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ORIGINAL RESEARCH

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## A circulating T<sub>H</sub>2 cytokines profile predicts survival in patients with resectable pancreatic adenocarcinoma

Geny Piro<sup>a,b,c</sup>, Francesca Simionato <sup>c,d</sup>, Carmine Carbone<sup>a,c</sup>, Melissa Frizziero<sup>c,d</sup>, Giuseppe Malleo<sup>c,e</sup>, Silvia Zanini<sup>c,d</sup>, Raffaella Casolino<sup>c,d</sup>, Raffaella Santoro<sup>a,c</sup>, Maria Mihaela Mina<sup>b,c</sup>, Camilla Zecchetto<sup>c,d</sup>, Valeria Merz<sup>c,d</sup>, Aldo Scarpa<sup>c,f</sup>, Claudio Bassi<sup>c,e</sup>, Giampaolo Tortora<sup>b,c,d,†</sup>, and Davide Melisi <sup>a,c,d,†</sup>

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### ABSTRACT

Surgery is the only potentially curative option for patients with pancreatic ductal adenocarcinoma (PDAC), but metastatic relapse remains common. We hypothesized that the expression levels of inflammatory cytokines could predict recurrence of PDAC, thus allowing to select patients who most likely could benefit from surgical resection.

We prospectively collected plasma at diagnosis from 287 patients with pancreatic resectable neoplasms. The expression levels of 23 cytokines were measured in 90 patients with PDAC by using a multiplex analyte profiling assay. Levels higher than cutoff identified of the T<sub>H</sub>2 cytokines interleukin (IL)4, IL5, IL6 of macrophage inflammatory protein (MIP)1 $\alpha$ , granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein (MCP)1, and of IL17 $\alpha$ , IFN $\gamma$ -induced protein (IP)10, and IL1b were significantly associated with a shorter median OS. In particular, levels of IL4 and IP10 higher than cutoff identified, and level of T<sub>H</sub>1 cytokines TNF $\alpha$  and INF $\gamma$ , and of IL9 and IL1R $\alpha$  lower than cutoff identified were significantly associated with a shorter DFS. In the multivariate analysis, high IP10 was confirmed as negatively associated with OS (HR = 3.097,  $p$  = 0.014) and IL4 and TNF $\alpha$  remain negatively (HR = 2.75,  $p$  = 0.002) and positively (HR = 0.224,  $p$  = 0.049) associated with DFS, respectively. Simultaneous expression of low IL4 and high TNF $\alpha$  identified patients with best prognosis (HR = 0.313,  $p$  < 0.0001). In conclusion, we demonstrated that, among a series of cytokines, IL4 is the most significant independent prognostic factor for DFS in resectable PDAC patients, and it could be useful to select patients with high risk of early recurrence who may avoid an unnecessary resection.

### ARTICLE HISTORY

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### KEYWORDS

circulating cytokine profile; IL4; pancreatic adenocarcinoma; prognostic biomarker; T<sub>H</sub>2 cytokines

### Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a deadly disease, with the lowest 5-y relative survival rate among solid tumors at 7%,<sup>1</sup> and is projected to become the second leading cause of cancer-related death by 2030.<sup>2</sup> Surgery is the only potentially curative option for PDAC patients, but metastatic relapse remains common and no more than 20% of patients undergoing surgery and post-surgical therapy achieve long-term survival.<sup>3</sup> Thus, the identification of biologic markers able to predict metastatic recurrence of PDAC remains critical to select patients most likely to benefit from surgical resection.<sup>4</sup>


Tumor microenvironment contains both innate and adaptive immune cells that communicate with each other by means of cytokines and chemokines production to control tumor growth and spread.<sup>5</sup> In this “immune contexture,” the cytokine

expression profile may be more relevant than its specific immune cell content, and provide malignant cells with continuous supply of growth and survival signals.<sup>6-8</sup>

In PDAC, a dysfunctional immune system aids rather than controls cancer development and progression.<sup>9</sup> However, it is still unclear which cytokines or chemokines are critical for metastasis and prognosis of established tumors.<sup>10</sup> Previous studies examined the association between serum levels of several proinflammatory cytokines and overall survival (OS) in cohorts of patients with mostly advanced PDAC. In these studies, only a high level of IL6 was consistently demonstrated as an independent prognostic factor for poor OS.<sup>11-14</sup> However, comprehensive cytokine profiles have not been performed in early PDAC to date, therefore it is still unclear the potential value of cytokines or chemokines in predicting recurrence in this disease.

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 Supplemental data for this article can be accessed on the [publisher's website](#).

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Here, we investigated whether the preoperative expression levels of 23 cytokines in a large and prospective cohort of patients with resectable PDAC could be predictive of their Disease free survival (DFS) or OS, thus, serving as potential biomarker to select patients more likely to benefit from an upfront surgical resection.

## Results

### Association of patients' characteristics with OS and DFS

Two-hundred-eighty-seven patients admitted at the Unit of General and Pancreatic Surgery of the Azienda Ospedaliera Universitaria Integrata of Verona between 2012 and 2014 with suspected PDAC were assessed for eligibility. Among them, a total of 90 treatment-naïve resectable patients with histologically proven non-metastatic PDAC were included in the study (Fig. 1). Patients' characteristics are shown in Table 1. The mean age was 63 y and 51% were male. Most of them had tumors in the head of pancreas (79%), T3 stage (96%), and positive nodes (86%). Radical resection (R0) was obtained in 46% of cases. The majority of patients received adjuvant chemotherapy (82%), mostly with a gemcitabine-based regimen (97%). After a median follow-up of 26.9 mo, the median DFS was 19.9 mo and the median OS was not reached (data not show). Compliance with REMARK guidelines is reported in Table S1, available at *Clinical Cancer Research* online.

Univariate analysis of correlation between clinical features and OS or DFS is shown in Table 1. Among the clinical parameters analyzed, patients with poorly differentiated tumor (G3) had a significantly shorter OS (HR = 3.986,  $p = 0.001$ ) and DFS (HR = 2.109,  $p = 0.012$ ) compared with patients with well and moderately differentiated tumors (G1/G2). Conversely, patients treated with adjuvant therapy had a significantly longer DFS than did untreated patients (HR = 0.502,  $p = 0.038$ ). Association between DFS and other commonly used prognostic parameters, such as microscopically infiltrated resection margins (R1) and positive lymph nodes (N+), although displayed a negative trend, was not statistically significant in this cohort (HR = 1.573,  $p = 0.121$ , and HR = 1.435,  $p = 0.408$ , respectively).

**Table 1.** Characteristics of patients involved in the study.

Patients characteristics	N°	%	$p$ (OS)	HR	$p$ (DFS)	HR
Age (y)						
Median	63		0.319	1.572	0.772	1.088
Range	37–77					
Gender						
Female, $n$ (%)	44	49				
Male, $n$ (%)	46	51	0.784	0.892	0.1	0.626
Tumor stage						
T1, $n$ (%)	1	1	ND		ND	
T2, $n$ (%)	2	2				
T3, $n$ (%)	86	96				
T4, $n$ (%)	1	1				
Nodal stage						
N0, $n$ (%)	13	14				
N+, $n$ (%)	77	86	0.441	1.768	0.408	1.435
Metastasis stage						
M0, $n$ (%)	90	100				
M+, $n$ (%)	0	0	ND		ND	
Location						
Head, $n$ (%)	71	79				
Body/tail, $n$ (%)	19	21	0.490	0.720	0.346	0.732
Resection margins						
R0, $n$ (%)	41	46				
R1, $n$ (%)	49	54	0.394	1.441	0.121	1.573
Adjuvant therapy						
No, $n$ (%)	16	18				
Yes, $n$ (%)	74	82	0.157	0.510	0.038	0.502
Non-gemcitabine based, $n$ (%)	2	3				
Gemcitabine-based, $n$ (%)	72	97	ND		ND	
Radiotherapy						
No, $n$ (%)	65	72				
Yes, $n$ (%)	25	28	0.383	0.643	0.430	0.775
Tumor grade						
G1, $n$ (%)	7	8				
G2, $n$ (%)	58	64				
G3, $n$ (%)	25	28	0.001	3.986	0.012	2.109

HR, hazard ratio; R1, resection denotes a microscopically positive margin; T, Tumor; N, node; G, grade.

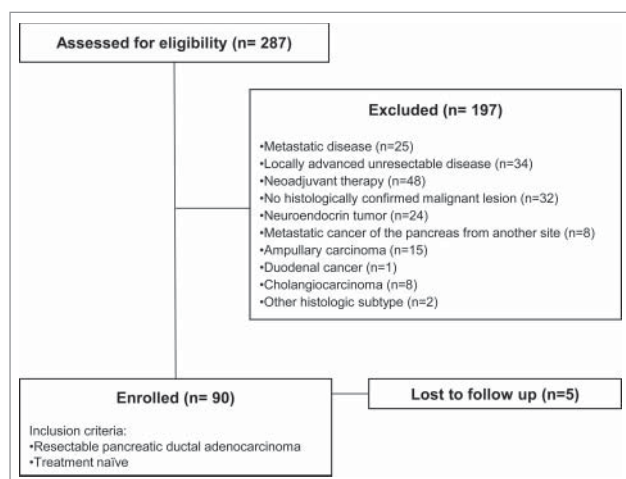
### Association of circulating cytokines and chemokines levels with OS and DFS

To determine whether patterns of circulating cytokines and chemokines could predict patients outcome, we measured the concentration of a panel of 23 different  $T_{H1}$ ,  $T_{H2}$ ,  $T_{H9}$ ,  $T_{H17}$  cytokines, chemokines, and growth factors in preoperative plasma samples from 90 treatment naïve patients with non-metastatic PDAC (Table 2). The optimal cutoff thresholds able to significantly predict patients' outcome were evaluated for each cytokine (Table 2 and Fig. 2A and B).

Concentration of the  $T_{H2}$  cytokines IL4, IL5, IL6 of the monocyte/macrophage infiltration cytokines MIP1a, GM-CSF, and MCP-1, and of IL17a, IP10, and IL1b at level higher than cutoff were significantly associated with a shorter patient's median OS.

Concentration of IL4 and IP10 higher than cutoff were significantly associated with shorter median DFS. ON the contrary, concentration of  $T_{H1}$  cytokines  $TNF\alpha$  and interferon (IFN) $\gamma$  and of IL9 and IL1Ra higher than cutoff were significantly associated with an increased median DFS. A summary of the overall findings of the study is reported in Fig. 3B.

An additional analysis demonstrated that patients whose DFS exceeded 8 mo had significantly less circulating IL4 level than did patients with DFS < 8 mo ( $p = 0.016$ ) (Fig. 2C). The optimal cutoff threshold of 9.365 pg/mL had a sensitivity of



**Figure 1.** Strobe diagram of the study.

**Table 2.** Pre-surgical circulating cytokines levels significantly correlated with OS and DFS.

Soluble factor	N°	mean pg/mL (Lower–upper 95%CI)	Median pg/mL (range)	Association with OS ( <i>p</i> )	cutoff (pg/mL)	Association with DFS ( <i>p</i> )	cutoff (pg/mL)
<b>T<sub>H</sub>2 cytokines</b>							
IL4	90	9.739 (8.12–11.36)	7.42 (1.28–45.48)	0.025	9.365	0.01	9.365
IL5	90	15.08 (11.64–18.52)	9.31 (0–74.02)	0.047	5.255	0.34	—
IL6	90	36.8 (26.92–46.67)	23.02 (2.96–319.2)	0.038	23.92	0.41	—
IL13	90	36.22 (29.2–43.25)	28.63 (0.5–223.4)	0.17	—	0.058	—
<b>T<sub>H</sub>1 cytokines</b>							
IFN $\gamma$	90	574.4 (440.1–708.6)	392.5 (10.8–3418)	0.56	—	0.004	129
IL12(p70)	90	45.05 (30.01–60.1)	27.63 (0–561.6)	0.07	—	0.24	—
TNF $\alpha$	90	94.17 (66.74–121.6)	69.27 (0–1069)	0.76	—	0.003	22.04
IL2	90	21.22 (8.47–33.97)	0 (0–426.2)	0.14	—	0.096	—
<b>T<sub>H</sub>9 cytokines</b>							
IL9	90	39.22 (23.51–54.92)	20.69 (0.3–610)	0.66	—	0.021	5.48
<b>T<sub>H</sub>17 cytokines</b>							
IL17 $\alpha$	90	175.6 (134.8–216.4)	109.6 (0–913)	0.03	360.4	0.59	—
<b>Chemokines</b>							
MIP1 $\alpha$	90	6.44 (5.39–7.49)	5.11 (0.8–34.68)	0.042	10.14	0.37	—
MCP1	90	129.3 (104.6–154)	107.9 (10.31–809.7)	0.032	109.3	0.15	—
MIP1b	90	107.3 (73.39–141.3)	82.13 (26.81–1577)	0.94	—	0.3	—
IP10	90	1734 (1289–2179)	1143 (376.2–17964)	0.003	2958	0.04	2958
IL8	90	79.2 (60.05–98.35)	47.62 (8.85–484.6)	0.14	—	0.054	—
eotaxin	90	175.1 (103.8–246.4)	102.7 (0–2912)	0.19	—	0.062	—
<b>Other cytokines and growth factors</b>							
G-CSF	90	243.5 (191.4–295.5)	155.9 (19.9–1076)	0.17	—	0.064	—
GM-CSF	90	70.95 (50.91–91)	49.48 (0–547.6)	0.035	134	0.38	—
VEGF	90	82.82 (63.04–102.6)	53.82 (0–471.9)	0.17	—	0.9	—
IL7	90	19.85 (15.88–23.83)	14.17 (0–121.2)	0.12	—	0.11	—
IL15	90	<OOR	<OOR	<OOR	—	<OOR	—
IL1 $\beta$	90	7.639 (5.526–9.751)	5.255 (0–59.64)	0.018	7.92	0.44	—
IL1R $\alpha$	90	715.1 (480–950.2)	356 (4.44–8552)	0.66	—	0.039	115.5

60% (95% CI = 38.7%–78.1%) and a specificity of 75.7% (95% CI = 64.5%–84.2%). In particular, an early relapse within 8 mo occurred in 12 out of 29 (41.4%) patients with a plasma concentration of IL4 higher than cutoff, and only in 8 out of 61 (13.1%) patients with IL4 lower than cutoff. The same association was not proven for the other cytokines (Fig. S1, available at *Clinical Cancer Research* online).

### Multivariate analysis of correlation between prognostic factors, including plasma cytokines, and OS and DFS

To confirm our findings and select the best prognostic cytokines, we performed a multivariate analysis including clinical features that had univariate significance ( $p < 0.05$ ), and the most significant prognostic cytokines at univariate analysis ( $p < 0.01$ ). In this analysis, high IP10 was confirmed as negatively associated with OS (HR = 3.097,  $p = 0.014$ ) and IL4 and TNF $\alpha$  remain negatively (HR = 2.753,  $p = 0.002$ ) and positively (HR = 0.224,  $p = 0.049$ ) associated with DFS, respectively (Table 3).

Since the multivariate analysis revealed both IL4 and TNF $\alpha$  as independent predictors of DFS, we tried to determine whether the two factors could interact to affect the prognosis of patients. Indeed, concurrent plasma concentrations of IL4 and TNF $\alpha$  lower and higher than their respective cutoffs, identified patients with best prognosis (HR = 0.313,  $p < 0.0001$ ) (Fig. 3A).

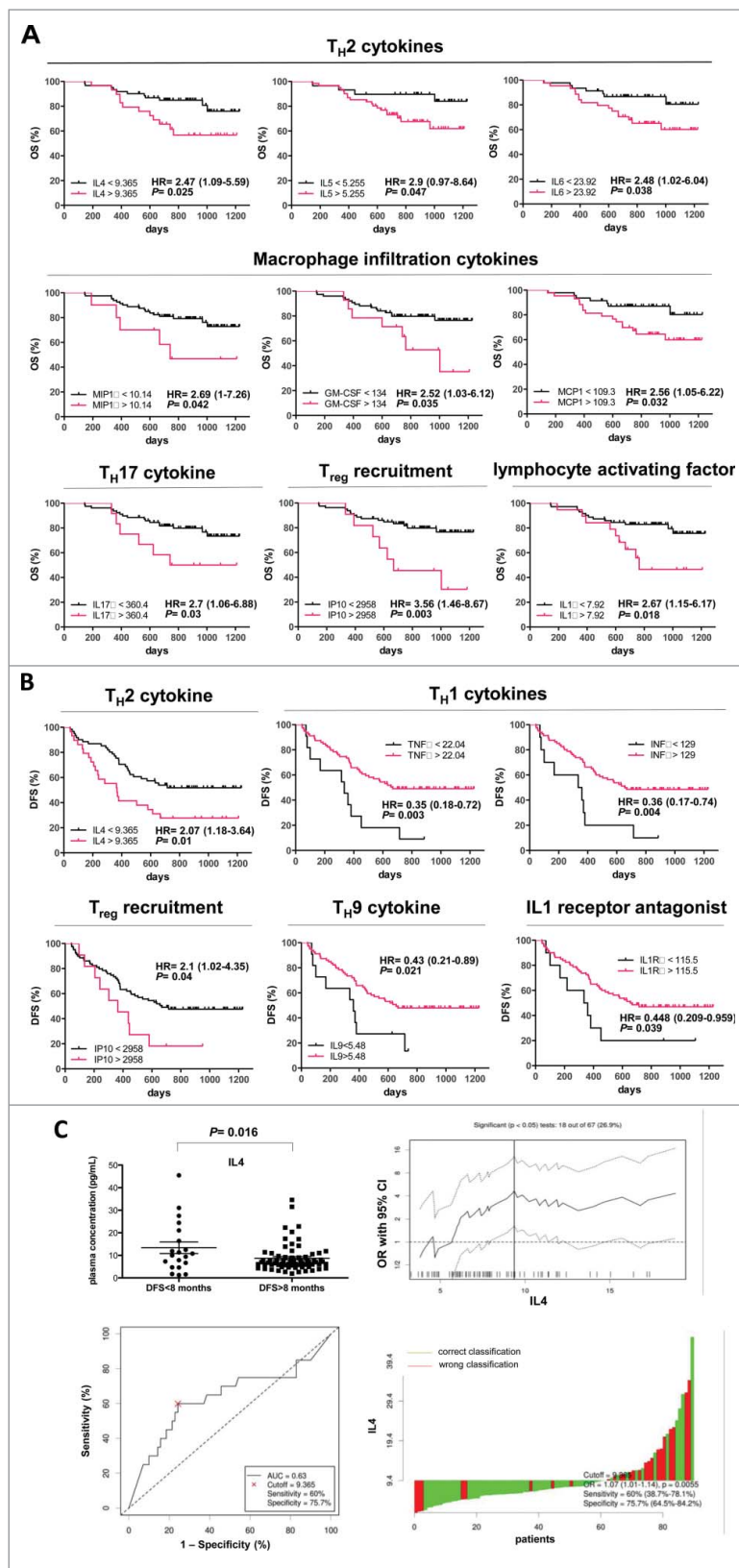
### Discussion

To our knowledge, this study represents the most comprehensive profiling of cytokines in the largest prospective cohort of resectable PDAC patients to date. We demonstrated that IL4 is

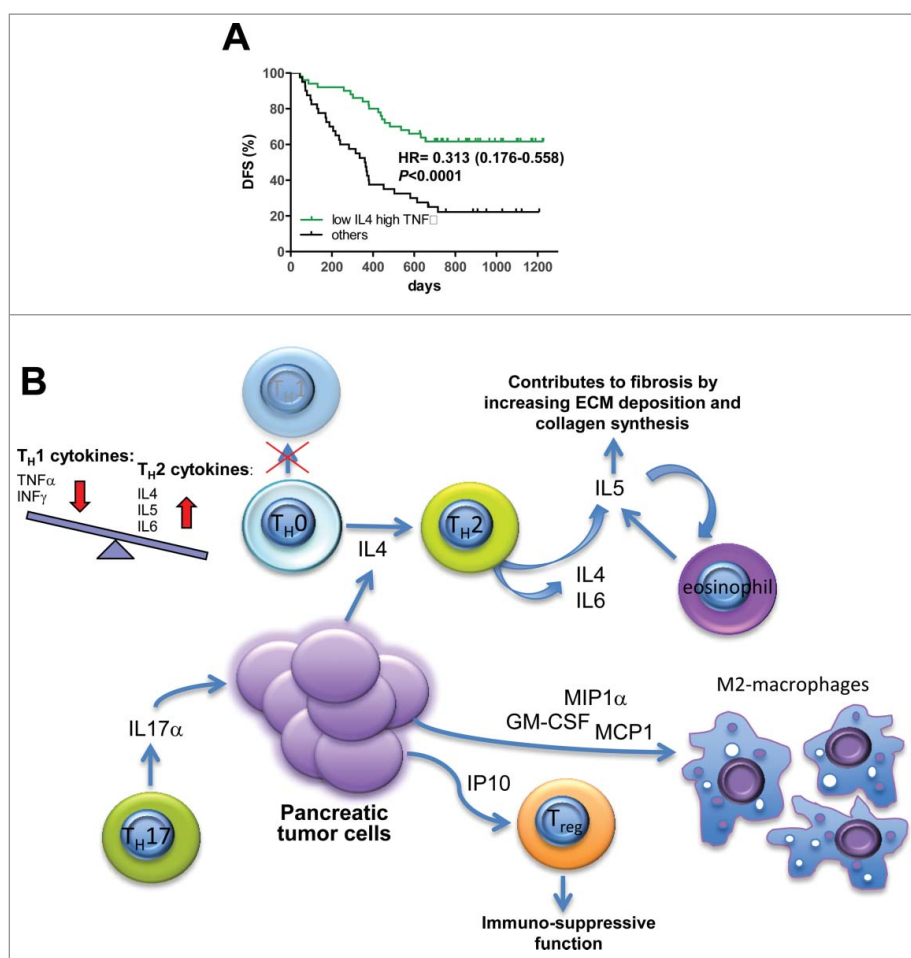
the most significant independent prognostic factor for DFS in resectable PDAC patients among a series of cytokines, representing a potential biomarker to stratify patients suited for surgery from patients with high risk of early recurrence who may avoid unnecessary resections.

T<sub>H</sub>2 immune response is defined by the cytokines IL4, IL5, IL9, and IL13, which induce in turn a complex inflammatory response characterized by T<sub>H</sub>2 subset of CD4<sup>+</sup> helper T cells, eosinophils, mast cells, basophils, and alternatively activated macrophages. In particular, IL4 is the signature cytokine of the T<sub>H</sub>2 effector cells, by acting as both an inducer and an effector cytokine of these cells.<sup>15</sup>

In most solid tumors, it is generally conceived that a T<sub>H</sub>2 inflammation promotes tumorigenesis and tumor growth. In particular, several studies provided evidence for a general T<sub>H</sub>2 shift in PDAC with a predominance of T<sub>H</sub>2 cytokines in the plasma of patients (reviewed in<sup>16</sup>). Important studies mainly by the group of Protti and colleagues<sup>17</sup> provided evidence on the mechanisms underlying these observations. They identified a cross talk between PDAC cells and microenvironment components, resulting in thymic stromal lymphopoietin production by activated cancer-associated fibroblasts that, in turn, induced a T<sub>H</sub>2 cell polarization through myeloid dendritic cell conditioning. The T<sub>H</sub>2 (GATA-3<sup>+</sup>)/T<sub>H</sub>1 (T-bet<sup>+</sup>) lymphoid cells ratio was independently predictive of DFS and OS in a population of resected PDAC patients. More recently, they demonstrated that basophils recruited in tumor-draining lymph nodes of PDAC patients regulate tumor promoting T<sub>H</sub>2 inflammation, being the early source of IL4 necessary for the full stabilization of the T<sub>H</sub>2 phenotype.<sup>18</sup> Our study contributes to this field by providing evidence, through an inductive approach, for



**Figure 2.** OS and DFS of patients with PDAC stratified according to cytokines levels. Kaplan–Meier curves for OS (A) and DFS (B) by significant cytokines cutoff concentration in plasma samples. Cytokines concentration expressed as pg/mL. (C) upper left, IL4 level in patients stratified around an early relapse cutoff of 8 mo; upper right, determination of cutoff thresholds of IL4 level for PDAC patients dichotomized according to early relapse of 8 mo. All possible cutoff thresholds were considered and the corresponding odds ratios (OR) were calculated and plotted. Each data point in the line gives the corresponding OR and 95% confidence interval (dotted lines) on the y axis. Lower left, receiver operator characteristic (ROC) curves for IL4 level in patients stratified around early relapse cutoff of 8 mo; lower right, waterfall plot, green and red bars represent cases with correct or wrong classification, respectively.



**Figure 3.** Combined cytokine signature predicts DFS. (A) patients were stratified for DFS on the basis of simultaneous expression of low IL4 and high TNF $\alpha$ . (B) tumor-immune network.

a T<sub>H</sub>2 shift in those PDAC patients for which we expect an early recurrence of disease. We examined a comprehensive immune circulating biomarkers panel demonstrating that high pre-surgical plasma levels of the T<sub>H</sub>2 cytokines IL4, IL5, IL6, and low plasma levels of T<sub>H</sub>1 cytokines TNF $\alpha$  and INF $\gamma$  were significantly associated with worst patients' outcome. More importantly, in the multivariate analysis, we confirmed IL4 as the strongest independent prognostic factor for DFS, a clinical end point directly correlated with tumor aggressiveness that

could be not corrupted by the effect of subsequent lines of therapy.

IL4 was identified as the original inducer of the polarization of the alternatively activated M2 macrophages,<sup>19</sup> which are generally conceived to suppress antitumor immunity and to favor growth and spreading in solid tumors.<sup>20</sup> However, recent studies correlating the infiltration of M2-polarized CD163<sup>+</sup> macrophages and prognosis in patients affected by resectable PDAC reached opposite conclusions.<sup>21,22</sup> In this regard, our study demonstrated that high plasma levels of the cytokines involved in macrophage recruitment MIP1 $\alpha$ , GM-CSF, and MCP1 were significantly associated with shorter patients' survival after surgery.

Beside T<sub>H</sub>2 inflammatory cells, a FOXP3<sup>+</sup> regulatory T cells (T<sub>reg</sub>) enriched pancreatic tumor infiltrate has been found to correlate with shorter patient survival.<sup>23,24</sup> This cell subtype can be recruited in the tumor microenvironment by the chemokine IP10 expressed by pancreatic stellate cells, leading to immunosuppressive and tumor-promoting effects.<sup>25,26</sup> Consistently with these observations, we demonstrated that high IP10 plasma level were negatively associated with patients' OS.

In conclusion, our present study prospectively demonstrated through an inductive approach that circulating markers of a T<sub>H</sub>2 immune response, and macrophages and T<sub>reg</sub> recruitment could be predictive of early metastatic relapse and poor prognosis in resectable PDAC patients. The simple measurement of

**Table 3.** Multivariate analysis of factors influencing OS and DFS in patients with resectable pancreatic cancer.

Variable	HR	95% CI		p
		Lower	Upper	
OS				
Tumor grade G3	3.698	1.602	8.535	0.002
IP10	3.097	1.257	7.632	0.014
DFS				
Tumor grade G3	2.472	1.339	4.564	0.004
Adjuvant therapy	0.609	0.312	1.186	0.145
IL4	2.753	1.465	5.175	0.002
TNF $\alpha$	0.224	0.051	0.995	0.049
INF $\gamma$	0.864	0.195	3.833	0.847

HR, hazard ratio; CI, confidential interval.

these cytokines by a non-invasive, blood-based assay, and the interpretation of their significance based on the cutoff thresholds here determined could represent a significant advantage over the assessment of the immune cells infiltration and differentiation in preoperative tumor biopsies, which often provide insufficient material to generate intratumor immune profiles and could not completely recapitulate the heterogeneity of these tumors. IL4 emerged among several other cytokines as the most significant independent prognostic factor for DFS in resectable PDAC patients. The expression of this T<sub>H</sub>2 cytokine could be useful to select patients with high risk of early recurrence who may avoid an unnecessary resection.

## Patients and methods

### Patients

Inclusion criteria for this study were histopathological confirmation of PDAC, no prior neo-adjuvant therapy, no evidence of metastatic disease, eligible for surgical resection. Peripheral blood samples were prospectively collected from all patients before surgical resection using EDTA-containing tubes. Plasma was isolated from each sample by centrifugation and stored at  $-20^{\circ}\text{C}$ . The variables evaluated included age, gender, tumor location, tumor size, differentiation status, lymph node involvement and TNM stage,<sup>27</sup> patterns of resection margins, patterns of recurrence. DFS was determined from the time of surgery until local or metastatic PDAC tumor recurrence. OS was defined as the time of surgery to death. Informed consent was obtained from all subjects. This study was performed in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association.

### Multiplex cytokines profiling

Using a 23-plex kit from Bio-Rad, all plasma specimens were analyzed for interleukin (IL)1 $\beta$ , IL2, IL4, IL5, IL6, IL7, IL8 (CXCL8), IL9, IL-12p70, IL13, IL15, IL17a, eotaxin (CCL11), IL1 $\alpha$ , granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN $\gamma$ , IP10 (CXCL10), monocyte chemoattractant protein (MCP1; CCL2), macrophage inflammatory protein 1 $\alpha$  (MIP1 $\alpha$ ; CCL3), MIP1 $\beta$  (CCL4), TNF $\alpha$ , and vascular endothelial growth factor (VEGF). All Luminex assays were performed according to the instructions provided by the manufacturer (Bio-Rad Laboratories). All Luminex assays were performed according to the instructions provided by the manufacturer (Bio-Rad Laboratories). Median fluorescence intensities were collected on a Luminex-200 instrument, using Bio-Plex Manager software version 6.2. Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kits.

Cytokine concentrations in samples were determined from the standard curve using a 5-point regression to transform mean fluorescence intensities into concentrations.

### Statistical analysis

Survival curves were drawn by Kaplan–Meier estimates and compared by log rank test. Univariate and multivariate analyses

of DFS and OS, with stepwise variable selection, were conducted by Cox's proportional hazard regression models. Multivariate analysis was conducted using the clinical-pathologic variables with a  $p$ -value  $< 0.05$  and the strongest significant molecular variables in univariate analysis ( $p$ -value  $< 0.01$ ). The optimal cutoff thresholds for soluble biomarkers were obtained based on the maximization of the Youden's statistics  $J = \text{sensitivity} + \text{specificity} - 1$ <sup>28</sup> using an  $R$ -based software as described in Budczies et al.<sup>29</sup> Statistical analyses were performed using SPSS 24.0 statistical software (SPSS, Inc.), GraphPad Prism software program (version 6.0; GraphPad Software), and the statistical language R.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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## References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; 65:5-29; PMID:25559415; <https://doi.org/10.3322/caac.21254>
2. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; 74:2913-21; PMID:24840647; <https://doi.org/10.1158/0008-5472.CAN-14-0155>
3. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, Padbury R, Moore MJ, Gallinger S, Mariette C et al. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. *Jama* 2010; 304:1073-81; PMID:20823433; <https://doi.org/10.1001/jama.2010.1275>
4. Melisi D, Calvetti L, Frizziero M, Tortora G. Pancreatic cancer: systemic combination therapies for a heterogeneous disease. *Curr Pharmaceutical Design* 2014; 20:6660-9; PMID:25341938; <https://doi.org/10.2174/1381612820666140826154327>
5. Giraldo NA, Becht E, Remark R, Damotte D, Sautes-Fridman C, Fridman WH. The immune contexture of primary and metastatic human tumours. *Curr Opin Immunol* 2014; 27:8-15; PMID:24487185; <https://doi.org/10.1016/j.coi.2014.01.001>
6. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; 140:883-99; PMID:20303878; <https://doi.org/10.1016/j.cell.2010.01.025>
7. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* 2013; 19:1423-37; PMID:24202395; <https://doi.org/10.1038/nm.3394>

8. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; 12:298-306; PMID:22419253; <https://doi.org/10.1038/nrc3245>
9. Sideras K, Braat H, Kwelkeboom J, van Eijck CH, Peppelenbosch MP, Sleijfer S, Bruno M. Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies. *Cancer Treat Rev* 2014; 40:513-22; PMID:24315741; <https://doi.org/10.1016/j.ctrv.2013.11.005>
10. Roshani R, McCarthy F, Hagemann T. Inflammatory cytokines in human pancreatic cancer. *Cancer Lett* 2014; 345:157-63; PMID:23879960; <https://doi.org/10.1016/j.canlet.2013.07.014>
11. Ebrahimi B, Tucker SL, Li D, Abbruzzese JL, Kurzrock R. Cytokines in pancreatic carcinoma: correlation with phenotypic characteristics and prognosis. *Cancer* 2004; 101:2727-36; PMID:15526319; <https://doi.org/10.1002/cncr.20672>
12. Mitsunaga S, Ikeda M, Shimizu S, Ohno I, Furuse J, Inagaki M, Higashi S, Kato H, Terao K, Ochiai A. Serum levels of IL-6 and IL-1beta can predict the efficacy of gemcitabine in patients with advanced pancreatic cancer. *Br J Cancer* 2013; 108:2063-9; PMID:23591198; <https://doi.org/10.1038/bjc.2013.174>
13. Bellone G, Smirne C, Mauri FA, Tonel E, Carbone A, Buffolino A, Dughera L, Robecchi A, Pirisi M, Emanuelli G. Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. *Cancer Immunol Immunother* 2006; 55:684-98; PMID:16094523; <https://doi.org/10.1007/s00262-005-0047-0>
14. Farren MR, Mace TA, Geyer S, Mikhail S, Wu C, Ciombor K, Tahiri S, Ahn D, Noonan AM, Villalona-Calero M et al. Systemic immune activity predicts overall survival in treatment-naive patients with metastatic pancreatic cancer. *Clin Cancer Res* 2016; 22:2565-74; PMID:26719427; <https://doi.org/10.1158/1078-0432.CCR-15-1732>
15. Wynn TA. Type 2 cytokines: mechanisms and therapeutic strategies. *Nat Rev Immunol* 2015; 15:271-82; PMID:25882242; <https://doi.org/10.1038/nri3831>
16. Wormann SM, Diakopoulos KN, Lesina M, Algul H. The immune network in pancreatic cancer development and progression. *Oncogene* 2014; 33:2956-67; PMID:23851493; <https://doi.org/10.1038/onc.2013.257>
17. De Monte L, Reni M, Tassi E, Clavenna D, Papa I, Recalde H, Braga M, Di Carlo V, Doglioni C, Protti MP et al. Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. *J Exp Med* 2011; 208:469-78; PMID:21339327; <https://doi.org/10.1084/jem.20101876>
18. De Monte L, Wormann S, Brunetto E, Heltai S, Magliacane G, Reni M, Paganoni AM, Recalde H, Mondino A, Falconi M et al. Basophil recruitment into tumor-draining lymph nodes correlates with Th2 inflammation and reduced survival in pancreatic cancer patients. *Cancer Res* 2016; 76:1792-803; PMID:26873846; <https://doi.org/10.1158/0008-5472.CAN-15-1801-T>
19. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1992; 176:287-92; PMID:1613462; <https://doi.org/10.1084/jem.176.1.287>
20. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* 2010; 11:889-96; PMID:20856220; <https://doi.org/10.1038/ni.1937>
21. Tjomsland V, Niklasson L, Sandstrom P, Borch K, Druid H, Bratthall C, Messmer D, Larsson M, Spångeus A. The desmoplastic stroma plays an essential role in the accumulation and modulation of infiltrated immune cells in pancreatic adenocarcinoma. *Clin Dev Immunol* 2011; 2011:212810; PMID:2190968; <https://doi.org/10.1155/2011/212810>
22. Kurahara H, Shinchi H, Mataka Y, Maemura K, Noma H, Kubo F, Sakoda M, Ueno S, Natsugoe S, Takao S. Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. *J Surgical Res* 2011; 167:e211-9; PMID:19765725; <https://doi.org/10.1016/j.jss.2009.05.026>
23. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 2006; 12:5423-34; PMID:17000676; <https://doi.org/10.1158/1078-0432.CCR-06-0369>
24. Yamamoto T, Yanagimoto H, Satoi S, Toyokawa H, Hirooka S, Yamaki S, Yui R, Yamao J, Kim S, Kwon AH. Circulating CD4+CD25+ regulatory T cells in patients with pancreatic cancer. *Pancreas* 2012; 41:409-15; PMID:22158072; <https://doi.org/10.1097/MPA.0b013e3182373a66>
25. Lunardi S, Jamieson NB, Lim SY, Griffiths KL, Carvalho-Gaspar M, Al-Assar O, Yameen S, Carter RC, McKay CJ, Spoletini G, et al. IP-10/CXCL10 induction in human pancreatic cancer stroma influences lymphocytes recruitment and correlates with poor survival. *Oncotarget* 2014; 5:11064-80; PMID:25415223; <https://doi.org/10.18632/oncotarget.2519>
26. Lunardi S, Lim SY, Muschel RJ, Brunner TB. IP-10/CXCL10 attracts regulatory T cells: implication for pancreatic cancer. *Oncoimmunology* 2015; 4:e1027473; PMID:26405599; <https://doi.org/10.1080/2162402X.2015.1027473>
27. Sobin LH, Compton CC. TNM seventh edition: what's new, what's changed: communication from the International Union against cancer and the american joint committee on cancer. *Cancer* 2010; 116:5336-9; PMID:20665503; <https://doi.org/10.1002/cncr.25537>
28. Youden WJ. Index for rating diagnostic tests. *Cancer* 1950; 3:32-5; PMID:15405679; [https://doi.org/10.1002/1097-0142\(1950\)3:1<32::AID-CNCR2820030106>3.0.CO;2-3](https://doi.org/10.1002/1097-0142(1950)3:1<32::AID-CNCR2820030106>3.0.CO;2-3)
29. Budczies J, Klauschen F, Sinn BV, Gyorffy B, Schmitt WD, Darb-Esfahani S, Denkert C. Cutoff finder: a comprehensive and straightforward Web application enabling rapid biomarker cutoff optimization. *PloS One* 2012; 7:e51862; PMID:23251644; <https://doi.org/10.1371/journal.pone.0051862>