



# Assessment of soluble angiogenic markers in pancreatic cancer

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Angiogenic markers such as VEGF/basic FGF (bFGF) can enlarge the diagnostic biomarkers panel for pancreatic cancer. **Materials & methods:** Serum samples from 32 stage I–IV pancreatic cancer patients and 20 controls were analyzed for soluble VEGF/bFGF by ELISA and xMAP array. **Results:** VEGF/bFGF serum levels were significantly increased in patients compared with controls ( $p < 0.0001$ ). We report a correlation with tumor diameter ( $p < 0.01/p < 0.05$ ), stage ( $p < 0.001$ ), Ki67LI ( $p < 0.005/p < 0.05$ ) and carbohydrate 19–9 antigen ( $p < 0.005/p < 0.001$ ). VEGF/bFGF levels analyzed by xMAP array were comparable with the pattern (patient/control) outline obtained by ELISA tests. We obtained a good correlation between these two soluble angiogenic markers ( $p < 0.001$ ). **Conclusion:** Data obtained for angiogenic markers qualifies them as important candidates in the pancreatic cancer biomarker panel.

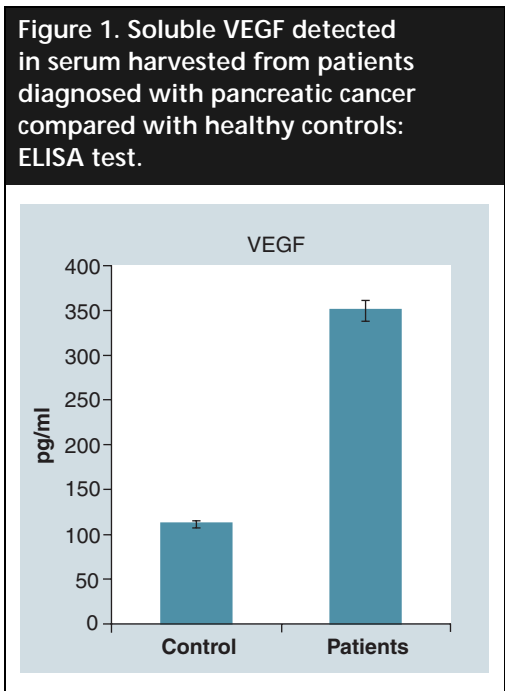
Exocrine pancreatic carcinoma is now the fifth leading cause of cancer in the USA, Japan and Europe, with an overall 5-year survival rate of less than 5%. One of the major causes of death is peritoneal dissemination and liver metastasis [1]. Although pancreatic cancer is not a highly vascularized tumor, its growth depends on the development of an adequate blood supply through angiogenesis at both primary and secondary sites. The pivotal role of angiogenesis in primary tumor growth and metastasis has been recognized for many years [2,3]. Activation of the angiogenic switch during early stages of tumor development suggests that regulation of the angiogenic process is likely to be a rate-limiting step in the progression from small lesion to extensive disease. Several growth factors and their specific receptors have been detected at the tumor site: the VEGF family, FGF family, PDGFs and TGFs. The VEGF family is an important group of signaling proteins involved in pathological angiogenesis. The VEGF family is secreted by tumor cells in the majority of cancers and acts on endothelial cells, promoting new blood-vessel formation. VEGF expression is driven by many factors that are characteristic of tumors, including oncogene expression and hypoxia. Moreover, VEGF mRNA expression can be induced by some of the principal pro-angiogenic regulators that include growth factors (e.g., FGF, PDGF, TGF and EGF) and cytokines [4–7]. The binding of VEGF to its specific receptor activates intracellular signaling pathways leading to cell proliferation, migration, survival, sprouting and tube formation;

upregulation of the molecules involved in degradation of the extracellular matrix further allows proliferating cells to migrate [7].

The FGF family exerts several biological effects, including tumor growth and angiogenesis [8]. These factors stimulate the proliferation of fibroblasts and endothelial cells that give rise to angiogenesis. Basic FGF (bFGF), known as FGF-2, is highly involved in the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures, thus promoting angiogenesis [3]. bFGF, along with VEGF or PDGF, is a potent angiogenic factor.

The extent of angiogenesis depends on the balance between proangiogenic and antiangiogenic factors released by tumor and host cell [9]. The expression of VEGF, at present regarded as the major proangiogenic factor for most types of human cancers, is strongly induced by EGFs and TGFs. Human pancreatic cancer cells secrete the proangiogenic molecules VEGF and bFGF [10]. It is well established that human pancreatic cancer tissue and cell lines overexpress VEGF [2,11–13].

There is no effective early screening test in pancreatic cancer, and symptoms only appear during late-stage disease when the tumor has metastasized and invaded surrounding tissues. Aiming to enlarge the panel of possible markers detecting the evolution of pancreatic cancer, we have studied soluble VEGFs and bFGFs in sera harvested from patients diagnosed with stage I–IV pancreatic cancer. We have focused on the correlation between soluble VEGF, bFGF and the disease stage in relation to conventional



markers. The previously mentioned angiogenic factors were quantified using both ELISA and xMAP technology for simultaneous detection of parameters.

Materials & methods

Subjects

The study included 32 patients with pancreatic cancer diagnosed in the Surgical Department of Fundeni Clinical Institute (2003–2007); mean

age was 55 years (range: 32–74 years); and 22 men and ten women took part. Tumor staging was based on radiological reports, operative findings and pathology reports in accordance with the TNM system. None of the patients had undergone any treatment when tested.

The control group consisted of 20 healthy volunteers (12 men and eight women; mean age: 57 years; range: 25–70 years). The absence of disease was confirmed by physical examination, clinical history and routine laboratory tests, including liver and renal function tests. All patients and healthy controls provided informed, written consent and the study was approved by the Ethics Committee of Fundeni Clinical Institute and the Victor Babes National Institute of Pathology.

Sample collection

Blood samples were collected into serum-separating tubes (Vacutainer Systems, Becton Dickinson). The blood was allowed to clot for 30 min before centrifugation for 10 min at 1000 *g*. Serum was removed and stored in aliquot at -80°C until assayed.

VEGF & bFGF: ELISA

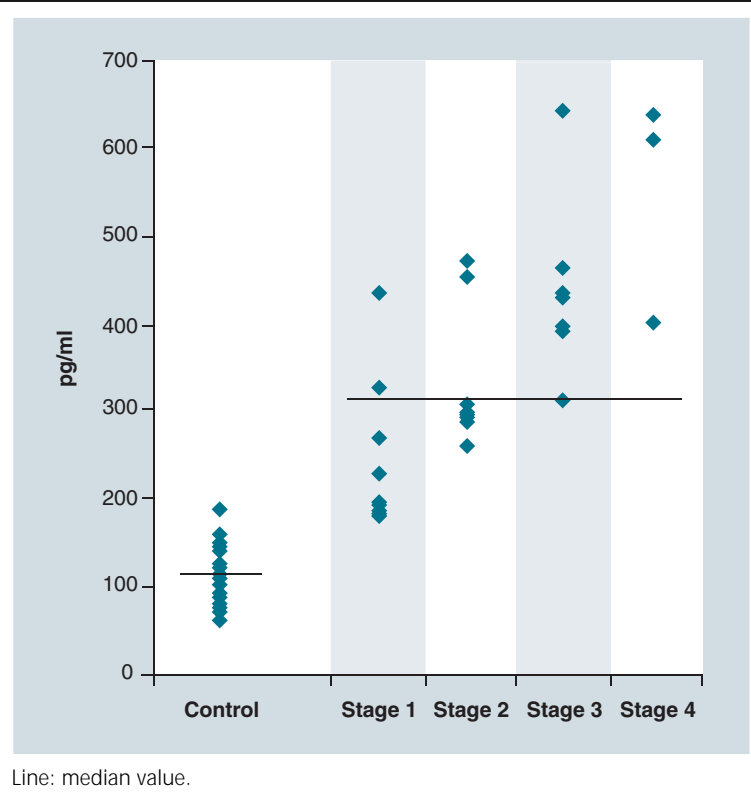
Serum levels of angiogenic markers VEGF and bFGF were measured by the immunoenzymatic ELISA method (Quantikine Human VEGF Immunoassay and Quantikine Human FGF Basic Immunoassay, R&D Systems, Minneapolis, MS, USA) according to manufacturer

**Table 1. Correlation between VEGF and basic FGF level and clinicopathological features, Ki67 and carbohydrate 19–9 antigen.**

		VEGF				bFGF			
		<300	≥300	$\chi^2$	<i>p</i> value	<25	≥25	$\chi^2$	<i>p</i> value
Gender	M	11	11	0.27	<i>p</i> = 0.6	11	11	0.27	<i>p</i> = 0.6
	F	4	6			4	6		
Age	<60	10	13	0.37	<i>p</i> = 0.54	9	14	1.96	<i>p</i> = 0.16
	≥60	5	4			6	3		
Diameter	<3	13	7	7.03	<i>p</i> < 0.01	12	8	3.69	<i>p</i> < 0.05
	≥3	2	10			3	9		
Stage	1+2	15	6	14.78	<i>p</i> < 0.001	14	7	9.61	<i>p</i> < 0.001
	3+4	0	11			1	10		
Ki67 LI	<20	9	2	8.21	<i>p</i> < 0.005	8	3	4.5	<i>p</i> < 0.05
	≥20	6	15			7	14		
CA 19-9	<40	8	1	8.87	<i>p</i> < 0.005	9	0	14.2	<i>p</i> < 0.001
	≥40	7	16			6	17		
CEA	<2.5	12	8	5.72	<i>p</i> < 0.01	13	7	7.03	<i>p</i> < 0.01
	≥2.5	2	10			2	10		

bFGF: basic FGF; CA 19–9: Carbohydrate 19–9 antigen; CEA: Carcinoembryonic antigen; F: Female; LI: Labeling index; M: Male.

**Figure 2. Soluble VEGF detected in correlation with the stage of pancreatic cancer.**



protocol. The absorbance at 450 nm was measured and concentrations were determined by interpolation of a standard calibration curve. As stated by the supplier, the lower limit of detection is 9 pg/ml for VEGF and 0.22 pg/ml for bFGF. All samples were assayed in duplicate and the mean value of VEGF/bFGF was taken into account.

#### *Carcinoembryonic antigen & carbohydrate 19–9 antigen: ELFA*

The serum concentrations for carcinoembryonic antigen (CEA) and carbohydrate 19–9 antigen (CA 19–9) were measured by enzyme-linked fluorescent immunoassay technique ([ELFA], bioMérieux, Marcy l'Etoile, France). The intensity of the fluorescence is proportional to the concentration of CEA and CA 19–9 present in the sample. Test values above 2.5 UI/ml for CEA and above 37 UI/ml for CA 19–9 were considered as positive.

#### *VEGF & bFGF: Luminex xMAP technology*

In order to extend to high-throughput testing, in addition we have used Luminex xMAP® technology (Luminex® 200™, Austin, TX, USA).

The xMAP assay for VEGF and bFGF was performed according to the protocol of R&D-Fluorokine MAP Human. Plates were analyzed by the Luminex 200 array system.

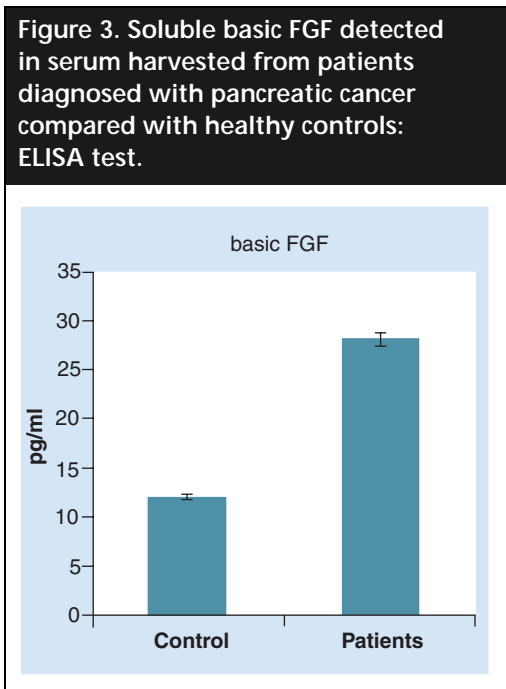
#### *Statistical analysis*

Data were expressed as the mean  $\pm$  standard error of the mean; minimum and maximum values were provided when necessary. The Kolmogorov–Smirnov test was used to assess the distribution of data. Differences between groups were analyzed by unpaired Student's t-test. p-values less than 0.05 were considered to be statistically significant. The chi-square test ( $\chi^2$ , p) and Pearson correlation (r, p) were used to explore the association between angiogenic marker expression and tumoral markers or clinical parameters. Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

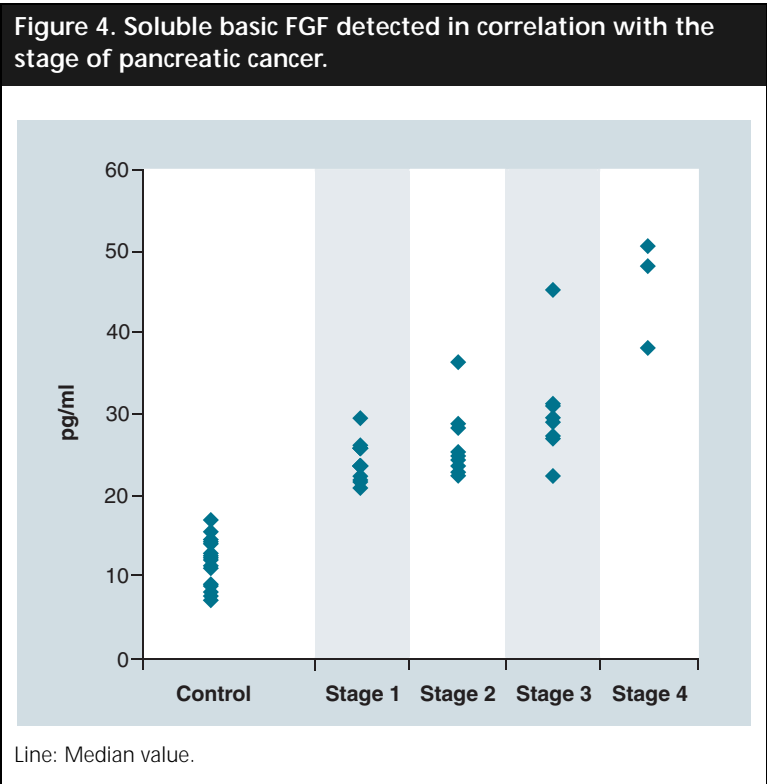
#### *Results*

##### *VEGF levels in pancreatic cancer: correlation with disease stage & conventional tumor markers*

No matter the disease stage, we found a mean level of 349.28 pg/ml VEGF (range: 181–637 pg/ml), significantly higher than in controls, namely 115.06 pg/ml (range: 62.16–189 pg/ml;  $p < 0.0001$ ) (Figure 1). Similar high values of soluble VEGF in pancreatic cancer patients were found earlier in a comparable study [14], in which the authors found a mean value of 505 pg/ml in pancreatic cancer patients compared with the 193 pg/ml found in the control group. Another group previously reported that they had used a cut-off level of 149.5 pg/ml VEGF to discriminate pancreatic cancer [15]. With slightly overlapping values compared with controls, a group studying the correlation of VEGF with the stage of pancreatic cancer obtained a mean value of 294.8 pg/ml VEGF [16]. We found that serum levels of VEGF correlated significantly with the disease stage and with the standard proliferative marker, Ki67. As the disease stage increased, we found higher VEGF levels ( $\chi^2 = 14.78$ ;  $p < 0.001$ ) (Figure 2) correlated with Ki67 labeling index (LI) equal to or greater than 20% ( $\chi^2 = 8.21$ ;  $p < 0.005$ ) (Table 1). A dependence of VEGF level on tumor diameter was found, hence patients with tumors larger than 3 cm in diameter had high VEGF serum levels ( $\chi^2 = 7.03$ ;  $p < 0.01$ ) (Table 1). Other studies correlating hepatocellular carcinoma tumor diameter with VEGF



serum values proved that patients bearing tumors over 3 cm diameter have high soluble VEGF levels [17,18]. We found that when compared with conventional markers, soluble VEGF correlated with an increased level of CA 19-9 ( $\chi^2 = 8.87$ ;  $p < 0.005$ ) and CEA ( $\chi^2 = 5.72$ ;  $p < 0.01$ ), but no correlation with patient gender or age was recorded (Table 1). In all



our correlation studies, the threshold employed was 300 pg/ml VEGF, a value chosen in accordance with our median value and previous reported values.

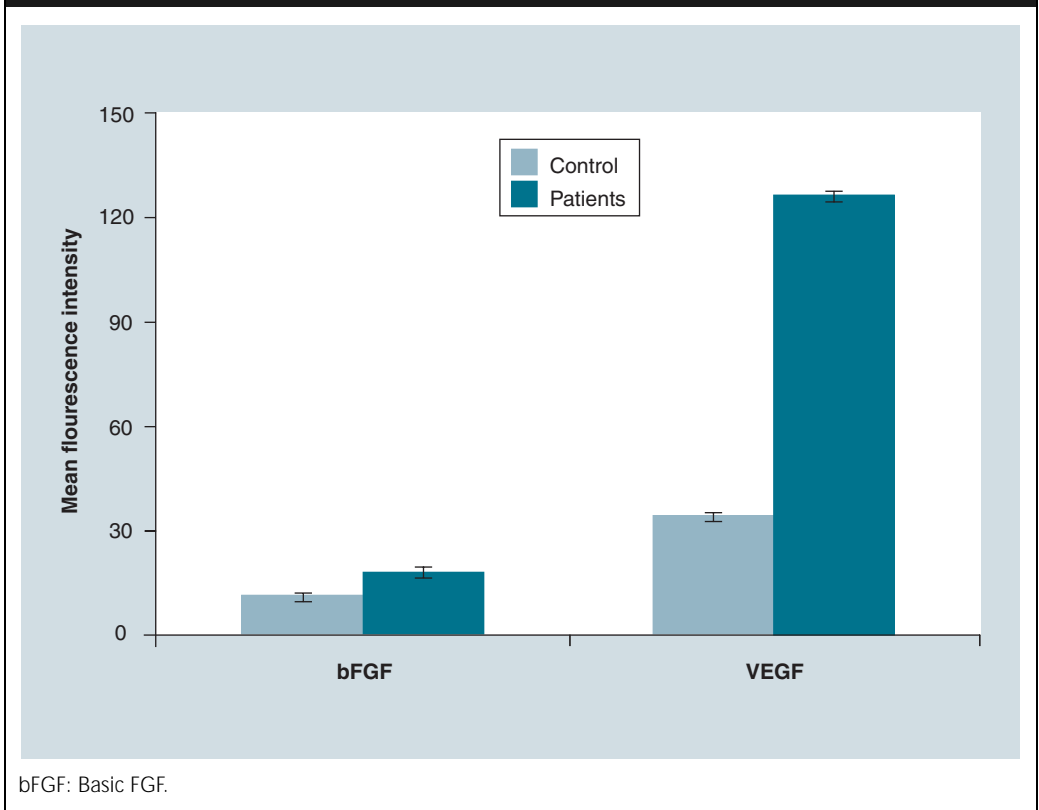
*bFGF levels in pancreatic cancer: correlation with disease stage & conventional tumor markers*

Soluble bFGF in the serum of pancreatic cancer patients had a mean value of 28.19 pg/ml (range: 20.8–50.6 pg/ml), significantly higher compared with control values at 11.76 pg/ml (range: 7.16–15.4 pg/ml;  $p < 0.0001$ ) on bFGF (Figure 3). Although there are fewer reports than VEGF, there are various soluble bFGF values reported, thus the authors state that 5 pg/ml soluble bFGF is found in endometrial cancer [19], approximately 15 pg/ml in colorectal cancer [20] and 60 pg/ml in B-cell chronic lymphocytic leukemia [21]. The different value obtained in our studies can be attributed to pancreatic cancer being a more aggressive type of neoplasia. We have obtained, as in the case of soluble VEGF, bFGF serum levels significantly correlating with the stage of disease and proliferative marker, Ki67. Following the VEGF pattern, bFGF levels increased in association with the disease stage ( $\chi^2 = 9.61$ ;  $p < 0.001$ ) (Figure 4) and with Ki67 LI equal to or greater than 20% ( $\chi^2 = 4.5$ ;  $p < 0.05$ ). As with VEGF, we found a positive linear correlation with the tumor diameter ( $\chi^2 = 3.69$ ;  $p < 0.05$ ) and with increased levels of classical markers CA 19-9 ( $\chi^2 = 14.2$ ;  $p < 0.001$ ) and CEA ( $\chi^2 = 7.03$ ;  $p < 0.01$ ). No correlation was found between bFGF levels and the patients' gender or age (Table 1). In current practice, CA 19-9 assays are not optimal for detecting small pancreatic lesions, but correlating this marker with new angiogenic markers can strengthen its power in pancreatic cancer.

In all correlation studies, the threshold used was 25 pg/ml, based on our median value and the reported values in previous studies.

*Luminex xMAP technology for angiogenic marker analysis*

A subset of serum samples was also analyzed for VEGFs and bFGFs using Luminex xMAP technology. Comparing the control and patient groups, the VEGF/bFGF values obtained by the xMAP array were comparable to the outline obtained by the ELISA tests (Figure 5). The differences obtained between the control groups and patients were somewhat higher in ELISA tests;

**Figure 5. Soluble VEGF/basic FGF detected by xMAP technology in patients diagnosed with pancreatic cancer compared with controls.**

the mean value for soluble VEGF in patients versus controls was approximately 1.60-fold greater in Luminex and approximately twofold greater in ELISA analysis. Soluble bFGF mean value was approximately 3.5-fold in patients versus control when Luminex technology was used, and approximately threefold in ELISA quantification. Comparing the absolute values obtained in both techniques, soluble VEGF detected by xMAP technology was reduced five times compared with ELISA detection, while bFGF was three-times lower.

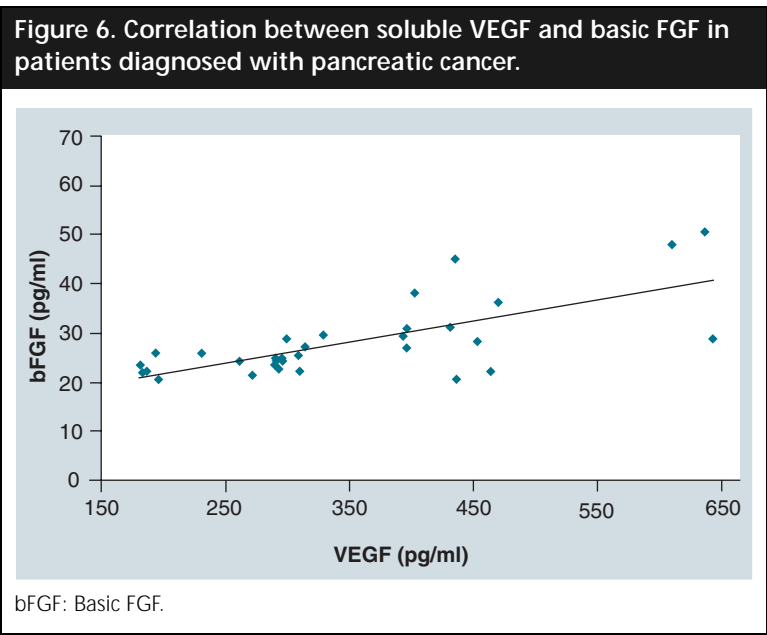
The high number of samples analyzed, the information gathered concomitantly and the shorter protocol time are obvious advantages in xMAP technology. Nevertheless, interferences may occur, leading to a decrease in the quantified parameters. Another explanation of the differences obtained compared with the ELISA technique is that the monoclonal antibodies used in the kits could have differing specificities [22]. The broader range of values for multiparameter detection (5–10,000 pg/ml) compared with ELISA (VEGF: 0–2000 pg/ml; bFGF: 0–64 pg/ml) can contribute equally to a different detection capacity in samples with high/low marker concentrations.

We have obtained a good correlation between the soluble angiogenic markers VEGF and bFGF ( $r = 0.7$ ;  $p < 0.001$ ; Pearson correlation) (Figure 6), along with a positive correlation with the classical serum marker CA 19–9 (for VEGF:  $r = 0.73$ ,  $p < 0.001$ ; for bFGF:  $r = 0.70$ ,  $p < 0.001$ ) (Table 1). It can be noted that both angiogenic markers follow pancreatic cancer stages from I–IV.

#### Discussion

Early diagnosis of pancreatic cancer increases the survival rate of patients, but current clinical pancreatic tumor markers lack the sensitivity and specificity required for early detection. There is still no specific tumor marker for pancreatic cancer; therefore, finding new serum tumor markers of pancreatic cancer has become an important topic in pancreatic surgery [23–26].

The aforementioned simultaneous increase of soluble VEGF and bFGF in pancreatic cancer could be explained by the closely related angiogenic factor pathways sharing common downstream signaling pathways. It seems that the EGFR pathway upregulates VEGF and bFGF, enhancing angiogenesis [10]. The



correlation of the soluble level with the disease stage obtained and, moreover, with the tumor diameter, is in concordance with results obtained in tissue samples where the disease stage is correlated with the expression of VEGF/bFGF [27–29].

The predictive power of the abovementioned angiogenic markers were reported by several groups and related to different cancers (Table 2). Thus, high serum VEGF levels predicted poor survival results, independent of clinicopathological features in patients with different types of cancer undergoing resection or receiving chemotherapy [14,18,30].

To our knowledge, this study reports the combined testing of soluble angiogenic markers in pancreatic cancer for the first time. Nevertheless, important reports have studied bFGF in combination with VEGF in skin melanomas [31], meningiomas [32], chronic lymphocytic leukemia [33] and nonsmall cell lung cancer [34]. Circulating levels of angiogenic cytokines were reported as having predictive power in neuroendocrine carcinoma of gastro–entero–pancreatic systems [35].

In pancreatic cancer, our results are consistent with other groups that have found significantly higher serum VEGF levels correlated with disease stage and metastases. These findings suggested that serum VEGF concentrations may reflect pancreatic cancer progression, and highlights it as an important marker [14,36]. Later, other groups [15,16] confirmed the high VEGF serum levels in pancreatic cancer patients and pointed it out as a marker to be looked for when antineoplastic agents are used. There is no information regarding soluble bFGF involvement in pancreatic cancer, therefore our study provides data showing a correlation with disease stage, proliferation markers and conventional serum markers. Moreover, we have established a relation between both angiogenic markers and clinicopathological parameters. Furthermore, we detected the angiogenic markers by two systems: the classical ELISA and a newer technology, xMAP. The results obtained displayed a similar pattern regarding controls versus patients.

Comparing ELISA with the xMAP technique, we confirmed that each system has its own merits with regards to screening pancreatic cancer

Table 2. Angiogenic biomarkers: correlation with clinical outcome.				
Material	VEGF	bFGF	Samples	Ref.
Chronic lymphocytic leukemia	↑	↑	Serum	[29]
Nonsmall cell lung cancer	↑	↑	Serum	[30]
Melanoma, stage III	↓	↑	Serum	[27]
Melanoma, stage IV	↑	↑	Serum	[27]
Ovarian cancer	↑	–	Serum	[26]
Endometrial cancer	–	↑	Serum	[15]
Hepatocellular carcinoma	↑	–	Serum	[14]
Colorectal cancer	–	↑	Serum	[16]
Pancreatic cancer	↑	–	Serum/plasma	[11]
Pancreatic cancer	↑	–	Serum	[10,12,25,32]
Pancreatic cancer	↑	↑	Serum	Present results
Pancreatic adenocarcinomas	↑	↑	Tissue	[23]
Pancreatic ductal carcinoma	↑	↑	Tissue	[24]

bFGF: Basic FGF.



patients, and that different systems display results that cannot be combined [37]. We emphasize that, as recently reported [38], although ELISA and xMAP results are different, the patterns of angiogenic markers generally agree.

Following the development of antiangiogenic drugs, angiogenic-factor testing is useful in order to quantify the response to therapy. Serum VEGF/bFGF levels were reported to be good indicators of antiangiogenic therapy in nonpancreatic cancers [33,34,39].

Our study focused on the relationship of soluble angiogenic factors, tumor stage and conventional markers. In this respect, soluble VEGFs and bFGFs could be included in the panel of pancreatic cancer biomarkers. To our knowledge, this is the first study correlating the two soluble angiogenic markers in pancreatic cancer. Moreover, we have focused on the high throughput nature of the testing, therefore comparatively using xMAP technology for soluble VEGFs and bFGFs.

### Conclusion

Biomarker discovery for pancreatic cancer is still ongoing, and multiple approaches have been developed for the identification of serum biomarkers. Although angiogenic-soluble factors do not possess the requisite specificity for pancreatic cancer, they can be applied within a panel of protein biomarkers.

VEGFs and bFGFs play important roles in tumor growth and progression. We postulate that blood levels of angiogenic factors may become a tool for the detection of disease recurrence, monitoring of antiangiogenic therapy and selection of patients subjected to VEGF/bFGF-targeted therapies.

In conclusion, this study has demonstrated high soluble VEGF and bFGF levels in pancreatic cancer patients when compared with healthy controls, which correlate with disease stage and tumor progression.

### Future perspective

Early detection of pancreatic adenocarcinoma, diagnosed in its preinvasive state, may greatly impact the treatment and prognosis of patients with fatal malignancy. VEGFs and bFGFs, which probably share common intracellular signaling pathways, and play important roles in tumor growth and progression through the exertion of both indirect and direct effects on tumor cells. Although these angiogenic factors may lack specificity for pancreatic cancer but, in addition to conventional tumor markers, they have the potential to enlarge the panel of protein biomarkers.

The inclusion of angiogenic markers in the pancreatic cancer biomarkers panel could assist in:

- Earlier diagnosis
- Higher prognosis, recurrence and patient survival rates
- Improved therapy follow-up and individual antiangiogenic therapy

### Financial & competing interests disclosure

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*No writing assistance was utilized in the production of this manuscript.*

### Ethical conduct of research

*For investigation involving human subjects, informed consent has been obtained from the participants involved. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.*

### Executive summary

- Our findings regarding soluble VEGFs/basic FGFs (bFGFs) in pancreatic cancer suggests that these markers may reflect disease progression, and their quantification could be clinically useful.
- VEGF/bFGF angiogenic markers have a positive correlation with proliferative markers, stage of disease and tumor diameter, and the fact that they relate to conventional markers, such as as carbohydrate 19-9 antigen and carcinoembryonic antigen, makes them important candidates in the pancreatic cancer biomarker panel.
- VEGFs and bFGFs may qualify as markers for prognosis and therapy control in patients with pancreatic cancer.

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