Placental growth factor supports neuroendocrine tumor growth and predicts disease prognosis in patients

Georg Hilfenhaus^{1,2}, Andreas Göhrig^{1,2}, Ulrich-Frank Pape², Tabea Neumann², Henning Jann², Dietmar Zdunek³, Georg Hess³, Jean Marie Stassen⁴, Bertram Wiedenmann², Katharina Detjen^{1,2}, Marianne Pavel^{2,*} and Christian Fischer^{1,2,*}

¹Experimental and Clinical Research Center, a joint cooperation between the Charité Medical Faculty and the Max-Delbrück Center for Molecular Medicine, Lindenberger Weg 80, 13125 Berlin, Germany

²Medizinische Klinik mit Schwerpunkt Hepatologie und Gastroenterologie, Charité-Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany

³Roche Diagnostics, Nonnenwald 2, 82377 Penzberg, Germany

⁴ThromboGenics NV, Gaston Geenslaan 1, 3001 Leuven, Belgium

* (M Pavel and C Fischer contributed equally to this work)

Correspondence should be addressed to C Fischer
Email
christian.fischer@charite.de

Abstract

Placental growth factor (PIGF), a VEGF-homolog implicated in tumor angiogenesis and adaptation to antiangiogenic therapy, is emerging as candidate target in malignancies. Here, we addressed the expression, function, and prognostic value of PIGF in neuroendocrine tumors (NETs). PIGF was determined in NET patients' sera collected retrospectively ($n=88$) and prospectively ($n=87$) using Roche-Elecsys and correlated with clinicopathological data. Tumoral PIGF was evaluated by immunohistochemistry, effects of PIGF on proliferation and migration *in vitro* were assessed using different NET cell lines and effects on tumor growth *in vivo* in orthotopic xenografts. Circulating and tumoral PIGF was elevated in patients with pancreatic NETs (pNETs) compared with control sera and respective healthy tissue. *De novo* PIGF expression occurred primarily in the tumor stroma, suggesting paracrine stimulatory circuits. Indeed, PIGF enhanced NET proliferation and migration *in vitro* and, conversely, neutralizing antibodies to PIGF reduced tumor growth *in vivo*. Elevated circulating PIGF levels in NET patients correlated with advanced tumor grading and were associated with reduced tumor-related survival in pNETs. Subsequent determinations confirmed and extended our observation of elevated PIGF levels in a prospective cohort of grade 1 and grade 2 pNETs ($n=30$) and intestinal NETs ($n=57$). In low-grade pNETs, normal circulating PIGF levels were associated with better survival. In intestinal NETs, circulating PIGF above median emerged as an independent prognostic factor for shorter time-to-progression in multivariate analyses. These data assign to PIGF a novel function in the pathobiology of NETs and propose PIGF as a prognostic parameter and therapeutic target.

Key Words

- neuroendocrine tumor
- NET
- placental growth factor
- PIGF
- prognostic biomarker

Endocrine-Related Cancer
(2013) 20, 305–319

Introduction

Neuroendocrine tumors (NETs) are rare neoplasms with an estimated annual incidence of 2–5/100 000, and are typically diagnosed at an advanced/metastatic stage of the disease. Despite dissemination however, the clinical course is relatively variable, including periods of stable disease (Baudin 2007, Modlin *et al.* 2008). Pancreatic NETs (pNETs) and intestinal NETs constitute distinct molecular, pathologic, and clinical subgroups, and consequently different diagnostic and therapeutic algorithms have been developed. Parameters such as tumor size, locoregional or distant metastasis, and Ki67-based grading are currently used to separate prognostic subgroups (Pape *et al.* 2008), but the individual course of the disease remains difficult to predict. Eventually, the majority of NET patients will experience disease progression despite therapeutic interventions, resulting in an overall 5-year survival of 30–65% (Modlin *et al.* 2008, Pape *et al.* 2008, Panzuto *et al.* 2011). Hence, advanced NETs constitute a malignant disease with unsolved diagnostic and therapeutic medical needs. Recently, two targeted therapies, the tyrosine-kinase inhibitor sunitinib and the mTOR inhibitor everolimus, have been shown to be effective in prolonging progression-free survival in both pNETs and intestinal NETs, and have been approved for treatment of pNETs (Pavel *et al.* 2011, Raymond *et al.* 2011, Yao *et al.* 2011b). Both drugs act at least in part via inhibition of tumor angiogenesis, promoting interest in the vascular features of NETs (Alexandraki & Kaltsas 2012). Well-established angiogenic growth factors, such as VEGF-A (Terris *et al.* 1998) or angiopoietins (Srirajaskanthan *et al.* 2009, Detjen *et al.* 2010), are present in NETs. In addition, characteristic neuroendocrine secretion products, including chromogranin A fragments (Corti 2010) and serotonin (Asada *et al.* 2009), affect angiogenesis and vascular permeability, altogether creating a unique stromal microenvironment.

Placental growth factor (PlGF) is an angiogenic growth factor of the VEGF family, which might be important in NETs for several reasons: PlGF selectively supports pathological angiogenesis, thereby promoting tumor growth, progression, and dissemination (Carmeliet *et al.* 2001, Fischer *et al.* 2007). Thus, PlGF expression in tumor tissue and/or circulating PlGF levels correlated with tumor stage, vascularity, metastasis, survival, and recurrence in several human malignancies (Parr *et al.* 2005, Wei *et al.* 2005, 2009, Fischer *et al.* 2008, Maae *et al.* 2012). Moreover, PlGF induction occurred as a result of anti-angiogenic therapies in human cancer patients and in mouse models, and (in the latter situation) constituted a

functionally relevant mechanism of resistance development (Batchelor *et al.* 2007, Fischer *et al.* 2007, 2008, Rini *et al.* 2008, Willett *et al.* 2009). Finally, changes in circulating PlGF following treatment initiation are emerging as predictors of therapy response for selected antiangiogenic treatment modalities in clinical trials (Bass *et al.* 2010). With regard to NETs, preliminary data released from biomarker determinations in the RADIANT-3 trial revealed a small transient reduction of pretreatment circulating PlGF in patients receiving everolimus (ESMO abstract Yao *et al.* (2011c)). Though preliminary, this observation highlights the demand for a systematic study of PlGF function in NETs.

Mechanistically, PlGF functions as a pleiotropic cytokine that is expressed by and affects a wide range of different cell types within the tumor microenvironment. PlGF binds to Flt1 (VEGFR1), and to neuropilin-1 (NRP1) and -2 (NRP2), but not to the prototype VEGF receptor Flk1 (VEGFR2) (Fischer *et al.* 2008). Thereby, PlGF stimulates endothelial cell migration, growth and survival, and increases the proliferation of cancer-associated fibroblasts and smooth-muscle cells (Fischer *et al.* 2008). Moreover, PlGF recruits endothelial and other angiogenesis-competent bone-marrow progenitors as well as tumor-associated macrophages, which promote tumor angiogenesis, growth, and metastasis (Lyden *et al.* 2001, Fischer *et al.* 2008). Besides, PlGF also directly affects tumor cells, which express the PlGF receptors Flt1, NRP1, or NRP2 (Fischer *et al.* 2008, Bagri *et al.* 2009). Thereby, PlGF can activate AKT and ERK-mediated canonical signaling pathways, ultimately leading to enhanced tumor cell survival, proliferation, migration, and invasiveness (Fischer *et al.* 2008). Conversely, blocking PlGF using anti-PlGF antibodies emerged as therapeutic strategy to inhibit growth and metastasis in various preclinical tumor models (Fischer *et al.* 2007, Coenegrachts *et al.* 2010, Van de Veire *et al.* 2010, Schmidt *et al.* 2011, Yao *et al.* 2011a, Heindryckx *et al.* 2012).

Here, we addressed the role of PlGF in NETs. The study characterizes PlGF as a growth factor that promotes proliferation and migration of NET cells *in vitro* and tumor growth *in vivo*, and furthermore proposes circulating PlGF as a prognostic biomarker.

Materials and methods

Patients and samples

Sera and tissue samples were obtained from individuals with NETs treated at Charité-Universitätsmedizin Berlin,

Department of Gastroenterology, from 1998 to 2012. Tumor staging with TNM classification performed at the time of blood sampling, as well as histopathological diagnosis and grading, obtained from pathology reports, were established according to ENETS guidelines (Rindi *et al.* 2006, 2007). Clinical parameters were obtained from systematic review of the medical records. Patient informed consent and local ethics committee approval was obtained.

A retrospective cohort of patients with pNETs ($n=88$) and a prospective cohort including also intestinal NETs (57 intestinal NETs, 30 pNETs; Table 1) were studied. In the retrospective cohort, 65 out of 88 patients were chemotherapy-naïve and five patients were off treatment for at least 3 months before blood sampling. Out of 65, 10

chemotherapy-naïve patients received somatostatin analogs (SSA), one patient had undergone radioreceptor therapy 4 months before blood sampling, and one patient had undergone transarterial chemoembolization (TACE) within a month of blood sampling. Of the pNET patients in the validation cohort, 15 were therapy-naïve, six received SSA, seven had had chemotherapy, one patient had a combination of SSA and chemotherapy, and one patient received everolimus. Out of 57, 33 intestinal NET patients were treated with SSA and one patient with a combination of SSA and everolimus. Furthermore, one patient each received everolimus, IFN α , and the study drug oral angiogenesis inhibitor PTK787/ZK 222584 (PTK/ZK) in the intestinal NET cohort, and one patient received chemotherapy. All intestinal NET patients

Table 1 Patients baseline characteristics.

Parameters	Retrospective cohort ($n=88$)	Prospective cohort ($n=87$)	
	pNET ($n=88$)	pNET ($n=30$)	Intestinal NET ($n=57$)
Sex (female/male)	37/51	16/14	32/25
Age, median (range)	61 (24–84) years	54 (30–82) years	59 (20–78) years
pNET subtype			
Nonfunctional	82	25	
Gastrinoma	3	1	
Insulinoma	1	1	
Glucagonoma	1	1	
VIPoma	1	2	
Carcinoid syndrome	0	0	41
Midgut NET subtype			
Lower jejunum/ileum			52
Appendix			1
Caecum			4
TNM-stage			
Stage I	2	0	0
Stage II (A/B)	2 (0/2)	0	0
Stage III (A/B)	8 (2/6)	3 (0/3)	1 (0/1)
Stage IV	72	27	56
Grading			
G1	11	7	30
G2	47	23	27
G3	16	0	0
Ki67 sub-division G2			
2–5%	15	12	20
> 5–10%	14	7	4
> 10–15%	4	3	0
> 15–20%	7	1	1
PIGF levels (pg/ml)			
Median	19.51	16.53	17.57
Range	7.27–75.09	5.79–44.05	9.80–100.91
sFlt1 levels (pg/ml)			
Median	83.62	85.40	80.40
Range	32.60–245.60	13.6–159.00	48.10–155.60
PIGF:sFlt1 ratio			
Median	0.22	0.19	0.23
Range	0.07–0.99	0.07–1.00	0.13–0.66

Unless otherwise stated values indicate numbers (n) of subjects.

included in time-to-progression (TTP) analyses were chemotherapy-naïve. The time frame of the prospective cohort was May 2009–December 2012, and blood sampling and follow-up visits with imaging studies were performed every 3–6 months. Tumor progression was determined based on multi-phasic computed tomography or magnetic resonance imaging. Healthy controls were blood donors without medical history of malignant disease and consisted of 58 women and 29 men, with a median age of 45 years (range 41–56 years).

Materials

Antibodies were from ReliaTech (Braunschweig, Germany; to PIGF). Secondary antibodies were from Dianova (Hamburg, Germany). Recombinant human PIGF was from R&D Systems (Minneapolis, MN, USA). Everolimus and sunitinib were obtained from Sigma–Aldrich. Neutralizing antibodies to murine PIGF (5D11D4) and to human PIGF (16D3), as well as the IgG₁ control antibody (1C8) for use *in vivo* were supplied by ThromboGenics NV (Leuven, Belgium) (Fischer *et al.* 2007).

Cell lines and culture

BON cells were a generous gift from C M Townsend (Department of Surgery, University of Texas Medical Branch, Galveston). KRJ-I intestinal NET cells were generated by R Pfragner (Institute of Pathophysiology and Immunology, Medical University of Graz, Austria) (Pfragner *et al.* 1996) and kindly provided by I Modlin (Department of Surgery, Yale University School of Medicine, New Haven). BON and KRJ-I cells were authenticated by short tandem repeat DNA-typing and confirmed as unique cell lines. QGP-1 cells were from the Health Science Research Resources Bank (Osaka, Japan); H727 cells were from Banca Biologica, Istituto Nazionale per la Ricerca sul Cancro (Genova, Italy).

Determination of PIGF and sFlt1 levels in serum and culture supernatants

Concentrations of PIGF and sFlt1 were determined from frozen serum samples using Elecsys PIGF and sFlt1 immunoassays (Schiettecatte *et al.* 2010). As circulating PIGF might be increased in patients with ischemic cardiomyopathy (Nakamura *et al.* 2009), parallel proBNP determinations served as a surrogate marker of myocardial function. Patients with elevated proBNP (> 500 pg/ml) were not excluded from the analysis. However, the

outcome of the analyses of grading, survival, and TTP in the overall cohorts and subgroups remained unchanged, if these patients were excluded. PIGF levels from cell culture supernatants were measured using Quantikine ELISA kits (R&D Systems) according to the manufacturer.

Immunohistochemical analyses

Cryostat-sections were fixed in 4% paraformaldehyde (PFA). Immunoperoxidase-staining was performed using Vectastain Elite ABC-kit (Vector Laboratories, Wertheim-Bettingen, Germany) and AEC as substrate chromogen (DAKO, Hamburg, Germany). The antibody to PIGF was diluted 1:100, and was omitted in negative controls. For a semi-quantitative evaluation of immunoreactivity, the immunoreactive area was determined relative to the total field measured using AxioVision (Zeiss, Jena, Germany).

Growth assays

In 24-well dishes, 10⁵ cells/well were plated and allowed to attach for 6 h. Following stimulation with PIGF for 48 h, cell numbers were counted using a Neubauer hemocytometer.

Migration assays

A number of 2 × 10⁵ cells/insert were placed in serum-free medium in the upper well of a chemotaxis chamber and allowed to migrate for 20 h toward PIGF added to the bottom well. Migrated cells were stained and quantified by counting 12 standardized fields at 200× magnification (refer to [Supplementary Methods](#), see section on [supplementary data](#) given at the end of this article for details).

BON NET orthotopic tumor model

Female NMRI^{nu/nu} mice (21–25 g) were from Taconics (Bomholt, Denmark). Animal care followed institutional guidelines and experiments were approved by local animal research authorities. Mice were anesthetized by i.p. administration of Ketanest (100 mg/kg) and Rompun (10 mg/kg). For tumor induction, the pancreas was exposed and 1 × 10⁶ BON cells were injected into the head of the pancreas as previously described (Detjen *et al.* 2010). Treatment of engrafted tumors was initiated at week 3 following tumor cell implantation. Antibodies to PIGF and isotype-matched IgG₁ control antibodies were administered by i.p. injection (25 mg/kg; twice weekly).

Mice were sacrificed after 9 weeks, and primary tumors were removed and weighted.

Statistical analysis

Data are presented as mean $\pm$ s.e.m., circulating levels of PIGF and sFlt1 as median with interquartile ranges. Statistical significance was determined by *t*-test, Fisher's exact test, and Mann–Whitney *U* test using SPSS (v18.0) and GraphPad Prism (v5.0; San-Diego, CA, USA). Tumor-related survival and TTP were calculated based on the date of blood sampling and analyzed using the Kaplan–Meier method and Log-rank test. Cox proportional hazards regression model was used for multivariate analysis. **P* < 0.05 values were considered significant; all tests were two-sided.

Results

PIGF serum levels are elevated in patients with pNETs

Given that PIGF is released into systemic circulation, we initially determined circulating PIGF in sera of patients with pNETs and healthy controls (Table 1). Median circulating PIGF concentration was found elevated to 19.5 pg/ml in pNET patients compared with 12.3 pg/ml in healthy controls (Fig. 1A). Receiver operating characteristic (ROC) analysis displayed 79.5% sensitivity and 82.8% specificity for the discrimination between patients with pNETs and controls at a cut-off level of 15.35 pg/ml (Fig. 1B), indicating that pNETs are associated with an increase in circulating PIGF.

As binding of PIGF to its soluble receptor Flt1 (sFlt1) may diminish its biological activity, we also determined sFlt1 levels. Though we found circulating sFlt1 increased in pNET patients compared with controls (Fig. 1C and D), the ratio PIGF:sFlt1 remained significantly elevated in patients with pNETs (Fig. 1E and F).

Elevated PIGF serum levels correlate with tumor grading of pNETs

We next hypothesized that circulating PIGF levels in patients with pNETs reflect histopathological properties and/or the clinical course of the disease. No correlation of PIGF with TNM classification was obtained. In particular, circulating PIGF did not discriminate between localized vs metastatic disease (Fig. 2A). However median PIGF levels progressively increased from grade 1 (G1) to 3 (G3) tumors (Fig. 2C).

Levels of circulating sFlt1 correlate with metastatic disease in pNETs

As sFlt1 has been proposed as a surrogate marker of tumor progression, we tested the association of sFlt1 levels with clinical parameters. Different from PIGF, sFlt1 levels correlated with metastatic disease (Fig. 2B). Median sFlt1 levels also progressively increased with grading (Fig. 2D).

Elevated PIGF serum levels predict reduced tumor-related survival in pNETs

The association of circulating PIGF and sFlt1 with tumor grade raised the question, whether these serum parameters reflected the prognosis. Accordingly, patients were grouped by high (>50th percentile) or low ($\leq$ 50th percentile) levels of circulating PIGF, respectively, and Kaplan–Meier curves based on tumor-related survival were generated. Circulating PIGF levels >50th percentile were associated with poor prognosis and predicted shorter tumor-related survival with a median survival of 4.52 years, whereas median survival in patients with low-PIGF serum concentrations had not yet been reached (hazard ratio: 2.35; 95% CI: 1.08–5.10; Log-rank *P* = 0.031; Fig. 2E). Subsequent multivariate analysis confirmed tumor grade, but not circulating PIGF as an independent prognostic parameter in the overall cohort (not shown), which is in accordance with the correlation of PIGF levels and grading described above. In contrast to PIGF, neither sFlt1 nor the PIGF:sFlt1 ratio correlated with tumor-related survival (Fig. 2F, and not shown).

Prospective evaluation of PIGF as prognostic biomarker in NETs

As our retrospective analysis suggested circulating PIGF as prognostic biomarker, we next sought to determine PIGF and sFlt1 in an independent validation cohort. Clinically, the availability of a prognostic biomarker would benefit the management of patients with lower grade tumors (G1 and G2), which often require a personalized therapeutic strategy to accompany them for decades. In order to be able to focus on lower grade tumors, yet recruit a prospective cohort within a reasonable time frame, we opened our analyses to intestinal NETs. Accordingly, patients with either low-grade pNETs or intestinal NETs were enrolled in a prospective study (Table 1). PIGF levels were found elevated in the overall cohort (*n* = 87), as well as in the pNET and intestinal subgroups (Fig. 3A), confirming our initial observation. We furthermore

Figure 1

Elevated circulating PIGF and sFlt1 levels in patients with pNETs. (A) PIGF serum concentrations in healthy controls ($n=87$) and patients with pNET ($n=88$). Shown is the scatter dot plot with the median and interquartile range, $*P<0.0001$, Mann–Whitney U test. (B) Panel depicts corresponding ROC curve of circulating PIGF. AUC=0.85, 95% CI: 0.80–0.91, $*P<0.0001$. (C) sFlt1 serum concentrations in healthy controls ($n=87$) and patients with pNET ($n=88$). Shown is the scatter dot plot with a median of 73.9 pg/ml in healthy controls vs 83.6 pg/ml in pNET patients and interquartile range,

$*P<0.0001$, Mann–Whitney U test. (D) Panel depicts corresponding ROC curve of circulating sFlt1. AUC=0.70, 95% CI: 0.62–0.78, $P<0.0001$. (E) PIGF:sFlt1 ratios in healthy controls ($n=87$) and patients with pNET ($n=88$). Shown is the scatter dot plot with the median of 0.17 in healthy controls vs 0.23 in pNET patients and interquartile range, $*P<0.0001$, Mann–Whitney U test. (F) Panel depicts corresponding ROC curve of PIGF:sFlt1 ratio. AUC=0.76, 95% CI: 0.69–0.84, $*P<0.0001$.

confirmed increased sFlt1 concentrations as well as an elevated PIGF:sFlt1 ratio in NETs compared with healthy controls (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Due to their slow progression, we were unable to determine tumor-related survival in the subgroup of intestinal NETs, and hence had to restrict survival analyses to pNET patients. Different from the retrospective analysis,

a distinct cut-off value rather than the cohort-dependent median was required. As PIGF values from low-grade pNETs revealed considerable overlap with healthy controls, we choose the cut-off that best separated NET patients from healthy controls in our ROC analyses (15.35 pg/ml; Fig. 1B). Kaplan–Meier analyses using this cut-off indicated favorable tumor-related survival for individuals with non-pathologic circulating PIGF when

Figure 2

Elevated circulating PIGF levels in patients with pNETs correlate to tumor grading and reduced survival. Correlation of circulating PIGF and sFlt1 levels with TNM stage and grading was tested. (A) No significant correlation (NS) was observed between serum PIGF levels in patients with localized (TNM stage I-III) vs metastatic disease (TNM stage IV). Shown is the scatter dot plot with the median and interquartile range; $*P=0.482$ for TNM stage IV vs stage I-III, Mann-Whitney U test. (B) Serum levels of sFlt1 are significantly elevated in pNET patients with metastatic disease (TNM stage IV) compared with localized disease (TNM stage I-III). Shown is the scatter dot plot with the median and interquartile range; $*P<0.005$ for TNM stage IV vs stage I-III, Mann-Whitney U test. (C) Correlation between serum PIGF levels and histological tumor grading as determined by Ki67 labeling index; G1 < 2%, $n=11$; G2 2–20%, $n=47$; G3 > 20%, $n=16$. Shown is the scatter dot plot with median PIGF values of 16.6, 20.9, and 22.5 pg/ml for grade 1, 2, and 3, and interquartile range; $*P=0.036$ for G1 vs. G2,

$*P=0.002$ for G1 vs G3, Mann-Whitney U test. (D) Correlation between serum sFlt1 levels and histological tumor grading (groups as in C). Median sFlt1 was 74.8 pg/ml for G1, 84.6 pg/ml for G2, and 96.7 pg/ml for G3, respectively. Shown is the scatter dot plot with the median and interquartile range; $*P=0.013$ for G1 vs G3, $*P=0.032$ for G2 vs G3, Mann-Whitney U test. (E) Kaplan-Meier survival curve of pNET patients with high vs low levels of circulating PIGF. Patients were allocated to a group with high (> 50th percentile, corresponds to > 19.3 pg/ml, $n=43$) or low ($\leq$ 50th percentile, $n=44$) PIGF serum levels, and Kaplan-Meier analyses were conducted; hazard ratio: 2.35; 95% CI: 1.08–5.10; Log-rank $P=0.031$. (F) Kaplan-Meier analysis of tumor-related survival of pNET patients with high vs low levels of circulating sFlt1. Patients were allocated to a group with high (> 50th percentile, corresponds to > 83.6 pg/ml, $n=43$) or low ($\leq$ 50th percentile, $n=44$) sFlt1 serum levels, and Kaplan-Meier analyses were conducted; hazard ratio: 1.25; 95% CI: 0.57–2.71; Log-rank $P=0.576$.

compared with those with circulating PIGF > 15.35 pg/ml (Log-rank $P=0.025$; Fig. 3B). Likewise, circulating PIGF levels furthermore allowed to separate prognostic subgroups within the group of G2 pNETs (Log-rank $P=0.032$; Fig. 3C).

As was expected for the subgroup of intestinal NETs, survival is still pending in the prospective cohort and

therefore we evaluated TTP instead. TTP could be determined in 47 out of 57 patients with G1 and G2 intestinal NETs, which were enrolled with stable disease. Kaplan-Meier analysis separating patients based on normal vs pathologic PIGF levels did not reveal significant differences. However, TTP was significantly reduced in patients with circulating PIGF above median (hazard ratio:

Figure 3

Prospective confirmation of elevated circulating PIGF levels and its prognostic relevance in patients with pancreatic and intestinal NETs.

(A) PIGF serum concentrations in healthy controls ($n=87$) and a prospective cohort of patients with pNET ($n=30$) and intestinal NETs ($n=57$). Shown is the scatter dot plot with a median of 12.3 pg/ml in healthy controls vs 16.5 pg/ml in pNETs and 17.6 pg/ml in intestinal NETs, and the respective interquartile range, $*P=0.001$ and $\#P<0.0001$, Mann-Whitney U test.

(B) Pathologic PIGF levels were associated with reduced tumor-related survival in low-grade pNETs of the prospective cohort. Patients were allocated to a group with pathological (>15.35 pg/ml, $n=52$) or normal (<15.35 pg/ml, $n=18$) serum PIGF levels, and Kaplan-Meier analyses were performed to compare tumor-related survival; Log-rank $P=0.025$.

(C) Similar results were obtained with the subgroup of G2 pNETs when using

the cut-off value of 15.35 pg/ml; Log-rank $P=0.032$. (D) Circulating PIGF values above median identify patients with rapid disease progression in intestinal NETs. Shown is a Kaplan-Meier analysis of time-to-progression in patients with intestinal NETs allocated to a group with high (>50 th percentile, corresponds to >17.1 pg/ml, $n=23$) or low (≤ 50 th percentile, $n=24$) PIGF serum levels; TTP 15 months vs undefined; hazard ratio: 4.01; 95% CI: 1.38–11.65; Log-rank $P=0.011$. (E) Elevated circulating PIGF levels discriminate prognostic subgroups within the group of patients with grade 2 intestinal NETs. Patients were allocated to a group with high (>50 th percentile, corresponds to >17.8 pg/ml, $n=12$) or low (≤ 50 th percentile, $n=13$) serum PIGF levels, and Kaplan-Meier analyses were performed to compare time-to-progression; TTP 10 months vs undefined; hazard ratio: 4.95; 95% CI: 1.25–19.60; Log-rank $P=0.023$.

4.01; 95% CI: 1.38–11.65; Log-rank $P=0.011$; Fig. 3D). Conversely, patients experiencing disease progression within 6 months presented higher median baseline PIGF levels than patients with stable disease throughout this period (26.7 vs 16.6 pg/ml; $P=0.009$). Moreover, median PIGF levels further stratified the clinically heterogeneous group of G2 intestinal NETs into prognostic subgroups

(hazard ratio: 4.95; 95% CI: 1.25–19.60; Log-rank $P=0.023$; Fig. 3E). Finally, PIGF, but not grading or biotherapy with SSAs (29 out of 47 patients), constituted an independent prognostic parameter in the multivariate analysis of the intestinal NET cohort (Table 2). As grading is expected to emerge as an independent prognostic parameter in intestinal NETs, we inspected Ki67 values

Table 2 Multivariate analysis for time-to-progression (TTP) in intestinal NETs ($n=47$).

Covariate	P value	HR (95% CI)
Age (years)	0.601	0.99 (0.93–1.04)
Sex (female/male)	0.450	0.64 (0.20–2.02)
TNM-stage	0.988	0.00 ^a
Grading	0.737	1.21 (0.40–3.71)
Biotherapy (SSA)	0.349	1.76 (0.54–5.78)
Serum PIGF levels (> median to ≤ median)	0.029	4.87 (1.18–20.13)

HR, hazard ratio; SSA, somatostatin analogs.

^aOut of 47 subjects 46 were TNM-stage IV.

in our cohort in more detail. Indeed, 16 of the 21 G2 intestinal NETs had Ki67 values that were lower or equal to 5%, four had Ki67 values between 5 and 10%, and only one tumor had more than 10% Ki67 positive cells. We therefore speculate that this low-proliferative activity accounted for the comparable TTP in G1 and G2 subgroups.

Taken together, our prospective approach confirmed elevated circulating PIGF levels in pNETs and intestinal NETs and suggested PIGF levels as a prognostic parameter.

pNETs are associated with *de novo* expression of PIGF

As PIGF may be expressed and bound by cells from various tumor compartments, we aimed to further specify the

abundance and location of PIGF in pNETs. Based on histomorphological criteria, pNET samples ($n=23$) consistently displayed strong PIGF expression in stromal cells, such as endothelial and inflammatory cells (Fig. 4A, B, C and E), and occasionally weak immunoreactivity for PIGF in the tumor cell compartment. In contrast, no staining was observed in non-transformed endocrine or exocrine pancreatic tissues, including their stroma (Fig. 4D and E), indicating *de novo* expression of PIGF in pNETs.

PIGF enhances proliferation and motility of NET cells and is induced by targeted therapies *in vitro*

To functionally address the role of PIGF, we initially determined effects of PIGF stimulation on NET cell biology *in vitro*. A panel of pancreatic (BON and QGP-1), ileal (KRJ-I), and bronchial (H727) NET cell lines was used. PIGF determination from supernatants of these tumor cell cultures was at (BON) or below (QGP-1, H727) the detection limit (not shown), and thus in agreement with the sparse immunohistochemical reactivity of epithelial tumor cells. However, expression analysis readily revealed the presence of at least one of the known PIGF receptors, Flt1, NRP1, and NRP2 in these NET cell lines (Supplementary Figure 2, see section on supplementary data given at the end of this article), thereby enabling responsiveness to paracrine PIGF stimulation. Indeed, recombinant PIGF enhanced proliferation of all four NET cell lines tested

Figure 4

De novo expression of PIGF occurs in human pNETs. (A, B, C and D) Representative immunohistochemistry for PIGF on cryosections of clinical specimens from patients with pNETs (A, B and C) and respective adjacent non-transformed tissue (D). Scale bars = 100 μ m. (E) Semiquantitative analysis of overall PIGF expression in tumor epithelial and stromal cells of

clinical specimens ($n=23$). Immunoreactivity for PIGF was determined from the percentage of immunoreactive area/total tumor area, without additional assessment of staining intensity. Data represent mean $\pm$ S.E.M.

* $P<0.05$; t -test.

(Fig. 5A, B and C; H727 not shown). In addition to this mitogenic action, PIGF substantially enhanced directed migration of BON and QGP-1 cells (Fig. 5D, E, F and G). Taken together, exogenous PIGF stimulation elicited biological responses that were consistent with a more aggressive tumor phenotype.

In view of the biological action of PIGF on NET cells, we next tested, whether recently approved targeted therapies affect PIGF production of NET cells *in vitro*. In order to allow for paracrine tumor cell/stroma interactions, we determined PIGF production from different endothelial cell and fibroblast preparations, including NET-derived tumor fibroblasts. PIGF was consistently detected in supernatants from endothelial cells, but not from fibroblast cultures (not shown), prompting us to choose mixed spheroid cultures of BON cells with endothelial cells as an *in vitro* approach (see [Supplementary Methods](#)). Incubation of spheroid co-cultures with the tyrosine-kinase inhibitor sunitinib, but not with the mTOR-inhibitor everolimus over 72 h elevated PIGF levels in the supernatant ($264.3 \pm 38.7\%$ of control; $P=0.02$; and $122.3 \pm 10.5\%$ of control; $P=0.13$). Thus, PIGF release increased in response to sunitinib, suggesting that PIGF induction in NETs may occur following antiangiogenic treatment.

Blocking PIGF inhibits growth of orthotopic NETs

Finally, we decided to investigate the function of PIGF *in vivo*, using an established preclinical NET model. Accordingly, BON orthotopic pNET xenografts were grown in NMRI^{nu/nu} mice and engrafted tumors treated with either isotype-matched control IgG₁ or a combination of neutralizing antibodies to mouse and human PIGF from week 3 following tumor cell implantation. By the end of the experiment in week 9, functional inactivation of tumor cell and stroma-derived PIGF by the neutralizing antibodies had resulted in a significant reduction of tumor weight, indicating that PIGF supports NET growth *in vivo* and constitutes a therapeutic target (Fig. 5H, I and J).

Discussion

The current study highlights PIGF as a novel, stroma-derived growth factor in pancreatic and intestinal NETs. *In vitro*, PIGF enhanced proliferation and migration of NET cell lines, suggesting that PIGF directly affects NET cell biology. *In vivo*, blocking PIGF using neutralizing antibodies reduced growth of orthotopic BON NET

xenograft tumors, indicating that PIGF represents a therapeutic target in preclinical NET models. In the clinical situation, elevated circulating PIGF levels were associated with reduced tumor-related survival and/or shorter TTP in NETs, suggesting circulating PIGF as an easily accessible candidate prognostic biomarker.

The therapeutic concept of antiangiogenesis has created much interest in tumor-type specific angioregulatory growth factors. Studies conducted in NETs found that VEGF-A, the prototype angiogenic factor in cancer, is highly expressed in intestinal NETs (Terris *et al.* 1998) but was found less abundant in pNETs, inversely correlated to microvascular density, and did not convey prognostic information in pNETs (Terris *et al.* 1998, Marion-Audibert *et al.* 2003, Couvelard *et al.* 2005). Here, we find that PIGF, a member of the VEGF family, is prominently induced in the stroma of pNETs. Although *de novo* expression of PIGF reportedly may occur in the tumor cell compartment (Fischer *et al.* 2008), our analysis suggests that this was rarely the case in pNETs. Rather, the tumor stroma of pNETs uniformly revealed abundant PIGF immunoreactivity. Genetic and pharmacological evidence attests to the functional relevance of stroma-derived PIGF in that inactivation of stromal PIGF impaired (Carmeliet *et al.* 2001, Schmidt *et al.* 2011), whereas transgene overexpression of stromal PIGF stimulated tumor growth, angiogenesis, and metastasis in mouse models (Marcellini *et al.* 2006, Fischer *et al.* 2007, 2008). Given that the known PIGF receptors, Flt1, and neuropilins were shown expressed in NETs (Terris *et al.* 1998, von Marschall *et al.* 2003), our current observation of stromal PIGF expression suggests the presence of auto-/paracrine signaling loops. Functionally, PIGF stimulated proliferation and migration of NET cells *in vitro*, which is in line with published results revealing stimulatory actions of PIGF on proliferation of pancreatic tumor cells (Fischer *et al.* 2007), as well as on migration of glioma, breast, and lung cancer cells (Fischer *et al.* 2008, Taylor *et al.* 2010). More importantly, we were able to reduce the growth of BON NET xenograft tumors using neutralizing antibodies to functionally inactivate tumor cell- and stroma-derived PIGF.

A second line of evidence strongly implicates PIGF in the pathology of NETs: circulating PIGF levels were elevated in pNET patients, correlated with tumor grade, and predicted poor clinical outcome. In contrast, PIGF did not correlate with TNM stages and did not differ between localized (TNM stages I–III) and metastatic disease (TNM stage IV). We therefore favor the concept that high circulating PIGF levels reflected a more aggressive tumor biology rather than a more extensive tumor load.

Figure 5

PIGF enhances proliferation and directed migration of neuroendocrine tumor cells *in vitro*, and conversely, blocking PIGF inhibits growth of orthotopic NETs *in vivo*. (A) PIGF (100 ng/ml) stimulates growth of BON cells to $124.7 \pm 3.8\%$ of vehicle control over 48 h. (B and C) Similarly, PIGF increased cell numbers of QGP-1 (B) and KRJ-I (C) cultures to 113.5 ± 0.8 , and $134.5 \pm 9.2\%$ of controls respectively. All experiments were at least conducted three times, each in duplicates. Data represent means $\pm$ S.E.M. * $P < 0.05$; *t*-test. (D, E, F and G) PIGF (100 ng/ml) functions as chemoattractant and stimulates the directed migration of BON (D, E and F) and QGP-1 (G) NET cells in transwell assays to 186.0 ± 19.4 and $183.7 \pm 17.0\%$ of

vehicle controls respectively. Representative fields from membranes show migrated cells after staining with crystal violet (D and E). All experiments were at least conducted three times, each in duplicates. Data represent means $\pm$ S.E.M. * $P < 0.05$; *t*-test. (H, I and J) Effects of anti-PIGF antibodies on growth of BON NET tumors. NMRI^{nu/nu} mice with engrafted orthotopic pancreatic NET tumors were treated with either isotype-matched control IgG₁ antibodies ($n = 13$; illustrated in H) or a combination of neutralizing antibodies to mouse and human PIGF ($n = 16$; illustrated in I). Bar graph represents tumor weight at the end of the experiment as means $\pm$ S.E.M. (J; $P = 0.031$).

Although expression of PIGF in tumor tissues was proposed as prognostic marker for progression and survival in gastric, colorectal, NSCLC, hepatocellular, and breast cancer (Parr *et al.* 2005, Wei *et al.* 2005, Fischer *et al.* 2008),

relatively few studies evaluated circulating levels of PIGF. Results linked high circulating PIGF to adverse prognosis in renal (Matsumoto *et al.* 2003) and oral squamous cell carcinoma (Cheng *et al.* 2012) but not in hepatocellular

cancer (Nagaoka *et al.* 2010), and high preoperative PIGF levels predicted reduced recurrence-free and overall survival in colorectal cancer patients undergoing curative surgery (Wei *et al.* 2009, Rahbari *et al.* 2011). The latter data fit with our observation that PIGF levels >50th percentile correlated with shorter TTP in the prospective intestinal NET cohort. Indeed, we identified circulating PIGF as a prognostic indicator of TTP in an intestinal NET collective with low overall proliferative activity, in which grading was unable to separate prognostic subgroups. Furthermore, pathologic PIGF levels identified distinct subgroups regarding tumor-related survival within the heterogeneous group of G2 pNETs in our prospective approach. Currently, we cannot pinpoint the cause of the more robust separation of prognostic subgroups of G2 intestinal NETs by median PIGF levels compared with the separation of G2 pNET prognostic subgroups by pathologic PIGF levels. Difference in end points (tumor-related survival vs TTP), differences in NET types (pNET vs intestinal NET), or differences in treatment modalities offer potential explanations that will have to be thoroughly evaluated in future prospective studies.

Irrespective of such adjustments, our current data established the link from circulating PIGF to disease prognosis, which could have considerable translational impact on the management of NETs based on three considerations:

First, advanced G2 tumors comprise a large and clinically heterogeneous group of patients, in which the course of disease and resultant treatment decisions are variable, with choices currently based on empirical knowledge rather than objective parameters. In the current study, high PIGF levels in patients with G2 tumors specifically characterized individuals with an unfavorable prognosis. Notably, absolute Ki67 values within the G2 subgroup did not correlate to PIGF levels ($r=0.156$; $P=0.215$), suggesting that PIGF conveyed prognostic information that is distinct from Ki67-based grading. Hence, circulating PIGF may serve to further stratify patients with G2 tumors and help to optimize clinical management, as it could identify patients that benefit from immediate/more aggressive treatment and/or shorter monitoring intervals.

Second, our preclinical results in the orthotopic NET xenograft model characterize PIGF as a therapeutic target in NETs. Compelling preclinical evidence established blocking PIGF using anti-PIGF antibodies as effective strategy to inhibit tumor growth and metastasis (Fischer *et al.* 2007, Coenegrachts *et al.* 2010, Van de Veire *et al.* 2010, Schmidt *et al.* 2011, Yao *et al.* 2011a, Heindryckx *et al.* 2012). As PIGF is a disease-specific factor and redundant for physiological vessel growth (Carmeliet

et al. 2001, Fischer *et al.* 2007), inhibition of PIGF is likely to exhibit less side effects than VEGF-A/VEGFR2-based therapies (Fischer *et al.* 2007, Verheul & Pinedo 2007). In NET disease, which often requires long-term treatment regimens, prevention of co-morbidity, which might be of greater risk to patients than the malignancy itself, is of crucial importance. So far, a phase I clinical trial with the humanized anti-PIGF antibody TB-403 has proven lack of toxicity (Martinsson-Niskanen *et al.* 2011, Lassen *et al.* 2012). Moreover the recent approval of sunitinib for the treatment of pNETs (Raymond *et al.* 2011) might have substantial implications on considering PIGF as therapeutic target in these tumors, as sunitinib efficiently blocks Flt1 signaling (Fischer *et al.* 2008).

Third, changes in circulating PIGF may occur as a consequence of treatment. Patients with NETs may undergo decades of therapy. Apart from side effects of long-term treatments, resistance development therefore constitutes a major concern, especially when considering antiangiogenic treatments (Paez-Ribes *et al.* 2009). In this context, mouse models revealed that circulating and tumoral PIGF levels increase upon VEGF inhibition and conventional chemotherapies, resulting in recruitment of proangiogenic macrophages and angiogenic escape (Fischer *et al.* 2007). Similarly, chronic exposure of colorectal cancer cells *in vitro* to bevacizumab increased the expression of PIGF and consequently enhanced tumor cell migration and invasion, and metastatic potential *in vivo* (Fan *et al.* 2011). Of clinical relevance, induction of circulating PIGF occurred in patients receiving VEGF inhibitors for treatment of colorectal and renal cell cancers and glioblastoma (Motzer *et al.* 2006, Batchelor *et al.* 2007, Fischer *et al.* 2007, Willett *et al.* 2009, de Groot *et al.* 2011). Thus, it is tempting to speculate that early and/or transient increases in circulating PIGF may predict therapy response, while long-term induction of circulating PIGF might constitute a functional mechanism and/or indicator of resistance development. Given the recent approvals of sunitinib and everolimus for treatment of pNETs, circulating PIGF may represent an easily accessible candidate biomarker for monitoring therapy response and/or disease progression. While sunitinib-dependent effects on circulating PIGF levels have not yet been determined in NET patients, PIGF measurements from the RADIANT-3 trial reported a transient reduction in response to everolimus, which is difficult to interpret. At a first glance, the decrease of circulating PIGF levels seems counterintuitive in view of the antiangiogenic activity attributed to everolimus, which might be expected to induce a compensatory rise of PIGF. However, reported

values give a snapshot of circulating PIGF levels obtained at 28 days of treatment, and the moderate antiangiogenic action of everolimus may require a longer treatment period for efficient induction of an angiogenic escape. Furthermore, PIGF reduction may have resulted from reduced tumor burden in treatment responders.

With the advent of novel therapies for NETs, a personalized therapy may have come into reach, but a definition of biomarker profiles that are useful for the stratification of subgroups is mandatory. PIGF holds promise for NETs in multiple ways: as a prognostic marker, as potential therapeutic target, and – possibly – as a response marker for selected systemic therapies. Our current analyses provide an intriguing link from high PIGF serum levels to poor outcome that deserves an urgent prospective follow up in larger cohorts of pancreatic and intestinal NETs.

Thus, our data provide a compelling basis for a more extensive prospective evaluation of PIGF in larger and more diverse NET cohorts. Finally, our observations strongly advocate an inclusion of PIGF into the portfolio of biomarkers in ongoing and/or further prospective NET trials.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-12-0223>.

Declaration of interest

G Hess was previously an employee of Roche Diagnostics and is currently a consultant to Roche Diagnostics. JM Stassen was previously an employee of ThromboGenics NV and is currently a scientific advisor to ThromboGenics NV. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

G Hilfenhaus was supported by a Gunther Speidel scholarship. C Fischer was supported by a grant from Experimental and Clinical Research Center, Berlin, Germany. This work was supported by a grant from Berliner Krebsgesellschaft e.V., Berlin, Germany.

Acknowledgements

We thank M Welzel for outstanding technical assistance, and R Pfragner and I Modlin for the opportunity to use the KRJ-I cells.

References

- Alexandraki KI & Kaltsas G 2012 Gastroenteropancreatic neuroendocrine tumors: new insights in the diagnosis and therapy. *Endocrine* **41** 40–52. (doi:10.1007/s12020-011-9562-2)
- Asada M, Ebihara S, Yamanda S, Niu K, Okazaki T, Sora I & Arai H 2009 Depletion of serotonin and selective inhibition of 2B receptor suppressed tumor angiogenesis by inhibiting endothelial nitric oxide synthase and extracellular signal-regulated kinase 1/2 phosphorylation. *Neoplasia* **11** 408–417.
- Bagri A, Tessier-Lavigne M & Watts RJ 2009 Neuropilins in tumor biology. *Clinical Cancer Research* **15** 1860–1864. (doi:10.1158/1078-0432.CCR-08-0563)
- Bass MB, Sherman SI, Schlumberger MJ, Davis MT, Kivman L, Khoo HM, Notari KH, Peach M, Hei YJ & Patterson SD 2010 Biomarkers as predictors of response to treatment with motesanib in patients with progressive advanced thyroid cancer. *Journal of Clinical Endocrinology and Metabolism* **95** 5018–5027. (doi:10.1210/jc.2010-0947)
- Batchelor TT, Sorensen AG, di Tomaso E, Zhang WT, Duda DG, Cohen KS, Kozak KR, Cahill DP, Chen PJ, Zhu M et al. 2007 AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell* **11** 83–95. (doi:10.1016/j.ccr.2006.11.021)
- Baudin E 2007 Gastroenteropancreatic endocrine tumors: clinical characterization before therapy. *Nature Clinical Practice. Endocrinology & Metabolism* **3** 228–239. (doi:10.1038/ncpendmet0425)
- Carmeliet P, Moons L, Luttun A, Vincenti V, Compernelle 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. *Nature Medicine* **7** 575–583. (doi:10.1038/87904)
- Cheng SJ, Lee JJ, Cheng SL, Chen HM, Chang HH, Wang YP, Kok SH, Kuo MY & Chiang CP 2012 Increased serum placenta growth factor level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma. *Oral Oncology* **48** 424–428. (doi:10.1016/j.oraloncology.2011.12.007)
- Coenegrachts L, Maes C, Torrekens S, Van Looveren R, Mazzone M, Guise TA, Bouillon R, Stassen JM, Carmeliet P & Carmeliet G 2010 Anti-placental growth factor reduces bone metastasis by blocking tumor cell engraftment and osteoclast differentiation. *Cancer Research* **70** 6537–6547. (doi:10.1158/0008-5472.CAN-09-4092)
- Corti A 2010 Chromogranin A and the tumor microenvironment. *Cellular and Molecular Neurobiology* **30** 1163–1170. (doi:10.1007/s10571-010-9587-8)
- Couvelard A, O'Toole D, Turley H, Leek R, Sauvaget A, Degott C, Ruszniewski P, Belghiti J, Harris AL, Gatter K et al. 2005 Microvascular density and hypoxia-inducible factor pathway in pancreatic endocrine tumours: negative correlation of microvascular density and VEGF expression with tumour progression. *British Journal of Cancer* **92** 94–101. (doi:10.1038/sj.bjc.6602245)
- Detjen KM, Rieke S, Deters A, Schulz P, Rexin A, Vollmer S, Hauff P, Wiedenmann B, Pavel M & Scholz A 2010 Angiopoietin-2 promotes disease progression of neuroendocrine tumors. *Clinical Cancer Research* **16** 420–429. (doi:10.1158/1078-0432.CCR-09-1924)
- Fan F, Samuel S, Gaur P, Lu J, Dallas NA, Xia L, Bose D, Ramachandran V & Ellis LM 2011 Chronic exposure of colorectal cancer cells to bevacizumab promotes compensatory pathways that mediate tumour cell migration. *British Journal of Cancer* **104** 1270–1277. (doi:10.1038/bjc.2011.81)
- 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-PIGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* **131** 463–475. (doi:10.1016/j.cell.2007.08.038)
- Fischer C, Mazzone M, Jonckx B & Carmeliet P 2008 FLT1 and its ligands VEGFB and PIGF: drug targets for anti-angiogenic therapy? *Nature Reviews. Cancer* **8** 942–956. (doi:10.1038/nrc2524)
- de Groot JF, Piao Y, Tran HT, Gilbert MR, Wu HK, Liu J, Bekele BN, Cloughesy T, Mehta MP, Robins HI et al. 2011 Myeloid biomarkers associated with glioblastoma response to anti-vascular endothelial growth factor therapy with aflibercept. *Clinical Cancer Research* **17** 4872–4881. (doi:10.1158/1078-0432.CCR-11-0271)

- Heindryckx F, Bogaerts E, Coulon SH, Devlies H, Geerts AM, Libbrecht L, Stassen JM, Carmeliet P, Colle IO & Van Vlierberghe HR 2012 Inhibition of the placental growth factor decreases burden of cholangiocarcinoma and hepatocellular carcinoma in a transgenic mouse model. *European Journal of Gastroenterology & Hepatology* **24** 1020–1032. (doi:10.1097/MEG.0b013e3283554219)
- Lassen U, Nielsen DL, Sorensen M, Winstedt L, Niskanen T, Stenberg Y, Pakola S, Stassen JM & Glazer S 2012 A phase I, dose-escalation study of TB-403, a monoclonal antibody directed against PIGF, in patients with advanced solid tumours. *British Journal of Cancer* **106** 678–684. (doi:10.1038/bjc.2011.609)
- Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L et al. 2001 Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nature Medicine* **7** 1194–1201. (doi:10.1038/nm1101-1194)
- Maae E, Olsen DA, Steffensen KD, Jakobsen EH, Brandslund I, Sorensen FB & Jakobsen A 2012 Prognostic impact of placenta growth factor and vascular endothelial growth factor A in patients with breast cancer. *Breast Cancer Research and Treatment* **133** 257–265. (doi:10.1007/s10549-012-1957-0)
- Marcellini M, De Luca N, Riccioni T, Ciucci A, Orecchia A, Lacal PM, Ruffini F, Pesce M, Cianfarani F, Zambruno G et al. 2006 Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor. *American Journal of Pathology* **169** 643–654. (doi:10.2353/ajpath.2006.051041)
- Marion-Audibert AM, Barel C, Gouysse G, Dumortier J, Pilleul F, Pourreyron C, Hervieu V, Poncet G, Lombard-Bohas C, Chayvialle JA et al. 2003 Low microvessel density is an unfavorable histoprognostic factor in pancreatic endocrine tumors. *Gastroenterology* **125** 1094–1104. (doi:10.1016/S0016-5085(03)01198-3)
- von Marschall Z, Scholz A, Cramer T, Schafer G, Schirner M, Oberg K, Wiedenmann B, Hocker M & Rosewicz S 2003 Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis. *Journal of the National Cancer Institute* **95** 437–448. (doi:10.1093/jnci/95.6.437)
- Martinsson-Niskanen T, Riisbro R, Larsson L, Winstedt L, Stenberg Y, Pakola S, Stassen JM & Glazer S 2011 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* **33** 1142–1149. (doi:10.1016/j.clinthera.2011.08.007)
- Matsumoto K, Suzuki K, Koike H, Okamura K, Tsuchiya K, Uchida T, Takezawa Y, Kobayashi M & 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 Research* **23** 4953–4958.
- Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP et al. 2008 Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncology* **9** 61–72. (doi:10.1016/S1470-2045(07)70410-2)
- Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE et al. 2006 Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *Journal of Clinical Oncology* **24** 16–24. (doi:10.1200/JCO.2005.02.2574)
- Nagaoka S, Yoshida T, Akiyoshi J, Akiba J, Hisamoto T, Yoshida Y, Abe M, Koga H, Toirimura T, Ueno T et al. 2010 The ratio of serum placenta growth factor to soluble vascular endothelial growth factor receptor-1 predicts the prognosis of hepatocellular carcinoma. *Oncology Reports* **23** 1647–1654. (doi:10.3892/or_00000807)
- Nakamura T, Funayama H, Kubo N, Yasu T, Kawakami M, Momomura S & Ishikawa SE 2009 Elevation of plasma placental growth factor in the patients with ischemic cardiomyopathy. *International Journal of Cardiology* **131** 186–191. (doi:10.1016/j.ijcard.2007.10.050)
- Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D & Casanovas O 2009 Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* **15** 220–231. (doi:10.1016/j.ccr.2009.01.027)
- Panzuto F, Boninsegna L, Fazio N, Campana D, Pia Brizzi M, Capurso G, Scarpa A, De Braud F, Dogliotti L, Tomassetti P et al. 2011 Metastatic and locally advanced pancreatic endocrine carcinomas: analysis of factors associated with disease progression. *Journal of Clinical Oncology* **29** 2372–2377. (doi:10.1200/JCO.2010.33.0688)
- Pape UF, Berndt U, Muller-Nordhorn J, Bohmig M, Roll S, Koch M, Willich SN & Wiedenmann B 2008 Prognostic factors of long-term outcome in gastroenteropancreatic neuroendocrine tumours. *Endocrine-Related Cancer* **15** 1083–1097. (doi:10.1677/ERC-08-0017)
- Parr C, Watkins G, Boulton M, Cai J & Jiang WG 2005 Placenta growth factor is over-expressed and has prognostic value in human breast cancer. *European Journal of Cancer* **41** 2819–2827. (doi:10.1016/j.ejca.2005.07.022)
- Pavel ME, Hainsworth JD, Baudin E, Peeters M, Horsch D, Winkler RE, Klimovsky J, Lebowitz D, Jehl V, Wolin EM et al. 2011 Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study. *Lancet* **378** 2005–2012. (doi:10.1016/S0140-6736(11)61742-X)
- Pfragner R, Wirsberger G, Niederle B, Behmel A, Rinner I, Mandl A, Wawrina F, Luo J, Adamiker D, Hoyer H et al. 1996 Establishment of a continuous cell line from a human carcinoid of the small intestine (KRJ-I). *International Journal of Oncology* **8** 513–520.
- Rahbari NN, Reissfelder C, Muhlhaber M, Weidmann K, Kahlert C, Buchler MW, Weitz J & Koch M 2011 Correlation of circulating angiogenic factors with circulating tumor cells and disease recurrence in patients undergoing curative resection for colorectal liver metastases. *Annals of Surgical Oncology* **18** 2182–2191. (doi:10.1245/s10434-011-1761-9)
- Raymond E, Dahan L, Raoul JL, Bang YJ, Borbath I, Lombard-Bohas C, Valle J, Metrakos P, Smith D, Vinik A et al. 2011 Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. *New England Journal of Medicine* **364** 501–513. (doi:10.1056/NEJMoa1003825)
- Rindi G, Kloppel G, Alhman H, Caplin M, Couvelard A, de Herder WW, Eriksson B, Falchetti A, Falconi M, Komminoth P et al. 2006 TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system. *Virchows Archiv* **449** 395–401. (doi:10.1007/s00428-006-0250-1)
- Rindi G, Kloppel G, Couvelard A, Komminoth P, Korner M, Lopes JM, McNicol AM, Nilsson O, Perren A, Scarpa A et al. 2007 TNM staging of midgut and hindgut (neuro) endocrine tumors: a consensus proposal including a grading system. *Virchows Archiv* **451** 757–762. (doi:10.1007/s00428-007-0452-1)
- Rini BI, Michaelson MD, Rosenberg JE, Bukowski RM, Sosman JA, Stadler WM, Hutson TE, Margolin K, Harmon CS, DePrimo SE et al. 2008 Antitumor activity and biomarker analysis of sunitinib in patients with bevacizumab-refractory metastatic renal cell carcinoma. *Journal of Clinical Oncology* **26** 3743–3748. (doi:10.1200/JCO.2007.15.5416)
- Schietecatte J, Russcher H, Anckaert E, Mees M, Leeseer B, Tirelli AS, Fiedler GM, Luthe H, Denk B & Smits J 2010 Multicenter evaluation of the first automated Elecsys sFlt-1 and PIGF assays in normal pregnancies and preeclampsia. *Clinical Biochemistry* **43** 768–770. (doi:10.1016/j.clinbiochem.2010.02.010)
- Schmidt T, Kharabi Masouleh B, Loges S, Cauwenberghs S, Fraisl P, Maes C, Jonckx B, De Keersmaecker K, Kleppe M, Tjwa M et al. 2011 Loss or inhibition of stromal-derived PIGF prolongs survival of mice with imatinib-resistant Bcr-Abl1(+) leukemia. *Cancer Cell* **19** 740–753. (doi:10.1016/j.ccr.2011.05.007)

- Srirajaskanthan R, Dancey G, Hackshaw A, Luong T, Caplin ME & Meyer T 2009 Circulating angiopoietin-2 is elevated in patients with neuroendocrine tumours and correlates with disease burden and prognosis. *Endocrine-Related Cancer* **16** 967–976. (doi:10.1677/ERC-09-0089)
- Taylor AP, Leon E & Goldenberg DM 2010 Placental growth factor (PlGF) enhances breast cancer cell motility by mobilising ERK1/2 phosphorylation and cytoskeletal rearrangement. *British Journal of Cancer* **103** 82–89. (doi:10.1038/sj.bjc.6605746)
- Terris B, Scoazec JY, Rubbia L, Bregeaud L, Pepper MS, Ruzsniowski P, Belghiti J, Flejou J & Degott C 1998 Expression of vascular endothelial growth factor in digestive neuroendocrine tumours. *Histopathology* **32** 133–138. (doi:10.1046/j.1365-2559.1998.00321.x)
- Van de Veire S, Stalmans I, Heindryckx F, Oura H, Tijeras-Raballand A, Schmidt T, Loges S, Albrecht I, Jonckx B, Vinckier S *et al.* 2010 Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. *Cell* **141** 178–190. (doi:10.1016/j.cell.2010.02.039)
- Verheul HM & Pinedo HM 2007 Possible molecular mechanisms involved in the toxicity of angiogenesis inhibition. *Nature Reviews. Cancer* **7** 475–485. (doi:10.1038/nrc2152)
- Wei SC, Tsao PN, Yu SC, Shun CT, Tsai-Wu JJ, Wu CH, Su YN, Hsieh FJ & Wong JM 2005 Placenta growth factor expression is correlated with survival of patients with colorectal cancer. *Gut* **54** 666–672. (doi:10.1136/gut.2004.050831)
- Wei SC, Liang JT, Tsao PN, Hsieh FJ, Yu SC & Wong JM 2009 Preoperative serum placenta growth factor level is a prognostic biomarker in colorectal cancer. *Diseases of the Colon and Rectum* **52** 1630–1636. (doi:10.1007/DCR.0b013e3181afbdaf)
- Willett CG, Duda DG, di Tomaso E, Boucher Y, Ancukiewicz M, Sahani DV, Lahdenranta J, Chung DC, Fischman AJ, Lauwers GY *et al.* 2009 Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: a multidisciplinary phase II study. *Journal of Clinical Oncology* **27** 3020–3026. (doi:10.1200/JCO.2008.21.1771)
- Yao J, Wu X, Zhuang G, Kasman IM, Vogt T, Phan V, Shibuya M, Ferrara N & Bais C 2011a Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy. *PNAS* **108** 11590–11595. (doi:10.1073/pnas.1109029108)
- Yao JC, Shah MH, Ito T, Bohas CL, Wolin EM, Van Cutsem E, Hobday TJ, Okusaka T, Capdevila J, de Vries EG *et al.* 2011b Everolimus for advanced pancreatic neuroendocrine tumors. *New England Journal of Medicine* **364** 514–523. (doi:10.1056/NEJMoa1009290)
- Yao JC, Tsuchihashi Z, Panneerselvam A, Winkler RE, Bugarini R & Pavel M 2011c 6573 POSTER Effect of everolimus treatment on markers of angiogenesis in patients with advanced pancreatic neuroendocrine tumours (pNET)—results from the phase III RADIANT-3 study. *European Journal of Cancer* **47** (Supplement 1) S463.

Received in final form 6 February 2013

Accepted 4 March 2013

Made available online as an Accepted Preprint

5 March 2013