

Circulating angiopoietin-2 is elevated in patients with neuroendocrine tumours and correlates with disease burden and prognosis

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Abstract

Angiogenesis is an essential process in the development and growth of tumours. There are a large number of angiogenic mediators including the angiopoietin (Ang) family and vascular endothelial growth factor, which play an important role in both physiological and pathological angiogenesis. This study examines serum levels of Ang-1 and Ang-2 in patients with neuroendocrine tumour (NET) compared healthy controls. ELISA for Ang-1 and Ang-2 was performed in 47 patients with histologically proven NETs and 44 healthy controls. Immunohistochemical staining for Ang-2 was performed in patients to demonstrate cellular location of Ang-2. Serum Ang-2 levels were significantly elevated in patients compared controls (median 4756 vs 2495 pg/ml, $P < 0.001$), while there was no significant difference in Ang-1 levels. The ratio of Ang-2:Ang-1 was significantly elevated in patients compared controls (0.13 vs 0.066, $P < 0.001$). Serum Ang-2 levels were significantly elevated in patients with distant metastases compared with those without metastasis (median 5080 vs 3360 pg/ml, $P = 0.01$). There was also a significant increase between Ang-2 levels and volume of liver metastases ($P = 0.014$). Time to disease progression was worse in patients with serum Ang-2 levels > 4756 pg/ml ($P = 0.04$). Serum Ang-2 but not Ang-1 is elevated in NET patients. Ang-2 may be a useful serum marker for monitoring and assessment of prognosis in patients with NETs.

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Introduction

Angiogenesis is an essential process in cancer growth, maintenance and metastasis. It is regulated by the balance between pro- and anti-angiogenic factors (Folkman *et al.* 1989, Holash *et al.* 1999). There are a large number of angiogenic mediators including the angiopoietin (Ang) family and vascular endothelial growth factor (VEGF), which play an important role in both physiological and pathological angiogenesis (Holash *et al.* 1999, Carmeliet & Jain 2000, Machein *et al.* 2004). To date, four Ang have been identified; termed Ang 1–4 (Lee *et al.* 2004). Of these, Ang-1 and Ang-2 are the most widely studied and function as

ligands for Tie-2, which is a receptor tyrosine kinase specifically expressed on endothelial cells (Suri *et al.* 1996, Oliner *et al.* 2004).

Ang-1 binds specifically to Tie-2 causing activation by phosphorylation. Ang-1 is produced by endothelial cells and pericytes and is widely expressed in adult tissue, where it appears to have a stabilizing effect on blood vessels (Davis *et al.* 1996). The role of Ang-1 in tumour development is complex and studies have shown both pro- and anti-angiogenic effects with this growth factor. Ang-2 is expressed at sites of vascular remodelling (Maisonpierre *et al.* 1997) and promotes vessel destabilization (Yu & Stamenkovic 2001).

This appears to be accomplished by Ang-2 binding to Tie-2 and therefore blocking Ang-1 binding. Ang-2 appears to be a non-signal transducing ligand and therefore disrupts normal Tie-2 activation (Bach *et al.* 2007). The endothelial cells are then acted upon by various angiogenic factors such as VEGF, which lead to proliferation (Wang *et al.* 2005).

In tumours, a shift in the balance pro- and anti-angiogenic factors is thought to occur; termed the 'angiogenic switch' resulting in an angiogenic phenotype (Folkman & Hanahan 1991, Tanaka *et al.* 2003). It has been proposed that a change in the ratio of Ang-1:Ang-2 in favour of Ang-2 might play a role in this switch (Tait & Jones 2004). Support comes from animal studies in colonic and gastric tumours transfected with Ang-2, which larger and heavier in nude mice compared those transfected with Ang-1 (Ahmad *et al.* 2001, Etoh *et al.* 2001).

Neuroendocrine tumours (NETs) are uncommon tumours that can occur in almost any organ and are thought to originate from neuroendocrine cells (Caplin *et al.* 1998, Modlin *et al.* 2008). The most common sites of origin is from gastroenteropancreatic tract, where there is a reported incidence of 2–5 per 100 000 population (Modlin *et al.* 2003) but the incidence and prevalence are increasing (Modlin *et al.* 2003). NETs are generally regarded as slow growing in comparison adenocarcinomas; however, can behave aggressively and often produce highly vascular liver metastases. To date, chromogranin A (CgA) is the best biochemical marker for NETs; however, it lacks specificity and sensitivity especially in patients with low-volume disease (Eriksson *et al.* 2000, de Herder 2007).

The serum levels of Ang have not been evaluated in NET patients. The aim of this study was to measure serum Ang-1 and Ang-2 levels in patients with NETs and assess their interrelationship and clinical significance.

Materials and methods

Patients

We enrolled 47 patients with NETs, between July 2007 and March 2008. All patients had histological confirmation, including assessment of morphology and immunohistochemical analysis for neuron-specific enolase, CgA, synaptophysin and PGP9.5. All cases were well or moderately differentiated and no patients enrolled in the study had poorly differentiated NET. The presence of necrosis, number of mitoses and Ki67 index evaluated. Tumours were graded using the system proposed by European Neuroendocrine

Tumour Society consensus group (Rindi *et al.* 2007, Eriksson *et al.* 2008). Using this classification, low-grade tumour was regarded as mitotic count <2 per 10 high-power fields (HPF) and Ki67 ≤2%, intermediate grade as having a mitotic count 2–20 per 10 HPF and Ki67 3–20%. All patients underwent imaging with CT or MRI and appropriate nuclear medicine imaging including somatostatin (SST) receptor scintigraphy using ¹¹¹In-DTPA-Phe I-Pentetreotide (Octreoscan, Mallinckrodt Inc, St Louis, MO, USA), within 2 months of blood sampling to enable staging of disease. Serum CgA was measured in all patients at the same time as serum collection for the study. All previous and current therapies that patients had received were recorded from patient records. The demographics of both groups are shown in Table 1.

Table 1 Demographic data

	NET	Control
Subject number	47	44
Age	61.4 (34–80)	59.65 (24–86)
Males	26	23
Females	21	21
Primary tumour		
Bronchial	3	
Pancreatic	17	
Jejunal	2	
Ileal	20	
Unknown	5	
Functional types		
Carcinoid syndrome	18	
Glucagonoma	1	
Non-functional	28	
Histological grade		
Low	24	
Intermediate	23	
Biochemical markers		
Chromogranin A > 60	35	
Chromogranin A 0–60	12	
CgA (pmol/l) (range)	Median 99 (17– > 1000)	
Concurrent SST analogues	29	
Previous therapies		
Previous chemotherapy	4	
Radiotargetted therapy	4	
Surgery	19	

All therapies are previous treatments that patients had undergone, except somatostatin (SST) analogues in all patients were currently on at the time of study. Histological grade was classified using the ENETS proposed system for grading (Rindi *et al.* 2007). The classification was expanded to include bronchial and tumours of unknown primary site. Chromogranin A measurements were made using commercial RIA kit (Roche), which measures the pancreastatin fragment of chromogranin A, normal range is 0–60 pmol/l.

Survival data and time to progression of disease were identified in all cases. Time to progression was assessed using radiological evidence of disease progression according to RECIST criteria (Therasse *et al.* 2000), and was calculated in days from time of blood collection. Patients underwent cross-sectional imaging for restaging of their disease on a 3–4 monthly basis.

As control subjects, 44 healthy volunteers who were age and sex matched were enrolled in the same period (Table 1). Most subjects in the control group were healthy relatives of patients and the remainder subjects were volunteers. All control patients had no previous history of cancer.

The study was approved by the Local Ethical Committee and all participants gave written informed consent prior to obtaining samples.

ELISAs

Serum samples were obtained from each individual, immediately placed on ice and allowed to stand for 30 min. Following this, the samples were centrifuged at 1000 *g* for 15 min. Sera were stored at -80°C . ELISA were used to measure Ang-1 and Ang-2 (Quantikine, R&D Systems, Minneapolis, MN, USA). Serum samples were diluted as appropriate prior to being added to separate microplates, each containing a specific antibody for Ang-1 or Ang-2. The mixtures were then incubated for 2 h on an orbital microplate shaker. Plates were then washed four times to remove unbound antigen. Enzyme-linked polyclonal antibodies specific for each angiogenic factor were then added and the mixture incubated for 2 h. Plates were then washed four times prior to the substrate solution being added to the wells. The colour was allowed to develop following which the stop solution was added. The optical density of each well was determined at 540 nm.

CgA was measured using a commercial RIA that measures the pancreastatin fragment of CgA.

Immunohistochemical analysis of Ang-2

Formalin-fixed paraffin-embedded tumour tissues were available from nine patients with a histologically confirmed diagnosis of NET in whom serum samples had been collected for Ang-2 analysis. All cases had been assessed for grade of tumour; including Ki67 proliferation index and number of mitoses per 10 HPF were available in all cases. The study population comprised of six primary pancreatic NETs and three midgut NETs.

Four micrometre sections of tumour tissue were dewaxed three times in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation in 0.5% hydrogen peroxide, diluted in acetone, for 10 min. Slides were microwaved for 10 min at 600 in citrate buffer and then transferred to a humidity chamber. Slides were incubated overnight in primary monoclonal Ang-2 antibody at a dilution 1:50 (Santa Cruz product no. Sc-74403). Slides were washed with Post Primary Block (Novolink Max Polymer RE7280 detection kit) for 30 min in a humidity chamber at room temperature. Slides were washed in TBS-t (Tris buffered saline) and left for 5 min. Novolink Polymer (RE7280-K) was added for 30 min in a humidity chamber for 30 min, following which slides were washed in TBS-t. The sections were then developed with Novolink 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution for 5 min by adding 50 μl of DAB chromagen to 1 ml of Novolink DAB substrate buffer. The slides are counterstained with Mayer's haematoxylin for 3 min. Positive controls for Ang-2 were renal tissue; negative controls included substitution of the primary antibody with normal sera. An experienced histopathologist (T V L) performed the interpretation of immunohistological staining for the antibody studied.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The values were generally not normally distributed, so non-parametric tests were used to compare marker levels between groups, i.e. the Mann–Whitney *U* test (for two groups) or Kruskal–Wallis test (for several groups). Multivariate linear regression analyses were used to examine Ang-1 and Ang-2 (on a logarithmic scale) in relation to several other factors. Spearman correlation coefficient was used to analyse correlations between parameters. Time to disease progression curves were plotted using the Kaplan–Meier method and the log-rank test applied.

We also evaluated the screening performance of the markers using the detection rate (sensitivity), defined as the proportion of NET cases that had levels above a specified cut-off, and the false-positive rate, defined as the proportion of controls that had levels above the same cut-off. The likelihood ratio quantifies the 'power' of the marker and is the detection rate divided by the false-positive rate; the higher the value the better the marker.

Results

Serum Ang-1 and -2 levels in patients with NETs

Figure 1 shows scatterplots of the distributions of Ang-1, Ang-2 and the ratio of the two. Patients with NETs had significantly higher serum Ang-2 levels than the control group (median 4756 vs 2495 pg/ml, $P < 0.001$). There was still a highly statistically significant difference when allowing for age and sex ($P < 0.001$). Median serum Ang-1 levels were not different between the two groups (39 135 pg/ml controls versus 39 405 pg/ml cases; $P = 0.60$). The ratio of Ang-2 to Ang-1 was significantly increased in patients (median 0.133 compared with 0.066 in controls $P < 0.001$), but this probably largely reflects the difference associated with Ang-2. There was no evidence of a correlation between serum Ang-2 and Ang-1 ($r = 0.11$, $P = 0.49$); Ang-1 and plasma CgA ($r = -0.22$, $P = 0.13$); or Ang-2 and plasma CgA ($r = 0.03$, $P = 0.8$). We conducted a multivariate analysis to look at the association between each of Ang-1, Ang-2 and the ratio, and three prognostic factors: histological grade, CgA and stage. There was some evidence of an association between histological grade and Ang-2, even after allowing for stage and CgA ($P = 0.003$); the geometric mean was 4140 and 6668 pg/ml in the low and intermediate grades respectively. There was no evidence of an association between either Ang-1 or Ang-2:Ang-1 ratio and the prognostic factors.

To confirm that there was no significant daily variation in Ang-2 levels, we obtained two matched serum samples from 17 control patients separated by 24 h. There was no significant difference between Ang-2 levels ($P = 0.143$) or Ang-1 ($P = 0.662$).

Ang levels and type of NET

Compared to controls (median 2495 pg/ml), serum Ang-2 was significantly elevated in both midgut NETs (median 4790 pg/ml, $P < 0.001$) and foregut primary NETs (median 4900 pg/ml, $P < 0.001$). When comparing levels between foregut and midgut primary tumours, there was no evidence of a difference in serum Ang-2 (median 4900 vs 4790 pg/ml, $P = 0.59$), serum Ang-1 (median 39 100 vs 33 750 pg/ml, $P = 0.28$) or the ratio of Ang-2 to Ang-1 (median 0.133 vs 0.147, $P = 0.68$). There was no significant difference in Ang-2 levels between patients with functional tumours ($n = 19$) and non-functional tumours ($n = 28$; median 4503 vs 4867 pg/ml, $P = 0.58$). Furthermore, there was no difference in serum Ang-2 levels between the 29 patients currently

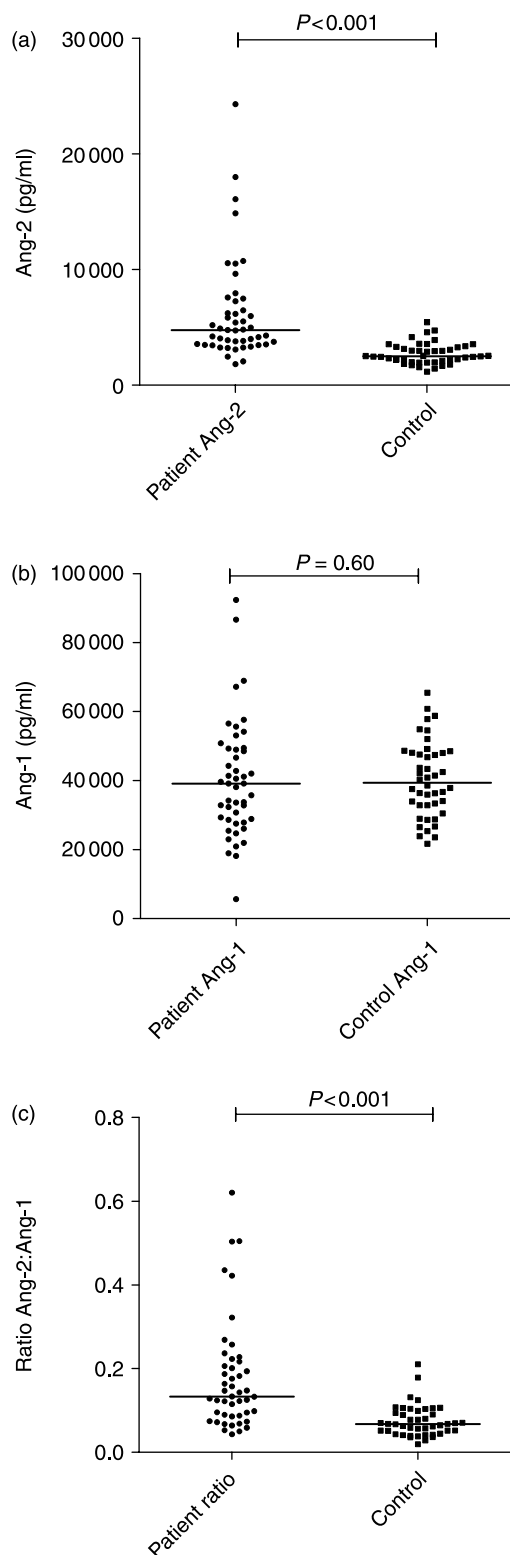


Figure 1 Comparisons of Ang-2, Ang-1 and Ang-2:Ang-1 ratios in patients with NETs ($n = 47$) and controls ($n = 44$). (a) Ang-2 levels, (b) Ang-1 levels and (c) ratio of Ang-2 to Ang-1.

on SST analogues and 18 patients who were not (median 4755 vs 4500 pg/ml, $P=0.82$). Similarly for Ang-1 levels (median 40 550 vs 33 900 pg/ml, $P=0.24$).

Ang levels and stage of disease

Ang-2 levels were elevated in patients with metastatic disease compared with those with localized disease without distant metastases (median 5081 vs 3359 pg/ml, $P=0.01$); see Fig. 2. Ang-2 levels were significantly higher in patients with localized non-metastatic disease ($n=6$), compared healthy controls (3360 vs 2495 pg/ml, $P=0.02$; Fig. 3).

From radiological assessment of CT or MRI scans performed within 2 months of blood sampling, visual assessments were made of the volume of liver metastases present. Four categories were created; localized disease without metastases, low-volume liver metastases (involving <25% of liver), intermediate- (involving 25–50% of liver) and large-volume liver metastases (>50% of liver), see Fig. 4. There was a statistically significant difference between the groups – when comparing localized disease, low-, intermediate- and large-volume liver metastases – ($P=0.014$, one-way ANOVA test), with a suggestion that increased Ang-2 is associated with increased tumour burden.

Serum Ang-2 as a marker of disease

Receiver operator curves for serum Ang-2 were constructed to determine the cut-off values for specificity and sensitivity of Ang-2 and Ang-2:Ang-1

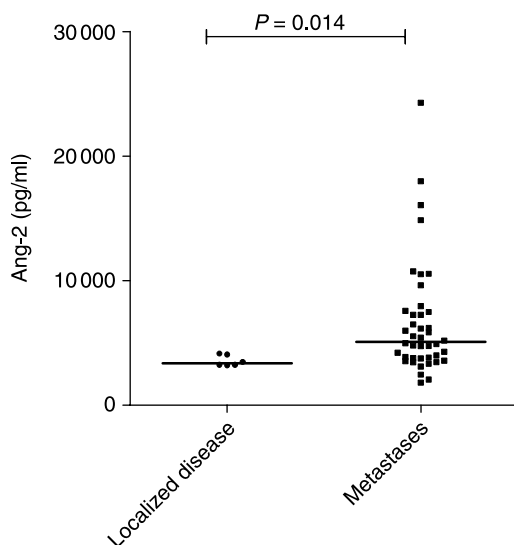


Figure 2 Comparison of serum Ang-2 levels in patients with liver metastases ($n=41$ cases) and those with localized disease ($n=6$).

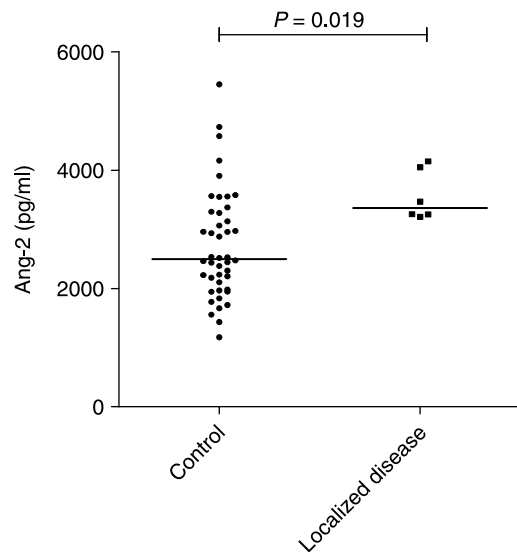


Figure 3 Serum Ang-2 levels are significantly raised in patients with localized non-metastatic disease ($n=6$ cases) compared healthy controls ($n=44$).

ratio. The area under the curve for serum Ang-2 was 0.88 and was greater than Ang-1 (0.53; Fig. 5). Detection rate, false-positive rate and likelihood ratio for Ang-2 and the ratio of Ang-2 to Ang-1 are shown in Table 2. Serum Ang-2 was a better marker than the ratio, with a sensitivity of 85%, for a false-positive rate of 22.7%. The CgA assay has been previously validated and a cut-off of 60 pmol/ml has been defined. Using this cut-off in our study, CgA had a sensitivity of 80.9%.

Immunohistochemical staining

Staining for Ang-2 was identified in five out of the nine tumour samples. In all cases with positive Ang-2 staining, the corresponding serum Ang-2 levels were greater than the control population, see Table 3. Of the four samples that were negative for Ang-2 staining, the serum Ang-2 was raised above the >4756 pg/ml (median Ang-2 value for patient cohort) in three cases. The staining pattern was predominantly cytoplasmic with clear tumour staining present. There was no staining of the background liver tissue and staining was localized only to tumour cells and endothelium. In one case, there was strong staining in 10% of tumour cells metastatic to the gallbladder; however, there was only moderate 1% staining of the surrounding tumour in liver metastases, see Fig. 6. Ang-2 staining was seen in low- and intermediate-grade tumours as well as pancreatic and midgut tumours. The immunohistochemical studies demonstrate there is intra-tumoural variation in staining patterns for Ang-2.

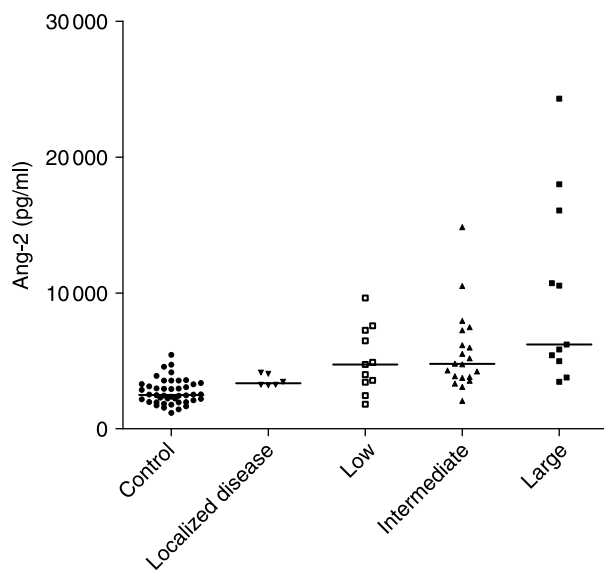


Figure 4 Serum Ang-2 levels in controls ($n=44$), patients without metastatic disease ($n=6$) and those with low- (<25% liver metastases) ($n=11$), intermediate- (25–50% liver metastases) ($n=19$) and large-volume (>50% liver metastases) ($n=11$) liver metastases.

Survival data

Owing to the indolent nature of these tumours, survival data alone are difficult to obtain (only four patients died during the 8 month follow-up). We therefore examined time to disease progression. Patients with progressive disease within 2 months prior to blood sampling were excluded from progression analysis. The median serum Ang-2 level in the patient group as a whole was used to divide the patients into two groups (cut-off value was 4756 pg/ml). The median follow-up period was 6 months, range 4–8 months. NET patients with serum Ang-2 levels >4756 pg/ml ($n=14$) had a worse prognosis than those with Ang-2 levels \leq 4756 pg/ml ($n=22$; Fig. 7). Both groups had similar previous treatments and a similar number of patients on SST analogues (see Fig. 7b).

Discussion

This is the first study to demonstrate elevated serum Ang-2 levels and Ang-2:Ang-1 ratio in patients with NETs compared healthy controls. Similar findings have been reported in a number of studies looking at serum Ang-2 levels in other cancers including colonic, lung and ovarian cancer (Wong et al. 2000, Shim et al. 2002, Machein et al. 2004, Mi et al. 2006, Park et al. 2007). Furthermore, Ang-2 mRNA levels are elevated in the tumour tissue of a number of other cancers, including human hepatocellular carcinoma, prostate

cancer and breast cancer (Ahmad et al. 2001, Etoh et al. 2001, Mitsuhashi et al. 2003, Sfiligoi et al. 2003, Stoeltzing et al. 2003, Lind et al. 2005). Our findings

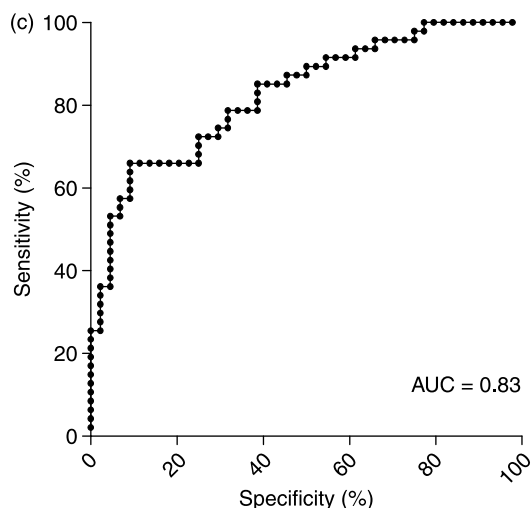
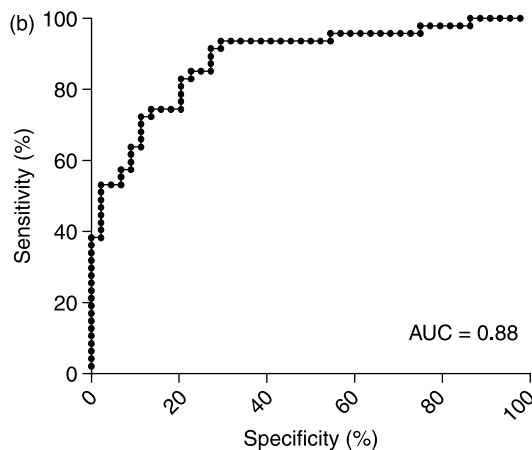
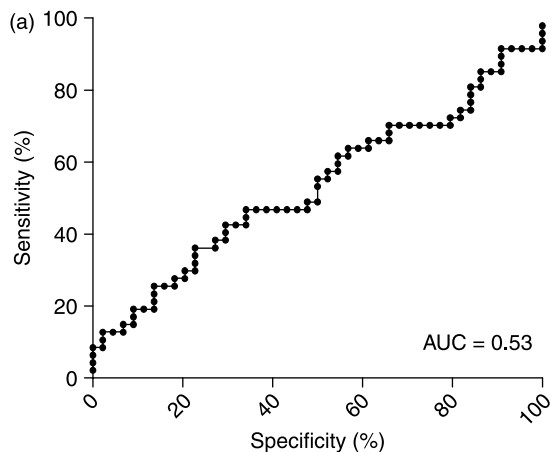


Figure 5 Receiver operator curves for Ang-1 (a), Ang-2 (b) and the ratio of Ang-2 to Ang-1 (c). AUC, area under the curve.

Table 2 Detection rate (sensitivity), false-positive rates and likelihood ratio for Ang-2 and the ratio of Ang-2 to Ang-1 in NETs

Detection rate (sensitivity)	Ang-2				Angiopietin 2:1 ratio			
	Cut-off	FPR (%)	CI (%)	LR	Cut-off	FPR (%)	CI (%)	LR
70%	>3770	11.4	3.6–24.6	6.1	>0.095	25.0	13.2–40.3	2.8
75%	>3565	15.4	5.2–27.4	4.9	>0.088	31.8	18.6–47.6	2.4
80%	>3447	21.5	9.8–35.3	3.7	>0.074	38.6	24.4–55.6	2.1
85%	>3313	22.7	11.5–37.8	3.7	>0.071	38.4	24.4–52.2	2.3
90%	>3172	27.3	15.0–42.8	3.3	>0.0644	55.6	38.8–69.4	1.7
95%	>2447	55.8	38.8–69.6	1.7	>0.0527	65.9	50.1–79.6	1.5

The cut-off values for Ang-2 are in pg/ml. FPR, false-positive rate; CI, confidence interval; LR, likelihood ratio.

suggest that Ang-2 was superior to Ang-1 for differentiating NET patients from control samples, and distinguishing patients with distant metastasis from those without and appears to increase in relation to increasing disease burden. In addition, our multivariate analysis suggests that high levels of Ang-2 are associated with higher grade tumours.

NETs often progress at a relatively slow rate; however, occasionally they may grow rapidly. During periods of growth or progressive disease, there may well be a shift in proangiogenic factors including Ang-2. This could be a reflection on the biology and natural course of NETs. Interpretation of Ang-1 levels is difficult due to the wide range of values expressed and the complex behaviour of this growth factor. Interestingly, high levels of Ang-1 were not correlated with increased Ang-2 levels. However, the higher ratio of Ang-2 is more commonly associated with high tumour load, i.e. >50% liver metastases rather than low-volume disease or patients without metastasis. Our results support the hypothesis that elevation of Ang-2 being an important aspect of tumour angiogenesis.

This study has shown Ang-2 as a marker, with high sensitivity, however, lacking specificity for use as a

single marker for NETs since it is raised in a number of cancers and patients with cirrhosis. However, it could potentially have a role as an additional marker for monitoring the development of recurrent disease or distant metastases, although further validation of this marker will be required. Since CgA lacks sensitivity in cases with low tumour volume (Eriksson *et al.* 2000), Ang-2 levels could be useful since there was a significant difference in Ang-2 levels in controls and patients without metastatic (i.e. low volume) disease, however, the number of patients with localized disease were small ($n=6$).

In our study, while Ang-1 did not have a significant prognostic implication, serum Ang-2 was more promising as a potential prognosticator; with levels >4756 pg/ml associated with earlier time to disease progression. The exact value for the cut-off will require prospective validation in another data set. Whether the level of serum Ang-2 is also a predictor of survival will require re-evaluation after prolonged follow-up.

Currently, there are no accurate biochemical markers to aid in the diagnosis of progressive disease in patients with NETs. Even though CgA has been reported to have prognostic value especially in midgut

Table 3 Immunohistochemical staining for Ang-2 performed in nine patients

Primary	Grade	Functional	Biopsy site	Ang-2 (pg/ml)	Staining pattern
Midgut	Low grade	Yes	Liver	6800	Negative
Midgut	Low grade	Yes	Liver	13 708	Moderate staining, granular staining 10% and strong staining in cytoplasm 1% of cells
Midgut	Low grade	Yes	Liver	6205	Negative
Pancreas	Low grade	No	Pancreas	5983	Negative
Pancreatic	Low grade	No	Liver	6152	Moderate staining in 1% cells
Pancreas	Low	Yes	Liver	3815	Strong positivity in 1% cells
Pancreas	Intermediate	Yes	Liver	10 540	Strong positivity in 1% cells
Pancreas	Low	No	Liver	3752	Strong positivity in 1% cells
Pancreatic	Intermediate	No	Gall bladder/liver	14 846	5% strong positive in gall bladder tumour, 1% positive staining in liver

Table shows site of primary, location and grade of tumour biopsy and staining pattern for Ang-2.

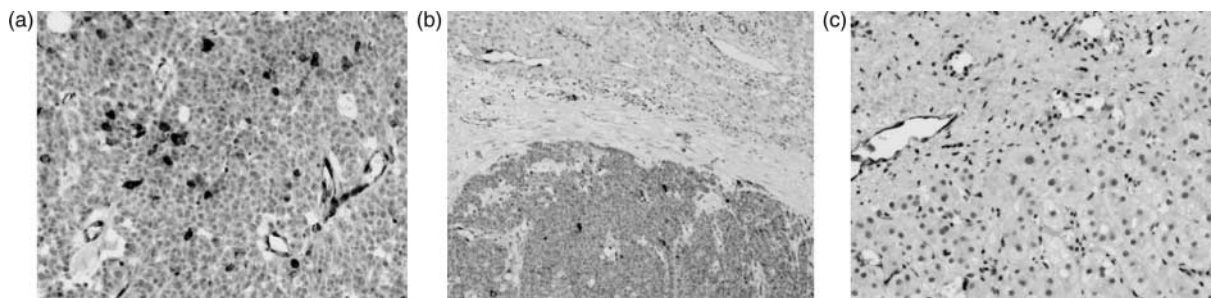


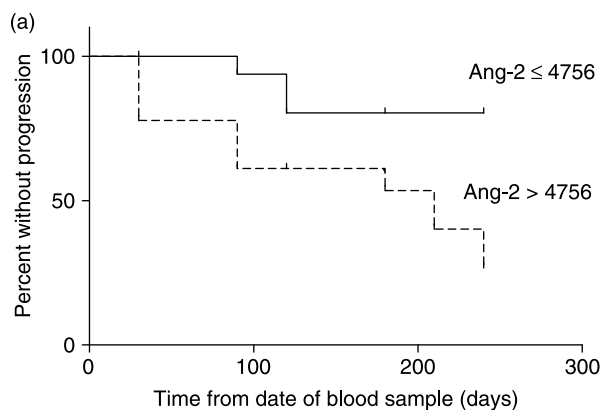
Figure 6 (a) Ang-2 staining in NET invading the gall bladder shows strong Ang-2 positivity in tumour cells and endothelial cells (internal control), ×20 magnification. (b) The top part of the image is normal liver with negative Ang-2 staining and the lower part of the image is liver metastatic NET, with scattered Ang-2 positive staining, ×10 magnification. (c) Normal liver showing only endothelial positive staining for Ang-2, ×20 magnification.

carcinoid tumours (Janson *et al.* 1997), it is not 100% sensitive. Therefore, the use of Ang-2 in patients with NET could serve as a marker indicating likelihood of developing progressive disease.

There was no significant difference in Ang-2 levels between patients treated with SST analogues and those not. Since SST analogues are thought to inhibit angiogenesis through a number of pathways, the lack of difference between the groups at first may seem surprising. However, there may be a number of reasons

no difference is seen, most importantly is the heterogeneity of the groups. The tumour load of patients on SST analogues is often greater than those not on SST analogue therapies, since these patients all have metastatic tumours and hence syndromic. We have demonstrated that patients with large-volume disease generally had high Ang-2 levels.

We have demonstrated by immunohistochemistry that a minority of NET cells strongly express Ang-2 in contrast to the normal liver. We did not find any clear



(b)

Therapy	Ang-2 ≤ 4756 (n = 22)	Ang-2 > 4756 (n = 14)
Concurrent SST analogues	14 (63.6%)	8 (57.1%)
Prior therapy		
Chemotherapy	2 (9.1%)	3 (21.4%)
Interferon	2 (9.1%)	0 (0%)
Radiotargeted therapy	5 (22.7%)	2 (14.3%)
Right hepatectomy	4 (18.2%)	1 (7.1%)
Other surgery	3 (13.6%)	5 (35.7%)
None	3 (13.6%)	1 (7.1%)

Figure 7 (a) Kaplan–Meier curves of time to progression of NETs according to Ang-2 levels. Log rank $P=0.03$. (b) Treatments undergone by patients in the two groups during the course of their illness and the number of patients on current somatostatin analogue therapy.

correlation between tumour staining and serum levels. There are three explanations for this; first, there may be heterogeneity of expression within the tumour, as we have demonstrated clearly in one case, and one small sample may not be representative. Second, the Ang-2 serum level appears to be related to tumour burden. Hence, a small tumour with high Ang-2 expression may result in a lower serum level than a bulky metastatic tumour with a lower Ang-2 expression. Finally, we cannot exclude the possibility that the Ang-2 arises from another source, but its release is associated with tumour.

There were limitations with our study; first, the study incorporated a rather diverse group of patients including patients with NETs with a foregut, midgut and those with unknown primaries. Second, the study group had a number of different previous treatments and some patients had stable disease without any evidence of radiological progression, while others had progressive disease at time of serum collection. This may in part explain the heterogeneity identified with Ang-1 and Ang-2 levels.

In conclusion, this is the first study reporting that serum Ang-2 is elevated in patients with NETS and that it may serve as a useful marker in NETs for monitoring and prognostication. A greater understanding of the role of Ang-1 and -2 in the pathogenesis of NETs may provide an opportunity for the development of effective therapy.

Declaration of interest

The authors declare that there is conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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