Annals of Oncology Advance Access published April 9, 2015

original article

Annals of Oncology 00: 1–7, 2015
doi:10.1093/annonc/mdv133

A randomized, placebo-controlled phase III trial of masitinib plus gemcitabine in the treatment of advanced pancreatic cancer

G. Deplanque[1*], M. Demarchi[2], M. Hebbar[3], P. Flynn[4], B. Melichar[5], J. Atkins[6], E. Nowara[7], L. Moyé[8], D. Piquemal[9], D. Ritter[9], P. Dubreuil[10,11,12,13,14], C. D. Mansfield[14], Y. Acin[14], A. Moussy[14], O. Hermine[14,15,16,17,18,19,20] & P. Hammel[21]

[1]Department of Medical Oncology, Saint Joseph Hospital, Paris; [2]Department of Medical Oncology, University Hospital of Besançon, Besançon; [3]Department of Medical Oncology, University Hospital, Lille, France; [4]Metro-Minnesota Community Clinical Oncology Program, Park Nicollet Institute, Minneapolis, USA; [5]Department of Oncology, Palacký University Medical School & Teaching Hospital, Olomouc, Czech Republic; [6]Southeastern Medical Oncology Center, Goldsboro, USA; [7]Department of Clinical and Experimental Oncology, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland; [8]Department of Biostatistics, University of Texas School of Public Health, Houston, USA; [9]Clinical Development, Acobiom, Montpellier; [10]Signaling, Hematopoiesis and Mechanism of Oncogenesis, Inserm U1068, CRCM, Marseille; [11]Institut Paoli-Calmettes, Marseille; [12]Aix-Marseille University, UM 105, Marseille; [13]CNRS, UMR7258, CRCM, Marseille; [14]Clinical Development, AB Science, Paris; [15]Department of Clinical Hematology, Necker Hospital, Paris; [16]INSERM UMR 1163, Laboratory of Cellular and Molecular Mechanisms of Hematological Disorders and Therapeutic Implications, Paris; [17]Paris Descartes – Sorbonne Paris Cité University, Imagine Institute, Paris; [18]CNRS ERL 8254, Paris; [19]Laboratory of Excellence GR-Ex, Paris; [20]National Reference Center on Mastocytosis (CEREMAST), Paris; [21]Department of Gastroenterology, Hôpital Beaujon, Clichy, France

Received 2 December 2014; revised 5 February 2015; accepted 18 February 2015

Background: Masitinib is a selective oral tyrosine–kinase inhibitor. The efficacy and safety of masitinib combined with gemcitabine was compared against single-agent gemcitabine in patients with advanced pancreatic ductal adenocarcinoma (PDAC).

Patients and methods: Patients with inoperable, chemotherapy-naïve, PDAC were randomized (1 : 1) to receive gemcitabine (1000 mg/m^2) in combination with either masitinib (9 mg/kg/day) or a placebo. The primary endpoint was overall survival (OS) in the modified intent-to-treat population. Secondary OS analyses aimed to characterize subgroups with poor survival while receiving single-agent gemcitabine with subsequent evaluation of masitinib therapeutic benefit. These prospectively declared subgroups were based on pharmacogenomic data or a baseline characteristic.

Results: Three hundred and fifty-three patients were randomly assigned to receive either masitinib plus gemcitabine ($N = 175$) or placebo plus gemcitabine ($N = 178$). Median OS was similar between treatment-arms for the overall population, at respectively, 7.7 and 7.1 months, with a hazard ratio (HR) of 0.89 (95% CI [0.70; 1.13]. Secondary analyses identified two subgroups having a significantly poor survival rate when receiving single-agent gemcitabine; one defined by an overexpression of acyl–CoA oxidase-1 (*ACOX1*) in blood, and another via a baseline pain intensity threshold (VAS > 20 mm). These subgroups represent a critical unmet medical need as evidenced from median OS of 5.5 months in patients receiving single-agent gemcitabine, and comprise an estimated 63% of patients. A significant treatment effect was observed in these subgroups for masitinib with median OS of 11.7 months in the '*ACOX1*' subgroup [HR = 0.23 (0.10; 0.51), $P = 0.001$], and 8.0 months in the 'pain' subgroup [HR = 0.62 (0.43; 0.89), $P = 0.012$]. Despite an increased toxicity of the combination as compared with single-agent gemcitabine, side-effects remained manageable.

Conclusions: The present data warrant initiation of a confirmatory study that may support the use of masitinib plus gemcitabine for treatment of PDAC patients with overexpression of *ACOX1* or baseline pain (VAS > 20mm). Masitinib's effect in these subgroups is also supported by biological plausibility and evidence of internal clinical validation.

Trial Registration: ClinicalTrials.gov:NCT00789633.

Key words: pancreatic cancer, PDAC, tyrosine–kinase inhibitor, pain, genetic biomarker, *ACOX1*

introduction

Pancreatic cancer continues to be a disease with high unmet medical need, requiring new active agents. For over a decade, single-agent gemcitabine has been the standard first-line treatment for unresectable, locally advanced or metastatic pancreatic ductal adenocarcinoma (PDAC). Median overall survival (OS) is between 6 and 7 months and 1-year survival rates range between 17% and 25% [1, 2]. Numerous gemcitabine-based combination regimens evaluated in randomized trials have either failed to demonstrate significant improvement in OS or

*Correspondence to: Dr Gaël Deplanque, Service d'Oncologie, Groupe Hospitalier Paris Saint Joseph, 185 rue Raymond Losserand, Paris 75674, France. Tel: +33-(0)1-44-12-76-43. Fax: +33-(0)144-12-32-59. E-mail: gdeplanque@hpsj.fr

have shown statistically significant but rather modest survival benefits compared with gemcitabine alone; e.g. nab-paclitaxel plus gemcitabine recently reported a significant median OS gain of +1.8 months when compared with single-agent gemcitabine [1–3].

The potential therapeutic benefit of masitinib in combination with gemcitabine for the treatment of advanced PDAC has been previously reported in preclinical studies, wherein masitinib was shown to enhance the antiproliferative activity of gemcitabine in gemcitabine–refractory pancreatic cancer cell lines, and also in a clinical phase II trial [4, 5]. Exploratory analysis from the clinical study revealed two distinct patient subgroups with respect to masitinib treatment susceptibility, as evidenced by a plateau in the OS Kaplan–Meier curve between 9 and 17 months (see section A of the Supplementary Material, available at *Annals of Oncology* online). This observation could not be explained by patient–disease status leading to a hypothesis that there may be at least one subgroup of PDAC patients with particularly poor survival and susceptibility to masitinib plus gemcitabine treatment, the said subgroup being identifiable via a gene expression profile and/or another biological or clinical marker. Hence, future trials of masitinib in this indication would need to perform prospectively declared secondary subgroup analyses.

This observation is consistent with evidence that heterogeneity in tumor biology and microenvironment may be an important determinant of survival difference amongst groups of PDAC patients (i.e. aggressive versus relatively slow disease progression, as seen in routine clinical practice), which in turn leads to variability in terms of treatment susceptibility and potential failure of targeted drugs in the overall population [1, 6, 7]. It has been reported that such heterogeneity in PDAC patients may be associated with increased mast cell infiltration into the tumor or tumor microenvironment, both of which are prognostic factors for poor survival in PDAC [8, 9]. Masitinib is a potent oral tyrosine–kinase inhibitor (TKI) that targets a limited number of receptor tyrosine kinases including c-Kit, Lyn and Fyn, making it a highly selective inhibitor of mast cell function and activity [10].

methods

study design

The present study was a prospective, multicenter, randomized, double-blind, two-parallel group, placebo-controlled phase III trial evaluating the safety and efficacy of masitinib plus gemcitabine against placebo plus gemcitabine in chemotherapy-naïve PDAC patients. Masitinib (9 mg/kg/day) was administered orally in two daily doses, while gemcitabine (1000 mg/m^2) was administered according to standard clinical practice. The composition and dispensing of masitinib and placebo capsules were identical except for the amount of the active ingredient contained. Treatments were administered until progression, intolerance, or patient withdrawal, with disease progression assessed via CT scan according to RECIST criteria every 8 weeks. In the event of a treatment-related grade 3 or 4 adverse event (AE), treatment interruption or blinded dose reduction was permitted according to predefined criteria. The investigation was carried out in accordance with the Declaration of Helsinki and approved by the national health authorities and local ethics committees.

patients and randomization

Eligible patients were chemotherapy-naïve with histologically or cytologically confirmed inoperable advanced or metastatic PDAC. Other eligibility criteria included: age 18 years or older; Eastern Cooperative Oncology Group (ECOG) performance status ≤1; a life-expectancy of >12 weeks; bilirubin <3×ULN, adequate renal, cardiac, and hepatic functions. At baseline, patients were centrally randomized to treatments groups (1:1) using an Interactive Voice Response System (IVRS), with treatment allocated according to a modified minimization method. Stratification was done according to geographic region and disease status (locally advanced versus metastatic). The investigators, patients, data analysts, and the trial sponsor were blinded to the randomization sequence and treatment assignment.

statistical analysis

Safety was assessed throughout the study in all patients who received at least one dose of masitinib or placebo using the Common Terminology Criteria for Adverse Events version 3 (CTCAE v3) for classification of AE. Quality of life (QoL) was assessed using the EORTC QLQ-C30 questionnaire.

The primary endpoint was OS in the modified intent-to-treat (mITT) population, i.e. all randomized patients, excluding those withdrawn prematurely from the study for a well-documented non-treatment related cause, with OS measured from the date of randomization to the date of death. It was estimated that at least 320 patients were required to detect a difference in median OS between treatment-arms with a power of 80% using a two-sided log-rank test and significance level of 0.05 (assuming 264 events after 12 months follow-up). Comparative analyses were based on an alpha of 5% (two-sided), with results presented according to a two-sided 95% confidence interval (CI), unless otherwise stated.

Consistent with study rationale, secondary analyses on OS were pre-specified in the protocol with the objectives of: (a) characterizing a subgroup based upon pharmacogenomic data with poorer survival while under gemcitabine standard-of-care, (b) evaluating the therapeutic benefit of added masitinib in this genetic subgroup, (c) characterizing a subgroup based upon a baseline variable that negatively impacts survival while under gemcitabine standard-of-care, and (d) evaluating the therapeutic benefit of added masitinib in this baseline variable subgroup. Sample size for the prospectively declared subgroup analyses was predefined prior to unblinding. For the subgroup based on a baseline variable predictive of poor survival, it was estimated that 220 patients would be needed for 80% power to detect a hazard ratio (HR) of 0.66 (masitinib plus gemcitabine versus placebo plus gemcitabine) using a two-sided log-rank test with a significance level of 0.05. Overall survival was investigated in patients from the placebo plus gemcitabine treatment-arm according to each baseline variable (a total of 16 baseline characteristics were tested) through a univariate analysis, thereby, identifying characteristics that impact OS independently of treatment (see section B of the Supplementary Material, available at *Annals of Oncology* online). Multivariate analysis of OS was performed using a Cox proportional-hazard model to evaluate the treatment effect with adjustment for the stratification factors. For the subgroup based on pharmacogenomic data, it was estimated that 100 patients per treatment-arm would be required for 80% power to detect a HR of 0.50 (masitinib plus gemcitabine versus placebo plus gemcitabine) using a two-sided log-rank test with a significance level of 0.05.

pharmacogenomic analysis

Prospectively declared secondary subgroup analyses included pharmacogenomic examination of the RNA expression in peripheral blood samples collected using the PAXgene Blood RNA System prior to treatment. Genome-wide analysis of RNA expression using a high-throughput method of next-generation sequencing was performed by Acobiom, Montpellier, France. The methodology used for identification of the genetic biomarker subgroup is described in section C of the Supplementary Material, available at *Annals of Oncology* online.

results

A total of 353 patients from 73 active centers (predominantly located in France, United States and the Czech Republic) were randomly assigned to receive masitinib plus gemcitabine or placebo plus gemcitabine. The safety population comprised all randomized patients who received at least one dose of either masitinib or placebo ($N = 349$). A CONSORT flow diagram for the study population as well as subgroups of interest and description of patient baseline characteristics are provided in sections D and E of the Supplementary Material, available at *Annals of Oncology* online. Baseline characteristics were generally well-balanced. The average number of post-study treatments was similar between treatment-arms at 1.1 ± 1.3 for the masitinib plus gemcitabine treatment-arm, and 1.0 ± 1.0 for the placebo plus gemcitabine arm, with the majority of patients receiving either single-agent gemcitabine (25% and 11%, respectively) or no additional treatment-line (27% and 31%) upon study discontinuation. Median exposure to masitinib or placebo in the safety population was 1.6 and 3.7 months, respectively, while median exposure to gemcitabine in the masitinib or placebo treatment-arms was 1.4 and 3.3 months, respectively; $P = 0.001$. At the data cut-off date, corresponding to a median follow-up of 26 months, one patient was ongoing treatment in the masitinib plus gemcitabine treatment-arm.

A summary of safety data is presented in Table 1. Overall toxicity increased for masitinib combined with gemcitabine when compared with single-agent gemcitabine. A higher frequency of serious and severe (grade 3 and 4) AEs, discontinuations, temporary interruptions and dose reductions was reported in the masitinib plus gemcitabine treatment-arm, although the occurrence of AE related deaths was lower in this treatment-arm than in the placebo plus gemcitabine arm. Hematological AEs contributed strongly to the discrepancy between treatment-arms, with the higher frequency reported for masitinib-treated patients due predominantly to an increase in neutropenia. No deaths were reported due to neutropenia in the masitinib plus gemcitabine treatment-arm, moreover, the occurrence of febrile neutropenia was similar between treatment-arms (1.7% for masitinib plus gemcitabine versus 0.6% for placebo plus gemcitabine), as were infections (30.6% versus 37.5%, respectively). Non-hematological AEs were typical of previously reported toxicity for masitinib, including vomiting, nausea and rash, but these generally resolved without sequelae and were not associated with any deaths.

Patient QoL at baseline was similar between the treatment-arms (mean global health score of 53.5 ± 22.4 versus 53.9 ± 21.1 for the masitinib plus gemcitabine and placebo plus gemcitabine treatment-arms, respectively), as well as at the last patient visit (46.3 ± 23.7 versus 49.7 ± 21.7, respectively). The combination of masitinib plus gemcitabine did not, therefore, accelerate the decline in QoL with respect to single-agent gemcitabine.

The median OS for the overall population, the primary efficacy analysis, was similar for both treatment-arms; 7.7 months [95% CI (6.1; 10.6)] for masitinib plus gemcitabine and 7.0 months [95% CI (6.1; 10.6)] for placebo plus gemcitabine (all results reported hereafter relate to the multivariate analysis unless otherwise stated) (Table 2). The corresponding HR was 0.89 [95% CI (0.70; 1.13)]. Secondary analyses on surrogate survival endpoints of the overall population, e.g. progression-free survival or time-to-progression, were also similar between treatment-arms (data not shown).

Secondary analyses on OS did, however, show two subgroups of patients having particularly poor survival with single-agent gemcitabine, which was consistent with the study's hypothesis and prospectively declared subgroup analysis. These subgroups comprised patients with a genetic biomarker (overexpression of *ACOX1* in blood), and patients with baseline pain intensity above a threshold of 20 mm as measured on a 100 mm visual

Table 1. Safety according to the number of patients with at least one reported adverse reaction (safety population)

Number of patients (%)	M + G (n = 173)	P + G (n = 176)	P-value[a]
Summary of AE			
All grades	173 (100%)	173 (98%)	0.248
Severe non-hematological[b]	132 (76%)	124 (71%)	0.010
Severe hematological[b]	109 (63%)	73 (42%)	<0.001
Non-fatal serious	107 (68%)	94 (53%)	0.111
Deaths[c]	14 (8%)	19 (11%)	0.388
AE leading to:			
Study discontinuation[d]	73 (42%)	48 (27%)	0.003
Temporary interruption[d]	129 (75%)	90 (51%)	<0.001
Dose reduction[d]	28 (16%)	16 (9%)	0.046
AEs of interest[e]			
Back pain	10 (6%)	27 (15%)	0.004[f]
Constipation	38 (22%)	62 (35%)	0.006[f]
Pulmonary embolism	4 (2%)	12 (7%)	0.044[f]
Vomiting	87 (50%)	57 (37%)	<0.001
Nausea	100 (58%)	82 (47%)	0.036
Rash	60 (35%)	22 (13%)	<0.001
Thrombocytopenia	83 (48%)	48 (27%)	<0.001
Thrombosis	8 (5%)	0 (0%)	0.003
Hypokalemia	34 (20%)	16 (9%)	0.005
Pyrexia	70 (41%)	48 (27%)	0.009
Neutropenia	87 (50%)	65 (37%)	0.012
Anemia	105 (61%)	84 (48%)	0.015

Adverse Events (AE) classified according to the Common Terminology Criteria for Adverse Events version 3.

[a]The Fisher exact test or Chi-squared test was used for comparison of qualitative variables; analysis of variance was used for comparison of quantitative variables.

[b]Severe adverse events correspond to CTCAE v3 grade 3 and 4 adverse events.

[c]Toxicity related deaths under study treatment.

[d]Adverse events leading to discontinuation (except death), interruption or dose reduction of study drug (masitinib or placebo).

[e]Adverse events reported with a significantly higher frequency in one treatment-arm.

[f]Adverse event reported at a statistically significant higher frequency in placebo plus gemcitabine-treated patients than in the masitinib plus gemcitabine-treated patients.

AE, adverse event; GEM, gemcitabine; P + G, placebo plus gemcitabine; M + G, masitinib plus gemcitabine.

Table 2. Summary of treatment effect according to overall survival for masitinib plus gemcitabine versus placebo plus gemcitabine in the mITT population (primary analysis) and also in two subgroups with a demonstrated poor survival while under standard-of-care, comprised patients with a genetic biomarker ('ACOX1 subgroup') and patients with baseline pain intensity of VAS > 20 ('pain subgroup')

	N	Median OS [95% CI] (months)	[a]Median OS Gain (months)	HR [95% CI]	*P*-value
Overall (mITT)	348				
P + G	175	7.0 [6.1;10.6]	+0.7	0.89 [0.70;1.13]	0.695
M + G	173	7.7 [6.1;10.6]			
'ACOX1' subgroup	40				
P + G	20	5.6 [3.7;12.9]	+6.1	0.23 [0.1;0.51]	0.001
M + G	20	11.7 [8.3;19.9]			
'Pain' subgroup	137				
P + G	73	5.4 [4.5;8.0]	+2.6	0.62 [0.43;0.89]	0.012
M + G	64	8.0 [5.8;11.5]			

Median follow-up of 26 months; multivariate model.
[a]Difference in median OS between treatment-arms (M + G minus P + G).
OS, overall survival; HR, hazard ratio of death; P + G, placebo plus gemcitabine; M + G, masitinib plus gemcitabine; mITT, modified intent-to-treat population.

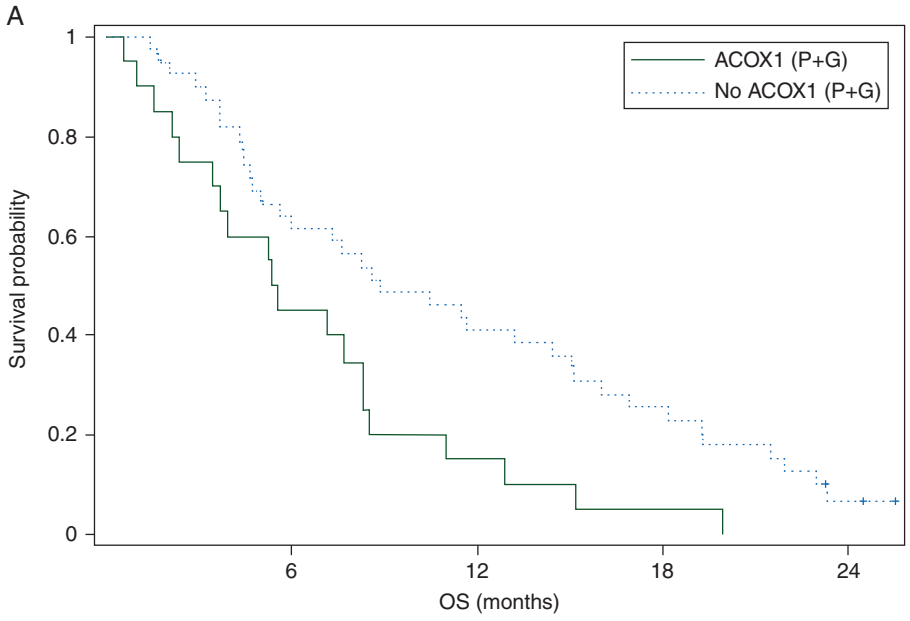
analog scale (VAS). In both cases, the placebo plus gemcitabine patient cohorts divided into two distinct subgroups with survival reflecting aggressive or relatively slow disease progression (Figure 1), thus characterizing the defining variables of the prospectively declared secondary subgroup analysis (for further subgroup description see sections C and F of the Supplementary Material, available at *Annals of Oncology* online). Subsequent evaluation of the interaction between these variables and the combination of masitinib plus gemcitabine revealed a significant treatment benefit in both subgroups with respect to the placebo plus gemcitabine treatment-arm (Table 2).

Considering the patient cohort with pharmacogenomic data, 119 patients enrolled for the study had peripheral blood samples collected at baseline and were randomly assigned to the masitinib plus gemcitabine or placebo plus gemcitabine treatment-arms ($n = 60$ and $n = 59$ patients, respectively). The *ACOX1* subgroup was determined following a pre-specified methodology as patients with overexpression of *ACOX1* in blood defined as a delta cycle threshold (DCt) value of ≤ 3.05 (see section C of the Supplementary Material, available at *Annals of Oncology* online). In the overall pharmacogenomic population, a total of 40/119 patients (34%) were identified as being in the *ACOX1* subgroup while 79/119 patients (66%) were assigned to its complement subgroup (i.e. absence of *ACOX1* overexpression or non-*ACOX1*). In the *ACOX1* subgroup, median exposure to masitinib or placebo was 1.8 and 2.4 months, respectively, while median exposure to gemcitabine in the masitinib or placebo treatment-arms was 2.1 and 1.9 months, respectively; $P = 0.78$. In the placebo plus gemcitabine treatment-arm, patients without *ACOX1* overexpression ($n = 39$) had a significantly longer median OS compared with patients having *ACOX1* overexpression ($n = 20$); 8.8 months [95% CI (5.6; 15.0)] versus 5.5 months [95% CI (3.4; 8.3)] (univariate model). The corresponding HR was 0.46 [95%CI (0.26; 0.82)], $P = 0.007$ (Figure 1A).

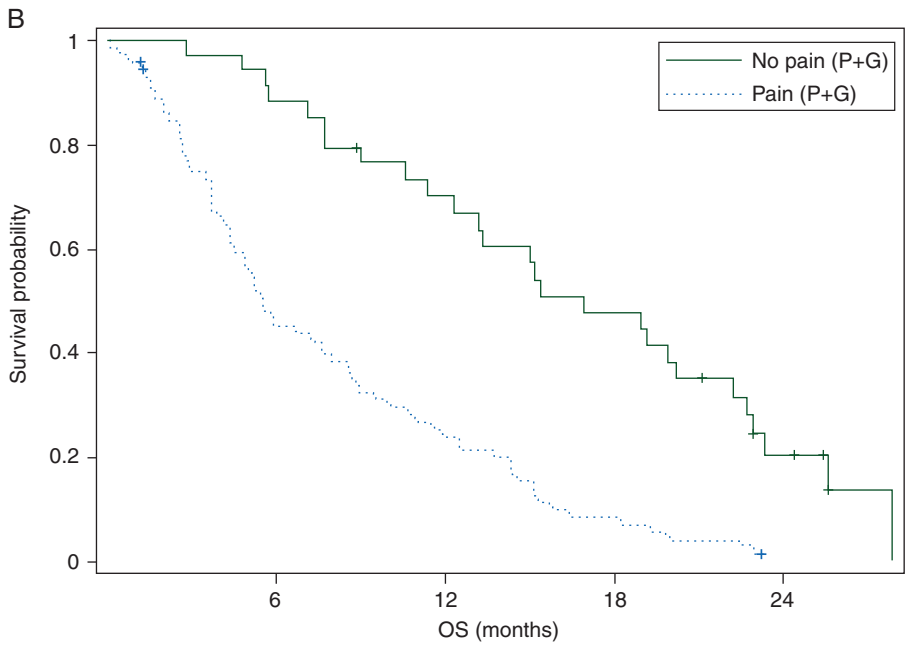
In the aforementioned *ACOX1* subgroup, those patients treated with masitinib plus gemcitabine ($n = 20$) had a median OS of 11.7 months [95% CI (8.3; 19.9)] compared with a median OS of 5.6 months [95% CI (3.7; 12.9)] for the placebo plus gemcitabine treatment-arm ($n = 20$) (multivariate model), a statistically significant OS gain of + 6.1 months. The corresponding HR was 0.23 [95% CI (0.10; 0.51), $P < 0.001$] (Table 2). Overall survival rates at 6, 12, 18, and 24 months were respectively, 82%, 48%, 15%, and 11%, in masitinib plus gemcitabine treatment-arm versus 45%, 8%, 0.6%, and 0.3%, in the placebo plus gemcitabine treatment-arm. Safety in the *ACOX1* subgroup was similar to the overall safety population (data not shown).

Considering the prospectively declared subgroup based on a baseline clinical characteristic, i.e. pain intensity tested once at baseline, 312 patients from the mITT population had VAS data available. The 'pain' subgroup, 137/312 patients (44%), included all patients reporting a VAS score of >20 mm, this threshold being consistent with established precedent and defined prior to unblinding (see section F of the Supplementary Material, available at *Annals of Oncology* online). Comparison was made against patients reporting negligible baseline pain intensity, defined by a VAS < 5 and not requiring opioid analgesics to manage disease-related pain, referred to hereafter as the 'no pain' subgroup ($n = 68/312$ patients, 22%). All remaining patients, i.e. those with a baseline VAS ≥ 5 but <20 or VAS < 5 but taking analgesics opioids ($n = 107/312$, 34%) formed a third subgroup. In the 'pain' subgroup, median exposure to masitinib or placebo was 1.5 and 2.5 months, respectively, while median exposure to gemcitabine in the masitinib or placebo treatment-arms was 1.4 and 2.3 months, respectively; $P = 0.17$. In the placebo plus gemcitabine treatment-arm, patients from the 'no pain' subgroup ($n = 34$) had a significantly longer median OS than patients in the 'pain' subgroup ($n = 73$), 16.9 months [95% CI (13.2; 22.2)] versus 5.6 months [95% CI (4.4; 8.0)] (univariate model). The corresponding HR was 0.30 [95% CI (0.18; 0.48), $P < 0.001$] (Figure 1B).

In the aforementioned 'pain' subgroup those patients treated with masitinib plus gemcitabine ($n = 64$) had a median OS of 8.0 months [95% CI (5.8; 11.5)] compared with a median OS of 5.4 months [95% CI (3.7; 8.3)] for the placebo plus gemcitabine



	No. of subjects	Event	Censored	Median survival (95% CI)
ACOX1 (P+G)	20	100% (20)	0% (0)	5.5 (3.4; 8.3)
No ACOX1 (P+G)	39	92% (36)	8% (3)	8.8 (5.6; 15.0)



	No. of subjects	Event	Censored	Median survival (95% CI)
No pain (P+G)	34	79% (27)	21% (7)	16.9 (13.2; 22.2)
Pain (P+G)	73	96% (70)	4% (3)	5.6 (4.4; 8.0)

Figure 1. (**A**) Overall survival analysis in patients with advanced PDAC and treated with placebo plus gemcitabine (standard-of-care) according to subgroups defined via pharmacogenomic data (i.e. the 'ACOX1' subgroup versus its complement 'non *ACOX1*' subgroup); corresponding HR was 0.46 [95% CI (0.26; 0.82), $P = 0.007$]. (**B**) Overall survival analysis in patients with advanced PDAC and treated with placebo plus gemcitabine according to subgroups defined via a baseline variable (i.e. the 'pain' subgroup versus the 'no pain' subgroup); corresponding HR was 0.30 [95% CI (0.18; 0.48), $P < 0.001$]. These data demonstrate the prognostic value of *ACOX1* overexpression in blood and baseline pain intensity, thereby revealing two patient subgroups with remarkably poor survival and a critical unmet medical need. Median follow-up of 26 months; univariate model.

treatment-arm ($n = 73$) (multivariate model), a statistically significant OS gain of + 2.6 months. The corresponding HR was 0.62 [95% CI (0.43; 0.89), $P = 0.012$] (Table 2). Overall survival rates at 6, 12, and 18 months, were respectively, 58%, 32%, and 18%, in the masitinib plus gemcitabine treatment-arm versus 44%, 18%, and 8%, in the placebo plus gemcitabine treatment-arm. Safety in the pain subgroup was similar to the overall safety population (data not shown). One also notes that the frequency of patients reporting back pain as an AE during treatment was significantly lower ($P = 0.004$) in the masitinib plus gemcitabine treatment-arm than in the placebo plus gemcitabine treatment-arm of the safety population (Table 1).

Internal validation of masitinib's effect in patients from the 'pain' subgroup is provided through analysis of survival data in patients consuming high doses of opioid analgesics at baseline (>1 mg/kg/day), referred to hereafter as the 'high opioid' subgroup ($n = 34$). Briefly, it is a fair assumption that such patients were experiencing moderate to severe cancer-related pain to justify initiation of such pain management measures and are, therefore, comparable to patients from the 'pain' subgroup. Patients in the exploratory 'high opioid' subgroup and treated with masitinib plus gemcitabine ($n = 20$) had a median OS of 8.5 months [95% CI (6.0; NA)], whereas patients treated with placebo plus gemcitabine ($n = 14$) had a median OS of 6.0 months [95% CI (3.5; NA)]. This corresponds to a survival benefit of 2.5 months and HR of 0.43 [0.17; 1.06]; $P = 0.23$.

discussion

Although no discernible difference between treatment-arms was observed for the primary endpoint in the overall population, this study did identify subgroups with remarkably poor survival while under single-agent gemcitabine. Patients with overexpression of *ACOX1* or baseline pain (VAS > 20 mm) had a worse prognosis (median OS of 5.6 and 5.4 months, respectively) with respect to the overall population (median OS of 7.0 months) and historical median OS data for gemcitabine-treated patients (typically 6.5 months) [1]. Such data illustrate that the markers of *ACOX1* expression in blood and baseline pain intensity may have prognostic value, with patients from these subgroups experiencing aggressive disease progression while receiving single-agent gemcitabine. It is estimated that together, these subgroups encompass 63% of the entire PDAC population (i.e. 34% of patients who were identified as belonging to the '*ACOX1*' subgroup and 29% of patients who were identified as belonging to the 'pain' subgroup with no overexpression of *ACOX1*, amounting to 63% of patients in one or the other subgroup).

Both parameters of *ACOX1* and baseline pain (VAS > 20 mm) also suggested predictive value with the masitinib plus gemcitabine treatment-arm showing a statistically significant median OS gain of + 6.1 months [HR = 0.23 (0.10; 0.51)] in the *ACOX1* subgroup and + 2.6 months [HR = 0.62 (0.43; 0.89)] in the pain subgroup when compared with single-agent gemcitabine. Although there was increased toxicity with the addition of masitinib to gemcitabine, safety remained within acceptable limits with application of appropriate risk management measures and there was no overall detrimental effect on QoL. Therefore, the combination of masitinib and gemcitabine for the treatment of advanced PDAC appears to exhibit a positive benefit–risk ratio for these subpopulations. Of note, the pharmacogenomic examination of RNA expression in peripheral blood samples also identified a set of ten genes with high discriminatory power, albeit ambiguous biological plausibility, with *ACOX1,* representing the single most important gene to explain OS (see section C of the Supplementary Material, available at *Annals of Oncology* online).

There is an emerging consensus that under certain circumstances it is possible for a subgroup to be considered of clinical significance (see section G of the Supplementary Material, available at *Annals of Oncology* online). The present study has met these criteria. For example, internal consistency supporting the clinical plausibility of each subgroup is provided from independent patient samples (see sections C and F of the Supplementary Material, available at *Annals of Oncology* online). Considering biological plausibility, it is thought that the presence of baseline pain (VAS > 20 mm) or an overexpression of *ACOX1* effectively identifies those patients with a pro-tumoral T-helper cell type-2 (Th2) immune response, a condition caused in part by increased mast cell activity in the tumor microenvironment or by transcriptional or physiological alterations favoring M2-polarization of tumor-associated macrophages (TAM) (see section H of the Supplementary Material, available at *Annals of Oncology* online). For instance, mast cells have been implicated with the development of neuropathic pain in PDAC patients and skewing macrophage polarization towards a pro-tumoral M2-type [11, 12]. Furthermore, recent preclinical data from KrasG12D driven mouse models of PDAC with pain or spontaneous chronic pancreatitis show that pancreatic tumor lesions of masitinib-treated mice have decreased mast cell count and reduced intra-tumoral vascularization and innervation when compared with control mice (Dubreuil P, 2014; personal communication). Other nascent research suggests masitinib may induce the recruitment of macrophages with a potential for antitumoral activity within the tumor (Hermine O, 2014; personal communication). Thus, mechanisms of action associated with masitinib apparently converge towards favoring a preferential accumulation of antitumoral M1-macrophages in the tumor microenvironment with concomitant reduction of oxidative stress effects. Presentation of these supportive data fall beyond the scope of the current clinical paper with additional translational research needed to fully elucidate such mechanisms; as such, these preclinical data will be reported in full elsewhere.

In conclusion, the survival benefit observed for PDAC patients with overexpression of *ACOX1* in blood or reporting baseline pain of VAS > 20 mm when treated with masitinib plus gemcitabine, coupled with manageable toxicity suggests a positive benefit–risk ratio. This has led to the initiation of a confirmatory study that may support the use of masitinib plus gemcitabine as a new treatment option for these two subgroups of PDAC patients.

acknowledgements

We thank the patients who participated in this trial and their families, all the participating investigators and clinical staff, and also members of the Data Review and Central Review Committees. For pharmacogenomic analysis we would like to thank Florian Noguier (DGE and real-time qualitative PCR experiments) Roman

Bruno (Biostatistics), Laurent Manchon (Bioinformatics), Fabien Pierrrat (Bioinformatics), Bernadette Trentin (DGE experiments), and Marine Morent (DGE experiments). We thank the Montpellier GenomiX (MGX) and Q-PCR facility, and the HPC@LR Center for high-performance computing. We also thank Thierry Conroy (Institut de Cancérologie de Lorraine), Franck Bonnetain (University Hospital of Besançon), and the Institut de Biologie Computationnelle (IBC, Montpellier, France) for fruitful discussions.

funding

This study was financially supported by AB Science, Paris, France (no grant number) and by Acobiom, Montpellier, France (no grant number). The Sponsors were involved in the study design; data processing (monitoring, collection, analysis, and interpretation); manuscript preparation and submission. The corresponding author had full access to all the data and had final responsibility for the decision to submit for publication. All authors approved the final manuscript.

disclosure

Masitinib is under clinical development by the study sponsor, AB Science (Paris, France). AM, CM, and YA are an employees and shareholders of the study sponsor AB Science. OH and PD are consultants and shareholders of AB Science. LM served as a paid consultant to AB Science. DP and DR are employees and shareholders of the study sponsor Acobiom. All other authors declare no conflicts of interest.

references

1. Heinemann V, Boeck S, Hinke A et al. Meta-analysis of randomized trials: evaluation of benefit from gemcitabine-based combination chemotherapy applied in advanced pancreatic cancer. BMC Cancer 2008; 8: 82.
2. Von Hoff DD, Ervin T, Arena FP et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 2013; 369(18): 1691–1703.
3. Moore MJ, Goldstein D, Hamm J et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 2007; 25(15): 1960–1966.
4. Humbert M, Castéran N, Letard S et al. Masitinib combined with standard gemcitabine chemotherapy: *in vitro* and *in vivo* studies in human pancreatic tumour cell lines and ectopic mouse model. PLoS ONE 2010; 5: e9430.
5. Mitry E, Hammel P, Deplanque G et al. Safety and activity of masitinib in combination with gemcitabine in patients with advanced pancreatic cancer. Cancer Chemother Pharmacol 2010; 66(2): 395–403.
6. Zelvyte I, Ohlsson B, Axelson J, Janciauskiene S. Diverse responses between human pancreatic cancer cell lines to native alpha 1-antitrypsin and its C-terminal fragment. Anticancer Res 2003; 23(3B): 2267–2273.
7. Winter JM, Tang LH, Klimstra DS et al. A novel survival-based tissue microarray of pancreatic cancer validates MUC1 and mesothelin as biomarkers. PLoS One 2012; 7(7): e40157.
8. Chang DZ, Ma Y, Ji B et al. Mast cells in tumor microenvironment promotes the in vivo growth of pancreatic ductal adenocarcinoma. Clin Cancer Res 2011; 17(22): 7015–7023.
9. Protti MP, De Monte L. Immune infiltrates as predictive markers of survival in pancreatic cancer patients. Front Physiol 2013; 4: 210.
10. Dubreuil P, Letard S, Ciufolini M et al. Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. PLoS One 2009; 4(9): e7258.
11. Demir IE, Schorn S, Schremmer-Danninger E et al. Perineural mast cells are specifically enriched in pancreatic neuritis and neuropathic pain in pancreatic cancer and chronic pancreatitis. PLoS One 2013; 8(3): e60529.
12. Maltby S, Khazaie K, McNagny KM. Mast cells in tumor growth: angiogenesis, tissue remodelling and immune-modulation. Biochim Biophys Acta 2009; 1796(1): 19–26.

Online Supplementary Material for the article 'A randomized, placebo-controlled phase III trial of masitinib plus gemcitabine in the treatment of advanced pancreatic cancer.'

Gaël Deplanque, Martin Demarchi, Mohamed Hebbar, Patrick Flynn, Bohuslav Melichar, James Atkins, Elzbieta Nowara, Lem Moyé, David Piquemal, Didier Ritter, Patrice Dubreuil, Colin Mansfield, Yolène Acin, Alain Moussy, Olivier Hermine, Pascal Hammel

Corresponding author:
Dr Gaël DEPLANQUE, Service d'Oncologie, Groupe Hospitalier Paris Saint Joseph, 185 rue Raymond Losserand, 75674 Paris, France. Tel.: +33 (0)1 44 12 76 43. Fax: +33 (0)1 44 12 32 59. E-mail: gdeplanque@hpsj.fr.

Table of Contents

A. The potential therapeutic benefit of masitinib in combination with gemcitabine for the treatment of advanced pancreatic cancer has been previously reported in a phase II study.

Mitry et al. previously reported exploratory data from a phase II study in which 22 advanced pancreatic ductal adenocarcinoma (PDAC) patients were treated with masitinib in combination with gemcitabine.[1] Findings revealed two distinct patient subgroups with respect to masitinib plus gemcitabine treatment susceptibility. A plateau in the Kaplan-Meier survival curve between 9 to 17 months divided patients into those having an overall survival (OS) of less than 9 months (short survival subgroup) or an OS of greater than 17 months (long survival subgroup) (Figure S1). No patient had an OS of between 9 and 17 months.

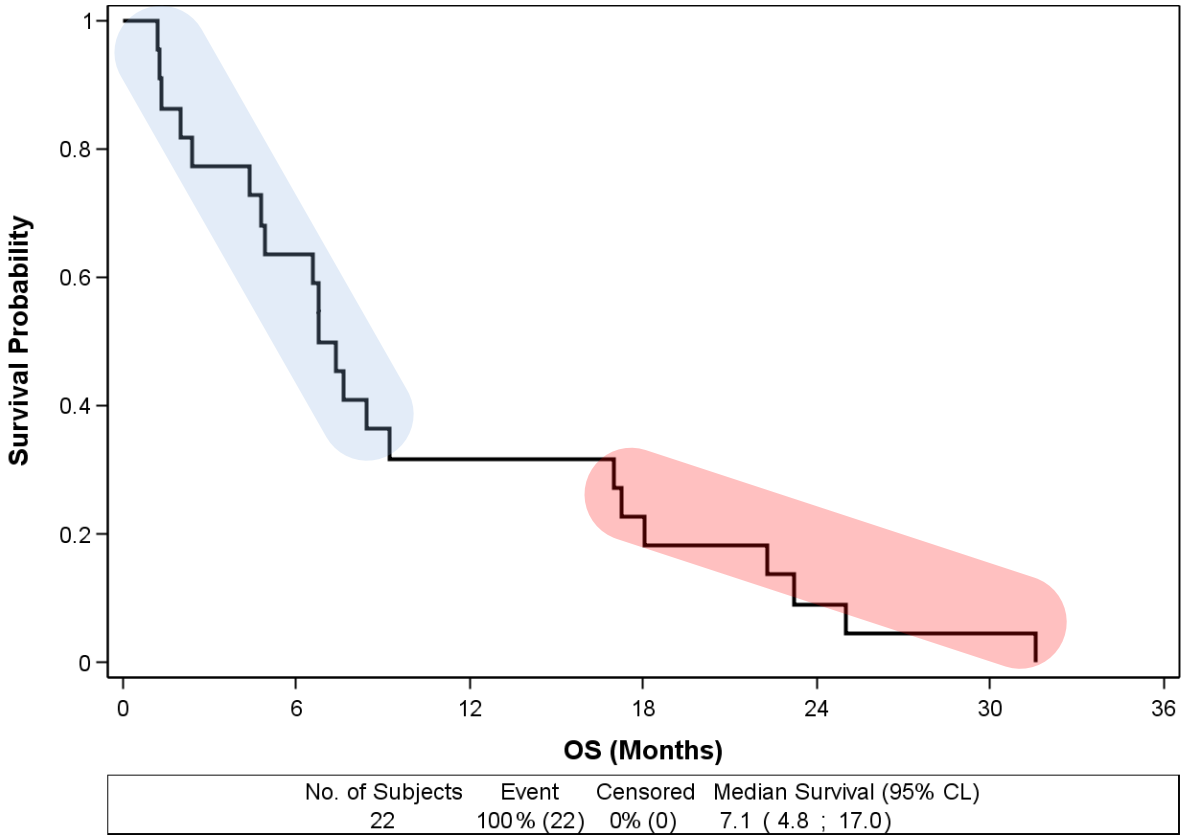
Assessment of patient disease status, i.e. locally advanced versus metastatic, a parameter commonly associated with different survival times, did not explain this observed dichotomous patient susceptibility to the masitinib plus gemcitabine combination (Table S1). The 'long survival subgroup' included patients with metastasis for whom one would have expected a short survival; with metastatic patients also reported in the 'short survival subgroup' indicating that masitinib treatment was not beneficial for all patients with metastatic disease. Similarly, patients with a locally advanced PDAC were found in both subgroups, indicating that masitinib treatment was not beneficial for all patients with locally advanced disease. Therefore, disease status cannot explain the OS benefit of masitinib plus gemcitabine in a specific subgroup.

Table S1: Disease status and median OS for each of the 22 patients included in the phase II study (AB05034)

Patient No.	Disease status	Median OS (months)	
0204	Metastatic	1.2	
0205	Metastatic	1.3	
1201	Metastatic	1.3	
0103	Locally advanced	2.0	
0101	Locally advanced	2.4	
0801	Locally advanced	4.4	
0501	Metastatic	4.8	Patients experiencing aggressive disease progression (short survival subgroup)
0403	Locally advanced	4.9	
0401	Metastatic	6.5	
0202	Metastatic	6.8	
0903	Metastatic	6.8	
1301	Metastatic	7.4	
0203	Metastatic	7.6	
0601	Locally advanced	8.4	
0901	Metastatic	9.2	
0404	Locally advanced	17.0	
0102	Locally advanced	17.2	
0904	Metastatic	18.0	Patients experiencing relatively slow disease progression (long survival subgroup)
0902	Metastatic	22.3	
0201	Locally advanced	23.2	
0402	Locally advanced	25.0	
1202	Metastatic	31.6	

Taken together, these observations implied that the overall PDAC population is comprised of various subgroups, with tumor biology and microenvironment possibly being an important driver of survival groups (i.e. aggressive versus relatively slow disease progression). Observations from this phase II study therefore generated hypotheses for the phase III study's secondary analyses, namely, that there exists at least one subgroup of PDAC patients for whom treatment with masitinib plus gemcitabine will generate a benefit in survival. Such subgroups could be identifiable via a gene expression profile and/or another variable such as a baseline characteristic, both biomarkers effectively indicating the onset of a disease mechanism susceptible to treatment with masitinib.

Figure S1: Overall survival in patients from the phase II study (AB05034) showing two distinct survival trends with a plateau between 9 to 17 months



B. Analysis and development of multivariate Cox model in the overall population for identification of the most discriminatory baseline characteristic to explain overall survival in gemcitabine treated patients

The methodology used to determine which baseline characteristic was the most discriminatory to explain overall survival in gemcitabine treated patients was prospectively declared. Randomization was stratified on two variables provided at time of inclusion: country and clinical classification defined as locally advanced or metastatic. Primary analysis was a stratified log-rank test, using a re-randomization method. Stratification variables were planned to be those used at randomization for balancing treatment groups.

Overall survival was investigated in patients according to each baseline characteristic through a univariate analysis in patients having received the placebo plus gemcitabine treatment, to determine variables that may impact overall survival independently from the treatment. As expected, the two variables used for stratification (locally advanced/metastatic tumors and country) were shown to have a univariate effect on overall survival. However, it was observed also that these stratification variables did not rank as the most important factors in terms of impact on overall survival for univariate analysis (Table S2). The variable with the greatest impact on overall survival among other potential prognostic factors was pain. Since several variables clearly showed an impact on overall survival in patients treated with the placebo plus gemcitabine combination treatment, it was expected that any differences in baseline characteristics between both combination treatment-arms would also impact overall survival. A univariate model, even if stratified on two variables, was therefore not suitable. Instead a multivariate Cox model, taking into account any discrepancy in baseline characteristics between the two treatment-arms, was necessary for identifying the effect of the combination treatment on overall survival.

Consequently, as planned for, a multivariate model was performed on all patients. In order to construct the multivariate model, variables were selected through a "stepwise" procedure, using 5% thresholds for both entry and maintenance of the variables. Interactions of all factors with the treatment-arm were not included in this model at this stage. The variables of tumor stage (locally advanced/metastatic), primary tumor localization (head), albumin level (normal/ abnormal), and pain intensity (VAS score) achieved statistical significance at 5% in the overall population (Table S3). Interactions of those factors with the treatment-arm were included in a subsequent step without any procedure for selection of variables. If significant, these interactions were graphically validated via Kaplan-Meier estimates, by treatment-arm and by factor modality. The final multivariate model factors included: treatment-arm whatever its level of significance, advanced/metastatic cancer, localization of primary tumor in the body of the pancreas, albumin level, and VAS pain intensity score.
Table S3 summarizes the statistically significant variables identified by the multivariate analysis Cox model for the overall (mITT) population.

The final multivariate Cox model revealed a significant impact on OS of four variables:

- Pain (p-value <0.001).
- Albumin level (p-value <0.001). Significant interaction with albumin was explored graphically and was considered as non clinically relevant.
- Clinical classification as metastatic or locally advanced (p-value=0.015),
- Localization of the primary tumor in the body of the pancreas (p-value=0.023).

Through this multivariate analysis we arrive at baseline pain as being the variable of greatest importance to explain the difference in overall survival within the gemcitabine cohort and between treatment-arms. To the author's knowledge it has never been demonstrated that pain is such an important factor for overall survival in pancreatic cancer patients treated with gemcitabine and the impact of pain on overall survival is therefore considered a notable finding.

Table S2: Median overall survival in patients treated with placebo plus gemcitabine according to each baseline characteristic – univariate analysis, mITT population

Baseline characteristic	N	Patients censored	Δ Median OS (months)	Median OS (months)
All	175	12 (6.9%)		8.2 [6.8; 9.6]
VAS > 20	**73**	**3 (4.1%)**		**5.6 [4.4; 8.0]**
VAS]5-20]	**36**	**1 (2.8%)**	**1.2***	**6.8 [5.4; 8.3]**
VAS ≤ 5	**48**	**8 (16.7%)**	**7.6***	**13.2 [10.8;16.9]**
Locally advanced	24	4 (16.7%)	6.2	13.8 [8.6;18.2]
Metastatic	151	8 (5.3%)		7.6 [5.7; 8.8]
Normal albumin at baseline (≥ 32 g/L)	159	15 (9.4%)	6	8.6 [7.7;10.8]
Abnormal albumin	16	0 (0.0%)		2.6 [1.4; 2.9]
Metastases: Liver	122	8 (6.6%)	4.9	7.0 [5.6; 8.3]
No Metastases: Liver	53	7 (13.2%)		11.9 [8.6;14.6]
ECOG 0	61	4 (6.6%)	4.4	11.4 [8.2;12.9]
ECOG 1	113	8 (7.1%)		7.0 [5.3; 8.3]
Head of pancreas	94	9 (9.6%)	1.7**	8.3 [6.7;10.8]
Body of pancreas	59	3 (5.1%)		10.0 [7.1;12.6]
Tail of pancreas	49	2 (4.1%)	4.3**	5.7 [4.4; 8.2]
France	110	8 (7.3%)		8.4 [6.7;11.7]
United States	28	1 (3.6%)	0.8***	7.6 [5.4; 9.6]
Lebanon	6	0 (0.0%)	0.3***	8.1 [2.9;13.8]
Romania	5	1 (20.0%)	4.1***	4.3 [2.8; NA]
Poland	4	0 (0.0%)	4.2***	12.6 [7.6;22.9]
Czech Republic	22	2 (9.1%)	1.4***	7.0 [5.0;10.7]
Normal GGT at baseline (≤ 3*ULN or ≤5*ULN if liver metastases)	138	10 (7.2%)	2.9	8.6 [7.0;10.7]
Abnormal Gamma GT at baseline	36	2 (5.6%)		5.7 [4.4; 8.3]
CA 19-9 clinically significant	45	3 (6.7%)	1.3	7.1 [5.7; 8.5]
CA 19-9 not clinically significant	106	7 (6.6%)		8.4 [6.7;10.7]
Weight ≤65 kg	75	4 (5.3%)	0.2	8.4 [5.5;11.7]
Weight >65 kg	100	9 (9.0%)		8.2 [6.0; 9.6]
≤65 years old	112	7 (6.3%)	0.5	8.3 [6.0;10.1]
>65 years old	63	5 (7.9%)		7.8 [5.6;10.7]
Male	102	7 (6.9%)	0.3	8.2 [5.7; 9.6]
Female	73	5 (6.8%)		8.5 [7.0;11.9]
BMI ≤20 kg/m²	27	1 (3.7%)	0.2	8.4 [5.5;15.1]
BMI >20 kg/m²	148	11 (7.4%)		8.2 [6.7;10.0]

VAS = visual analogue scale of pain intensity.*ΔMedian OS was calculated with respect to VAS >20. ** ΔMedian OS was calculated with respect to body. *** ΔMedian OS was calculated with respect to France. NA = not assessable. OS = overall survival. CA19-9 = carbohydrate antigen 19-9. ECOG = Eastern Cooperative Oncology Group Performance Status. mITT = modified intent-to-treat population. BMI = body mass index. ULN = upper limit of normal. Gamma GT = gamma-glutamyl transferase.

Table S3: Development and analysis (overall survival) of multivariate Cox model, mITT population

	Univariate model		Multivariate stepwise selection	Final multivariate Cox model with treatment-arm	
Baseline characteristic	HR [95% CI]	χ^2 *P*-value	χ^2 p-value	HR [95% CI]	χ^2 *P*-value
Treatment-arm (masitinib/placebo)	1.00 [0.80;1.24]	0.982	Not selected	0.89 [0.70; 1.13]	0.326
Sex (Female/Male)	0.79 [0.64; 0.99]	0.039	Not selected		
Age (>65 years Yes/No)	0.99 [0.79; 1.24]	0.927	Not selected		
Metastatic/Locally Advanced	1.53 [1.10; 2.13]	0.012	0.017	1.55 [1.09; 2.21]	0.015
ECOG (1/0)	1.56 [1.24; 1.96]	<0.001	Not selected		
Country (France Yes/No)	0.75 [0.60;0.94]	0.012	Not selected		
Pain VAS (mm) – continuous	1.01 [1.00; 1.01]	0.001	Not selected		
Pain VAS - by class	**1.67 [1.27;2.19]***	**<0.001**	**<0.001**	**1.55 [1.21; 1.98]****	**<0.001**
Clinically significant CA 19-9 (Yes/No)	1.17 [0.90; 1.52]	0.253	Not selected		
Liver Metastases (Yes/No)	1.35 [1.07; 1.72]	0.012	Not selected		
Metastases Lymph Nodes (Yes/No)	1.42 [1.08; 1.87]	0.013	Not selected		
Weight (>65 kg Yes/No)	1.04 [0.83; 1.29]	0.735	Not selected		
Localization Head (Yes/No)	1.00 [0.80; 1.24]	0.982	Not selected		
Localization Body (Yes/No)	0.83 [0.66; 1.06]	0.135	0.028	0.74 [0.57; 0.96]	0.023
Localization Tail (Yes/No)	1.10 [0.87; 1.40]	0.434	Not selected		
BMI (kg/m²) - continuous	1.00 [0.98; 1.02]	0.956	Not selected		
Gamma GT (Normal/Abnormal)	0.69 [0.53; 0.90]	0.006	Not selected		
Albumin (Normal/Abnormal)	0.30 [0.20; 0.43]	<0.001	<0.001	0.30 [0.20 ; 0.45]	<0.001

VAS = visual analogue scale of pain intensity. HR = hazard ratio of death. * HR was calculated with respect to VAS> 20 versus VAS [0;5]). ** HR was calculated with respect to VAS> 20 versus VAS [0;20]). ECOG = Eastern Cooperative Oncology Group Performance Status. mITT; modified intent-to-treat population. BMI = body mass index. Gamma GT = gamma-glutamyl transferase.

C. The genetic biomarker (ACOX1) subgroup as defined according to pharmacogenomic data

Pharmacogenomic analysis for the current study (AB07012) was performed on peripheral whole blood samples collected prior to administration of the first study treatment. Enrollment for this patient cohort only commenced once the necessary funding was secured, approximately 9 months after initial patient accrual began, and also for those patients consenting to the procedure. As a result the patient cohort with pharmacogenomic data comprised 119 patients. Findings showed that patients overexpressing *ACOX1* (defined as a Delta Cycle Threshold value of ≤ 3.05 relative to the expression level of housekeeping genes *B2M* and *GAPDH*), experienced comparatively poor survival if treated with single-agent gemcitabine, thus defining the relevant subpopulation (Table S4). A summary of the predefined methodology used to characterize the ACOX1 subgroup is presented below. To the best of our knowledge, findings from this study represent one of the first demonstrations that RNA expression from whole blood samples is capable of predicting survival in patients depending upon the treatment received.

Additionally, a related genetic biomarker (GBM) subgroup comprised of 10 genes was identified in which *ACOX1* was the single most discriminatory factor for masitinib efficacy. Patients belonging to this GBM subgroup possessed alterations in expression levels of at least one of six possible gene combinations, which in total comprised ten differentially expressed genes; referred to hereafter as the 10-gene GBM. The divergence in survival between the 10-gene GBM subgroup and its complement subgroup (i.e. non 10-gene GBM) when treated with single-agent gemcitabine was even more pronounced than that observed for the ACOX1 subgroup with a difference in median OS of 9.6 months, $P<0.0001$. Likewise for the observed masitinib treatment efficacy in patients harboring the 10-gene GBM as compared against the placebo treatment-arm with a HR of 0.17(95%CI[0.09;0.33]. However, the methodology behind derivation of the 10-gene GBM remains a novel approach in the pancreatic cancer research field with mechanistic understanding of how masitinib impacts on survival undoubtedly being complex and multifaceted, requiring further confirmatory study and translational research to establish biological plausibility. Conversely, preclinical data and findings reported in the scientific literature provide strong biological and clinical plausibility for the ACOX1 subgroup. More detailed commentary on the 10-gene GBM and the methodology applied to analyze pharmacogenomic data from this study for identification the ACOX1 and its related 10-gene GBM subgroups will be reported elsewhere. Similarly, any in-depth discussion on the possible mechanisms of action associated with *ACOX1* overexpression or the 10-gene GMB is also beyond the scope of this current paper and will be reported in full elsewhere.

Methodology for identification of genetic biomarker (ACOX1) subgroup

Differential gene expression for detection of novel gene expression patterns
Because it was not possible to pre-define the genes of the genetic subgroup at the time of study initiation, only the methodology to characterize the genes of this genetic signature was pre-specified. Characterization of the ACOX1 subgroup was therefore based on pre-defined genome-wide methodology and a cross-validation strategy. The patient cohort with pharmacogenomic data comprised 119 patients who were randomly assigned (1:1 ratio) to the masitinib plus gemcitabine or placebo plus gemcitabine treatment-arms (n=60 and n=59 patients, respectively). RNA blood samples were collected from these patients at baseline prior to treatment administration. The DGE (Digital Gene Expression) method used in this study is a high throughput sequencing approach for transcriptomic analysis. This approach provides a digital measure of RNA abundance represented by the sequence read counts in a region of interest as opposed to an indirect, analog signal from microarrays. In addition, it has a broader dynamic range, and is not dependent on having pre-existing knowledge about the transcriptome under study. This approach therefore has the ability to comprehensively detect novel transcripts and mRNA variants resulting from alternative promoter usages, splice sites, and polyadenylation.

In general, differential gene expression presents several challenges in terms of reproducibility and detection of truly novel gene expression patterns. For example, analysis from Blood RNA samples is subject to various errors due to experimental and interindividual variability. Also, pre-selection of genes of interest could be hampered by the need of pre-existing knowledge of the transcriptome. The following measures were adopted to address these problems and to ensure optimal reproducibility:

- The usage of PAXgene™ Blood RNA collection system avoids degradation of the RNA and differences in sample quality due to different collection standards among sites.
- Inherent gene-specific and interindividual expression variability were taken into account through edgeR Bioconductor analysis and pooling of RNA samples.
- The DGE methodology does not rely on a pre-existing knowledge of the transcriptome of interest and can therefore be applied to any patient group of interest.
- For the qPCR experiment, the current state of the art platform was used, which complies with industry and research standards.

Digital Gene Expression library construction and analysis
In a first step, the full human genome was analyzed for possible correlation between RNA expression levels, overall survival (OS), and treatment-arm. Blood samples were selected according to survival (long-term >15 months and short-term < 4 months) and treatment-arm, i.e. four survival profiles. A total of 12 DGE libraries were constructed, three for each survival profile, using Illumina's DGE Tag Profiling kit as per the manufacturer's protocol. Each DGE library was comprised of three pooled samples with technical duplicates of each library also constructed. Sequencing analysis and base calling were performed using the Illumina pipeline, and sequence tags were obtained after purity filtering. Data (DGE-tag) were annotated using UniGene clusters. In order to select genes, a differential expression test between conditions was performed, with the response variable being the number of RNA sequences expressed per gene. DGE libraries were analyzed with R software and the edgeR package (edgeR v 2.4.6). Default parameters were used for normalization. Three analyses were conducted for identification of biomarkers: one based on long-term survivors from both treatment-arms produced 113 markers (P <0.01); another based on short-term survivors from both treatment-arms produced 38 markers (P <0.01); and a third based on long-term and short-term survivors from the masitinib treatment-arm produced 18 markers (P <0.01).

Gene selection via qPCR
A total of 169 genes were selected following DGE library analyses. DGE-tags were then analyzed individually to restrict mRNAs' selection to optimal genetic biomarkers. Several bioinformatics filters were used to ensure optimal real-time PCR results for each target:

- Discard ambiguous or non-assigned DGE-tags on RNA sequence due to presence of repeated or conserved sequences (e.g. SINE; Short Interspersed Nucleotide Element).
- Discard weakly expressed biomarkers to remain within a good dynamic and to optimize real-time PCR assay (the output of DGE analysis consists of a count of each mRNA).
- Discard uncommon or unknown RNA sequences to optimize PCR primers design for real-time PCR assay.

A total of 64 genes were retained following qPCR analysis. Variations in the Delta Cycle Threshold (DCt) value were calculated for each of these 64 genes with relative gene expression, i.e. down-regulation or up-regulation of a given gene, quantified with respect to the expression level of two housekeeping genes (B2M and GAPDH). These house-keeping genes showed stable expression in the DGE analysis throughout the blood RNA samples. DCt are defined for each gene under investigation by subtracting the Ct values from the geometric mean of the Ct values of the reference genes.[2] DCt values are inversely proportional to the level of gene expression.

Identification of most discriminatory gene biomarker
The selection was performed on DCt values from the 64 pre-selected genes, for both technical duplicates. The dataset was then randomly allocated into a Training set and a Test set (1:1 ratio) using a bootstrap method (1000 iterations without replacement) was used to randomly divide the dataset into a Training set and a Test set. The treatment effect of masitinib with respect to placebo was calculated for each gene using a Cox-model with the following factors: treatment-arm, stage, localization of primary tumor, and baseline albumin level. Six different Cox models were run for each gene corresponding to DCt thresholds of greater than and less than the median, first and third quartiles. A given gene and its associated DCt cut-off was considered to be cross-validated at 10% level of significance when the treatment effect was significant in favor of masitinib (hazard ratio <1) in the Training set and was repeated in the Test set.

ACOX1 was found to be the gene with the most important impact on overall survival among patients receiving placebo plus gemcitabine (Table S4) and was thus identified as the most discriminatory gene biomarker. *ACOX1* also had the highest discriminatory power for treatment efficacy among all genes (Table S5). This positive treatment effect was confirmed 567 times out of 1000 iterations, with the best cross-validation observed in the subgroup of highly over-expressed ACOX1 (i.e. DCt ≤Q1) with an averaged cut-off DCt value for Q1 of 3.02 (90%CI=[2.98; 3.09]). As a final step, the ACOX1 gene and associated Q1 cut-off were retained with a final DCt cut-off of ≤3.05 selected to optimize the subgroup population (n=40) while maintaining HR and P-value.

Hence, the single gene (*ACOX1*) subgroup was arrived at because ACOX1 represented the single most important gene to explain overall survival in both the placebo and masitinib treatment groups. In addition, the ACOX1 subgroup has biological plausibility for the observed masitinib treatment effect, which is an important factor for interpretation of subgroup validity.

Table S4: Impact of the expression level of numerous genes on overall survival of patients from the placebo plus gemcitabine treatment-arm of study AB07012.

Gene	Chi2 p-value (Duplicate 1)	HR (Duplicate 1)	Chi2 p-value (Duplicate 2)	HR (Duplicate 2)
ACOX1	0.0013	0.209	0.0292	0.312
LYN	0.0089	0.415	0.0031	0.343
ABCC3	0.0550	0.677	0.0453	0.660
UBE2H	0.3465	0.624	0.7430	0.845
HIF1A	0.3601	0.764	0.2040	0.689
RPS23	0.9978	1.000	0.8000	1.025
TNFRSF10B	0.9865	1.003	0.5421	1.101
IGJ	0.0452	1.305	0.1146	1.214

Duplicate 1 and 2 refer to the technical duplicates of the Digital Gene Expression libraries constructed.

Table S5: Discriminatory power of genes for treatment efficacy (overall survival)

Subpopulation	N (M+G vs. P+G)	HR [95% CI]	*P* (Log-rank)
ACOX1 ≤ 3.05	20 vs. 20	0.23 [0.10; 0.51]	0.00106673
IGJ > 7.05	17 vs. 10	0.24 [0.07; 0.79]	0.02008567
Other genes	-	-	> 0.05

HR = hazard ratio of death. P+G = placebo plus gemcitabine. M+G = masitinib plus gemcitabine.

Robustness of ACOX1 DCt cut-off

The Delta Cycle Threshold method reflects differences of expression between a gene of interest and reference gene(s).[2] When comparing patient populations a difference in DCt values reflects dissimilar expression levels of said gene with respect to the populations because no difference should exist for the reference gene. DCt values are inversely proportional to the level of gene expression; therefore, in the case of up-regulated genes a lower DCt value indicates a greater level of expression. Table S6 shows that responsiveness to masitinib plus gemcitabine compared with placebo plus gemcitabine is increasingly significant with increasing over-expression of *ACOX1*. Moreover, applying a range of DCt cut-off values from 2.8 to 3.2 maintains a statistically significant treatment effect for approximately 15% to 55% of the population, respectively. Consequently, a relatively wide margin of error is associated with the *ACOX1* DCt threshold of ≤3.05, which in turn mitigates risk associated with treating a false-positive patient.

Hence, the *ACOX1* DCt cut-off ≤3.05 is robust because patients with surrounding cut-offs also experience a significant masitinib treatment effect.

Table S6: Overall-survival analysis (multivariate, mITT) stratified by *ACOX1* DCt values in masitinib plus gemcitabine and placebo plus gemcitabine treatment-arms

Subpopulation	*N*	% population	Treatment	Median (months)	HR [95% CI]	*P*-value
ACOX1 ≤ 2.8	17	14.3%	P+G	5.3 [1.5; NR]	0.07 [0.01; 0.41]	0.004
			M+G	NR [11.7; NR]		
ACOX1 ≤ 2.9	28	23.5%	P+G	6.1 [5.3; NR]	0.27 [0.10; 0.75]	0.010
			M+G	NR [11.0; NR]		
ACOX1 ≤ 3	36	30.3%	P+G	5.6 [3.7; 17.1]	0.20 [0.08; 0.49]	<0.001
			M+G	11.2 [8.3; NR]		
ACOX1 ≤ 3.05	40	33.6%	P+G	5.6 [3.7; 12.9]	0.23 [0.10; 0.51]	0.001
			M+G	11.7 [8.3; 19.9]		
ACOX1 ≤ 3.1	46	38.7%	P+G	6.4 [3.9; 14.3]	0.42 [0.21; 0.81]	0.019
			M+G	11.2 [7.7; 17.6]		
ACOX1 ≤ 3.2	68	57.1%	P+G	6.0 [4.6; 11.7]	0.55 [0.32; 0.95]	0.050
			M+G	8.7 [6.2; 15.6]		

HR = hazard ratio of death. P+G = placebo plus gemcitabine. M+G = masitinib plus gemcitabine. mITT = modified intent-to-treat population. NR = not reached.

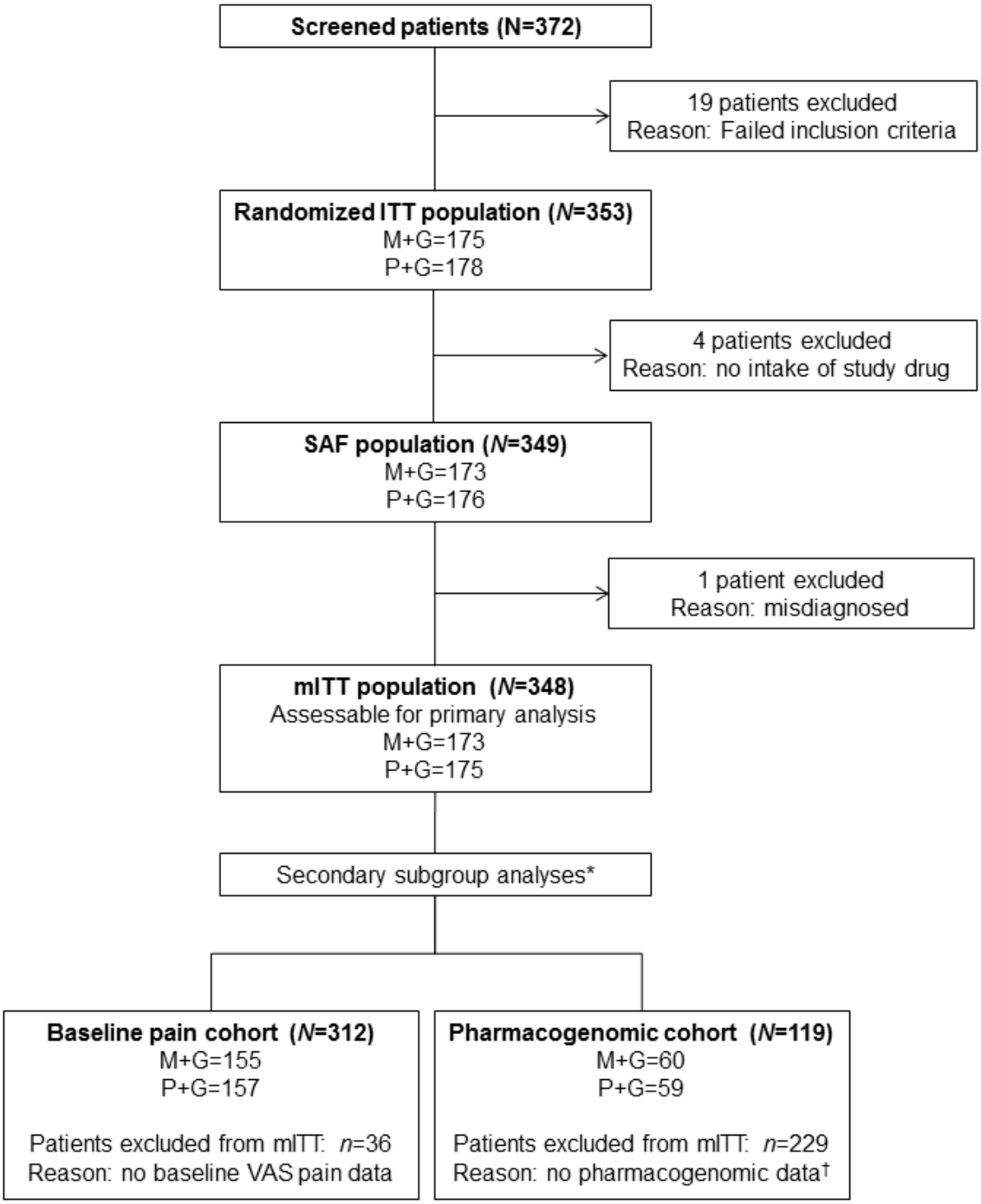
Internal validation of masitinib's effect in patients from the ACOX1 subgroup

A measure of internal validation related to masitinib's effect in patients from the ACOX1 subgroup is provided through analysis of survival data in patients with relatively high monocyte count in blood at baseline ($\geq 0.9*10^3$ cells/µL), referred to hereafter as the 'high monocyte' subgroup. Monocytes represent the primary source of *ACOX1* mRNA among peripheral blood mononuclear cells,[3] the altered gene expression of which is indicative of various diseases.[4] Furthermore, it has been shown that circulating inflammatory monocytes are recruited to the tumor microenvironment through CCL2 whereupon they are preferentially differentiated into protumoral M2-polarized macrophages. Therefore, one may reason that patients from the 'high monocyte' subgroup have analogous pathophysiology with the 'ACOX1' subgroup.

For patients in the 'high monocyte' subgroup median OS was significantly increased in the masitinib plus gemcitabine treatment-arm (n=25) when compared with the placebo plus gemcitabine treatment-arm (n=31), with median OS of 5.4 months (95%CI[3.8;22.5]) versus 3.7 months (95%CI[2.7;8.5]), respectively. This corresponds to a HR of 0.52[0.27;1.00]; P=0.05.

D. Trial profile for study AB07012

Figure S2: CONSORT flow diagram for the overall study population and subgroups of interest



*Secondary analyses used predefined methodology to characterize a subgroup based either on pharmacogenomic data or a baseline variable that negatively impacts survival with the power for each subgroup set at 80%. †Collection of samples for pharmacogenomic analysis commenced once the necessary funding had been secured, approximately 9 months after initiation of patient accrual, and therefore this cohort comprised all consenting patients enrolled on to the study (mITT) after that date.

E. Baseline patient characteristics

Table S7: Baseline patient characteristics (mITT population)

Baseline characteristic	M+G (*n*=173)	P+G (*n*=175)	*P*-value[†]
Gender (Female)	87 (50%)	73 (42%)	0.11
Age (years); median (range)	62.6 (36.0–84.0)	61.7 (31.0–79.0)	0.42
Body mass index; mean (SD)	24.1 (4.5)	24.1 (4.2)	0.90
Geographical region, France	110 (64%)	110 (63%)	N/A
CA 19-9 (U/mL); mean (SD)	57256 (365383)	20563 (71190)	0.20
Albumin (g/L); mean (SD)	40.6 (6.7)	40.5 (6.6)	0.89
QLQ-C30 Global; mean (SD)	53.5 (22.4)	53.9 (21.1)	0.87
ECOG PS			
ECOG [0]	66 (38%)	61 (35%)	0.52
ECOG [1]	106 (62%)	113 (65%)	0.42
VAS score at baseline (mm)			0.64
Pain 0<VAS≤5	53 (34.2%)	48 (31%)	
Pain 5<VAS≤20	38 (24.5%)	36 (23%)	
Pain VAS >20	64 (41.3%)	73 (47%)	
Monocyte count (per µL); median (range)	0.6 (0.1–2.9)	0.6 (0.0–3.6)	0.48
Time since diagnosis (months); mean (SD)	1.5 (3.6)	1.8 (3.6)	0.55
Tumor localization[‡]			
Head	93 (54%)	94 (54%)	0.99
Body	50 (29%)	59 (34%)	0.33
Tail	54 (31%)	49 (28%)	0.51
Clinical Classification			0.784
Locally advanced	22 (13%)	24 (14%)	
Metastatic	151 (87%)	151 (86%)	
Liver Metastases	114 (66%)	122 (70%)	0.45

Unless stated otherwise, data are number of patients (%). [†]The Fisher exact test or Chi-squared test was used for comparison of qualitative variables; analysis of variance was used for comparison of quantitative variables. [‡]Patients presenting tumors in more than one location are included in both categories. CA19-9; carbohydrate antigen 19-9. ECOG PS; Eastern Cooperative Oncology Group Performance Status. mITT; modified intent-to-treat population. M+G; masitinib plus gemcitabine. P+G; placebo plus gemcitabine. N/A; not applicable. QLQ-C30 Global; European Organization for Research and Treatment of Cancer (EORTC) quality of life questionnaire core 30 item global health status. SD; standard deviation. VAS; visual analogue scale of pain intensity.

F. The 'pain' subgroup as defined according to visual analog scale pain intensity at baseline

While pain has been identified as a variable having an impact on OS of PDAC patients,[6, 11, 12] it has not previously been demonstrated that pain is such an important baseline factor for OS. Biological plausibility of the observed masitinib treatment-effect in this subgroup is discussed in section H of this Supplementary Material document. Any in-depth discussion on the mechanisms of action associated with the observed treatment effect of masitinib in advanced PDAC based on the predictive value of baseline pain intensity, along with supporting preclinical data, is however beyond the scope of this current paper and will be reported in full elsewhere.

The visual analog scale for assessment of baseline pain intensity
Considering the hypothesized subgroup defined via a baseline clinical characteristic, the parameter with the greatest impact on overall survival (OS) of patients receiving the standard-of-care, i.e. single-agent gemcitabine, was pain. Pain intensity was assessed via a visual analog scale (VAS) at baseline. This linear scale provides a visual representation of pain as perceived by the patient. Pain intensity was represented by a 100 mm long, continuous line free of any internal reference marks. One extremity indicated an absence of pain (0-value) and the other extremity indicated very severe pain (100-value). The question asked to the patient was the following: "How severe is your pain today? Please place a vertical mark on the line below to indicate how bad you feel your pain is today." The corresponding VAS value in millimeters was recorded upon collection of the case report form. With respect to definition of the pain-related subgroups, this VAS pain intensity was only tested once at baseline.

Regarding the administration of analgesics opioids, pain in PDAC patients was managed according to the usual practice with monitoring of analgesic consumption throughout the study. Evolution of VAS score and consumption of opioid analgesics generally increased over the study, as is to be expected with disease progression, and there was no significant difference reported between treatment-arms in the overall population or subgroups (data not shown).

A total of 312 patients from the mITT population had baseline VAS data available from which three subgroups were characterized as follows:

- The VAS threshold for the 'pain' subgroup (n=137/312, 44%) was set to VAS ≥20 mm.
- The antithesis of the 'pain' subgroup, i.e. the 'no pain' subgroup, comprised those patients with negligible baseline pain intensity and no use of analgesic opioids at baseline (n=68/312, 22%). A cut-off of VAS ≤5 mm was used because the VAS scale extremities (0- and 100-values) were vertical lines with patients having no pain typically drawing a vertical line or cross on the horizontal, slightly to the right-hand side of the 0-value line in order to make it visible.
- A third subgroup was formed from all remaining patients, i.e. those with a baseline VAS >5 mm but <20 mm, or VAS ≤5 mm but taking analgesics opioids (n=107/312, 34%) (data not shown).

The threshold of VAS >20 mm is consistent with established precedent
The 'pain' subgroup included all patients reporting a baseline VAS score of >20 mm, with this threshold being defined prior to unblinding and consistent with established precedent from the scientific literature, including numerous studies in PDAC and cancer related pain. [5-10] For example, the VAS >20 threshold for pain has previously been used for treatment assessment in pancreatic cancer, including:

- A gemcitabine study in 5-FU-refractory pancreatic cancer patients used a baseline pain intensity score of >20 mm (on a VAS of 100 mm) as a patient inclusion criterion with the median baseline pain intensity score of 29 (range 3 – 68).[5]
- Pivotal data for the approval of erlotinib plus gemcitabine in advanced pancreatic cancer used pain intensity as a baseline stratification factor with a threshold of 20 mm (on a VAS of 100 mm). The median baseline VAS pain intensity score was 22.2 (range 0 – 100).[6]
- A study to investigate effective treatment of drug-resistant oncological pain of the visceral/neuropathic type, including patients pancreatic cancer, defined VAS >20 (on a VAS of 100 mm) as the upper pain threshold indicating 'more intense pain' relative to 'slight pain'.[7]
- A phase II randomized placebo controlled study of apricoxib in combination with erlotinib and gemcitabine in pancreatic cancer patients stratified patients according to baseline pain intensity using the threshold of 20 mm on a VAS of 100 mm.[8]
- Finally, pivotal data for the approval of gemcitabine in advanced pancreatic cancer was based on improvement in specific disease-related signs and symptoms (clinical benefit), which included a baseline pain intensity score of >20 mm (on a VAS of 100 mm) as one of its three main inclusion criteria.[9]

Internal validation of masitinib's effect in patients from the pain subgroup

Internal validation of masitinib's effect in patients from the 'pain' subgroup via analysis of survival data in patients consuming high doses of opioid analgesics at baseline has been presented in the Results section of the main article associated with this Supplementary Material. In summary, those patients receiving pain management through high doses of opioid analgesics (>1 mg/kg/day), regardless of their VAS score, represent an independent patient sample having comparable pathophysiology with the 'pain' (VAS >20) subgroup. The hazard ratio of death for masitinib plus gemcitabine was equivalent between the 'pain' and 'high opioid' subgroups at 0.62 [0.43;0.89] and 0.43 [0.17;1.06], respectively. Reported median OS gains were also well-matched at +2.6 and +2.5 months, respectively. This exploratory analysis suggests internal consistency thereby supporting clinical plausibility of the pain subgroup.

G. Criteria for demonstration of efficacy in subgroups when the primary analysis has failed in the overall population

Successful demonstration of efficacy in subgroups when the primary analysis has failed in the overall population presents challenges for interpretation of clinical relevance. A statistically significant treatment effect in the overall study population has conventionally been considered necessary for any formal proof of efficacy. However, there is an emerging consensus that under certain circumstances it is possible for a subgroup to be considered credible, in particular for the clinical setting with high unmet medical need.[13-15] This approach is particularly relevant for diseases comprising a range of biological phenotypes, such as PDAC,[16, 17] and for targeted therapies that are likely to act in a more selective manner than standard chemotherapies. Draft EMA (European Medicines Agency) guidelines on the investigation of subgroups in confirmatory clinical trials stress the importance of sound biological plausibility for said subgroup, internal consistency, and that the estimated subgroup treatment-effect should be more pronounced in absolute terms (i.e. indicating a greater benefit) than that observed in the overall population.[18] Each of these aspects has been satisfied by the present study as discussed in relevant sections of the document herein (sections C, F and H) or the main article associated with this Supplementary Material.

Additional factors to consider include: unmet medical need; reduced sample size; pre-specified subgroup population with associated statistical hypotheses and alpha risk; conservative method used for alpha risk adjustment; and that the power was adequate for detection of meaningful treatment effects.

Critical need has been established for the 'ACOX1' and 'pain' subgroups via OS comparison in patients receiving single-agent gemcitabine with respect to their relevant complement subgroups (i.e. non ACOX1 or no pain), as evidenced from the statistically significant hazard ratios of 0.46 (95%CI[0.26;0.82] and 0.30 (95%CI[0.18;0.48], respectively (univariate model). Survival analyses according to treatment-arm also showed a median OS of 5.6 and 5.4 months among PDAC patients receiving single-agent gemcitabine for the 'ACOX1' and 'pain' subgroups, respectively (multivariate model). These subgroups therefore present a worse prognosis compared to the study's overall population (median OS of 7.0 months), or historical median OS data for gemcitabine-treated patients (typically 6.5 months).[19] Furthermore, masitinib is the only treatment to date demonstrating a survival benefit in patients belonging to the 'ACOX1' and 'pain' subgroups with respect to single-agent gemcitabine; therapeutic options such as FOLFIRINOX or nab-paclitaxel having never been specifically assessed in these subgroups. In addition to the lack of any reported benefit from these drugs there is also an absence of mechanistic rationale to support the use of FOLFIRINOX or nab-paclitaxel (each of which belongs to the same class of agents as gemcitabine, i.e. inhibitors of DNA synthesis) in the identified subgroups. Even in the hypothetical scenario where FOLFIRINOX or nab-paclitaxel can benefit such patients, the more stringent exclusion criteria practiced with these drugs in comparison with masitinib (e.g. patient age, peripheral neuropathy, gastrointestinal disorders, high bilirubin level, route of administration to age, and tumor stage) is likely to generate a sizeable unmet medical need. Masitinib would therefore still represent an option for those patients failing to meet the aforementioned inclusion criteria of alternative other available therapies. Finally, a statistically significant reduction of symptoms associated with PDAC such as back pain or constipation was evident in the masitinib plus gemcitabine treatment-arm when compared with the placebo plus gemcitabine treatment-arm of the safety population. This further supports the notion that masitinib targets a population with an unmet medical need.

The reduced sample available for subgroup analysis can also be problematic. However, in a life-threatening and rare disease it is acceptable for the size of the subgroup to be on the order of 40% of the total cohort sample size for the confirmatory analyses to be executed successfully.[15] This criterion was met for both the 'pain' subgroup with 137/312 patients (44%), and the 'ACOX1' subgroup with 40/119 patients (34%). Regarding masitinib's therapeutic benefit, the pre-specified power for each subgroup of 80% was sufficient to detect a clinically meaningful treatment effect in each prospectively declared subgroup with the observed median OS benefit greater in absolute terms than that seen in the overall population. Because of the critical unmet medical need identified in the two subgroups, it is considered acceptable to analyze the primary and two secondary analyses at the same alpha risk provided a conservative approach is used to protect against type I error for multiple tests. Adjusting for multiplicity of testing using Bonferroni the correction, the adjusted statistical significance corresponds to an alpha risk of 0.0167 (i.e. α/number of individual tests=0.05/3). Therefore, after adjusting for multiplicity the treatment effect OS benefit in both subgroups remains statistically significant.

Hence, it is considered that the criteria for credible demonstration of efficacy in subgroups when the primary analysis has failed in the overall population have been met by this study.

H. Biological plausibility for the observed treatment effect of masitinib plus gemcitabine in PDAC patients with baseline pain (VAS>20) or *ACOX1* over-expression in blood samples

Regulatory guidelines and scientific consensus on the investigation of subgroups in confirmatory clinical trials stress the importance of there being a sound biological plausibility for the subgroup. There is compelling evidence from the scientific literature and recent preclinical studies in support of biological plausibility for the observed masitinib treatment-effect. Briefly, it is thought that the presence of baseline pain (VAS>20) or an over-expression of *ACOX1* in blood effectively identifies those patients with a pro-tumoral T-helper cell type-2 (Th2) immune response, favored respectively by an increased mast cell activity in the tumor microenvironment or by transcriptional or physiological alterations favoring M2-polarization of tumor-associated macrophages (TAM). Growing evidence indicates that masitinib's mechanism of action in pancreatic cancer can exert leverage on such patients through its activity against mast cells and by inducing dendritic cell-mediated natural killer cell activation.[20] Although detailed reporting of recent preclinical studies pertaining to masitinib's mechanism of action in pancreatic cancer fall beyond the scope of this clinical paper, there are relevant data from KrasG12D driven mouse models of PDAC with pain and also spontaneous chronic pancreatitis indicating that pancreatic tumor lesions of masitinib-treated mice had a decrease in mast cell count and reduced intra-tumoral vascularization and innervation when compared with control mice (Dubreuil P, 2014; personal communication). Other research suggests masitinib may induce the recruitment of macrophages with a potential antitumoral activity within the tumor (Hermine O, 2014; personal communication); although additional translational research is needed to fully elucidate such mechanisms.

Considering further the 'pain' subgroup, cumulative evidence indicates that the presence of pain in PDAC potentially flags an increased mast cell activity within the tumor microenvironment where they can promote disease progression via release of numerous pro-tumoral factors,[21-24] down-regulate the immune response to tumors,[25, 26] and skew polarization of TAMs towards a pro-tumoral macrophage type-2 (M2).[27] Specifically, the prognostic importance of pain in PDAC is well-established;[28-31] for example, pain intensity correlates to disease progression and significantly poorer survival rate,[11, 12] with considerable neural remodeling of intrapancreatic nerves also observed in PDAC patients experiencing pain.[32] Mast cell infiltration is strongly implicated with development of neuropathic pain in PDAC patients.[33] This was evidenced by the specific enrichment of mast cells around intrapancreatic nerves in neuropathic pain due to PDAC, suggesting the presence of mast cell induced visceral hypersensitivity in the pancreas. Furthermore, increased mast cell infiltration into the tumor is known to promote disease progression and is itself a prognostic factor for poor survival in PDAC patients;[34-39] for example, it has been shown that mast cells are actively recruited to the tumor site where they contribute to tumor growth, with inhibition of mast cell function leading to an *in vivo* suppression of tumor growth and increased survival.[40] Mast cells also contribute to tumorigenesis by suppression of the immune response;[21, 25] for example, via secretion of histamine and immunosuppressive cytokines such as IL-10 and TNF-α, and through interaction with regulatory T-cells. Finally, mast cells are an important source of intratumoral prostaglandin D2 and histamine, both of which inhibit IL-12 and enhance IL-10 release from human dendritic cell, leading to increased Th2-polarization with subsequent skewing of the balance of Th differentiation towards a Th2-immune response. This in turn drives TAMs towards a pro-tumoral M2-polarization.[27] Macrophage infiltration at the PDAC tumor site with preferential M2-polarization has been associated with poor survival and TAMs have also been shown to stimulate chemoresistance by promoting the expression of cytidine deaminase, the enzyme responsible for the inactivation of gemcitabine by cancer cells.[41-43] Furthermore, the quantity of Th2-cells with respect to Th1-cells present in the tumor stroma of PDAC has a direct correlation with prognosis in surgically resected patients, the ratio of Th2/Th1 tumor infiltrating lymphocytes being an independent predictive marker of patient survival.[44] Hence, the highly selective inhibition of human mast cell survival and activation by masitinib can be expected to be of therapeutic benefit by impacting on mast cell related remodeling of the tumor microenvironment, thereby inhibiting tumor growth, differentiation, and survival of tumor cells, and also redirecting the immune system toward an anti-tumoral Th1-type response. Together, this evidence provides strong biological plausibility for the treatment effect observed with administration of masitinib plus gemcitabine in PDAC patients with baseline pain (VAS>20).

Considering the ACOX1 subgroup, evidence indicates that *ACOX1* over-expression in blood samples from PDAC patients may flag the presence of pro-tumoral M2-polarization in the tumor microenvironment. For instance, *ACOX1* over-expression induces an abnormal activation of the transcription factor Nuclear Factor-kappa B (NF-κB) through the production of hydrogen peroxide, with NF-κB known to drive TAMs towards a pro-tumoral M2-polarization.[45-47] It has also been shown that over-expression of peroxisome proliferative-activated receptor gamma (PPARγ) in pancreatic tissue is linked to poor survival in PDAC patients.[48] PPARγ activates the promoter of *ACOX1*, which encodes the rate-limiting enzyme of the peroxisomal β-oxidation pathway.[49] Hence, over-expression of PPARγ leads to over-expression of *ACOX1* with subsequent increased

oxidative stress and up-regulation of NF-κB, which in turn contributes further to a pro-tumoral M2-polarization state, with M2-polarized macrophage recruitment to the tumor microenvironment being associated with poor survival in PDAC patients and gemcitabine resistance.[41-43] Although the biomarker *ACOX1* is expressed by peripheral blood mononuclear cells there are precedents to show that pathophysiological changes occurring in organs can be revealed as abnormal gene expression of mononuclear cells, e.g. in pancreatic cancer and renal cell carcinoma,[50, 51] with such expression changes possibly reflecting activation of specific immune responses of circulating cells. Consistent with this, blood monocytes (CD14) are a primary source of *ACOX1* with a 5-fold greater expression of *ACOX1* mRNA than the median tissue level in humans,[3] suggesting the observed *ACOX1* over-expression is most likely associated to an up-regulation of its expression in monocytes and/or an increase in number of circulating monocytes.

These pieces of evidence provide strong biological plausibility for the observed treatment effect of masitinib plus gemcitabine in PDAC patients with baseline pain (VAS>20) or *ACOX1* over-expression in blood.

References for Supplementary Material

1. Mitry E, Hammel P, Deplanque G, et al. Safety and activity of masitinib in combination with gemcitabine in patients with advanced pancreatic cancer. *Cancer Chemother Pharmacol.* Jul 2010;66(2):395-403.

2. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* Dec 2001;25(4):402-408.

3. Genomics Institute of the Novartis Research Foundation. Gene expression pattern of the ACOX1 gene. URL://commons.wikimedia.org/wiki/File:PBB_GE_ACOX1_209600_s_at_tn.png. Accessed 16 July, 2014.

4. Frampton AE, Fletcher CE, Gall TM, et al. Circulating peripheral blood mononuclear cells exhibit altered miRNA expression patterns in pancreatic cancer. *Expert Rev Mol Diagn.* Jun 2013;13(5):425-430.

5. Rothenberg ML, Moore MJ, Cripps MC, et al. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol.* Apr 1996;7(4):347-353.

6. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol.* May 20 2007;25(15):1960-1966.

7. Marineo G. Untreatable pain resulting from abdominal cancer: new hope from biophysics? *JOP.* Jan 2003;4(1):1-10.

8. Keogh GP, Langdon RM, Stella PJ. APRICoT-P: A phase II randomized placebo controlled study of apricoxib, a potent COX-2 inhibitor in combination with erlotinib and gemcitabine in pancreatic cancer patients. *J Clin Oncol.* 2010;28:Abstract TPS224.

9. Burris HA, 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol.* Jun 1997;15(6):2403-2413.

10. Kai-hoi Sze F, Wong E, Lo R, Woo J. Do pain and disability differ in depressed cancer patients? *Palliat Med.* 2000;14:11–17.

11. Lindsay TH, Jonas BM, Sevcik MA, et al. Pancreatic cancer pain and its correlation with changes in tumor vasculature, macrophage infiltration, neuronal innervation, body weight and disease progression. *Pain.* Dec 15 2005;119(1-3):233-246.

12. Watanabe I, Sasaki S, Konishi M, et al. Onset symptoms and tumor locations as prognostic factors of pancreatic cancer. *Pancreas.* Mar 2004;28(2):160-165.

13. Alosh M, Huque MF. A flexible strategy for testing subgroups and overall population. *Stat Med.* Jan 15 2009;28(1):3-23.

14. Grouin JM, Coste M, Lewis J. Subgroup analyses in randomized clinical trials: statistical and regulatory issues. *J Biopharm Stat.* 2005;15(5):869-882.

15. Moye LA, Deswal A. Trials within trials: confirmatory subgroup analyses in controlled clinical experiments. *Control Clin Trials.* Dec 2001;22(6):605-619.

16. Winter JM, Tang LH, Klimstra DS, et al. A novel survival-based tissue microarray of pancreatic cancer validates MUC1 and mesothelin as biomarkers. *PLoS One.* 2012;7(7):e40157.

17. Zelvyte I, Ohlsson B, Axelson J, Janciauskiene S. Diverse responses between human pancreatic cancer cell lines to native alpha 1-antitrypsin and its C-terminal fragment. *Anticancer Res.* May-Jun 2003;23(3B):2267-2273.

18. European Medicines Agency. EMA/CHMP/539146/2013, Guideline on the investigation of subgroups in confirmatory clinical trials. www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/02/WC500160523.pdf (Accessed 16 September 2014) 2014.

19. Heinemann V, Boeck S, Hinke A, Labianca R, Louvet C. Meta-analysis of randomized trials: evaluation of benefit from gemcitabine-based combination chemotherapy applied in advanced pancreatic cancer. *BMC Cancer.* 2008;8:82.

20. Adenis A, Blay J-Y, Bui-Nguyen B, et al. Masitinib in advanced gastrointestinal stromal tumor (GIST) after failure of imatinib: A randomized controlled open-label trial. doi:10.1093/annonc/mdu237. *Annals of Oncology.* 2014.

21. Dyduch G, Kaczmarczyk K, Okon K. Mast cells and cancer: enemies or allies? *Pol J Pathol.* Mar 2012;63(1):1-7.

22. Khazaie K, Blatner NR, Khan MW, et al. The significant role of mast cells in cancer. *Cancer Metastasis Rev.* Mar 2011;30(1):45-60.

23. Liu CY, Xu JY, Shi XY, et al. M2-polarized tumor-associated macrophages promoted epithelial-mesenchymal transition in pancreatic cancer cells, partially through TLR4/IL-10 signaling pathway. *Lab Invest.* Jul 2013;93(7):844-854.

24. Theoharides TC. Mast cells and pancreatic cancer. *N Engl J Med.* Apr 24 2008;358(17):1860-1861.

25. Evans A, Costello E. The role of inflammatory cells in fostering pancreatic cancer cell growth and invasion. *Front Physiol.* 2012;3:270.

26. Maltby S, Khazaie K, McNagny KM. Mast cells in tumor growth: angiogenesis, tissue remodelling and immune-modulation. *Biochim Biophys Acta.* Aug 2009;1796(1):19-26.

27. Christy AL, Brown MA. The multitasking mast cell: positive and negative roles in the progression of autoimmunity. *J Immunol.* Sep 1 2007;179(5):2673-2679.

28. Kelsen DP, Portenoy R, Thaler H, Tao Y, Brennan M. Pain as a predictor of outcome in patients with operable pancreatic carcinoma. *Surgery.* Jul 1997;122(1):53-59.

29. Morizane C, Okusaka T, Morita S, et al. Construction and validation of a prognostic index for patients with metastatic pancreatic adenocarcinoma. *Pancreas.* Apr 2012;40(3):415-421.

30. Okusaka T, Okada S, Ueno H, et al. Abdominal pain in patients with resectable pancreatic cancer with reference to clinicopathologic findings. *Pancreas.* Apr 2001;22(3):279-284.

31. Vickers MM, Powell ED, Asmis TR, et al. Comorbidity, age and overall survival in patients with advanced pancreatic cancer - results from NCIC CTG PA.3: a phase III trial of gemcitabine plus erlotinib or placebo. *Eur J Cancer.* Jul 2012;48(10):1434-1442.

32. Ceyhan GO, Demir IE, Rauch U, et al. Pancreatic neuropathy results in "neural remodeling" and altered pancreatic innervation in chronic pancreatitis and pancreatic cancer. *Am J Gastroenterol.* Oct 2009;104(10):2555-2565.

33. Demir IE, Schorn S, Schremmer-Danninger E, et al. Perineural mast cells are specifically enriched in pancreatic neuritis and neuropathic pain in pancreatic cancer and chronic pancreatitis. *PLoS One.* 2013;8(3):e60529.

34. Cai SW, Yang SZ, Gao J, et al. Prognostic significance of mast cell count following curative resection for pancreatic ductal adenocarcinoma. *Surgery.* Apr 2011;149(4):576-584.

35. Chang DZ, Ma Y, Ji B, et al. Mast cells in tumor microenvironment promotes the in vivo growth of pancreatic ductal adenocarcinoma. *Clin Cancer Res.* Nov 15 2011;17(22):7015-7023.

36. Protti MP, De Monte L. Immune infiltrates as predictive markers of survival in pancreatic cancer patients. *Front Physiol.* 2013;4:210.

37. Ribatti D, Vacca A, Nico B, Crivellato E, Roncali L, Dammacco F. The role of mast cells in tumour angiogenesis. *Br J Haematol.* Dec 2001;115(3):514-521.

38. Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med.* Oct 2007;13(10):1211-1218.

39. Strouch MJ, Cheon EC, Salabat MR, et al. Crosstalk between mast cells and pancreatic cancer cells contributes to pancreatic tumor progression. *Clin Cancer Res.* Apr 15 2010;16(8):2257-2265.

40. Ma Y, Hwang RF, Logsdon CD, Ullrich SE. Dynamic mast cell-stromal cell interactions promote growth of pancreatic cancer. *Cancer Res.* Jul 1 2013;73(13):3927-3937.

41. Amit M, Gil Z. Macrophages increase the resistance of pancreatic adenocarcinoma cells to gemcitabine by upregulating cytidine deaminase. *Oncoimmunology.* Dec 1 2013;2(12):e27231.

42. Kurahara H, Takao S, Kuwahata T, et al. Clinical significance of folate receptor beta-expressing tumor-associated macrophages in pancreatic cancer. *Ann Surg Oncol.* Jul 2012;19(7):2264-2271.

43. Sanford DE, Belt BA, Panni RZ, et al. Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis. *Clin Cancer Res.* Jul 1 2013;19(13):3404-3415.

44. De Monte L, Reni M, Tassi E, 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.* Mar 14 2011;208(3):469-478.

45. Crittenden MR, Cottam B, Savage T, Nguyen C, Newell P, Gough MJ. Expression of NF-kappaB p50 in tumor stroma limits the control of tumors by radiation therapy. *PLoS One.* 2012;7(6):e39295.

46. Dijkgraaf EM, Heusinkveld M, Tummers B, et al. Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment. *Cancer Res.* Apr 15 2013;73(8):2480-2492.

47. Li Y, Tharappel JC, Cooper S, Glenn M, Glauert HP, Spear BT. Expression of the hydrogen peroxide-generating enzyme fatty acyl CoA oxidase activates NF-kappaB. *DNA Cell Biol.* Feb 2000;19(2):113-120.

48. Kristiansen G, Jacob J, Buckendahl AC, et al. Peroxisome proliferator-activated receptor gamma is highly expressed in pancreatic cancer and is associated with shorter overall survival times. *Clin Cancer Res.* Nov 1 2006;12(21):6444-6451.

49. Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, Wahli W. Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell.* Mar 6 1992;68(5):879-887.

50. Baine MJ, Chakraborty S, Smith LM, et al. Transcriptional profiling of peripheral blood mononuclear cells in pancreatic cancer patients identifies novel genes with potential diagnostic utility. *PLoS One.* 2011;6(2):e17014.

51. Twine NC, Stover JA, Marshall B, et al. Disease-associated expression profiles in peripheral blood mononuclear cells from patients with advanced renal cell carcinoma. *Cancer Res.* Sep 15 2003;63(18):6069-6075.