Inhibition of the placental growth factor decreases burden of cholangiocarcinoma and hepatocellular carcinoma in a transgenic mouse model

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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 proven 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 (PIGF) could offer a therapeutic option in mice with hepatocellular carcinoma and cholangiocarcinoma. PIGF is a homologue of the vascular endothelial growth factor, which 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 are treated with murine monoclonal antibodies targeting PIGF.

Results  This study has shown for the first time that inhibiting PIGF decreases the burden of cholangiocarcinoma, by affecting both angiogenesis and inflammation.


Keywords: angiogenesis, biliary tumours, placental growth factor, prolyl hydroxylase containing domain, vascular endothelial growth factor

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PIGF, 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, PIGF binds to VEGF receptor 1 (VEGFR1) and its coreceptors neuropilin-1 and neuropilin-2 [18]. Therefore, PIGF exerts its proangiogenic potential through two mechanisms, directly, by inducing signal transduction through its receptors [19], and indirectly, by preventing the binding of VEGF to its decoy receptor VEGFR1 [20]. In contrast to VEGF, genetic studies have shown that PIGF is specifically involved in the pathological angiogenesis [14,15]. Therefore, its inhibition would not affect healthy blood vessels, providing an attractive drug candidate with a good safety profile. Treatment with PIGF antibodies has shown beneficial effects in several mouse models for chronic liver disease, including portal hypertension [11], cirrhosis [12] and HCC [13]. Its influence on CC remains unknown.
Although antiangiogenic treatment for HCC has been established as the standard of care in advanced HCC patients, its potential in CC patients is less investigated. However, the role of the Ras–Raf–Mek–Erk pathway [5] and VEGF in biliary carcinomas has already been clarified in several studies [21,22]. 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) [23]. The combination of bevacizumab and erlotinib may offer a therapeutic alternative in patients with advanced biliary cancer, but may also be limited because of the occurrence of adverse effects (NCT00356889) [24]. In addition, studies have shown that inducing an ischaemic injury by transarterial chemoembolization in HCC leads to an increased occurrence of a mixed hepatoceliangiocellular tumour phenotype, emphasizing the need for new therapeutics to manage this aggressive form of primary liver cancer [25,26].

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 [27]. 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.

### Table 1  Immunohistochemistry protocols

<table>
<thead>
<tr>
<th>Target</th>
<th>Antigen retrieval</th>
<th>Blocking</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
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</thead>
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<tr>
<td>CD105</td>
<td>Citrate 30’ (99°C)</td>
<td>15’ 3% H2O2</td>
<td>Goat polyclonal anti-endoglin (AF1320; R&amp;D systems) 1/50</td>
<td>18 h (RT) LSAB-kit (K0690; Dako, Heverlee, Belgium) Manufacturer’s protocol TBS</td>
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<td>F4/80</td>
<td>Target retrieval solution (S1899; Dako) 20’ (99°C)</td>
<td>15’ 3% H2O2</td>
<td>Rat monoclonal anti-F4/80 (MCA497; Serotec, Düsseldorf, Germany) 1/300</td>
<td>18 h (RT) Rabbit anti-rat (E0468; Dako) 1/300 Vectorstain kit TNB</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Target Retrieval solution (S1899; Dako) 20’ (99°C)</td>
<td>15’ 3% H2O2</td>
<td>Rat monoclonal anti-HLA-DR (sc-59297; Santa Cruz; Boechout, Belgium) 1/300</td>
<td>1 h (37°C) LSAB-kit (K0690; Dako) Manufacturers protocol PBS</td>
</tr>
<tr>
<td>CD206</td>
<td>Target retrieval solution (S1899; Dako) 20’ (99°C)</td>
<td>15’ 3% H2O2</td>
<td>Rabbit polyclonal anti-CD206 (orb4941; Biorbyt, Cambridge, UK) 1/400</td>
<td>1 h (37°C) LSAB-kit (K0690; Dako) Manufacturers protocol PBS</td>
</tr>
<tr>
<td>CD163</td>
<td>Target retrieval solution (S1899; Dako) 20’ (99°C)</td>
<td>15’ 3% H2O2</td>
<td>Rabbit polyclonal anti-CD163 (orb13303; Biorbyt) 1/400</td>
<td>1 h (37°C) LSAB-kit (K0690; Dako) Manufacturers protocol PBS</td>
</tr>
</tbody>
</table>

RT, room temperature; TBS, tris buffered saline; TNB, Tris-NaCl-blocking buffer.

### Table 2  Genes and primer sets

<table>
<thead>
<tr>
<th>Gene name (ID)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Marker</th>
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<tr>
<td>Hprt (15452)</td>
<td>GTTAAGCAGTACACGCCCAA</td>
<td>AGGGCATATCCAAACACAACTT</td>
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<tr>
<td>Hnbs (15288)</td>
<td>AAGGGCGTTCTGCAGGGCACCC</td>
<td>AGTTGCACATCTTCTATCTACTG</td>
<td>Reference gene</td>
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<tr>
<td>Sdha (66948)</td>
<td>CTTGAGAAGCCTGAGCTGTTG</td>
<td>ATCCATAAACCTGTCTCTGTT</td>
<td>Reference gene</td>
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<tr>
<td>Gapdh (14433)</td>
<td>CATGGGCTTGCTGCTGCTCTA</td>
<td>GCCGGCACTGCATCGAATCA</td>
<td>Reference gene</td>
</tr>
<tr>
<td>Mmp9 (17395)</td>
<td>GAGACGGGTATCCCTTGT</td>
<td>TGACATGGGGTCACCGTGG</td>
<td>Metastasis</td>
</tr>
<tr>
<td>Itgav (19140)</td>
<td>CAATTGGCTGCTGCCCATTTG</td>
<td>GATTGGAGATGGGACCGAAG</td>
<td>Metastasis</td>
</tr>
<tr>
<td>Icam (15898)</td>
<td>GCCCTTGAGAGGTCGAGTGAG</td>
<td>GACCGAGCTGGAAAGGTGTGA</td>
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</tr>
<tr>
<td>Vegf (22339)</td>
<td>ACTCGGATGCCGACAGGGGA</td>
<td>CCTCGGCTTTGCTTGCACCC</td>
<td>Angiogenesis</td>
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</tbody>
</table>

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Materials and methods

Genotyping

PHD2+/– mice were obtained from the Vesalius Research facility (KU Leuven, Belgium). A heterogenic couple was used for breeding and the offspring was genotyped with the following primers at a concentration of 10^2 mol/l: ACCTATGATCTCAGCATTTGGGAG; TCAGGACAGTGAAGCTAGAAACT and AAATTCTAATCGTAGCTGATGTGAGC; the latter being used as a reversed primer in both the wild-type and 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 and 340 bp.

Hepatocellular carcinoma and cholangiocarcinoma induction

Five-week-old heterozygous PHD2 knockout male mice (PHD2+/–) with an 129S2/SvPasCtrl background received
intraperitoneal injections once a week with DEN (Sigma-Aldrich, Bornem, Belgium) or saline as described previously [27,28]. After 25 weeks, mice developed both HCC and CC. The Ethical Committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocol.

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 DEN for 25 weeks were subsequently treated with 25 mg/kg αPlGF (2×/week, n = 8) for 5 weeks or IgG (2×/week, n = 8) as a control substance. During the treatment, mortality was noted to obtain survival curves.

Sampling and histology
Animals were killed after administration of DEN for 25 weeks followed subsequently by 5 weeks of treatment [αPlGF (n = 6) or IgG (n = 5)] with isoflurane (Forene, Abbott, Hoofddorp, the Netherlands) anaesthesia; blood was obtained from the ophthalmic artery. The liver was prelevated first, and after washing with sterile 5% NaCl, it was 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 (ref: 4078.9020; Klinipath, Olen, Belgium) and embedded in paraffin, as previously described [28]. Tumour lesions and nontumour tissue were separately collected and snap frozen in liquid nitrogen for subsequent analysis. Haematoxylin–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 as it is closely linked to cholangiofibrosis. CC lesions were also staged by distinguishing normal bile ducts (stage 0), cholangioma (stage 1), mixed cholangioma and CC (stage 2) and CC (stage 3), as previously described [29]. 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 376.665.37 mm². Tumour area was calculated by manually selecting the tumour’s perimeter using the Olympus CellID software (Olympus, Zoeterwoude, the Netherlands).

Immunohistochemistry
Immunohistochemistry was used to quantify protein levels inside hepatic tumours and in surrounding nontumour tissue. As a marker for angiogenesis, a monoclonal antibody was used targeting CD105 or endoglin (ref: AF1320; R&D systems, Abingdon, UK) [30]. Intercapillary distance was used as a marker for microvessel density by measuring the average distance between vessels in HCC nodules on CD105-stained slides. F4/80 (ref: MCA497G; AbD serotec, Dusseldorf, Germany) was used as a pan-macrophage marker. CD206 and CD163 were used as M2-macrophage markers, whereas HLA-DR was used as an M1-macrophage marker. Stainings were performed as described in Table 1 and quantified using Olympus CellID software.

Enzyme-linked immunosorbent assay
VEGF protein levels were measured (Mouse VEGF Quantikine ELISA Kit, R&D Biosystems, Abingdon, UK) in liver tissue and serum.

Quantitative real-time polymerase chain reaction
Total RNA was extracted from 20 mg of frozen tumour or nontumour lesions from IgG or αPIGF-treated mice following the manufacturer’s guidelines (RNeasy Mini Kit, ref: 74104; Qiagen, Venlo, the Netherlands) and diluted to a concentration of 100 ng/µl. The purity of RNA was evaluated using spectrophotometry. cDNA was obtained from 10µl RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Nazareth-Eke, Belgium) with oligo (dT) primers, according to the manufacturer’s protocol (170-8890; Bio-Rad, Nazareth, Belgium). Expression levels were measured using quantitative real-time PCR, with 3µl cDNA and the LightCycler 480 Green Master Mix (04707516001; Roche, Vilvoorde, Belgium).

Table 3 Liver tumours

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>SEM</th>
<th>αPlGF</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of HCCs (n)</td>
<td>8.5</td>
<td>0.65</td>
<td>3.8</td>
<td>0.49</td>
<td>***</td>
</tr>
<tr>
<td>Area of HCC (m²)</td>
<td>5633168.02</td>
<td>1479988.35</td>
<td>467461.56</td>
<td>63513.22</td>
<td>***</td>
</tr>
<tr>
<td>HCC burden (n×m²)</td>
<td>4788192.17</td>
<td>961992.4275</td>
<td>1776533.928</td>
<td>31121.4778</td>
<td>**</td>
</tr>
<tr>
<td>Number of dysplastic lesions (n)</td>
<td>5.67</td>
<td>0.67</td>
<td>3.4</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Cholangiocarcinoma (n)</td>
<td>4.77</td>
<td>0.33</td>
<td>2.4</td>
<td>0.98</td>
<td></td>
</tr>
</tbody>
</table>

HCC, hepatocellular carcinoma.
*P<0.05.
**P<0.01.
***P<0.001.
Microscopic tumour burden. (a, b) Representative H&E-stained slide of IgG-treated livers showing severe hepatocellular and cholangiocellular damage. (c, d) H&E-stained slide of αPlGF-treated liver. (e) The percentage of Sirius Red, used as a marker for cholangiofibrosis, showed a decrease after treatment with αPlGF. Asterisks represent significant P-values (∗P<0.05; ∗∗P<0.01; ∗∗∗P<0.001). Scale bars = 100 μm. CC, cholangiocarcinoma; H&E, haematoxylin–eosin; HCC, hepatocellular carcinoma; PlGF, placental growth factor.
Belgium. Genes and corresponding primer sets are listed in Table 2. The efficiency is calculated from the slope of the standard curve according to the following formula: 

\[ E = 10^{-\left(\frac{1}{\text{slope}}\right)} - 1 \]

All reactions were run in duplicate and normalized to a set of reference genes that showed stable expression in all samples. The comparative \( C_t \) method was used to determine the number of transcripts.

**Statistics**

Data were analysed using SPSS 19 (SPSS Inc., Chicago, Illinois, USA). After testing for normality and homoscedasticity, data were either subjected to Student’s \( t \)-test or Mann–Whitney \( U \)-test. A \( P \)-value of less than 0.05 was considered statistically significant. Data mentioned in the manuscript are expressed as averages±SEM. \( n = 6 \) for \( \alpha \)-PIGF and \( n = 5 \) for IgG in all the experiments.
Results

Macroscopic evaluation

A significant increase in weight ($P < 0.05$) was observed in DEN-induced PHD2$^{+/−}$ mice treated with αPIGF (25.37±0.95 g) compared with the IgG controls (22.16±0.46 g; Fig. 1). Macroscopic evaluation of the liver revealed that DEN-induced PHD2$^{+/-}$ mice treated with αPIGF had less hepatic tumours (7.80±2.20 liver tumours) compared with those who received control (22.17±4.22 liver tumours). No significant difference was seen in relative liver or spleen weight (Fig. 1). Treatment with αPIGF also did not significantly improve survival (Fig. 1). Although DEN-injected IgG mice did not show any extrahepatic metastasis, two out of eight (25%) αPIGF-treated mice developed tumours in the lungs and vesicula seminalis. However, this was not significant.

Microscopic evaluation

Hepatocellular carcinoma

H&E staining showed that all PHD2$^{+/−}$ mice developed HCC. 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

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Hepatic inflammation. (a) The level of fibrosis was measured using the Metavir score and was significantly decreased after treatment with PIGF antibodies. (b) αPIGF treatment significantly decreased macrophage recruitment in the surrounding nontumoural tissue. (c) No significant difference was seen in macrophage recruitment inside CC lesions. (d) No significant difference was seen in macrophage recruitment inside HCC lesions. (e) F4/80-stained liver treated with IgG. (f) F4/80-stained liver treated with αPIGF. Asterisks represent significant $P$-values ($^{*}P<0.05$). Scale bars = 100 µm. CC, cholangiocarcinoma; HCC, hepatocellular carcinoma; PIGF, placental growth factor.
confirmed as HCC using reticulin and H&E staining. 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 467 000±64 000 μm² vs. IgG 5 633 000±1 480 000 μm²; *P* < 0.001) of HCC lesions. Consequently, the tumour burden was significantly decreased (*P* < 0.01; Fig. 2; Table 3).

**Cholangiocarcinoma**

H&E staining showed that all PHD2 +/− mice had developed CC (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, whereas control-treated livers had a mixed occurrence of cholangioma and CC, characterized 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 there was a decrease in cholangiofibrosis in mice that received αPlGF compared with mice that received IgG as a control (Fig. 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±1.50% F4/80 vs. IgG 7.02±0.52% F4/80; *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 nontumour tissues and in CC lesions, however a nonsignificant 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).

**Angiogenesis**

No significant difference was seen between αPlGF-treated and IgG-treated mice (Fig. 3). Yet, αPlGF-treated HCC lesions showed a trend towards lower vascularization, measured by determining both the intercapillary distances and the percentages of endoglin (CD105) staining inside the hepatocellular tumours (Fig. 7). Treatment with αPlGF significantly decreased the vascularization of fibrotic, nontumour 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, as no significant difference in VEGF concentration was seen in tumour and nontumour tissues between αPlGF-treated and IgG-treated livers (Fig. 7). Furthermore, treatment with αPlGF significantly decreased serum levels of VEGF compared with treatment with IgG controls (αPlGF 206.35±12.70 pg/ml vs. IgG 528.94±210.56 pg/ml; *P* < 0.05; Fig. 7).

**Metastasis**

Expression levels of the prometastatic markers **Mmp9**, **Itgav** and **Icam** showed that αPlGF significantly decreased the pro-metastatic potential. In the tissue surrounding the tumour, a significant decrease in **Itgav** (*P* < 0.05) and **Icam** (*P* < 0.05) was seen, whereas a trend towards lower **Mmp9** expression was observed (Fig. 9). Inside tumour lesions, a significant
decrease in Icam (P < 0.05) was observed and a nonsignificant trend towards higher Itgav expression was seen.

Discussion
Treatment with PlGF antibodies has shown beneficial effects in several mouse models for chronic liver disease, including portal hypertension [11], cirrhosis [12] and HCC [13]; yet, its influence on CC remains unknown.

In a previous study, we have shown that heterozygous PHD2 knockout mice develop both HCC and CC after chronic administration of DEN [27]. 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 of CC. This might be due to inhibition of the proliferative effect of PlGF on macrophages [16,31] and endothelial cells [14,16]; however, further investigation is needed to assess the exact mechanism of how (anti)PlGF influences hepatocarcinogenesis.

Macrophage histology. (a) Staining for the M1 marker HLA-DR in IgG-treated liver. (b) Staining for the M1 marker HLA-DR in αPIGF-treated liver. (c) Staining for the M2 marker CD206 in IgG-treated liver. (d) Staining for the M2 marker CD206 in αPIGF-treated liver. (e) Staining for the M2 marker CD163 in IgG-treated liver. (f) Staining for M2 marker CD163 in αPIGF-treated liver. Scale bars = 100 μm. PlGF, placental growth factor.
As PlGF is known to affect the tumour-associated macrophage niche [17], we assessed the M1 and M2 macrophages using three markers (HLA-DR, CD206 and CD163). A significant decrease in CD206+ macrophages was observed in CC lesions, suggesting a possible reduction in M2-macrophage infiltration. These macrophages are known to have a protumoural effect and could possibly enhance metastasis. Recent studies using microarrays and immunohistochemistry have shown that PlGF expression is increased in Japanese nonfluke-related Hepatic angiogenesis. (a) Although not significant, a trend towards lower vascularization of the HCC lesions was seen after treatment with αPlGF. (b) The surrounding tissue was also not significantly less vascularized, yet a trend towards lower expression of CD105 was seen after treatment with αPlGF. (c) As a marker for microvessel density, intercapillary distances were measured, and they showed a trend towards a decreased vascularization of αPlGF-treated HCC lesions. (d) Treatment with αPlGF significantly decreased serum levels of VEGF. (e) Treatment with αPlGF did not induce an upregulation of VEGF inside the tumours. (f) Treatment with αPlGF did not induce an upregulation of VEGF in the surrounding nontumoural tissue. Asterisks represent significant P-values (*P<0.05). HCC, hepatocellular carcinoma; PlGF, placental growth factor; VEGF, vascular endothelial growth factor.

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CC [32] and that its expression is correlated with loss of liver–intestine cadherine [33], which may reflect that PlGF can be correlated with development of metastasis in CC. In our study, RNA analysis showed a significant decrease in the prometastatic markers Itgav and Icam in the tissue surrounding the tumour. However, in the tumour tissue only Icam was significantly decreased, whereas there was a trend towards higher Itgav expression. In addition, the occurrence of extrahepatic tumours in some αPlGF-treated mice needs further investigation in mouse models more suitable for metastasis research.

Possibly because of the small sample size, only a positive trend but no significant difference was observed between vascularization in αPlGF-treated and IgG-treated HCC lesions; however, previous studies have shown that PlGF antibodies decrease tumour induced vascularization in and around HCC lesions [13]. Several studies have shown that targeting the VEGF pathway often induces ‘angiogenic escape’ by upregulating other angiogenic factors [34,35]. Therefore, we have measured expression levels of VEGF, as angiogenic escape of PlGF treatment is likely to act through this factor [15]. Nevertheless, no significant differences were seen in protein VEGF levels after treatment with αPlGF. 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.

**Conclusion**

This study has shown for the first time that inhibition of 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. As clinical studies using sorafenib (Nexavar) for treatment of CC have shown that it provides only minimal therapeutic benefit, accompanied by severe adverse effects (SWOG 0514) [23], the use of monoclonal antibodies targeting PlGF can serve as a potential systemic treatment method in patients having this aggressive liver tumour.

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**Conflicts of interest**

ThromboGenics NV developed PlGF inhibitors for antiangiogenic treatment and Jean Marie Stassen is the Senior Director of Research and Development at ThromboGenics NV.
Fig. 9

Metastasis. (a) Although not significant, a relevant trend towards lower expression of the prometastatic marker Mmp9 was seen in the tissue surrounding the tumour after treatment with αPIGF. (b) A trend towards lower expression of the prometastatic marker Mmp9 was seen in the tumour tissue treated with αPIGF. (c) Treatment with αPIGF significantly decreased the expression of the prometastatic marker Itgav in the surrounding tissue. (d) A trend towards higher expression of the prometastatic marker Itgav was seen in the αPIGF-treated tumour tissue. (e) Treatment with αPIGF significantly decreased the expression of the prometastatic marker Icam in the surrounding tissue. (f) Treatment with αPIGF significantly decreased the expression of the prometastatic marker Icam in tumours. Asterisks represent significant P-values (*P<0.05). PlGF, placental growth factor.

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