao yu fen zi mian yi xue za zhi = Chinese journal of cellular and molecular immunology. 2011 Feb;27(2):199–202.

162. Tamesa T, Iizuka N, Mori N, Okada T, Takemoto N, Tangoku A, et al. High serum levels of vascular endothelial growth factor after hepatectomy are associated with poor prognosis in hepatocellular carcinoma. Hepato-gastroenterology. 56(93):1122–6.

163. Morbidelli L, Birkenhaeger R, Roeckl W, Granger HJ, Kaerst U, Weich HA, et al. Distinct capillary density and progression promoted by vascular endothelial growth factor-A homodimers and heterodimers. Angiogenesis. 1997;1(1):117–30.

164. Brodsky SV, Mendelev N, Melamed M, Ramaswamy G. Vascular density and VEGF expression in hepatic lesions. Journal of gastrointestinal and liver diseases : JGLD. 2007 Dec;16(4):373–7.

165. Tseng P-L, Tai M-H, Huang C-C, Wang C-C, Lin J-W, Hung C-H, et al. Overexpression of VEGF is associated with positive p53 immunostaining in hepatocellular carcinoma (HCC) and adverse outcome of HCC patients. Journal of surgical oncology. 2008 Oct 1;98(5):349–57.

166. Ho M-C, Chen C-N, Lee H, Hsieh F-J, Shun C-T, Chang C-L, et al. Placenta growth factor not vascular endothelial growth factor A or C can predict the early recurrence after radical resection of hepatocellular carcinoma. Cancer letters. 2007 Jun 8;250(2):237–49.

167. Nagaoka S, Yoshida T, Akiyoshi J, Akiba J, Hisamoto T, Yoshida Y, et al. The ratio of serum placenta growth factor to soluble vascular endothelial growth factor receptor-1 predicts the prognosis of hepatocellular carcinoma. Oncology reports. 2010 Jun;23(6):1647–54.

168. Xu H-X, Zhu X-D, Zhuang P-Y, Zhang J-B, Zhang W, Kong L-Q, et al. Expression and prognostic significance of placental growth factor in hepatocellular carcinoma and peritumoral liver tissue. International journal of cancer. Journal international du cancer. 2011 Apr 1;128(7):1559–69.

169. Kato A, Miyazaki M, Ambiru S, Yoshitomi H, Ito H, Nakagawa K, et al. Multidrug resistance gene (MDR-1) expression as a useful prognostic factor in patients with human hepatocellular carcinoma after surgical resection. Journal of surgical oncology. 2001 Oct;78(2):110–5.

170. Huang M, Liu G. The study of innate drug resistance of human hepatocellular carcinoma Bel7402 cell line. Cancer letters. 1999 Jan 8;135(1):97–105.

171. Di Maio M, De Maio E, Perrone F, Pignata S, Daniele B. Hepatocellular carcinoma: systemic treatments. Journal of clinical gastroenterology. 35(5 Suppl 2):S109–14.

172. Samant RS, Shevde LA. Recent advances in anti-angiogenic therapy of cancer. Oncotarget. 2011 Mar;2(3):122–34.

173. Kesisis G, Broxterman H, Giaccone G. Angiogenesis inhibitors. Drug selectivity and target specificity. Current pharmaceutical design. 2007 Jan;13(27):2795–809.

174. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer research. 2006 Dec 15;66(24):11851–8.

175. Huynh H, Ngo VC, Koong HN, Poon D, Choo SP, Thng CH, et al. Sorafenib and rapamycin induce growth suppression in mouse models of hepatocellular carcinoma. J Cell Mol Med. 2009;13(8B):2673–83.

176. Rimassa L, Santoro A. Sorafenib therapy in advanced hepatocellular carcinoma: the SHARP trial. Expert review of anticancer therapy. 2009 Jun;9(6):739–45.

177. Siegel AB, Cohen EI, Ocean A, Lehrer D, Goldenberg A, Knox JJ, et al. Phase II trial evaluating the clinical and biologic effects of bevacizumab in unresectable hepatocellular carcinoma. J Clin Oncol. 2008;26(18):2992–8.

178. Xiong YQ, Sun HC, Zhu XD, Zhang W, Zhuang PY, Zhang JB, et al. Bevacizumab enhances chemosensitivity of hepatocellular carcinoma to adriamycin related to inhibition of survivin expression. J Cancer Res Clin Oncol. 2010;

179. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, et al. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003 Jan;9(1):327–37.

180. Zhu AX, Sahani DV, Duda DG, di Tomaso E, Ancukiewicz M, Catalano OA, et al. Efficacy, safety, and potential biomarkers of sunitinib monotherapy in advanced hepatocellular carcinoma: a phase II study. J Clin Oncol. 2009;27(18):3027–35.

181. Faivre S, Raymond E, Boucher E, Douillard J, Lim HY, Kim JS, et al. Safety and efficacy of sunitinib in patients with advanced hepatocellular carcinoma: an open-label, multicentre, phase II study. The lancet oncology. 2009 Aug;10(8):794–800.

182. J. L. Raoul Y. K. Kang, J. W. Park, R. Harris, V. Coric, M. Donica, I. Walters RSF. An open-label phase II study of first- and second-line treatment with brivanib in patients with hepatocellular carcinoma (HCC), . In: ASCO. 2009.

183.   Huynh H, Ngo VC, Fargnoli J, Ayers M, Soo KC, Koong HN, et al. Brivanib alaninate, a dual inhibitor of vascular endothelial growth factor receptor and fibroblast growth factor receptor tyrosine kinases, induces growth inhibition in mouse models of human hepatocellular carcinoma. Clin Cancer Res. 2008;14(19):6146–53.

184.   Bradley DP, Tessier JL, Checkley D, Kuribayashi H, Waterton JC, Kendrew J, et al. Effects of AZD2171 and vandetanib (ZD6474, Zactima) on haemodynamic variables in an SW620 human colon tumour model: an investigation using dynamic contrast-enhanced MRI and the rapid clearance blood pool contrast agent, P792 (gadomelitol). NMR Biomed. 2008;21(1):42–52.

185.   Giannelli G, Azzariti A, Sgarra C, Porcelli L, Antonaci S, Paradiso A. ZD6474 inhibits proliferation and invasion of human hepatocellular carcinoma cells. Biochemical pharmacology. 2006 Feb 14;71(4):479–85.

186.   Murakami M, Kobayashi S, Marubashi S, Tomimaru Y, Noda T, Wada H, et al. Tyrosine kinase inhibitor PTK/ZK enhances the antitumor effects of interferon-α/5-fluorouracil therapy for hepatocellular carcinoma cells. Annals of surgical oncology. 2011 Feb;18(2):589–96.

187.   Liu Y, Poon RT, Li Q, Kok TW, Lau C, Fan ST. Both antiangiogenesis- and angiogenesis-independent effects are responsible for hepatocellular carcinoma growth arrest by tyrosine kinase inhibitor PTK787/ZK222584. Cancer Res. 2005;65(9):3691–9.

188.   Yau T, Chan P, Pang R, Ng K, Fan ST, Poon RT. Phase 1-2 trial of PTK787/ZK222584 combined with intravenous doxorubicin for treatment of patients with advanced hepatocellular carcinoma: implication for antiangiogenic approach to hepatocellular carcinoma. Cancer. 2010 Nov 1;116(21):5022–9.

189.   Villanueva A, Llovet JM. Targeted therapies for hepatocellular carcinoma. Gastroenterology. 2011 May;140(5):1410–26.

190.   Badamtseren B, Odkhuu E, Koide N, Haque A, Naiki Y, Hashimoto S, et al. Thalidomide inhibits interferon-γ-mediated nitric oxide production in mouse vascular endothelial cells. Cellular immunology. 2011 Jan;270(1):19–24.

191.   D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proceedings of the National Academy of Sciences of the United States of America. 1994 Apr 26;91(9):4082–5.

192.   Kenyon BM, Browne F, D'Amato RJ. Effects of thalidomide and related metabolites in a mouse corneal model of neovascularization. Experimental eye research. 1997 Jun;64(6):971–8.

193.   Pinter M, Wichlas M, Schmid K, Plank C, Muller C, Wrba F, et al. Thalidomide in advanced hepatocellular carcinoma as antiangiogenic treatment approach: a phase I/II trial. Eur J Gastroenterol Hepatol. 2008;20(10):1012–9.

194.   Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nature medicine. 2002 Feb;8(2):128–35.

195.   Yoon DH, Ryoo BY, Ryu MH, Lee SG, Hwang S, Suh DJ, et al. Sorafenib for recurrent hepatocellular carcinoma after liver transplantation. Japanese journal of clinical oncology. 2010;40(8):768–73.

196.   Kim JW, Lee JO, Han SW, Oh DY, Im SA, Kim TY, et al. Clinical Outcomes of Sorafenib Treatment in Patients With Metastatic Hepatocellular Carcinoma Who Had Been Previously Treated With Fluoropyrimidine Plus Platinum-based Chemotherapy. American journal of clinical oncology. 2010;

197.   Malka D, Van den Eynde M, Boige V, Dromain C, Ducreux M. Splenic infarction and bevacizumab. The lancet oncology. 2006 Dec;7(12):1038.

198.   Zhu AX, Blaszkowsky LS, Ryan DP, Clark JW, Muzikansky A, Horgan K, et al. Phase II study of gemcitabine and oxaliplatin in combination with bevacizumab in patients with advanced hepatocellular carcinoma. J Clin Oncol. 2006;24(12):1898–903.

199.   Hsu CH, Yang TS, Hsu C, Toh HC, Epstein RJ, Hsiao LT, et al. Efficacy and tolerability of bevacizumab plus capecitabine as first-line therapy in patients with advanced hepatocellular carcinoma. Br J Cancer. 2010;102(6):981–6.

200.   Wich C, Agaimy A, Strobel D, Wissniowski TT, Hartmann A, Ocker M. Sustained treatment response of metastatic hepatocellular carcinoma with bevacizumab and sorafenib. World journal of gastroenterology : WJG. 2010 Jul 28;16(28):3592–6.

201.   Hsu C, Chen CN, Chen LT, Wu CY, Hsieh FJ, Cheng AL. Effect of thalidomide in hepatocellular carcinoma: assessment with power doppler US and analysis of circulating angiogenic factors. Radiology. 2005;235(2):509–16.

202.   Hsu C, Chen C-N, Chen L-T, Wu C-Y, Yang P-M, Lai M-Y, et al. Low-dose thalidomide treatment for advanced hepatocellular carcinoma. Oncology. 2003 Jan;65(3):242–9.

203.   Schwartz JD, Sung M, Schwartz M, Lehrer D, Mandeli J, Liebes L, et al. Thalidomide in advanced hepatocellular carcinoma with optional low-dose interferon-alpha2a upon progression. The oncologist. 2005 Oct;10(9):718–27.

204.   Han SH, Park SH, Kim JH, Lee JJ, Kwon SY, Kwon OS, et al. Thalidomide for treating metastatic hepatocellular carcinoma: a pilot study. Korean J Intern Med. 2006;21(4):225–9.

205. Chen SC, Tsai HJ, Jan CM, Wang YH, Chen LT. Low dose of thalidomide can be effective in advanced hepatocellular carcinoma. J Gastroenterol Hepatol. 2006;21(12):1868–9.

206. Shiah H-S, Chao Y, Chen L-T, Yao T-J, Huang J-D, Chang J-Y, et al. Phase I and pharmacokinetic study of oral thalidomide in patients with advanced hepatocellular carcinoma. Cancer chemotherapy and pharmacology. 2006 Nov;58(5):654–64.

207. Chang JY, Ka WS, Chao TY, Liu TW, Chuang TR, Chen LT. Hepatocellular carcinoma with intra-atrial tumor thrombi. A report of three cases responsive to thalidomide treatment and literature review. Oncology. 2004;67(3-4):320–6.

208. Philip PA, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, et al. Phase II study of Erlotinib (OSI-774) in patients with advanced hepatocellular cancer. J Clin Oncol. 2005;23(27):6657–63.

209. Thomas MB, Morris JS, Chadha R, Iwasaki M, Kaur H, Lin E, et al. Phase II trial of the combination of bevacizumab and erlotinib in patients who have advanced hepatocellular carcinoma. J Clin Oncol. 2009;27(6):843–50.

210. Thomas MB, Chadha R, Glover K, Wang X, Morris J, Brown T, et al. Phase 2 study of erlotinib in patients with unresectable hepatocellular carcinoma. Cancer. 2007;110(5):1059–67.

211. Le Tourneau C, Raymond E, Faivre S. Sunitinib: a novel tyrosine kinase inhibitor. A brief review of its therapeutic potential in the treatment of renal carcinoma and gastrointestinal stromal tumors (GIST). Ther Clin Risk Manag. 2007;3(2):341–8.

212. Ebos JML, Lee CR, Christensen JG, Mutsaers AJ, Kerbel RS. Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy. Proceedings of the National Academy of Sciences of the United States of America. 2007 Oct 23;104(43):17069–74.

213. Treiber G. mTOR inhibitors for hepatocellular cancer: a forward-moving target. Expert Rev Anticancer Ther. 2009;9(2):247–61.

214. Sahin F, Kannangai R, Adegbola O, Wang J, Su G, Torbenson M. mTOR and P70 S6 kinase expression in primary liver neoplasms. Clin Cancer Res. 2004;10(24):8421–5.

215. Rizell M, Lindner P. Inhibition of mTOR suppresses experimental liver tumours. Anticancer Res. 2005;25(2A):789–93.

216. Newell P, Toffanin S, Villanueva A, Chiang DY, Minguez B, Cabellos L, et al. Ras pathway activation in hepatocellular carcinoma and anti-tumoral effect of combined sorafenib and rapamycin in vivo. Journal of hepatology. 2009 Oct;51(4):725–33.

217. Villanueva A, Chiang DY, Newell P, Peix J, Thung S, Alsinet C, et al. Pivotal role of mTOR signaling in hepatocellular carcinoma. Gastroenterology. 2008;135(6):1972–83, 1983 e1–11.

218. Rizell M, Andersson M, Cahlin C, Hafstrom L, Olausson M, Lindner P. Effects of the mTOR inhibitor sirolimus in patients with hepatocellular and cholangiocellular cancer. Int J Clin Oncol. 2008;13(1):66–70.

219. Huynh H, Ngo VC, Koong HN, Poon D, Choo SP, Toh HC, et al. AZD6244 enhances the anti-tumor activity of sorafenib in ectopic and orthotopic models of human hepatocellular carcinoma (HCC). J Hepatol. 2010;52(1):79–87.

220. Huynh H, Soo KC, Chow PK, Tran E. Targeted inhibition of the extracellular signal-regulated kinase kinase pathway with AZD6244 (ARRY-142886) in the treatment of hepatocellular carcinoma. Mol Cancer Ther. 2007;6(1):138–46.

221. Hui IC-F, Tung EK-K, Sze KM-F, Ching Y-P, Ng IO-L. Rapamycin and CCI-779 inhibit the mammalian target of rapamycin signalling in hepatocellular carcinoma. Liver international : official journal of the International Association for the Study of the Liver. 2010 Jan;30(1):65–75.

222. Kudo K, Arao T, Tanaka K, Nagai T, Furuta K, Sakai K, et al. Antitumor activity of BIBF 1120, a triple angiokinase inhibitor, and use of VEGFR2+pTyr+ peripheral blood leukocytes as a pharmacodynamic biomarker in vivo. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011 Mar 15;17(6):1373–81.

223. Morelli MP, Brown AM, Pitts TM, Tentler JJ, Ciardiello F, Ryan A, et al. Targeting vascular endothelial growth factor receptor-1 and -3 with cediranib (AZD2171): effects on migration and invasion of gastrointestinal cancer cell lines. Mol Cancer Ther. 2009;8(9):2546–58.

224. Huynh H. Tyrosine kinase inhibitors to treat liver cancer. Expert Opin Emerg Drugs. 2010;15(1):13–26.

225. Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, et al. AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. Cancer research. 2005 May 15;65(10):4389–400.

226. Alberts SR, Fitch TR, Kim GP, Morlan BW, Dakhil SR, Gross HM, et al. Cediranib (AZD2171) in Patients With Advanced Hepatocellular Carcinoma: A Phase II North Central Cancer Treatment Group Clinical Trial. American journal of clinical oncology. 2011 Mar 17;

227. Curwen JO, Musgrove HL, Kendrew J, Richmond GH, Ogilvie DJ, Wedge SR. Inhibition of vascular endothelial growth factor-a signaling induces hypertension:

examining the effect of cediranib (recentin; AZD2171) treatment on blood pressure in rat and the use of concomitant antihypertensive therapy. Clin Cancer Res. 2008;14(10):3124–31.

228. Zhu AX, Stuart K, Blaszkowsky LS, Muzikansky A, Reitberg DP, Clark JW, et al. Phase 2 study of cetuximab in patients with advanced hepatocellular carcinoma. Cancer. 2007 Aug 1;110(3):581–9.

229. Louafi S, Boige V, Ducreux M, Bonyhay L, Mansourbakht T, de Baere T, et al. Gemcitabine plus oxaliplatin (GEMOX) in patients with advanced hepatocellular carcinoma (HCC): results of a phase II study. Cancer. 2007;109(7):1384–90.

230. Asnacios A, Fartoux L, Romano O, Tesmoingt C, Louafi S S, Mansoubakht T, et al. Gemcitabine plus oxaliplatin (GEMOX) combined with cetuximab in patients with progressive advanced stage hepatocellular carcinoma: results of a multicenter phase 2 study. Cancer. 2008 Jun 15;112(12):2733–9.

231. Chen WC, Chen W, Tseng GC, Lai HC, Shih CM, Hsia TC. Gefitinib as an effective therapy for advanced hepatocellular carcinoma with lung metastasis? Liver Int. 2010;

232. Jung HS, Byun GW, Lee KE, Mun YC, Nam SH, Kwon JM, et al. Gefitinib Trial in a Fanconi's Anemia Patient with Multiple Squamous Cell Carcinomas and Hepatocellular Carcinoma. Cancer Res Treat. 2005;37(6):370–3.

233. Schiffer E, Housset C, Cacheux W, Wendum D, Desbois-Mouthon C, Rey C, et al. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. Hepatology. 2005;41(2):307–14.

234. Ramanathan RK, Belani CP, Singh DA, Tanaka M, Lenz H-J, Yen Y, et al. A phase II study of lapatinib in patients with advanced biliary tree and hepatocellular cancer. Cancer chemotherapy and pharmacology. 2009 Sep;64(4):777–83.

235. Linifanib. Drugs in R&D. 2010 Jan;10(2):111–22.

236. Yau TC, Chen P-J, Chan P, Curtis CM, Murphy PS, Suttle AB, et al. Phase I Dose-Finding Study of Pazopanib in Hepatocellular Carcinoma: Evaluation of Early Efficacy, Pharmacokinetics, and Pharmacodynamics. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011 Aug 10;

237. Zhu X-D, Zhang J-B, Fan P-L, Xiong Y-Q, Zhuang P-Y, Zhang W, et al. Antiangiogenic effects of pazopanib in xenograft hepatocellular carcinoma models: evaluation by quantitative contrast-enhanced ultrasonography. BMC cancer. 2011 Jan;11:28.

238. Yau T, Pang R, Chan P, Poon RT. Molecular targeted therapy of advanced hepatocellular carcinoma beyond sorafenib. Expert opinion on pharmacotherapy. 2010 Sep;11(13):2187–98.

239.   Spratlin JL, Mulder KE, Mackey JR. Ramucirumab (IMC-1121B): a novel attack on angiogenesis. Future oncology (London, England). 2010 Jul;6(7):1085–94.

240.   Neal J, Wakelee H. AMG-386, a selective angiopoietin-1/-2-neutralizing peptibody for the potential treatment of cancer. Current opinion in molecular therapeutics. 2010 Aug;12(4):487–95.

241.   Eder JP, Shapiro GI, Appleman LJ, Zhu AX, Miles D, Keer H, et al. A phase I study of foretinib, a multi-targeted inhibitor of c-Met and vascular endothelial growth factor receptor 2. Clin Cancer Res. 2010;16(13):3507–16.

242.   Matsui J, Yamamoto Y, Funahashi Y, Tsuruoka A, Watanabe T, Wakabayashi T, et al. E7080, a novel inhibitor that targets multiple kinases, has potent antitumor activities against stem cell factor producing human small cell lung cancer H146, based on angiogenesis inhibition. International journal of cancer. Journal international du cancer. 2008 Feb 1;122(3):664–71.

243.   Kanai F, Yoshida H, Tateishi R, Sato S, Kawabe T, Obi S, et al. A phase I/II trial of the oral antiangiogenic agent TSU-68 in patients with advanced hepatocellular carcinoma. Cancer chemotherapy and pharmacology. 2011 Feb;67(2):315–24.

244.   Basic Search - ClinicalTrials.gov [Internet]. [cited 2011 Nov 1];Available from: http://www.clinicaltrials.gov/ct2/search

245.   Jain RK, Duda DG, Willett CG, Sahani DV, Zhu AX, Loeffler JS, et al. Biomarkers of response and resistance to antiangiogenic therapy. Nat Rev Clin Oncol. 2009;6(6):327–38.

246.   Ebos JML, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009 Aug 15;15(16):5020–5.

247.   Ebos JML, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer cell. 2009 Mar 3;15(3):232–9.

248.   Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell. 2009;15(3):220–31.

249.   Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nature reviews. Cancer. 2008 Aug;8(8):592–603.

250.   Brahimi-Horn C, Pouysségur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. Bulletin du cancer. 2006 Aug;93(8):E73–80.

251.   Du G, Han G, Zhang S, Lin H, Wu X, Wang M, et al. Baicalin suppresses lung carcinoma and lung metastasis by SOD mimic and HIF-1alpha inhibition. European journal of pharmacology. 2010 Mar 25;630(1-3):121–30.

252.   Subarsky P, Hill RP. The hypoxic tumour microenvironment and metastatic progression. Clinical & experimental metastasis. 2003 Jan;20(3):237–50.

253.   Kerbel RS, Yu J, Tran J, Man S, Viloria-Petit A, Klement G, et al. Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. Cancer metastasis reviews. 2001 Jan;20(1-2):79–86.

254.   Hlushchuk R, Riesterer O, Baum O, Wood J, Gruber G, Pruschy M, et al. Tumor recovery by angiogenic switch from sprouting to intussusceptive angiogenesis after treatment with PTK787/ZK222584 or ionizing radiation. The American journal of pathology. 2008 Oct;173(4):1173–85.

255.   van der Schaft DWJ, Seftor REB, Seftor EA, Hess AR, Gruman LM, Kirschmann DA, et al. Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells. Journal of the National Cancer Institute. 2004 Oct 6;96(19):1473–7.

256.   Kamba T, Tam BYY, Hashizume H, Haskell A, Sennino B, Mancuso MR, et al. VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. American journal of physiology. Heart and circulatory physiology. 2006 Feb;290(2):H560–76.

257.   Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. 2005 Dec 8;438(7069):820–7.

258.   Farber E. The multistep nature of cancer development. Cancer research. 1984 Oct;44(10):4217–23.

259.   Diwan BA, Rice JM, Ohshima M, Ward JM. Interstrain differences in susceptibility to liver carcinogenesis initiated by N-nitrosodiethylamine and its promotion by phenobarbital in C57BL/6NCr, C3H/HeNCrMTV- and DBA/2NCr mice. Carcinogenesis. 1986 Mar;7(2):215–20.

260.   Voskoglou-Nomikos T, Pater JL, Seymour L. Clinical predictive value of the in vitro cell line, human xenograft, and mouse allograft preclinical cancer models. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003 Sep 15;9(11):4227–39.

261.   Heindryckx F, Colle I, Van Vlierberghe H. Experimental mouse models for hepatocellular carcinoma research. Int J Exp Pathol. 2009;90(4):367–86.

262.   Pitot HC, Dragan YP. Facts and theories concerning the mechanisms of carcinogenesis. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1991 Jun;5(9):2280–6.

263.   Binato M, Kruel Schmidt M, Silveira Volkweis B, Behrend Silva Ribeiro G, Isabel Edelweiss M, Ricachenevsky Gurski R. Mouse model of diethylnitrosamine-induced gastric cancer. The Journal of surgical research. 2008 Aug;148(2):152–7.

264.   Qi Y, Chen X, Chan C-yan, Li D, Yuan C, Yu F, et al. Two-dimensional differential gel electrophoresis/analysis of diethylnitrosamine induced rat hepatocellular carcinoma. International journal of cancer. Journal international du cancer. 2008 Jun 15;122(12):2682–8.

265.   Kolaja KL, Xu Y, Walborg EF, Stevenson DE, Klaunig JE. Vitamin E modulation of dieldrin-induced hepatic focal lesion growth in mice. Journal of toxicology and environmental health. Part A. 1998 Mar 27;53(6):479–92.

266.   Kawanishi S, Hiraku Y, Murata M, Oikawa S. The role of metals in site-specific DNA damage with reference to carcinogenesis. Free radical biology & medicine. 2002 May 1;32(9):822–32.

267.   Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-biological interactions. 2006 Mar 10;160(1):1–40.

268.   Chen B, Liu L, Castonguay A, Maronpot RR, Anderson MW, You M. Dose-dependent ras mutation spectra in N-nitrosodiethylamine induced mouse liver tumors and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induced mouse lung tumors. Carcinogenesis. 1993 Aug;14(8):1603–8.

269.   Stahl S, Ittrich C, Marx-Stoelting P, Köhle C, Altug-Teber O, Riess O, et al. Genotype-phenotype relationships in hepatocellular tumors from mice and man. Hepatology (Baltimore, Md.). 2005 Aug;42(2):353–61.

270.   Tada M, Omata M, Ohto M. [Detection of ras gene mutations of primary hepatic malignant tumors by polymerase chain reaction and direct sequencing method]. Nihon Shokakibyo Gakkai zasshi = The Japanese journal of gastro-enterology. 1990 Mar;87(3):811–5.

271.   Wang Q, Lin ZY, Feng XL. Alterations in metastatic properties of hepatocellular carcinoma cell following H-ras oncogene transfection. World journal of gastroenterology : WJG. 2001 Jun;7(3):335–9.

272.   Williams GM, Iatropoulos MJ, Jeffrey AM. Mechanistic basis for nonlinearities and thresholds in rat liver carcinogenesis by the DNA-reactive carcinogens 2-acetylaminofluorene and diethylnitrosamine. Toxicologic pathology. 28(3):388–95.

273. Lee JS, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, et al. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. Nature genetics. 2004;36(12):1306–11.

274. Lee JS, Grisham JW, Thorgeirsson SS. Comparative functional genomics for identifying models of human cancer. Carcinogenesis. 2005;26(6):1013–20.

275. Teoh NC, Dan YY, Swisshelm K, Lehman S, Wright JH, Haque J, et al. Defective DNA strand break repair causes chromosomal instability and accelerates liver carcinogenesis in mice. Hepatology (Baltimore, Md.). 2008 Jun;47(6):2078–88.

276. Pegg AE. Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. Cancer research. 1990 Oct 1;50(19):6119–29.

277. Rao KV, Vesselinovitch SD. Age- and sex-associated diethylnitrosamine dealkylation activity of the mouse liver and hepatocarcinogenesis. Cancer research. 1973 Jul;33(7):1625–7.

278. Vesselinovitch SD. Certain aspects of hepatocarcinogenesis in the infant mouse model. Toxicologic pathology. 1987 Jan;15(2):221–8.

279. Nakatani T, Roy G, Fujimoto N, Asahara T, Ito A. Sex hormone dependency of diethylnitrosamine-induced liver tumors in mice and chemoprevention by leuprorelin. Japanese journal of cancer research : Gann. 2001 Mar;92(3):249–56.

280. Buchmann A, Bauer-Hofmann R, Mahr J, Drinkwater NR, Luz A, Schwarz M. Mutational activation of the c-Ha-ras gene in liver tumors of different rodent strains: correlation with susceptibility to hepatocarcinogenesis. Proceedings of the National Academy of Sciences of the United States of America. 1991 Mar 1;88(3):911–5.

281. Bird AP, Wolffe AP. Methylation-induced repression--belts, braces, and chromatin. Cell. 1999 Nov 24;99(5):451–4.

282. Goldsworthy TL, Fransson-Steen R. Quantitation of the cancer process in C57BL/6J, B6C3F1 and C3H/HeJ mice. Toxicologic pathology. 30(1):97–105.

283. Park TJ, Kim JY, Oh SP, Kang SY, Kim BW, Wang HJ, et al. TIS21 negatively regulates hepatocarcinogenesis by disruption of cyclin B1-Forkhead box M1 regulation loop. Hepatology (Baltimore, Md.). 2008 May;47(5):1533–43.

284. Hacker HJ, Mtiro H, Bannasch P, Vesselinovitch SD. Histochemical profile of mouse hepatocellular adenomas and carcinomas induced by a single dose of diethylnitrosamine. Cancer research. 1991 Apr 1;51(7):1952–8.

285. Vesselinovitch SD, Mihailovich N, Rao KV. Morphology and metastatic nature of induced hepatic nodular lesions in C57BL x C3H F1 mice. Cancer research. 1978 Jul;38(7):2003–10.

286. Frey S, Buchmann A, Bursch W, Schulte-Hermann R, Schwarz M. Suppression of apoptosis in C3H mouse liver tumors by activated Ha-ras oncogene. Carcinogenesis. 2000 Mar;21(2):161–6.

287. Zimmers TA, Jin X, Gutierrez JC, Acosta C, McKillop IH, Pierce RH, et al. Effect of in vivo loss of GDF-15 on hepatocellular carcinogenesis. Journal of cancer research and clinical oncology. 2008 Jul;134(7):753–9.

288. Liu X-en, Dewaele S, Vanhooren V, Fan Y-D, Wang L, Van Huysse J, et al. Alteration of N-glycome in diethylnitrosamine-induced hepatocellular carcinoma mice: a non-invasive monitoring tool for liver cancer. Liver international : official journal of the International Association for the Study of the Liver. 2010 Sep;30(8):1221–8.

289. Shiota G, Harada K, Ishida M, Tomie Y, Okubo M, Katayama S, et al. Inhibition of hepatocellular carcinoma by glycyrrhizin in diethylnitrosamine-treated mice. Carcinogenesis. 1999 Jan;20(1):59–63.

290. Finnberg N, Stenius U, Högberg J. Heterozygous p53-deficient (+/-) mice develop fewer p53-negative preoplastic focal liver lesions in response to treatment with diethylnitrosamine than do wild-type (+/+) mice. Cancer letters. 2004 Apr 30;207(2):149–55.

291. Waxman DJ, Azaroff L. Phenobarbital induction of cytochrome P-450 gene expression. The Biochemical journal. 1992 Mar 1;281 (Pt 3:577–92.

292. Imaoka S, Osada M, Minamiyama Y, Yukimura T, Toyokuni S, Takemura S, et al. Role of phenobarbital-inducible cytochrome P450s as a source of active oxygen species in DNA-oxidation. Cancer letters. 2004 Jan 20;203(2):117–25.

293. Watson RE, Goodman JI. Effects of phenobarbital on DNA methylation in GC-rich regions of hepatic DNA from mice that exhibit different levels of susceptibility to liver tumorigenesis. Toxicological sciences : an official journal of the Society of Toxicology. 2002 Jul;68(1):51–8.

294. Loeppen S, Schneider D, Gaunitz F, Gebhardt R, Kurek R, Buchmann A, et al. Overexpression of glutamine synthetase is associated with beta-catenin-mutations in mouse liver tumors during promotion of hepatocarcinogenesis by phenobarbital. Cancer research. 2002 Oct 15;62(20):5685–8.

295. Aydinlik H, Nguyen TD, Moennikes O, Buchmann A, Schwarz M. Selective pressure during tumor promotion by phenobarbital leads to clonal outgrowth of beta-catenin-mutated mouse liver tumors. Oncogene. 2001 Nov 22;20(53):7812–6.

296. Mao TL, Chu JS, Jeng YM, Lai PL, Hsu HC. Expression of mutant nuclear beta-catenin correlates with non-invasive hepatocellular carcinoma, absence of portal vein spread, and good prognosis. The Journal of pathology. 2001 Jan;193(1):95–101.

297. Hsu HC, Jeng YM, Mao TL, Chu JS, Lai PL, Peng SY. Beta-catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. The American journal of pathology. 2000 Sep;157(3):763–70.

298. Lee GH, Ooasa T, Osanai M. Mechanism of the paradoxical, inhibitory effect of phenobarbital on hepatocarcinogenesis initiated in infant B6C3F1 mice with diethylnitrosamine. Cancer research. 1998;58(8):1665–9.

299. Diwan BA, Rice JM, Ward JM, Ohshima M, Lynch PH. Inhibition by phenobarbital and lack of effect of amobarbital on the development of liver tumors induced by N-nitrosodiethylamine in juvenile B6C3F1 mice. Cancer letters. 1984 Jun;23(2):223–34.

300. Weghorst CM, Klaunig JE. Phenobarbital promotion in diethylnitrosamine-initiated infant B6C3F1 mice: influence of gender. Carcinogenesis. 1989 Mar;10(3):609–12.

301. Klaunig JE, Pereira MA, Ruch RJ, Weghorst CM. Dose-response relationship of diethylnitrosamine-initiated tumors in neonatal balb/c mice: effect of phenobarbital promotion. Toxicologic pathology. 1988 Jan;16(3):381–5.

302. Kanamura S, Kanai K, Watanabe J. Fine structure and function of hepatocytes during development. Journal of electron microscopy technique. 1990 Mar;14(2):92–105.

303. Counts JL, Goodman JI. Hypomethylation of DNA: a nongenotoxic mechanism involved in tumor promotion. Toxicology letters. 1995 Dec;82-83:663–72.

304. Lee GH. Paradoxical effects of phenobarbital on mouse hepatocarcinogenesis. Toxicologic pathology. 28(2):215–25.

305. Kostka G, Urbanek-Olejnik K, Wiadrowska B, Bańkowski R. [Phenobarbital-induced hypermethylation of the p53 promoter region in the liver of Wistar rats]. Roczniki Państwowego Zakładu Higieny. 2008 Jan;59(4):455–65.

306. Farber E, Solt D, Cameron R, Laishes B, Ogawa K, Medline A. Newer insights into the pathogenesis of liver cancer. The American journal of pathology. 1977 Nov;89(2):477–82.

307. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology (Baltimore, Md.). 2006 Feb;43(2 Suppl 1):S45–53.

308.   Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. European journal of cancer (Oxford, England : 1990). 2004 Apr;40(6):827–36.

309.   Troiani T, Schettino C, Martinelli E, Morgillo F, Tortora G, Ciardiello F. The use of xenograft models for the selection of cancer treatments with the EGFR as an example. Critical reviews in oncology/hematology. 2008 Mar;65(3):200–11.

310.   Kornek M, Raskopf E, Tolba R, Becker U, Klöckner M, Sauerbruch T, et al. Accelerated orthotopic hepatocellular carcinomas growth is linked to increased expression of pro-angiogenic and prometastatic factors in murine liver fibrosis. Liver international : official journal of the International Association for the Study of the Liver. 2008 Apr;28(4):509–18.

311.   Sun FX, Tang ZY, Lui KD, Ye SL, Xue Q, Gao DM, et al. Establishment of a metastatic model of human hepatocellular carcinoma in nude mice via orthotopic implantation of histologically intact tissues. International journal of cancer. Journal international du cancer. 1996 Apr 10;66(2):239–43.

312.   Shao DM, Wang QH, Chen C, Shen ZH, Yao M, Zhou XD, et al. N-acetylglucosaminyltransferase V activity in metastatic models of human hepatocellular carcinoma in nude mice. Journal of experimental & clinical cancer research : CR. 1999 Sep;18(3):331–5.

313.   Frese KK, Tuveson DA. Maximizing mouse cancer models. Nature reviews. Cancer. 2007 Sep;7(9):645–58.

314.   Calvisi DF, Thorgeirsson SS. Molecular mechanisms of hepatocarcinogenesis in transgenic mouse models of liver cancer. Toxicologic pathology. 2005 Jan;33(1):181–4.

315.   Tuveson DA, Jacks T. Technologically advanced cancer modeling in mice. Current opinion in genetics & development. 2002 Mar;12(1):105–10.

316.   Wu CG, Salvay DM, Forgues M, Valerie K, Farnsworth J, Markin RS, et al. Distinctive gene expression profiles associated with Hepatitis B virus x protein. Oncogene. 2001 Jun 21;20(28):3674–82.

317.   Avantaggiati ML, Natoli G, Balsano C, Chirillo P, Artini M, De Marzio E, et al. The hepatitis B virus (HBV) pX transactivates the c-fos promoter through multiple cis-acting elements. Oncogene. 1993 Jun;8(6):1567–74.

318.   Hu KQ, Yu CH, Vierling JM. Up-regulation of intercellular adhesion molecule 1 transcription by hepatitis B virus X protein. Proceedings of the National Academy of Sciences of the United States of America. 1992 Dec 1;89(23):11441–5.

319.   Babinet C, Farza H, Morello D, Hadchouel M, Pourcel C. Specific expression of hepatitis B surface antigen (HBsAg) in transgenic mice. Science (New York, N.Y.). 1985 Dec 6;230(4730):1160–3.

320.   Chisari FV, Pinkert CA, Milich DR, Filippi P, McLachlan A, Palmiter RD, et al. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. Science (New York, N.Y.). 1985 Dec 6;230(4730):1157–60.

321.   Araki K, Miyazaki J, Hino O, Tomita N, Chisaka O, Matsubara K, et al. Expression and replication of hepatitis B virus genome in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America. 1989 Jan;86(1):207–11.

322.   Xiong J, Yao Y-C, Zi X-Y, Li J-X, Wang X-M, Ye X-T, et al. Expression of hepatitis B virus X protein in transgenic mice. World journal of gastroenterology : WJG. 2003 Jan;9(1):112–6.

323.   Burk RD, DeLoia JA, elAwady MK, Gearhart JD. Tissue preferential expression of the hepatitis B virus (HBV) surface antigen gene in two lines of HBV transgenic mice. Journal of virology. 1988 Feb;62(2):649–54.

324.   Lakhtakia R, Kumar V, Reddi H, Mathur M, Dattagupta S, Panda SK. Hepatocellular carcinoma in a hepatitis B "x" transgenic mouse model: A sequential pathological evaluation. Journal of gastroenterology and hepatology. 2003 Jan;18(1):80–91.

325.   Takada S, Tsuchida N, Kobayashi M, Koike K. Disruption of the function of tumor-suppressor gene p53 by the hepatitis B virus X protein and hepatocarcinogenesis. Journal of cancer research and clinical oncology. 1995 Jan;121(9-10):593–601.

326.   Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T, et al. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. Hepatology (Baltimore, Md.). 1994 Apr;19(4):810–9.

327.   Zheng Y, Chen W-ling, Louie SG, Yen TSB, Ou J-hsiung J. Hepatitis B virus promotes hepatocarcinogenesis in transgenic mice. Hepatology (Baltimore, Md.). 2007 Jan;45(1):16–21.

328.   Lee TH, Finegold MJ, Shen RF, DeMayo JL, Woo SL, Butel JS. Hepatitis B virus transactivator X protein is not tumorigenic in transgenic mice. Journal of virology. 1990 Dec;64(12):5939–47.

329.   Sell S, Hunt JM, Dunsford HA, Chisari FV. Synergy between hepatitis B virus expression and chemical hepatocarcinogens in transgenic mice. Cancer research. 1991 Feb 15;51(4):1278–85.

330. Pecic V, Stankovic-Djordjevic D, Nestorovic M, Radojkovic M, Marjanovic H, Ilic B, et al. Hepatitis C virus-related hepatocellular carcinoma and liver cirrhosis. Journal of B.U.ON. : official journal of the Balkan Union of Oncology. 16(2):277–81.

331. Lan K-H, Sheu M-L, Hwang S-J, Yen S-H, Chen S-Y, Wu J-C, et al. HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. Oncogene. 2002 Jul 18;21(31):4801–11.

332. Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, et al. NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. The Journal of general virology. 2006 Jun;87(Pt 6):1703–13.

333. Feng X, Zhang H, Liu H, Song X, Wang G, Chen K, et al. Cancerogenic effect of different fragments of the hepatitis C virus core protein. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP). 2007 Aug;16(4):304–11.

334. Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology. 2002 Feb;122(2):352–65.

335. Kawamura T, Furusaka A, Koziel MJ, Chung RT, Wang TC, Schmidt EV, et al. Transgenic expression of hepatitis C virus structural proteins in the mouse. Hepatology (Baltimore, Md.). 1997 Apr;25(4):1014–21.

336. Tanaka N, Moriya K, Kiyosawa K, Koike K, Aoyama T. Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor alpha in transgenic mice: implications for HCV-associated hepatocarcinogenesis. International journal of cancer. Journal international du cancer. 2008 Jan 1;122(1):124–31.

337. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nature medicine. 1998 Sep;4(9):1065–7.

338. Naas T, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, et al. Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. The Journal of general virology. 2005 Aug;86(Pt 8):2185–96.

339. Kamegaya Y, Hiasa Y, Zukerberg L, Fowler N, Blackard JT, Lin W, et al. Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. Hepatology (Baltimore, Md.). 2005 Mar;41(3):660–7.

340. Thorgeirsson SS, Santoni-Rugiu E. Transgenic mouse models in carcinogenesis: interaction of c-myc with transforming growth factor alpha and hepatocyte growth factor in hepatocarcinogenesis. British journal of clinical pharmacology. 1996 Jul;42(1):43–52.

341. Harada N, Miyoshi H, Murai N, Oshima H, Tamai Y, Oshima M, et al. Lack of tumorigenesis in the mouse liver after adenovirus-mediated expression of a dominant stable mutant of beta-catenin. Cancer research. 2002 Apr 1;62(7):1971–7.

342. Harada N, Oshima H, Katoh M, Tamai Y, Oshima M, Taketo MM. Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. Cancer research. 2004 Jan 1;64(1):48–54.

343. Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH, Merlino GT. TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell. 1990 Jun 15;61(6):1137–46.

344. Ohgaki H, Sanderson ND, Ton P, Thorgeirsson SS. Molecular analyses of liver tumors in c-myc transgenic mice and c-myc and TGF-alpha double transgenic mice. Cancer letters. 1996 Aug 23;106(1):43–9.

345. Borlak J, Meier T, Halter R, Spanel R, Spanel-Borowski K. Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. Oncogene. 2005 Mar 10;24(11):1809–19.

346. Nicholes K, Guillet S, Tomlinson E, Hillan K, Wright B, Frantz GD, et al. A mouse model of hepatocellular carcinoma: ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice. The American journal of pathology. 2002 Jun;160(6):2295–307.

347. Geller SA, Nichols WS, Kim S, Tolmachoff T, Lee S, Dycaico MJ, et al. Hepatocarcinogenesis is the sequel to hepatitis in Z#2 alpha 1-antitrypsin transgenic mice: histopathological and DNA ploidy studies. Hepatology (Baltimore, Md.). 1994 Feb;19(2):389–97.

348. Horie Y, Suzuki A, Kataoka E, Sasaki T, Hamada K, Sasaki J, et al. Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. The Journal of clinical investigation. 2004 Jun;113(12):1774–83.

349. Watanabe S, Horie Y, Kataoka E, Sato W, Dohmen T, Ohshima S, et al. Non-alcoholic steatohepatitis and hepatocellular carcinoma: lessons from hepatocyte-specific phosphatase and tensin homolog (PTEN)-deficient mice. Journal of gastroenterology and hepatology. 2007 Jun;22 Suppl 1:S96–S100.

350. Dubois N, Bennoun M, Allemand I, Molina T, Grimber G, Daudet-Monsac M, et al. Time-course development of differentiated hepatocarcinoma and lung metastasis in transgenic mice. Journal of hepatology. 1991 Sep;13(2):227–39.

351. Lou D-Q, Molina T, Bennoun M, Porteu A, Briand P, Joulin V, et al. Conditional hepatocarcinogenesis in mice expressing SV 40 early sequences. Cancer letters. 2005 Nov 8;229(1):107–14.

352. Araki K, Hino O, Miyazaki J, Yamamura K. Development of two types of hepatocellular carcinoma in transgenic mice carrying the SV40 large T-antigen gene. Carcinogenesis. 1991 Nov;12(11):2059–62.

353. Sepulveda AR, Finegold MJ, Smith B, Slagle BL, DeMayo JL, Shen RF, et al. Development of a transgenic mouse system for the analysis of stages in liver carcinogenesis using tissue-specific expression of SV40 large T-antigen controlled by regulatory elements of the human alpha-1-antitrypsin gene. Cancer research. 1989 Nov 1;49(21):6108–17.

354. Cullen JM, Sandgren EP, Brinster RL, Maronpot RR. Histologic characterization of hepatic carcinogenesis in transgenic mice expressing SV40 T-antigens. Veterinary pathology. 1993 Mar;30(2):111–8.

355. Dupuy AJ, Akagi K, Largaespada DA, Copeland NG, Jenkins NA. Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. Nature. 2005 Jul 14;436(7048):221–6.

356. Keng VW, Villanueva A, Chiang DY, Dupuy AJ, Ryan BJ, Matise I, et al. A conditional transposon-based insertional mutagenesis screen for genes associated with mouse hepatocellular carcinoma. Nature biotechnology. 2009 Mar;27(3):264–74.

Chapter II
Aims

1   General Aims

The importance of angiogenesis in the development and progression of HCC has long been established. It is therefore not surprising that inhibiting angiogenesis, has opened up the world of therapeutic targets against HCC. These angiogenic inhibitors are unique cancer-fighting agents, inhibiting the growth of blood vessels, without directly targeting the tumour cells. Unfortunately, recent studies have shown that this tactic also has a negative side, since hypoxia can induce a more aggressive tumour phenotype and that tumours tend to escape their anti-angiogenic therapy through other mechanisms. Furthermore, classic anti-angiogenic treatment targets both physiologic as pathologic angiogenesis, resulting in numerous adverse effects.

To assess new anti-angiogenic drugs, a **representative mouse model for HCC** had to be developed. It was important that it resembled human HCC, not only molecularly, but also in its development on a background of chronic liver damage. Therefore, a chemically induced mouse model for HCC was established in the first part of our study.

Since we will mainly focus on tumour growth and vascularisation, several **innovative imaging techniques** will be validated. Non-invasive technology, such as PET-CT imaging, will be verified as techniques to identify small HCC-lesions. Furthermore, techniques for visualizing the tumours microvasculature will be established.

The placental growth factor is the new kid on the angiogenesis block. Its mere involvement in pathological angiogenesis, offers a unique potential in targeting tumour blood vessels without affecting healthy tissue. Whether PlGF is involved in the pathogenesis of HCC remains unknown and is thus, one of the key research questions that have been investigated in the doctoral thesis.

After the **role of PlGF in the hepatocarcinogenesis** has been established in a transgenic *Pgf* **knock out model**, our goal is to assess whether monoclonal **antibodies targeting PlGF** could serve as a potential therapy against hepatocellular carcinoma. Since inhibiting PlGF is likely to alter the genomic expression of tumours, a **genomic expression signature** of anti-PlGF treated tumours will be compared to WT tumours. **The prognostic and predictive value** of the PlGF-gene-profile will be validated, using genomic datasets of human HCC.

Contradictory findings regarding the role of prolyl hydroxylase containing domains in tumour progression and vascularisation, has made this factor a hot research topic. Whether PHD2 plays a role in the pathogenesis of HCC, is unknown and has been investigated in our research project. The effect of PHD2-silencing will be assessed using a **heterozygous *Phd2* knock out model**, in which we will focus on angiogenesis, tumour development and hepatic progenitor cells.

2   Specific aims

2.1   Develop a representative mouse model for HCC.

- Develop an orthotopic **mouse model** in which hepatocellular tumours develop as a result of chronic liver damage.

- Validate innovative **imaging techniques** for investigating tumour vascularisation, vascular morphology and tumour progression in an orthothopic mouse model for HCC.

- Confirm the presence of **angiogenesis** in this mouse model.

These objectives were assessed in the following manuscript:

Heindryckx F, Mertens K, Charette N, Vandeghinste B, Casteleyn C, Van Steenkiste C, et al. **Kinetics of angiogenic changes in a new mouse model for hepatocellular carcinom**a. *Molecular cancer.* 2010 Jan; 9:219.

2.2   Determine the role of PlGF in the pathogenesis of HCC

- Assess whether administration of **PlGF-antibodies** could serve as a potential therapy against hepatocellular carcinoma.

- Investigate the involvement of PlGF in the tumorigenesis of HCC through a **transgenic *Pgf*-knock out model**.

- Confirm the importance of **PlGF in human** HCC- and cirrhotic patients.

- Determine the **gene expression profile** of PlGF-regulated angiogenesis in anti-PlGF treated mice.

- Evaluate **the prognostic and predictive value** of the PlGF-gene-profile, using genomic datasets of human HCC.

These objectives were assessed in the following manuscripts:

Van de Veire, S., Stalmans, I., Heindryckx, F., Oura, H., Tijeras Raballand, A., Schmidt, T., Loges, S., et al. (2010). **Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease**. *Cell*, *141*(1), 178-190.

Heindryckx F., Coulon S., Terrie E., Casteleyn C., Laukens D., Colle I., Stärkel P., et al, **The placental growth factor as a target against hepatocellular carcinoma,** submitted for publication in *Hepatology*

2.3   Determine the effect of PHD2 silencing on the pathogenesis of hepatocellular carcinoma.

- Assess the effect of PHD2 silencing and up-regulation of HIF on the hepatocarcinogenesis, using a **transgenic *Phd2*-heterozygous knock out mouse model**.

- Determine the effect of decreased degradation of HIF on the differentiation of **hepatic progenitor cells**.

These objectives were assessed in the following manuscript:

Heindryckx, F., Kuchnio, A., Casteleyn, C., Coulon, S., Olievier, K., Colle, I., Geerts, A., et al. **Effect of prolyl hydroxylase domain-2 haplodeficiency on hepatocarcinogenesis in mice**. *Journal of Hepatology,* accepted 20 feb. 12.

2.4   Determine the effect of PlGF inhibition on the pathogenesis of cholangiocarcinoma.

- Assess whether administration of **PlGF-antibodies** could serve as a potential therapy against cholangiocarcinoma.

This objective was assessed in the following manuscript:

Heindryckx F., Bogaerts E., Coulon S., Devlies H., Geerts A., Libbrecht L., Carmeliet P. et al, **Inhibition of the placental growth factor decreases cholangiocarcinoma and hepatocellular carcinoma burden in a transgenic mouse model**, *European journal of gastroenterology and hepatology, accepted 27 apr. 12.*

Chapter III
Results

1 Angiogenic changes in a new mouse model for HCC

MOLECULAR CANCER

1.1   Abstract

Background: The increasing incidence of hepatocellular carcinoma in Western countries has led to an expanding interest of scientific research in this field. Therefore, a vast need of experimental models that mimic the natural pathogenesis of hepatocellular carcinoma (HCC) in a short time period is present. The goal of our study was ① to develop an efficient mouse model for HCC research, in which tumours develop in a natural background of fibrosis and ② to assess the time-dependent angiogenic changes in the pathogenesis of HCC. Methods: Weekly intraperitoneal injections with the hepatocarcinogenic compound N-nitrosodiethylamine was applied as induction method and samples were taken at several time points to assess the angiogenic changes during the progression of HCC. Results: The N-nitrosodiethylamine-induced mouse model provides well vascularised orthotopic tumours after 25 weeks. It is a representative model for human HCC and can be used as an excellent platform for the development of new therapeutic targets.

1.2   Introduction

An efficient and representative mouse model is the cornerstone of a successful experiment. The growing incidence of hepatocellular carcinoma (HCC) in Western countries has resulted in an expanding interest of scientific research in this field. Therefore, a vast need of experimental models that mimic the natural pathogenesis of HCC in a short time period is present.

Several genetically modified mouse models (GMM) develop HCC in relatively short time periods. They often represent only one or a few specific mutation(s), while natural tumours are a dynamic environment consisting of a heterogenic cell population with different genotypes which change over time as a response to variable external conditions [1-3]. Xenograft models are relevant for fast drug screening and proof-of-principle experiments [4], but face similar limitations as the GMMs, since only one cell phenotype is assessed, while tumours exist of a large variety of phenotypes. Results should always be interpreted with care, because introducing foreign cells in an animal system, as done in a xenograft mouse model, creates an altered physiological interaction

between tumour and environment [5], leading to spectacular results that can seldom be confirmed in cancer patients [6].

A compound often used for the chemical induction of HCC is N-nitrosodiethylamine (DEN). DEN is metabolised by cytochrome P450 enzymes, which are abundantly present in the liver, leading to the formation of the reactive ethyl diazonium ion [7]. The latter holds the potential to alkylate DNA structures, causing alterations in the expression levels of tumour promoting and/or suppressing genes [8]. Single injections of DEN, sometimes in combination with phenobarbital treatment, are frequently used for the induction of HCC in mice and rats and have been validated as a genetically representative model for human HCC [9]. However, it does not induce fibrosis.

The goal of our study was ① to develop an efficient mouse model for hepatocellular carcinoma (HCC) research, in which HCC develops in a natural background of fibrosis and ② to assess the time-dependent angiogenic changes in the pathogenesis of HCC [10-11] since anti-angiogenic molecules are currently a hot topic in research concerning therapies for non-resectable HCC [12-14].

1.3 Materials & Methods

1.3.1 Animals

4-week-old male mice (129S2/SvPasCrl) were purchased from Charles River laboratories (Brussels, Belgium). They were kept under constant temperature and humidity in a 12h controlled dark/light cycle. Mice were fed *ad libitum* on a standard pellet diet. The Ethical Committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocols.

1.3.2 HCC induction

5-week-old male mice (n = 45) received intraperitoneal injections once per week with DEN (35 mg/kg bodyweight) diluted in saline using a 0,5 mL syringe

with a 29G needle. If mice suffered from weight loss ≥15% compared to the previous week, an injection was omitted. The control group was injected with an equal volume of saline and injections were randomly passed over in a comparable quantity as in the DEN-group.

1.3.3 Tissue sampling & histology

After 4, 16, 20, 25 and 30 weeks, 8 animals per group were sacrificed under isoflurane (Forene®) anaesthesia while blood was obtained from the carotic artery. After macroscopic evaluation, all organs were sampled in 4% phosphate buffered formaldehyde (Klinipath, ref: 4078.9020) and embedded in paraffin. HCC-lesions and non-HCC-tissue were separately collected and snap frozen in liquid nitrogen. Haematoxilin-eosin staining (H&E) was performed to evaluate the morphological changes inflicted by the DEN-treatment. Sirius Red staining was carried out to score the fibrotic stage of the liver. Reticulin staining was performed to help identifying HCC-nodules. All stainings were done using standard histology protocols and evaluated by an experienced pathologist.

1.3.4 Immunohistochemistry

Immunohistochemical stainings (IHC) were used to quantify protein expression levels inside HCC-nodules, tissue surrounding HCC-nodules and non-HCC tissue. As a marker for angiogenesis specific monoclonal antibodies were used against vascular endothelial growth factor (VEGF) (Santa Cruz biotechnology, ref sc-152), CD105 (R&D systems, ref AF1320) and Tie2 (BD Bioscience, ref 557039). Tumour hypoxia was evaluated by staining for hypoxia inducible factor 1 alpha (HIF1α) (Santa Cruz ref sc-53546) and macrophages were visualised using F4/80-staining (AbD serotec, ref MCA497G). A negative marker for HCC, Fatty Acid Binding Protein (FABP, Hycult biotechnology, ref HP8010) was used [15]. Stainings were performed as previously described [15-17] and were semi-quantitatively measured by Olympus Cell^D software. Intercapillary distance (ICD) was used as a marker for microvessel density, by measuring the average distance between vessels in HCC-nodules on CD105-stained slides.

1.3.5   Medical imaging

Additional *in vivo* tests were performed using microPET imaging as non-invasive technology. The latter acquisitions were performed using a GE FLEX Triumph micro-PET/SPECT/CT scanner (Gamma Medica-Ideas). This state-of-the-art scanner consists of a micro-PET module (LabPET8) with $2 \times 2 \times 10$ mm^3 LYSO/LGSO scintillators in an 8-pixel, quad-APD detector module arrangement. This system is capable of delivering 1 mm spatial resolution in rodents at a sensitivity of 4%, thereby covering a field-of-view of 10 cm transaxially by 8 cm axially, while the CT part can scan structures down to 10-15 µm. The micro-CT part consists of a high-resolution micro-CT tube with a focal spot size switchable between 10 or 50 µm, combined with a flat-panel CsI detector.

Animals were injected with 18,5 MBq of [18F]-fluoromethylcholine ([18F]FMCH) (Laboratory of Radiopharmacy, Ghent, Belgium) [18-19] immediately prior to their microPET scan on the camera bed at the start of a dynamic acquisition. Frames of 15x20sec and 5x5min were accordingly sequentially recorded. For anatomical localisation, a microCT-scan was sequentially acquired using 256 projections over 360 degrees at 70 kVp/180 µA and 1,3 magnification with a spot size of 50 µm. This results in a scanning time below 2 minutes, whilst keeping the radiation dose at 20 – 25 mGy. This radiation dose is sufficiently low enough to allow for follow-up studies. The resulting PET data were reconstructed using 30 iterations of the Maximum Likelihood Expectation Maximisation algorithm in 160x160x63 images of 0,5 x 0,5 x 1,175 mm voxel size and no *posteriori* 3D filtering was applied. CT-reconstruction was straightforward analytical. All images were fused and analysed with VIVID (Amira ®, San diego, USA).

Vascular corrosion casts (VCC) were obtained by perfusing Batson n°17 (Polysciences ref: 07349) through the aorta (arterial casts) or vena ileocolica (venous cast); and dissolving soft tissue in KOH. The VCC were then scanned in the micro-CT scanner, after which the reconstructed dataset was segmented before generating 3D surface models. In order to assess the VCC with scanning electron microscopy (SEM), the relevant parts were cut off and coated with platinum.

1.3.6 Protein expression

Alterations in protein levels of angiogenic factors in HCC-tissue were measured by ELISA [20]. Placental growth factor (PlGF) (Mouse PlGF-2 Quantikine ELISA Kit, R&D Biosystems) and VEGF (Mouse VEGF Quantikine ELISA Kit, R&D Systems) were measured in liver tissue and in serum. Protein levels of soluble VEGF receptor 1 (sVEGFR1) (Mouse sVEGF R1/Flt-1 Quantikine ELISA Kit R&D Biosystems) were measured in serum. To normalise the total protein concentration in the samples, a normalisation factor was determined by a Biorad RC/DC Protein Assay kit.

1.3.7 Statistics

Data were statistically analysed with SPSS16. Datasets were tested for normality using the Kolmogorov-Smirnov test before further analysis. Parametric data were subjected to a student's t-test to evaluate the difference between the DEN and control group. Data that did not show a normal distribution were tested with the non-parametric Mann-Whitney-U test. A p value of <0.05 was considered statistically significant. Correlations were calculated using the Pearson correlation test.

1.4 Results

1.4.1 Macroscopic evaluation

Significant lower weight (p<0,001) was observed between adult DEN-mice (25,26 g (± 0,49)) and control mice (31,28 g (± 0,91)) at 25W. Macroscopic evaluation of the liver revealed tumours at 20W (2,6 (±2,66) tumours/liver), 25W (6,81 (± 1,69) tumours/liver) and 30W (12,9 (± 2,17) tumours/liver) (figure 1).
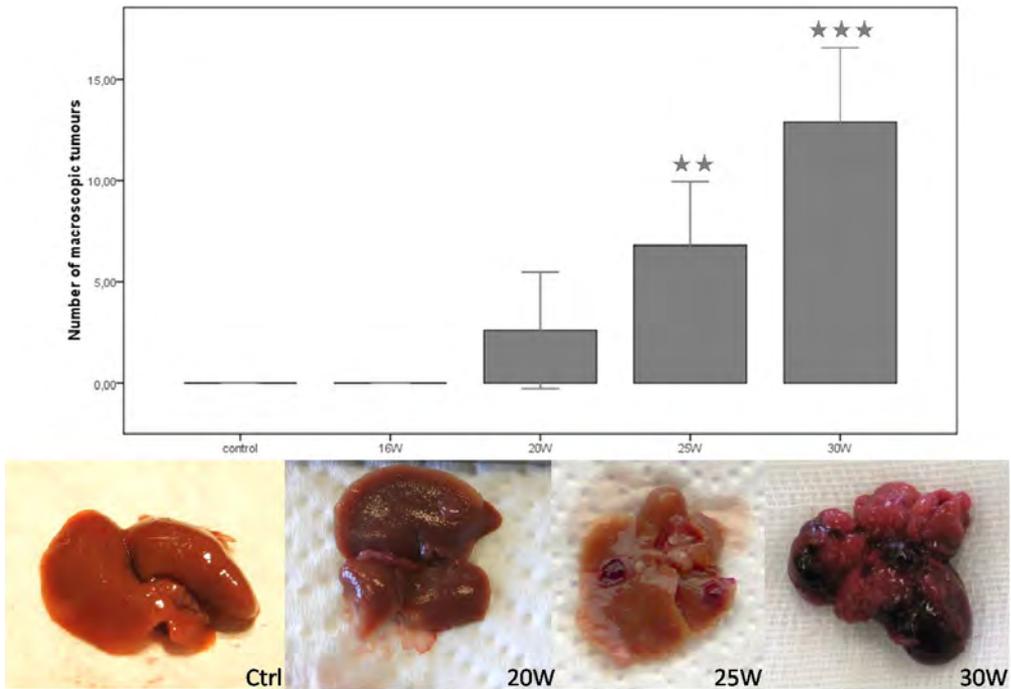
Fig 1: macroscopic HCC-lesions at different time points throughout the experiment. Asterisks (★) represent the significant P-values of the control group compared to DEN-groups (★★ = p<0,01 and ★★★ = p < 0,001).

1.5   Microscopic evaluation

1.5.1   HCC progression

H&E staining showed dysplastic changes at 20W, 25W and 30W. Small cell dysplasia was frequently found throughout the liver and readily distinguishable nodules of neoplasia were seen at 25W and 30W (figure 2). These nodules were confirmed as HCC using reticulin staining and HE (and immunohistochemistry for FABP). HCC nodules were identified and measured on reticulin and H&E stained histological slides. Tumour burden (size x number) increased significantly during DEN-treatment (figure 2). Sirius red staining demonstrated the fibrotic action of DEN, with time-dependent increase of Metavir-score (figure 3), while controls did not show any fibrotic septa.
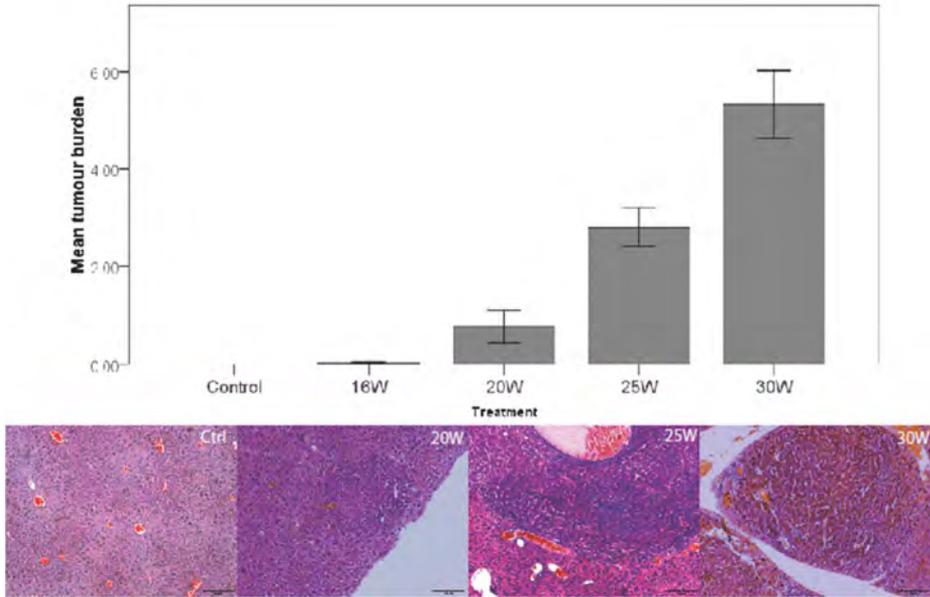
Figure 2: *Mean HCC burden at different time points and representative pictures of H&E stained HCC-nodules*
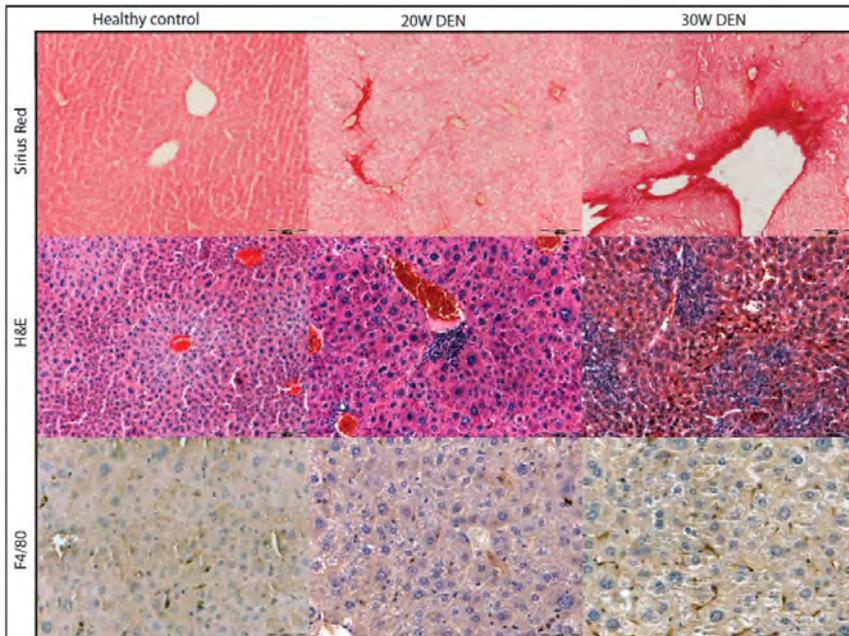

Figure 3: *representative pictures of fibrosis in non–tumorous tissue on sirius red staining (top), inflammatory foci on H&E staining (middle) and macrophages on F4/80 immunohistochemistry (bottom)*

A time-dependent increase in FABP-negative spots was seen throughout the experiment (figure 4). While parts of the liver were FABP-negative, some hepatocytes had an increased FABP-expression in the DEN-treated groups whereas control livers showed a homogenous expression (figure 4). FABP (p<0,001) was significantly down regulated in all the DEN-treated groups compared to the control group (figure 4).
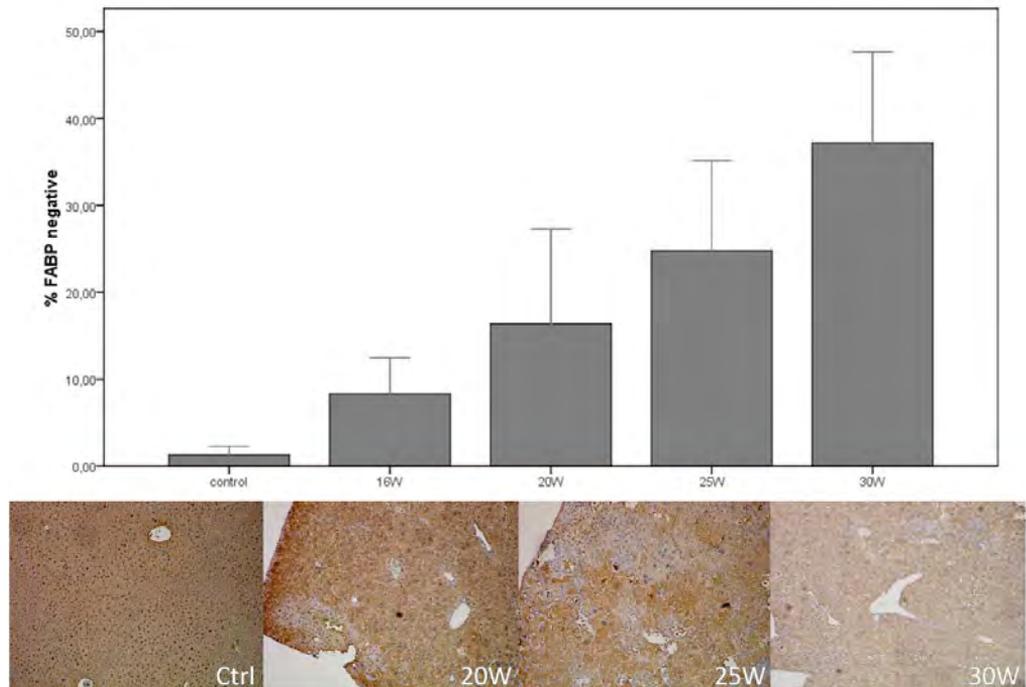

Figure 4: *percentage of FABP-negative sites and representative pictures of the IHC staining. Asterisks (★) represent the significant p-value of the control group compared to DEN-groups (★ ★ ★ = p < 0,001).*

1.5.2   Inflammation and fibrosis

The number and size of inflammatory foci significantly increases after 20W (p<0,001 and p<0,05), 25W (p<0,001 and p<0,05) and 30W DEN (p<0,001 and p<0,05) compared to control livers (figure 3 and 5). This was accompanied by an increased abundance of Kuppfer cells in and around HCC, as well as in non-HCC tissue (figure 3). Compared to non-HCC tissue, the expression of F4/80 was

significantly upregulated after 16W (p<0,01), 20W (p<0,05), 25W (p<0,001) and 30W (p<0,001) in HCC, and in the surrounding matrix of HCC-lesions (p<0,001) (figure 5).
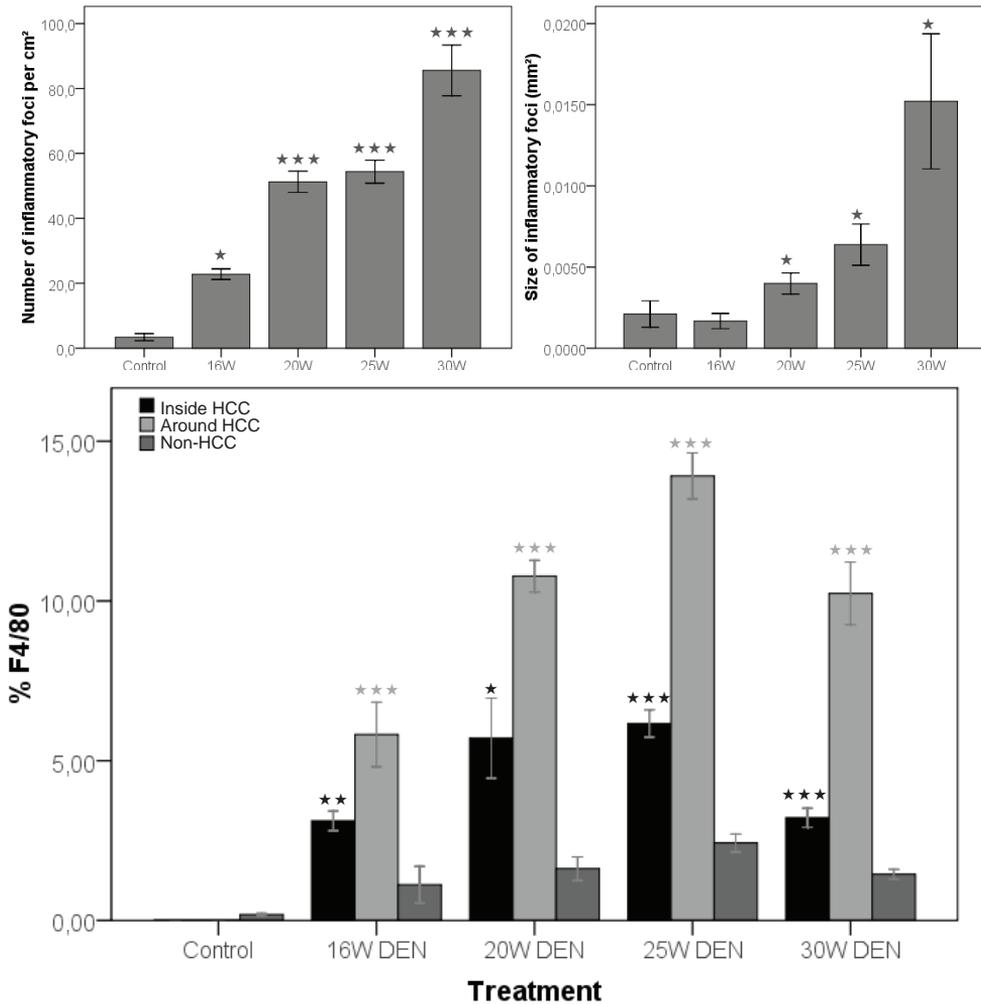

Figure 5: Top: mean number (left) and size (right) of inflammatory foci at different time points. Bottom: expression of F4/80 on immunohistochemistry. Asterisks (★) represent the significant P-value of the difference between expression inside and around HCC-nodules compared to non-tumour tissue in DEN-treated livers (★ = p < 0,05, ★★ = p < 0,01, ★★★ = p < 0,001).

1.5.3 Angiogenesis

VEGF-expression was more prominent inside HCC-nodules (figure 6 and 7) than in the surrounding matrix, yet both were significantly increased (p<0,01) after 20W, 25W and 30W DEN compared to adjacant non-HCC tissue. VEGF-levels were significantly increased inside HCC-nodules compared to controls after 25W (p<0,05) and 30W DEN (p<0,05) (table 1). The expression of VEGF was correlated (r=0,68, p<0,001) with HIF1-α levels. HIF1-α levels were significantly upregulated in and around tumour tissue at 20W (p<0,001), 25W (in: p<0,001; around: p<0,05) and 30W DEN (p<0,001) compared to adjacant non-HCC tissue (figure 6 and 7). Non-HCC tissue was hypoxic after 16W (p<0,01), 25W (p<0,01) and 30W (p<0,001) of DEN compared to healthy controls (table 1). The increased HIF1-α expression was more prominent inside HCC-nodules than in surrounding matrix (figure 6), the latter being characterised by a major increase in vascularisation after 20W, 25W and 30W of DEN compared to non-tumour tissue (p<0,001). CD105 was also increased inside HCC-lesions after 20W (p<0,05), 25W (p<0,001) and 30W (p<0,01) (figure 6 and 7), implicating an increased intratumoural neovascularisation, confirmed by ICD (figure 8). The ICD decreased at 20W and 25W, verifying neovascularisation; an increase was seen at 30W (figure 8). Differences of ICD were statistically significant between all groups (p<0,001). DEN-induced HCC-lesions showed an increased expression of Tie2 which was higher in HCC-surrounding tissue than inside HCC-nodules (figure 6). Tie2 was significantly up-regulated around HCC after 20W (p<0,01), 25W (p<0,001) and 30W (p<0,01) compared to non-HCC tissue, and inside HCC after 25W (p<0,001) and 30W DEN (p<0,01 (figure 7, table 1). TIE2-expression was correlated (r = 0,58, p<0,001) with macrophage recruitment (F4/80-staining).

	Inside tumour											
	16W			20W			25W			30W		
	Av (%)	SEM	p	Av (%)	SEM	p	Av (%)	SEM	p	Av (%)	SEM	p
TIE2	0,75	0,68	NS	8,81	2,52	NS	13,24	1,53	<0,01	9,17	1,84	NS
VEGF	6,74	2,09	<0,05	16,91	3,28	<0,001	27,72	3,42	<0,001	33,50	8,16	<0,01
HIF1-a	3,79	0,57	<0,001	5,49	0,48	<0,001	7,63	0,37	<0,001	11,47	0,50	<0,001
CD105	6,29	2,88	NS	10,40	1,70	<0,001	15,08	1,44	<0,001	12,57	1,75	<0,001
F4/80	3,12	0,30	<0,001	5,70	1,25	<0,001	6,16	0,46	<0,001	3,21	0,30	<0,001
	Surrounding tumour											
	16W			20W			25W			30W		
	Av (%)	SEM	p	Av (%)	SEM	p	Av (%)	SEM	p	Av (%)	SEM	p
TIE2	0,36	0,68	NS	12,35	2,37	<0,05	17,63	2,52	<0,01	13,30	2,89	<0,05
VEGF	8,06	2,09	<0,01	10,35	1,92	<0,001	26,56	4,23	<0,001	30,01	7,39	<0,001
HIF1-a	1,51	0,70	<0,01	1,43	0,17	<0,001	1,67	0,19	<0,001	3,20	0,17	<0,001
CD105	5,17	2,49	NS	13,68	0,88	<0,001	17,14	1,77	<0,001	21,94	3,24	<0,001
F4/80	5,82	0,37	<0,001	10,78	0,50	<0,001	13,92	0,72	<0,001	10,24	0,98	<0,001
	Non-tumour											
	16W			20W			25W			30W		
	Av (%)	SEM	p	Av (%)	SEM	p	Av (%)	SEM	p	Av (%)	SEM	p
TIE2	4,77	1,75	NS	4,33	1,36	NS	3,90	0,62	NS	2,95	0,83	NS
VEGF	4,50	0,88	NS	2,80	0,73	NS	4,19	0,85	NS	8,72	1,60	<0,01
HIF1-a	1,04	0,16	<0,01	0,38	0,12	NS	0,98	0,19	<0,01	2,53	0,08	<0,001
CD105	3,74	0,68	NS	6,06	0,74	<0,05	5,36	0,48	<0,001	4,40	0,31	<0,05
F4/80	0,87	0,27	NS	1,62	0,37	<0,05	2,43	0,28	<0,01	1,44	0,16	<0,01

Table 1: *Summary of % IHC-positive staining of TIE2, VEGF, HIF1-a, CD105 and F4/80 in tumour, peritumoural and non-tumour tissue of mice injected with DEN for 16W, 20W and 25W.*
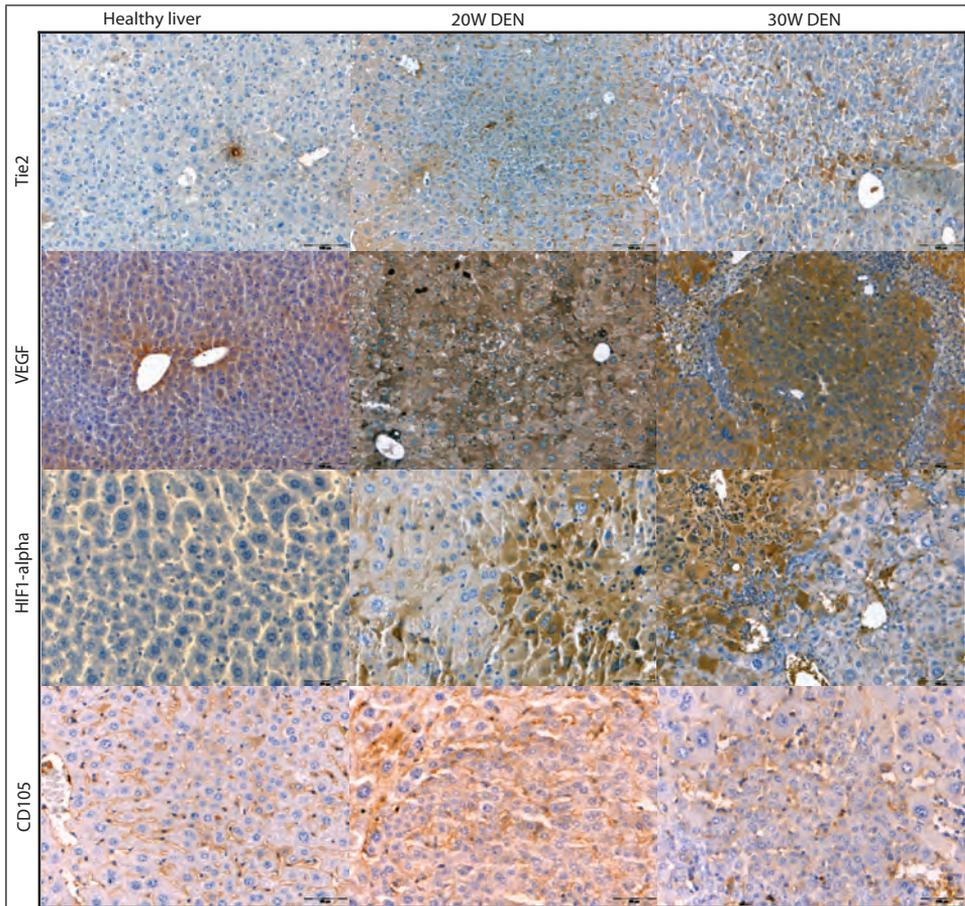
Figure 6: *Representative pictures of immunohistochemical stainings for Tie2, VEGF, HIF1α and CD105 in healthy liver, 20W DEN (tumour tissue) and 30W DEN (tumour tissue).*
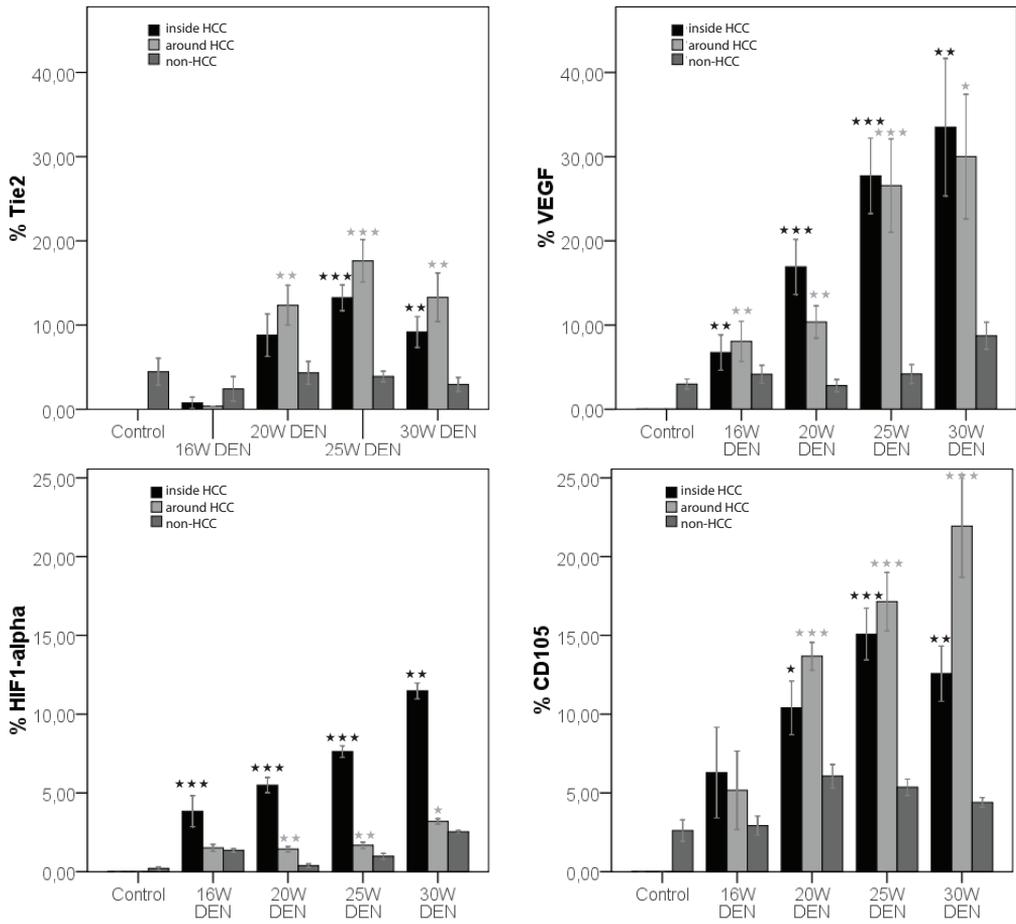
Figure 7: Top: Tie2 expression (left) and VEGF expression (right). Bottom: HIF1-α (left) and CD105 (right). Asterisks (★) represent the significant P-value of the difference between expression inside and around HCC-nodules compared to non-tumour tissue in DEN-treated livers (★ = p < 0,05, ★★ = p < 0,01, ★★★ = p < 0,001).
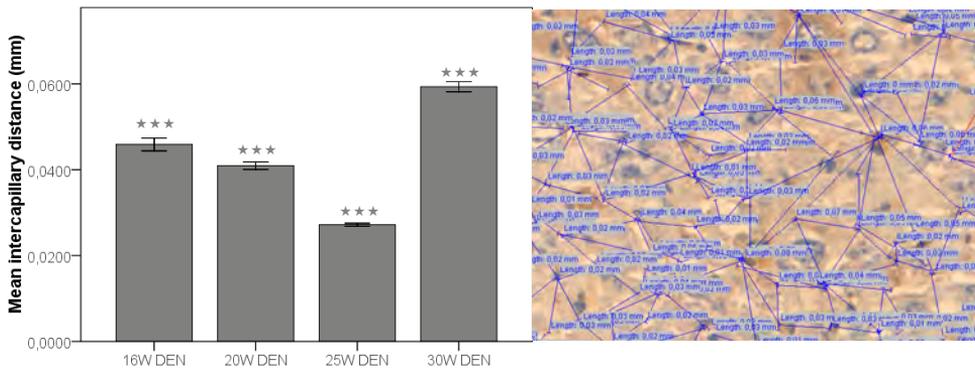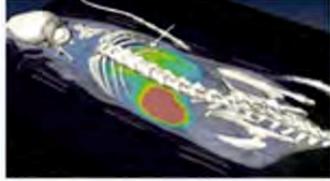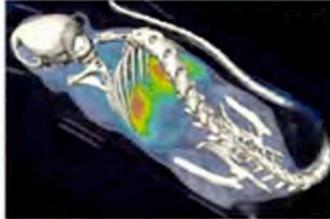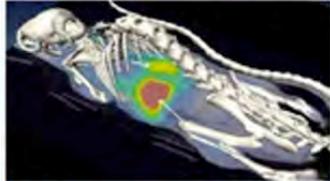
Figure 8: *Left: mean intercapillary distances at different time points, ★ ★ ★ = p < 0,001. Right: measurement of ICD.*

1.5.4   Medical imaging

Preliminary results, illustrated by Figure 9 suggest that these tumours could be visualised by [18F]FMCH PET imaging; and that this technique is useful for follow up studies. A short follow study (25W – 35W) was conducted on mice. An increased uptake was seen at the sequential measurements, which correlates with their increased tumour burden. However, further confirmation in larger groups is needed.

The combination of VCC and CT-scanning revealed the chaotic pattern and dishierarchical organisation of tumour vessels (figure 10). The SEM-images provide further insight in the microvasculature (figure 10), showing the tumour vessel abnormality and visualising two mechanisms of neovascularisation, pruning and intussusceptive angiogenesis (figure 11).

| Macroscopic evaluation	PET/CT image
Animal 1: 2 tumour located close together on the left liver lobe and 1 tumour close to the gallbladder, just above the right kidney	
Animal 2: 12 small HCC-lesions symmetrically spread over both liver lobes	
Animal 3: large tumour located on the left liver lobe and 3-4 smaller lesions on the right liver lobe	
Animal 4: no macroscopic lesions	
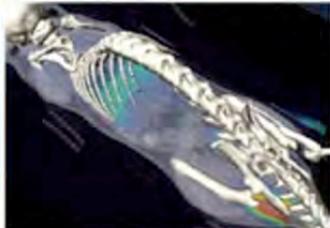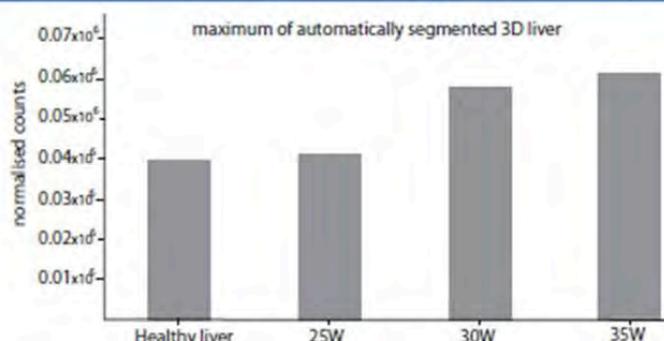
Figure 9: Top: PET-images of mice at 30 weeks, static reconstruction of a 30 min acquisition. Bottom: normalised counts of F[18]MCH uptake during a follow-up study (n=1).
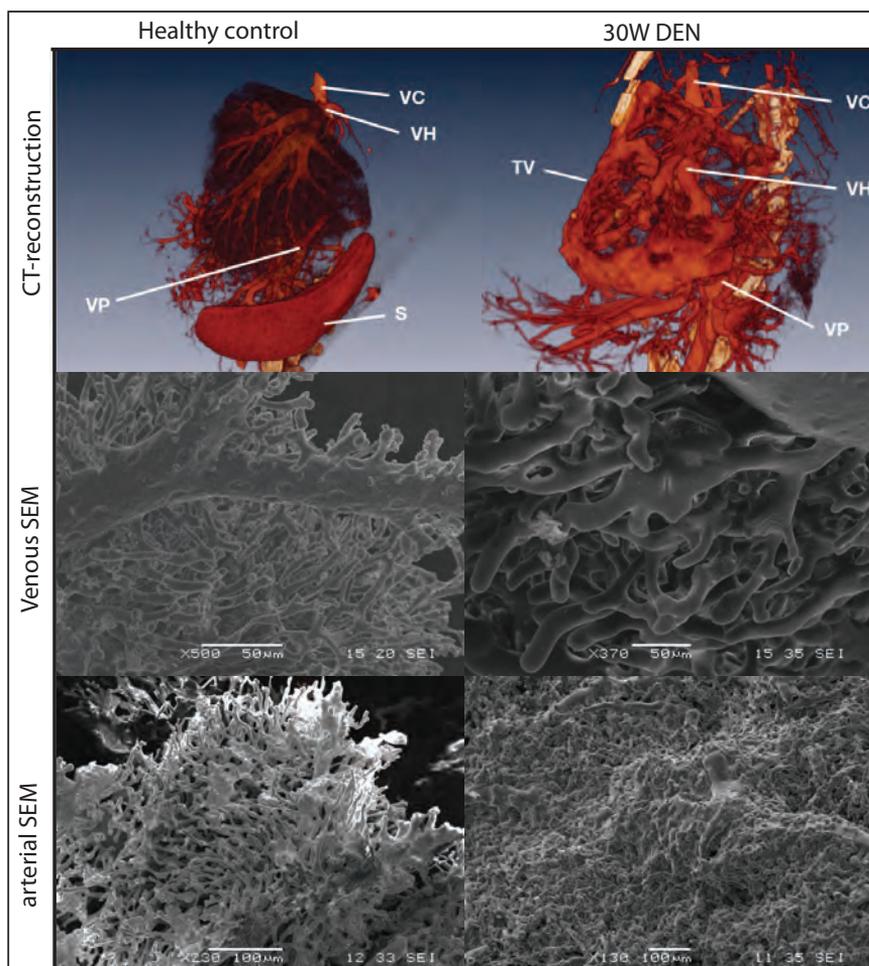
Figure 10: *Top: 3D reconstructions of vascular corrosion castings. Middle: SEM-images of venous corrosion castings. Bottom: SEM-images of arterial corrosion castings.*
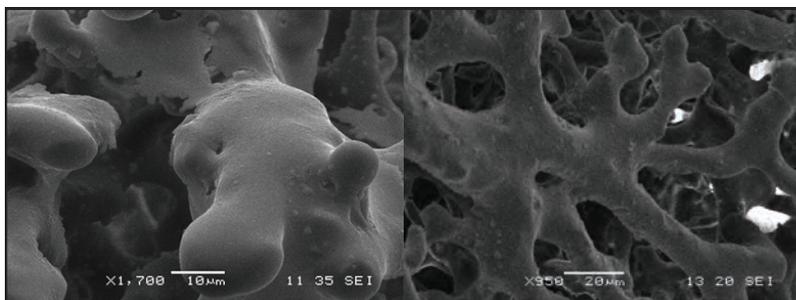

Figure 11: *Left: sprouting angiogenesis Right: intussusceptive angiogenesis*

1.5.5 Protein expression

PlGF and VEGF are important factors involved in tumour angiogenesis and were significantly upregulated in liver tissue at 16W (respectively $p<0,05$ and $p<0,001$), 20W ($p<0,05$ and $p<0,01$) and tumour tissue samples at 25W DEN ($p<0,05$ and $p<0,01$). The up-regulation of PlGF was more prominent in tumour tissue at 30W with an average of 422,28 pg/mg (±76,99) PlGF compared to the 2,90 pg/mg (±0,77) PlGF detected in the control group ($p<0,001$) (figure 12).

In serum, the PlGF protein levels were significantly upregulated at 16W ($p<0,05$), 25W ($p<0,05$) and 30W ($p<0,01$) with similar results as seen in liver tissue (figure 12). VEGF levels were upregulated at 25W ($p<0,05$) and 30W ($p<0,05$). There was a slight increase of sVEGFR1 at 30W DEN treatment, but no significant difference was seen between controls and 25W DEN (figure 12).
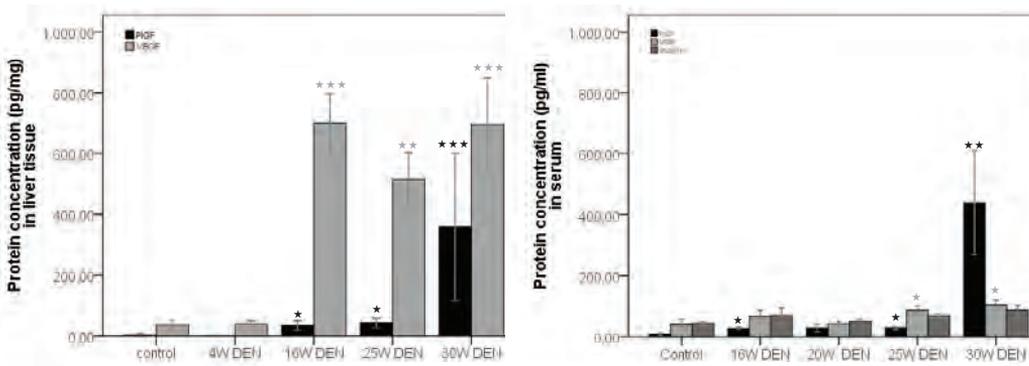

Figure 12: Left: protein concentrations in liver tissue, determined by ELISA. Right: protein concentrations in serum, determined by ELISA. Asterisks (★) represent the significant P-value of the difference between DEN-treated groups and controls. (★ = p < 0,05, ★★ = p < 0,01, ★★★ = p < 0,001).

1.6   Discussion

Animal models can provide essential knowledge about the pathogenesis of hepatocellular carcinoma, particularly when they mimic the environment in which human tumours develop. N-nitrosodiethylamine has been shown to induce tumours, which are molecularly similar to human HCC with poor prognosis [9]. Yet, the major drawback is the long time needed for tumour progression [21-26]. The macroscopic and microscopic evaluation of tumours, PET-CT images and histopathological analysis confirmed the presence of HCC after 25 weeks in our mouse model. While most DEN-induced models take at least 1 year to develop tumours, weekly intraperitoneal injections with 35 mg/kg DEN give rise to relatively fast tumour occurrence in a background of fibrosis. Since HCC is known to be a hypervascularised tumour, and recent therapies have focused on inhibiting angiogenesis, we have studied the time-dependent changes in angiogenic and inflammatory factors in this mouse model.

Previous studies with several DEN-induced rat and mice models, have shown that the expression level of FABP is decreased in DEN-induced liver lesions [15, 27]. L-FABP is induced in mitotic hepatocytes and studies with transfected hepatoma cell lines have shown that a high expression of L-FABP stimulates DNA-synthesis. However, L-FABP also binds growth inhibitory prostaglandins with high affinity and the inhibitory potency of these substances correlated with their binding affinity to L-FABP. Previous data thus seem contradictory, with an elevated expfression of L-FABP in AAF-induced early HCC and decreased L-FABP levels at later stages of liver carcinogenesis, including hepatocellular carcinomas. However, in a DEN-model it has been shown that that L-FABP-negative foci have a larger growth potential compared to other foci in DEN-treated rats [15].

Consequently, the FABP-expression was measured as a parameter for HCC-development. Even after a relatively short period of DEN administration (16W) significant decrease in FABP-expression is seen compared to controls. After 25W DEN large FABP-negative lesions appear throughout the liver, alternating with small FAPB-positive regions, while control tissue showed a homogenous expression. However, keeping in mind the previous contradictory results on L-

FABP as a marker for HCC, more studies are needed to confirm the use of FABP as a negative marker for HCC.

DEN-injections also gave rise to increased inflammation, confirmed by H&E staining, as well as F4/80, which is a marker for Kuppfer cells. Activation of Kupffer cells leads to the release of cytokines, reactive oxygen species, and platelet activating factors, which are involved in the angiogenic switch. Furthermore, the chronic inflammation causes hepatocyte damage, leading to fibrosis as seen on the sirius red staining. In our study we showed that Tie2 is upregulated in the fibrotic matrix surrounding the HCC-lesions, which was also characterised by increased macrophage infiltration, confirming Tie2's correlation with hepatic inflammation. In addition, Tie2 is a receptor for angiopoetins, which play an important role in angiogenesis. Furthermore, the presence of fibrosis creates an increased intrahepatic vascular resistance and impairs oxygen diffusion, resulting in hypoxia, which is the onset for the angiogenic switch. This is supported by our findings HIF1-α and VEGF levels were increased in the fibrotic non-HCC tissue after DEN-treatment. The increased expression of HIF1α inside tumour nodules, confirms the vast oxygen need of the malignant hepatocytes. To fulfil that requirement, tumour cells start expressing angiogenic factors, such as VEGF and PlGF, inducing the angiogenic switch.

Up till now, VEGF was one of the key targets in angiogenesis research, but recently PlGF gained interest because its association with pathological conditions. PlGF levels are known to be elevated in a variety of cancers [28-33] and is associated with poor prognosis in HCC [34]. The upregulation of PlGF in this DEN-model supports the theory that PlGF plays an essential role in the angiogenesis of HCC. Protein levels of sVEGFR1, an important inhibitor for VEGF and PlGF, did not significantly alter, suggesting that the increase of angiogenic factors was physiologically relevant. Thus, the increase of angiogenic factors (VEGF, PlGF and Tie2) resulted in an increased neovascularisation. The IHC-results of the CD105-staining revealed a low CD105-expression in the normal endothelial cells of the liver and was upregulated in the malignant liver lesions at 20W, consistent with previous studies [17]. The intercapillary distances significantely decreased after 25W DEN, verifying neovascularisation; although an increase was seen at 30W supporting the theory that malignant hepatocytes tend to outgrow their vascular supply and become accostumed to hypoxia. This

upregulation of CD105 was more prominent around HCC-lesions, suggesting that the newly formed vessels form a circumferential mantle around the tumours. This was confirmed by the CT-reconstructions of the vascular casts. SEM-images demonstrate the presence of neo-angiogenesis, by visualising budding and intussusceptive angiogenesis. Furthermore, the images illustrate the overall microvascular abnormality of DEN-treated livers. Thus, the combination of casting and micro-CT imaging provides unique data on the hepatic circulation and neovascularisation. It allows to digitally visualise the complex architecture of the liver blood vessels and to provide high-resolution data for qualitative morphological analysis.

During this study we have established a new mouse model for HCC, which is considerably faster than current chemically induced models and has the advantage of tumour progression occurring in a background of inflammation and fibrosis. Furthermore, angiogenic factors were assessed at different time points, to provide important information about the kinetic changes of angiogenic factors during HCC progression. Moreover, several innovative imaging techniques were applied, not only to assess tumour growth, but also to provide further insight in microvascular alterations HCC livers.

1.7   Acknowledgments

1.8   References

1. Egeblad M, Ewald AJ, Askautrud HA, Truitt ML, Welm BE, Bainbridge E, Peeters G, Krummel MF, Werb Z: **Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy.** *Dis Model Mech* 2008, **1:**155-167; discussion 165.
2. Bissell MJ, Radisky D: **Putting tumours in context.** *Nature Reviews Cancer* 2001, **1:**46-54.
3. Gopinathan A, Tuveson DA: **The use of GEM models for experimental cancer therapeutics.** *Dis Model Mech* 2008, **1:**83-86.
4. Richmond A, Su Y: **Mouse xenograft models vs GEM models for human cancer therapeutics.** *Dis Model Mech* 2008, **1:**78-82.
5. Talmadge JE, Singh RK, Fidler IJ, Raz A: **Murine models to evaluate novel and conventional therapeutic strategies for cancer.** *Am J Pathol* 2007, **170:**793-804.
6. Kerbel RS: **Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: better than commonly perceived-but they can be improved.** *Cancer Biol Ther* 2003, **2:**S134-139.
7. Swenberg JA, Hoel DG, Magee PN: **Mechanistic and statistical insight into the large carcinogenesis bioassays on N-nitrosodiethylamine and N-nitrosodimethylamine.** *Cancer Res* 1991, **51:**6409-6414.
8. Everhart JE, Ruhl CE: **Burden of digestive diseases in the United States Part III: Liver, biliary tract, and pancreas.** *Gastroenterology* 2009, **136:**1134-1144.
9. Lee JS, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, Thorgeirsson SS: **Application of comparative functional genomics to identify best-fit mouse models to study human cancer.** *Nat Genet* 2004, **36:**1306-1311.
10. Amarapurkar AD, Vibhav, Kim V: **Angiogenesis in liver cirrhosis and hepatocellular carcinoma.** *Indian J Pathol Microbiol* 2008, **51:**323-328.
11. Pang RW, Joh JW, Johnson PJ, Monden M, Pawlik TM, Poon RT: **Biology of hepatocellular carcinoma.** *Annals of Surgical Oncology* 2008, **15:**962-971.
12. Finn RS, Zhu AX: **Targeting angiogenesis in hepatocellular carcinoma: focus on VEGF and bevacizumab.** *Expert Rev Anticancer Ther* 2009, **9:**503-509.
13. Pang R, Poon RT: **Angiogenesis and antiangiogenic therapy in hepatocellular carcinoma.** *Cancer Lett* 2006, **242:**151-167.
14. Ribatti D, Vacca A, Nico B, Sansonno D, Dammacco F: **Angiogenesis and anti-angiogenesis in hepatocellular carcinoma.** *Cancer Treat Rev* 2006, **32:**437-444.
15. Thullberg M, Grasl-Kraupp B, Hogberg J, Garberg P: **Changes in liver fatty acid-binding protein in rat enzyme-altered foci.** *Cancer Lett* 1998, **128:**1-10.
16. Vanheule E, Geerts AM, Van Huysse J, Schelfhout D, Praet M, Van Vlierberghe H, De Vos M, Colle I: **An intravital microscopic study of the hepatic microcirculation in cirrhotic mice models: relationship between fibrosis and angiogenesis.** *Int J Exp Pathol* 2008, **89:**419-432.
17. Geerts AM, De Vriese AS, Vanheule E, Van Vlierberghe H, Mortier S, Cheung KJ, Demetter P, Lameire N, De Vos M, Colle I: **Increased angiogenesis and**

permeability in the mesenteric microvasculature of rats with cirrhosis and portal hypertension: an in vivo study. *Liver Int* 2006, **26:**889-898.

18. Beheshti M, Vali R, Waldenberger P, Fitz F, Nader M, Hammer J, Loidl W, Pirich C, Fogelman I, Langsteger W: **The Use of F-18 Choline PET in the Assessment of Bone Metastases in Prostate Cancer: Correlation with Morphological Changes on CT.** *Mol Imaging Biol* 2009, **6:** 446-454.

19. DeGrado TR, Baldwin SW, Wang S, Orr MD, Liao RP, Friedman HS, Reiman R, Price DT, Coleman RE: **Synthesis and evaluation of (18)F-labeled choline analogs as oncologic PET tracers.** *J Nucl Med* 2001, **42:**1805-1814.

20. Soininen H, Kosunen O, Helisalmi S, Mannermaa A, Paljarvi L, Talasniemi S, Ryynanen M, Riekkinen P, Sr.: **A severe loss of choline acetyltransferase in the frontal cortex of Alzheimer patients carrying apolipoprotein epsilon 4 allele.** *Neurosci Lett* 1995, **187:**79-82.

21. Chen B, Liu L, Castonguay A, Maronpot RR, Anderson MW, You M: **Dose-Dependent Ras Mutation Spectra in N-Nitrosodiethylamine Induced Mouse-Liver Tumors and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Induced Mouse Lung-Tumors.** *Carcinogenesis* 1993, **14:**1603-1608.

22. Teoh NC, Dan YY, Swisshelm K, Lehman S, Wright JH, Haque J, Gu Y, Fausto N: **Defective DNA strand break repair causes chromosomal instability and accelerates liver carcinogenesis in mice.** *Hepatology* 2008, **47:**2078-2088.

23. Frey S, Buchmann A, Bursch W, Schulte-Hermann R, Schwarz M: **Suppression of apoptosis in C3H mouse liver tumors by activated Ha-ras oncogene.** *Carcinogenesis* 2000, **21:**161-166.

24. Zimmers TA, Jin XL, Gutierrez JC, Acosta C, McKillop IH, Pierce RH, Koniaris LG: **Effect of in vivo loss of GDF-15 on hepatocellular carcinogenesis.** *Journal of Cancer Research and Clinical Oncology* 2008, **134:**753-759.

25. Shiota G, Harada K, Ishida M, Tomie Y, Okubo M, Katayama S, Ito H, Kawasaki H: **Inhibition of hepatocellular carcinoma by glycyrrhizin in diethylnitrosamine-treated mice.** *Carcinogenesis* 1999, **20:**59-63.

26. Hacker HJ, Mtiro H, Bannasch P, Vesselinovitch SD: **Histochemical Profile of Mouse Hepatocellular Adenomas and Carcinomas Induced by a Single Dose of Diethylnitrosamine.** *Cancer Research* 1991, **51:**1952-1958.

27. Finnberg N, Stenius U, Hogberg J: **Heterozygous p53-deficient (+/-) mice develop fewer p53-negative preoplastic focal liver lesions in response to treatment with diethylnitrosamine than do wild-type (+/+) mice.** *Cancer Lett* 2004, **207:**149-155.

28. Matsumoto K, Suzuki K, Koike H, Okamura K, Tsuchiya K, Uchida T, Takezawa Y, Kobayashi M, Yamanaka H: **Prognostic significance of plasma placental growth factor levels in renal cell cancer: an association with clinical characteristics and vascular endothelial growth factor levels.** *Anticancer Res* 2003, **23:**4953-4958.

29. Lacal PM, Failla CM, Pagani E, Odorisio T, Schietroma C, Falcinelli S, Zambruno G, D'Atri S: **Human melanoma cells secrete and respond to placenta growth factor and vascular endothelial growth factor.** *Journal of Investigative Dermatology* 2000, **115:**1000-1007.

30.   Chen CN, Hsieh FJ, Cheng YM, Cheng WF, Su YN, Chang KJ, Lee PH: **The significance of placenta growth factor in angiogenesis and clinical outcome of human gastric cancer.** *Cancer Lett* 2004, **213:**73-82.

31.   Donnini S, Machein MR, Plate KH, Weich HA: **Expression and localization of placenta growth factor and PlGF receptors in human meningiomas.** *J Pathol* 1999, **189:**66-71.

32.   Zhang L, Chen J, Ke Y, Mansel RE, Jiang WG: **Expression of Placenta growth factor (PlGF) in non-small cell lung cancer (NSCLC) and the clinical and prognostic significance.** *World J Surg Oncol* 2005, **3:**68.

33.   Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, et al: **Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions.** *Nat Med* 2001, **7:**575-583.

34.   Ho MC, Chen CN, Lee H, Hsieh FJ, Shun CT, Chang CL, Lai YT, Lee PH: **Placenta growth factor not vascular endothelial growth factor A or C can predict the early recurrence after radical resection of hepatocellular carcinoma.** *Cancer Lett* 2007, **250:**237-249.

2 Pharmacological PlGF inhibition phenocopies PlGF deficiency

Phamacological PlGF inhibition phenocopies PlGF deficiency in cancer and ocular neovascularisation.

S. Van de Veire, I. Stalmans, F. Heindryckx, H. Oura, A. Tijeras-Raballand, T. Schmidt, S. Loges, I Albrecht, B. Jonckx, S. Vinckier, C. Van Steenkiste, S. Tugues, C. Rolny, M. De Mol, D. Dettori, P. Hainaud, L. Coenegrachts, JO. Contreres, T. Van Bergen, H. Cuervo, WH. Xiao, C. Le Henaff, I. Buysschaert, B. Kharabi, A. Geerts, T. Schomber, P. Bonnin, V. Lambert, J. Haustraete, S. Zacchigna, JM. Rakic, W. Jiménez, A. Noël, M. Giacca, I. Colle, JM Foidart, G. Tobelem, M. Moralez-Ruiz, J. Vilar, P. Maxwell, SA. Vinores, G. Carmeliet, M. Dewerchin, L. Claesson-Welsh, E. Dupuy, H. Van Vlierberghe, G. Christofori, M. Mazzone, M. Detmar, D. Collen & P. Carmeliet. (2010)

2.1   Introduction

Placental growth factor (PlGF) is a VEGF-homologue. Genetic studies in two independently generated PlGF$^{-/-}$ mouse lines identified a role for PlGF in ischemic, inflammatory and malignant disease (Carmeliet et al., 2001; Cheng and Sefton, 2009; Cheung et al., 2009; Fischer et al., 2008; Luttun et al., 2002a; Oura et al., 2003; Van Steenkiste et al., 2009; Yano et al., 2008; Yoo et al., 2009). PlGF induces responses in endothelial, malignant, immune, marrow progenitor and other cells (Fischer et al., 2008). PlGF binds to VEGFR1 and its co-receptor neuropilin-1 (NP1). While VEGFR1 may act as a trap for VEGF (Park et al., 1994), it also transmits signals in response to PlGF via its tyrosine kinase (TK) domains (Autiero et al., 2003; Landgren et al., 1998). The role of Flt1 in cancer remains controversial, but the majority of studies report that inhibition or silencing of VEGFR1 blocks tumour growth and metastasis. In mice expressing a signalling-dead *VEGFR1* without (TK) domains (*Flt1-TK$^{-/-}$*), tumour growth and metastasis were inhibited (Hiratsuka et al., 2001; Hiratsuka et al., 2002; Kerber et al., 2008; Murakami et al., 2008) or not affected (Dawson et al., 2009).

An increasing number of clinical studies shows that PlGF levels correlate with poor prognosis of diverse types of cancers, including hepatocellular (Ho et al., 2006), colorectal (Wei et al., 2005), renal (Matsumoto et al., 2003) and other cancer types. In some cancers, PlGF expression can be epigenetically silenced (Xu and Jain, 2007). PlGF levels are also upregulated in cancer patients treated with VEGF-inhibitors (Ebos et al., 2007; Willett et al., 2009). Elevated PlGF expression in tumour or stromal cells promotes tumour growth (Li et al., 2006; Marcellini et al., 2006).

Anti-PlGF mAbs were generated and reduced tumour growth and metastasis, in part by inhibiting angiogenesis and inflammation (Fischer et al., 2007). However, anti-PlGF mAbs, generated by Bais *et al*, were ineffective (Bais et al., 2009), begging for the question whether the anti-cancer activity of 5D11D4 could be reproduced by independently generated anti-PlGF mAbs. In addition, questions were raised about the high doses used and whether the effects of 5D11D4 were related to an off-target activity. Furthermore, Bais *et al* argued that little genetic evidence supported a role for PlGF in cancer, overall

questioning the therapeutic value of anti-PlGF strategies. In this study, we addressed these outstanding questions in a mouse model for HCC.

2.2　Materials and methods

2.2.1　DEN-Induced HCC Model

Five-week-old male WT and PlGF$^{-/-}$ mice (129/Sv) received weekly i.p. injections with DEN (35 mg/kg). Wild-type mice were treated with 5D11D4 or IgG (20 mg/kg; 2×/week).

2.2.2　Morphometry of tumour microvessel density

Stained pancreata sections were viewed on a Nikon Diaphot 300 fluorescence microscope (Nikon, Egg, Switzerland) using Openlab 3.1.7. Software (Improvision, Coventry, England). Tumor vessel density was quantified using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007) and displayed as % of stained intratumoral area to total tumor area.

2.2.3　Immunohistology and morphometry

All methods for histology and immunostaining have been described (Carmeliet et al., 1998; Fischer et al., 2007; Luttun et al., 2002b). Immunohistological stainings were performed using the following primary antibodies: rat anti-F4/80 (MCA497G, Serotec Cruz Biotechnology) and rabbit anti-VEGFR1 (sc316, Santa Cruz). Sections were then incubated with the appropriate peroxidase labeled IgGs (Dako), followed by amplification with the proper tyramide signal amplification systems (Perkin Elmer, Life Sciences) when necessary. Morphometric analyses were performed using a Zeiss Axio Imager Z1 microscope with customized macros developed in KS300 image analysis software. Neovascular growth was determined on sections as the endoglin area and vessel density as endoglin vessels per mm; inflammation was determined as F4/80^{+} area.

2.3   Results

2.3.1   Transgenic and carcinogen-induced hepatocellular carcinoma model

To exclude that germline *Pgf* deficiency induces compensatory changes that favour tumour inhibition independently of PlGF deficiency, we tested whether genetic (PlGF$^{-/-}$ mice) and pharmacologic PlGF silencing inhibited growth of hepatocellular carcinoma (HCC) models.

We used a carcinogen-induced HCC model by treating mice with diethylnitrosamine (DEN). In this model, fibrosis and dysplastic lesions appear at 16 weeks, while hypervascularised HCC tumours become visible by 25 weeks. Hepatic PlGF levels were not acutely upregulated by DEN (not shown) and remained initially low (pg/mg: 2.9 ± 0.7 in healthy vs 1.5 ± 0.4 after 4 weeks DEN; N=6; P=NS), but increased to 423 ± 77 pg/mg in end stage tumour nodules (N=6; P<0.05). After 30 weeks of DEN-treatment, 58% of WT-mice (N=24) but only 5% of PlGF$^{-/-}$ mice (N=21) succumbed (P=0.002, Log-rank) and fewer tumour nodules were present in PlGF$^{-/-}$ mice (nodules/liver: 17.9 ± 2.2 in WT vs 5.9 ± 2.4 in PlGF$^{-/-}$; N=18; P=0.002). When WT-mice with established HCC were treated with 5D11D4 (20 mg/kg; 2x/wk) from 25 weeks onwards for 5 weeks, 45% of mice receiving control IgG died while only 23% died in the 5D11D4 group (N=25-23; P<0.05). Also, 5D11D4-treated mice developed fewer nodules per liver (21.6 ± 2.9 after IgG vs 11.1 ± 2.6 after 5D11D4; N=15; P=0.02). After 10 weeks of mAb treatment, mortality was 90% in the control group, but only 41% after 5D11D4 (N=11; P<0.05).
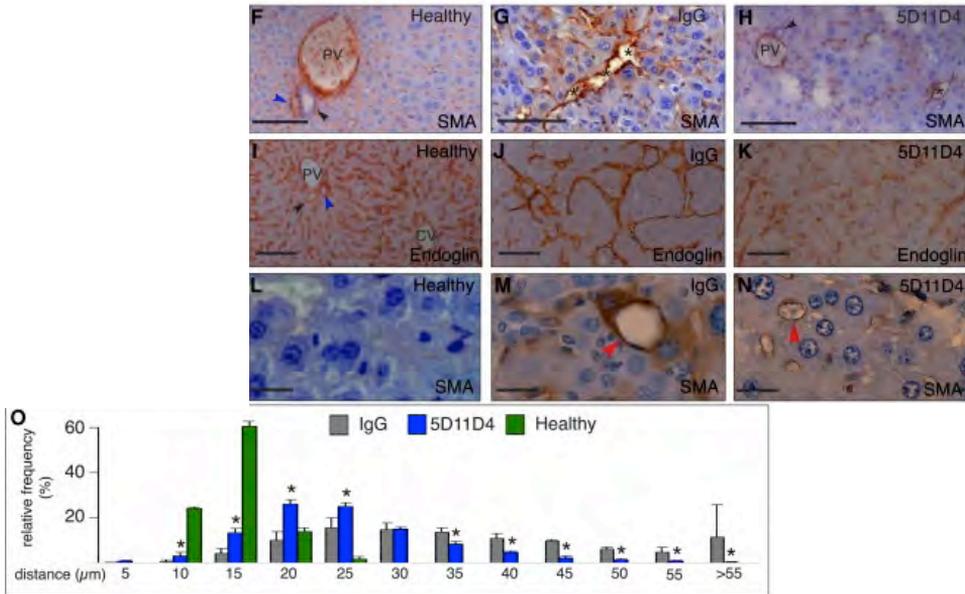
Fig. 1 *(F – H) SMA staining, revealing a portal triad with paired hepatic artery in a healthy liver (F), large, smooth muscle cell-coated unpaired arteries (asterisks) in HCC mice treated with IgG (G), and a portal triad and small unpaired artery (asterisk) in HCC liver after 5D11D4 (H). Black arrowhead (bile duct); blue arrowhead (hepatic paired artery); PV (portal vein).(I – K) Immunostaining for the endothelial marker endoglin, showing portal vein (PV) and central vein (CV) amidst a sinusoidal network in healthy liver (I); black arrowhead (bile duct); blue arrowhead (hepatic paired artery). In IgG-treated HCC nodules, the capillary network is chaotically organized, with tortuous vessels and cords, laying at large distances from each other (J), in contrast to the more regular pattern, size, and shape of capillaries and shorter intercapillary distances in 5D11D4-treated HCC (K).(L – N) Immunostaining for SMA, showing undetectable expression in HSCs around sinusoids in healthy liver (L). In HCC, tumour capillaries (red arrowhead) are surrounded by strongly SMA*-stained HSCs in IgG-treated mice (M) but not in 5D11D4-treated mice (N).(O) Histogram of intercapillary distances, showing shorter distances in 5D11D4-treated HCC; values in healthy liver are shown for comparisonBars: 100 μm (F – K); 20 μm (L – N). Data are mean ± SEM.*

We then explored the underlying mechanisms of the responsiveness or resistance of the spontaneous tumour model to PlGF blockage. In contrast to the largely portal venous blood supply in healthy livers, the hepatic arterial network expanded substantially in HCC (Fernandez et al., 2009). In the DEN-induced HCC model, 5D11D4-treated HCC-livers contained fewer and smaller

unpaired arteries, identified by their presence of smooth muscle actin (SMA)[+] smooth muscle cells (SMA[+] unpaired vessels: relative area, % and number/mm^2: 5.8 ± 1.0 and 11.3 ± 2.9 after IgG vs 1.4 ± 0.3 and 6.3 ± 0.3 after 5D11D4; N=7-3; P<0.001 and P<0.05; Figure 1).
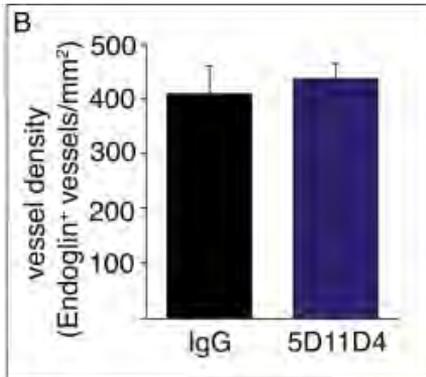

Figure 2: (B) Endothelial staining for endoglin, revealing comparable capillary density in tumour nodules of control and PlGF-blocked HCC in the DEN-induced HCC model (n = 5 – 8; p = NS).Data are mean ± SEM.

PlGF-blockage also affected angiogenesis within tumour nodules. In healthy liver, sinusoids are fenestrated, devoided of basement membrane and covered by quiescent hepatic stellate cells (HSCs), which function as pericytes but lack SMA-expression (Fernandez et al., 2009); staining for the endothelial marker endoglin and for SMA confirmed these findings (Figure 1). In HCC, sinusoids are known to loose their typical characteristics and develop into capillaries ("sinusoidal capillarisation") (Fernandez et al., 2009). HCC-capillaries had an abnormal shape and size (ranging from dilated tortuous lakes to cords without recognizable lumen), and were chaotically organized at large inter-capillary distances (Figure 1). Unlike in other tumour types, where capillaries often become devoid of mural cells, HCC vessels instead become surrounded by activated HSCs, that express increased amounts of SMA, deposit matrix, and release angiogenic and tumorigenic factors (Johnson et al., 1998); increased SMA-expression by perivascular cells was also observed in control HCC tumours (Figure 1). Such "vessel abnormalisation" increases the resistance to blood flow and oxygen delivery (Fernandez et al., 2009; Paternostro et al.), leading in turn to hypoxia which fuels HCC malignancy (Wu et al., 2007). PlGF-blockage did not

124

affect the relative tumour vessel area (Figure 2), but induced partial normalization of tumour capillaries. Indeed, in PlGF-blocked tumours, there were fewer tortuous capillaries and non-luminised cords, and the vascularisation pattern was more regular (tortuous vessels/mm^2 and cords/mm^2: 44 ± 44 and 237 ± 21 in IgG vs 22 ± 2 and 181 ± 15 in 5D11D4; N=4-5; P<0.001 and P=0.05; Figure 1); intercapillary distances were also shorter (Figure 1). These changes were functionally relevant, as PlGF-blocked HCC nodules expressed lower levels not only of HIF-2a (HIF-2a$^+$ area, % of nodular area: 28.1 ± 4.3 for IgG versus 14.6 ± 2.6 for 5D11D4; n = 4–5; p < 0.05) but also of other hypoxia-inducible genes (table 1). Further, fewer capillaries were covered by SMA$^+$ mural cells (SMA$^+$ area around capillaries, %: 5.9 ± 1.3 after IgG vs 2.2 ± 0.6 after 5D11D4; N=6-7; P=0.02; Figure 1). Overall, PlGF-blockage counteracted the highly abnormal nature of HCC capillaries. As a result, PlGF-blocked HCC nodules were less hypoxic, as suggested by the reduced expression of hypoxia-inducible genes (table 1); the weaker staining of tumour capillaries for the hypoxia-inducible endoglin further suggests a less hypoxic microenvironment. Notably, malignant hepatocyte proliferation was higher in zones with large inter-capillary distances in IgG-treated mice than in zones with short inter-capillary distances in 5D11D4-treated mice (PCNA$^+$ cells/mm^2: 90 ± 18 after IgG vs 36 ± 2 after 5D11D4; N=4; P=0.02).

Consistent with the SMA$^+$ HSC coverage around HCC capillaries, activated HSCs expressed *VEGFR1* (not shown) and responded to PlGF by increased proliferation (BrdU$^+$ cells, % of total: 3.4 ± 0.8 after control vs 20.5 ± 3.0 after PlGF; N=4; P<0.05) and migration (migrated cells: 5.3 ± 1.2 after control vs 39 ± 7.9 after PlGF; N=4; P<0.05). That activated HSCs may also stimulate (abnormal) capillary growth is suggested by findings that they produced substantial amounts of PlGF and that tumoural capillaries, but not healthy sinusoids, expressed high levels of Flt1. In line with reports that macrophage accumulation promotes HCC (Roberts et al., 2007), PlGF-blockage reduced hepatic macrophage numbers in the PlGF knock out model (Figure 1). PlGF itself was, however, not a mitogen for cultured hepatoma cells (not shown).

TARGET GENE	IgG-TREATED MICE	5D11D4 –TREATED MICE
GLUT1	0.54 ± 0.05	0.18 ± 0.05*
PHOSPHOFRUCTOKINASE	1.26 ± 0.24	0.64 ± 0.16#
PROLYL-HYDROXYLASE DOMAIN 3 (PHD3)	1.33 ± 0.33	0.45 ± 0.10*
PYRUVATE DEHYDROGENASE KINASE (PDK1)	0.76 ± 0.15	0.29 ± 0.08*

*Table 1: RT-PCR analysis of expression of hypoxia-inducible glycolytic genes in HCC livers (DEN model) showing reduced expression upon 5D11D4 treatment. The data (mean ± SEM) are expressed as mRNA copies / 10_3 mRNA copies ß-actin (control vs PlGF-blocked; N=4; *P<0.05). Since tumour nodules could not be dissected separately, contaminating healthy liver parenchyma could not be avoided (explaining why a tendency was observed for PFK; #P=0.08). These findings suggest that 5D11D4-treated HCC tumours may be less glycolytic because of improved oxygenation; such metabolic reprogramming counteracts the Warburg effect, which is well known to increase cancer malignancy. In agreement with this metabolic reprogramming, PlGF-blockage lowered the expression levels of PDK1, which inhibits the activity of the pyruvate dehydrogenase complex (PDC) required for the conversion of pyruvate to acetyl CoA and thus the entry of glycolytic intermediates into the Krebs cycle for oxidative metabolism, suggesting that PlGF-blocked HCC tumours had a more oxidative metabolism due to higher oxygenation.*

2.4 Discussion

Bais *et al* raised questions regarding the disease role of PlGF and therapeutic potential of anti-PlGF mAbs (Bais et al., 2009). A first comment relates to the lack of genetic evidence for a cancer role of PlGF. We show that PlGF deficiency delays carcinogen-induced HCC consistent with reports that PlGF levels correlate with a poor outcome in HCC patients (Ho et al., 2006) and are elevated in hepatitis (Salcedo Mora et al., 2005), a risk factor for malignancy.

A second concern was whether the 5D11D4 effects are specific. Independent evidence argues for a specific activity. First, the anti-cancer effects by 5D11D4 are phencopied by the loss of PlGF. In the transgenic mouse model. Similar findings were obtained independently in pediatric brain tumours (R. Jain, personal communication). Furthermore, the safety profile of 5D11D4 in mice (Fischer et al., 2007) and of the humanized anti-human PlGF mAb TB403 in

humans (Lassen et al., 2009; Riisbro et al., 2009) resembles the lack of disease development in PlGF^{-/-} mice (Carmeliet et al., 2001). Overall, when *PlGF*^{-/-} and 5D11D4 effects were compared, 5D11D4 mimicked each PlGF^{-/-} phenotype analysed so far, supporting a specific activity of 5D11D4.

Another argument for the specificity of 5D11D4 is that delivery of this mAb and loss of PlGF induced similar mechanisms, albeit with contextual differences. For instance, angiogenesis was inhibited comparably by loss or inhibition of PlGF in choroidal neovascularisation (CNV). In HCC, silencing or inhibition of PlGF reduced arterialisation and tumour vessel abnormalisation. Angiogenic defects were also observed in skin papillomas, reminiscent of those seen after PlGF loss / inhibition in implanted tumours and other diseases (Carmeliet et al., 2001; Fischer et al., 2007) and in ischemic retina in an independently generated PlGF^{-/-} model (Cheung et al., 2009). Arterialization defects in PlGF-blocked HCC tumours resemble those in portal hypertension and ischemic limb revascularization in PlGF^{-/-} mice (Carmeliet et al., 2001; Van Steenkiste et al., 2009). Also, in HCC, loss, silencing or inhibition of PlGF blocked macrophage infiltration, as in PlGF-blocked atherosclerosis (Roncal et al., 2009), arthritis (Yoo et al., 2009) and skin inflammation (Oura et al., 2003), in line with numerous reports on a role of PlGF in inflammation. In papilloma, PlGF loss / inhibition did not prevent macrophage accumulation, but since these cells promote angiogenesis by releasing PlGF, blockage of macrophage-derived PlGF can also impair papilloma growth. As a word of caution, short-term PlGF inhibition at an advanced stage in a chronic malignant process should not necessarily induce similar effects as permanent germline *Pgf* gene-inactivation.

Third, the dose of 5D11D4 was criticized. In initial studies, we used 50 mg/kg, even though signs of efficacy were noticed at 12.5 mg/kg (Fischer et al., 2007). This is higher than the dose used for other mAbs against (un)related targets (1 to 10 mg/kg), but comparable to the dose used for DC101 or anti-Flt1 (40 mg/kg). Anti-VEGF mAbs are used clinically at 5 to 15 mg/kg. In more recent studies, we obtained anti-disease activity with 5D11D4 at 20-25 mg/kg in atherosclerosis, CNV and HCC, delivered two and not three times per week (this study and (Roncal et al., 2009)). Furthermore, 16D3 inhibited human cancer xenografts at 12.5 mg/kg, while statistically significant tumour growth inhibition has been observed with the humanized anti-human PlGF mAb TB-403 in a human RCC xenograft at doses as low as 3 mg/kg (Rizzo, C. personal

communication). Furthermore, disease stabilization for 12 months has been observed with TB403 at 5 mg/kg in two out of 23 cancer patients with advanced disease refractory to standard therapy (Lassen et al., 2009; Riisbro et al., 2009). Anti-PlGF and anti-VEGF mAbs are circulating in vast excess over their ligands. Indeed, in cancer patients and mice, comparable levels have been reported for VEGF and PlGF (10 to 100 pg/ml) and for anti-VEGF and anti-PlGF mAbs delivered at effective doses (300 to 600 µg/ml) (Gordon et al., 2001; Lassen et al., 2009; Riisbro et al., 2009). Of note, gene transfer elevated plasma human PlGF-2 levels to >200-300 ng/ml, i.e. >1,000-fold higher than endogenous mouse PlGF levels in tumour-bearing mice. Nonetheless, the precise reason for the need of a higher dose of 5D11D4 than of anti-VEGF mAbs needs to be clarified.

While we do not question the findings by Bais *et al*, we also do not have a unifying explanation and additional studies will be required to resolve the matters arising. For instance, an outstanding question is how our PlGF$^{-/-}$ data can be reconciled with *Flt1:TK*$^{-/-}$ data. Controversial findings on tumour growth in *Flt1:TK*$^{-/-}$ mice will need to be resolved as well (Autiero et al., 2003; Dawson et al., 2009; Hiratsuka et al., 2001; Hiratsuka et al., 2002; Kerber et al., 2008; Landgren et al., 1998; Murakami et al., 2008).

HCC is a "hypervascular" cancer. Indeed, a marked arterialization in response to intra-tumoural hypoxia takes place in an effort to provide more oxygen to the highly proliferative cancer cells. However, this increase in proximal blood supply is counteracted by the sinusoidal capillarisation and capillary vessel abnormalisation distally in the vascular tree, which impair oxygen delivery. Other vascular changes including shunts, which already develop in cirrhotic livers prior to becoming neoplastic, may aggravate this situation. Notably, tumour endothelial cells in HCC exhibit marked phenotypic abnormalities and loose their cobblestone appearance, typical of quiescent normal cells (Xiong et al., 2009), reminiscent of the capillary aberrancies in the HCC model. By inducing partial vessel normalization distally, 5D11D4 may restore the balance between oxygen supply and demand, and thereby reduce the need for arterialization. As hypoxia promotes HCC malignancy and chemo-resistance (Wu et al., 2007), it will be interesting to asses whether anti-PlGF treatment will also improve chemotherapy. Furthermore, given the possibility that VEGF-inhibitors fuel metastasis by evoking hypoxia, the effect of anti-PlGF treatment

on metastasis of this cancer warrants further investigation.

Finally, the results of the phase I clinical trial with TB403 have been reported very recently (Lassen et al., 2009; Riisbro et al., 2009). These first-in man anti-PlGF studies show that TB403 was well tolerated without increased risk of adverse effects in healthy volunteers and terminal cancer patients, but with signs of disease stabilization in six of 23 cancer patients. Future clinical studies will be required to assess the therapeutic potential of anti-PlGF strategies in cancer and non-oncology indications but, by their very nature, such developments represent complex challenges.

2.5   References

Ambati, J., Anand, A., Fernandez, S., Sakurai, E., Lynn, B.C., Kuziel, W.A., Rollins, B.J., and Ambati, B.K. (2003). **An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice**. Nat Med *9*, 1390-1397.

Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F.*, et al.* (2003). **Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1**. Nat Med *9*, 936-943.

Bais, C., Wu, X., Yao, J.Y., Crawford, Y., McCutcheon, K., Tan, C., Koluman, G., Vernes, J.M., Easthma-Anderson, J., Haughney, P.*, et al.* (2009). **PlGF blockade does not inhibit angiogenesis during primary tumor growth.** Cell *submitted*.

Bocci, G., Man, S., Green, S.K., Francia, G., Ebos, J.M., du Manoir, J.M., Weinerman, A., Emmenegger, U., Ma, L., Thorpe, P.*, et al.* (2004). I**ncreased plasma vascular endothelial growth factor (VEGF) as a surrogate marker for optimal therapeutic dosing of VEGF receptor-2 monoclonal antibodies**. Cancer Res *64*, 6616-6625.

Bonnin, P., Pournaras, J.A., Lazrak, Z., Cohen, S.Y., Legargasson, J.F., Gaudric, A., Levy, B.I., and Massin, P. (2009). **Ultrasound assessment of short-term ocular vascular effects of intravitreal injection of bevacizumab (Avastin) in neovascular age-related macular degeneration**. Acta Ophthalmol.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P.*, et al.* (1998). **Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis**. Nature *394*, 485-490.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H*., et al.* (2001). **Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions**. Nat Med *7*, 575-583.

Cheng, D., and Sefton, M.V. (2009). **Dual delivery of placental growth factor and vascular endothelial growth factor from poly(hydroxyethyl methacrylate-co-methyl methacrylate) microcapsules containing doubly transfected luciferase-expressing l929 cells.** Tissue Eng Part A *15*, 1929-1939.

Cheung, E., Myers, K., Yancopoulos, G., Lobov, I.B., and Wiegand, S.J. (2009). **Placental growth factor (PlGF) deficient mice show delays in normal retinal vascular development and reduced vascular abnormalities in oxygen-induced retinal ischemia**. ARVO abstract.

Davidoff, A.M., Nathwani, A.C., Spurbeck, W.W., Ng, C.Y., Zhou, J., and Vanin, E.F. (2002). **rAAV-mediated long-term liver-generated expression of an angiogenesis inhibitor can restrict renal tumor growth in mice.** Cancer Res *62*, 3077-3083.

Dawson, M.R., Duda, D.G., Chae, S.S., Fukumura, D., and Jain, R.K. (2009). **VEGFR1 activity modulates myeloid cell infiltration in growing lung metastases but is not required for spontaneous metastasis formation.** PLoS One *4*, e6525.

Dupuy, E., Hainaud, P., Villemain, A., Bodevin-Phedre, E., Brouland, J.P., Briand, P., and Tobelem, G. (2003). **Tumoral angiogenesis and tissue factor expression during hepatocellular carcinoma progression in a transgenic mouse model**. J Hepatol *38*, 793-802.

Ebos, J.M., Lee, C.R., Christensen, J.G., Mutsaers, A.J., and Kerbel, R.S. (2007). **Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy.** Proc Natl Acad Sci U S A *104*, 17069-17074.

Fernandez, M., Semela, D., Bruix, J., Colle, I., Pinzani, M., and Bosch, J. (2009). **Angiogenesis in liver disease.** J Hepatol *50*, 604-620.

Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., Chorianopoulos, E., Liesenborghs, L., Koch, M., De Mol, M*., et al.* (2007). **Anti-PlGF Inhibits Growth of VEGF(R)-Inhibitor-Resistant Tumors without Affecting Healthy Vessels.** Cell *131*, 463-475.

Fischer, C., Mazzone, M., Jonckx, B., and Carmeliet, P. (2008). **FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy?** Nat Rev Cancer *8*, 942-956.

Gerber, H.P., Wu, X., Yu, L., Wiesmann, C., Liang, X.H., Lee, C.V., Fuh, G., Olsson, C., Damico, L., Xie, D*., et al.* (2007). **Mice expressing a humanized form of VEGF-A may provide insights into the safety and efficacy of anti-VEGF antibodies.** Proc Natl Acad Sci U S A *104*, 3478-3483.

Gordon, M.S., Margolin, K., Talpaz, M., Sledge, G.W., Jr., Holmgren, E., Benjamin, R., Stalter, S., Shak, S., and Adelman, D. (2001). **Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer**. J Clin Oncol *19*, 843-850.

Hawighorst, T., Velasco, P., Streit, M., Hong, Y.K., Kyriakides, T.R., Brown, L.F., Bornstein, P., and Detmar, M. (2001). **Thrombospondin-2 plays a protective role in multistep carcinogenesis: a novel host anti-tumor defense mechanism.** EMBO J *20*, 2631-2640.

Hiratsuka, S., Maru, Y., Okada, A., Seiki, M., Noda, T., and Shibuya, M. (2001). **Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis**. Cancer Res *61*, 1207-1213.

Hiratsuka, S., Nakamura, K., Iwai, S., Murakami, M., Itoh, T., Kijima, H., Shipley, J.M., Senior, R.M., and Shibuya, M. (2002). **MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis**. Cancer Cell *2*, 289-300.

Ho, M.C., Chen, C.N., Lee, H., Hsieh, F.J., Shun, C.T., Chang, C.L., Lai, Y.T., and Lee, P.H. (2006). **Placenta growth factor not vascular endothelial growth factor A or C can predict the early recurrence after radical resection of hepatocellular carcinoma.** Cancer Lett.

Jeganathan, V.S., and Verma, N. (2009). **Safety and efficacy of intravitreal anti-VEGF injections for age-related macular degeneration**. Curr Opin Ophthalmol *20*, 223-225.

Johnson, S.J., Burr, A.W., Toole, K., Dack, C.L., Mathew, J., and Burt, A.D. (1998). **Macrophage and hepatic stellate cell responses during experimental hepatocarcinogenesis**. J Gastroenterol Hepatol *13*, 145-151.

Kami, J., Muranaka, K., Yanagi, Y., Obata, R., Tamaki, Y., and Shibuya, M. (2008). **Inhibition of choroidal neovascularization by blocking vascular endothelial growth factor receptor tyrosine kinase.** Jpn J Ophthalmol *52*, 91-98.

Kerber, M., Reiss, Y., Wickersheim, A., Jugold, M., Kiessling, F., Heil, M., Tchaikovski, V., Waltenberger, J., Shibuya, M., Plate, K.H.*, et al.* (2008). **Flt-1 signaling in macrophages promotes glioma growth in vivo**. Cancer Res *68*, 7342-7351.

Klein, K.S., Walsh, M.K., Hassan, T.S., Halperin, L.S., Castellarin, A.A., Roth, D., Driscoll, S., and Prenner, J.L. (2009). **Endophthalmitis after anti-VEGF injections.** Ophthalmology *116*, 1225 e1221.

Landgren, E., Schiller, P., Cao, Y., and Claesson-Welsh, L. (1998). **Placenta growth factor stimulates MAP kinase and mitogenicity but not phospholipase C-gamma and migration of endothelial cells expressing Flt 1**. Oncogene *16*, 359-367.

Lassen, U., Nielsen, D., Sorensen, M., Ronnengart, E., Eldrup, K., Bentzon, K., Winstedt, L., Niskanen, T., Stenberg, Y., Pakola, S.*, et al.* (2009). **A phase I, dose escalation study of TB-403, a monoclonal antibody directed against PlGF, in patients with solid tumors.** . Mol Cancer Ther *8*.

Li, B., Sharpe, E.E., Maupin, A.B., Teleron, A.A., Pyle, A.L., Carmeliet, P., and Young, P.P. (2006). **VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization.** Faseb J *20*, 1495-1497.

Luttun, A., Brusselmans, K., Fukao, H., Tjwa, M., Ueshima, S., Herbert, J.M., Matsuo, O., Collen, D., Carmeliet, P., and Moons, L. (2002a). **Loss of placental growth factor protects mice against vascular permeability in pathological conditions**. Biochem Biophys Res Commun *295*, 428-434.

Luttun, A., Tjwa, M., Moons, L., Wu, Y., Angelillo-Scherrer, A., Liao, F., Nagy, J.A., Hooper, A., Priller, J., De Klerck, B.*, et al.* (2002b). **Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis** by anti-Flt1. Nat Med *8*, 831-840.

Marcellini, M., De Luca, N., Riccioni, T., Ciucci, A., Orecchia, A., Lacal, P.M., Ruffini, F., Pesce, M., Cianfarani, F., Zambruno, G.*, et al.* (2006). **Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor.** Am J Pathol *169*, 643-654.

Matsumoto, K., Suzuki, K., Koike, H., Okamura, K., Tsuchiya, K., Uchida, T., Takezawa, Y., Kobayashi, M., and Yamanaka, H. (2003). **Prognostic significance of plasma placental growth factor levels in renal cell cancer: an association with clinical characteristics and vascular endothelial growth factor levels.** Anticancer Res *23*, 4953-4958.

Murakami, M., Zheng, Y., Hirashima, M., Suda, T., Morita, Y., Ooehara, J., Ema, H., Fong, G.H., and Shibuya, M. (2008). **VEGFR1 tyrosine kinase signaling promotes lymphangiogenesis as well as angiogenesis indirectly via macrophage recruitment.** Arterioscler Thromb Vasc Biol *28*, 658-664.

Nozawa, H., Chiu, C., and Hanahan, D. (2006). **Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis.** Proc Natl Acad Sci U S A *103*, 12493-12498.

Oura, H., Bertoncini, J., Velasco, P., Brown, L.F., Carmeliet, P., and Detmar, M. (2003). **A critical role of placental growth factor in the induction of inflammation and edema formation.** Blood *101*, 560-567.

Park, J.E., Chen, H.H., Winer, J., Houck, K.A., and Ferrara, N. (1994). Placenta growth factor. **Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR.** J Biol Chem *269*, 25646-25654.

Paternostro, C., David, E., Novo, E., and Parola, M. **Hypoxia, angiogenesis and liver fibrogenesis in the progression of chronic liver diseases**. World J Gastroenterol *16*, 281-288.

Riisbro, R., Larsson, L., Winstedt, L., Niskanen, T., Pakola, S., Stassen, J.M., Lassen, U., and Glazer, S. (2009). **A first-in-man phase I dose escalation study of TB403, a monoclonal antibody directed atainst PlGF in healthy male subjects**. Mol Cancer Ther *8*, A3.

Roberts, R.A., Ganey, P.E., Ju, C., Kamendulis, L.M., Rusyn, I., and Klaunig, J.E. (2007). **Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis**. Toxicol Sci *96*, 2-15.

Roncal, C., Buysschaert, I., Gerdes, N., Georgiadou, M., Ovchinnikova, O., Fischer, C., Stassen, J.M., Moons, L., Collen, D., De Bock, K.*, et al.* (2009). **Short-term delivery of anti-PlGF antibody delays progression of atherosclerotic plaques to vulnerable lesions.** Cardiovasc Res.

Salcedo Mora, X., Sanz-Cameno, P., Medina, J., Martin-Vilchez, S., Garcia-Buey, L., Borque, M.J., and Moreno-Otero, R. (2005). **Association between angiogenesis soluble factors and disease progression markers in chronic hepatitis C patients**. Rev Esp Enferm Dig *97*, 699-706.

Taylor, A.P., and Goldenberg, D.M. (2007). **Role of placenta growth factor in malignancy and evidence that an antagonistic PlGF/Flt-1 peptide inhibits the growth and metastasis of human breast cancer xenografts**. Mol Cancer Ther *6*, 524-531.

Van Steenkiste, C., Geerts, A., Vanheule, E., Van Vlierberghe, H., De Vos, F., Olievier, K., Casteleyn, C., Laukens, D., De Vos, M., Stassen, J.M.*, et al.* (2009). **Role of placental growth factor in mesenteric neoangiogenesis in a mouse model of portal hypertension.** Gastroenterology *137*, 2112-2124 e2111-2116.

Wei, S.C., Tsao, P.N., Yu, S.C., Shun, C.T., Tsai-Wu, J.J., Wu, C.H., Su, Y.N., Hsieh, F.J., and Wong, J.M. (2005). **Placenta growth factor expression is correlated with survival of patients with colorectal cancer.** Gut *54*, 666-672.

Willett, C.G., Duda, D.G., di Tomaso, E., Boucher, Y., Ancukiewicz, M., Sahani, D.V., Lahdenranta, J., Chung, D.C., Fischman, A.J., Lauwers, G.Y.*, et al.* (2009). **Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: a multidisciplinary phase II study.** J Clin Oncol *27*, 3020-3026.

Wu, X.Z., Xie, G.R., and Chen, D. (2007). **Hypoxia and hepatocellular carcinoma: The therapeutic target for hepatocellular carcinoma.** J Gastroenterol Hepatol *22*, 1178-1182.

Xiong, Y.Q., Sun, H.C., Zhang, W., Zhu, X.D., Zhuang, P.Y., Zhang, J.B., Wang, L., Wu, W.Z., Qin, L.X., and Tang, Z.Y. (2009). **Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells.** Clin Cancer Res *15*, 4838-4846.

Xu, L., and Jain, R.K. (2007). **Down-regulation of placenta growth factor by promoter hypermethylation in human lung and colon carcinoma.** Mol Cancer Res *5*, 873-880.

Yano, K., Okada, Y., Beldi, G., Shih, S.C., Bodyak, N., Okada, H., Kang, P.M., Luscinskas, W., Robson, S.C., Carmeliet, P.*, et al.* (2008). **Elevated levels of placental growth factor represent an adaptive host response in sepsis**. J Exp Med *205*, 2623-2631.

Yoo, S.A., Yoon, H.J., Kim, H.S., Chae, C.B., De Falco, S., Cho, C.S., and Kim, W.U. (2009). **Role of placenta growth factor and its receptor flt-1 in rheumatoid inflammation: a link between angiogenesis and inflammation**. Arthritis Rheum *60*, 345-354.

3 The placental growth factor as a potential target against HCC

The placental growth factor as a target against hepatocellular carcinoma in an orthotopic mouse model

F. Heindryckx, S. Coulon, E. Terrie, C. Casteleyn, JM Stassen, A. Geerts, L. Libbrecht, J. Allemeersch, P. Carmeliet, I. Colle & H. Van Vlierberghe

3.1   Abstract

The placental growth factor (PlGF) is member of the vascular endothelial growth factor (VEGF)-family known to stimulate endothelial cell growth, migration and survival, attract angiocompetent macrophages and bone marrow progenitor cells and determine the metastatic niche. Unlike VEGF, genetic studies have shown that PlGF is specifically involved in the pathologic angiogenesis, thus its inhibition would not affect healthy blood vessels, providing an attractive drug candidate with a good safety profile. In this study, we assess whether inhibition of PlGF could be used as a potential therapy against hepatocellular carcinoma (HCC), by using PlGF-knock out mice and monoclonal antibodies targeting PlGF in an orthotopic mouse model for HCC. In addition, the effect of PlGF-antibodies is compared to that of sorafenib, as well as the combination of both therapies. In our study we have found that both in a transgenic knock out model as in a treatment model, silencing or inhibition of PlGF significantly decreased tumour burden. This was achieved not only by inhibiting neo-vascularisation, but also by decreasing hepatic macrophage recruitment and by normalizing the remaining blood vessels, thereby decreasing hypoxia and thus, reducing the pro-metastatic potential of HCC. **Conclusion:** considering its favourable safety profile and its pleiotropic effect on vascularisation, metastasis and inflammation, targeting PlGF could become a valuable therapeutic strategy against HCC.

3.2   Introduction

Hepatocellular carcinoma (HCC) is a primary liver tumour that originates in a background of cirrhosis. Every year almost 500.000 patients are diagnosed with HCC, making it the 5th most common and the 3rd most deadly cancer worldwide. The underlying liver disease often conceals the presence of liver tumours; therefore most tumours are discovered in an advanced stage. At this point no curative option exists and systemic treatment is limited due to the high drug resistance of HCC (1).

Significant progress on the treatment of advanced HCC has been made by the introduction of sorafenib (2). Sorafenib is a small molecular inhibitor targeting tyrosine protein kinases in the Raf/MEK/ERK-pathway (anti-proliferative effect)

(3); the platelet derived growth factor and the vascular endothelial growth factor receptor 1 and receptor 2 (VEGFR1 and VEGFR2) (anti-angiogenic effect). However, patients receiving sorafenib often encounter adverse effects such as diarrhoea, weight loss, hand–foot skin reaction, anorexia, and fatigue (4,5).

Another weakness of current anti-angiogenic therapies is tumours tend to escape their treatment by upregulating alternative angiogenic pathways and becoming resistant. As a result, several cases have been reported of resistance against sorafenib (6,7), sunitinib (8,9) and VEGF(R)-inhibitors (10–14). Furthermore, the hypoxic conditions created by anti-angiogenic therapy, may select for more invasive tumour variants, better adapted to survive and proliferate under reduced oxygen tension (15–17). Intratumoural hypoxia has been linked with metastasis, regulating several steps of the metastatic process, such as invasion through the basement membrane and extracellular matrix, increasing production of fibronectin; increased intravasation and regulation of genes that promote the pre-metastatic niche (18).

The placental growth factor (PlGF) is member of the VEGF-family known to stimulate endothelial cell growth, migration and survival (19–21). It also attracts angiocompetent macrophages and bone marrow progenitor cells and determines the metastatic niche (19,20). Unlike VEGF, PlGF binds to VEGF receptor 1 (VEGFR1) and its co-receptors neuropilin-1 and 2. Therefore, anti-PlGF exerts its anti-angiogenic potential directly by preventing signal transduction through its receptors (22) and indirectly, by preventing the binding of VEGF on its decoy receptor VEGFR1 (23). Furthermore, PlGF is known to alter the tumour associated macrophage (TAM) niche (24). In non-progressing or regressing tumours, TAMs are characterised by an M1-like program, resulting in pro-inflammatory activity and tumour inhibition. In malignant tumours, TAMs resemble alternatively activated macrophages (M2-type), that stimulate angiogenesis, metastasis and tumour growth; by suppressing anti-tumour immunity (25).

Genetic studies have shown that PlGF is specifically involved in pathologic angiogenesis (19,21). Therefore, its inhibition would not affect healthy blood vessels, providing an attractive drug candidate with a good safety profile (26). In this study, we assess whether inhibition of PlGF could serve as a therapeutic

agent in an orthotopic mouse model for HCC, focussing on the effect on angiogenesis, metastasis, inflammation and vascular morphology.

3.3 Material & methods

3.3.1 HCC induction

Mice received intraperitoneal injections of diethylnitrosamine (DEN) (Sigma-Aldrich, Bornem, Belgium) once a week or saline as previously described (27). The Ethical Committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocol.

3.3.2 PlGF inhibition

An—anti-PlGF monoclonal antibody (clone 5D11D4; referred to as αPlGF) specifically recognizing mouse PlGF-2 was obtained from Thrombogenics, Belgium. Mice that received DEN for 25 weeks had developed HCC, were subsequently treated with 25mg/kg αPlGF (2x/week, intraperitoneal), 10mg/kg sorafenib (daily, intragrastric), combination of αPlGF + sorafenib, or 25mg/kg IgG (2x/week, intraperitoneal) for 5 weeks. *Pgf*-knock out mice (PlGFKO) were obtained from the laboratory of Angiogenesis & Neurovascular link (Leuven) and injected with DEN or saline for 20W, 25W and 30W, after which they were sacrificed and compared to their WT-counterparts.

3.3.3 Sampling & histology

Eight animals per group were sacrificed under isoflurane (Forene®, Hoofddorp, The Netherlands) anaesthesia while blood was obtained from the ophthalmic artery. After macroscopic evaluation and quantification of hepatic tumours, organs were sampled as previously described (25). Haematoxilin-eosin staining was performed to evaluate the morphological changes. Sirius Red staining was used to determine the Metavir-score for fibrosis. Reticulin staining was performed to identify HCC-nodules. Stainings were done using standard histology protocols and evaluated by an experienced pathologist.

3.3.4   Immunohistochemistry

Immunohistochemistry (IHC) was used to quantify protein levels inside hepatic tumours and surrounding non-tumour tissue. Stainings were used to quantify vascularisation, macrophages (M1 and M2), activated hepatic stellate cells and hypoxia, as described in **supplementary table 1.** A semi-quantitatively analysis was performed with Olympus Cell^D software (Zoeterwoude, The Netherlands) by using automatic DAB-staining detection in tumour and non-tumour lesions. Intercapillary distances (ICD) were used as a marker for microvessel density.

3.3.5   Vascular corrosion casting

Vascular corrosion casts were obtained as previously described. To provide insight in the microvasculature of the livers the peritumoural fragments of the casts were qualitatively assessed with scanning electron microscopy (SEM) (Jeol 5600-LV, Zaventem, Belgium).

	Antigen retrieval		Blocking			Primary Antibody		Secundary Antibody		Buffer	
	Product (reference)	Time	Peroxidase	Product (dilution)	Time	Product (ref) dilution	Time	Product (ref) dilution	Time	Buffer	Marker
VEGF	Trypsin	7' (37°C)	15' 3% H2O2	Goat serum (1/5)	45' (RT)	Rabbit polyclonal anti-VEGF (sc-152, Santa Cruz) 1/250	18h (4°C)	Goat anti-Rabbit (P0448, Dako) 1/300	45' (RT) + Vectastain kit	TNB	Angiogenesis
CD105	Citrate	30' (99°C)	15' 3% H2O2	BSA (0,1%)	30' (RT)	Goat polyclonal anti-Endoglin (AF1320, R&D systems) 1/50	18h (RT)	LSAB-kit (K0690, Dako)	Manufacturers protocol	TBS	Angiogenesis
F4/80	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	Rabbit serum (1/5)	45' (RT)	Rat monoclonal anti-F4/80 (MCA497, Serotec) 1/300	18h (RT)	Rabbit anti-Rat (E0468, Dako) 1/300	45' (RT) + Vectastain kit	TNB	Macrophages
αSMA	Trypsin	10' (37°C)	15' 3% H2O2	BSA (1%)	45' (RT)	Mouse monoclonal anti-actin (ab18146, Abcam) 1/200	30' (37°C)	LSAB-kit (K0690, Dako)	Manufacturers protocol	TBS	Activated HSC
HIF1α	Citrate	30' (99°C)	15' 3% H2O2	BSA (1%)	30' (37°C)	Rabbit polyclonal anti-HIF1alpha (sc-10790, Santa Cruz) 1/400	1h (37°C)	LSAB-kit (K0690, Dako)	Manufacturers protocol	PBS	Hypoxia
HLA-DR	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	BSA (1%)	30' (RT)	Rat monoclonal anti-HLA-DR (sc-59257, Santa Cruz) 1/800	1h (37°C)	LSAB-kit (K0690, Dako)	Manufacturers protocol	PBS	M1 Macrophages
CD206	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	BSA (1%)	30' (RT)	Rabbit Polyclonal anti-CD206 (orb4941, Biorbyt) 1/400	1h (37°C)	LSAB-kit (K0690, Dako)	Manufacturers protocol	PBS	M2 Macrophages
CD163	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	BSA (1%)	30' (RT)	Rabbit Polyclonal anti-CD163 (orb13303, Biorbyt) 1/400	1h (37°C)	LSAB-kit (K0690, Dako)	Manufacturers protocol	PBS	M2 Macrophages

Supplementary table 1: *IHC-staining protocols.*

3.3.6   ELISA

PlGF (R&D Biosystems ,Mouse PlGF-2 Quantikine ELISA Kit, Abingdon, UK) and VEGF (R&D Biosystems, Mouse VEGF Quantikine ELISA Kit, Abingdon, UK) levels were measured in liver tissue and serum. Human serum of HCC-patients (n=17), cirrhotic patients (n=17) and healthy controls (n=8) were collected after informed consent and PlGF-serum levels were determined (R&D Biosystems, human PlGF-2 Quantikine ELISA Kit, Abingdon, UK). Cirrhotic patients were matched with HCC-patients regarding sex, age and etiology. PlGF-levels were tested for correlation with the following parameters: presence of HCC, concentrations of AFP, bilirubin, AST/ALT and alcohol intake.

3.3.7   Microarrays

Gene expression analysis was carried out using Agilent SurePrint G3 Mouse GE 8x60K Microarrays (Agilent Technologies, Diegem, Belgium). This specific array represents 41,000+ mouse genes and transcripts, providing full coverage of mouse genes and transcripts. Following conditions were assessed: 25W DEN + 5W αPlGF tumour tissue (n=5), 25W DEN + 5W αPlGF surrounding tissue (n = 4), 25W DEN + 5W IgG tumour tissue (n=5), 25W DEN + 5W IgG surrounding tissue (n = 4), 25W saline + 5W αPlGF (n=3) and 25W saline + 5W IgG (n=3). The data are accessible on the GEO-database (GSE35289). Expression data for sorafenib-treated mice were obtained from the GEO-database (GSE33621) consisting of 6 sorafenib and 6 control-treated xenograft HCC-tumours. Human expression data were obtained from the GEO-database (GSE25097), consisting of whole genome-expression profiles of 268 HCC, 243 adjacent non-tumour, 40 cirrhotic and 6 healthy liver samples.

3.3.8   Quantitative Real Time PCR (qPCR)

RNA was extracted from 20mg frozen liver tissue, following manufacturer's guidelines (Qiagen, RNeasy Mini Kit ref: 74104, Venlo, Netherlands) and diluted to a concentration of 100 ng/µl. RNA Quality was evaluated using spectrophotometry. cDNA was obtained from 10µl RNA with the iScript cDNA Synthesis Kit using Oligo(Dt) primers, according to manufacturer's protocol (Bio-Rad, 170-8890, Nazareth, Belgium). Expression levels were measured using

140

qRT-PCR, with 3µl cDNA and the LightCycler 480 Green Master Mix (Roche, 04707516001, Vilvoorde, Belgium). Genes and corresponding primersets are provided in **supplementary table 2.** The efficiency of each primer pair was calculated using the formula $^{10-1/slope}$. All reactions were run in duplicate and normalised to a set of reference genes that had a stable expression in all samples. The comparative Ct method was used for determining the number of transcripts.

Gene name (ID)	Forward primer	Reverse primer	Marker
Hprt (15452)	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT	Reference gene
Hmbs (15288)	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG	Reference gene
Sdha (66945)	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT	Reference gene
Gapdh (14433)	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	Reference gene
Pfk1 (18641)	GCCGGCTCAGTGAGACAAG	TGGCACCTTCAGCAACAATG	Hypoxia
Glut1 (20512)	GCTTATGGGCTTCTCCAAAC	GTGACACCTCTCCCACATAC	Hypoxia
Mmp9 (17395)	GAGACGGGTATCCCTTCGAC	TGACATGGGGCACCATTTGAG	Metastasis
Itgav (16410)	CAATTGCTGCTCCCTATGGT	GATTTGAGATGGCACCGAAT	Metastasis
Icam (15898)	GCCTTGGTAGAGGTGACTGAG	GACCGGAGCTGAAAAGTTGTA	Metastasis
Vegfa (22339)	ACTCGGATGCCGACACGGGA	CCTGGCCTTGCTTGCTCCCC	Angiogenesis

Supplementary table 2: genes and primer sets

3.3.9   Statistics

Data were analysed with SPSS19. After testing for normality and homoscedasticity, data were subjected to a student's t-test or a Mann-Whitney-U test. A P-value of <0.05 was considered statistically significant.

For the microarray data, statistical differences between the conditions were calculated using the log2-intensities with the limma package (25,26) of Bioconductor by estimating the contrasts of interest between combinations that were plotted in advance. Whether this contrast is significantly deviating from 0 was tested using a moderated t-statistic (implemented in limma) and a stringent cut-off of p<0,001 was applied to prevent false positive results.

3.4   Results

3.4.1   PlGF knock out study

Silencing of *Pgf* in the PlGFKO-model significantly improved survival (P<0,01), 80% of the WT-mice died, while only 23% of the PlGFKO-mice succumbed to the disease. Furthermore, PlGFKO-mice suffered less from weight loss compared to WT-mice (25W, P<0,05), suggesting an improved wellbeing. This was possibly a result of a decreased tumour number, as macroscopically seen after 20W (P<0,001), 25W (P<0,01) and 30W (P<0,05) DEN (**fig1A**) and confirmed by a significant decrease in histological HCC-burden after 25W and 30W (P<0,05) (**fig1B**). Small cell dysplasia was frequently found throughout the liver starting from 20W DEN in WT-mice and readily distinguishable nodules of neoplasia were seen at 25W and 30W, while only small tumours were seen in PlGFKO-mice (**supplementary figure 1**). Tumours were significantly less vascularised after 30W (P<0,05) and the surrounding non-tumour tissue also showed a decreased expression of the endothelial cell marker endoglin (**fig1C-D**). Measurements of ICD confirmed that PlGFKO-tumours had a lower microvessel density (20W, P<0,05) (**supplementary figure 1**). VEGF-ELISA showed that PlGF-silenced tumours had lower VEGF-levels (30W, P<0,05) (**fig1E-F**). SEM-analysis of vascular casts showed that capillaries of WT-mice injected with 30W DEN had an abnormal shape and chaotic organisation, resulting in a tortuous and disorganised vasculature typically seen in tumours (**Fig2A**), while complete blockage of PlGF induced partial normalization (**Fig2B**). PlGF-blockage reduced the expression of the hypoxia-inducible genes *Glut1* (P<0,05) and *Pfk* (not significant) after 30W DEN in tumour (no significant differences were seen at other time points) (**Fig2C**) and *Glut1* in surrounding tissue after 25W DEN. IHC also showed a decreased staining of HIF1α at 20W and 30W in tumour (20W P<0,01; 30W P<0,01) and surrounding tissue (20W P<0,001; 30W P<0,01) (**Fig2F**). Furthermore, PlGF-deficient livers expressed less pro-metastatic markers Mmp-9 (20W, P<0,05; 25W P<0,01), Itgav (25W, P<0,05; 30W, P<0,05) and Icam (20W, P<0,01; 25W, P<0,01) compared to WT-mice with HCC (**fig2D-E**).

F4/80-staining revealed less infiltration of macrophages in PlGFKO-tumours at 30W (P<0,05) (**supplementary figure 1, Fig3A**). A similar trend was seen in

surrounding non-tumour tissue. The M2-markers CD206 and CD163 were significantly decreased surrounding tissue of 20W DEN (respectively P<0,01 and P<0,05), while tumour samples showed fewer CD163+macrophages in 30W DEN (P<0,05) (**Fig3B-C, supplementary figure 2)**. However, the M1-marker HLA-DR was also significantly decreased 20W DEN surrounding tissue (P<0,05). Less activation of HSC was seen in tumour (25W, P<0,05; 30W, P<0,01) and non-tumour tissue (25W, P<0,05; 30W, P<0,01) (**Fig3A, Fig3D-F**). Inhibition of PlGF slightly decreased Metavir-score, but not statistically significant.
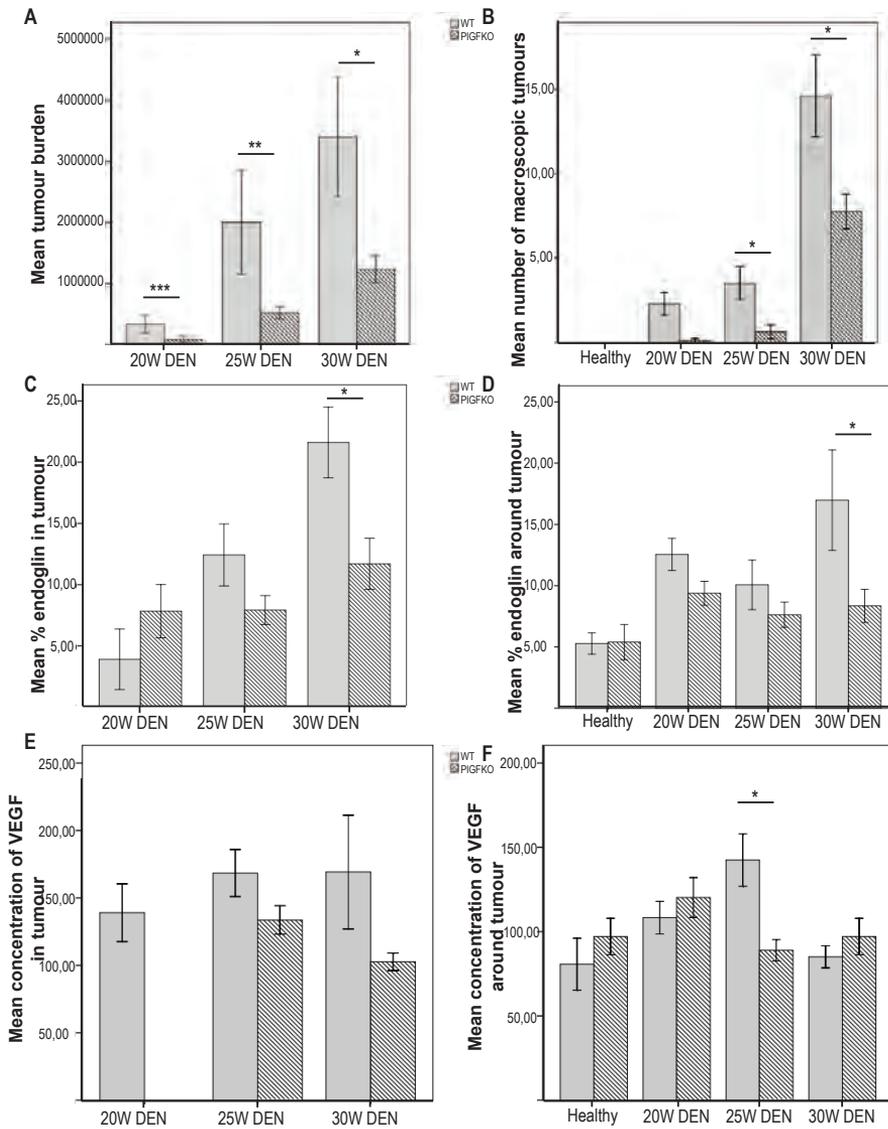
Figure 1: *PlGF knock out study. Silencing of PlGF significantly decreased macroscopic tumour numbers (fig1A) and histological HCC-burden (P<0,05) (fig1B). Tumours were significantly less vascularised after 30W and the surrounding non-tumour tissue showed a decreased expression of the endothelial cell marker Endoglin (fig1C-D). VEGF-ELISA showed that PlGF-silenced tumours had lower VEGF-levels after 30W (fig1E-F). Asterisks represent p-values (* = P<0,05, ** = P<0,01 and *** = P<0,001).*
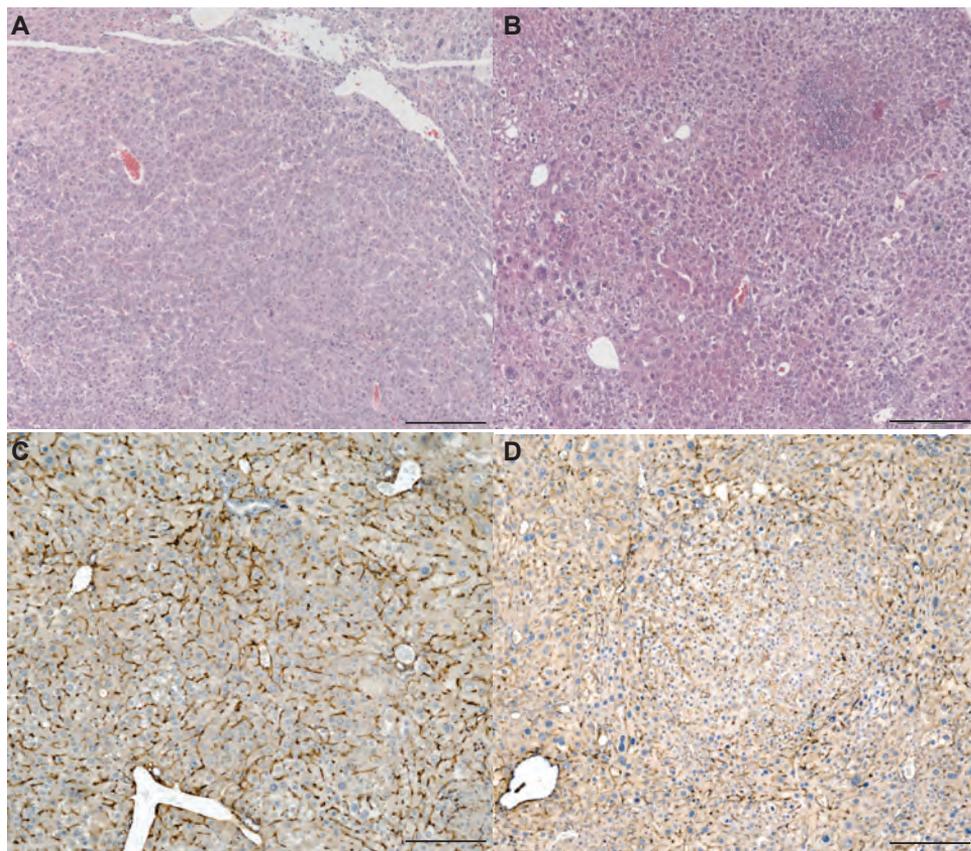
Supplementary figure 1: *HE-stained slides of WT (A) and PlGFKO (B) HCC-lesions. Endoglin-stained slides of WT (C) and PlGFKO (D) HCC-lesions, showing tumour vascularisation. Scale bars = 200 µm.*
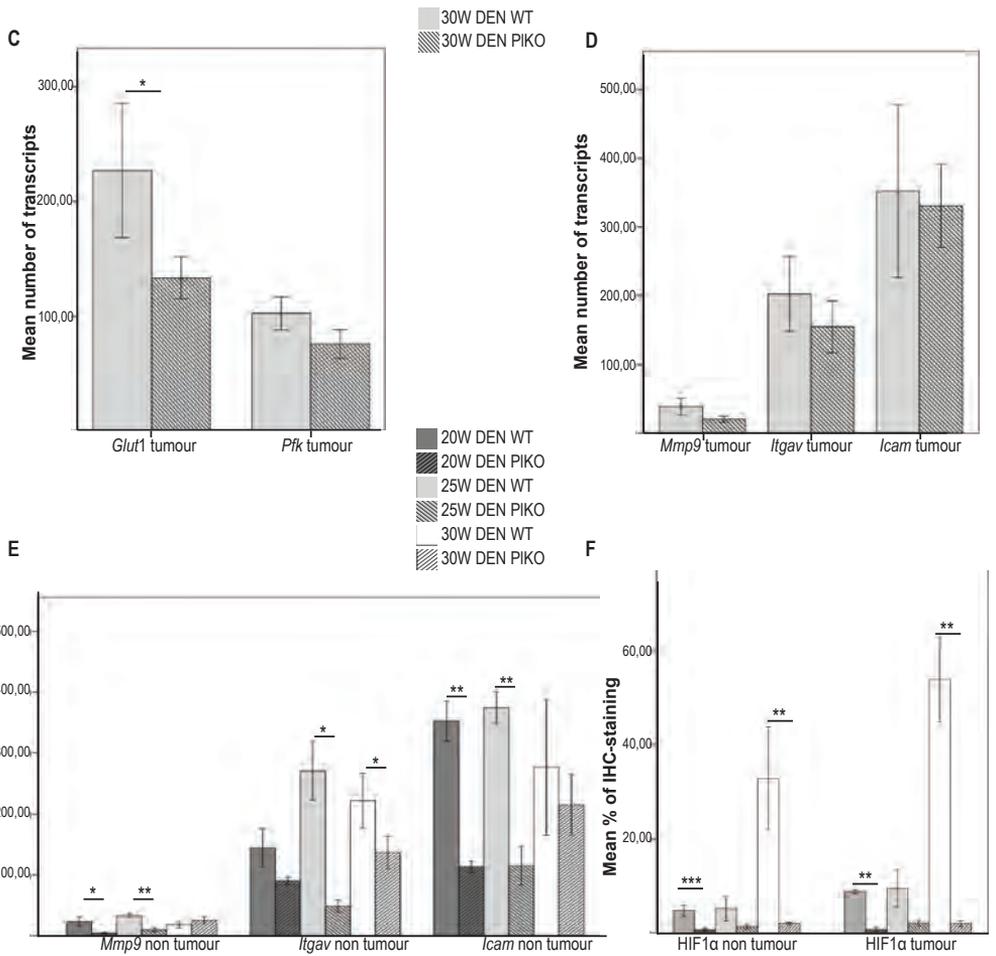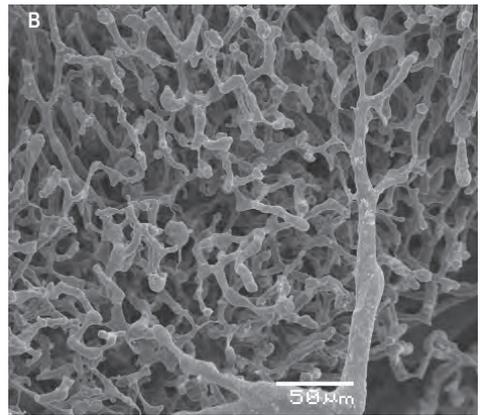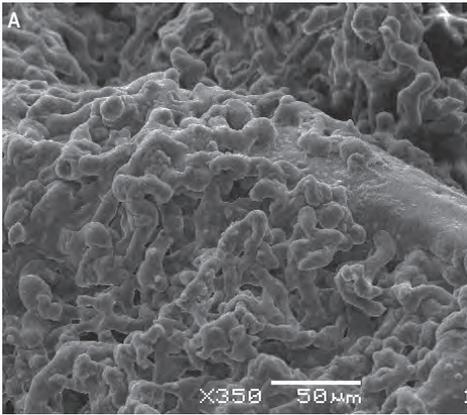
Figure 2 (previous page): Vascular normalisation and metastasis in the PlGFKO-model. Capillaries of WT-mice injected with 30W DEN had an abnormal shape and chaotic organisation of the microvasculature (Fig2A) and complete blockage of PlGF induced partial normalization of tumour capillaries (Fig2B). PlGF-blocked HCC-nodules and the surrounding non-tumour tissue were therefore less hypoxic, as suggested by the reduced expression of hypoxia-inducible genes of Glut1 and PFK (not significant) (Fig2C). PlGF-deficient livers expressed less pro-metastatic markers Mmp-9, Itgav and Icam compared to WT-mice with HCC (fig2D-E). HIF1α-staining showed a significant decrease in PlGF-deficient livers compared to WT-mice (Fig2F). Asterisks represent p-values (* = P<0,05, ** = P<0,01 and *** = P<0,001)
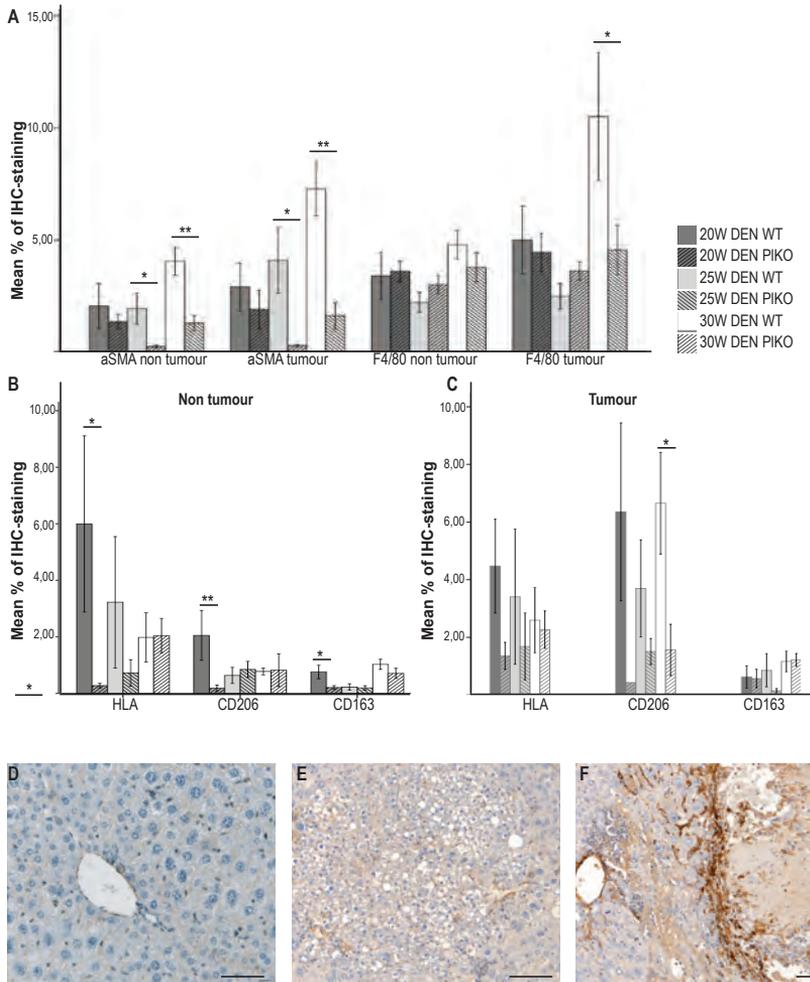
Figure 3: tumour associated macrophage niche. F4/80-staining revealed less infiltration of macrophages in PlGFKO-tumours at 30W and a decreased activation of HSC, both in tumour as non-tumour tissue (fig3A). The M2-markers CD206 and CD163 and the M1-marker HLA-DR were significantly decreased 20W DEN surrounding tissue (Fig3B). Tumour samples showed fewer CD163+macrophages in 30W DEN (P (Fig3C). Nearly no αSMA-expression was seen in healthy livers (Fig3D). Staining with αSMA showed that there were less activated HSC in 30W DEN PlGFKO-mice (Fig3E), compared to 30W DEN WT-mice (Fig3F). Scale-bars = 100µm. Asterisks represent p-values (* = P<0,05, ** = P<0,01 and *** = P<0,001).
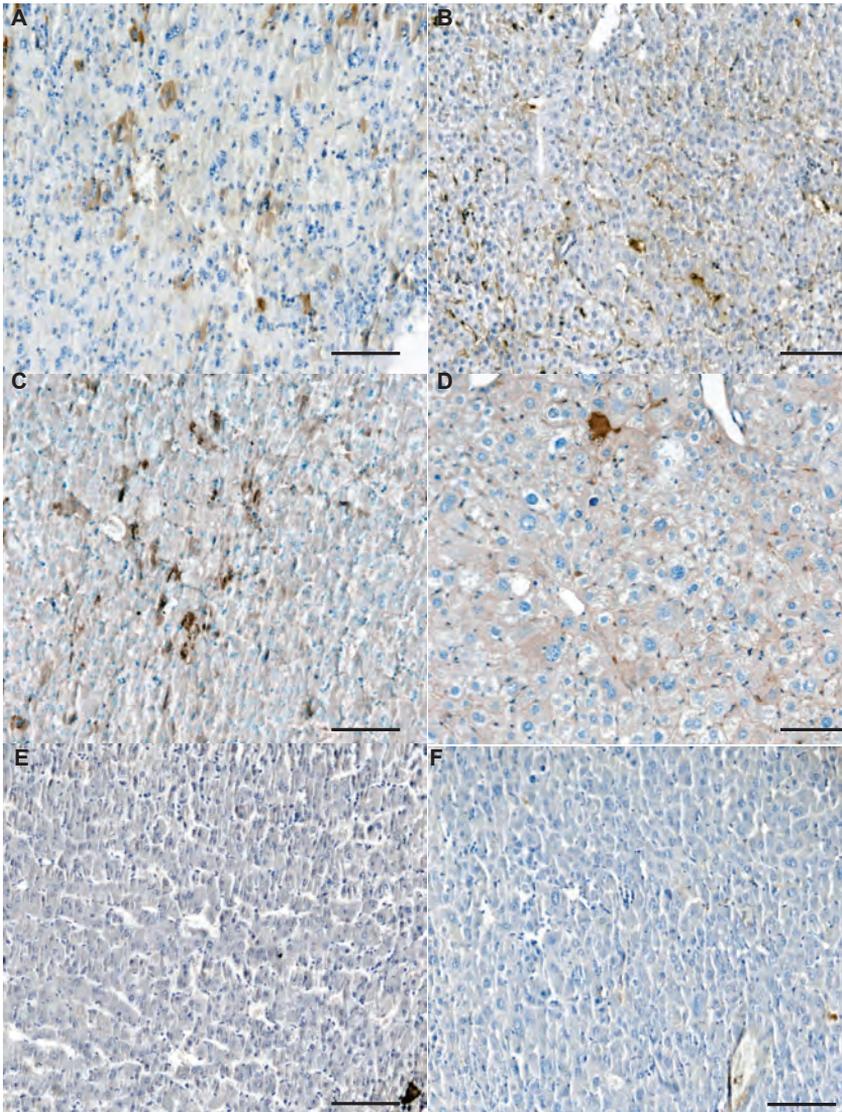
Supplementary figure 2: Tumour associated macrophage niche. Representative stainings of HLA-DR in 30W DEN WT-livers (Supplementary figure2A) and PlGFKO-livers (Supplementary figure2B); CD206 in 30W DEN WT-livers (Supplementary figure2C) and PlGFKO-livers (Supplementary figure2D), and CD163 in 30W DEN WT-livers (Supplementary figure2E) and PlGFKO-livers (Supplementary figure2F). These results show a switch from an M2-phenotype towards M1-macrophages. Scale-bars = 100µm.

3.5 Monotherapy: αPlGF and sorafenib

Treatment with sorafenib or αPlGF improved survival, since 25% of the IgG-treated-mice died, while 8,3% of αPlGF and 16% of sorafenib-treated mice succumbed during the 5W of treatment. Treatment with αPlGF and sorafenib in a monotherapeutic setting significantly decreased macroscopic tumour number (αPlGF, P<0,01; sorafenib, P<0,01) (**Fig4A)** and microscopic HCC-burden (αPlGF, P<0,01; sorafenib, P<0,01) (**Fig4B, supplementary figure 3**). However, no significant difference was seen between treatments.
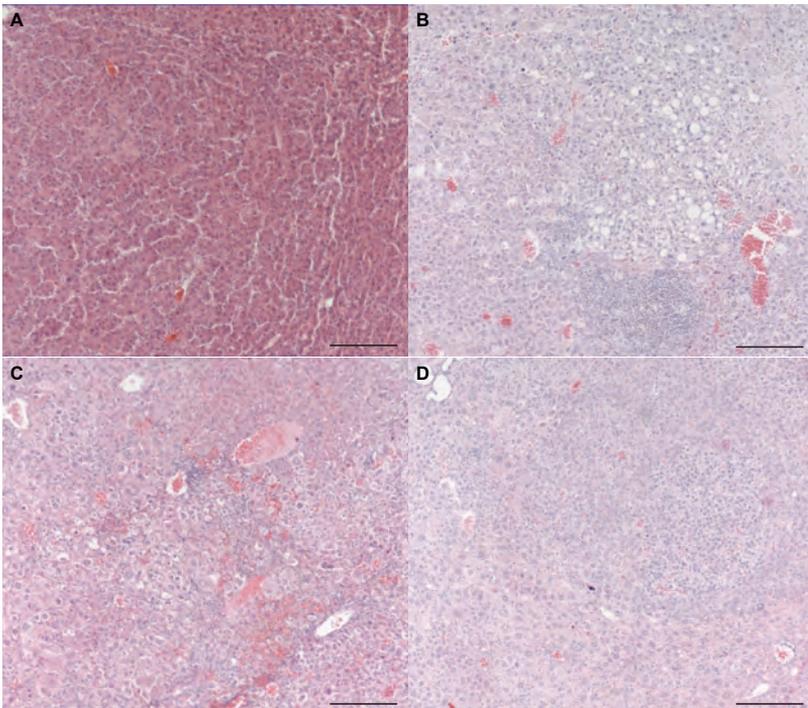
Supplementary figure 3: *H&E-stained slides of mice treated with IgG (A), αPlGF (B), sorafenib (C) and αPlGF+sorafenib (D), showing HCC-lesions.*

ELISA confirmed that VEGF-levels in serum and non-tumorous tissue were unaltered after treatment with αPlGF and sorafenib. Nevertheless, a significant downregulation of VEGF was seen in HCC-tissue treated with αPlGF (P<0,01) and sorafenib (P<0,01), (**Fig4C),** and no significant difference was seen on RNA-transcription (**Fig4D)**. There was a trend towards higher PlGF-levels in

sorafenib-treated tumours (**Fig4C)** and sorafenib-treated livers were characterised by decreased expression of *Vegf* (**Fig4D**). Endoglin-staining showed that tumours (αPlGF, P<0,05; sorafenib, not significant) and the surrounding fibrotic matrix (αPlGF, P<0,05; sorafenib, P<0,05) were less vascularised after treatment (**Fig4E),** however no significant difference was seen between treatments (**supplementary figure 5)**. Treatment with αPlGF improved the hepatic vasculature surrounding HCC-lesions (**supplementary figure 6),** while sorafenib-treated livers still had a chaotically organised vasculature, characterised by several small sprouting vessels and a the lack of hierarchic branching.
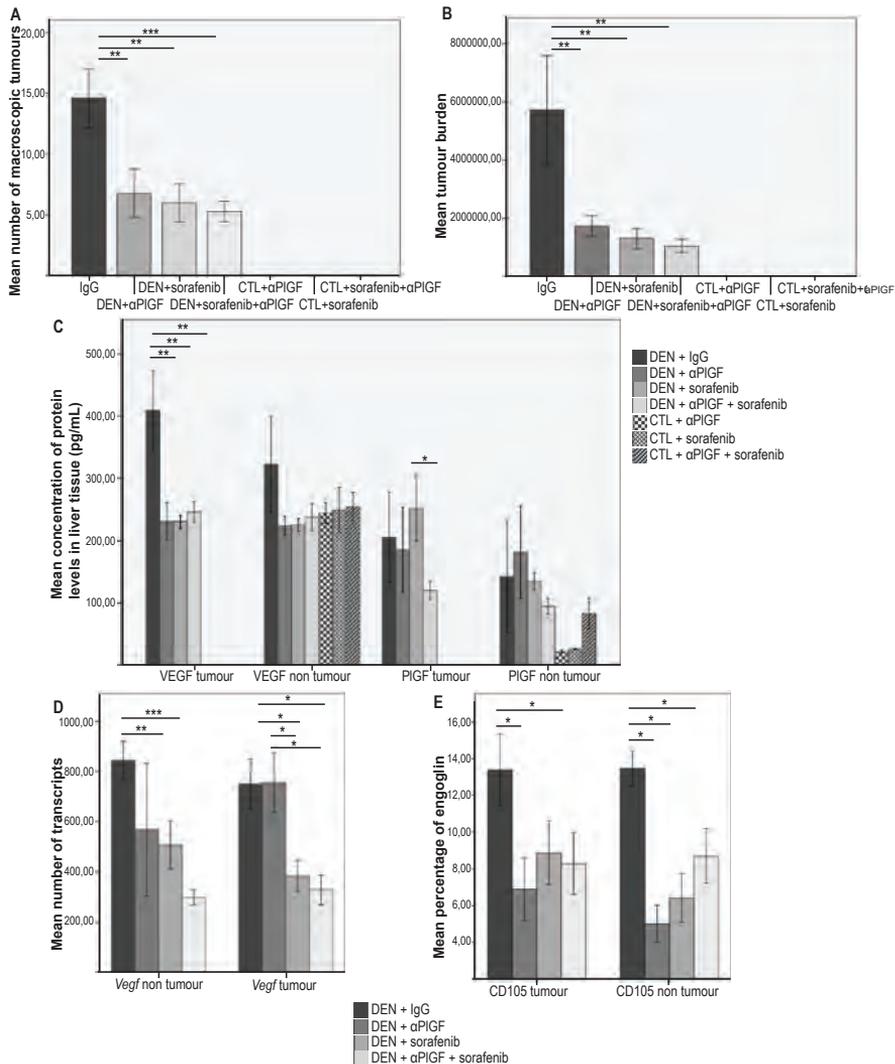
Figure 4: *Both combination and monotherapies significantly decreased macroscopic tumour number (Fig4A) and microscopic tumour burden (Fig4B). ELISA confirmed that VEGF-levels in serum and non-tumorous tissue were unaltered after treatment with αPlGF and sorafenib, however, a significant down regulation was seen in treated HCC-tissue (Fig4C), but no significant difference was seen on RNA-transcription (Fig4D). A trend towards higher expression of PlGF was seen in sorafenib-treated tumours, which was diminished in the combination treatment (Fig4C). On RNA-levels, sorafenib-treated livers had less expression of VEGF (Fig4D). Endoglin-staining showed that tumours and the surrounding fibrotic matrix were less vascularised in the treatment-regimes (Fig4E). Asterisks represent p-values (* = P<0,05, ** = P<0,01 and *** = P<0,001)*
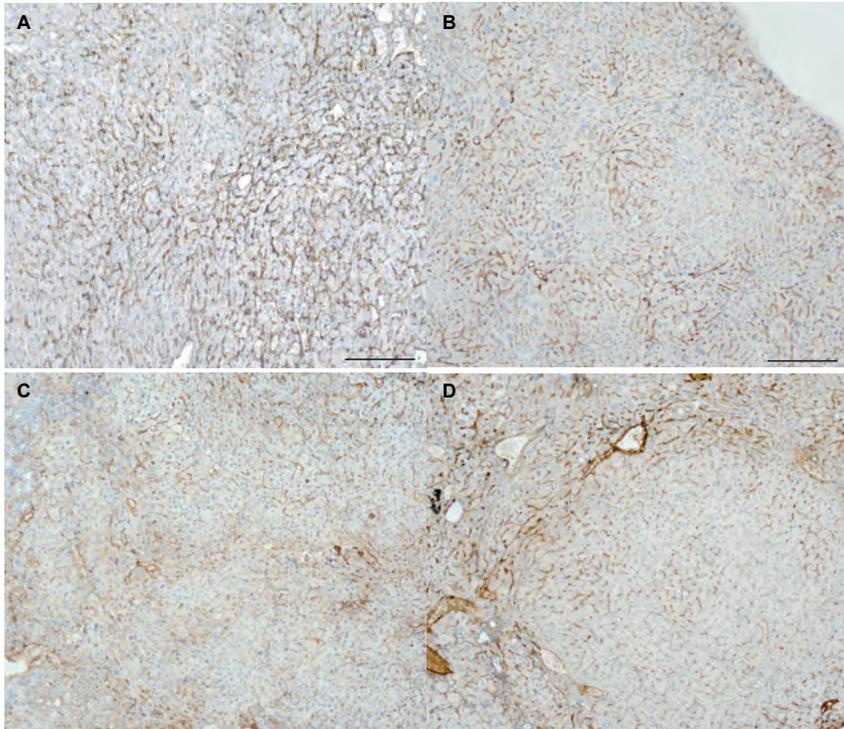
Supplementary figure 4: *Endoglin-stained slides of IgG-treated (A), αPlGF-treated (B), sorafenib-treated and αPlGF+sorafenib-treated livers, showing that treatment with αPlGF and sorafenib decreased HCC-vascularisation.*

Sorafenib caused a significant upregulation of the hypoxic marker *Pfk* (P<0,05) and a trend towards higher *Glut1*-expression **(Fig5C)**. There was a significant increase of HIF1α in the surrounding tissue and in tumour tissue of mice treated with sorafenib, compared to those treated with αPlGF (non-tumour: P<0,05; tumour P<0,01) **(Fig5D)**. Treatment with αPlGF also significantly decreased tumoural hypoxia compared to IgG-controls (P<0,01). The metastatic marker *Mmp9* was upregulated (P<0,05) in the tumour's surrounding compared to IgG-treated mice. Other metastatic markers were not altered (**Fig5E-F**). F4/80-staining revealed a significantly lower macrophage infiltration in surrounding non-tumour tissue (αPlGF, P<0,05; sorafenib P<0,05), and a lowered trend inside HCC-lesions (**Fig6A**). The M2-marker CD206 was significantly decreased in surrounding and tumour tissue of αPlGF-treated mice,

compared to IgG-controls. The M1-marker HLA-DR was significantly increased in αPlGF-treated tumours (P<0,05). No differences were seen on overall macrophage recruitment or HSC-activation between treatments. However, treatment with αPlGF resulted in a significantly increased number of HLA+-macrophages in the surrounding (P<0,05) and tumour (P<0,055) tissue compared to sorafenib (**Fig6B-C**). Furthermore, less activated HSC were found in tumour (αPlGF, P<0,01; sorafenib, P<0,01) as in non-tumour tissue (αPlGF, P<0,05; sorafenib, P<0,01). Inhibition of PlGF slightly improved fibrosis, however not significantly (**Fig6D**).
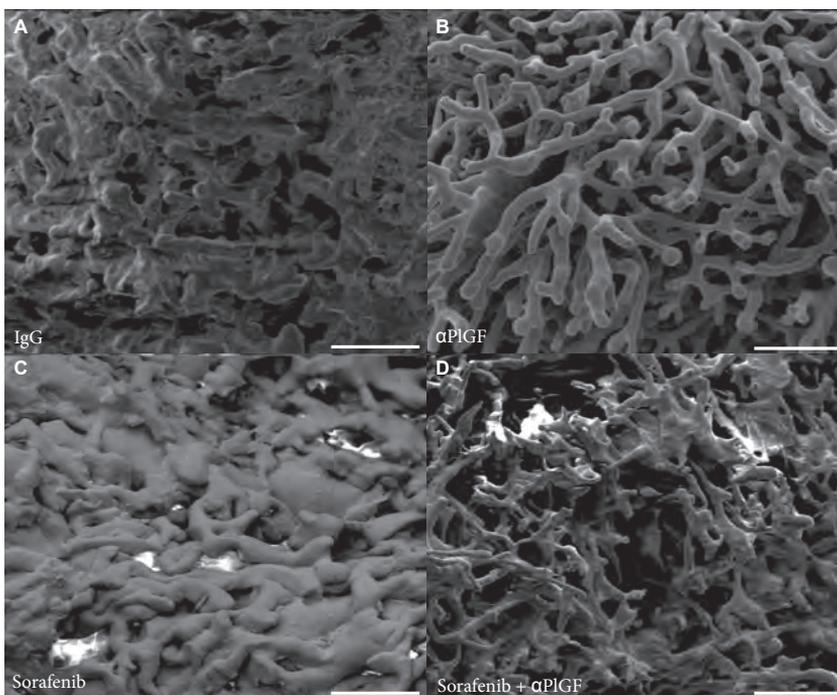
Supplementary figure 5: *SEM-images showing the microvasculature of IgG (A), αPlGF (B), sorafenib (C) and sorafenib+αPlGF (D) treated peritumoural vessels.*

Microarrays showed that αPlGF-treatment significantly altered expression-levels 129 gene-probes in surrounding tissue (**Fig5A**) and of 115 gene-probes in tumour tissue (**Fig5B**). Genes involved in tumour development, angiogenesis and metastasis were selected for further analysis (**table 1**). The angiogenic genes *Sema3g, Sds, Igfbp2, Ccl28* and *Apln* had a decreased expression in

tumour tissue of αPlGF-treated mice compared to IgG-treated mice with HCC (p<0,001). Furthermore, surrounding liver tissue of αPlGF-treated mice had a lower expression of the pro-angiogenic genes *Kdr*, *Thsd7a* and *Prcp* (p<0,001). Both in tumour and surrounding tissue, no alternative angiogenic pathways were upregulated after treatment with αPlGF (**table 1**). 15 genes in tumour and 14 genes in surrounding tissue that are involved in tumour progression and had a different expression between αPlGF- and IgG-treated mice. Most of these alterations showed that αPlGF could decrease the hepatocarcinogenic process (**table 1**). The metastatic suppressor *Cd82* was significantly upregulated in αPlGF-treated tumours (P<0,001), whereas pro-metastatic genes *Actc1, Ptprb, Gjc1* and *Igfbp2* were significantly downregulated (P<0,001) compared to IgG-treated mice. This decrease of the pro-metastatic niche was also found in the surrounding tissue of αPlGF-treated mice, where the pro-metastatic gene *Ptprb* was downregulated and the metastatic-suppressor gene *Klf17* was upregulated. However, the metastatic-suppressors *Erdr1* and *Akap12* were significantly downregulated and the pro-metastatic gene *Camk1d* was overexpressed in αPlGF-treated tumours (P<0,001) (**table 1**).

Analysis of the GSE33621-dataset showed that the metastatic suppressor *Kiss1* was significantly downregulated (P<0,001) and the pro-metastatic gene *Mta1* was significantly upregulated (P<0,001) after treatment with sorafenib. Furthermore, expression of *Bad* was downregulated (P<0,001). Sorafenib suppressed tumour progression by downregulating *Pkb-alpha, Il-8, Muc18* and *P110beta* (P<0,001), however three important tumour suppressor genes *Rb1, P27Kip1* and *Casper/Flip,* were also significantly downregulated.

A Surrounding: IgG vs αPlGF

B Tumour: IgG vs αPlGF

C

Legend:
- DEN + IgG
- DEN + αPlGF
- DEN + sorafenib
- DEN + αPlGF + sorafenib

Glut1 non tumour   *Glut1* tumour   *Pfk1* non tumour   *Pfk1* tumour

Mean number of transcripts

D

Legend:
- DEN + IgG
- DEN + αPlGF
- DEN + sorafenib
- DEN + αPlGF + sorafenib
- CTL + αPlGF
- CTL + sorafenib
- CTL + αPlGF + sorafenib

Mean % IHC-staining

HIF1α non tumour   HIF1α tumour

E Non tumour

Mean number of transcripts

Mmp9   *Itgav*   *Icam*

F tumour

Mean number of transcripts

Mmp9   *Itgav*   *Icam*

Legend:
- DEN + IgG
- DEN + αPlGF
- DEN + sorafenib
- DEN + αPlGF + sorafenib

156

Figure 5 (previous page): **hypoxia and metastasis in the treatment study.** *Gene expression profiling revealed that treatment with αPlGF significantly altered the expression of 115 gene-probes in the tumour tissue (Fig5A) and 129 gene-probes in the surrounding non-tumour tissue (Fig5B). Treatment with sorafenib caused a significant upregulation of the hypoxic marker PFK (P<0,05) and a trend towards higher Glut1 expression (Fig5C). HIF1α-staining showed a significant decrease in PlGF-deficient livers compared to those treated with sorafenib (Fig5D). The pro-metastatic marker MMP9 was significantly upregulated in the tumour's surrounding compared to IgG-treated mice, however no significant difference was seen between the other tested metastatic markers (Fig5E-F). Asterisks represent p-values (* = P<0,05, ** = P<0,01 and *** = P<0,001).*

	Gene	aFGF	IgG	p-value	Function
Tumour tissue	Cd82	11,32262362	10,3205116	5,83E-06	Anti-metastatic
	Actc1	2,94360859	5,746879282	0,000264959	Cell motility
	Ptprb	2,941555092	4,4147304	0,000719374	Cell spreading and migration
	Gjc1	1,969745953	4,10946648	3,40E-05	Pro-metastatic
	Erdr1	10,68560564	12,32152879	0,000532221	Anti-metastatic
	Akap12	7,800377961	9,103115799	0,000155914	Anti-metastatic
	Camk1d	10,46507258	8,417571631	0,000817848	Pro-metastatic
	Igfbp2	11,96987373	14,91463022	0,000407379	Pro-metastasic and pro-angiogenic
	Sema3g	3,317762963	6,087361833	0,000641693	Interacts with neuropilin
	Sds	2,319141919	5,645935511	0,00025411	Associated with hypoxia
	Ccl28	1,768524008	3,56918628	0,000715052	Chemoresistance and pro-angiogenic
	Apln	3,386074216	5,718111483	0,000252562	Vascularisation and tumour growth
	Saa2	16,43385725	12,94100384	0,000790312	Inflammatory
	Saa1	16,50476705	13,1982873	0,000718441	Inflammatory
	Hist1h1c	12,58495171	11,17582426	0,000786575	Cel growth
	Csrp3	10,10254127	7,965980017	2,95E-05	Cell differentiation
	Fryl	2,147350179	3,935702291	9,17E-05	Cell fate
	Igfbp2	11,79719471	14,85805604	0,000316272	Chemoresistance and cell growth
	Lcp1	11,78188757	10,44976147	0,000485338	Expressed in tumours
	Ccdc37	2,220600282	4,34710114	0,000860019	Expressed in tumours
	Foxn2	2,272566846	3,995211378	6,98E-05	Might be involved in tumorigenesis
	Fam26f	7,517755359	5,154391333	4,31E-05	Might be involved in tumorigenesis
	Ifna12	4,630404796	3,408593743	0,000172986	Tumour suppressor
	Sfxn2	10,57364591	11,72440017	0,000128068	Tumour marker
	S100pbp	2,021279746	3,627055461	0,000181348	Tumour marker, correlated with tumour agressiveness
	Casz1	5,419481734	4,162747453	0,000346735	Tumour suppressor
	Extl1	7,964363498	6,180440917	0,000301712	Tumour suppressor
	Per1	10,12105345	11,28903258	7,89E-05	Circadian clock gene, tumour promotor
Surrounding tissue	Ptprb	2,353250134	3,948066425	0,000966469	Cell spreading and migration
	Klf17	3,66613759	2,200691406	0,000652595	Anti-metastasic
	Kdr	9,727835341	10,73885097	0,000865578	Pro-angiogenic
	Thsd7a	2,77926104	4,804056739	0,000639571	Pro-angiogenic
	Prcp	4,639719282	6,389373506	0,00054792	Pro-angiogenic
	Nsd1	10,46916694	11,47207781	0,000538778	Chromogene integrity and cellular transformation
	Taok2	2,174860245	4,075178946	1,02E-05	Upregulated during cell damage
	Dcc	15,23946929	17,04420232	0,000983316	Tumour supressor
	Ahrr	3,152750174	2,033422346	0,000691642	Tumour supressor
	Specc1	1,664845557	3,044047708	0,000591178	Tumour marker
	Brd7	7,107010451	8,122823566	0,000228486	Tumour supressor
	Zfp612	8,092251952	9,458517486	0,000135993	Might be involved in tumorigenesis
	Sdad1	7,352384829	9,140246686	0,000568129	Might be involved in tumorigenesis
	Per1	9,940612605	11,19206578	0,000126739	Circadian clock gene, tumour promotor
	Gcm1	15,04849311	17,02734644	0,000411643	Cell fusion in cancer
	Mucl1	2,883424064	1,695993993	0,00019326	Expressed in tumours
	Nkx2-4	2,971674486	1,804238408	1,13E-07	Tumour suppressor
	Homer3	1,990967395	3,691589245	0,000168722	Expressed in tumours
	Edaradd	2,942614415	1,648383944	0,000351932	Tumour supressor

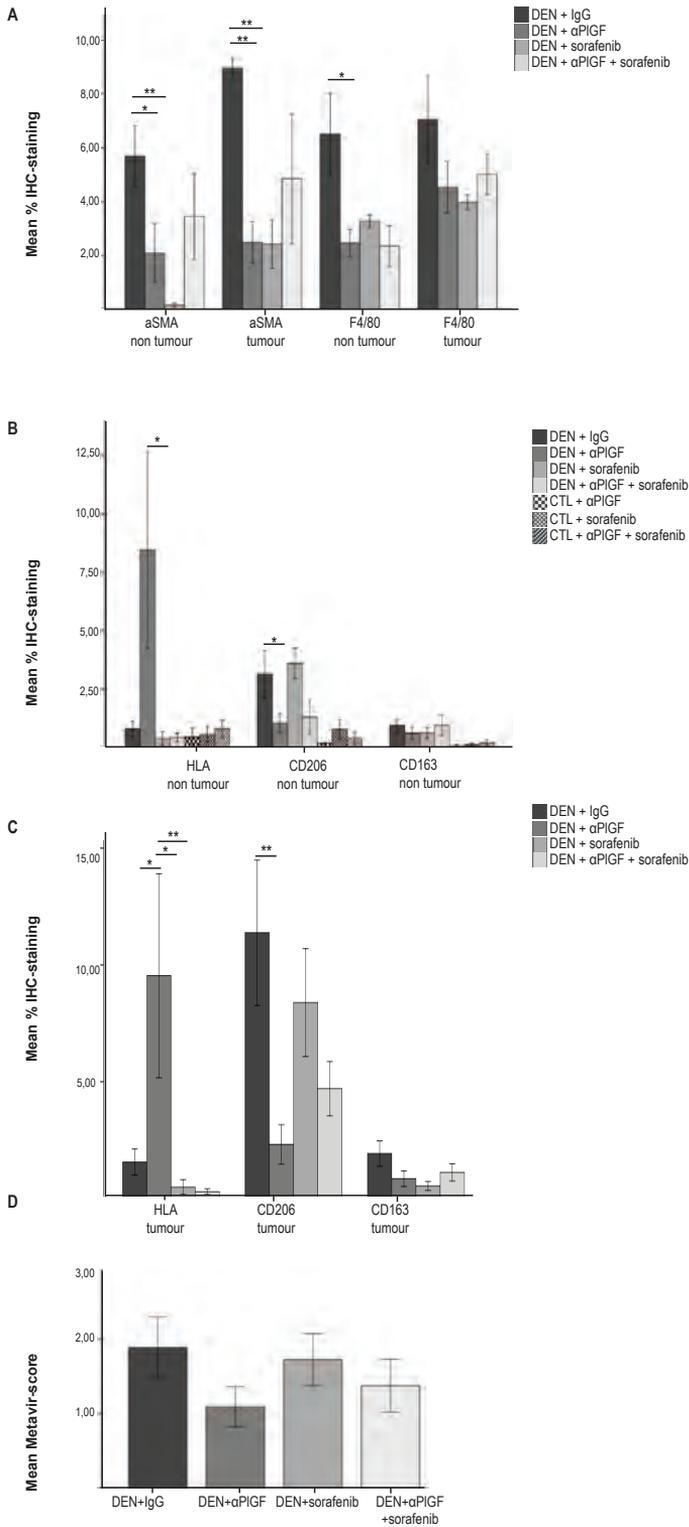**Table 1:** *Summary of the data obtained by gene expression profiling.*

3.5.1   Combination therapy: sorafenib and αPlGF

The combination-treatment improved survival, only 7,7% of mice died while receiving the combination-therapy. The combination of αPlGF and sorafenib significantly decreased macroscopic tumour number (P<0,001) (**Fig4A**) and microscopic tumour burden (P<0,01) compared to IgG-controls (**Fig4B & supplementary figure 3**). However, no significant improvement was seen between the combination and the monotherapeutic set-up. RNA-levels of *Vegf* were significantly downregulated in the combination therapy compared to the αPlGF-monotherapy and IgG-controls (**Fig4D**), yet this was not reflected in protein levels (**Fig4C**). Protein levels of PlGF were significantly downregulated in the tumour compared to mice treated with sorafenib (P<0,05) and IgG-controls (P<0,05). The αPlGF-sorafenib-combination led to a significantly decreased vascularisation of tumours (P<0,05) and surrounding (fibrotic) tissue (P<0,05), measured by endoglin-staining (**supplementary figure 5**). No significant difference was observed between monotherapy and combination-therapy (**Fig5B**). While no significant differences were seen in *Glut1* and *PfK*-transcription levels, HIF1α was significantly increased in tumours treated with the combination-therapy, compared αPlGF (**Fig5C-D**). Compared to sorafenib-treated mice, the pro-metastatic genes *Mmp-9* (P<0,05) and *Itgav* (P<0,05) were significantly decreased in the combination treatment group's surrounding tissue, and a trend towards lower *Icam* expression was observed (**Fig5E-F**). Pro-metastatic gene expression in the tumour was not altered compared to IgG-controls.

F4/80 staining showed a trend towards lower macrophage recruitment in the αPlGF-Sorafenib-combination treatment. No significant difference was seen compared to monotherapy or IgG-controls (**Fig6A**). However, the combination-treated mice had significantly less M1-macrophages in tumour tissue compared to αPlGF-treated (P<0,01) and a similar trend was seen in the surrounding tissue (**Fig6B-C).**

3.5.2   Human PlGF

PlGF-serum levels were significantly upregulated in cirrhotic patients (P<0,05) and HCC-patients (P<0,001) compared to healthy controls (**Fig6**). HCC-patients had significantly higher serum levels of PlGF compared to cirrhotic patients (P<0,05), and PlGF-levels were correlated to the presence of HCC (P<0,05; *corr* 0,41), AST/ALT (P<0,05; *corr* 0,40) and CRP (P<0,001; *corr* 0,81). Also, gene-expression-levels of *PGF* were significantly higher in HCC compared to cirrhotic (P<0,05) and non-tumour tissue (P<0,001) (**Fig7B)**. While *ENG*-expression was significantly decreased in HCC-patients (P<0,001), a significant increased was seen in the vascular marker *VWF* compared to healthy (P<0,05) and non-tumour tissue (P<0,001) (**Fig7C)**. The pro-metastatic markers *MMP9* and *ITGAV* were significantly increased in HCC-patients compared to non-tumour (P<0,001) and cirrhotic tissue (respectively (P<0,05 and P<0,01) (**Fig7D)**. However, the pro-metastatic marker *ICAM* was significantly decreased in HCC-tissue compared to surrounding non-tumour (P<0,001) and cirrhotic tissue (P<0,001). This led to a significant correlation between the RNA-levels of *PGF* with *MMP9* (P<0,001; *Corr* 0,48), *ITGAV* (P<0,001, *Corr* 0,50) and VWF (P<0,001; *Corr* 0,44*)*
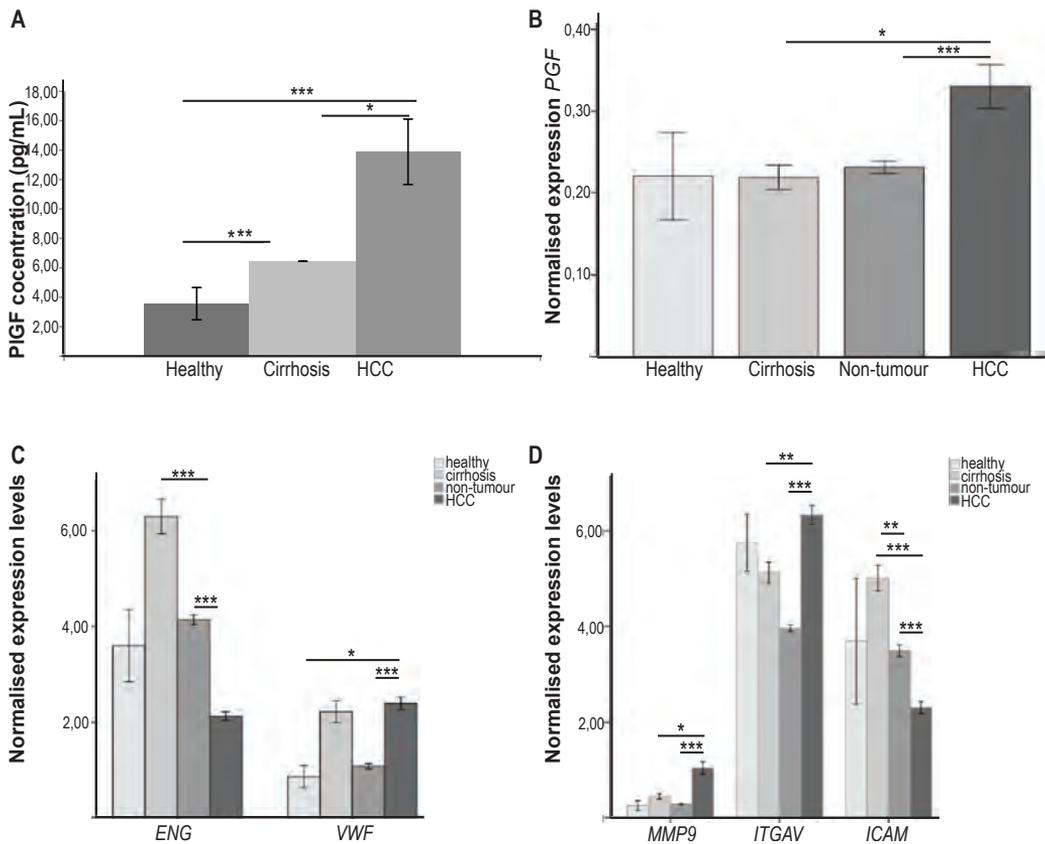
Figure 7: *Human PlGF-data. PlGF-serum levels were significantly up regulated in cirrhotic patients and HCC-patients compared to healthy controls. Asterisks represent p-values (* = P<0,05, ** = P<0,01 and *** = P<0,001). Also, gene-expression-levels of PGF were significantly higher in HCC compared to cirrhotic and non-tumour tissue (Fig7B). While ENG-expression was significantly decreased in HCC-patients, a significant increased was seen in the vascular marker VWF compared to healthy and non-tumour tissue (Fig7C). The pro-metastatic markers MMP9 and ITGAV were significantly increased in HCC-patients (Fig7D). Asterisks represent p-values (* = P<0,05, ** = P<0,01 and *** = P<0,001).*

3.6   Discussion

Anti-angiogenic treatment has opened a new era in the field of anti-cancer therapy. While most studies have focussed on inhibiting VEGF and its receptors, we have chosen to investigate the role of the VEGF-homologue PlGF, known for its sole involvement in pathologic angiogenesis, in the pathogenesis of HCC.

HCC is a solid tumour greatly depending on neovascularisation to support its growth. Therefore, anti-angiogenic treatment with sorafenib has shown substantial improvement for patients with advanced HCC. In our study, we have shown that PlGF is involved in the pathogenesis of HCC, both in mice and human. In the transgenic knock out model as well as in the treatment model, silencing or inhibition of PlGF significantly decreased tumour burden, however, blockage of PlGF did not result in a complete stop of tumour growth. PlGF-inhibition decreased vascularisation and reduced hepatic macrophage recruitment, in line with previous reports showing that PlGF is a potent attractor for endothelial cells and macrophages (20,24). Other *in vivo* and *in vitro* studies have found that PlGF exerts part of its effect by recruiting pro-angiogenic macrophages (24)(26). Although the results in our study were not always consistent (the PlGFKO-study showed a decrease of the M1-marker at 20W DEN), there was a general trend in the knock out and treatment study of a switch towards the M1-macrophage phenotype. Furthermore, we did not see an increase of M1-macrophages in mice treated with sorafenib. M1-macrophages are known to promote a tumouricidal response (30), thus this could have contributed to the decreased tumour burden in PlGF-inhibited mice.

Compared to sorafenib, αPlGF did not provoke a significant improvement on survival, tumour burden, and hepatic stellate cell activation. However, sorafenib and other VEGF-VEGFR-targeted therapies cause a number of adverse effects, including teratogenicity, pruning of healthy vessels, thrombosis, hypertension, bleeding, disrupted wound healing and skin toxicity. In our study, sorafenib-treated mice were more prone to bleeding. PlGF-expression is negligible in quiescent tissues in baseline conditions and a full knock out does not cause any noteworthy abnormalities in mice. So far, a phase I clinical trial with a humanised PlGF-antibody (TB403) has shown good safety (NCT00702494). In line with previous reports PlGF-levels of HCC-patients were

significantly upregulated compared to cirrhotic and healthy controls, confirming PlGF could play a role in human hepatocarcinogenesis (31,32). Although other studies have shown a correlation between PlGF-levels and tumour stage, we were not able to show this in our patient-population, perhaps due to the small sample size (33). However, microarray expression data from the GSE25097-dataset revealed a correlation between *PGF*-expression and the pro-metastatic markers *MMP9* and *ITGAV,* as well as vascular marker *VWF,* suggesting a possible involvement of PlGF in tumour-angiogenesis and metastasis. Yet, further analysis in larger datasets should be performed to provide additional insight into which patients would potentially benefit the most from αPlGF-treatment. Larger studies in human patients, thereby specifically focussing on those receiving anti-angiogenic treatment are needed in the future.

Even though HCC is a "hypervascular" cancer, it is characterised by hypoxia, and its growth is promoted by hypoxia (34). While hypoxic HCC cells try to improve their oxygenation by stimulating the arterial blood supply, their oxygen delivery is often impaired by the formation of abnormal tumour vessels. This initiates a reinforcing vicious circle of vascularisation and promoting malignancy. Anti-angiogenic therapy is expected to prune tumour vasculature, depriving the tumour from oxygen. The hypoxic conditions created by anti-angiogenic therapy, may select for more invasive tumour variants better adapted to survive and proliferate under reduced oxygen tension, leading to increased intravasation and metastatic dissemination (18). Furthermore, this increased hypoxia often induces an additional angiogenic switch, thereby allowing tumours to provoke angiogenic-escape mechanisms by upregulating alternative angiogenic pathways. Indeed, PlGF-inhibited tumours were characterized by a decreased infiltration of M2-like TAMs which induce vessel abnormalisation, while M1-like TAMs are not or less involved (25,36). αPlGF did not only inhibit the formation of tumour blood vessels, but also normalized remaining blood vessels as seen on the SEM-images of the vascular corrosion casts. PlGF-inhibition did not lead to an upregulation of other pro-angiogenic factors such as VEGF. A decrease of VEGF was seen in HCC-tissue treated with αPlGF, perhaps due to increased binding to the decoy receptor VEGFR1, since this was not reflected in the RNA-transcription. By switching TAMs away from the M2- to a tumour-inhibiting M1-like phenotype, PlGF-inhibition could promote anti-

tumour immune responses and vessel normalization (20,24,35). Indeed, PlGF-inhibited tumours were characterized by a decreased infiltration of M2-macrophages, which are also known to produce angiogenic factors. Thus, switching the TAMs towards an M1-phenotype could have a positive influence on the pathogenesis of HCC and could partially explain the vessel normalization and the decreased angiogenic response seen in PlGF-silenced tumours (24). However, the complex, highly heterogeneous nature of HCC makes it unlikely that targeting one pathway will achieve optimal disease control. Even though no evidence was found towards angiogenic escape, further investigation using long-term administration of PlGF-antibodies is necessary to exclude this phenomenon.

Microarray analysis showed that PlGF-inhibition possibly selects for a less metastatic tumour phenotype, possibly by normalising the vasculature and subsequently decreasing hypoxia (37). Indeed, αPlGF–treated mice had decreased expression of the pro-metastatic genes *Actc1, Ptprb, Gjc1, Ptprb* and *Igfbp2* and the increased expression of the metastatic suppressor gene *Klf17.* qPCR-analysis showed that treatment with sorafenib increased the expression of *Mmp9* and *Icam*, two pro-metastatic genes, probably due to the increased hypoxia. Analysis of the sorafenib-expression-data showed that the metastatic-suppressor gene *Kiss1* is decreased after treatment with sorafenib. However, since this DEN-model does not induce extrahepatic metastasis and not al pro-metastatic markers were consistently decreased, further investigation using more suitable metastatic models for HCC, would provide valuable insight on the influence of αPlGF and sorafenib on the metastatic potential.

Inhibition of PlGF leads to an increased expression of tumour-suppressor-genes and a decrease of tumour-promoting-genes, possibly suggesting a less aggressive tumour environment. This effect was not observed in the sorafenib-treated xenografts. Furthermore, studies have shown vessel normalisation could improve drug delivery and efficacy (38–40). Whereas analysis of the GSE33621-dataset showed that sorafenib possibly selects for a more chemoresistant phenotype by downregulating *Bad*. Thereby, treatment with αPlGF could open new therapeutic opportunities for this otherwise chemoresistent tumour. Combining αPlGF with classic chemotherapeutic agents, would hold great potential for future research.

To conclude, both in the transgenic knock out model as in the treatment model, silencing or inhibition of PlGF significantly decreased tumour burden, not only by inhibiting the vascularisation, but also by decreasing hepatic macrophage recruitment and inducing a switch towards M1-macrophage polarization. The use of αPlGF holds great potential, since it does not only inhibit the formation of tumour blood vessels, but also normalizes the remaining blood vessels, thereby decreasing hypoxia and reducing the pro-metastatic potential of HCC.

3.7 Acknowledgements

3.8 References

1. Huang M, Liu G. The study of innate drug resistance of human hepatocellular carcinoma Bel7402 cell line. Cancer letters. 1999;135:97-105.

2. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J-F, et al. Sorafenib in advanced hepatocellular carcinoma. The New England journal of medicine. 2008;359:378-90.

3. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer research. 2006;66:11851-8.

4. Lee WJ, Lee JL, Chang SE, Lee MW, Kang YK, Choi JH, et al. Cutaneous adverse effects in patients treated with the multitargeted kinase inhibitors sorafenib and sunitinib. The British journal of dermatology. 2009;161:1045-51.

5. Yang C-H, Lin W-C, Chuang C-K, Chang Y-C, Pang S-T, Lin Y-C, et al. Hand-foot skin reaction in patients treated with sorafenib: a clinicopathological study of cutaneous manifestations due to multitargeted kinase inhibitor therapy. The British journal of dermatology. 2008;158:592-6.

6.   Scholl S, Spies-Weisshart B, Klink A, Muegge L-O, Fricke H-J, Hochhaus A. Secondary resistance to sorafenib in two patients with acute myeloid leukemia (AML) harboring FLT3-ITD mutations. Annals of hematology. 2011;90:473-5.

7.   Chen K-F, Chen H-L, Tai W-T, Feng W-C, Hsu C-H, Chen P-J, et al. Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. The Journal of pharmacology and experimental therapeutics. 2011;337:155-61.

8.   Bender C, Ullrich A. PRKX, TTBK2 and RSK4 expression causes sunitinib resistance in kidney carcinoma- and melanoma cell lines. International journal of cancer. Journal international du cancer. 2011;

9.   Marijon H, Dokmak S, Paradis V, Zappa M, Bieche I, Bouattour M, et al. Epithelial-to-mesenchymal transition and acquired resistance to sunitinib in a patient with hepatocellular carcinoma. Journal of hepatology. 2011;54:1073-8.

10.   Kerbel RS, Yu J, Tran J, Man S, Viloria-Petit A, Klement G, et al. Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. Cancer metastasis reviews. 2001;20:79-86.

11.   Dempke WCM, Heinemann V. Resistance to EGF-R (erbB-1) and VEGF-R modulating agents. European journal of cancer (Oxford, England : 1990). 2009;45:1117-28.

12.   Tamaskar I, Dhillon J, Pili R. Resistance to angiogenesis inhibitors in renal cell carcinoma. Clinical advances in hematology & oncology : H&O. 2011;9:101-10.

13.   Cao Y, Zhong W, Sun Y. Improvement of antiangiogenic cancer therapy by understanding the mechanisms of angiogenic factor interplay and drug resistance. Semin Cancer Biol. 2009;19:338-343.

14.   Ebos JML, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15:5020-5.

15.   Ebos JML, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer cell. 2009;15:232-9.

16.   Höckel M, Vaupel P. Biological consequences of tumor hypoxia. Seminars in oncology. 2001;28:36-41.

17.   Brahimi-Horn C, Pouysségur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. Bulletin du cancer. 2006;93:E73-80.

18.   Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16:5928-35.

19.   Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nature medicine. 2001;7:575-583.

20.   Adini A, Kornaga T, Firoozbakht F, Benjamin LE. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. Cancer research. 2002;62:2749-52.

21.   Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell. 2007;131:463-475.

22.   Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. 2005;438:820-7.

23.   Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. The Journal of biological chemistry. 1994;269:25646-54.

24.   Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer cell. 2011;19:31-44.

25.   Qian B-Z, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141:39-51.

26.   Van de Veire S, Stalmans I, Heindryckx F, Oura H, Tijeras-Raballand A, Schmidt T, et al. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. Cell. 2010;141:178-190.

27.   Heindryckx F, Mertens K, Charette N, Vandeghinste B, Casteleyn C, Van Steenkiste C, et al. Kinetics of angiogenic changes in a new mouse model for hepatocellular carcinoma. Molecular cancer. 2010;9:219.

28. Wettenhall JM, Smyth GK. limmaGUI: a graphical user interface for linear modeling of microarray data. Bioinformatics (Oxford, England). 2004;20:3705-6.

29. Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics (Oxford, England). 2005;21:2067-75.

30. Watkins SK, Egilmez NK, Suttles J, Stout RD. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. Journal of immunology (Baltimore, Md. : 1950). 2007;178:1357-62.

31. Nagaoka S, Yoshida T, Akiyoshi J, Akiba J, Hisamoto T, Yoshida Y, et al. The ratio of serum placenta growth factor to soluble vascular endothelial growth factor receptor-1 predicts the prognosis of hepatocellular carcinoma. Oncology reports. 2010;23:1647-54.

32. Ho M-C, Chen C-N, Lee H, Hsieh F-J, Shun C-T, Chang C-L, et al. Placenta growth factor not vascular endothelial growth factor A or C can predict the early recurrence after radical resection of hepatocellular carcinoma. Cancer letters. 2007;250:237-49.

33. Xu H-X, Zhu X-D, Zhuang P-Y, Zhang J-B, Zhang W, Kong L-Q, et al. Expression and prognostic significance of placental growth factor in hepatocellular carcinoma and peritumoral liver tissue. International journal of cancer. Journal international du cancer. 2011;128:1559-69.

34. Huang G-W, Yang L-Y, Lu W-Q. Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival. World journal of gastroenterology : WJG. 2005;11:1705-8.

35. Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. Cell. 2009;136:839-851.

36. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nature reviews. Cancer. 2004;4:71-8.

37. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nature reviews. Drug discovery. 2011;10:417-27.

38. Campbell NE, Greenaway J, Henkin J, Moorehead RA, Petrik J. The thrombospondin-1 mimetic ABT-510 increases the uptake and effectiveness of

cisplatin and paclitaxel in a mouse model of epithelial ovarian cancer. Neoplasia (New York, N.Y.). 2010;12:275-83.

39.   McGee MC, Hamner JB, Williams RF, Rosati SF, Sims TL, Ng CY, et al. Improved intratumoral oxygenation through vascular normalization increases glioma sensitivity to ionizing radiation. International journal of radiation oncology, biology, physics. 2010;76:1537-45.

40.   Verreault M, Strutt D, Masin D, Anantha M, Yung A, Kozlowski P, et al. Vascular normalization in orthotopic glioblastoma following intravenous treatment with lipid-based nanoparticulate formulations of irinotecan (Irinophore C^{TM}), doxorubicin (Caelyx®) or vincristine. BMC cancer. 2011;11:124.

4 Effect of PHD2 haplodeficiency on hepatocarcinogenesis in mice.

Effect of prolyl hydroxylase domain-2 haplodeficiency on hepatocarcinogenesis in mice

F. Heindryckx, A. Kuchnio, C. Casteleyn, S. Coulon, K. Olievier, I. Colle, A. Geerts, L. Libbrecht , I. Colle & H. Van Vlierberghe (2012)

4.1   Abstract

Background and Aims: The two major primary liver cancers in adults are hepatocellular carcinoma and cholangiocarcinoma. These tumours rapidly outgrow their vascular supply and become hypoxic, resulting in the production of hypoxia inducible factors. Recently, interest has grown in the regulators of these factors. Several reports have been published describing the role of prolyl hydroxylase domains – the key oxygen sensor responsible for the degradation of hypoxia inducible factors – in tumour progression and vascularisation. The effect of prolyl hydroxylase domain 2 on the pathogenesis of liver cancer has never been studied. *Methods:* a diethylnitrosamine induced mouse model was used in this study, allowing primary hepatic tumours to occur as a result of chronic liver damage. Several parameters of prolyl hydroxylase domain 2-haplodeficient mice were compared to those of wild type-mice, thereby focussing on the expression of angiogenic factors and on hepatic progenitor cell activation and differentiation. *Results:* this study shows that inhibiting prolyl hydroxylase domain 2 increases the hepatocarcinogenesis and stimulates the development of cholangiocarcinoma. Furthermore, PHD2-deficiency and the accompanying continuous HIF-activation, selected for a more metastatic tumour phenotype. *Conclusion:* The effect of prolyl hydroxylase domain 2-deficency on the hepatocarcinogenesis hold a great potential for therapeutic intervention, since hypoxia and the selection for a more aggressive cholangiocarcinoma-phenotype might also have a repercussion on patients receiving long-term treatment with anti-angiogenic compounds.

4.2   Background

Hepatocellular carcinoma (HCC) is a primary malignancy that mostly emerges on a background of chronic liver diseases. This process is characterised by increased hypoxia due to the formation of fibrotic septa, as well as sinusoidal capillarisation, resulting in an increased resistance to blood flow and decreased oxygen delivery [1]. Furthermore, HCC is a fast growing tumour that rapidly exceeds its blood supply and becomes hypoxic. Through the expression of the hypoxia inducible factor (HIF), several angiogenic factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), are regulated. HIF is tightly controlled by prolyl hydroxylase domain (PHD)

molecules, and particularly PHD2 plays an essential role as the key oxygen sensor [2]. Proline hydroxylation regulates the stability of the α-subunits of HIF. In the event of hypoxia, PHD2 is unable to hydroxylate HIF, preventing its degradation and inducing an angiogenic switch. This leads to an uncoordinated production of angiogenic factors, inducing a poorly functional vasculature incapable of meeting tumoural oxygen demands.

The main rationale behind the intensive interest in prolyl hydroxylases is their potential to serve as therapeutic targets. Recent studies have highlighted the importance of PHD2 in tumorigenesis [3–5] and shown a differential role of PHD2 in tumoural and stromal compartments. Some studies suggest that PHD2-haplodeficiency normalises vessel morphology, therefore improving tumour perfusion and oxygenation, resulting in a less aggressive tumour phenotype [5]. However, this is in contrast to other findings describing an increased capillary density, vessel branching and an increased tumour growth as a result of PHD2 silencing in the tumour environment [3]. Furthermore, studies in pancreatic cancer have shown that PHD2 serves as a potential tumour inhibitor [6] and results in glioma tumour cells support the positive effect of PHD2-expression on mice survival [4]. Most of these studies use different mouse models to assess the effect of PHD2-inhibition on tumour development, which might explain part of the discrepancies [7] and also the distinct role of PHD2 in specific tumour compartments will influence the outcome. The effect of PHD2-deficiency on the development and progression of primary liver tumours, has never been studied. Therefore, we used a DEN-induced mouse model and compared PHD2-heterozygous knock out mice with their wild type (WT) counterparts, thereby focusing on angiogenesis, tumour development and tumour phenotype.

4.3   Material and methods

4.3.1   HCC induction

5-week-old male mice received intraperitoneal injections once per week with DEN (35 mg/kg bodyweight) diluted in saline, as previously described [8]. PHD2$^{+/-}$-mice were obtained from the Vesalius Research Centre (KULeuven, Belgium). A heterogenic couple was used for breeding and the offspring was

genotyped. The Ethical Committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocols.

4.3.2 Sampling & histology

8 mice per group were sacrificed by isoflurane (Forene®, Abott, Wavre, Belgium) anaesthesia while blood was obtained from the ophthalmic venous sinus. After macroscopic evaluation and quantification of the number of tumours, all organs were sampled as previously described [8,9]. Tumour-lesions and non-tumour-tissue were separately collected. Haematoxilin-eosin staining was performed to evaluate the morphological changes and to identify HCC and CC-lesions. Sirius Red was used to stain CC-lesions and was carried out to score CC-burden, which was also staged by distinguishing normal bile ducts (stage 0), pre-malignant cholangioma (stage 1), mixed cholangioma-cholangiocarcinoma (stage 2) and cholangiocarcinoma (stage 3), on haematoxilin-eosin-stained slides as previously described [10]. An experienced pathologist used histological characteristics of haematoxylin-eosin-stained slides and immunohistochemistry to distinguishing HCC and CC. Pre-malignant hepatocellular lesions were separated from HCC using the absence reticulin and micropathologiccal characteristics as diagnostic criteria [11]. Fibrosis was quantified using Metavir-score on Sirius Red-stained slides [12,13], analysing 5 ROI's per slide. Stainings were done using standard histology protocols and blindly evaluated by an experienced pathologist.

4.3.3 Protein levels

Immunohistochemistry was used to quantify protein levels inside hepatic tumours and in surrounding non-tumour tissue. Immunohistochemical staining protocols for HPC-markers (CK19, LIF and Oct4) [14], angiogenesis (CD105 and VEGF), macrophage recruitment (F4/80) and hypoxia (HIF1α) are provided in **supplementary table 1** and were quantified as previously described [13]. Intercapillary distance (ICD) was used as a marker for microvessel density, by measuring the average distance between vessels in HCC-nodules on CD105-stained slides. Protein levels of PlGF (R&D Biosystems, Mouse PlGF-2

Quantikine ELISA Kit, Abingdon, UK) and VEGF (R&D Biosystems, Mouse VEGF Quantikine ELISA Kit, Abingdon, UK) were measured in liver tissue.

	Antigen retrieval		Peroxidase	Blocking		Primary Antibody		Secundary Antibody		Buffers
	Product (reference)	Time		Product (dilution)	Time	Product (ref) dilution	Time	Product (ref) dilution	Time	
VEGF	Trypsin	7' (37°C)	15' 3% H2O2	Goat serum (1/5)	45' (RT)	Rabbit polyclonal anti-VEGF (sc-152, Santa Cruz) 1/250	18h (4°C)	Goat anti-Rabbit (P0448, Dako) 1/300	45' (RT) + Vectastain kit	TNB
CD105	Citrate	30 ' (99°C)	15' 3% H2O2	BSA (0,1%)	30' (RT)	Goat polyclonal anti-Endoglin (AF1320, R&D systems) 1/50	18h (RT)	LSAB-kit (K0690, Dako)	Manufacturers protocol	TBS
HIF1-alpha	Citrate	30' (99°C)	15' 3% H2O2	BSA (1%)	30' (37°C)	Rabbit polyclonal anti-HIF1alpha (sc-10790, Santa Cruz) 1/400	1h (37°C)	LSAB-kit (K0690, Dako)	Manufacturers protocol	PBS
F4/80	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	Rabbit serum (1/5)	45' (RT)	Rat monoclonal anti-F4/80 (MCA497, Serotec) 1/300	18h (RT)	Rabbit anti-Rat (E0468, Dako) 1/300	45' (RT) + Vectastain kit	TNB
CK19	Proteinase K (S302080, Dako)	10' (RT)	15' 3% H2O2	BSA (2%)	30' (RT)	Rat anti-TROMA III (DSHB) 1/500	1,5h (RT)	Rabbit anti-Rat (E0468, Dako) 1/300	45' (RT) + Vectastain kit	PBS
LEF-1	Proteinase K (S302080, Dako)	10' (RT)	15' 3% H2O2	BSA (2%)	15 ' (RT)	Goat polyclonal anti-Lef1 (sc-8592, Santa Cruz) 1/200	18h (RT)	LSAB-kit (K0690, Dako)	Manufacturers protocol	PBS 0,1% BSA 0,01% Tween20
Oct4	Proteinase K (S302080, Dako)	10' (RT)	15' 3% H2O2	BSA (2%)	15 ' (RT)	Rabbit polyclonal anti-oct4 (ab18976, Abcam) 1/50	18h (4°C)	Goat anti-Rabbit (P0448, Dako) 1/50	45' (RT) + Vectastain kit	PBS 0,1% BSA 0,01% Tween20

Supplementary table 1: *immunohistochemistry protocols*

4.3.4   Quantitative Real Time PCR (qPCR)

RNA was extracted from 20mg frozen liver tissue (macroscopic tumour or non-tumour) preserved on RNA-latter, following manufacturer's guidelines (Qiagen, RNeasy Mini Kit, Venlo, Netherlands) and diluted to a concentration of 100ng/µl. RNA-Quality was evaluated using spectrophotometry. cDNA was obtained from 10µl RNA with the iScript cDNA Synthesis Kit using random hexamere primers, according to manufacturer's protocol (Bio-Rad, Eke, Belgium). Expression was measured using qRT-PCR, with 3µl cDNA and the LightCycler 480 Green I Master Mix (Roche, Vilvoorde, Belgium). Primers are shown in **supplementary table 2.**

Gene name (ID)	Forward Primer	Reverse Primer
Hprt (15452)	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT
Hmbs (15288)	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG
Sdha (66945)	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT
Gapdh (14433)	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA
Notch2 (18129)	ATGTGGACGAGTGTCTGTTGC	GGAAGCATAGGCACAGTCATC
Jag2 (16450)	CCAAAGTGGACAACCGCGCC	CTGGCTATTCCCTGCCAGCACG
PFK1 (18641)	GCCGGCTCAGTGAGACAAG	TGGCACCTTCAGCAACAATG
Glut1 (20512)	GCTTAT GGGCTTCTCCAAAC	GTGACACCTCTCCCACATAC
Endoglin (13805)	CCACCGGCCATGAACTTGTCCC	AGTGGGGTGAGGAGATCCCAAGG

Supplementary table 2: *qPCR primers*

4.3.5   Human gene expression

Gene expression data of HCC- and CC-lesions were obtained from the GSE15765 dataset [15]. 70 HCC-patients and 13 CC-patients were included and expression of the Notch-pathway was assessed using robust multichip averages (RMA).

4.3.6   Vascular corrosion casting

Vascular corrosion casts were created by perfusing Batson n°17 (Polysciences ref: 07349, Eppelheim, Germany) through the *aorta abdominalis* and *vena ileocolica*; followed by dissolving soft tissue in 25% KOH. Casts were evaluated with scanning electron microscopy (Jeol JSM5600LV, Zaventem, Belgium).

4.3.7   Statistics

Data were analysed with SPSS16. After testing for normality and homoscedasticity, data were either subjected to a student's t-test or a Mann-Whitney-U test. P<0.05 was considered statistically significant.

4.4   Results

4.4.1   Macroscopic evaluation

No significant difference was seen in mortality between PHD2-inhibited mice and WT (**Fig. 1.**). Nevertheless, while WT-mice displayed a steady increase in mortality that starts early in the experiment, in the PHD2$^{+/-}$-group 80% of the mice died in the last 5 weeks of the experiment. PHD2$^{+/-}$-mice suffered from a significantly increased weight loss compared to their WT-counterparts at 20W (P<0,01) and 25W (P<0,001) (**Fig. 1.**). Furthermore, there were an increased number of macroscopic liver tumours after 25W (P<0,05) (**Fig. 1.**); in PHD2-inhibited mice and at 30W this resulted in a higher liver weight (P<0,05) (**Fig. 1.**). No ascites was present and no extrahepatic metastases were observed.
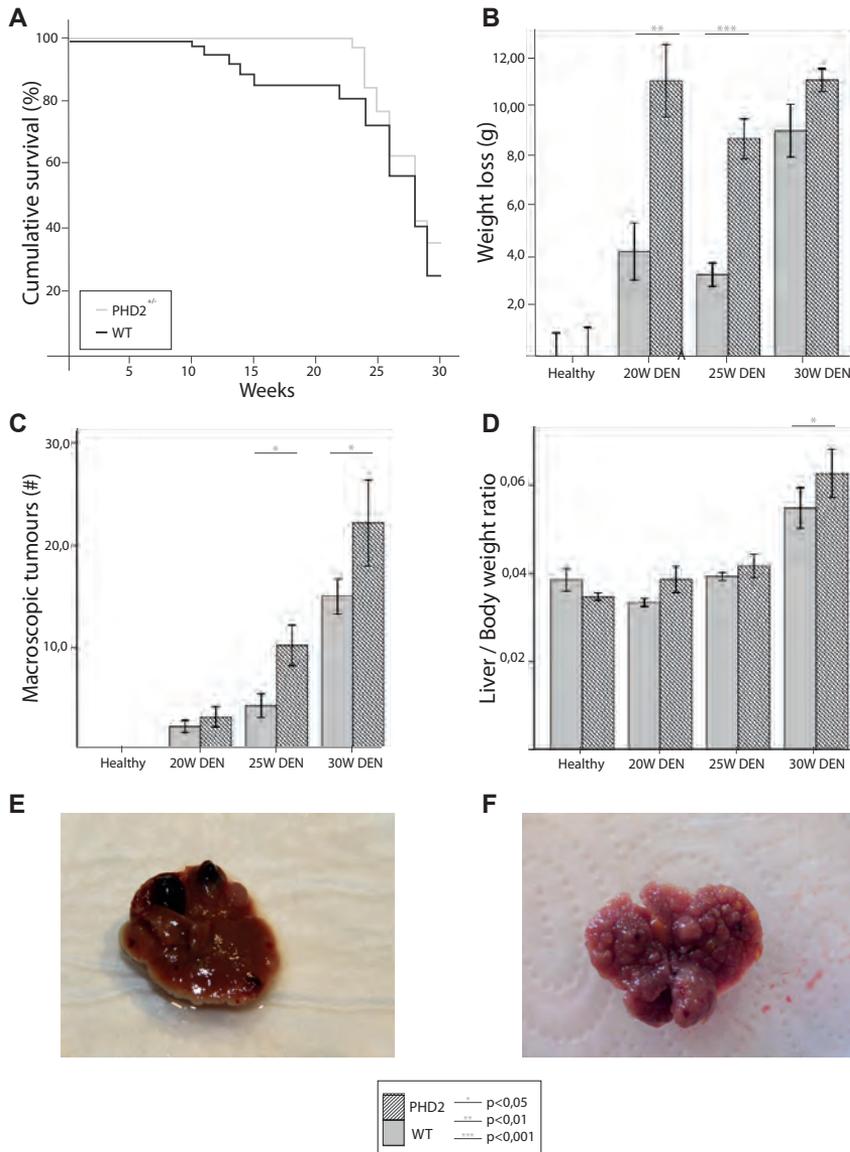
Fig. 1.: *Macroscopic parameters. Mortality of PHD$^{2+/-}$ mice and WT-mice injected with DEN did not yield a significant difference. However, a sudden increase of mortality of PHD2$^{+/-}$ mice is seen in the last 5 weeks (A). PHD2$^{+/-}$ mice suffered from more weight loss compared to WT-mice, suggesting that PHD2-deficiency negatively influenced the mice's wellbeing (B). The number of macroscopic liver tumours was significantly increased in PHD2$^{+/--}$ mice after 25W and 30W DEN (C). The liver to bodyweight ratio, was significantly increased after 30W DEN in PHD2$^{+/-}$-mice, correlating with tumour burden (D). Livers of WT (E) and PHD2$^{+/-}$ (F) mice after 30W DEN.*

4.4.2 Histological evaluation

Microscopic analyses revealed that tumours in WT-mice are predominantly HCC, while PHD2$^{+/-}$-mice are characterised by both HCC and CC (**Fig. 2.**). Starting from 20W DEN all PHD2$^{+/-}$-mice had developed pre-malignant dysplastic nodules and HCC-burden was significantly increased at 20W and 25W DEN (**Fig. 2.**). Interestingly, dysplastic foci were predominantly of the small-cell type, yet large dysplastic hepatocytes were also found in both groups. No vascular invasion was seen in tumour lesions. Sirius Red showed there was a significant increase of CC-lesions at 25W and 30W (**Fig. 2.**). Evaluation of CC-stage revealed that WT-mice occasionally displayed pre-malignant cholangioma-lesions at 25W and 30W DEN (**Fig. 2**.). At 20W DEN, PHD2$^{+/-}$-livers showed several biliary hyperplastic and cholangioma-lesions, lined by flattened epithelium (stage 1) (**Fig. 2.**), suggesting a pre-malignant state (**Table 1)**. These progressed further at 25W DEN to a mixed occurrence of cholangioma and CC, characterised by the appearance of some goblet-like cells as well as flattened epithelium and biliary dysplasia (stage 2) (**Fig. 2**.). After 30W DEN several CCs are present, tumour cells remain heterogeneous even within the same gland but often display an intestinal goblet cell like phenotype (stage 3) (**Fig. 2.**). Percentages of malignant and pre-malignant lesions from all groups are provided in **table 1.**

	Dysplastic nodules	HCC	Cholangioma	Cholangiocarcinoma
20W DEN WT	86 %	14 %	0 %	0 %
20W DEN PHD2$^{+/-}$	100 %	43 %	43 %	14 %
25W DEN WT	100 %	88 %	13 %	0 %
25W DEN PHD2$^{+/-}$	100 %	100 %	47 %	50 %
30W DEN WT	100 %	100 %	20 %	0 %
30W DEN PHD2$^{+/-}$	100 %	100 %	100 %	75 %

Table 1: percentages of pre-malignant HCC-lesions (dysplastic nodules), HCC-lesions, pre-malignant CC-lesions (cholangioma) and CC-lesions in mice treated with DEN.
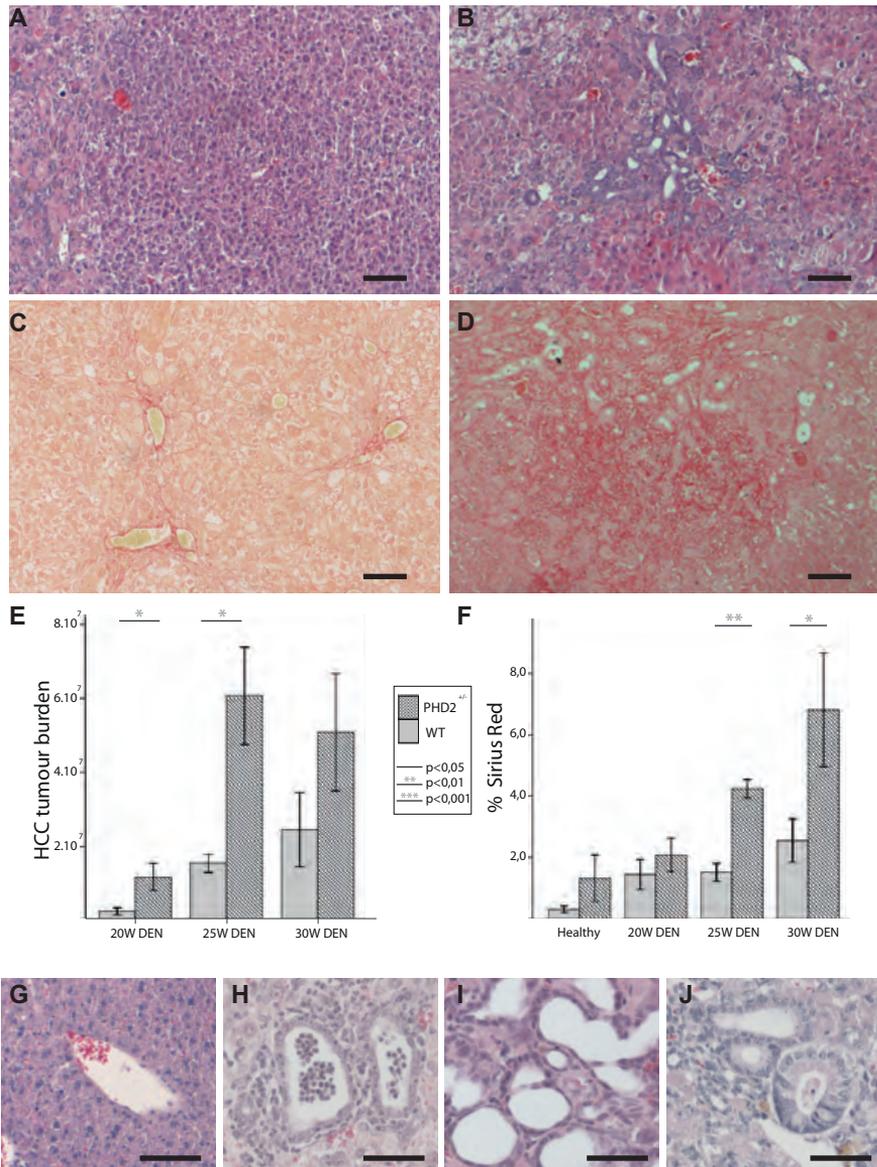
Fig. 2.: *H&E-staining of WT (A) and PHD2$^{+/-}$-livers (B) respectively showing HCC-lesions and CC-lesions. Sirius Red staining showing some fibrotic septa in WT-mice (C) and a CC-lesion in PHD2$^{+/-}$ mice (D). HCC-burden was significantly increased in PHD2$^{+/-}$-mice after 20W and 25W of DEN (E). Percentage of Sirius Red showed that PHD2-deficiency significantly increased CC-burden (F). Staging of CC happened as followed: stage 0 = healthy biliary ducts (G); stage 1 = ductular reaction and cholangioma with flattened epithelium (H), stage 2 = lesions characterised some goblet-like cells but also flattened epithelium (I) and stage 3 = cholangiocarcinoma (J). Scale-bars = 100µm.*

HIF1α staining was significantly increased in PHD2^{+/-}-livers and expression data of *Pfk* and *Glut1*, confirmed that PHD2-inhibition increased the activation of the HIF-pathway (**Fig. 3.**). PHD2-inhibition did not significantly alter VEGF-levels in liver tissue, but decreased the production of PlGF in liver tissue. PlGF-levels were significantly lower (P<0,05) in surrounding non-tumorous tissue of healthy and 25W DEN PHD2^{+/-} compared to WT, yet at 20W and 30W a trend towards higher PlGF-levels was seen (**Table 2**). No PlGF was measured in PHD2^{+/-}-tumours, while a time dependent increase was noted in WT-tumours, differing significantly at 30W DEN (P<0,05). aSMA staining showed that PHD2^{+/-}-mice had less activated HSC in HCC-lesions after 25W of DEN**.** The CC-lesions were characterised by a high activation of HSCs (not shown). No significant difference was seen in Metavir-score.

	Concentration of **PlGF** in tumour					Concentration of **PlGF** in non tumour tissue				
	WT		HE			WT		HE		
	Mean	SEM	Mean	SEM	P	Mean	SEM	Mean	SEM	P
Healthy	.	.	.	.		6,67	1,83	1,33	0,65	*
20W DEN	11,82	2,68	12,22	4,67		10,39	5,31	29,08	11,46	
25W DEN	101,27	46,92	25,19	9,17		66,35	31,59	11,85	4,93	*
30W DEN	1497,04	264,28	20,37	9,25	*	73,33	22,02	106,29	50,63	
	Concentration of **VEGF** in tumour					Concentration of **VEGF** in non tumour tissue				
	WT		HE			WT		HE		
	Mean	SEM	Mean	SEM	P	Mean	SEM	Mean	SEM	P
Healthy						110,39	10,49	106,77	8,13	
20W DEN	144,86	23,80	106,79	20,78		116,25	6,30	68,24	9,86	*
25W DEN	145,96	31,00	139,61	14,65		143,79	18,32	118,15	19,16	
30W DEN	202,31	16,39	173,88	15,50		106,87	20,52	105,33	10,23	

Table 2: Protein levels of PlGF and VEGF in tumour and non-tumour tissue of PHD2^{+/-} and WT-mice injected with DEN.

F4/80-staining showed macrophage recruitment was significantly increased in non-tumour tissue of the PHD2$^{+/-}$-groups after 20W and 25W DEN (P<0,05) (**Fig. 3.**). HCC-lesions occurring after 30W DEN in the PHD2$^{+/-}$-mice were characterised by higher macrophage infiltration compared to similar WT-lesions (P<0,05). In CC-lesions there was also a high F4/80-expression (not shown). PHD2$^{+/-}$-tumours were characterised by a higher microvessel density (20W, P<0,01; 30W P<0,05) determined by the ICD (**Fig. 3.**) and confirmed by CD105-staining in tumour tissue (**Fig. 3.**). Scanning electron microscopy images of the vascular corrosion casts and histological evaluation of the number of abnormal vessels on CD105-stained slides revealed that PHD2-inhibition did not improve the vessel morphology after 30W DEN (**Fig. 3.**). On the contrary, PHD2$^{+/-}$-livers were characterised by more vascular buds in the microvasculature and a more irregular organisation (**Supplementary Fig. 1**), supporting the increased vascularisation in CD105-stained slides (**Supplementary Fig. 2)**.
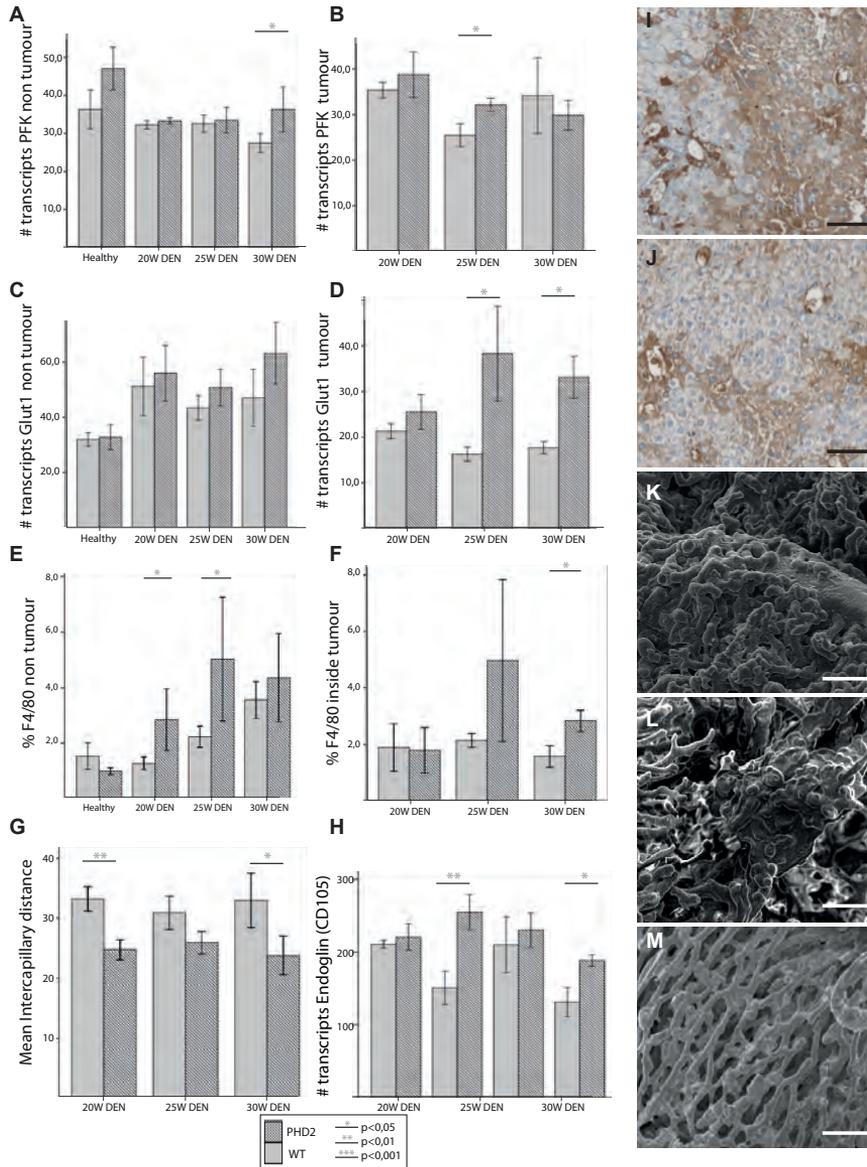
Fig. 3.: *PFK-expression was significantly increased in non-tumoural (A) and tumour (B) tissue of PHD2+/-. A trend towards higher Glut1-expression was seen in non-tumoural (C) and a significant upregulation was seen inside tumours (D) of PHD2+/-mice. F4/80 showed increased macrophage recruitment inside HCC (E) and in non-tumoural tissue (F) of PHD2+/--mice. ICD showed that HCC-lesions of PHD2+/- were more vascularised (G), confirmed by expression of CD105 in tumour tissue (H). PHD2+/- (I) and WT (J) livers, stained for HIF1α. SEM-images of PHD2+/- (K) and WT (L) and healthy livers (M), showing no vascular normalisation. Black scale bars = 100µm, white scale bars = 50 µm*
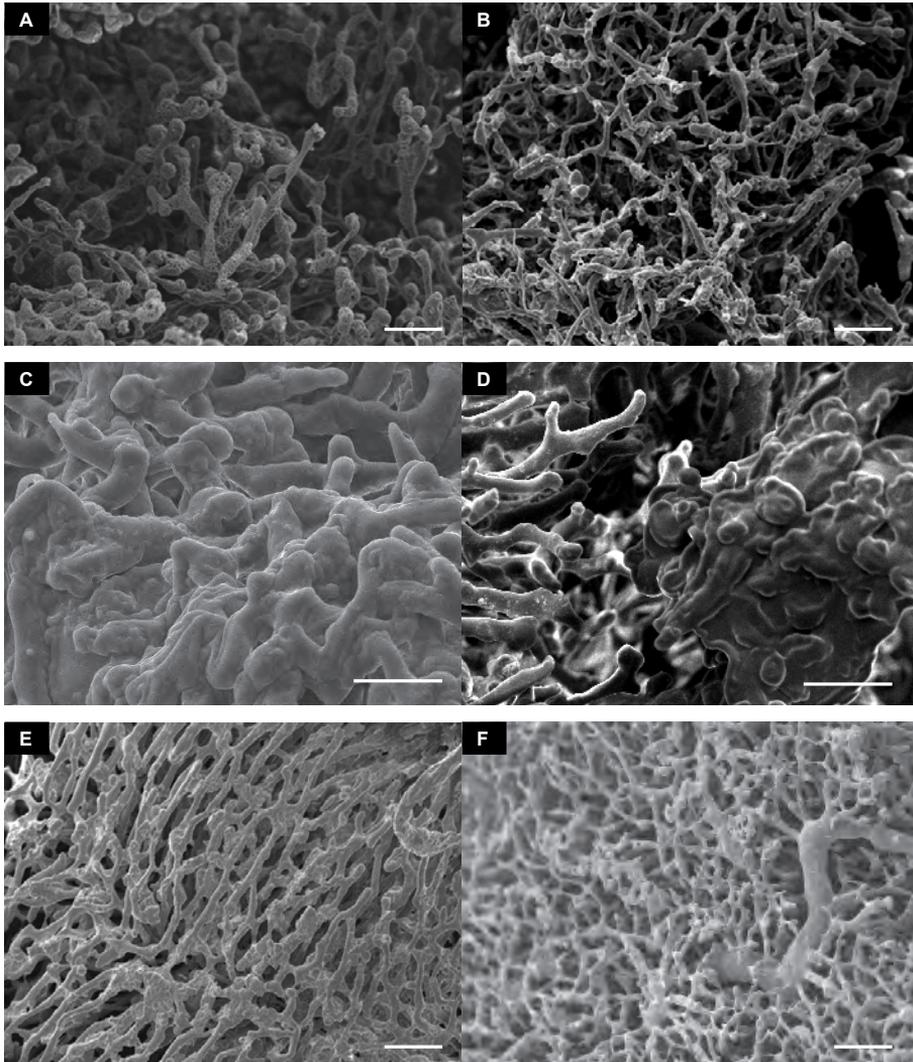
Supplementary figure 1: *SEM-images of vascular corrosion casts, showing that the overall organization of WT (A) and PHD2*$^{+/-}$ *(B) is clearly disrupted compared to healthy WT (E) and PHD2*$^{+/-}$ *(F) mice (scale bars = 50µm). A more detailed view shows that vascular buds are found more frequently in PHD2*$^{+/-}$*-mice*$^-$ *(D) then in WT-mice (C) (scale bars = 20µm). These images show that PHD*$^{+/-}$ *did not normalize the vasculature, on the contrary, a more disorganised vessel-organisation was found in PHD2*$^{+/-}$ *mice treated with DEN.*
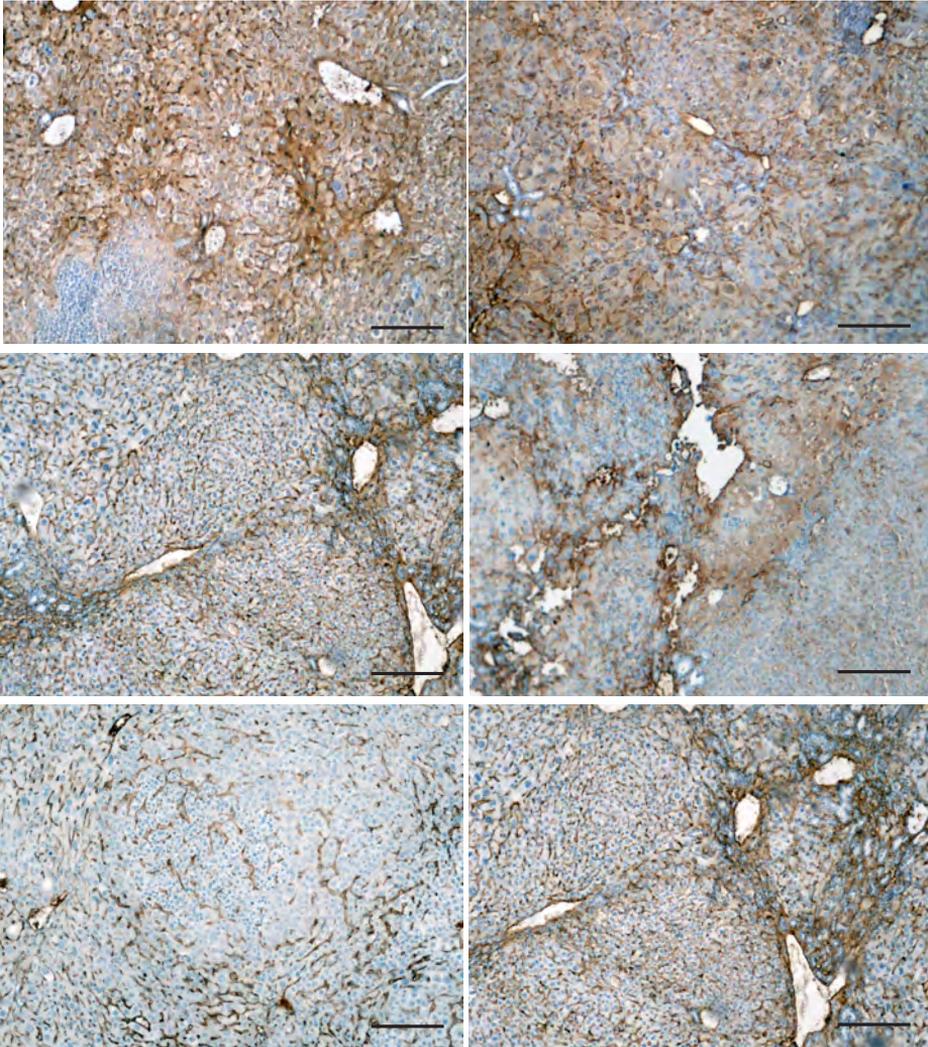
Supplementary figure 2: CD105-stained slides of 20W DEN WT (A), 20W DEN PHD2$^{+/-}$ (B), 25W DEN WT (C), 25W DEN PHD2$^{+/-}$ (D), 30W DEN WT (E) and 30W DEN PHD2$^{+/-}$ (F) mice, verifying that PHD2-deficiency did not lead to a more normalized vasculature. Scale bars = 200µm.

4.4.3   Hepatic progenitor cells (HPC)

Occurrence of both HCC and CC could imply involvement of HPCs, thus, the HPC-niche was immunohistochemically assessed using HPC-markers CK19, LIF and Oct4 (**Supplementary Fig. 3.**). CK19-staining showed that HCC and CC were characterised by CK19+ cells. In WT-mice, activation of HPCs mostly occurred in small HCC-lesions and in surrounding non-tumour tissue. While CC-lesions of PHD2$^{+/-}$ mice remained CK19+ at all time, larger HCC-lesions tended to have a lower CK19-expression compared to small HCC-lesions. The majority of WT HCC-lesions were Oct4+, while little expression was seen in PHD2$^{+/-}$ CC-lesions, although their surroundings showed several Oct4+ cells especially in highly inflammated regions. LIF was expressed both in HCC and CC in respectively WT and PHD2$^{+/-}$livers, however CC-lesions often showed a stronger LIF+ staining than HCC lesions.

PHD2$^{+/-}$-mice showed a significant higher expression of receptors and ligands of the Notch-pathway, especially during early stages *Jagged2* was significantly increased (P<0,05) in non-tumour and early tumour lesions at 20W DEN compared to WT's (**Fig. 4.**). *Notch2* was significantly increased in non-tumour tissue at 20W and 30W of DEN administration (P<0,05) (**Fig. 4.**).

Furthermore, a comparison of human gene expression data between HCC and CC patients, confirmed that CC have a higher expression of *NOTCH1* and *JAGGED1* (P<0,001) and levels of *PFK* showed an increased activation of the HIF-pathway (**Fig. 4.**).
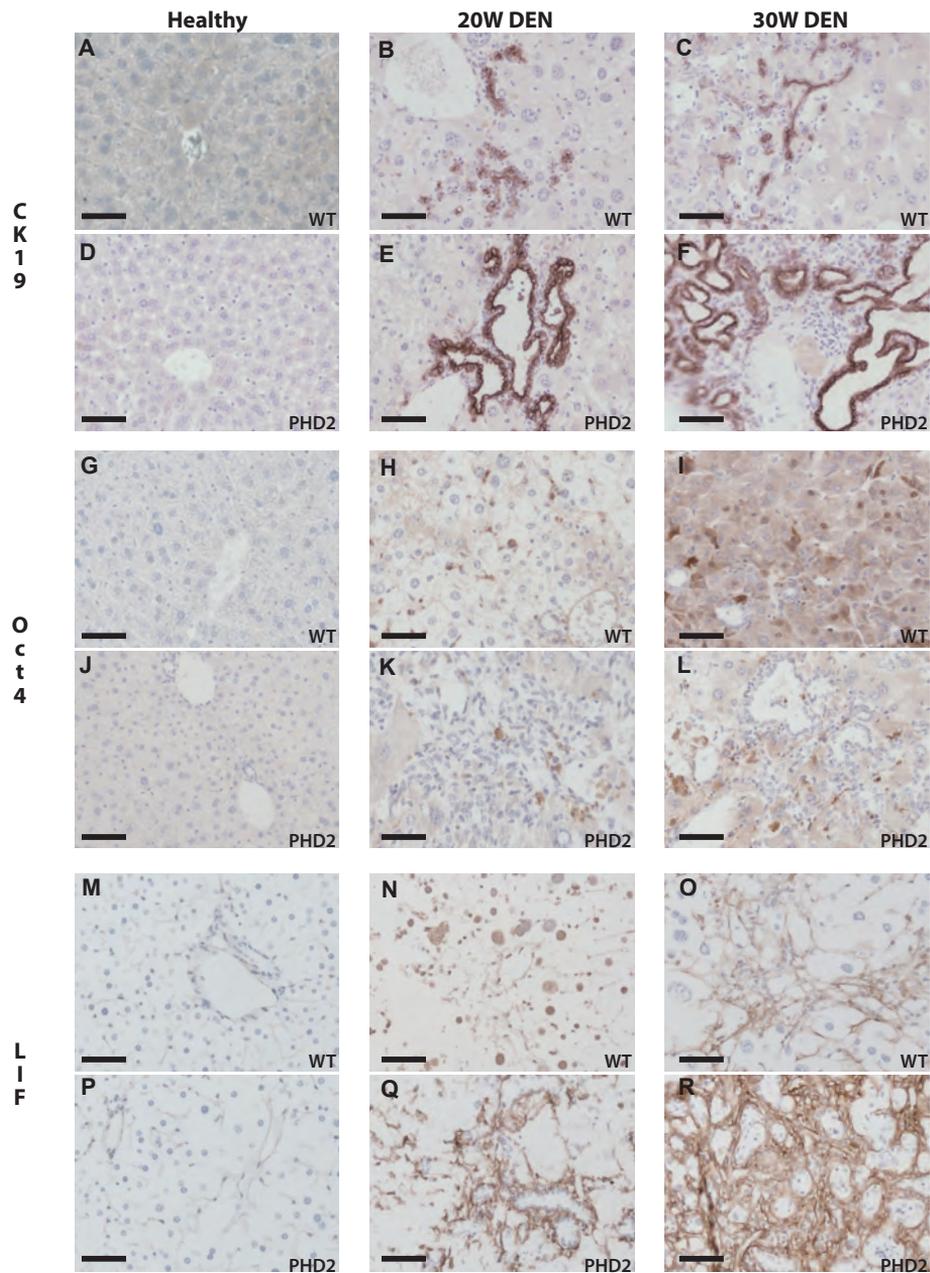
Supplementary figure 3: immunohistochemistry describing the HPC-niche in PHD2$^{+/-}$ -mice and WT-mice in healthy, 20W DEN and 30W DEN, by using markers for CK19 (A-F), Oct4 (G-L) and LIF (M-R), showing that both CC as HCC-lesions express HPC-markers. Scale-bars = 100μm.

187

4.4.4 Metastasis

The occurrence of extrahepatic tumours in the DEN-induced mouse model is very rare. Therefore, we used pro-metastatic markers, to assess the influence of PHD2-deficiency on the metastatic potential. Indeed, after 30W of DEN, PHD2$^{+/-}$ expressed significantly higher levels of the pro-metastatic markers *Icam* (p<0,05) and *Mmp9* (p<0,05) in tumour tissue, whereas *Itgav* was not significantly altered (**Fig. 4.**). No significant difference in expression of these metastastic markers was seen in the surrounding tissue (**Fig. 4.**).
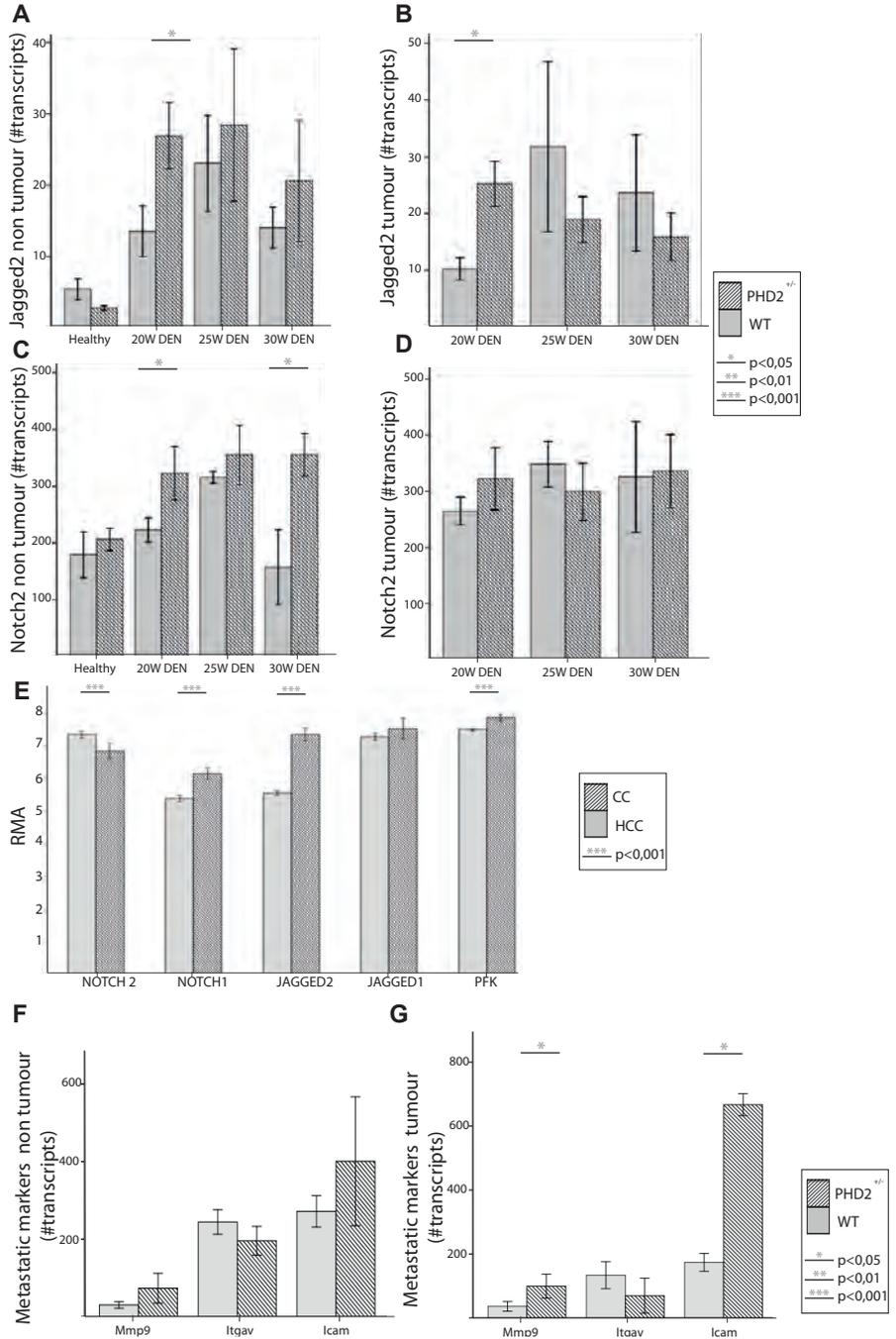
189

Fig. 4 *(previous page): Expression levels of ligands of the Notch-pathway were increased in surrounding (A,C) and tumour (B,D) tissue of PHD2+/--mice. Human expression levels showed as the robust multichip average (RMA), verified that NOTCH1, JAGGED1, JAGGED2 and PFK were also significantly upregulated in CC-lesions of patients compared to HCC. However, NOTCH2 was downregulated in human CC (E). Expression of metastatic markers Mmp9 and Icam were significantly increased in PHD2+/--tumours. No significant difference was seen in Itgav-levels (F) or in non-tumour lesions (G). No extrahepatic metastasis were observed.*

4.5 Discussion

Two main primary liver cancers in adults are HCC and CC. These tumours rapidly outgrow their vascular supply and become hypoxic, initiating the production of several angiogenic factors [8,9,16]. The identity and regulation of factors responsible for these angiogenic processes are areas of active investigation as these pathways could provide valuable targets for therapeutic intervention, especially in HCC where sorafenib (Nexavar®) is currently the standard of care for advanced HCC-patients. HIF and VEGF have gathered extensive attention for their roles in mediating tumour angiogenesis. Recently, interest has grown in regulators of HIF and in alternative mechanisms of pathological angiogenesis. Thus, several reports have been published describing the role of PHD2 in tumour progression and vascularisation, leading to contradictory findings. In our study we used an orthotopic model for liver cancer in which PHD2 is silenced both in the tumour as in the stroma, while the study of Giacca *et al* used PHD2-silenced tumour cells and in the study of Mazzone *et al* PHD2 was silenced only in the tumour environment. Macroscopic evaluation showed that PHD2$^{+/-}$-mice developed more tumours compared to their WT-counterparts. This was also found in other studies [3,17] . Microscopic analysis confirmed an increased HCC-burden at 20W and 25W, yet HCC-progression stagnates after 30W, perhaps due to the increased presence of CC, possibly resulting in a competition for nutrients between tumours [18].

We found that PHD2-inhibition did not significantly alter VEGF-levels. This corresponds to *in vitro* studies showing that PHD2 does not influence VEGF-expression, but exerts its proangiogenic potential through other factors [3]. In addition, studies have also shown that CC-lesions are characterised by less

expression of angiogenic factors compared to HCC [19] . Our research suggests that this is also independent of PlGF in tumour lesions, which can be partially explained by the decrease of HSC-activation in HCC-lesions, the main source of PlGF in chronic liver diseases. Studies have shown that PlGF exerts its main angiogenic effects in the non-tumorous surrounding [20], and indeed here we see an up-regulation in DEN-treated PHD2$^{+/-}$-mice compared to healthy livers.

Previous studies have shown that tumours implanted in PHD2-haplodeficient mice are characterised by a morphologically normalised vasculature, enhancing tumour oxygenation and promoting a less aggressive tumour phenotype [5,21]. This effect was not observed in our study, however, only advanced tumours were assessed using vascular corrosion casting and in certain cancer models vascular normalisation only occurs at an early stage. Earlier tumour stages were histologically evaluated using CD105-stained slides and no improvement of the vascular morphology was seen. Furthermore, the PHD2$^{+/-}$ tumour lesions were characterised by a higher expression of pro-metastatic markers, thus providing evidence that PHD2-deficiency did not select for a less aggressive hepatic tumour phenotype. This is in line with multiple reports showing a positive correlation between HIF-activation and metastasis [22]. Histologically we were not able to identify the loss of epithelial characteristics and gain of mesenchymal attributes, as typically seen during epithelial-mesenchymal transition. However, expression of *Mmp9* and *Icam* is known to be involved in this process, thus PHD2 might influence this event.

An interesting finding was that PHD2-inhibition influenced hepatic carcinogenesis, resulting in a shift from HCC to CC. Indeed, the prolonged administration of DEN-induced inflammation and fibrosis around cancer foci or in non-cancer liver tissues and could thereby influence ductular reaction. PHD2-deficiency increased the infiltration of macrophages, however, no significant difference in Metavir-score, a fibrosis-staging system, was seen between WT and PHD2$^{+/-}$ mice, suggesting the ductular reaction is not merely a result of increased inflammation. Since CC and HCC could be derived from HPCs [23–25], PHD2 might have altered their differentiation. HPC-proliferation is one of the earliest responses to DEN, however, contradictory results have been found on whether DEN-induced tumours HPC-derived [25,26]. Staining of CK19, Oct4 and LEF in the surrounding tissue and tumour lesions of DEN-injected WT and PHD2$^{+/-}$ could suggest an HPC-origin of these tumours.

In this study, we propose that the switch from HCC towards CC might be a result of the continuous HIF-activation in PHD2^{+/-}-mice. Current literature showed that HPC-differentiation and embryonic stem cells towards cholangiocytes is regulated through the Notch-pathway [14,27]. This supports our findings that PHD2^{+/-}-livers have a higher expression of *Jagged2* and *Notch2* in the DEN-model, especially in the pre-malignant lesions and surrounding non-tumoural tissue. PHD2^{+/-}-mice have a higher level of HIFs stimulating the Notch-pathway, possibly causing HPCs to differentiate towards a cholangiocytic-lineage. Additional studies using transgenic mice models will be needed to support this hypothesis, but human expression data also show a significant upregulation of the Notch-pathway in CC-lesions. However, the difference is not as distinct as in the mouse model, perhaps because only established HCC and CC were assessed in the human study, while a follow-up study was conducted in the mouse model. It also needs to be clarified that the pre-malignant "cholangioma-lesions" are not often found in patients, while they are abundant in this and other rodent models [10].

Alternatively, the occurrence of CC-lesions after continuous activation of HIF could also be a consequence of resistance of cholangiocytes to ischemic conditions and their potential to proliferate under these conditions [28,29]. Liver ischemia is known to stimulate ductular reaction, by inducing proliferation of the bile duct epithelium, providing a functional response to biliary dysfunction [28]. Further studies will be needed to clarify the exact influence of hypoxia on different types of liver-cells. Conditional knock out models that fully silence PHD2-expression in specific hepatic cell types could provide additional insight on how PHD2 influences the hepatocarcinogenesis.

These results provide interesting insight in the effect of long-term HIF-activation on hepatic tumours, and could hold a great potential for therapeutic intervention. Recent studies have shown that inducing ischemic injury by transarterial chemoembolization (TACE) leads to an increased occurrence of a mixed hepatocholangiocellular phenotype [30,31]. This biliary phenotype of HCC is more aggressive and could possibly be derived from HPCs, activated through the TACE-induced hypoxia to differentiate towards a cholangiocytic lineage. A possible link between hypoxia and the selection for a more aggressive tumour phenotype might also have a repercussion on patients receiving long-term treatment with anti-angiogenic compounds, such as

sorafenib, which create a more hypoxic environment [32]. Since recent studies have shown an association between the epidermal growth factor pathway and CK19-expression in liver tumours, this could open a possible role for therapeutic agents targeting this pathway, such as erlotinib and gefitinib, in patients with this aggressive class of HCC or as an adjuvant therapy for those receiving long-term administration of anti-angiogenic treatments [33].

To conclude, this study provides new insight in the role of PHD2, in the pathogenesis of primary liver cancer. We have shown that PHD2-inhibition aggravates HCC growth and induces the presence of CC, possibly through the Notch-pathway. Future research is necessary to clarify the exact role of PHD2 or HIF-stimuli on the differentiation of liver progenitor cells, especially for patients receiving long-term administration of anti-angiogenic therapy.

4.6   Acknowledgements

4.7   References

[1]   Coulon S, Heindryckx F, Geerts A, Van Steenkiste C, Colle I, Van Vlierberghe H. Angiogenesis in chronic liver disease and its complications. Liver Int 2011;31:146-62.

[2]   Takeda K, Cowan A, Fong GH. Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system. Circulation 2007;116:774-781.

[3]   Chan DA, Kawahara TLA, Sutphin PD, Chang HY, Chi J-T, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. Cancer Cell 2009;15:527-38.

[4]   Henze AT, Riedel J, Diem T, Wenner J, Flamme I, Pouysseggur J, et al. Prolyl hydroxylases 2 and 3 act in gliomas as protective negative feedback regulators of hypoxia-inducible factors. Cancer Res 2010;70:357-366.

[5]   Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. Cell 2009;136:839-851.

[6]   Su Y, Loos M, Giese N, Metzen E, Buchler MW, Friess H, et al. Prolyl hydroxylase-2 (PHD2) exerts tumor-suppressive activity in pancreatic cancer. Cancer 2011; ePub.

[7]   Chan DA, Giaccia AJ. PHD2 in tumour angiogenesis. Brit J Cancer 2010;103:1-5.

[8]   Heindryckx F, Mertens K, Charette N, Vandeghinste B, Casteleyn C, Van Steenkiste C, et al. Kinetics of angiogenic changes in a new mouse model for hepatocellular carcinoma. Mol cancer 2010;9:219.

[9]   Van de Veire S, Stalmans I, Heindryckx F, Oura H, Tijeras-Raballand A, Schmidt T, et al. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. Cell 2010;141:178-190.

[10]   Yang H, Li TW, Peng J, Tang X, Ko KS, Xia M, et al. A mouse model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression. Gastroenterology 2011;141:378-88, 388 e1-4.

[11]   Rogers AB, Theve EJ, Feng Y, Fry RC, Taghizadeh K, Clapp KM, et al. Hepatocellular carcinoma associated with liver-gender disruption in male mice. Cancer Res 2007;67:11536-46.

[12]   Pilette C, Rousselet MC, Bedossa P, Chappard D, Oberti F, Rifflet H, et al. Histopathological evaluation of liver fibrosis: quantitative image analysis vs semi-quantitative scores. Comparison with serum markers. J Hepatol 1998;28:439-46.

[13]   Van Steenkiste C, Ribera J, Geerts A, Pauta M, Tugues S, Casteleyn C, et al. Inhibition of placental growth factor activity reduces the severity of fibrosis, inflammation, and portal hypertension in cirrhotic mice. Hepatology 2011;53:1629-1640.

[14]   Spee B, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borght S, Gaudio E, et al. Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. Gut 2010;59:247-257.

[15]   Woo HG, Lee JH, Yoon JH, Kim CY, Lee HS, Jang JJ, et al. Identification of a cholangiocarcinoma-like gene expression trait in hepatocellular carcinoma. Cancer Res 2010;70:3034-3041.

[16]   El-Khoueiry AB, Rankin CJ, Ben-Josef E, Lenz H-J, Gold PJ, Hamilton RD, et al. SWOG 0514: a phase II study of sorafenib in patients with unresectable or metastatic gallbladder carcinoma and cholangiocarcinoma. Invest New Drugs 2011; [epub ahead of print]

[17]   Lee KA, Lynd JD, O'Reilly S, Kiupel M, McCormick JJ, LaPres JJ. The biphasic role of the hypoxia-inducible factor prolyl-4-hydroxylase, PHD2, in modulating tumor-forming potential. Mol Cancer Res 2008;6:829-842.

[18]   Elser JJ, Nagy JD, Kuang Y. Biological stoichiometry: An ecological perspective on tumor dynamics. Bioscience 2003;53:1112-1120.

[19]   Kawahara N, Ono M, Taguchi K, Okamoto M, Shimada M, Takenaka K, et al. Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma. Hepatology 1998;28:1512-7.

[20]   Schmidt T, Kharabi Masouleh B, Loges S, Cauwenberghs S, Fraisl P, Maes C, et al. Loss or Inhibition of Stromal-Derived PlGF Prolongs Survival of Mice with Imatinib-Resistant Bcr-Abl1(+) Leukemia. Cancer cell 2011;19:740-53.

[21]   Ameln AK, Muschter A, Mamlouk S, Kalucka J, Prade I, Franke K, et al. Inhibition of HIF prolyl hydroxylase-2 blocks tumor growth in mice through the antiproliferative activity of TGFbeta. Cancer Res 2011;71:3306-3316.

[22]   Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. Clin Cancer Res 2010;16:5928-35.

[23]   Roncalli M, Park YN, Di Tommaso L. Histopathological classification of hepatocellular carcinoma. Digest Liver Dis 2010;42 Suppl 3:S228-34.

[24]   Roskams T. Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene 2006;25:3818-3822.

[25]   Anilkumar TV, Golding M, Edwards RJ, Lalani EN, Sarraf CE, Alison MR. The resistant hepatocyte model of carcinogenesis in the rat: the apparent independent development of oval cell proliferation and early nodules. Carcinogenesis 1995;16:845-53.

[26]   Bralet M-P, Pichard V, Ferry N. Demonstration of direct lineage between hepatocytes and hepatocellular carcinoma in diethylnitrosamine-treated rats. Hepatology 2002;36:623-30.

[27]   Jin LF, Ji SH, Yang JF, Ji WZ. Notch signaling dependent differentiation of cholangiocyte-like cells from rhesus monkey embryonic stem cells. Zoo Res 2011;32:391-395.

[28]   Beaussier M, Wendum D, Fouassier L, Rey C, Barbu V, Lasnier E, et al. Adaptative bile duct proliferative response in experimental bile duct ischemia. J Hepatol 2005;42:257-65.

[29]   Fouassier L, Beaussier M, Schiffer E, Rey C, Barbu V, Mergey M, et al. Hypoxia-induced changes in the expression of rat hepatobiliary transporter genes. AMJ Phys 2007;293:G25-35.

[30]   Sapisochin G, Fidelman N, Roberts JP, Yao FY. Mixed hepatocellular cholangiocarcinoma and intrahepatic cholangiocarcinoma in patients undergoing transplantation for hepatocellular carcinoma. Liver Transplant 2011;17:934-942.

[31]   Zen C, Zen Y, Mitry RR, Corbeil D, Karbanova J, O'Grady J, et al. Mixed phenotype hepatocellular carcinoma after transarterial chemoembolization and liver transplantation. Liver Transplant 2011;17:943-954.

[32]   Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 2009;15:220-231.

[33]   Yoneda N, Sato Y, Kitao A, Ikeda H, Sawada-Kitamura S, Miyakoshi M, et al. Epidermal growth factor induces cytokeratin 19 expression accompanied by increased growth abilities in human hepatocellular carcinoma. Lab Invest 2011;91:262-272.

5 Inhibition of PlGF decreases cholangiocarcinoma and HCC burden in a transgenic mouse model

Inhibition of the placental growth factor decreases cholangiocarcinoma and hepatocellular carcinoma burden in a transgenic mouse model

F. Heindryckx, E. Bogaerts, S. Coulon, H. Devlies, A. Geerts, L. Libbrecht, JM Stassen, P. Carmeliet, I. Colle & H. Van Vlierberghe (2012)

5.1   Abstract

Objectives: Hepatocellular carcinoma and cholangiocarcinoma form the majority of primary hepatic tumours and are the third most common cause of cancer-related deaths. These liver tumours rapidly outgrow their vascular supply and become hypoxic, resulting in the production of hypoxia inducible factors and triggering the angiogenic switch. Therefore, inhibiting angiogenesis has proved to be a valuable therapeutic strategy in hepatocellular carcinoma, yet less is known about its use in cholangiocarcinoma. In this study, we assess whether inhibiting the placental growth factor could offer a therapeutic option in mice with hepatocellular carcinoma and cholangiocarcinoma. The placental growth factor (PlGF) is a homologue of the vascular endothelial growth factor that is only involved in pathological angiogenesis, therefore, its inhibition does not induce adverse effects. *Methods:* We have used a chemically induced transgenic mouse model in which both hepatocellular carcinoma and cholangiocarcinoma develop after 25 weeks and treated these tumours with murine monoclonal antibodies targeting PlGF. *Results:* This study has shown for the first time that inhibiting PlGF decreases cholangiocarcinoma burden, by affecting both angiogenesis and inflammation. *Conclusion:* The use of monoclonal antibodies targeting PlGF could thus offer a potential systemic treatment for patients who suffer from primary liver tumours.

5.2   Introduction

Almost all primary liver carcinomas are broadly classified either as hepatocellular carcinoma (HCC), derived from hepatocytes, or cholangiocarcinoma (CC), arising from intrahepatic bile duct epithelium. These tumours rapidly outgrow their vascular supply and become hypoxic, resulting in the production of hypoxia inducible factors and triggering the angiogenic switch [1], [2]. The identity and regulation of factors responsible for these angiogenic processes are areas of active investigation as these pathways could provide valuable targets for therapeutic intervention, especially in HCC where the multikinase inhibitor sorafenib (Nexavar®) is currently the standard of care for advanced HCC-patients [3–5]. The vascular endothelial growth factor (VEGF) has gathered extensive attention for its role in mediating tumour angiogenesis [6–9], but recently the interest has grown in the placental growth factor (PlGF)

[10–14]. PlGF is a VEGF-homologue that is only involved in pathologic angiogenesis, therefore its inhibition does not induce side effects [14], [15].

The placental growth factor (PlGF), a member of the VEGF family, is known to stimulate endothelial cell growth, migration and survival [15], [16]. It also attracts angiocompetent macrophages and bone marrow progenitor cells and determines the metastatic niche[16], [17]. Unlike VEGF, PLGF binds to VEGF receptor 1 (VEGFR1) and its co-receptors neuropilin-1 and 2 [18]. Therefore, PlGF exerts its anti-angiogenic potiential through two mechanisms, directly by inducing signal transduction through its receptors [19] and indirectly, by preventing the binding of VEGF on its decoy receptor VEGFR1 [20]. In contrast to VEGF, genetic studies have shown that PlGF is specifically involved in the pathologic angiogenesis [14], [15]. Therefore its inhibition would not affect healthy blood vessels, providing an attractive drug candidate with a good safety profile. Treatment with PlGF-antibodies has shown beneficial effects in several mouse models for chronic liver disease, including portal hypertension [21], cirrhosis [12] and HCC [13]. Its influence on CC remains unknown.

While anti-angiogenic treatment for HCC has been established as the standard of care for advanced HCC-patients, its potential for CC-patients is less investigated. Yet, the role of the Ras-Raf-Mek-Erk-pathway [5] and VEGF in biliary carcinomas has already been clarified in several studies [23], [24]. Furthermore, patients with biliary cancers receiving sorafenib (Nexavar®) had some therapeutic benefit. However, the severe adverse effects have forced an early stop to the phase II trial (SWOG 0514) [25]. The combination of bevacizumab and erlotinib may offer therapeutic alternative for patients with advanced biliary cancer, but may also be limited due to the occurrence of adverse effects (NCT00356889) [26]. Additionally, studies have shown that inducing ischemic injury by transarterial chemoembolization in HCC leads to an increased occurrence of a mixed hepatocholangiocellular tumour phenotype, emphasizing the need for new therapeutics to manage this aggressive form of primary liver cancer [27], [28].

In a previous study, we have shown that mice containing a heterozygous mutation silencing the prolyl hydroxylase containing domain 2 (PHD2) proteins develop both HCC and CC after 25 weeks of diethylnitrosamine (DEN) administration [22][21][20] [3]. In this study, we would like to use this model to

assess whether treatment with monoclonal antibodies targeting PlGF could offer a therapeutic option in mice with HCC and CC.

5.3 Material & Methods

5.3.1 Genotyping

PHD2$^{+/-}$mice were obtained from the Vesalius Research facility (KULeuven, Belgium). A heterogenic couple was used for breeding and the offspring was genotyped with following primers in a concentration of 10 µM: ACCTATGATCTCAGCATTTGGGAG; TCAGGACAGTGAAGCCTAGAAACT and AAATTCTAATCGTAGCTGATGTGAGC; the latter being used as a reversed primer in both the Wild Type as the MUTANT-reaction. The PCR-product was run on a 1,5% agarose gel and heterozygous mice were distinguished by the appearance of PCR-bands at 380 bp and 340 bp.

5.3.2 Hepatocellular carcinoma and cholangiocarcinoma induction

Five weeks old heterzygous PHD2 knock out male mice (PHD2$^{+/-}$) with an sv129-background received intraperitoneal injections once weekly with diethylnitrosamine (DEN) (Sigma-Aldrich, Bornem, Belgium) or saline as previously described [3], [17]. After 25 weeks, mice develop both hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). The Ethical Committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocol.

5.3.3 Placental Growth Factor inhibition

A murine anti-PlGF monoclonal antibody (clone 5D11D4; referred to as αPlGF) that specifically recognizes mouse PlGF-2 was obtained from Thrombogenics, Leuven (Belgium). Mice that received 25 weeks of DEN were subsequently treated with 25 mg/kg αPlGF (2x / week, n = 8) for 5 weeks or IgG (2x / week, n = 8) as a control substance. During the treatment mortality was noted to obtain survival curves.

5.3.4   Sampling & histology

Animals were sacrificed after 25 weeks of DEN subsequently followed by 5 weeks of treatment (αPlGF (n = 6) or IgG (n = 5)) under isoflurane (Forene®) anaesthesia while blood was obtained from the ophthalmic artery. The liver was prelevated first and after washing with sterile 5% NaCl, cut into 5 mm pieces for macroscopic quantification of the number of macroscopic (>1 mm) hepatic tumours. Subsequently, all organs were evaluated and sampled in 4% phosphate buffered formaldehyde (Klinipath, ref: 4078.9020) and embedded in paraffin, as previously described [29]. Tumour-lesions and non-tumour-tissue were separately collected and snap frozen in liquid nitrogen for subsequent analysis. Haematoxilin-eosin staining (H&E) was performed to evaluate the morphological changes inflicted by the treatment with αPlGF. Sirius Red staining was used to determine the Metavir score for fibrosis and to indirectly score the burden of CC-lesions since it is closely linked to cholangiofibrosis. CC-lesions were also staged by distinguishing normal bile ducts (stage 0), cholangioma (stage 1), mixed cholangioma-cholangiocarcinoma (stage 2) and cholangiocarcinoma (stage 3), as previously described [30]. Reticulin staining was performed to identify HCC-nodules. The HCC-burden was quantified by multiplying the size by the number of tumours in 10 random regions of interest of 376665,37 μm^2. Tumour area was calculated by manually selecting the tumour's perimeter in Olympus Cell^D software (Zoeterwoude, The Netherlands).

Stainings were done using standard histology protocols and evaluated by an experienced pathologist.

5.3.5   Immunohistochemistry

Immunohistochemistry was used to quantify protein levels inside hepatic tumours and in surrounding non-tumour tissue. As a marker for angiogenesis, a monoclonal antibody was used targeting CD105 or Endoglin (R&D systems, ref AF1320, Abingdon, UK) [31]. Intercapillary distance (ICD) was used as a marker for microvessel density, by measuring the average distance between vessels in HCC-nodules on CD105-stained slides. F4/80 (AbD serotec, ref MCA497G, Dusseldorf, Germany) was used as a pan-macrophage marker. CD206 and

CD163 were used as M2-macrophage markers, while HLA-DR was used as an M1-macrophage marker. Stainings were performed as described in **table 1** and quantified with Olympus Cell^D software (Zoeterwoude, The Netherlands).

Target	Antigen retrieval		Blocking			Primary antibody		Secondary antibody		Buffer
	Product	Time	Peroxidase	Product (dilution)	Time	Product (ref) dilution	Time	Product (ref) dilution	Time	
CD105	Citrate	30' (99°C)	15' 3% H2O2	BSA (0,1%)	30' (RT)	Goat polyclonal anti- Endoglin (AF1320, R&D systems) 1/50	18h (RT)	LSAB-kit (K0690, Dako)	Manufact. protocol	TBS
F4/80	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	Rabbit serum (1/5)	45' (RT)	Rat monoclonal anti-F4/80 (MCA497, Serotec) 1/300	18h (RT)	Rabbit anti-Rat (E0468, Dako) 1/300	45' (RT) + Vectastain kit	TNB
HLA-DR	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	BSA (1%)	30' (RT)	Rat monoclonal anti-HLA-DR (sc-59257, Santa Cruz) 1/800	1h (37°C)	LSAB-kit (K0690, Dako)	Manufact. Protocol	PBS
CD206	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	BSA (1%)	30' (RT)	Rabbit Polyclonal anti-CD206 (orb4941, Biorbyt) 1/400	1h (37°C)	LSAB-kit (K0690, Dako)	Manufact. Protocol	PBS
CD163	Target Retrieval solution (S1699, Dako)	20' (99°C)	15' 3% H2O2	BSA (1%)	30' (RT)	Rabbit Polyclonal anti-CD163 (orb13303, Biorbyt) 1/400	1h (37°C)	LSAB-kit (K0690, Dako)	Manufact. Protocol	PBS

Table 1: immunohistochemistry protocols

5.3.6 ELISA

Protein levels of VEGF (R&D Biosystems, Mouse VEGF Quantikine ELISA Kit, Abingdon, UK) were measured in liver tissue and serum.

5.3.7 Quantitative real time PCR (qPCR)

Total RNA was extracted from 20mg frozen tumour or non-tumour lesions from IgG or αPlGF treated mice, following manufacturer's guidelines (Qiagen, RNeasy Mini Kit ref: 74104, Venlo, Netherlands) and diluted to a concentration of 100 ng/µl. RNA Quality was evaluated using spectrophotometry. cDNA was

obtained from 10 µl RNA with the iScript cDNA Synthesis Kit using oligo(dT) primers, according to manufacturer's protocol (Bio-Rad, 170-8890, Nazareth, Belgium). Expression levels were measured using qRT-PCR, with 3 µl cDNA and the LightCycler 480 Green Master Mix (Roche, 04707516001, Vilvoorde, Belgium). Genes and corresponding primer sets are provided in **table 2.** The efficiency of each primer pair was calculated using the formula $10^{-1/slope}$. All reactions were run in duplicate and normalised to a set of reference genes that had a stable expression in all samples. The comparative Ct method was used for determining the number of transcripts.

Gene name (ID)	Forward primer	Reverse primer	Marker
Hprt (15452)	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT	Reference gene
Hmbs (15288)	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG	Reference gene
Sdha (66945)	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT	Reference gene
Gapdh (14433)	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	Reference gene
Mmp9 (17395)	GAGACGGGTATCCCTTCGAC	TGACATGGGGCACCATTTGAG	Metastasis
Itgav (16410)	CAATTGCTGCTCCCTATGGT	GATTTGAGATGGCACCGAAT	Metastasis
Icam (15898)	GCCTTGGTAGAGGTGACTGAG	GACCGGAGCTGAAAAGTTGTA	Metastasis
Vegfa (22339)	ACTCGGATGCCGACACGGGA	CCTGGCCTTGCTTGCTCCCC	Angiogenesis

Table 2: Genes and primer sets

5.3.8   Statistics

Data were analysed with SPSS 19. After testing for normality and homoscedasticity, data were either subjected to a Student's t-test or a Mann-Whitney-U test. A *p* value of <0.05 was considered statistically significant. Data mentioned in the manuscript are shown as averages ± standard error of mean. αPlGF n = 6 and for IgG n = 5 in all the experiments.

5.4   Results

5.4.1   Macroscopic evaluation

Significant increase of weight (p<0,05) was observed between DEN-induced PHD2$^{+/-}$ mice treated with αPlGF (25,37g ± 0,95) compared to the IgG-controls (22,16g ± 0,46) (**Fig. 1)**. Macroscopic evaluation of the liver revealed DEN-induced that PHD2$^{+/-}$ mice treated with αPlGF had less hepatic tumours (7,80 liver tumours ± 2,20) compared to those who received control (22,17 liver tumours ± 4,22). No significant difference was seen in relative liver or spleen weight (**Fig. 1)**. Treatment with αPlGF also did not significantly improve survival. While DEN-injected IgG mice did not show any extrahepatic metastasis, 2 out of 8 (25%) αPlGF-treated mice developed tumours in the lungs and *vesicula seminalis*. However, this was not significant.
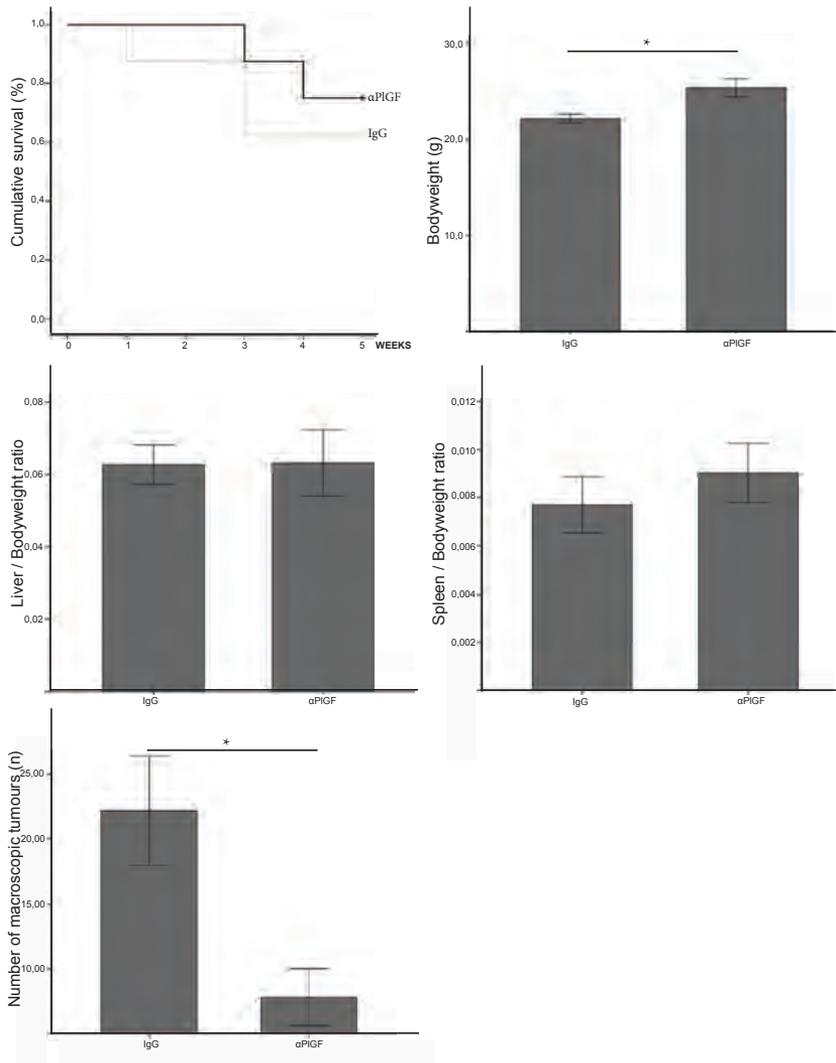
Figure 1: Upper left panel: No significant difference was seen on survival between αPlGF and IgG treated mice. Upper right panel: Administration of αPlGF causes significantly increased bodyweight, used as a marker for overall wellbeing. Middle left panel: The relative liver weight, calculated as the ratio of liver to bodyweight, did not significantly differ between treatment and control. Middle right panel: The relative spleen weight, an indirect marker for portal hypertension calculated as the ratio of spleen to bodyweight, did not significantly differ between treatment and control. Lower left panel: The macroscopic number of tumours is significantly decreased by treatment with PlGF-antibodies. Asterisks represent significant p-values (* = p < 0,05; ** = p < 0,01; *** = p < 0,001).

205

Hepatocellular carcinoma

H&E staining showed that all PHD2$^{+/-}$ mice had developed hepatocellular carcinoma. Besides HCC-lesions, small cell dysplasia was frequently found throughout the liver and readily distinguishable nodules of hepatic neoplasia were seen in both treatment types (table 3). These nodules were confirmed as HCC using reticulin staining and H&E. Treatment with αPlGF significantly decreased both the number (αPlGF 3,8 ± 0,49 vs IgG 8,5 ± 0,65; P < 0,001) and the size (αPlGF 467000 μm^2 ± 64000 vs IgG 5633000 ± 1480000; P < 0,001) of HCC-lesions. Consequently, the tumour burden was significantly decreased (P < 0,01) (**table 3**).

Cholangiocarcinoma

H&E staining showed that all PHD2+/- mice had developed cholangiocarcinoma (**Fig. 3**). Evaluation of CC-stage, revealed that αPlGF -treated mice were more likely to show biliary hyperplastic and cholangioma lesions, lined by flattened epithelium while control-treated livers had a mixed occurrence of cholangioma and CC, characterised by the appearance of some goblet-like cells as well as flattened epithelium and biliary dysplasia (**Fig. 3**). An increased uptake of Sirius Red was seen in the CC-lesions (**Fig. 3**). Measurement of the percentage of Sirius red staining, confirmed that αPlGF decreased the cholangiofibrosis, compared to mice that received IgG as a control (**Fig. 2**).

	IgG		aPlGF		
	Mean	*St. Err.*	*Mean*	*St. Err.*	*P*
Number of HCC (n)	8,5	0,65	3,8	0,49	***
Area of HCC (μm^2)	5633168,02	1479988,35	467461,56	63513,22	***
HCC burden (n x μm^2)	47881928,17	961992,42	1776353,92	31121,47	**
Number of dysplastic lesions (n)	5,67	0,67	3,4	0,51	
Cholangiocarcinoma (n)	4,77	0,33	2,4	0,98	*

Table 3: liver tumours
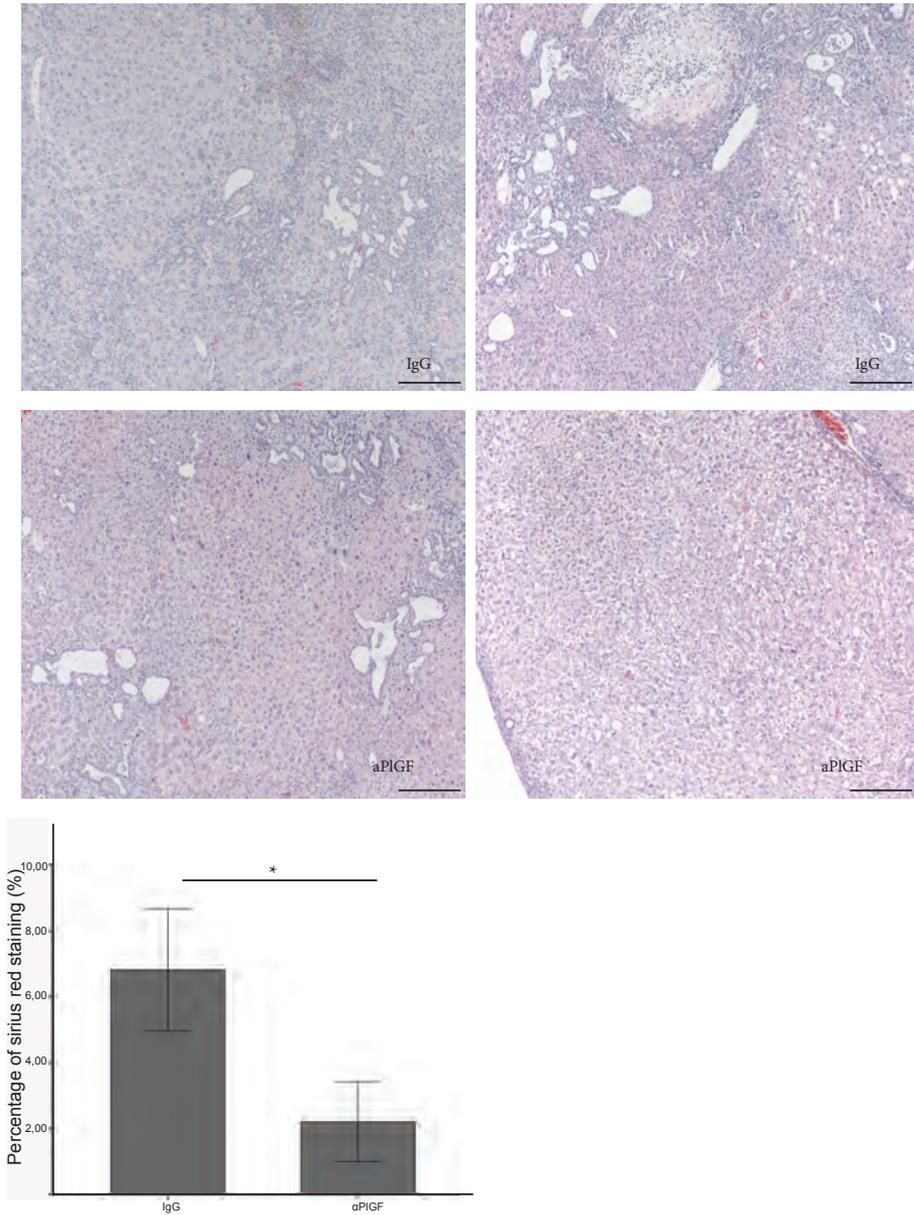
Figure 2: Microscopic tumour burden. Upper left and right panel: representative H&E stained slide of IgG-treated livers, showing severe hepatocellular and cholangiocellular damage. Middle left and right panel: H&E stained slide of αPlGF treated liver. Lower panel: The percentage of Sirius Red, used as a marker for cholangiofibrosis, showing a decrease after treatment with αPlGF. Asterisks represent significant p-values (* = p < 0,05; ** = p < 0,01; *** = p < 0,001). Scale bars represent 200 µm.

Figure 3: *Upper left panel: H&E stained slide of DEN-induced PHD2$^{+/-}$ IgG treated liver. Arrowheads represent cholangiocellu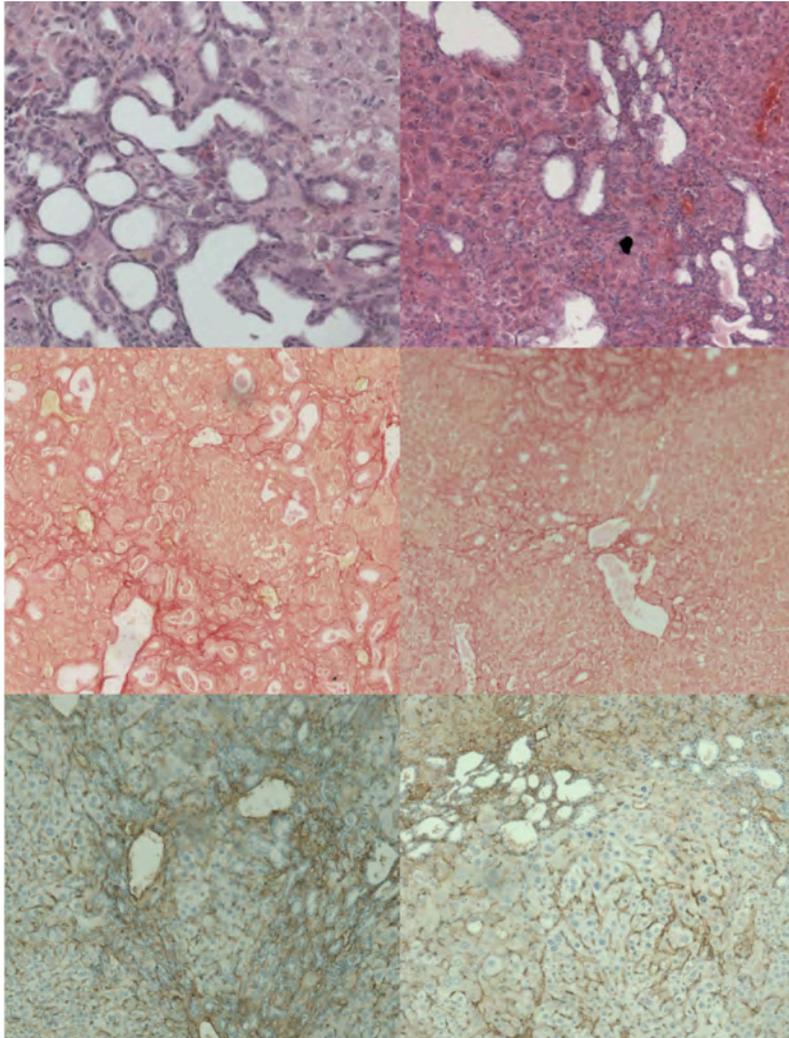lar carcinoma lesions, clearly showing thickened endothelium (200x). Upper right panel: H&E stained slide of DEN-induced PHD2$^{+/-}$ αPlGF treated liver (200x). Middle left panel: Sirius red stained slide of DEN-induced PHD2$^{+/-}$ IgG treated liver. Cholangiocellular carcinoma is characterized by an increased uptake of Sirius Red (100x). Middle right panel: Sirius red stained slide of DEN-induced PHD2$^{+/-}$ αPlGF treated liver. Arrowheads represent cholangiocellular lesions characterized by an increased uptake of Sirius Red (100x). Lower left panel: Endoglin staining showing vascularisation around cholangiocellular carcinoma lesions in DEN-induced PHD2$^{+/-}$ IgG treated liver (100x). Lower right panel: Endoglin staining showing vascularisation around cholangioma lesions in DEN-induced PHD2$^{+/-}$ αPlGF treated liver (100x).*

5.4.2 Inflammation and fibrosis

Fibrosis induced by chronic administration of DEN was significantly decreased (P < 0,05) by the PlGF-antibodies (Metavir scores: αPlGF 1,27 ± 0,40 vs IgG 2,73 ± 0,07) (**Fig. 4)**. Macrophage recruitment, measured using F4/80-staining, was significantly decreased in surrounding tissue of αPlGF-treated mice (αPlGF 4,02 % F4/80 ± 1,50 vs IgG 7,02 % F4/80 ± 0,52; P < 0,05) (**Fig. 4**). No significant difference was seen in percentage of F4/80 staining the HCC lesions, but a significant increase took place in the CC-lesions (**Fig. 4)**. αPlGF significantly decreased infiltration of CD206$^+$ macrophages (M2-marker) in non-tumour tissue and in CC-lesions , however a non-significant trend towards increase was seen in HCC-lesions (Fig. 5). No significant difference was seen in HLA-DR (M1-marker) or in CD163 (M2-marker) (Fig. 6).
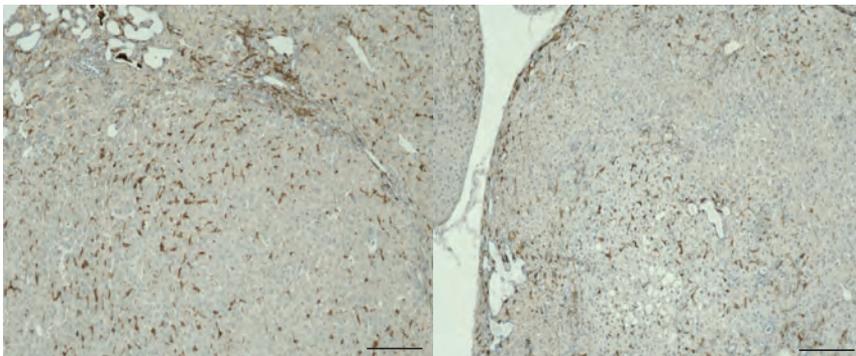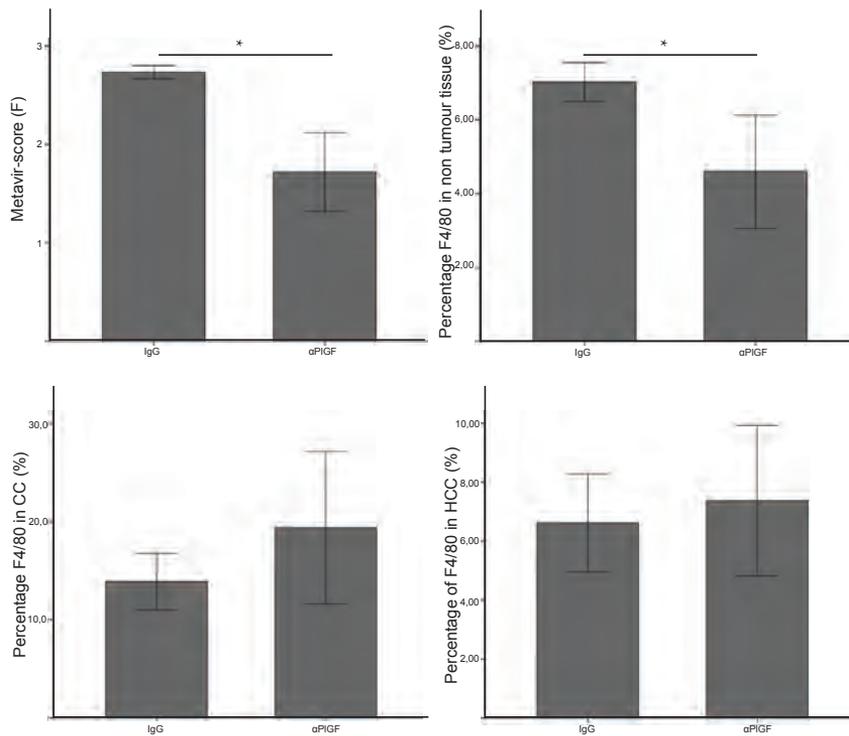
Figure 4: Upper left panel: fibrosis was measured using Metavir score and significantly decreased after treatment with PlGF-antibodies. Upper right panel: αPlGF-treatment significantly decreased macrophage recruitment in the surrounding tissue. Middle left panel: No significant difference was seen on macrophage recruitment inside CC-lesions. Middle right panel: No significant difference was seen on macrophage recruitment inside HCC-lesions. Lower left panel: F4/80 stained liver treated with IgG. Lower left panel: F4/80 stained liver treated with αPlGF. Asterisks represent significant p-values (* = p < 0,05; ** = p < 0,01; *** = p < 0,001). Scale bars represent 200 µm.
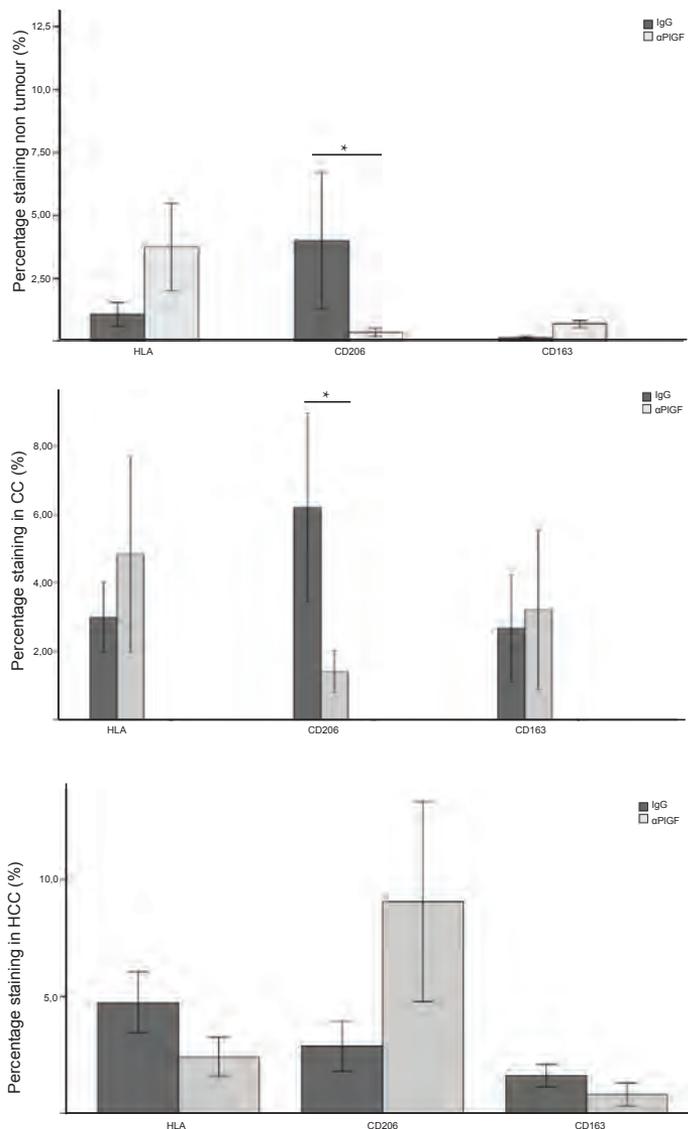
Figure 5: *tumour associated macrophage niche. Upper panel: Treatment with PlGF-antibodies significantly decreased the recruitments of CD206+ M2-macrophages in non tumour tissue. No significant differences were seen on the M1-marker HLA-DR or the M2-marker CD163. Middle panel: Treatment with PlGF-antibodies significantly decreased the recruitments of CD206+ M2-macrophages in CC tissue. No significant differences were seen on the M1-marker HLA-DR or the M2-marker CD163. Lower panel: No significant differences were seen in the M1/M2 macrophage niche in HCC-lesions treated with αPlGF or IgG.*
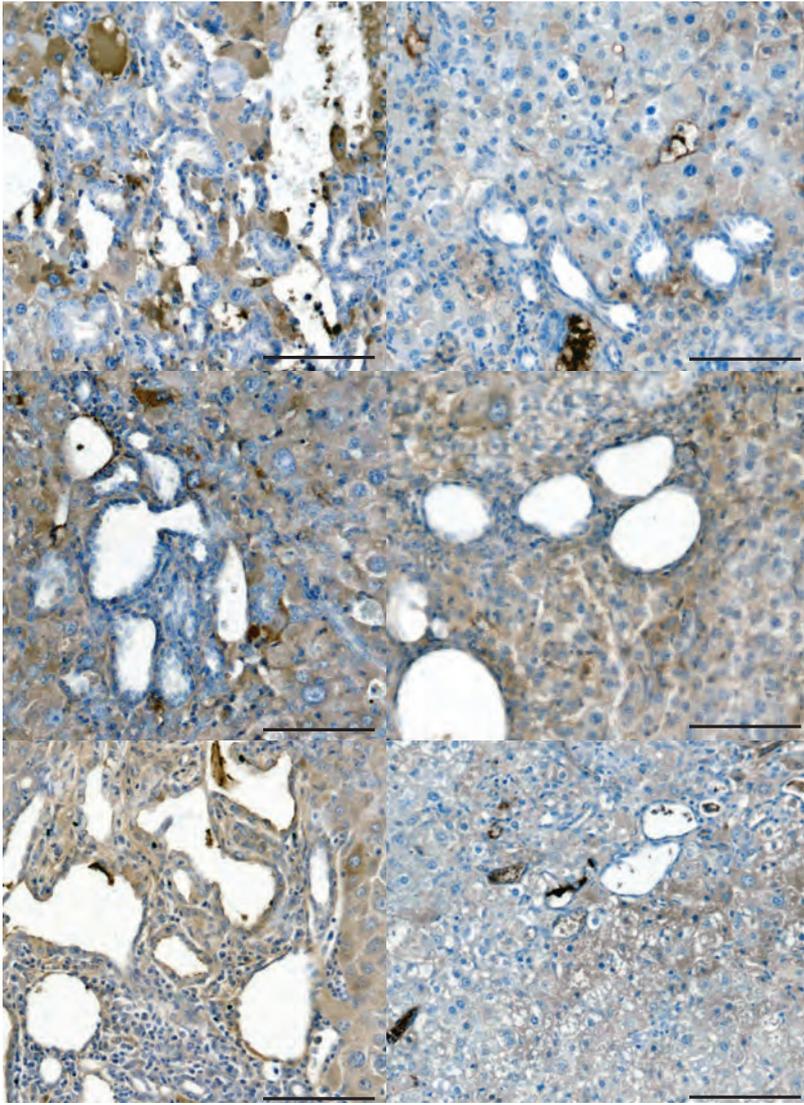
Figure 6: macrophage histology. Upper left panel: staining for M1-marker HLA-DR in IgG-treated liver. Upper right panel: staining for M1-marker HLA-DR in αPlGF-treated liver. Middle left panel: staining for M2-marker CD206 in IgG-treated liver. Middle right panel: staining for M2-marker CD206 in αPlGF-treated liver. Lower left panel: staining for M2-marker CD163 in IgG-treated liver. Lower right panel: staining for M2-marker CD163 in αPlGF-treated liver. Scale bars represent 200 μm.

5.4.3   Angiogenesis

No significant difference was seen between αPlGF and IgG treated mice (**Fig. 3**). Yet, αPlGF -treated HCC-lesions showed a trend towards lower vascularisation, measured both by determining the intercapillary distances as by the percentages of endoglin (CD105) staining inside the hepatocellular tumours (**Fig. 7**). Treatment with αPlGF significantly decreased the vascularisation of fibrotic, non-tumour tissue surrounding the CC and HCC lesions.–*Vegf* had a significantly higher expression in αPlGF-treated tumours, which was not seen in the surrounding tissue (**Fig. 8**). However, this increase was not translated into protein levels, since no significant difference was seen on VEGF-concentration in tumour and non-tumour tissue between αPlGF and IgG treated livers (**Fig. 7**). Furthermore, treatment with αPlGF significantly decreased serum levels of VEGF compared to IgG controls (αPlGF 206,35 pg/mL ± 12,70 vs IgG 528,94 pg/mL ± 210,56; p < 0,05) (**Fig. 7).**
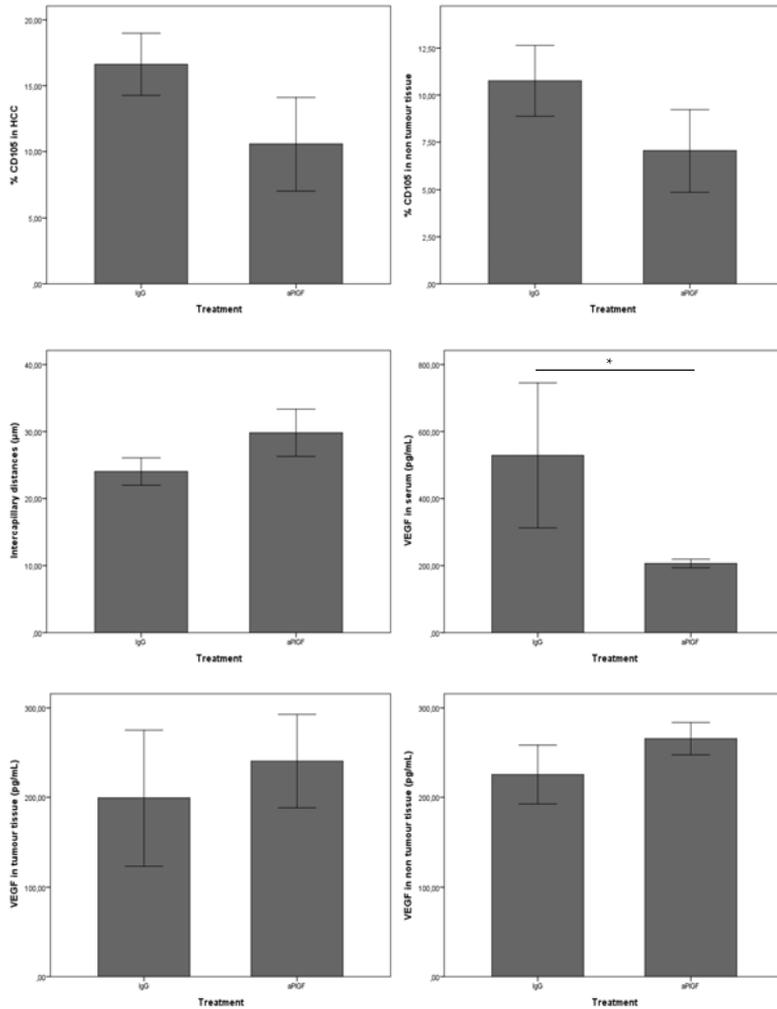
Figure 7: Hepatic angiogenesis. Upper left panel: Although not significant, a relevant trend towards lower vascularisation of the HCC-lesions was seen after treatment with αPlGF. Upper right panel: The surrounding tissue was also not significantly less vascularized, yet a trend towards lower expression of CD105 was seen after treatment with αPlGF. Middle left panel: As a marker for microvessel density, intercapilary distances were measured and showed a trend towards a decreased vascularisation of αPlGF-treated HCC-lesions. Middle right panel: Treatment with αPlGF significantly decreased serum levels of VEGF. Lower left panel: Treatment with αPlGF did not induce an angiogenic escape mechanism through up-regulation of VEGF inside the tumours. Lower right panel: Treatment with αPlGF did not induce an angiogenic escape mechanism through up-regulation of VEGF in the surrounding non-tumoural tissue. Asterisks represent significant p-values (* = p < 0,05; ** = p < 0,01; *** = p < 0,001).
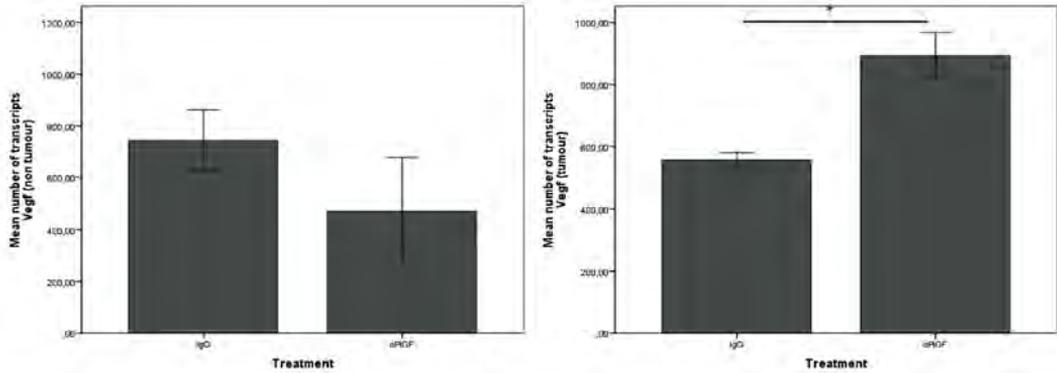
Fig. 8: *Vegf. Left panel: No significant difference was seen in Vegf-transcription between αPlGF-treated mice and IgG-treated mice in the surrounding tissue. Right panel: Treatment with αPlGF significantly increased Vegf-expression in tumour lesions. Asterisks represent significant p-values (* = p < 0,05; ** = p < 0,01; *** = p < 0,001).*

5.4.4   Metastasis

Expression levels of the pro-metastatic markers *Mmp9, Itgav* and *Icam* showed that αPlGF significantly decreased the pro-metastatic potential. In the tumour surrounding, a significant decrease of *Itgav* (p < 0,05) and *Icam* (p < 0,05) was measured, while a trend towards lower *Mmp9* expression was observed (**Fig. 9**). Inside tumour lesions, a significant decrease of *Icam* (p < 0,05) was found and a non-significant trend towards higher *Itgav*-expression was seen.
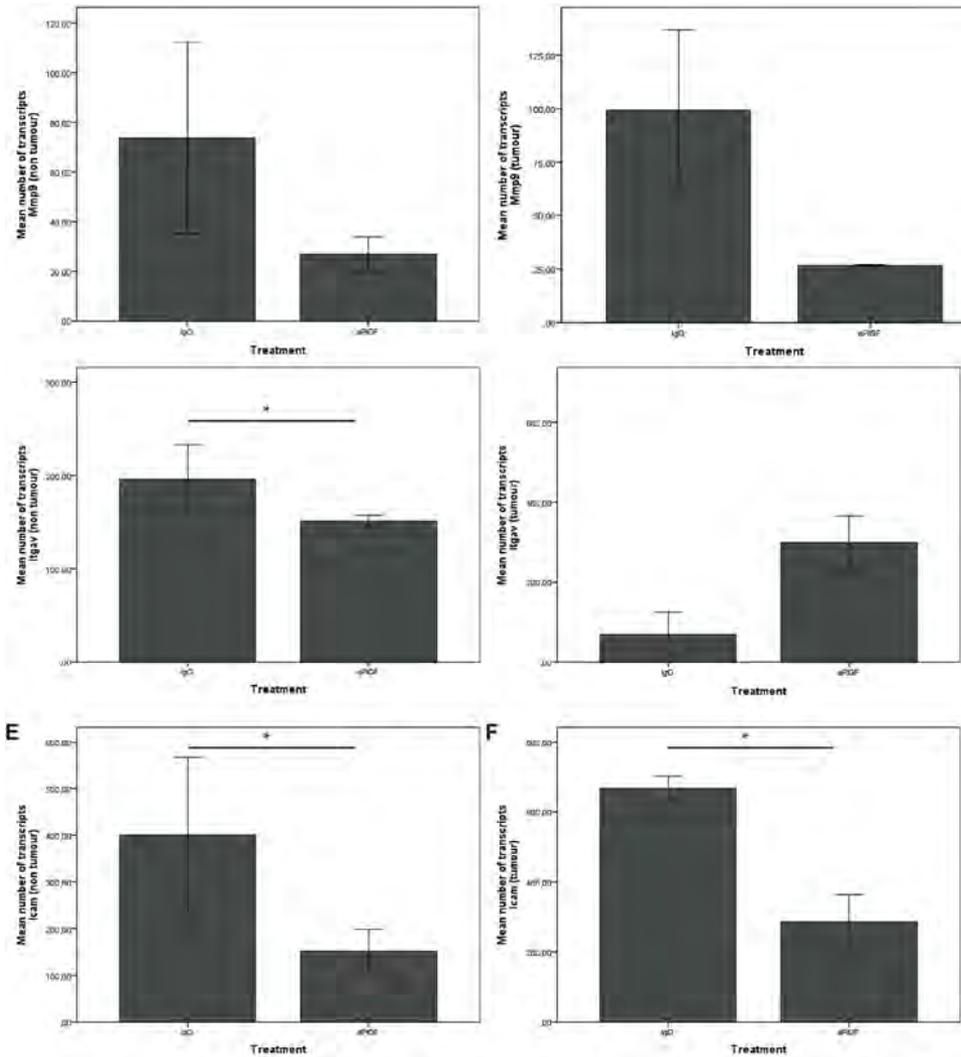
Figure 9: Metastasis. Upper left panel: Although not significant, a trend towards lower expression of the metastatic marker Mmp9 was seen in the surrounding tissue after treatment with αPlGF. Upper right panel: A trend towards lower expression of the pro-metastatic marker Mmp9 was seen in the tumour tissue treated with αPlGF. Middle left panel: Treatment with αPlGF significantly decreased expression of the pro-metastatic marker Itgav in the surrounding tissue. Middle right panel: A trend towards lower expression of the metastatic marker Itgav was seen in the αPlGF-treated tumour tissue. Lower left panel: Treatment with αPlGF significantly decreased the expression of the metastatic marker Icam in the surrounding tissue. Lower right panel: Treatment with αPlGF significantly decreased the expression of the metastatic marker Icam in tumours. Asterisks represent significant p-values (= p < 0,05; ** = p < 0,01; *** = p < 0,001).*

5.5   Discussion

Treatment with PlGF-antibodies has shown beneficial effects in several mouse models for chronic liver disease, including portal hypertension [21], cirrhosis [12] and HCC [13], yet its influence on CC remained unknown.

In a previous study we have shown that heterozygous PHD2 knock out mice develop both HCC as CC after chronic administration of DEN [22]. Therefore, we used this model to assess the effect of monoclonal antibodies targeting PlGF on the progression of HCC and CC.

Treatment with PlGF-antibodies significantly improved tumour burden, both of HCC and CC. This might be due to inhibiting PlGF's proliferative effect on macrophages [16], [34] and endothelial cells [14], [16], however further investigation is needed to assess the exact mechanism on how (anti-)PlGF influences the hepatocarcinogenesis

Since PlGF is known to affect the tumour-associated macrophage niche [17], we assessed the M1 and M2-macrophages using 3 markers (HLA-DR, CD206 and CD163). A significant decrease of CD163$^+$-macrophages was observed in CC-lesions, suggesting a possible reduction in M2-macrophage infiltration. These macrophages are known to have a pro-tumoural effect and could possibly enhance metastasis. Recent studies using microarrays and immunohistochemistry have shown that PlGF expression is increased in Japanese non-fluke related cholangiocarcinoma [32] and that its expression is correlated with loss of liver-intestine cadherine [33], which may reflect that PlGF could be correlated with metastasis development in cholangiocarcinoma. In our study, RNA-analysis showed a significant decrease of the pro-metastatic markers *Itgav* and *Icam* in the tumour's surrounding tissue. However, in the tumour tissue only *Icam* was significantly decreased, while there was a trend towards higher *Itgav*-expression. Also, the occurrence of extrahepatic tumours in some αPlGF-treated mice needs further investigation in mouse models more suitable for metastasis-research.

Possibly due to the small sample size only a positive trend but no significant difference was observed between vascularisation in αPlGF and IgG treated HCC-lesions, while previous studies have shown that PlGF-antibodies decrease tumour induced vascularisation in and around HCC-lesions [13]. Several studies have shown that targeting the VEGF-pathway often induces "angiogenic escape" by upregulating other angiogenic factors [35], [36]. Therefore, we have measured expression levels of VEGF, since escape of PlGF-treatment is likely to act through this factor [15]. No significant differences were seen in protein VEGF-levels after treatment with αPlGF, nevertheless. However, RNA-levels of *Vegf* showed a significant upregulation in tumour tissue. Thus, a longer follow up study would be needed to confirm that long term inhibition of PlGF does not induce angiogenic escape through VEGF. Treatment with αPlGF did significantly decrease the serum levels of VEGF, possibly due to an increased binding to its decoy receptor VEGFR1.

To conclude, this study has shown for the first time that inhibiting PlGF decreases HCC and CC burden in an orthotopic DEN-induced mouse model for these primary liver tumours. Unlike VEGF, genetic studies have shown that PlGF is specifically involved in the pathological angiogenesis [14], [15] thus providing an attractive drug candidate against CC and HCC with a good safety profile. Since clinical studies using sorafenib (Nexavar®) in CC have only provided minimal therapeutic benefit, accompanied by severe adverse effects (SWOG 0514) [25], the use of monoclonal antibodies targeting PlGF could offer a potential systemic treatment for patients that suffer from this aggressive liver tumour.

5.6   Acknowledgements

5.7   References

[1]   Thelen A, Scholz A, Weichert W, Wiedenmann B, Neuhaus P, Gessner R, et al. Tumor-associated angiogenesis and lymphangiogenesis correlate with progression of intrahepatic cholangiocarcinoma. *Am J Gastroenterol* 2010;**105**:1123-32.

[2]   Sugimachi K, Tanaka S, Terashi T, Taguchi K-ichi, Rikimaru T, Sugimachi K. The mechanisms of angiogenesis in hepatocellular carcinoma: angiogenic switch during tumor progression. *Surgery* 2002;**131**:S135-41.

[3]   Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J-F, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;**359**:378-90.

[4]   Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 2006;**66**:11851-8.

[5]   Sugiyama H, Onuki K, Ishige K, Baba N, Ueda T, Matsuda S, et al. Potent in vitro and in vivo antitumor activity of sorafenib against human intrahepatic cholangiocarcinoma cells. *J Gastroenterol* 2011;**46**:779-89.

[6]   Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* 2005;**69** Suppl 3:4-10.

[7]   Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, et al. AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. *Cancer Res* 2005;**65**:4389-400.

[8]   Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Yanase K, et al. Involvement of the vascular endothelial growth factor receptor-1 in murine hepatocellular carcinoma development. *J hepatol* 2004;**41**:97-103.

[9]   Huang G-W, Yang L-Y, Lu W-Q. Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival. *World J Gastroenterol* 2005;**11**:1705-8.

[10]   Xu H-X, Zhu X-D, Zhuang P-Y, Zhang J-B, Zhang W, Kong L-Q, et al. Expression and prognostic significance of placental growth factor in hepatocellular carcinoma and peritumoral liver tissue. *Int J Cancer* 2011;**128**:1559-69.

[11]   Van Steenkiste C, Geerts A, Vanheule E, Van Vlierberghe H, De Vos F, Olievier K, et al. Role of placental growth factor in mesenteric neoangiogenesis in a mouse model of portal hypertension. *Gastroenterology* 2009;**137**:2112-24.e1-6.

[12]   Van Steenkiste C, Ribera J, Geerts A, Pauta M, Tugues S, Casteleyn C, et al. Inhibition of placental growth factor activity reduces the severity of fibrosis, inflammation, and portal hypertension in cirrhotic mice. *Hepatology* 2011;53:1629-1640.

[13]   Van de Veire S, Stalmans I, Heindryckx F, Oura H, Tijeras-Raballand A, Schmidt T, et al. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. *Cell* 2010;**141**:178-190.

[14]   Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nature Med* 2001;**7**:575-583.

[15]   Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 2007;**131**:463-475.

[16]   Adini A, Kornaga T, Firoozbakht F, Benjamin LE. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res* 2002;**62**:2749-52.

[17]   Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. *Cancer cell* 2011;**19**:31-44.

[18]   Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;**92**:735-45.

[19]   Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 2005;**438**:820-7.

[20]   Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 1994;**269**:25646-54.

[21]   Van Steenkiste C, Geerts A, Vanheule E, Van Vlierberghe H, De Vos F, Olievier K, et al. Role of placental growth factor in mesenteric neoangiogenesis in a mouse model of portal hypertension. *Gastroenterology* 2009;**137**:2112-2116.

[22]   Heindryckx F, Kuchnio A, Casteleyn C, Coulon S, Olievier K, Colle I, et al. Effect of prolyl hydroxylase domain-2 haplodeficiency on hepatocarcinogenesis and progenitor cell activation. *J Hepatol* 2012; [Epub ahead of print]

[23]   Ogasawara S, Yano H, Higaki K, Takayama A, Akiba J, Shiota K, et al. Expression of angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in human biliary tract carcinoma cell lines. *Hepatology Res* 2001;**20**:97-113.

[24]   Enjoji M, Nakamuta M, Yamaguchi K, Ohta S, Kotoh K, Fukushima M, et al. Clinical significance of serum levels of vascular endothelial growth factor and its receptor in biliary disease and carcinoma. *World J Gastroenterol* 2005;**11**:1167-71.

[25]   El-Khoueiry AB, Rankin CJ, Ben-Josef E, Lenz H-J, Gold PJ, Hamilton RD, et al. SWOG 0514: a phase II study of sorafenib in patients with unresectable or metastatic gallbladder carcinoma and cholangiocarcinoma. *Invest new drugs* 2011; [Epub ahead of print]

[26]   Lubner SJ, Mahoney MR, Kolesar JL, Loconte NK, Kim GP, Pitot HC, et al. Report of a multicenter phase II trial testing a combination of biweekly bevacizumab and daily erlotinib in patients with unresectable biliary cancer: a phase II Consortium study. *J Clin Oncol* 2010;**28**:3491-7.

[27]   Zen C, Zen Y, Mitry RR, Corbeil D, Karbanova J, O'Grady J, et al. Mixed phenotype hepatocellular carcinoma after transarterial chemoembolization and liver transplantation. *Liver transplant* 2011;**17**:943-954.

[28]   Sapisochin G, Fidelman N, Roberts JP, Yao FY. Mixed hepatocellular cholangiocarcinoma and intrahepatic cholangiocarcinoma in patients undergoing transplantation for hepatocellular carcinoma. *Liver transplant* 2011;**17**:934-942.

[29]   Heindryckx F, Mertens K, Charette N, Vandeghinste B, Casteleyn C, Van Steenkiste C, et al. Kinetics of angiogenic changes in a new mouse model for hepatocellular carcinoma. *Mol cancer* 2010;**9**:219.

[30]   Yang H, Li TW, Peng J, Tang X, Ko KS, Xia M, et al. A mouse model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression. Gastroenterol 2011;**141**:378-88, 388 e1-4.

[31]   Yu D, Zhuang L, Sun X, Chen J, Yao Y, Meng K, et al. Particular distribution and expression pattern of endoglin (CD105) in the liver of patients with hepatocellular carcinoma. BMC cancer 2007;**7**:122.

[32]   Jinawath N, Chamgramol Y, Furukawa Y, Obama K, Tsunoda T, Sripa B, et al. Comparison of gene expression profiles between Opisthorchis viverrini and non-Opisthorchis viverrini associated human intrahepatic cholangiocarcinoma. *Hepatology* 2006;**44**:1025-38.

[33]   Takamura M, Yamagiwa S, Wakai T, Tamura Y, Kamimura H, Kato T, et al. Loss of liver-intestine cadherin in human intrahepatic cholangiocarcinoma promotes angiogenesis by up-regulating metal-responsive transcription factor-1 and placental growth factor. *Int J Oncol* 2010;**36**:245-54.

[34]   Khurana R, Moons L, Shafi S, Luttun A, Collen D, Martin JF, et al. Placental growth factor promotes atherosclerotic intimal thickening and macrophage accumulation. *Circulation* 2005;111:2828-36.

[35]   Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nature Rev* 2008;**8**:592-603.

[36]   Hlushchuk R, Makanya AN, Djonov V. Escape mechanisms after antiangiogenic treatment, or why are the tumors growing again? *Int J Dev Biol* 2011;**55**:563-7.

Chapter IV
Discussion

1 The development of a mouse model for HCC

1.1 Advantages of the mouse model

Animal models can provide essential knowledge about the pathogenesis of HCC, particularly when they mimic the natural environment in which human tumours develop. Diethylnitrosamine or "DEN" has been shown to induce hepatic tumours, which are molecularly similar to human HCC with poor prognosis. Yet, the major drawback is the long time needed for tumour progression, it can take over one year for tumours to develop. In our study, weekly intraperitoneal injections with 35 mg/kg DEN gave rise to tumour formation in a relatively short period of time and in a background of fibrosis. The macroscopic and microscopic evaluation, PET-CT images and histopathological analysis confirmed the presence of HCC after 25 weeks, thereby providing a relatively fast and economical model for HCC. The main advantage is that tumour progression occurred in a background of inflammation and fibrosis, thus mimicking the human setting and that the tumours were well vascularised, providing an excellent base for anti-angiogenic research.

1.2 Drawbacks of the mouse model

Although effective, this tumour model holds some weaknesses. The major drawback is the high labour intensity caused by these weekly injections of DEN. Furthermore, the continuous intraperitoneal injections cause peritoneal stress, inflammation, gastrointestinal perforations and urolithiasis, resulting in discomfort and mortality. The high and unpredictable mortality rates seen in the last few weeks, are certainly a disadvantage in this tumour model; and always need to be taken in account when planning future experiments. Also, even though 25 weeks is a short period of time for a chemically induced mouse model, the additional use of xenograft or transgenic mouse models for HCC (as described in our review (1)), or *in vitro* angiogenesis assays would be recommended for faster drug screening. To reduce the labour intensity and the animal's discomfort caused by intraperitoneal injections, DEN can be administrated in drinking water, resulting in tumour formation after 3 to 4

months in rats. Since rats are more prone to HCC, the dose and time should be validated for mice, however, by using non-invasive imaging techniques such as the combined PET-CT, the number of laboratory animals could be limited in a first pilot experiment.

1.3   Imaging-techniques

The combination of casting and micro-CT imaging provides unique data on the hepatic circulation and neovascularisation. It allows to digitally visualise the complex architecture of the liver blood vessels and to provide high-resolution data for qualitative morphological analysis. However, up till now, it has not been possible to quantitatively score the images, mainly due to the different camera-angles used in SEM. A possibility for the future would be combining video-capture and images, to quantify the number of branching points in vessels as a marker for microvessel density and vessel distribution.

1.4   Conclusion

Thus, during the first part of this study we have established a new mouse model for HCC, which is considerably faster than current chemically induced models and has the advantage of tumour progression occurring in a background of inflammation and fibrosis, resembling the human situation. However, the high mortality, the discomfort caused by the DEN-injections, and the almost 6-month time span, might provoke the need for alternative methods to assess HCC and would require additional research.

2 Role of PlGF in the pathogenesis of primary liver tumours

2.1 Effect on tumour growth and vascularisation

In the second part of our study, we have shown that PlGF is involved in the pathogenesis of HCC. Both in the transgenic knock out model as in the treatment model, silencing or inhibition of PlGF significantly decreased tumour burden, not only by inhibiting the vascularisation, but also by decreasing hepatic macrophage recruitment, in line with reports that state that PlGF exerts part of its effect by recruiting pro-angiogenic macrophages (2). Indeed, αPlGF acts also as an anti-inflammatory compound, since PlGF is known to attract VEGF-1^{+} macrophages (3,4). These macrophages are associated with tumour progression, metastasis and poor prognosis (2,5,6), thus the inhibition of PlGF could have an important influence on the establishment of the pre-metastatic niche, by preventing recruitment of tumour-associated macrophages.

Protein levels of HCC and cirrhotic patients confirm that PlGF is upregulated during the pathogenesis of HCC, thus an anti-human PlGF antibody (such as TB403) could become a valuable therapy in HCC-patients. Furthermore, our study using PHD2$^{+/-}$-mice injected with DEN, showed that inhibiting PlGF could also offer a therapeutic solution for patients with CC.

Besides the good safety profile of PlGF-antibodies, treatment with αPlGF holds great potential due to its effect on vascularisation and vascular morphology. Even though HCC is a "hypervascular" cancer, it is characterised by hypoxia, and its growth is promoted by hypoxia (7). While hypoxic HCC cells try to improve their oxygenation by stimulating the arterial blood supply, their oxygen delivery is often impaired by the formation of abnormal tumour vessels. This initiates a reinforcing vicious cycle of HCC-vascularisation, which promotes malignancy, especially since HIF is known to select for a more aggressive and pro-metastatic tumour phenotype. In line with previous studies, we have shown that αPlGF not only inhibits vessel growth, but also causes the selective regression of these existing abnormal tumour vessels. Therefore, the use of αPlGF holds great potential, since it does not only inhibit the formation of tumour blood vessels,

but also normalises the remaining vasculature, breaking this vicious circle. Indeed, in our study we have shown that PlGF-inhibited mice injected with DEN, express less pro-metastatic markers, revealing a possible shift towards a less aggressive tumour phenotype. However, further analysis in more suitable models for metastasis, is needed to confirm this hypothesis.

Aberrations in the tumour vasculature have great implications for tumour sensitivity to therapy. Hypoxia is a well-known mediator for cancer cell resistance to conventional radiotherapy and cytotoxics (8,9). Furthermore, the poor blood supply caused by the disorganized vasculature, impairs the delivery of systemically administered therapies (10–12). Indeed, besides improving the metabolic profile of the tumour microenvironment, vascular normalisation induced by αPlGF could also improve the delivery and efficacy of exogenously administered therapeutics, the efficacy of radiotherapy and decrease chemoresistance. This has not yet been tested in our study, but administrating classic chemotherapy in combination with αPlGF provides great opportunities for future research.

Combining anti-angiogenic therapies with other chemotherapeutic compounds has become an interesting field of research, resulting in several clinical trials. The combination of sorafenib with doxorubicin is presently being investigated in a phase III randomized trial, however because doxorubicin causes cell death through upregulation of the MEK/ERK pathway, and sorafenib has an opposite influence on the same cascade, co-treatment with these drugs has lead to an antagonistic effect in experimental models (13). However, other studies did report that pre-treatment of tumour cells with sorafenib significantly enhances the effects of chemotherapeutics through another mechanism. Sorafenib is able to inhibit DNA-repair proteins as a consequence tumour cells become more sensitive to DNA-damaging agents like chemotherapy and radiation treatment (14) The combination of vascular normalisation with αPlGF and the inhibition of DNA-repair proteins could become a potential strategy for decreasing chemoresistance in HCC.

2.2   Safety of αPlGF

Severe adverse effects such as teratogenicity, pruning of healthy vessels, thrombosis and hypertension often accompany inhibition of angiogenesis. For sorafenib, the main adverse effects in HCC-patients are diarrhoea, weight loss, hand–foot skin reaction, hair loss, anorexia, asthenia, fatigue, and voice changes. Since PlGF-expression is negligible in quiescent tissues in baseline conditions and its full knock out in mouse models does not cause any noteworthy abnormalities, generating a favourable safety profile. The safety of αPlGF or PlGF-silencing has been assessed in several mouse models, showing no effect on fetal development (15), pruning of healthy vessels (16) nor any other notable abnormalities. Previous studies in our research group have shown that PlGF deficiency and administration of αPlGF does not prune the vessels in the thyroid gland, while VEGF-inhibitors prune the mature quiescent vasculature in non-pathological organs such as the thyroid gland and the trachea (17). Also in this DEN-induced HCC-model, αPlGF was well tolerated and no drug-induced mortality or significant effects on the mice's well begin were observed. In addition, two phase I clinical trials with a humanised anti-human PlGF antibody (TB403) has shown acceptable safety (18)

However, our micro-array analysis revealed that 55 probes had an altered expression between αPlGF and IgG treated healthy control mice. Further analysis showed that several genes involved in neurogenesis, spermatogenesis and olfactory receptor development had a different expression after αPlGF-treatment. Although we were not able to show if this altered gene-expression had any relevant phenotypical consequences, it is noteworthy that these characteristics should be closely observed when further testing αPlGF (or TB403).

2.3   Angiogenic escape mechanisms

An important weakness of current anti-angiogenic therapies, is that tumours tend to escape their treatment by upregulating alternative angiogenic pathways, as discussed in the introduction. As a result, several cases have been reported of resistance against sorafenib-treatment (19,20).

In our study, we have used micro-arrays to assess whether treatment with αPlGF induces angiogenic escape on RNA-levels and we could not observe an increased expression of other angiogenic genes, in the contrary, several angiogenic factors were significantly downregulated after treatment with αPlGF. For instance, PlGF-silenced tumours were characterised lower protein levels of VEGF, while RNA-levels were unaltered, suggesting an increased binding to its decoy-receptor, VEGFR1, supporting the dual action of αPlGF (one dependent and one independent of VEGF).

One of the proposed mediators of angiogenic escape is the activation of HIF. By increasing tumour hypoxia, an additional angiogenic switch is induced after administration of anti-angiogenic drugs (21–23). Treatment with αPlGF does not result in a vast upregulation of hypoxic markers possibly explaining why no other angiogenic genes were upregulated. However, only a 5-week treatment with αPlGF was assessed, which does not provide information on the long-term therapeutic effects of PlGF-inhibition. In the PlGF-knock-out study tumours grew in a PlGF-silenced environment and indeed, after 30 weeks we did see a slight increase of tumour burden, which might be an indication of resistance against PlGF-inhibition.

Furthermore, we did not look at other angiogenic processes that work independent of HIF, such as vessel-cooption, vascular mimicry and a switch of angiogenic phenotype. Several authors have stated that administration of anti-angiogenic compounds causes a switch from sprouting angiogenesis towards intussusceptive angiogenesis. The latter has been proposed as one of the underlying mechanisms responsible for the normalizing the vasculature during anti-angiogenic treatment and thus this process needs to be investigated in the future. It is of uttermost importance that these processes are being assessed in the near future.

The complex, highly heterogeneous nature of HCC makes it unlikely that targeting only one pathway will achieve optimal disease control. Even though no evidence was found towards angiogenic escape mechanisms after treatment with αPlGF in our study, it is improbable that long-term administration of PlGF-antibodies would not induce resistance. Long term administration of variable dosages αPlGF and subsequent expression analysis, could provide additional insight in potential angiogenic-escape mechanisms against αPlGF. Furthermore,

combination therapies, new therapeutic targets, better patient selection based on tumour characteristics, and more sensitive imaging techniques are essential to obtain maximum response from αPlGF in patients.

2.4   αPlGF and sorafenib

In our study, we have used a monotherapeutic approach for αPlGF and sorafenib, as well as a combination therapy. We did not find a significant difference in survival, tumour burden and inflammation in αPlGF, sorafenib and the combination treatment. Yet, its primary advantage may lay in the fact that sorafenib and other VEGF-VEGFR-targeted therapies cause a number of adverse effects, while PlGF-targeted therapies are supposed to have a more favourable safety profile, as discussed before.

Another advantage of αPlGF is its effect on vascular normalisation, which consequently decreases hypoxia, selects for a less aggressive tumour phenotype and could potentially improve drug delivery and efficacy. In our study we did not observe improvement of the microvasculature after treatment with sorafenib and this resulted in a higher expression of hypoxic markers, consequently selecting for a more pro-metastatic tumour phenotype. Our micro-array analysis provides evidence that inhibition of PlGF selects for a less metastatic tumour phenotype, while qPCR-analysis showed that treatment with sorafenib tends to increase the expression pro-metastatic genes. Analysis of the GSE33621-dataset also showed that sorafenib-treatment decreases the metastatic-suppressor gene *Kiss1*. However, further investigation using metastatic models for HCC, could provide valuable insight on the influence of αPlGF and sorafenib on the metastatic potential.

Even though the combination of αPlGF and sorafenib did not provide a significant survival benefit compared to the monotherapies, it could still provide additional benefits. The administration of αPlGF could normalize the tumour vasculature, thereby possibly resulting in a better efficacy of low doses of sorafenib. By combining αPlGF and sorafenib, the dose of the latter could be decreased without losing functionality, yet reducing the adverse effects.

2.5   αPlGF in cholangiocarcinoma

Treatment with PlGF-antibodies significantly improved CC-burden, possibly by inhibiting PlGF's proliferative effect on macrophages and endothelial cells.

Recent studies have shown that inducing ischemic injury by transarterial chemoembolization leads to an increased occurrence of a mixed hepatocellular and cholangiocellular tumour phenotype (24,25). The mixed occurrence of HCC and CC is more aggressive, thus adjuvant αPlGF co-treatment could therefore offer a therapeutic solution for these patients. Recent studies using micro-arrays and immunohistochemistry have shown that PlGF expression is increased in Japanese non-fluke related cholangiocarcinoma (26) and that its expression is correlated with loss of liver-intestine cadherine (27), which may reflect that PlGF could be correlated with metastasis development in cholangiocarcinoma. Since clinical studies using sorafenib in CC have only provided minimal therapeutic benefit, accompanied by severe adverse effects (SWOG 0514), the use of monoclonal antibodies targeting PlGF could offer a potential systemic treatment for patients that suffer from this aggressive liver tumour. However, further investigation is needed using different models for CC, to assess the underlying mechanism of PlGF-inhibition on CC-development.

2.6   Future perspectives

In our study we have shown that inhibition of PlGF could potentially be used as a therapeutic strategy against primary liver tumours. While the work performed is very encouraging, many questions remain and will need further attention in the near future.

One of the main questions that still remain is the exact working mechanism of PlGF. We have shown that αPlGF can inhibit the infiltration of macrophages into the liver, however this effect in HCC was only limited compared to what is seen in other tumour models. A study by Rolny *et al.* (2), suggests that PlGF induces polarisation of tumour-associated macrophages to a pro-angiogenic M2-type phenotype, which is well known to promote tumour growth. Therapy with αPlGF in this case can prevent this polarisation to the M2-phenotype and promote macrophages to remain in the more dormant M1-phenotype. Taken

together, an effect on the number of macrophages, and polarisation of macrophages towards a more favourable phenotype can create an interesting interplay. Hopefully, future research will bring light onto the exact role of PlGF in macrophages, and define the molecular mechanisms at play.

We have shown that inhibiting PlGF normalises the remaining vasculature, thereby selecting for a less aggressive tumour phenotype. However, the DEN-induced HCC-model is not an optimal model to assess metastasis. Therefore, additional mouse models, more suitable for metastasis-research, would greatly expand our knowledge on how PlGF influences the metastatic-niche. A highly metastatic tumour model can be obtained by orthotopic implantation of metastatic tumours, either obtained from surgical specimens (28) or from metastatic cell lines that were ectopically implanted in SCID mice (29). Quantifying the number of lung metastasis, together with an extensive genome analysis on pro- and anti-metastatic markers, would provide valuable insight on the anti-metastatic effect of αPlGF on HCC.

In the present study, we have used micro-arrays to assess whether treatment with αPlGF induces angiogenic escape on RNA-levels and we could not observe an increased expression of other angiogenic genes. However, only a 5-week treatment with αPlGF was assessed, which does not provide information on the long-term therapeutic effects and the PlGFKO-study does give an indication that long-term inhibition of PlGF may cause tumours to find alternate ways to progress. Long-term administration of variable dosages αPlGF and followed by expression analysis, could provide additional insight in potential angiogenic-escape mechanisms against αPlGF.

Irregularities in the tumour vasculature have great implications for tumour sensitivity to therapy and in our study we have shown that PlGF-inhibition improves the vascular morphology, thus decreasing tumour hypoxia and could therefore reduce drug resistance (8,9). Testing gene-expression levels of multidrugresistance genes, such as MDR1, MDR2 and BCRP, in PlGF-inhibited tumours could strengthen this hypothesis. Additionally, administrating chemotherapy in combination with αPlGF or in PlGF knock out mice with HCC would provide an exciting new translational research topic.

In this study, we tested one dose of sorafenib (10 mg/kg) based upon studies found in the literature. This did not induce an additive effect when combined with αPlGF. However, it would be interesting to assess if giving αPlGF in combination with a lower dose of sorafenib would keep a similar level of functionality, but perhaps diminish the adverse effects.

Of course our study has only looked at the effect of PlGF-inhibition in mice and we can never be certain that these results are completely representable for the human setting. For one, humans express four isoforms of PlGF, while mice only express one. However, serum levels of PlGF in patients showed that PlGF-2, presumably the most important isoform, is upregulated in HCC and in cirrhotic patients, thereby providing some evidence that it might play a role in the human hepatocarcinogenesis. Currently the first human αPlGF studies show that TB403 was well tolerated without increased risk of adverse effects in healthy volunteers and terminal cancer patients, but with signs of disease stabilization in 6 out of 23 cancer patients (18). Future clinical studies are required to assess the overall therapeutic potential of αPlGF treatment strategies in hepatocellular carcinoma.

2.7   Conclusion

In the second part of our study, we have shown that PlGF is involved in the pathogenesis of HCC and CC. Both in the transgenic mouse model as in the treatment study, silencing or inhibition of PlGF significantly decreased tumour burden and prolonged survival. Its effect was induced not only by inhibiting neo-vascularisation, but also by decreasing hepatic macrophage recruitment. The use of αPlGF holds great potential, since it does not only inhibit the formation of tumour blood vessels, but also normalises the remaining blood vessels, thereby decreasing tumour hypoxia and thus reducing the pro-metastatic potential of HCC. Future research is needed to assess how treatment with αPlGF affects metastasis, multidrugresistance and angiogenic escape mechanisms. Since the first clinical trials using a human αPlGF are promising, we are eagerly awaiting the moment when αPlGF can be tested for patients with primary liver tumours.

3 Role of PHD2 in the pathogenesis of primary liver tumours

3.1 Effect on tumour growth and vascularisation

In the last part of our study, we used the DEN-model to compare the effect of PHD2-heterozygousity on HCC-development. Macroscopic evaluation showed that PHD2$^{+/-}$-mice developed more tumours compared to their WT-counterparts. This was not entirely unexpected, since several reports have shown that PHD2-deficiency could increase tumourigenesis (30–33). This was in contrast with what is seen in the study of Mazonne *et al* (34), who found that haplodeficiency of PHD2 did not increase tumour burden, but inhibited tumour cell invasion, intravasation and metastasis, by normalising the vasculature. In our study we used vascular corrosion casting and histological analysis to evaluate the vascular morphology and we were not able to detect any improvement, which was in line with other findings that observed an increased capillary density, vessel branching and an increased tumour growth as a result of PHD2-silencing in the tumour (31). All the studies mentioned above use different mouse models to assess the effect of PHD2-inhibition on tumour development, which might explain part of the discrepancies, but also the distinct role of PHD2 in specific tumour compartments could influence the outcome. In our study we have used the DEN-model in which spontaneous tumours occur as a result of chronic exposure to the hepatic carcinogen DEN, a situation that is more representable for the human hepatocarcinogenesis than the xenograft models used in most other studies. In addition, PHD2 was silenced both in the tumour as in the stroma, in contrast to xenograft studies were usually only one of those compartments has an altered PHD2-expression.

However, the different outcome in several studies, might also be a result of the dual role of PHD2 in tumour progression, as suggested in the study of Lee *et al* (30) using human malignant fibroblast cell strains at different transformation stages. Firstly, decreases of PHD2 were correlated with the increased malignancy and invasiveness of fibroblasts and cancer cell lines, supporting PHD2's involvement in the tumourigenesis. Interestingly, chemical inhibition of PHD2-activity in the cells decreased the tumour-forming potential of these

234

transformed cells; while overexpression of PHD2 in malignant fibroblasts also lead to an inhibition of tumour growth. This dual role will, of course, have severe consequences on drugs that target PHDs for cancer therapy. In our study, it is likely that hepatocytes that had amassed cellular mutations due to the DEN-injections were pushed to become more tumorigenic due to the partial blockage of PHD2. This could also imply that inhibiting PHD2 in established tumours would have a different outcome and is therefore an interesting topic for future research.

Microscopic analysis confirmed an increased HCC-burden at 20W and 25W, yet HCC-progression stagnates after 30W, perhaps due to the increased presence of CC, which might result in a competition for nutrients between tumours. The presence of both HCC and CC in PHD2$^{+/-}$-mice injected with DEN, was an interesting result. Indeed, the prolonged administration of DEN induced inflammation and fibrosis around cancer foci or in non-cancer liver tissues and could thereby influence ductular reaction. PHD2 deficiency increased the infiltration of macrophages, however, no significant difference of fibrosis was seen between WT and PHD2$^{+/-}$ mice, suggesting the ductular reaction is not merely a result of increased inflammation. Since both CC and HCC could be derived from HPCs, and our study showed that inhibition of PHD2 influences resulted in a shift from HCC to CC, perhaps PHD2-heterozygosity might have influenced progenitor cell activation and/or differentiation.

3.2 Effect on progenitor cell differentiation

We have shown that PHD2-inhibition leads to the occurrence of both HCC and CC in the liver. Several studies suggest that these hepatic tumours can be derived from HPCs which are bipotential stem cells that reside in the canals of Hering (35–37). HPCs are activated in the case of severe hepatic damage and the proliferation of progenitor cells is one of the earliest responses to DEN, however, contradictory results have been found on whether DEN-induced tumours are derived from HPCs. The expression of CK19, Oct4 and LEF in the surrounding tissue and tumour lesions of DEN-injected WT and PHD2$^{+/-}$ could suggest an HPC-origin of these tumours.

HPCs are able to differentiate towards biliary and hepatocytic lineages. However, the exact mechanism on how these cells are stimulated towards a certain lineage remains largely unknown. A possible regulator of HPC-differentiation is the Notch-pathway, whose activation will favour progression towards a cholangiocytic phenotype (36). The inhibition of PHD2 results in a decreased degradation of HIF. This increases the activation of the HIF-pathway, which could regulate the Notch-pathway thereby altering the differentiation of HPCs. Further investigation is needed to provide new insights in the influence of PHD2 and activation of the HIF-pathway in the tumour environment on the differentiation of HPCs and the selection of a more aggressive tumour phenotype during hypoxia.

The link between hypoxia in the tumour environment and the differentiation of HPCs holds a great potential for therapeutic intervention, especially since recent studies have shown that inducing ischemic injury by TACE leads to an increased occurrence of a mixed hepatocholangiocellular phenotype (38). This biliary phenotype of HCC is more aggressive and is likely derived from hepatic progenitor cells. Therefore, it could be that HPCs are stimulated through the TACE-induced hypoxia to differentiate towards a cholangiocytic lineage. This link between hypoxia and the selection for a more aggressive mixed HCC-CC phenotype might also have a repercussion on patients receiving long-term treatment with anti-angiogenic compounds, such as sorafenib which may induce a more hypoxic tumour environment. Further investigation is needed to support this hypothesis.

3.3   Future perspectives

We have shown that silencing PHD2 aggravates HCC growth and induces the presence of CC. Future research is necessary to clarify the exact role of PHD2 or HIF-stimuli on the hepatocarcinogenesis and specifically on the differentiation of hepatic progenitor cells.

In our study we have looked at the development of tumours in a PHD2-heterozygotic environment. Adding PHD2-inhibiting agent (such as DMOG) to mice with an established tumour would provide interesting information on how tumour cells react to a sudden inactivation of PHD2. A study of Henze *et al* (39)

found that inhibiting PHD2-activity by exposure to hypoxia or DMOG-administration reduced glioma tumour cell survival in an *in vitro* experiment and suggest a negative feedback loop to limit the hypoxic HIF-response. However, in our study we did not observe a negative feedback, on the contrary, the long-term silencing of PHD2 in the heterozygous knock out model, still led to an increased activation of the HIF-pathway. If would therefore be interesting to repeat Henze's experiment in our *in vivo* model for HCC.

In addition, studies have shown that PHD2 functions as a tumour suppressor and its loss is associated with increased malignancy (30,33). It would therefore be interesting to assess how overexpression of PHD2 influences the hepatocarcinogenesis. Furthermore, overexpression of PHD2 is likely to increase the hydroxylation of HIF, leading to less activation of the HIF-associated pathways, which are known to promote malignancy and drug resistance.

Even though several studies have shown that PHD2 is the uttermost important PHD-molecule involved in the regulation of HIF, a similar set-up with PHD1 and PHD3-mice could be valuable, especially since some studies have shown that PHD3 is involved in hypoxic survival mechanisms of tumour cells (40) and in the inflammatory-response (41). The absence of PHD1 has been associated with reduced neovascularisation and vascular permeability in a mouse model for ischemic retinopathy (42). If PHD1 or PHD2 play a role in the pathogenesis of HCC remains unknown and would provide an interesting field of research.

Whole genome expression profiling of CC and HCC-lesions and of their surrounding non-tumour tissue in the PHD2-deficient mouse model could provide new insights in the influence of PHD2 on the differentiation of HPCs and on the selection of a more aggressive tumour phenotype during hypoxia. Investigation of the characteristics and potential of HPCs from WT and PHD2$^{+/-}$ mice would clarify whether the combined appearance of HCC and CC is due to the intrinsic properties of PHD2$^{+/-}$ HPCs. By isolating HPCs from WT-mice and PHD2$^{+/-}$ -mice and subsequently subjecting them to gradients of hypoxia, we could further clarify their behaviour and possibly identify hypoxia as a stimulus for HPC-activation.

Our data suggest that the Notch-pathway may play an important role in the differentiation of HPC to cholangiocytes or hepatocytes. Using transgenic Notch1-lox or R26-lsl-NICD mice to conditionally alter the expression of the Notch-pathway in hepatocytes or HPC's in mice subjected to weekly injections of DEN, and subsequently isolating HPC, combined with FACS cell sorting (to determine the differentiation of cells) and histological analysis could be used to closely investigate the impact of this pathway at different phases of tumour progression.

3.4   Conclusion

In the last part of our study, we have shown that inhibition of PHD2 is involved in the hepatocarcinogenesis and that its inhibition aggravates tumour growth and induces the presence of cholangiocarcinoma, an aggressive liver tumour derived from biliary cells. This could possibly be explained by an altered HPC-differentiation through activation of the Notch-pathway. However, future research is necessary to clarify the exact role of PHD2 or HIF-stimuli on the differentiation of liver progenitor cells, especially for patients receiving long-term administration of anti-angiogenic therapy.

4 General discussion

The discovery of anti-angiogenic therapy has become a milestone in the combat against HCC, especially since the acknowledgement of sorafenib as the standard of care for advanced HCC patients. However, new compounds that can inhibit the tumour vasculature are being discovered every day and while the majority proves to be effective in murine cancer, only few make it to the clinic. Those that are approved as chemotherapeutics, still hold several important limitations.

One of the recent concerns is the occurrence of angiogenic escape mechanisms, which allow tumours to grow despite the administration of anti-angiogenic drugs, possibly even inducing a more aggressive tumour phenotype. The hypoxic conditions created by anti-angiogenic therapy, may select for more invasive tumour variants better adapted to survive and proliferate under reduced oxygen tension, leading to increased intravasation and metastatic dissemination (34,43–45). Anti-angiogenic therapies that inhibit a single target cause the upregulation of additional angiogenic factors, in a process called "angiogenic rescue" (46). Alternatively, these angiogenic escape mechanisms can also function independent from hypoxia, by inducing vascular mimicry, vessel cooption or switching the angiogenic phenotype.

In our study, we have shown that vascular normalization could potentially prevent the HIF-dependent angiogenic rescue mechanisms. Studies on vascular normalization have also shown that by correcting the abnormalities in structure and function of tumour vessels (rather than destroying vessels completely) the tumour microenvironment is normalized, resulting in better perfusion of cytotoxic drugs and controlling the tumour's progression (10,11). This effect is not unique for αPlGF, since pharmacological neutralisation of VEGF in mice has also decreased tumour vessel permeability and has impoved tumour perfusion, leading to better drug delivery. However, this effect is transient, since prolonged VEGF-inhibition prunes the majority of the vessels and thus dimishes this effect. Hence, dosage and duration of treatment seems to be an important factor in the process of vascular normalisation, which has to be further assessed, not only with αPlGF but also in other anti-angiogenic compounds.

A logical step could be to stop treatment when pruning of normal vessels occurs. However, mouse models have also shown that a rapid tumour growth may occur after stopping anti-angiogenic treatment. It thus seems that by continuing therapy, it increases the risk of inducing angiogenic-escape mechanisms, while discontinuation of treatment may lead to vessel regrowth. Thus, besides developing new anti-angiogenic drugs, it is needed to investigate how rendering a time schedule in drug delivery, combined with a multi-targeted approach to prevent anti-angiogenic escape, could provide additional benefit.

Future studies will be needed to assess whether combining vascular normalization, anti-angiogenic drugs and perhaps cytotoxic compounds could provide a long-term solution for HCC-patients.

5   General conclusions

In the first part of our study, we showed that weekly injections of DEN provide an excellent mouse model for HCC, which is considerably faster than current chemically induced models and has the advantage of tumour progression occurring in a background of inflammation and fibrosis. Furthermore, the assessment of several angiogenic factors showed that these tumours induce an angiogenic switch, providing an ideal base for further research on anti-angiogenic drugs. Moreover, several innovative imaging techniques were applied, not only to assess tumour growth, but also providing further insight in microvascular alterations of HCC livers.

After the establishment of a mouse model, we have used transgenic mouse models and monoclonal antibodies to assess the effect of PlGF and PHD2 deficiency on the hepatocarcinogenesis. In our study, we have shown that PlGF is involved in the pathogenesis of HCC. Both in the transgenic knock out model as in the treatment model, silencing or inhibition of PlGF significantly decreased tumour burden, not only by inhibiting the vascularisation, but also by decreasing hepatic macrophage recruitment. The use of αPlGF holds great potential, since it does not only inhibit the formation of tumour blood vessels, but also normalizes the remaining blood vessels, thereby decreasing hypoxia and thus reducing the pro-metastatic potential of HCC.

PHD2-deficiency increased the hepatocarcinogenesis and stimulated the development of cholangiocarcinoma. Since both CC and HCC could be derived from HPCs, PHD2 could possibly have altered their differentiation, possibly through up-regulating the Notch-pathway. In contrast to other studies, PHD2-deficiency did not normalise the vasculature, and thus did not select for a less aggressive tumour phenotype. On the contrary, PHD2-haplodeficiency and the accompanying continuous HIF-activation resulted in an increased expression of pro-metastatic markers and selected for a more aggressive combined HCC-CC tumour phenotype.

Administration of PlGF-antibodies to PHD2$^{+/-}$ mice that have developed HCC and CC after 25W of DEN, decreased the tumour burden of both HCC and CC, by affecting angiogenesis and inflammation.

6   References

1. Heindryckx F, Colle I, Van Vlierberghe H. Experimental mouse models for hepatocellular carcinoma research. Int J Exp Pathol. 2009;90(4):367–86.

2. Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer cell. 2011 Jan 18;19(1):31–44.

3. Autiero M, Luttun A, Tjwa M, Carmeliet P. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. Journal of thrombosis and haemostasis : JTH. 2003 Jul;1(7):1356–70.

4. Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den Heuvel J, et al. VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. Circulation research. 2003;92(4):378–85.

5. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nature reviews. Cancer. 2004 Jan;4(1):71–8.

6. Giraudo E, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. The Journal of clinical investigation. 2004 Sep;114(5):623–33.

7. Huang G-W, Yang L-Y, Lu W-Q. Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival. World journal of gastroenterology : WJG. 2005 Mar 21;11(11):1705–8.

8. He C, Sun X-P, Qiao H, Jiang X, Wang D, Jin X, et al. Downregulating Hypoxia-inducible factor-2α improves the efficacy of doxorubicin to treat hepatocellular carcinoma. Cancer science. 2011 Dec 6;

9. Jung EU, Yoon JH, Lee YJ, Lee JH, Kim BH, Yu SJ, et al. Hypoxia and retinoic acid-inducible NDRG1 expression is responsible for doxorubicin and retinoic acid resistance in hepatocellular carcinoma cells. Cancer letters. 2010;298(1):9–15.

10. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nature reviews. Drug discovery. 2011 Jun;10(6):417–27.

11. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science (New York, N.Y.). 2005 Jan 7;307(5706):58–62.

12. Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer research. 2004 Jun 1;64(11):3731–6.

13. Manov I, Pollak Y, Broneshter R, Iancu TC. Inhibition of Doxorubicin-induced autophagy in hepatocellular carcinoma Hep3B cells by Sorafenib: the role of extracellular signal-regulated kinase counteraction. The FEBS journal. 2011 Jul 26;278(18):3494–507.

14. Yadav A, Kumar B, Teknos TN, Kumar P. Sorafenib enhances the antitumor effects of chemoradiation treatment by downregulating ERCC-1 and XRCC-1 DNA repair proteins. Molecular cancer therapeutics. 2011 Jul;10(7):1241–51.

15. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nature medicine. 2001;7(5):575–83.

16. Kamba T, Tam BYY, Hashizume H, Haskell A, Sennino B, Mancuso MR, et al. VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. American journal of physiology. Heart and circulatory physiology. 2006 Feb;290(2):H560–76.

17. Steenkiste CV, Ribera J, Geerts A, Pauta M, Tugues S, Casteleyn C, et al. Inhibition of placental growth factor activity reduces the severity of fibrosis, inflammation, and portal hypertension in cirrhotic mice. Hepatology. 2011;53(5):1629–40.

18. Martinsson-Niskanen T, Riisbro R, Larsson L, Winstedt L, Stenberg Y, Pakola S, et al. Monoclonal antibody TB-403: a first-in-human, Phase I, double-blind, dose escalation study directed against placental growth factor in healthy male subjects. Clinical therapeutics. 2011 Sep;33(9):1142–9.

19. Scholl S, Spies-Weisshart B, Klink A, Muegge L-O, Fricke H-J, Hochhaus A. Secondary resistance to sorafenib in two patients with acute myeloid leukemia (AML) harboring FLT3-ITD mutations. Annals of hematology. 2011 Apr 1;90(4):473–5.

20.   Chen K-F, Chen H-L, Tai W-T, Feng W-C, Hsu C-H, Chen P-J, et al. Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. The Journal of pharmacology and experimental therapeutics. 2011 Apr;337(1):155–61.

21.   Ebos JML, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer cell. 2009 Mar 3;15(3):232–9.

22.   Ebos JML, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009 Aug 15;15(16):5020–5.

23.   Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell. 2009;15(3):220–31.

24.   Sapisochin G, Fidelman N, Roberts JP, Yao FY. Mixed hepatocellular cholangiocarcinoma and intrahepatic cholangiocarcinoma in patients undergoing transplantation for hepatocellular carcinoma. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2011;17(8):934–42.

25.   Lee JH, Chung GE, Yu SJ, Hwang SY, Kim JS, Kim HY, et al. Long-term prognosis of combined hepatocellular and cholangiocarcinoma after curative resection comparison with hepatocellular carcinoma and cholangiocarcinoma. Journal of clinical gastroenterology. 2011;45(1):69–75.

26.   Jinawath N, Chamgramol Y, Furukawa Y, Obama K, Tsunoda T, Sripa B, et al. Comparison of gene expression profiles between Opisthorchis viverrini and non-Opisthorchis viverrini associated human intrahepatic cholangiocarcinoma. Hepatology (Baltimore, Md.). 2006 Oct;44(4):1025–38.

27.   Takamura M, Yamagiwa S, Wakai T, Tamura Y, Kamimura H, Kato T, et al. Loss of liver-intestine cadherin in human intrahepatic cholangiocarcinoma promotes angiogenesis by up-regulating metal-responsive transcription factor-1 and placental growth factor. International journal of oncology. 2010 Jan;36(1):245–54.

28.   Sun FX, Tang ZY, Lui KD, Ye SL, Xue Q, Gao DM, et al. Establishment of a metastatic model of human hepatocellular carcinoma in nude mice via orthotopic implantation of histologically intact tissues. International journal of cancer. Journal international du cancer. 1996 Apr 10;66(2):239–43.

29.   Huynh H, Chow PKH, Tai WM, Choo SP, Chung AYF, Ong HS, et al. Dovitinib demonstrates antitumor and antimetastatic activities in xenograft models of hepatocellular carcinoma. Journal of hepatology. 2011 Oct 23;

30.   Lee KA, Lynd JD, O'Reilly S, Kiupel M, McCormick JJ, LaPres JJ. The biphasic role of the hypoxia-inducible factor prolyl-4-hydroxylase, PHD2, in modulating tumor-forming potential. Molecular cancer research : MCR. 2008;6(5):829–42.

31.   Chan DA, Kawahara TL, Sutphin PD, Chang HY, Chi JT, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. Cancer Cell. 2009;15(6):527–38.

32.   Takeda K, Cowan A, Fong GH. Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system. Circulation. 2007;116(7):774–81.

33.   Su Y, Loos M, Giese N, Metzen E, Buchler MW, Friess H, et al. Prolyl hydroxylase-2 (PHD2) exerts tumor-suppressive activity in pancreatic cancer. Cancer. 2011;

34.   Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. Cell. 2009;136(5):839–51.

35.   Libbrecht L. Hepatic progenitor cells in human liver tumor development. World journal of gastroenterology : WJG. 2006;12(39):6261–5.

36.   Spee B, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borght S, Gaudio E, et al. Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. Gut. 2010;59(2):247–57.

37.   Dolle L, Best J, Mei J, Al Battah F, Reynaert H, van Grunsven LA, et al. The quest for liver progenitor cells: A practical point of view. J Hepatol. 2009;

38.   Zen C, Zen Y, Mitry RR, Corbeil D, Karbanova J, O'Grady J, et al. Mixed phenotype hepatocellular carcinoma after transarterial chemoembolization and liver transplantation. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2011;17(8):943–54.

39.   Henze AT, Riedel J, Diem T, Wenner J, Flamme I, Pouyseggur J, et al. Prolyl hydroxylases 2 and 3 act in gliomas as protective negative feedback regulators of hypoxia-inducible factors. Cancer research. 2010;70(1):357–66.

40.   Högel H, Rantanen K, Jokilehto T, Grenman R, Jaakkola PM. Prolyl hydroxylase PHD3 enhances the hypoxic survival and G1 to S transition of carcinoma cells. PloS one. 2011 Jan;6(11):e27112.

41.   Walmsley SR, Chilvers ER, Thompson AA, Vaughan K, Marriott HM, Parker LC, et al. Prolyl hydroxylase 3 (PHD3) is essential for hypoxic regulation of neutrophilic inflammation in humans and mice. The Journal of clinical investigation. 2011 Mar 1;121(3):1053–63.

42.   Huang H, Van de Veire S, Dalal M, Parlier R, Semba RD, Carmeliet P, et al. Reduced retinal neovascularization, vascular permeability, and apoptosis in ischemic retinopathy in the absence of prolyl hydroxylase-1 due to the prevention of hyperoxia-induced vascular obliteration. Investigative ophthalmology & visual science. 2011 Sep;52(10):7565–73.

43.   Höckel M, Vaupel P. Biological consequences of tumor hypoxia. Seminars in oncology. 2001 Apr;28(2 Suppl 8):36–41.

44.   Brahimi-Horn C, Pouysségur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. Bulletin du cancer. 2006 Aug;93(8):E73–80.

45.   Du G, Han G, Zhang S, Lin H, Wu X, Wang M, et al. Baicalin suppresses lung carcinoma and lung metastasis by SOD mimic and HIF-1alpha inhibition. European journal of pharmacology. 2010 Mar 25;630(1-3):121–30.

46.   Kerbel RS, Yu J, Tran J, Man S, Viloria-Petit A, Klement G, et al. Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. Cancer metastasis reviews. 2001 Jan;20(1-2):79–86.

Chapter V
Summary

1   Summary

Hepatocellular carcinoma (HCC) is a primary liver tumour that originates in a background of cirrhosis. Every year almost 500.000 patients are diagnosed with HCC, making it the 5th most common and the 3rd most deadly cancer worldwide. The underlying liver disease often conceals the presence of liver tumours; therefore most tumours are discovered in an advanced stage. At this point no curative option is feasible and systemic treatment is limited due to the high drug resistance of HCC.

The importance of angiogenesis in the pathogenesis of HCC has been established for a long time. It is thus not surprising that inhibiting angiogenesis has opened up the world of therapeutic targets against HCC and the use of the angiogenic-inhibitor sorafenib has become the standard of care for advanced HCC patients. These angiogenic inhibitors are unique cancer-fighting agents, inhibiting the growth of blood vessels without directly targeting the tumour cells. Unfortunately, recent studies have shown that this tactic also has a negative side, since hypoxia can induce a more aggressive tumour phenotype and that tumours tend to escape their anti-angiogenic therapy. Furthermore, classic anti-angiogenic treatment targets both physiologic as pathologic angiogenesis, resulting in numerous adverse effects.

To assess new anti-angiogenic drugs, we developed a mouse model for HCC, by administrating weekly injections of diethylnitrosamine, a method that is considerably faster than current chemically induced models and has the advantage of tumour progression occurring in a background of inflammation and fibrosis. Furthermore, the assessment of several angiogenic factors showed that these tumours induce an angiogenic switch, providing an ideal base for further research on anti-angiogenic drugs. Moreover, several innovative imaging techniques were applied, not only to assess tumour growth, but also providing further insight in microvascular alterations of HCC livers.

The placental growth factor (PlGF) recently gained a lot of interest among angiogenesis-researchers. Its mere involvement in pathological angiogenesis, offers a unique potential in targeting tumour blood vessels, without affecting healthy tissue. We have used transgenic mouse models and monoclonal

antibodies to assess the effect of PlGF on the hepatocarcinogenesis. In our study, we have shown that PlGF is involved in the pathogenesis of HCC. Both in the transgenic knock out model as in the treatment model, silencing or inhibition of PlGF significantly decreased tumour burden, not only by inhibiting the vascularisation, but also by decreasing hepatic macrophage recruitment. The use of αPlGF holds great potential, since it does not only inhibit the formation of tumour blood vessels, but also normalizes the remaining blood vessels, thereby decreasing hypoxia and thus reducing the pro-metastatic potential of HCC.

Contradictory findings regarding the role of prolyl hydroxylase containing domains (PHD) in tumour progression and vascularisation, has made this factor a hot research topic. PHD2-deficiency increased the hepatocarcinogenesis and stimulated the development of cholangiocarcinoma (CC). Since both CC and HCC could be derived from HPCs, PHD2 could possibly have altered their differentiation, possibly through up-regulating the Notch-pathway. In contrast to other studies, PHD2- deficiency did not normalise the vasculature, and thus did not select for a less aggressive tumour phenotype. On the contrary, PHD2-haplodeficiency and the accompanying continuous HIF-activation resulted in an increased expression of pro-metastatic markers and selected for a more aggressive combined HCC-CC tumour phenotype.

In our study we have shown that PlGF and PHD2 are involved in the pathogenesis of HCC. Inhibiting PlGF could potentially be used as a therapeutic strategy against primary liver tumours, while silencing PHD2 aggravates HCC growth and induces the presence of CC. The work performed is very encouraging, however many questions remain and will need further attention in the near future.

2 Samenvatting

Hepatocellulair carcinoom (HCC) is een primaire levertumor die ontstaat in een achtergrond van cirrose. Elk jaar wordt bij ongeveer 500.000 patiënten HCC vastgesteld, waardoor dit de 5de meest voorkomende en 3de meest dodelijke kanker wereldwijd is. De onderliggende leverziekte verhult dikwijls de aanwezigheid van levertumoren, waardoor deze kanker vaak pas in een eindstadium vastgesteld wordt. Voor deze patiënten is geen genezing meer mogelijk en ook systemische therapie is beperkt door de hoge chemoresistentie van HCC.

Het belang van bloedvatvorming of *angiogenese* in de pathogenese van HCC is reeds lang vastgesteld. Het is daarom niet verwonderlijk dat het afremmen van de bloedvatvorming de deur geopend heeft voor tal van nieuwe behandelingen en het gebruik van de angiogenese-remmer sorafenib de standaardbehandeling is geworden voor patiënten met een vergevorderd HCC. Deze angiogenese-remmers zijn unieke behandelingen, vermits ze de vorming van bloedvaten afremmen zonder daarbij de tumorcellen direct te viseren. Helaas hebben recente studies aangetoond dat deze tactiek ook een negatieve kant heeft, namelijk door het ontstaan van zuurstofarmoede of *hypoxie* zal de groei van een agressiever tumortype gestimuleerd worden. Tevens hebben de huidige angiogenese-remmers zowel een invloed op de bloedvatvorming in tumoren als in gezonde weefsels, waardoor deze therapieën vaak gepaard gaan met talrijke bijwerkingen.

Om nieuwe angiogenese-remmers te onderzoeken, ontwikkelden we een muismodel voor HCC, waarbij wekelijkse injecties met de kankerverwekkende stof diethylnitrosamine zorgden voor de ontwikkeling van levertumoren na 25 weken. Deze methode is aanzienlijk sneller dan andere chemisch geïnduceerde modellen en heeft het voordeel dat tumoren ontstaan in een achtergrond van chronische leverschade. Verder heeft onderzoek naar factoren betrokken in de bloedvatvorming aangetoond dat deze tumoren de bloedvatvorming stimuleren, waardoor ze de perfecte basis vormen voor verder onderzoek naar angiogenese-remmers. Verder gebruikten we verschillende innovatieve beeldvormingstechnieken, waarbij niet enkel de tumorgroei maar ook de microvasculaire morfologie werd bestudeerd.

Recent trok de placentale groeifactor (PlGF) de aandacht van de wetenschappelijke wereld van angiogenese onderzoek. Zijn uitsluitende betrekking in de pathologische angiogenese, zorgde voor een unieke mogelijkheid om enkel de tumorbloedvaten te beïnvloeden, zonder daarbij het gezond weefsel aan te tasten. We gebruikten transgene muismodellen en monoclonale antilichamen om het effect van PlGF op de ontwikkeling van HCC te bestuderen. We konden in onze studie aantonen dat PlGF een rol speelde in de pathogenese van HCC. Zowel in het transgene *knock out* model, als in de therapeutische studie zagen we dat inhiberen van PlGF leidde tot een verminderd aantal tumoren, niet enkel door de bloedvatvorming af te remmen, maar ook door de ontstekingsreactie te verminderen. Het gebruik van PlGF-antilichamen is een veelbelovende therapie, aangezien niet enkel de vorming van tumorbloedvaten afgeremd wordt, maar ook de bestaande bloedvaten genormaliseerd worden, waardoor er minder hypoxie geïnduceerd wordt en een minder pro-metastatisch tumortype ontstaat.

Tegenstrijdige bevindingen rond de rol van *prolyl hydroxylase* bevattende eiwitten (PHD) in tumorontwikkeling en bloedvatvorming, zorgde voor een sterk toegenomen aandacht voor dit onderzoeksonderwerp. Vermindering van PHD2 zorgde voor een verhoogde tumorontwikkeling en stimuleerde de ontwikkeling van galwegkanker of *cholangiocarcinoom* (CC). Aangezien zowel CC en HCC kunnen ontstaan uit lever progenitor cellen, is het mogelijk dat PHD2 hun differentiatie zou beïnvloed hebben. In tegenstelling tot andere studies zagen wij geen normalisatie van de bloedvaten en kon dus geen selectie voor een minder agressief tumortype plaatsvinden. Integendeel, PHD2-heterozygote muizen hadden net meer hypoxie in de tumor, waardoor metastase-merkers een hogere expressie kenden.

We toonden in deze studie dat PlGF en PHD2 betrokken zijn in de pathogenese van HCC. Inhibitie van PlGF zou potentieel kunnen dienen als een therapie voor patiënten met primaire levertumoren, terwijl afremmen van PHD2 net de HCC-groei vermeerderde en tevens de ontwikkeling van CC stimuleerde. Hoewel deze resultaten veelbelovend zijn, blijven veel onderzoeksvragen open en verder onderzoek zal nodig zijn om de exacte rol van PlGF en PHD2 te achterhalen.

Chapter VI
Curriculum Vitae

1 Curriculum vitae

1.1 Personal information

Femke Heindryckx
Born: 31st December 1985
Nationality: Belgian
Marital status: Female, married

1.2 Education

Ghent University
Doctoral School of life science & medicine (2008 – 2012)
Post-graduate courses:
- Laboratory Animal Science course I and II (FELASA approved)
- Basic introduction SPSS
- Post-graduate course in applied statistics
- Advanced academic English: writing skills
- Effective slide design
- Effective poster design
- Biogazelle qBase course
- Applying for a post doctoral job
- Leadership foundation course
- Swedish language course (level A1)

Ghent University
Master of Science – Biology (2005-2007)
Master thesis:
"Role of metabolic stability in the determination
of the potential life span of *Caenorhabditis elegans*"
Research department:
Ageing physiology, molecular physiology and molecular evolution

1.3   Experience

Researcher
Gastroenterology - Hepatology
Faculty of health science and medicine
1st May 2008 – 30 September 2012:
Investigating the role of the placental growth factor and other
angiogenic factors in the pathogenesis of hepatocellular carcinoma.

Researcher
Virolory, Parasitology and Immunology
Faculty of Veterinary Medicine
1st October 2007 – 30th April 2008:
Role of the abomasal mucosa in achieving immunity
against gastrointestinal nematodes such as *Ostertagia ostertagi*.

1.4   Publications in journals with peer review

F. Heindryckx, I. Colle & H. Van Vlierberghe, Experimental mouse models for hepatocellular carcinoma research, *International Journal of Experimental Pathology* 2009, 90:4; 367-386

S. Van de Veire, I Stalmans; **F. Heindryckx** (*), H. Oura, A. Tijeras-Raballand *et al*, Further Pharmacological and Genetic Evidence for the Efficacy of PlGF Inhibition in Cancer and Eye Disease, *Cell* 2010, 141:1; 178-190
(*) Shared first author

F. Heindryckx, K. Mertens, N. Charette, B. Vandeghinste, C. Casteleyn, *et al*, Kinetics of angiogenic changes in a new mouse model for hepatocellular carcinoma, *Molecular Cancer* 2010, 9:219

S. Coulon, **F. Heindryckx** (*), A. Geerts, C. Van Steenkiste, I. Colle *et al*. Angiogenesis in chronic liver disease and its complications*, Liver International* 2010, 31:2: 146-162
(*) Shared first author

C. Van Steenkiste, J. Ribera, A. Geerts, C. Casteleyn, **F. Heindryckx**, *et al*, Inhibition of PlGF activity reduces the severity of fibrosis, inflammation and portal hypertension in cirrhotic mice, *Hepatology* 2011 May;53(5):1629-40.

B. Blomme, **F. Heindryckx** (*), JM Stassen, A. Geerts, I. Colle *et al*, Serum N-glycosylation patterns in diethylnitrosamine induced hepatocellular carcinoma mice and their evolution after inhibition of the Placental Growth Factor, submitted for publication in *Molecular and cellular biochemistry* (2012)
(*) Shared first author

F. Heindryckx, C. Casteleyn, S. Coulon, K. Olievier, I. Colle *et al*, Effect of prolyl hydroxylase domain-2 haplodeficiency on hepatocarcinogenesis and progenitor cell activation, *Journal of Hepatology,* 2012 [Epub ahead of print]

S. Coulon, S. Francque, A. Geerts, A. Verrijken, **F. Heindryckx** *et al*, Expression of inflammatory and angiogenic factors in the serum of a patients with steatosis and NASH, 2012 [accepted]

J. Best, L. Dollé, N. van Hul, LP. Bechmann, **F. Heindryckx** *et al*, Kupffer cells modulate the ductular reaction in a mouse model of sclerosing cholangitis, submitted for publication in *Gut* (2011)

F. Heindryckx, E. Bogaerts, S. Coulon, H. Devlies, A. Geerts *et al,* Inhibition of the placental growth factor decreases cholangiocarcinoma and hepatocellular carcinoma burden in a transgenic mouse model, *The European journal of Gastroenterology and Hepatology* 2012 [accepted]

F. Heindryckx, S. Coulon, E. Terrie, C. Casteleyn, JM. Stassen *et al,* The placental growth factor as a potential target against hepatocellular carcinoma in an orthotopic mouse model, submitted for publication in *Hepatology* (2012)

1.5   Abstracts of poster presentations

F. Heindryckx, B Vandeghinste, L Libbrecht *et al*, Angiogenic changes in a new mouse model for hepatocellular carcinoma, Signalling in the liver, FEB 18-20, 2010 Amsterdam THE NETHERLANDS

F. Heindryckx, B Vandeghinste, N Charette, *et al* Angiogenic changes in a new mouse model for hepatocellular carcinoma assessed with state-of-the-art imaging technology, 45th Annual Meeting of the European-Association-for-the-Study-of-Liver, APR 14-18, 2010 Vienna AUSTRIA

B. Blomme, **F. Heindryckx**, I. Colle, *et al* N-glycosylation patterns in HCC-mice and their evolution after treatment with anti-PlGF, Knowledge for growth, MAY 20, 2010 Ghent BELGIUM

F. Heindryckx, A Geerts, I Colle,*et al*, Effect of PlGF-inhibition on survival in mice with hepatocellular carcinoma, Knowledge for growth, MAY 20, 2010 Ghent BELGIUM

F. Heindryckx, B Vandeghinste, K Mertens, *et al* Angiogenic changes in a new mouse model for hepatocellular carcinoma assessed with innovative imaging technology, Knowledge for growth, MAY 20, 2010 Ghent BELGIUM

F. Heindryckx, L. Crapé, G. Cornelis *et al*, Inhibition of PlGF as a potential therapy against hepatocellular carcinoma , EASL monothematic: HCC from genomics to bedside, JUNE 25, 2010 Dubrovnik CROATIA

S. Coulon, A. Geerts, **F. Heindryckx** *et al* Presence of liver angiogenesis and upregulation of VEGF in a mice model of non-alcoholic steatohepatitis (NASH), The Liver Meeting (AASLD), SEPT 6, 2010 Boston USA

C. Casteleyn, C. Van Steenkiste, **F. Heindryckx** *et al* Vascular corrosion casting in cardiovascular research, 29th annual meeting of the Deutsche Gesellschaft für Klinische Mikrozirkulation und Hämorheologie, SEPT 17-19, 2010 Berlin GERMANY

F. Heindryckx, S. Coulon, L. Crapé, *et al*. A murine and human study demonstrating the role of PlGF in hepatocellular carcinoma. EASL, MAR 30 - APR 3, 2011 Berlin, GERMANY

S. Coulon, S. Francque, **F. Heindryckx** *et al* Expression of inflammatory and angiogenic factors in serum of patients with non-alcoholic fatty liver disease, Belgian Week of Gastroenterology, FEB 09 - 11, 2012 Oostende BELGIUM

S. Coulon, A. Geerts, **F. Heindryckx** *et al* Expression of inflammatory and angiogenic factors in serum of patients with non-alcoholic fatty liver disease, EASL APRIL 18 - 22, 2012 Barcelona SPAIN

S. Coulon, **F. Heindryckx**, B. Blomme *et al*, Inhibition of vascular endothelial growth factor receptor 2 (VEGFR2) attenuates steatosis and inflammation in a diet-induced mouse model for non-alcoholic steatohepatitis (NASH), EASL APRIL 18 - 22, 2012 Barcelona SPAIN

S. Coulon, N. Rohr-Udilova, **F. Heindryckx** *et al,* Role of angiogenesis and oxidative stress in a db/db mouse model for non-alcoholic steatohepatitis, EASL APRIL 18 - 22, 2012 Barcelona SPAIN

1.6   Abstracts from oral presentations

F. Heindryckx, B. Vandeghinste, N. Charette, *et al*, Angiogenic changes in a new mouse model for hepatocellular carcinoma assessed with state-of-the-art imaging technology, Belgian Week of Gastroenterology, MARCH 4-6, 2010 Antwerp BELGIUM

L. Libbrecht, S. Rogge, **F. Heindryckx** *et al*, clinicopathological features of hepatocellular adenomas classified according to the bordeaux criteria, Belgian Week of Gastroenterology, MAR 4-6, 2010 Antwerp BELGIUM

B. Blomme, **F. Heindryckx**, I. Colle, *et al* N-glycosylation patterns in HCC-mice chronically injected with den and their evolution after treatment with anti-PLGF Belgian Week of Gastroenterology, MAR 4-6, 2010 Antwerp BELGIUM

S. Coulon, A. Geerts, **F. Heindryckx** *et al*, presence of liver angiogenesis and upregulation of the vascular endothelial growth factor (VEGF) in a mice model of non-alcoholic steatohepatitis (NASH), Belgian Week of Gastroenterology, MAR 4-6, 2010 Antwerp BELGIUM

F. Heindryckx, S. Coulon, L. Libbrecht, *et al*, A human and murine study demonstrating the role of PlGF in the pathogenesis of HCC, Belgian Week of Gastroenterology, FEB 17-19, 2011 Liege BELGIUM

S. Coulon, **F. Heindryckx**, B. Blomme *et al*, Inhibition of vascular endothelial growth factor receptor 2 (VEGFR2) attenuates steatosis and inflammation in a diet-induced mouse model for non-alcoholic steatohepatitis (NASH), Belgian Week of Gastroenterology, FEB 09 - 11, 2012 Oostende BELGIUM

J. Best, L. Dollé, **F. Heindryckx**, *et al* Kupffercells modulate the ductular reaction in a mouse model of sclerosing cholangitis. Belgian week of Gastroenterology, FEB 9 – 11, 2012 Oostende BELGIUM

S. Coulon, N. Rohr-Udilova, **F. Heindryckx** *et al,* Role of angiogenesis and oxidative stress in a db/db mouse model for non-alcoholic steatohepatitis, Belgian Week of Gastroenterology, FEB 09 - 11, 2012 Oostende BELGIUM

1.7   Awards and scholarships

A PhD fellowship of the Scientific Research Foundation – Flanders (FWO)
Acquired in 2008 and renewed for 2 years in 2011

BASL research grant
Granted original research projects in the field of hepatology
Acquired in March 2009

EASL young investigators bursary
Granted for the participation in the EASL special conference
on Hepatocellular carcinoma
Acquired in June 2010

Best presentation award
Granted for a presentation on the Belgian Week of Gastroenterology 2011
Acquired in February 2011

1.8   Review activity

Reviewed manuscripts for the following international scientific journals:

- Acta Gastro-enterologica Belgica
- International Journal of Experimental Pathology
- British Journal of Cancer
- International Journal of Cancer
- Molecular Cancer Research

1.9   Student supervision and training

Nathalie Michels: Influence of liver cirrhosis on the nutritional state of rats.
Master in biomedical science (2007 – 2009) (nominated for best master thesis by the Fondation Pascal De Croos for distinct journalism in 2009)

Elke Debeleyr: Influence of portal hypertension on the nutritional state of rats.
Master in biomedical science (2007 – 2009)

Lare Crapé: PlGF antibodies in a therapeutic study on hepatocellular carcinoma in a DEN-induced mouse model.
Master in health science and medicine (2010 – 2011)

Gudrun Cornelis: Inhibition of PLGF and PHD2 in transgenic mouse models with DEN-induced HCC.
Master in health science and medicine (2010 – 2011)

Eliene Bogaerts: The role of glucose transporters in liver disease.
Master in biomedical science (2010 – 2012)

Aurélie Comhaire: Influence of hypoxia on the differentiation of progenitor cells in the liver.
Master in biomedical science (2011 – 2013)

Yves-Paul Vandewynckel: Impact on angiogenesis and ER stress and its effect on chemoresistance through PlGF inhibition in a mouse model of hepatocellular carcinoma.
Master in health science and medicine (2011 – 2012)

Supplements

Supplementary files can be downloaded using the following URL:

http://goo.gl/uYHLe

Or by scanning this QR-code: