Cancer Chemotherapy and Pharmacology (2019) 83:975–991
https://doi.org/10.1007/s00280-019-03807-4

ORIGINAL ARTICLE

TGFβ receptor inhibitor galunisertib is linked to inflammation- and remodeling-related proteins in patients with pancreatic cancer

Davide Melisi[1] · Rocio Garcia-Carbonero[2] · Teresa Macarulla[3] · Denis Pezet[4] · Gael Deplanque[5] · Martin Fuchs[6] · Jorg Trojan[7] · Mark Kozloff[8] · Francesca Simionato[1] · Ann Cleverly[9] · Claire Smith[9] · Shuaicheng Wang[10] · Michael Man[11] · Kyla E. Driscoll[11] · Shawn T. Estrem[11] · Michael M. F. Lahn[11] · Karim A. Benhadji[11] · Josep Tabernero[3]

Received: 22 October 2018 / Accepted: 1 March 2019 / Published online: 18 March 2019

Abstract

Purpose Galunisertib, the first small molecule transforming growth factor beta (TGFβ) receptor inhibitor, plus gemcitabine resulted in the improvement of survival in patients with unresectable pancreatic cancer, but markers to identify patients likely to respond are lacking.

Methods In the Phase 1b/2 JBAJ study, 156 patients were randomized 2:1 to galunisertib + gemcitabine ($N = 104$) or placebo + gemcitabine ($N = 52$). Clinical outcome data were integrated with baseline markers and pharmacodynamic markers while patients were on treatment, including circulating proteins using a multi-analyte panel, T cell subset evaluation, and miRNA profiling.

Results Baseline biomarkers associated with overall prognosis regardless of treatment included CA19-9 and TGF-β1. In addition, IP-10, FSH, MIP-1α, and PAI-1 were potential predictive proteins. Baseline proteins that were changed during treatment included amphiregulin, CA15-3, cathepsin D, P-selectin, RAGE, sortilin, COMP, eotaxin-2, N-BNP, osteopontin, and thrombospondin-4. Plasma miRNA with potential prognostic value included miR-21-5p, miR-301a-3p, miR-210-3p, and miR-141-3p, while those with potential predictive value included miR-424-5p, miR-483-3p, and miR-10b-5p.

Conclusions Galunisertib + gemcitabine resulted in improvement of overall survival, and 4 proteins (IP-10, FSH, MIP-1α, PAI-1) were potentially predictive for this combination treatment. Future studies should also include baseline evaluation of miR-424-5p, miR-483-3p, and miR-10b-5p.

Trial registration Clinicaltrials.gov NCT01373164.

Keywords Galunisertib · TGF-β1 · CA19-9 · Pancreatic cancer

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00280-019-03807-4) contains supplementary material, which is available to authorized users.

✉ Davide Melisi
davide.melisi@univr.it

[1] Digestive Molecular Clinical Oncology Research Unit, Section of Medical Oncology, Department of Medicine, Università degli studi di Verona, Piazzale L.A. Scuro, 10, 37134 Verona, Italy

[2] University Hospital Doce de Octubre, Institute of Health Research Hospital 12 de Octubre (imas12), UCM, CNIO, CIBERONC, Madrid, Spain

[3] Vall d'Hebron University Hospital, Vall d'Hebron Institute of Oncology, Autonomous University of Barcelona, CIBERONC, Barcelona, Spain

[4] Digestive Surgery Service, CHU Clermont-Ferrand, University Clermont Auvergne, Clermont-Ferrand, France

[5] Medical Oncology, Saint Joseph Hospital, Paris, France

[6] Hospital Bogenhausen, Municipal Hospital Munich GmbH, Munich, Germany

[7] Goethe University Medical Center, Frankfurt, Germany

[8] Ingalls Memorial Hospital, Harvey, IL, USA

[9] Eli Lilly and Company, Erl Wood, UK

[10] BioStat Solutions, Inc, Frederick, MD, USA

[11] Eli Lilly and Company, Indianapolis, IN, USA

Introduction

Pancreatic cancer has the lowest 5-year relative survival rate among solid tumors [1, 2], and it is projected to become the second leading cause of cancer-related death by 2030 in Western countries [3]. The poor prognosis for patients with pancreatic cancer could be mainly attributed to the early metastatic behavior demonstrated along the progression of the disease, its aggressive course, and, in particular, to the limited efficacy of currently approved classic chemotherapeutic treatments [4].

The TGF beta (TGFβ) signaling pathway has one of the most essential, but also complex and controversial, roles in cancer [5, 6]. TGFβ maintains homeostasis in normal tissue, however, being genetically unstable entities, cancer cells have the capacity to corrupt this suppressive influence, thus pathological forms of TGFβ signaling promote tumor growth, epithelial-to-mesenchymal transition (EMT), extracellular matrix remodeling, stemness, evasion of immune surveillance, and metastasis. In this regard, recent whole-genome or exome sequencing analyses confirmed TGFβ as the most recurrently mutated signal transduction pathway in pancreatic cancer [7].

We contributed to this field by demonstrating the therapeutic efficacy of the pharmacologic inhibition of the TGFβ pathway using the small molecule selective inhibitor of the type I TGFβ receptor (TGFβRI) LY2109761 in preclinical models of pancreatic cancer [8]. Based on the results of these preclinical studies, we recently conducted a Phase 1b/randomized Phase 2, double-blind study to evaluate the efficacy and safety of gemcitabine in combination with the small-molecule inhibitor of the TGFβRI serine/threonine kinase galunisertib or placebo in patients with unresectable pancreatic cancer (H9H-MC-JBAJ). In this study, we demonstrated that the combination therapy of galunisertib + gemcitabine resulted in a statistically significant improvement of overall survival (OS) and progression-free survival in patients with pancreatic cancer, with a manageable toxicity profile as compared to placebo + gemcitabine [9]. In this regard, the identification of biomarkers that could predict which patients would benefit more from the inhibition of TGFβ signaling remains of unique importance. In the present study, we measured several markers at baseline and during treatment in patients enrolled in the H9H-MC-JBAJ study to determine either potentially prognostic or predictive markers for the treatment with galunisertib in unresectable pancreatic cancer.

Materials and methods

The study was a multinational, 2-part study of oral galunisertib in combination with gemcitabine. The first part was a nonrandomized, open-label, multicenter, dose escalation phase (Phase 1b) [10]. The second part was a randomized, placebo and Bayesian-augmented controlled, double-blind study of galunisertib in combination with gemcitabine vs. gemcitabine plus placebo in patients with pancreatic cancer. Galunisertib was given 7 days before the first dose of gemcitabine. The biomarker work was limited to Part 2 and was not be included in Part 1 of this study, because patients in Part 1 had diverse tumor histologies. Phase 2 was a 2:1 randomized, double-blind, 2-arm study of galunisertib in combination with gemcitabine (Arm B) versus gemcitabine plus placebo (Arm A). A dynamic randomization procedure was used to minimize imbalance between treatment arms using the prognostic factors of Eastern Cooperative Oncology Group (ECOG) performance, disease stage (Stages II–IV), previous gemcitabine treatment, and investigator site [11].

Patients

Patients who were at least 18 years old and diagnosed with locally advanced (Stage II or III) or metastatic (Stage IV) adenocarcinoma of the pancreas not amenable to resection with curative intent were included in the study. Patients with previous radical surgery for pancreatic cancer were eligible after progression was documented. If they had received adjuvant chemotherapy or chemoradiotherapy with gemcitabine or other commonly used cytotoxic agents, they could be enrolled if the treatment had been completed at least 3 months before study entry. All patients had to have measurable or nonmeasurable disease, defined according to Response Evaluation Criteria in Solid Tumors. Patients with endocrine pancreatic tumors or ampullary cancer were excluded from the study.

Patients were required to have adequate hematologic, hepatic, and renal organ function, a performance status of ≤ 2 on the ECOG scale, and must have recovered from any Grade 3/4 toxicities of previous therapies.

Exclusion criteria included medically uncontrolled cardiovascular illness, medically significant electrocardiogram abnormalities and serious pre-existing medical conditions [12, 13], inability to swallow tablets, pregnancy or breastfeeding, medically significant illnesses that could not be adequately controlled with appropriate therapies, history of any other cancer (except non-melanoma skin cancer or carcinoma in situ), unless in complete remission and patient not taking all therapies for that disease for a minimum of 3 years, active infection that would interfere with the study objectives or influence study compliance, previous completion or withdrawal from this study or any other study investigating galunisertib or any other TGFβ inhibitor, and known allergy to galunisertib or gemcitabine or any ingredient of galunisertib or gemcitabine formulations.

Treatment: dose and dose levels

One cycle of treatment was defined as 28 days in all treatment arms. This study adopted intermittent treatment as described in the FHD study based on a pharmacokinetic/pharmacodynamic (PK/PD) model [14].

Patients were treated orally with galunisertib 300 mg/day (150-mg tablets twice a day, morning and evening) for 14 days followed by 14 days off in a 28-day cycle. All patients received gemcitabine at a dose of 1000 mg/m^2 once weekly for 7 weeks, followed by a week of rest from treatment. The initial dose of gemcitabine was administered 7 days (± 3 days) after the first dose of galunisertib or placebo. Subsequent cycles consisted of infusions once weekly for 3 consecutive weeks out of every 4 weeks.

At the time of the study, galunisertib had not been evaluated to determine whether food intake may change the PK profile; therefore, patients were instructed to take galunisertib on an empty stomach, and to wait at least 1 h (preferably 2 h) after taking galunisertib before eating a meal.

Sample size determination

The primary outcome was OS. The final analysis/evaluation of OS was to be performed after approximately 135 events (deaths) had been recorded or 18 months after the last patient was enrolled, whichever was sooner.

Biomarker and PD methods

Plasma samples from patients were analyzed for TGF-β1 levels by enzymelinked immunosorbent assay (ELISA) (R&D Systems, DB100B, Minneapolis, Minnesota, USA). Platelet factor 4 (PF4) levels were also assessed to determine possible platelet activation although it was expected that patients with pancreatic cancer would have elevated PF4 levels as part of their tumor-associated intravascular coagulopathy.

In circulating blood, carbohydrate antigen (CA) 19-9 kinetics were evaluated in both the per-protocol and the intent-to-treat populations.

The multi-analyte immunoassay panel (MAP) developed by Myriad RBM (Austin, Texas, USA) was used to assess plasma proteins.

T-cell subsets were determined by standard flow cytometry and also by utilizing epigenetic assessment of $CD3^+$ (Epiontis GmbH, Berlin, Germany) [15].

Plasma microRNAs (miRs) were extracted and measured using Exiqon miRCURY LNA Human miRNome PCR panel I and II V4 (752 miR assays) by Covance (Seattle, Washington, USA). Normalization was performed using GenEx software (Exiqon) using a global normalization based upon 52 miRs detected in all samples. The miR data were analyzed using both single-marker (Cox regression in combination with MaxChi method) and multimarker [16] approaches. There was no adjustment for multiplicity.

CA19-9 and TGF-β1

Prognostic and predictive evaluation of CA19-9 and TGF-β1 reduction

CA19-9 levels were assessed at baseline and every week after treatment. TGF-β1 levels were assessed at baseline and every 2 weeks after treatment, for the first 3 treatment cycles. The proportion of patients who achieved any, > 20%, or > 50% reduction in CA19-9 or TGF-β1 from baseline at any visit in the first 12 weeks of treatment was compared between the 2 treatment arms using a chi-squared test for each level of reduction. Responses based on CA19-9 reduction were assessed at 12 weeks to identify any late response and compared to the patients with reduced TGF-β1 levels in the same responder population [17]. The OS was summarized descriptively by treatment arm and response status for CA19-9 and TGF-β1 using the Kaplan–Meier method and log-rank test.

Changes in CA19-9 and TGF-β1 over time

For each treatment arm, changes in CA19-9 and TGF-β1 over time were calculated and summarized using the Kaplan–Meier method. The duration of decrease was calculated from the date of first occurrence of decrease (any, > 20%, or > 50%) to the date of first occurrence the patient no longer met decrease (i.e., increase above 0%, 20%, or 50% threshold). For patients who maintained a decrease at their last assessment, duration was censored at the last visit on which a sample was taken.

Changes in CA19-9 in the first 6 cycles after treatment with galunisertib were also evaluated using mixed-effect model repeated measures (MMRM) models. Data were $\log_e$-transformed prior to analysis and the ratio to baseline evaluated, with baseline CA19-9, ECOG performance status, disease stage, and previous gemcitabine treatment (randomization factors), as covariates, and fixed effect terms of treatment, visit, and the interaction of treatment and visit. An AR(1) variance–covariance structure was used to account for repeated measures within a patient. Similar MMRM analyses were not conducted for TGF-β1 levels because samples were not collected beyond Cycle 3. However, as an alternative, we considered the rate of decrease in the first 12 weeks.

The rate of decrease per week of CA19-9 and TGF-β1 during the first 12 weeks of treatment was estimated for each patient using a mixed-effects model. All observations available from baseline to the end of Cycle 3 (Week 12) were included in the model. The linear slope for the longitudinal

effect of CA19-9 decrease over time was considered appropriate following visual inspection of patient profile plots.

Data were $\log_e$-transformed prior to analysis and the ratio to baseline evaluated, with treatment as a fixed effect, week as a continuous effect, and treatment-by-week interaction and baseline levels as covariates. The rate of decrease was split into 2 groups at the median to represent high vs. low steepness of CA19-9 or TGF-β1 reduction, and the relationship to OS evaluated using the Kaplan–Meier method and log-rank test (further illustration of analysis methods are provided in Supplemental Fig. 1).

Multi-analyte immunoassay panel

Potential prognostic markers were measured at baseline and evaluated for their impact on OS. Each parameter was split into 2 groups (> median and ≤ median) and the markers with $P \leq 0.001$ were selected using univariate Cox models. Potential predictive markers, similarly split into 2 groups at the median, were evaluated to determine if baseline levels were predictive for a treatment response as assessed by OS. Cox models were used with terms including the interaction of baseline protein marker (> median, ≤median) and treatment arm (galunisertib + gemcitabine, placebo + gemcitabine), and a marker was identified to be potentially predictive if the interaction term $P \leq 0.05$. There was no adjustment for multiplicity.

Changes in the first 3 cycles after treatment with galunisertib were evaluated using MMRM models. Data were $\log_e$-transformed prior to analysis and the ratio to baseline evaluated, with baseline included as a covariate, and fixed effect terms of treatment, visit, and the interaction of treatment and visit.

Spearman's correlation was also estimated between CA19-9, TGF-β1, epigenetic $CD3^+$, and each of the prognostic markers to verify if any findings were associated to an underlying correlation.

Study approval

The study was conducted according to the principles of Good Clinical Practice, applicable laws and regulations, and the Declaration of Helsinki. Each institution's review board approved the study and all patients signed an informed consent document before study participation.

Results

Patient disposition

The Phase 2 study was conducted between July 2011 and February 2016 at 24 centers in 6 countries. Of the 199 patients who entered Phase 2, 156 were randomly assigned to study treatment and 155 received at least 1 dose of study treatment. Of the 155 patients who received at least 1 dose of study treatment, 52 received placebo + gemcitabine and 103 received LY2157299 300 mg/day galunisertib + gemcitabine.

CA19-9

Prognostic and predictive evaluation of CA19-9 reduction

The proportion of patients with CA19-9 decrease during treatment (any, > 20%, or > 50%) was similar between the galunisertib and placebo groups (64% vs. 73%; 60% vs. 63%; 39% vs. 37%, respectively) (Table 1). Among patients with reduction in CA19-9, median OS favored galunisertib + gemcitabine vs. placebo + gemcitabine, particularly in the subset of patients who achieved > 50% decrease: 15.6 months vs. 9.9 months, $P = 0.0658$ (Table 1).

Changes in CA19-9 over time

Time to first occurrence of CA19-9 decrease was similar for the 2 treatment arms. For patients achieving CA19-9 decrease > 20%, median time to first occurrence was approximately 4 weeks [interquartile range (IQR) 3–6 weeks] for galunisertib + gemcitabine and 3 weeks (IQR 2–5 weeks) for placebo + gemcitabine. Median time to first occurrence of CA19-9 decrease > 50% was approximately 6 weeks (IQR 4–10 weeks) for galunisertib + gemcitabine and 5 weeks (IQR 3–7 weeks) for placebo + gemcitabine.

Median duration of CA19-9 decrease > 20% was 5.8 months (95% CI 3.9, 7.4) for galunisertib + gemcitabine, and 2.5 months (95% CI 1.6, 5.7) for placebo + gemcitabine, $P = 0.1817$. Median duration of CA19-9 decrease > 50% was 3.7 months (95% CI 2.8, 5.8) for galunisertib + gemcitabine, and 5.6 months (95% CI 2.4, 19.7) for placebo + gemcitabine, $P = 0.5123$, and thus did not notably differ between the treatment arms.

In the evaluation of change from baseline CA19-9 levels by visit after treatment, mean decreases from baseline were observed for both treatment arms from Cycle 1 after an initial increase. From Cycle 3, mean decreases of approximately 50% from baseline in CA19-9 were observed for the galunisertib + gemcitabine arm (Fig. 1).

The rate of CA19-9 decrease per week was similar for both treatment arms, geometric mean 5.1% (95% CI 2.5%, 7.6%) for galunisertib + gemcitabine, and 5.0% (95% CI 1.3%, 8.5%) for placebo + gemcitabine (data not shown). Regardless of treatment, patients with a steeper rate of decrease (> 4.1%, where 4.1% = median cut) had improved OS relative to patients with a lower rate of decrease (≤4.1%), median OS = 12.6 months vs. 6.0 months ($P = 0.0012$) (data not shown). For patients who achieved a steeper rate

Table 1 Evaluation of CA19-9 and TGF-β1 reductions

	Frequency of CA19-9 and TGF-β1 reductions			Prognostic and predictive effect of CA19-9 and TGF-β1 reductions		
	Galunisertib + Gemcitabine ($N=104$)	Placebo + Gemcitabine ($N=52$)	P value[b]	Galunisertib + Gemcitabine ($N=104$)	Placebo + Gemcitabine ($N=52$)	P value[c]
				Median OS (95% CI)	Median OS (95% CI)	
CA19-9						
n	97	51				
Any decrease	62 (64%)	37 (73%)	0.2890	12.7 (10.7, 16.4)	9.9 (7.1, 15.4)	0.2913
No change or increase				5.5 (2.9, 8.2)	3.6 (1.5, 5.7)	0.4833
P value[a]				< 0.0001	0.0161	
Decrease > 20%	58 (60%)	32 (63%)	0.7267	13.7 (10.9, 16.7)	9.9 (7.2, 15.4)	0.2108
Decrease ≤ 20%				5.5 (2.9, 7.3)	3.7 (2.9, 6.6)	0.6340
P value[a]				< 0.0001	0.0706	
Decrease > 50%	38 (39%)	19 (37%)	0.8195	15.6 (9.0, 16.7)	9.9 (4.0, 12.6)	0.0658
Decrease ≤ 50%				8.2 (5.5, 10.2)	6.6 (3.6, 8.8)	0.5242
P value[a]				0.0040	0.9311	
TGF-β1						
n	93	50				
Any decrease	76 (82%)	35 (70%)	0.1088	10.9 (8.3, 13.7)	8.8 (6.6, 12.6)	0.5075
No change or increase				8.0 (3.6, 12.1)	3.6 (1.9, 8.1)	0.9734
P value[a]				0.1751	0.4938	
Decrease > 20%	64 (69%)	30 (60%)	0.2894	10.9 (8.2, 13.7)	9.8 (7.2, 15.5)	0.9389
Decrease ≤ 20%				9.0 (5.1, 11.8)	3.6 (2.8, 8.1)	0.2416
P value[a]				0.4961	0.1171	
Decrease > 50%	37 (40%)	20 (40%)	0.9800	10.7 (8.3, 15.7)	11.3 (7.6, 19.9)	0.4455
Decrease ≤ 50%				9.0 (6.7, 12.3)	4.0 (2.9, 7.9)	0.1038
P value[a]				0.7665	0.0337	
Both CA19-9 and TGF-β1						
n	91	50				
Any decrease	52 (57%)	31 (62%)	0.5750	13.7 (10.9, 16.7)	9.9 (7.1, 15.4)	0.1585
No change or increase				7.1 (3.8, 8.8)	3.7 (2.8, 7.9)	0.9059
P value[a]				< 0.0001	0.2003	
Decrease > 20%	40 (44%)	23 (46%)	0.8153	15.5 (10.9, 17.9)	10.1 (7.6, 15.5)	0.2691
Decrease ≤ 20%				8.2 (5.1, 10.2)	3.7 (2.9, 7.9)	0.7193
P value[a]				0.0011	0.1321	
Decrease > 50%	17 (19%)	8 (16%)	0.6900	15.9 (9.2, 16.7)	10.0 (7.2, 12.6)	0.2249
Decrease ≤ 50%				8.9 (7.1, 11.8)	6.6 (3.6, 8.8)	0.6397
P value[a]				0.1191	0.5776	

n number of patients with evaluable sample at baseline and after treatment

[a]*P* value calculated using the log-rank test for the within treatment comparison of the indicated decrease vs. no decrease comparison

[b]*P* value calculated using the chi-squared test for comparison between treatment arms

[c]*P* value calculated using the log-rank test for the between treatment comparison

of decrease of CA19-9, median OS was improved for galunisertib + gemcitabine compared to placebo + gemcitabine, median OS = 15.5 months vs. 9.9 months ($P = 0.1043$) (Fig. 2c). Evaluation of known prognostic factors appeared balanced between the treatment arms.

Taken together, these findings suggest that while the rate and timing of CA19-9 decrease may be similar among the treatment arms, the improved OS observed in the subset of patients with a steeper decrease for the galunisertib + gemcitabine arm may be due to longer duration or more consistent CA19-9 decrease in the experimental arm compared to the control arm.

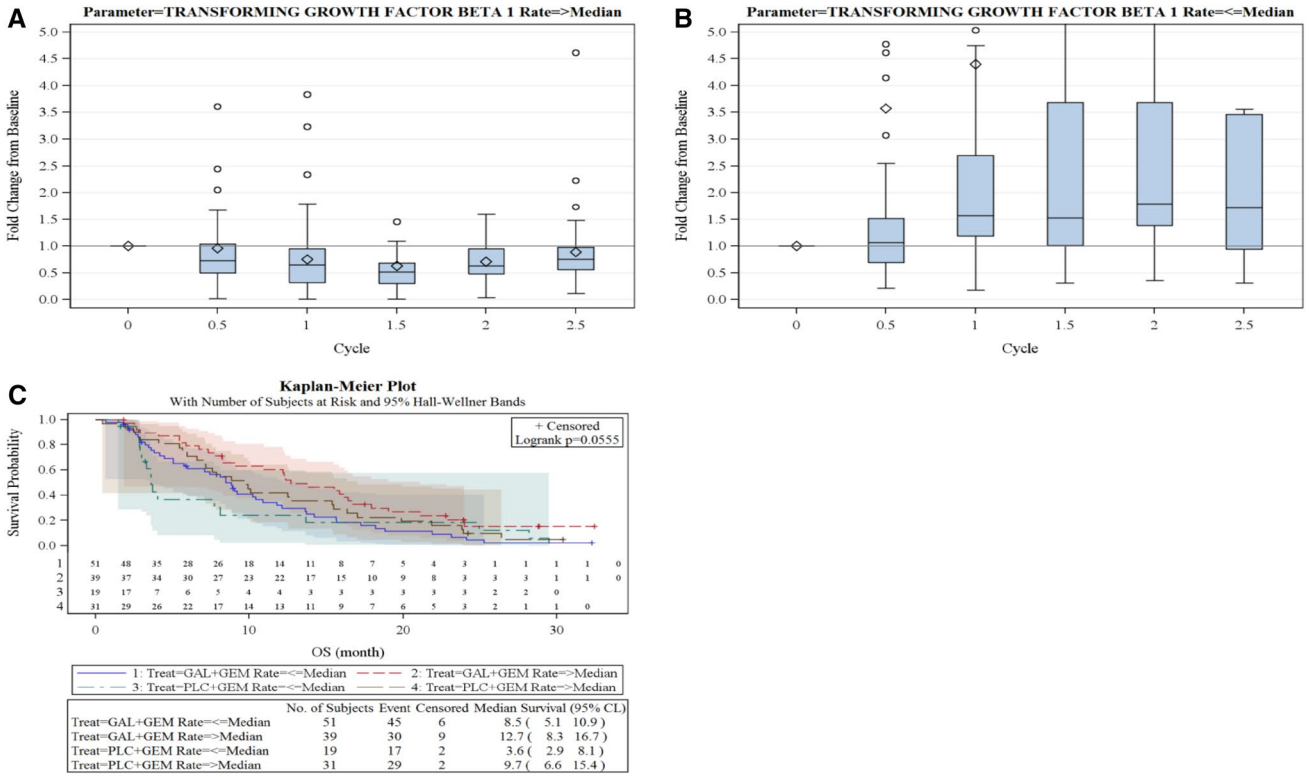

Fig. 1 Rate of decrease of TGF-β1. Patients with rate of decrease > median (**a**), patients with rate of decrease ≤ median (**b**), and OS by rate of decrease (**c**)

TGF-β1

Prognostic and predictive evaluation of TGF-β1 reduction

The proportion of patients with TGF-β1 decrease at any time during treatment was 82% for galunisertib + gemcitabine and 70% for placebo + gemcitabine, $P = 0.1088$. The proportion of patients with > 20% or > 50% decrease was similar between the two treatment arms (69% vs. 60% and 40% vs. 40%) (Table 1).

Reduction in TGF-β1 after treatment was not found to be a significant prognostic factor for OS; however, median OS generally favored patients who achieved a TGF-β1 decrease (Table 1). Among patients with a TGF-β1 decrease, median OS was similar for the two treatment arms (Table 1).

Changes in TGF-β1 over time

Data were variable for TGF-β1 levels as indicated by within patient coefficient of variation of approximately 100%. The linear model was able to adequately identify patients with more consistent TGF-β1 reductions by visual inspection of patient profile plots. Thus, the linear model was considered to be less prone to influential outliers and represent a general rate of change of TGF-β1 over the first 12 weeks of treatment.

Time to first occurrence of TGF-β1 decrease was similar for the 2 treatment arms. For patients achieving a TGF-β1 decrease > 20%, median time to first occurrence was approximately 2 weeks (IQR 2–4 weeks) for galunisertib + gemcitabine and 2 weeks (IQR 2–4 weeks) for placebo + gemcitabine. Median time to first occurrence of TGF-β1 decrease > 50% was approximately 2 weeks (IQR 2–6 weeks) for galunisertib + gemcitabine and 4 weeks (IQR: 2–6 weeks) for placebo + gemcitabine. Median duration of TGF-β1 decrease > 20% and decrease > 50% was approximately 1 month for both treatment arms.

TGF-β1 levels were evaluable for the first 3 cycles after treatment. No substantial increases or decreases from baseline were observed for either treatment arm, with no notable separation of the treatment arms, and thus treatment arms were combined for the evaluation of the rate of decrease (Supplemental Fig. 2). The rate of TGF-β1 decrease per week was similar for both treatment arms and did not indicate decreases in general after treatment, geometric mean rate of decrease/week = − 1.7% (95% CI: −4.9%, 1.4%) for galunisertib + gemcitabine, and − 0.2% (95% CI −4.9%, 4.3%) for placebo + gemcitabine. However, regardless of treatment, patients with a steeper

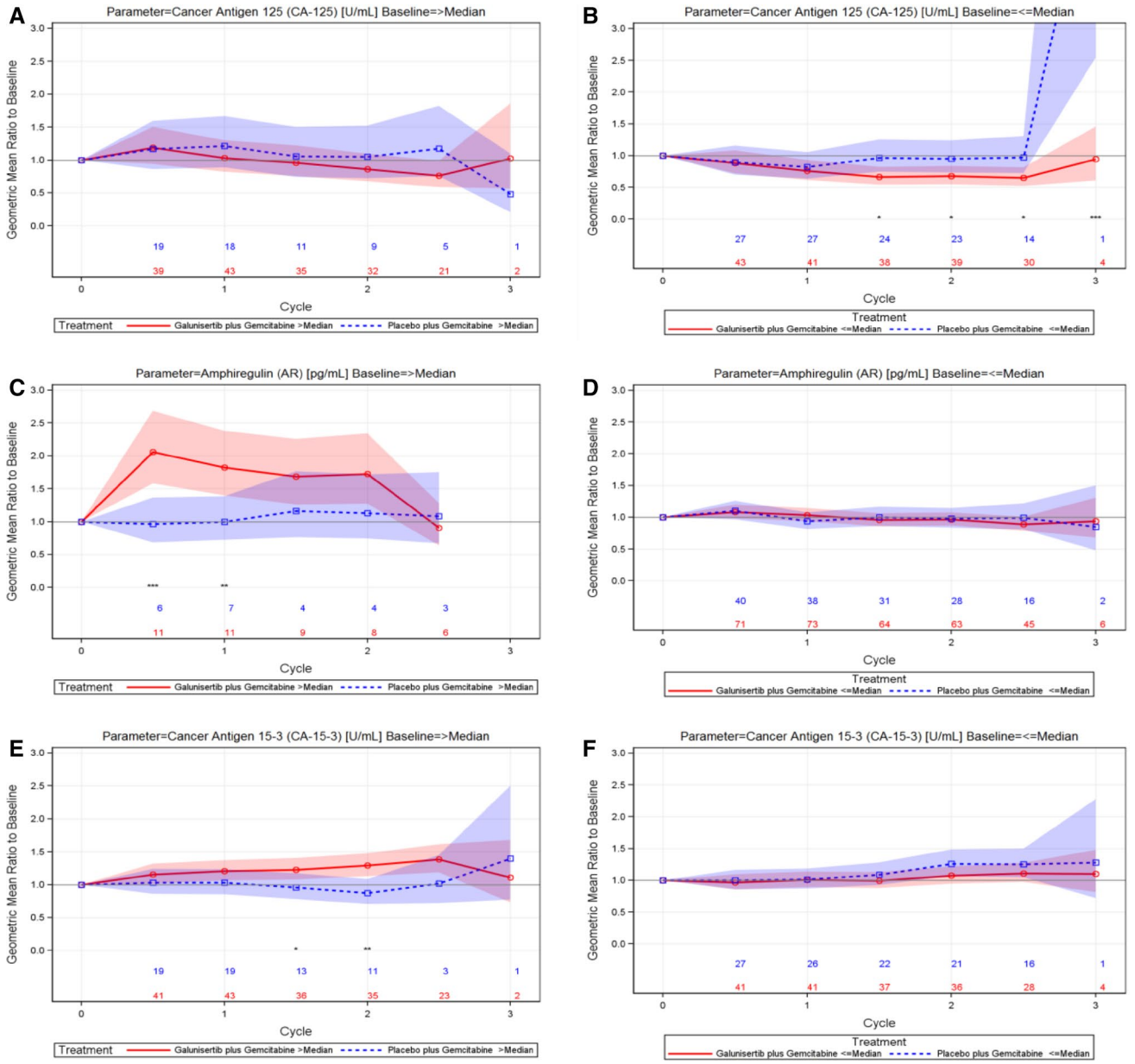

Fig. 2 Posttreatment changes for selected markers from MAP by treatment arm. Each circulating protein is represented by two panels: Panels on the left represent patients with baseline values greater than the median (**a, c, e, g, i, k, m, o, q, s, u, w**) and on the right patients whose baseline values were less or equal to the median (**b, d, f, h, j, l, n, p, r, t, v, x**). The red and the blue lines for each panel represent the geometric mean ratio to baseline. The colored band represent the 95% confidence interval associated with the mean ratio to baseline. Numbers at the bottom of each panel represent the measurement of patients evaluated. Comparison of galunisertib plus gemcitabine vs. placebo plus gemcitabine, $*P<0.05$, $**P<0.01$, $***P<0.001$

rate of decrease ($>-1.0\%$, where -1.0% = median cut) had improved OS relative to patients with a lower rate of decrease ($\leq -1.0\%$), median OS = 12.3 months vs. 7.7 months ($P=0.0146$) (data not shown). For patients who achieved a steeper rate of decrease of TGF-β1, median OS was numerically improved for galunisertib + gemcitabine compared to placebo + gemcitabine, 12.7 months vs. 9.7 months ($P=0.1892$) (Supplemental Fig. 2c). The rate of decrease of TGF-β1 was not correlated to the rate of decrease of CA19-9; and for patients with a steeper rate of decrease, evaluation of known prognostic factors appeared balanced between treatment arms. Taken together, these findings suggest that patients who are able to achieve a steeper rate or more consistent rate of TGF-β1 decrease after treatment may have improved survival and, while not statistically confirmed, the OS benefit may be enhanced in

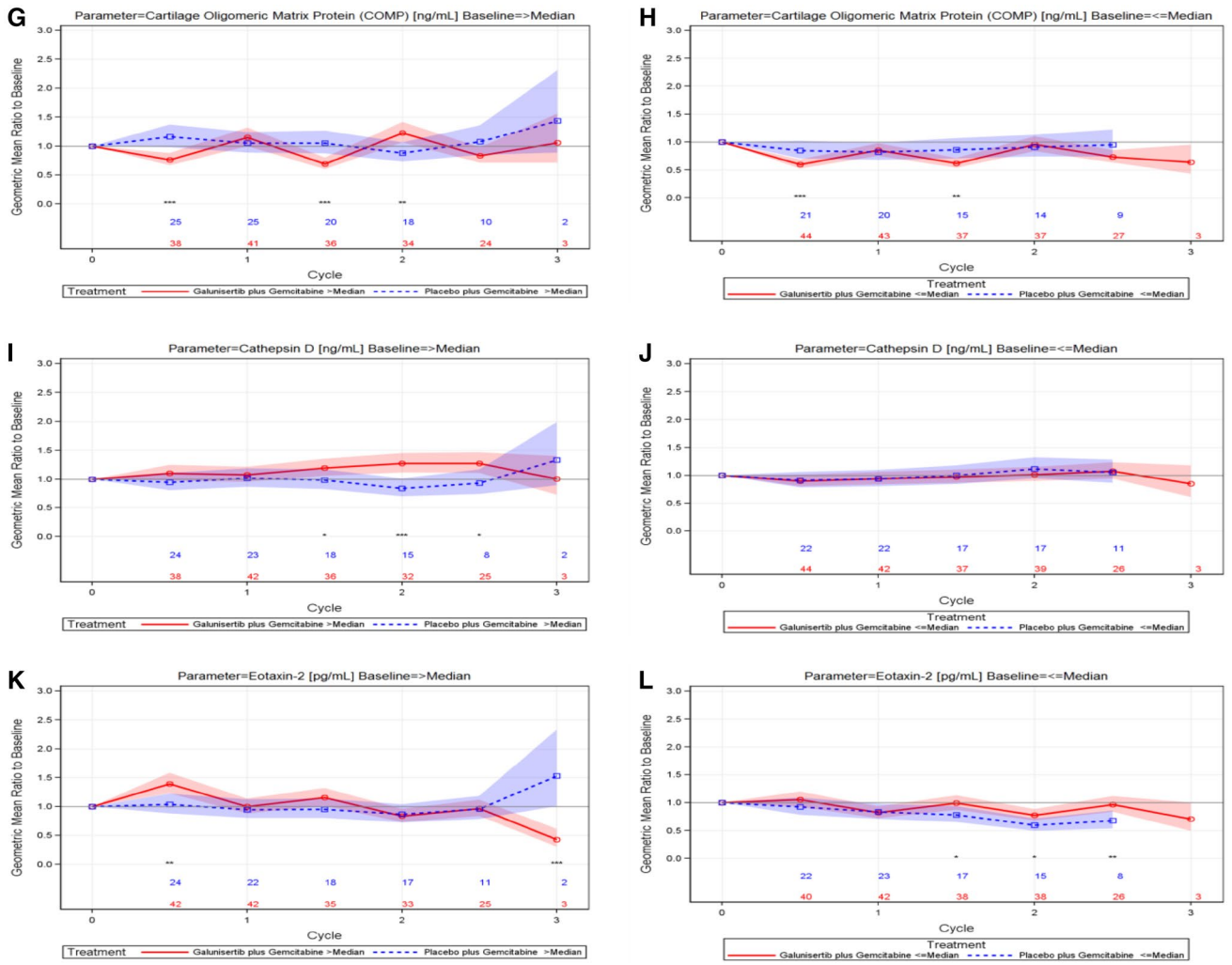

Fig. 2 (continued)

the galunisertib + gemcitabine vs. placebo + gemcitabine arm.

Multi-analyte immunoassay panel

The MAP developed by Myriad RBM consisted of approximately 279 plasma proteins, of which 30 proteins had > 50% of samples below the limit of quantification at baseline and were excluded, leaving 249 proteins evaluable for analysis. Of the 249 evaluable proteins, 31 were identified to be prognostic for OS ($P < 0.01$) using the univariate Cox regression model (Table 2). Osteocalcin and transthyretin were found to be associated with improved OS when baseline levels were high (> median) [18, 19]. All other parameters were associated with improved OS when baseline levels were low (≤ median).

Additionally, 18 proteins were identified to be potentially predictive for response to galunisertib (Table 3). The 4 statistically most significant proteins were interferon gamma induced protein 10 (IP-10) [pg/mL], follicle-stimulating hormone (FSH) [mIU/mL], macrophage inflammatory protein-1 alpha (MIP-1 alpha) [pg/mL], and plasminogen activator inhibitor 1 (PAI-1) [ng/mL] ($P < 0.01$) (Table 3).

Changes in each of the markers were evaluated after treatment with galunisertib.

Prognostic markers

For the 31 prognostic markers, there was a difference observed between the treatment arms for Cancer Antigen 125 (CA-125) [20], which showed mean decreases from baseline up to approximately 25% for the galunisertib + gemcitabine arm (i.e., <median group), which were not apparent for the placebo + gemcitabine arm ($P < 0.01$) (Supplemental Fig. 4a, b). Small decreases after treatment were observed in both treatment arms for proteins EN-RAGE,

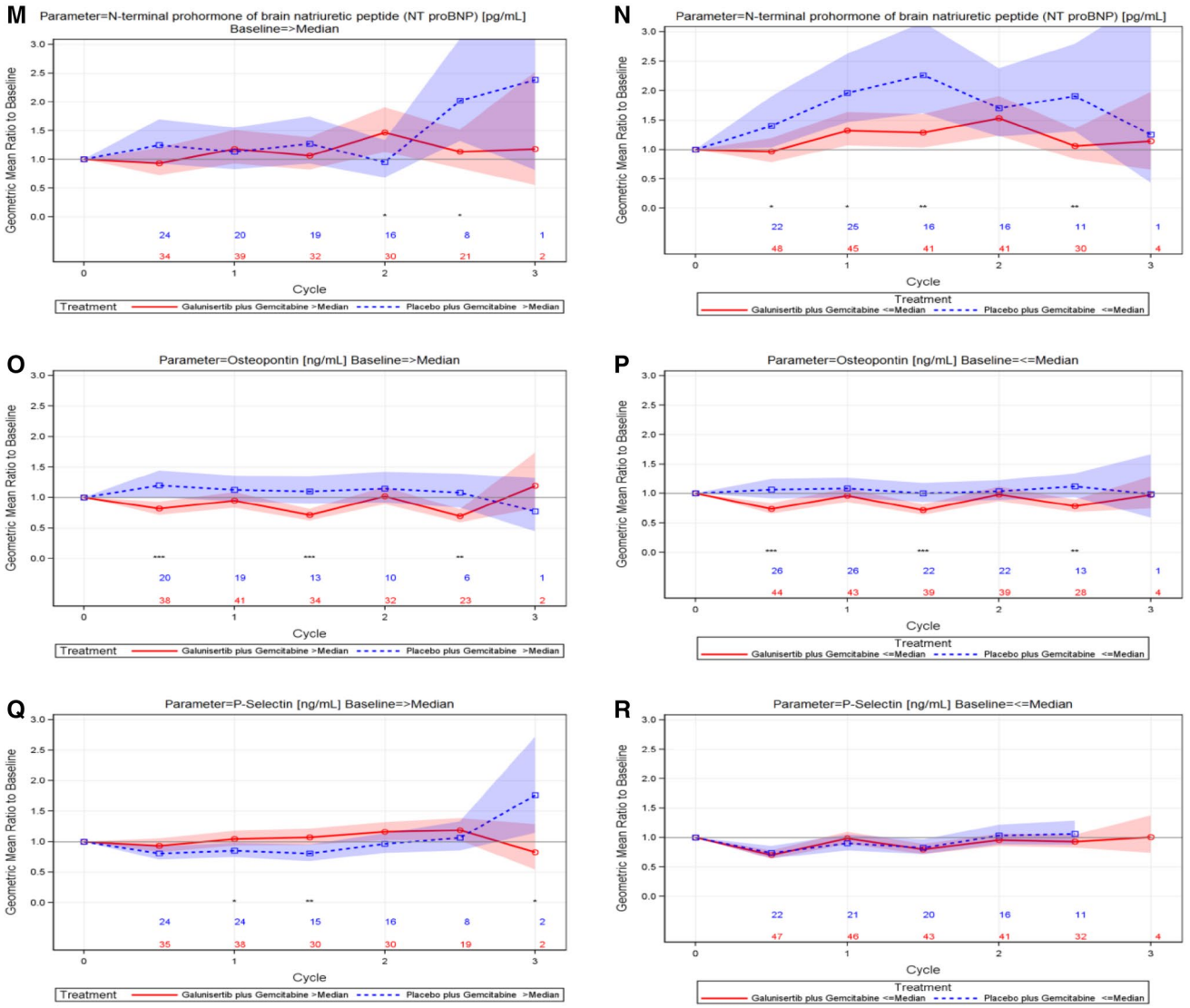

Fig. 2 (continued)

lectin-like oxidized LDL receptor 1 (LOX-1), osteocalcin, TNF-related apoptosis-inducing ligand receptor 3 (TRAIL R3), and transthyretin (TTR). Small increases after treatment were observed for both treatment arms for C-reactive protein (CRP), growth/differentiation factor 15 (GDF-15), human epididymis protein 4 (HE4), interleukin-2 receptor alpha (IL2 receptor alpha), interleukin-8 (IL8), macrophage colony-stimulating factor 1 (M-CSF), serum amyloid A protein (SAA), and tumor necrosis factor receptor I (TNF-R1) (data not shown).

Predictive markers

While there were no significant differences between the treatment arms for the 18 predictive markers measured at baseline, we then reviewed all proteins for possible changes independently of their association of treatment or OS when measured at baseline. Treatment differences were observed for markers among patients with high baseline levels (amphiregulin, CA15-3, cathepsin D, P-selectin, receptor for advanced glyosylation end products [RAGE]) and low baseline levels (sortilin), as well as among all patients regardless of baseline levels (cartilage oligomeric matrix protein, eotaxin-2, N-terminal prohormone of brain natriuretic peptide, osteopontin, and thrombospondin-4).

Because we observed inflammation-associated proteins, we also examined whether some of the plasma proteins were associated with TGF-β1 and T cells as determined by epigenetic $CD3^+$ expression. Modest correlation to TGF-β1 ($r \geq 0.3$) was observed for serum amyloid A protein (SAA), tissue inhibitor of metalloproteinases 1 (TIMP-1), EN-RAGE, alpha-1-antichymotrypsin (AACT), P-selectin,

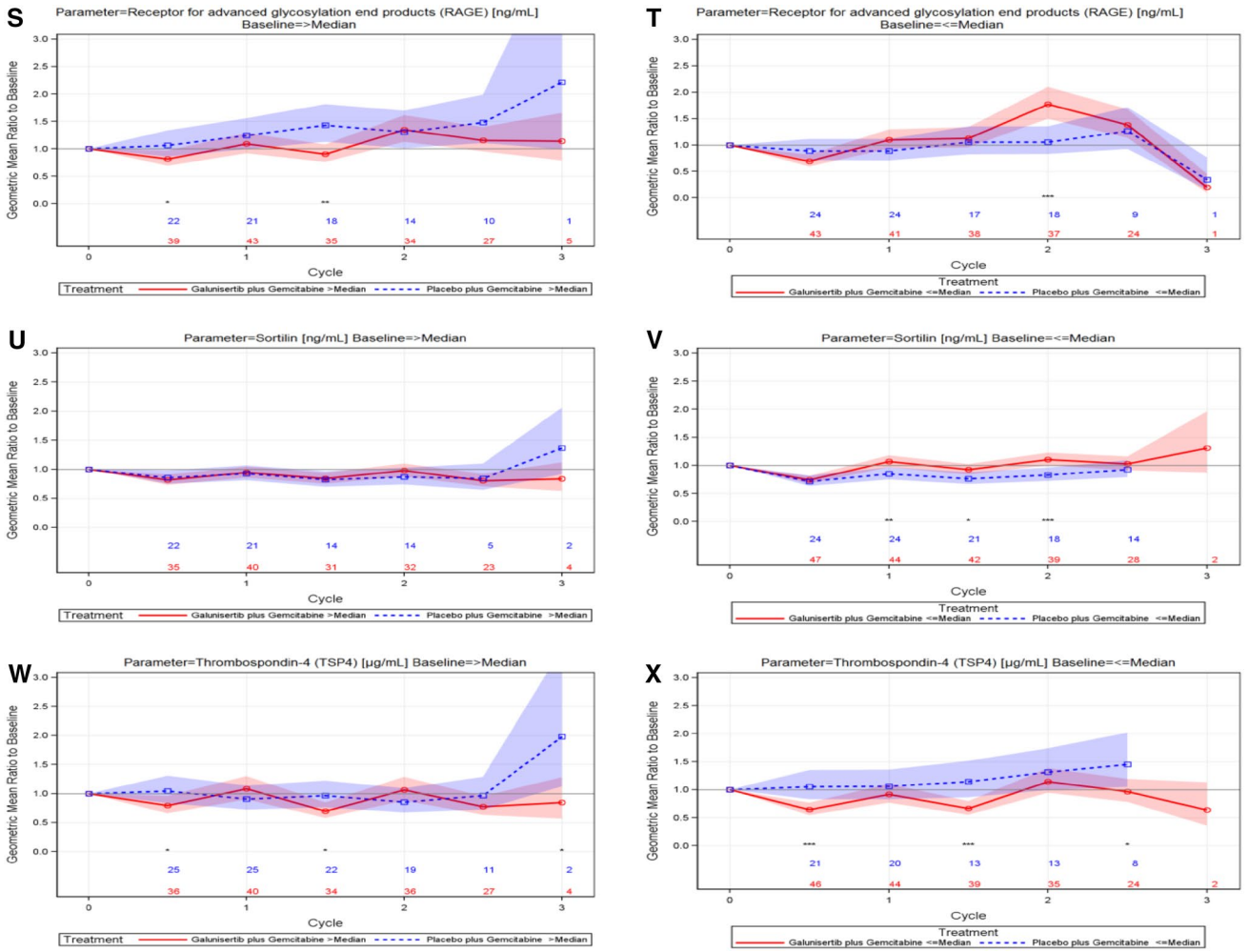

Fig. 2 (continued)

plasminogen activator inhibitor 1 (PAI-1), and osteocalcin. Correlation to CA19-9 was weak (< 0.3) for all parameters. $CD3^+$ epigenetic was negatively correlated to several prognostic markers (Table 4).

Plasma miR

The Exiqon miRCURY LNA Human miRNome PCR panel consisted of 752 miR specific assays, and 167 miRs were consistently detected (≥ 60% of samples with Cq ≤ 35) within the 135 baseline plasma samples from Phase 2 patients available for testing.

Prognostic markers

Focusing upon a median cutoff point only, the 167 consistently detected miRs were analyzed to identify circulating prognostic markers. The 3 most significant ($P < 0.01$) prognostic markers identified were miR-21-5p, miR-210-3p, and miR-148b-3p. High expression of each of these markers was associated with poor OS. Each of these had a hazard ratio (HR) of 2.13, 1.69, and 1.64, respectively.

Predictive markers

High expression of miR-424-5p was associated with better OS in the galunisertib + gemcitabine arm (upper quartile cutoff point, HR = 0.17, interaction $P = 0.004$). High expression of miR-18b-5p was associated with poor OS in the galunisertib + gemcitabine arm (upper quartile cutoff point, HR = 2.13, interaction $P = 0.008$). High expression of miR-10b-5p was also associated with better OS in the galunisertib + gemcitabine arm (lower quartile cutoff point, HR = 0.64, interaction $P = 0.009$). High expression of miR-324-5p was associated with poor OS in the galunisertib + gemcitabine arm (median cutoff point, HR = 1.43, interaction $P = 0.009$).

Table 2 Single-biomarker analysis of prognostic markers: OS comparison for high vs. low marker level at baseline for each treatment arm (italics indicates inflammation-associated proteins) (note that osteocalcin and transthyretin were high at baseline)

Marker	OS comparison high vs. low marker level at baseline HR (95% CI) (*P* value)
Cancer antigen 125 (CA-125) [U/mL]	3.23 (2.24, 4.67) (< 0.0001)
Human epididymis protein 4 (HE4) [pM]	*3.15 (2.19, 4.54) (< 0.0001)*
Interleukin-8 (IL-8) [pg/mL]	*2.66 (1.86, 3.81) (< 0.0001)*
Osteocalcin [ng/mL]	0.38 (0.27, 0.54) (< 0.0001)
Tissue inhibitor of metalloproteinases 1 (TIMP-1) [ng/mL]	2.50 (1.75, 3.56) (< 0.0001)
Osteoprotegerin (OPG) [pM]	2.45 (1.73, 3.49) (< 0.0001)
Tumor necrosis factor receptor I (TNF RI) [pg/mL]	*2.40 (1.69, 3.42) (< 0.0001)*
Fatty acid–binding protein, adipocyte (FABP, adipocyte) [ng/mL]	2.36 (1.65, 3.37) (< 0.0001)
Interleukin-2 receptor alpha (IL-2 receptor alpha) [pg/mL]	*2.26 (1.59, 3.22) (< 0.0001)*
Tumor necrosis factor receptor 2 (TNFR2) [ng/mL]	*2.29 (1.60, 3.27) (< 0.0001)*
Alpha-1-acid glycoprotein 1 (AGP-1) [µg/mL]	2.30 (1.60, 3.29) (< 0.0001)
Urokinase-type plasminogen activator receptor (uPAR) [ng/mL]	2.21 (1.56, 3.15) (< 0.0001)
Growth/differentiation factor 15 (GDF-15) [ng/mL]	2.18 (1.54, 3.09) (< 0.0001)
Macrophage colony-stimulating factor 1 (M-CSF) [ng/mL]	*2.16 (1.52, 3.09) (< 0.0001)*
EN-RAGE [ng/mL]	*2.11 (1.49, 2.99) (< 0.0001)*
Alpha-1-antichymotrypsin (AACT) [µg/mL]	2.10 (1.48, 2.97) (< 0.0001)
Alpha-1-antitrypsin (AAT) [mg/mL]	2.08 (1.46, 2.94) (< 0.0001)
Serum amyloid A protein (SAA) [ng/mL]	*2.01 (1.42, 2.83) (< 0.0001)*
Transthyretin (TTR) [mg/dL]	0.50 (0.35, 0.71) (< 0.0001)
Cystatin-B [ng/mL]	1.99 (1.41, 2.83) (0.0001)
C-reactive protein (CRP) [µg/mL]	*1.94 (1.38, 2.74) (0.0002)*
Leucine-rich alpha-2-glycoprotein (LRG1) [µg/mL]	1.94 (1.37, 2.74) (0.0002)
Trefoil factor 3 (TFF3) [µg/mL]	1.91 (1.35, 2.70) (0.0003)
TNF-related apoptosis-inducing ligand receptor 3 (TRAIL-R3) [ng/mL]	*1.91 (1.35, 2.72) (0.0003)*
Insulin-like growth factor binding protein 4 (IGFBP4) [ng/mL]	1.89 (1.33, 2.68) (0.0003)
P-selectin [ng/mL]	1.88 (1.33, 2.67) (0.0004)
Beta-2-microglobulin (B2M) [µg/mL]	*1.88 (1.32, 2.66) (0.0004)*
Hepatocyte growth factor (HGF) [ng/mL]	1.85 (1.31, 2.62) (0.0005)
Visfatin [ng/mL]	1.84 (1.30, 2.60) (0.0006)
Cathepsin D [ng/mL]	1.82 (1.29, 2.59) (0.0008)
Lectin-like oxidized LDL receptor 1 (LOX-1) [ng/mL]	1.81 (1.28, 2.55) (0.0008)

OS overall survival

Discussion

In the present study, we focused on the relationship of plasma or serum markers and OS in patients treated either with a combination of galunisertib and gemcitabine or gemcitabine and placebo as control. Previously, we had reported that the combination of galunisertib and gemcitabine resulted in significantly improved OS of approximately 2 months [9]. The tumor marker CA19-9 is a well-established marker for progression in pancreatic cancer [17]. In previous studies, the degree of CA19-9 reduction was determined and a reduction of at least 20% from baseline was found to be predictive for a positive treatment effect [17]. There are no such studies for the plasma levels of the cytokine TGF-β1 in patients with pancreatic cancer and subsequent treatment with either standard chemotherapy or a specific inhibitor against the TGF-β signaling pathway.

Comparing both treatment arms, we found no difference between the experimental arm and the control arm in serum CA19-9 and plasma TGF-β1 reduction (Table 1; Fig. 1a). Reduction in CA19-9 after treatment was a significant prognostic factor for OS, consistent with previous reports [17] (Table 1). While the reduction in both markers was similar in both treatment arms, the improved OS for patients achieving reductions compared to those without reductions was significantly different for CA19-9 and less for TGF-β1. This was unexpected because we had previously seen that a reduction in TGF-β1 levels was associated with increased OS in patients with hepatocellular carcinoma.

Table 3 Single-biomarker analysis of predictive markers: OS comparison for high vs. low marker level at baseline for each treatment arm

Marker	Median OS (95% CI)		OS Comparison high vs. low marker at baseline within each treatment arm		OS Comparison Gal + Gem vs. Pbo + Gem by high or low marker level at baseline		*P* value[a]
	High baseline	Low baseline	Gal + Gem: HR (95% CI)	Pbo + Gem: HR (95% CI)	High: HR (95% CI)	Low: HR (95% CI)	
Interferon gamma Induced Protein 10 (IP-10) [pg/mL]	Gal + Gem: 10.9 (8.1, 13.7) Pbo + Gem: 3.6 (2.7, 7.7)	Gal + Gem: 8.2 (5.5, 12.2) Pbo + Gem: 12.5 (7.1, 17.)	0.81 (0.53, 1.25)	2.78 (1.55, 4.99)	0.40 (0.23, 0.67)	1.35 (0.83, 2.21)	0.0010
Follicle-Stimulating Hormone (FSH) [mIU/mL]	Gal + Gem: 9.1 (7.1, 10.9) Pbo + Gem: 12.6 (7.1, 23.8)	Gal + Gem: 9.0 (5.5, 15.5) Pbo + Gem: 4.1 (3.0, 7.7)	1.20 (0.78, 1.85)	0.39 (0.22, 0.70)	1.40 (0.83, 2.37)	0.45 (0.27, 0.75)	0.0027
Males only ($n = 85$)	Gal + Gem: 10.4 (1.9, 23.1) Pbo + Gem: 13.7 (5.9, 17.0)	Gal + Gem: 11.8 (6.0, 15.6) Pbo + Gem: 5.5 (3.6, 7.7)	1.22 (0.54, 2.72)	0.58 (0.22, 1.53)	1.03 (0.32, 3.25)	0.49 (0.28, 0.83)	–
Females only (n = 71)	Gal + Gem: 9.1 (7.1, 12.1) Pbo + Gem: 11.3 (2.9, 24.9)	Gal + Gem: 7.0 (2.9, NE) Pbo + Gem: 2.9 (0.3, 9.9)	1.17 (0.36, 3.83)	0.20 (0.07, 0.62)	1.52 (0.84, 2.74)	0.26 (0.06, 1.19)	-
Macrophage Inflammatory Protein-1 alpha (MIP-1 alpha) [pg/mL]	Gal + Gem: 9.2 (5.5, 12.4) Pbo + Gem: 3.6 (2.5, 4.0)	Gal + Gem: 8.9 (6.0, 15.6) Pbo + Gem: 10.1 (7.2, 17.0)	1.48 (0.95, 2.29)	3.93 (2.14, 7.22)	0.38 (0.22, 0.66)	1.02 (0.63, 1.65)	0.0089
Plasminogen Activator Inhibitor 1 (PAI-1) [ng/mL]	Gal + Gem: 7.4 (3.8, 10.2) Pbo + Gem: 7.7 (2.9, 13.7)	Gal + Gem: 12.3 (8.5, 16.2) Pbo + Gem: 7.2 (3.6, 8.8)	1.97 (1.27, 3.04)	0.74 (0.42, 1.32)	1.45 (0.88, 2.41)	0.55 (0.33, 0.92)	0.0089
Immunoglobulin E (IgE) [U/mL]	Gal + Gem: 8.8 (5.9, 12.7) Pbo + Gem: 4.0 (2.9, 7.6)	Gal + Gem: 10.4 (7.1, 13.7) Pbo + Gem:12.6 (6.6, 19.9)	0.93 (0.61, 1.44)	2.38 (1.34, 4.23)	0.51 (0.31, 0.84)	1.30 (0.77, 2.19)	0.0110
Thrombin-Activatable Fibrinolysis (TAFI) [µg/mL]	Gal + Gem: 8.9 (6.7, 12.3) Pbo + Gem: 9.9 (5.7, 16.4)	Gal + Gem:10.7 (5.9, 14.2) Pbo + Gem: 5.5 (2.9, 7.9)	1.39 (0.90, 2.15)	0.55 (0.31, 0.98)	1.32 (0.79, 2.19)	0.52 (0.31, 0.87)	0.0129
Immunoglobulin M (IgM) [mg/mL]	Gal + Gem: 8.9 (6.0, 12.2) Pbo + Gem: 12.5 (5.7, 21.8)	Gal + Gem:10.9 (5.9, 14.2) Pbo + Gem: 5.5 (3.3, 7.9)	1.09 (0.71, 1.68)	0.44 (0.24, 0.80)	1.36 (0.79, 2.34)	0.55 (0.34, 0.89)	0.0149
Chemokine CC-4 (HCC-4) [ng/mL]	Gal + Gem: 12.2 (8.9, 15.5) Pbo + Gem: 6.6 (3.6, 9.9)	Gal + Gem: 7.8 (5.5, 9.1) Pbo + Gem: 7.6 (3.6, 15.4)	0.71 (0.46, 1.10)	1.71 (0.95, 3.08)	0.52 (0.31, 0.90)	1.26 (0.77, 2.05)	0.0194
6Ckine [pg/mL]	Gal + Gem: 8.9 (5.5, 12.1) Pbo + Gem: 4.1 (2.9, 7.2)	Gal + Gem: 10.9 (8.1, 15.5) Pbo + Gem: 13.7 (7.1, 23.8)	1.19 (0.77, 1.83)	2.73 (1.52, 4.90)	0.53 (0.32, 0.87)	1.21 (0.72, 2.04)	0.0250
Vascular endothelial growth factor C (VEGF-C) [ng/mL]	Gal + Gem: 7.1 (3.8, 10.2) Pbo + Gem: 8.1 (3.7, 13.7)	Gal + Gem: 12.2 (8.5, 15.6) Pbo + Gem: 6.6 (3.0, 9.9)	1.90 (1.23, 2.93)	0.84 (0.47, 1.48)	1.37 (0.82, 2.27)	0.60 (0.36, 1.00)	0.0260
Angiogenin [ng/mL]	Gal + Gem: 9.0 (5.5, 13.7) Pbo + Gem: 5.7 (3.0, 7.7)	Gal + Gem: 9.1 (7.1, 12.4) Pbo + Gem:12.5 (5.5, 23.8)	1.21 (0.78, 1.87)	2.73 (1.50, 4.95)	0.55 (0.33, 0.90)	1.24 (0.73, 2.09)	0.0273

Table 3 (continued)

Marker	Median OS (95% CI)		OS Comparison high vs. low marker at baseline within each treatment arm		OS Comparison Gal + Gem vs. Pbo + Gem by high or low marker level at baseline		*P* value[a]
	High baseline	Low baseline	Gal + Gem: HR (95% CI)	Pbo + Gem: HR (95% CI)	High: HR (95% CI)	Low: HR (95% CI)	
Macrophage inflammatory protein 3 beta (MIP-3 beta) [pg/mL]	Gal + Gem: 10.2 (6.0, 12.7) Pbo + Gem: 5.7 (2.9, 7.9)	Gal + Gem:8.5 (5.5, 13.7) Pbo + Gem: 9.9 (4.1, 17.0)	0.83 (0.54, 1.28)	1.83 (1.03, 3.25)	0.55 (0.33, 0.92)	1.21 (0.74, 1.99)	0.0304
Monokine induced by gamma interferon (MIG) [pg/mL]	Gal + Gem: 10.9 (8.1, 13.7) Pbo + Gem: 4.0 (2.9, 8.1)	Gal + Gem: 8.5 (5.7, 12.3) Pbo + Gem: 10.0 (6.6, 19.9)	0.96 (0.62, 1.48)	2.11 (1.19, 3.77)	0.56 (0.34, 0.92)	1.23 (0.73, 2.06)	0.0319
Collagen IV [ng/mL]	Gal + Gem: 8.5 (4.5, 10.7) Pbo + Gem: 8.8 (3.6, 12.6)	Gal + Gem: 13.6 (8.1, 16.2) Pbo + Gem: 7.2 (3.3, 9.9)	1.69 (1.09, 2.59)	0.77 (0.44, 1.37)	1.29 (0.78, 2.13)	0.59 (0.35, 0.98)	0.0333
Beta-2-Microglobulin (B2M) [µg/mL]	Gal + Gem: 8.5 (4.2, 10.9) Pbo + Gem: 3.6 (2.9, 5.9)	Gal + Gem: 10.9 (7.8, 15.9) Pbo + Gem:13.1 (7.6, 19.9)	1.46 (0.94, 2.26)	3.19 (1.77, 5.73)	0.52 (0.31, 0.89)	1.14 (0.70, 1.86)	0.0348
Thymus and activation-regulated chemokine (TARC) [ng/mL]	Gal + Gem: 7.8 (4.5, 10.4) Pbo + Gem: 5.9 (3.0, 12.5)	Gal + Gem: 12.3 (8.5, 16.2) Pbo + Gem: 8.8 (3.6, 12.6)	1.95 (1.25, 3.05)	0.89 (0.50, 1.59)	1.27 (0.75, 2.16)	0.58 (0.35, 0.96)	0.0376
Fatty acid-binding protein, liver (FABP, liver) [ng/mL]	Gal + Gem: 8.8 (5.5, 10.9) Pbo + Gem:7.6 (2.9, 16.4)	Gal + Gem: 10.9 (7.8, 12.7) Pbo + Gem: 7.2 (3.6, 9.9)	1.33 (0.87, 2.05)	0.61 (0.34, 1.11)	1.32 (0.76, 2.29)	0.60 (0.37, 0.97)	0.0398
Interleukin-31 (IL-31) [ng/mL]	Gal + Gem: 6.0 (2.7, 8.5) Pbo + Gem: 9.9 (3.6, 19.9)	Gal + Gem: 10.7 (8.5, 13.7) Pbo + Gem: 7.2 (3.6, 9.7)	1.94 (1.16, 3.23)	0.83 (0.44, 1.57)	1.69 (0.83, 3.41)	0.72 (0.48, 1.09)	0.0425

Gal galunisertib, *Gem* gemcitabine, *OS* overall survival, *Pbo* placebo

[a]*P* value for the interaction of treatment and baseline category using Cox regression model

Additional plasma markers and also T-cell subsets were examined to further understand the impact of adding galunisertib to gemcitabine treatment. First, we determined the baseline characteristics of all patients for plasma markers that may be considered prognostic (Table 2). We observed that at least 30 plasma proteins were associated with improved OS regardless of the subsequent treatment. Some of these plasma proteins are generally associated with inflammatory conditions, such as HE4, IL-8, TNF-RI, IL-2Ra, M-CSF, and CRP. Other proteins are associated with tissue remodeling, including TIMP-1 or uPAR, or tissue metabolism, such as AGP-1 or AAT. Interestingly, patients with high baseline levels of osteocalcin and transthyretin had a better OS, which suggests that in pancreatic cancer both these proteins play a possible protective role. Given the small size of this study these findings may be spurious or a result of an indirect effect. As a second step, we assessed whether any of the baseline proteins were associated with OS by either galunisertib + gemcitabine or placebo + gemcitabine treatment (Table 3). We found 18 plasma proteins, among which β2-microglobulin was also considered a prognostic protein. This group of 18 proteins appears to be limited to 2 groups: (1) inflammation-associated proteins (e.g., IP-10, MIP-1a, IgE, IgM, HCC-4, 6Ckine, MIP-3b, MIG, β-2M, TARC, and IL-31); (2) tissue remodeling (PAI-1, TAFI, VEGF-C, angiogenin, collagen IV, and FABP). The role of FSH in pancreatic cancer has been described before [21], but in this case may be influenced by sex. In addition, CA15-3 may play an important role as an immunogenic mucin for patients with pancreatic cancer. Two of the top 4

Table 4 Spearman's correlation of baseline prognostic and predictive markers to TGFβ and $CD3^+$ epigenetic

	Marker	Correlation to $CD3^+$ epigenetic	Correlation to TGFβ
Prognostic markers (Table 2)	Serum amyloid A protein (SAA) [ng/mL]	*−0.49*	*0.30*
	Alpha-1-antichymotrypsin (AACT) [μg/mL]	*−0.46*	*0.33*
	C-reactive protein (CRP) [μg/mL]	*−0.45*	*0.25*
	Leucine-rich alpha-2-glycoprotein (LRG1) [μg/mL]	*−0.44*	*0.29*
	EN-RAGE [ng/mL]	*−0.43*	*0.33*
	Alpha-1-acid glycoprotein 1 (AGP-1) [μg/mL]	*−0.43*	*0.29*
	Tumor necrosis factor receptor I (TNF RI) [pg/mL]	*−0.41*	*0.11*
	Cancer antigen 125 (CA-125) [U/mL]	*−0.38*	*0.25*
	Interleukin-2 receptor alpha (IL-2 receptor alpha) [pg/mL]	*−0.36*	*0.23*
	Tissue inhibitor of metalloproteinases 1 (TIMP-1) [ng/mL]	*−0.33*	*0.32*
	Visfatin [ng/mL]	*−0.33*	*0.28*
	TNF-related apoptosis-inducing ligand receptor 3 (TRAIL-R3) [ng/mL]	*−0.33*	*0.12*
	Interleukin-8 (IL-8) [pg/mL]	*−0.33*	*0.27*
	Tumor necrosis factor receptor 2 (TNFR2) [ng/mL]	*−0.31*	*0.10*
	Macrophage colony-stimulating factor 1 (M-CSF) [ng/mL]	*−0.31*	*0.17*
	Alpha-1-antitrypsin (AAT) [mg/mL]	−0.28	0.18
	Human epididymis protein 4 (HE4) [pM]	−0.28	0.03
	Urokinase-type plasminogen activator receptor (uPAR) [ng/mL]	−0.25	0.16
	Osteoprotegerin (OPG) [pM]	−0.25	0.18
	Cystatin-B [ng/mL]	−0.23	0.22
	Fatty acid–binding protein, adipocyte (FABP, adipocyte) [ng/mL]	−0.22	0.07
	Insulin-like growth factor binding protein 4 (IGFBP4) [ng/mL]	−0.20	−0.03
	Growth/differentiation factor 15 (GDF-15) [ng/mL]	−0.18	0.14
	Lectin-like oxidized LDL receptor 1 (LOX-1) [ng/mL]	−0.17	0.15
	Hepatocyte growth factor (HGF) [ng/mL]	−0.13	0.20
	Trefoil factor 3 (TFF3) [μg/mL]	−0.11	0.17
	Osteocalcin [ng/mL]	0.21	−0.30
	Transthyretin (TTR) [mg/dL]	0.27	−0.20
Predictive markers (Table 3)	Macrophage inflammatory protein 3 beta (MIP-3 beta) [pg/mL]	−0.17	0.12
	Plasminogen activator inhibitor 1 (PAI-1) [ng/mL]	−0.15	0.36
	Chemokine CC-4 (HCC-4) [ng/mL]	−0.14	−0.12
	6Ckine [pg/mL]	−0.12	0.08
	Angiogenin [ng/mL]	−0.11	0.01
	Collagen IV [ng/mL]	−0.11	0.21
	Immunoglobulin M (IgM) [mg/mL]	−0.10	0.16
	Macrophage inflammatory protein-1 alpha (MIP-1 alpha) [pg/mL]	−0.09	0.12
	Interferon gamma–induced protein 10 (IP-10) [pg/mL]	−0.08	−0.10
	Monokine induced by gamma interferon (MIG) [pg/mL]	−0.07	−0.11
	Fatty acid–binding protein, liver (FABP, liver) [ng/mL]	−0.06	0.16
	Interleukin-31 (IL-31) [ng/mL]	−0.02	0.07
	Vascular endothelial growth factor C (VEGF-C) [ng/mL]	−0.02	0.22
	Immunoglobulin E (IgE) [U/mL]	0.03	0.13
	Thymus and activation-regulated chemokine (TARC) [ng/mL]	0.03	0.22
	Follicle-stimulating hormone (FSH) [mIU/mL]	0.06	−0.03
	Thrombin-activatable fibrinolysis (TAFI) [μg/mL]	0.10	0.02
Prognostic and predictive (Tables 2, 3)	Beta-2-microglobulin (B2M) [μg/mL]	−0.21	0.05

Table 4 (continued)

	Marker	Correlation to $CD3^+$ epigenetic	Correlation to TGFβ
Additional markers identified of interest (Supplemental Fig. 4)	P-selectin [ng/mL]	−0.26	0.35
	Cancer antigen 15-3 (CA-15-3) [U/mL]	−0.25	0.07
	Osteopontin [ng/mL]	−0.21	0.10
	Sortilin [ng/mL]	−0.18	0.37
	N-terminal prohormone of brain natriuretic peptide (NT proBNP) [pg/mL]	−0.14	0.03
	Amphiregulin (AR) [pg/mL]	−0.12	0.11
	Cathepsin D [ng/mL]	−0.12	0.21
	Eotaxin-2 [pg/mL]	0.02	0.09
	Thrombospondin-4 (TSP4) [µg/mL]	0.12	0.04
	Receptor for advanced glycosylation end products (RAGE) [ng/mL]	0.15	−0.07
	Cartilage oligomeric matrix protein (COMP) [ng/mL]	0.16	−0.04
Correlation of CD3 and TGFβ			−0.17

Italics indicate prognostic markers with moderate correlation to $CD3^+$ epigenetic ($r < -0.3$). Given the exploratory nature of this study, a moderate correlation was considered to be of absolute magnitude 0.3–0.5

circulating proteins with a significant association with OS in the galunisertib + gemcitabine arm are known chemokines that influence T-cell migration. IP-10, a negative prognostic factor for pancreatic cancer, is associated with high stromal cell content and presence of intratumor regulatory T cells (Tregs) [22]. Additionally, TGF-β1 can induce expression of IP-10 and its receptor CXCR3 in some cell types [23, 24]. MIP1a/CCL3 the other chemokine significantly associated with OS is expressed by Tregs and influences the migration of $CD4^+$ and $CD8^+$ T cells [25]. TGF-β1 can induce expression of MIP1a in mouse macrophages and fibroblasts like synoviocytes [26, 27]. Therefore, it is possible that IP-10 and/or MIP1a may play a TGF-β-dependent role in pancreatic tumors establishing an immune-suppressive microenvironment. This could mechanistically explain how TGF-β inhibition mediates better tumor growth control in patients with high levels of IP-10 and MIP1a. Low levels of PAI1 and FSH were associated with better OS in the galunisertib + gemcitabine arm. PAI1 expression is increased by TGF-β1 and functions in cell migration, invasion, and EMT [28].

As a third step, we examined whether any of these baseline proteins were altered during the treatment with galunisertib + gemcitabine. We did not observe that galunisertib + gemcitabine treatment changed the levels of these 18 proteins. Perhaps patients who will benefit from the galunisertib + gemcitabine treatment must have a particular immune or inflammatory condition at baseline. Similar suggestions have been postulated for PD1 inhibitors, where chemokines at baseline and during treatment were associated with a better subsequent response to PD1 inhibition. However, in this study the presence of gemcitabine does not allow for a clear assessment, especially because gemcitabine may have an impact on immune cell function. In fact, we observed a reduction in most T-cell subsets in both treatment arms, which may explain why we were not able to observe a change in chemokines in association with galunisertib treatment (data not shown).

Because TGF-β signaling inhibition affects inflammatory pathways, we assessed all the plasma proteins during the treatment in both arms by grouping the plasma proteins either as those high or low at baseline. Interestingly, we observed modulation of 10 proteins that were associated mainly with the galunisertib + gemcitabine treatment. The following are the observed markers: amphiregulin, CA15-3 (Muc-1), cathepsin D. P-selectin, sortilin, RAGE, thrombospondin-4, COMP, eotaxin 2 (CCL24), NT-proBNP, and osteopontin. All these markers were altered during treatment with galunisertib + gemcitabine, mostly by being increased. At this time we do not know whether these markers are increased because the overall immune function is altered in patients or whether they reflect progression while patients are on treatment. For example, it is possible that some proteins are shed as a response to the treatment and hence they reflect an improvement of the microenvironment that enhances the immune response against the tumor.

Tumors often release miRs into circulation that can be measured and used as disease diagnostic and/or prognostic markers. Furthermore, the ease of collecting plasma coupled with the stability of plasma miRs led us to evaluate the utility of miRs as potential biomarkers predictive of galunisertib efficacy in patients with pancreatic cancer. Two of the four plasma miRs identified as candidate predictive markers—miR-424-5p and miR-10b-5p—were

previously determined to be overexpressed in pancreatic cancer [29, 30]. Furthermore, each has been linked with TGF-β biology in different cellular/disease models. TGF-β exposure increased miR-424 expression in mammary epithelial cells [31] and decreased protein expression of Smurf2, a negative regulator of TGF-β signaling in myofibroblast differentiation (during EMT in idiopathic pulmonary fibrosis) [32]. Additionally, miR-424-5p was overexpressed in gastric cancer, and was shown to target the TGF-β pathway protein Smad in this disease context [33]. Plasma miRNA miR-10b has been shown to promote TGF-β cancer promoting action in pancreatic cancer [34] and mediate TGF-β1 glioblastoma proliferation, migration, and EMT [35]. These observations indicate that it is possible that these miRs may modulate TGF-β biology in the context of pancreatic cancer. Additional experiments are needed to confirm the predictive and functional associations.

We also identified circulating miRs that were prognostic. The 3 most statistically significant prognostic miRs identified here (miR-21, miR-210, and miR-148b) were each previously shown to be prognostic in plasma or tumor tissue of patients with pancreatic cancer [36–39]. However, only miR-21 was directionally aligned with the published results, that is, both this report and previous reports indicate that high circulating miR-21 was associated with poor survival. In contrast with previous reports, we observed that elevated circulating levels of miR-210 are associated with poor survival. Published results indicate that high tumor expression of miR-210 was associated with poor survival [40], but higher plasma levels were associated with better survival [36]. Low tumor miR-148b expression was previously associated with poor survival [39]. These discrepancies may be explained by the use of different platforms to measure the miRs, different normalization strategies, or simply due to differences between tumor and plasma tissue.

In summary, we observed that patients with higher levels of a specific set of immune-suppressive chemokines at baseline benefit to a greater extent from treatment with galunisertib + gemcitabine. The exact mechanisms to explain this pre-existing condition remain to be explored, but our results indicate that some inflammation- and remodeling-associated proteins are involved in this response.

Acknowledgements The study team thanks patients and families for their willingness to participate in this study. We also thank all site staff and investigators at the institutions, and the trial personnel at Eli Lilly and Company and Covance. Work in the unit of the corresponding author was supported by the Investigator Grant no. 19111 through the Associazione Italiana per la Ricerca sul Cancro, Italy. Writing assistance was provided by Ananya Biswas (Eli Lilly Services India Pvt. Ltd., India).

Funding This study was funded by Eli Lilly and Company, Indianapolis, Indiana, USA (Grant Number 230-0061-07472).

Compliance with ethical standards

Conflict of interest Ann Cleverly, Ivelina Gueorguieva, Karim A. Benhadji, Shawn T. Estrem, Kyla Driscoll, and Michael Man are employees of Eli Lilly and Company, Indianapolis, Indiana, USA, and may hold company stock. Michael M. F. Lahn and Claire Smith are former employees of Eli Lilly and Company and hold company stock. Josep Tabernero has had an advisory role for Bayer, Boehringer Ingelheim, Genentech/Roche, Lilly, MSD, Merck Serono, Merrimack, Novartis, Peptomyc, Roche, Sanofi, Symphogen, and Taiho. Denis Pezet has had an advisory role for Sanofi, Novartis, and Roche.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Seufferlein T, Bachet JB, Van Custem E, Rougier P, ESMO Guidelines Working Group (2012) Pancreatic adenocarcinoma: ESMO-ESDO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 23(suppl 7):vii33–vii40
2. Von Hoff DD, Ervin T, Arena FP et al (2013) Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 369(18):1691–1703
3. National Comprehensive Cancer Network (NCCN) (2016) NCCN clinical practice guidelines in oncology: Pancreatic adenocarcinoma v.1.2014. http://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf. Accessed Oct 2018
4. Long J, Zhang Y, Yu X et al (2011) Overcoming drug resistance in pancreatic cancer. Expert Opin Ther Targets 15(7):817–828
5. Villanueva A, García C, Paules AB et al (1998) Disruption of the antiproliferative TGF-beta signaling pathways in human pancreatic cancer cells. Oncogene 17(15):1969–1978
6. Craven KE, Gore J, Wilson JL, Korc M (2016) Angiogenic gene signature in human pancreatic cancer correlates with TGF-beta and inflammatory transcriptomes. Oncotarget 7(1):323–341
7. Bailey P, Chang DK, Nones K et al (2016) Genomic analyses identify molecular subtypes of pancreatic cancer. Nature 531(7592):47–52
8. Javle M, Li Y, Tan D, Dong X et al (2014) Biomarkers of TGF-beta signaling pathway and prognosis of pancreatic cancer. PLoS One 9(1):e85942
9. Melisi D, Garcia-Carbonero R, Macarulla T et al (2018) Galunisertib plus gemcitabine vs. gemcitabine for first-line treatment of patients with unresectable pancreatic cancer. Br J Cancer 119(10):1208–1214
10. Kozloff M, Garcia-Carbonero R, Nadal T et al (2013) Phase Ib study evaluating safety and pharmacokinetics (PK) of the oral transforming growth factor-beta (TGF-β) receptor I kinase inhibitor LY2157299 monohydrate (LY) when combined with gemcitabine in patients with advanced cancer. In: 2013 ASCO annual meeting, Chicago **(J Clin Oncol 31(suppl; abstr 2563))**
11. Han B, Enas NH, McEntegart D (2009) Randomization by minimization for unbalanced treatment allocation. Stat Med 28(27):3329–3346
12. Kovacs RJ, Maldonado G, Azaro A et al (2015) Cardiac safety of TGF-beta receptor I kinase inhibitor LY2157299 monohydrate in

cancer patients in a first-in-human dose study. Cardiovasc Toxicol 15(4):309–323
13. Rodon J, Carducci MA, Sepulveda-Sánchez JM et al (2015) First-in-human dose study of the novel transforming growth factor-beta receptor I kinase inhibitor LY2157299 monohydrate in patients with advanced cancer and glioma. Clin Cancer Res 21(3):553–560
14. Gueorguieva I, Cleverly AL, Stauber A et al (2014) Defining a therapeutic window for the novel TGF-beta inhibitor LY2157299 monohydrate based on a pharmacokinetic/pharmacodynamic model. Br J Clin Pharmacol 77(5):796–807
15. Baron U, Floess S, Wieczorek G et al (2007) DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. Eur J Immunol 37(9):2378–2389
16. Loh WY, He X, Man M (2015) A regression tree approach to identifying subgroups with differential treatment effects. Stat Med 34(11):1818–1833
17. Chiorean EG, Von Hoff DD, Reni M et al (2016) CA19-9 decrease at 8 weeks as a predictor of overall survival in a randomized phase III trial (MPACT) of weekly nab-paclitaxel plus gemcitabine versus gemcitabine alone in patients with metastatic pancreatic cancer. Ann Oncol 27(4):654–660
18. Shevde LA, Samant RS (2014) Role of osteopontin in the pathophysiology of cancer. Matrix Biol 37:131–141
19. Chen J, Chen LJ, Xia YL et al (2013) Identification and verification of transthyretin as a potential biomarker for pancreatic ductal adenocarcinoma. J Cancer Res Clin Oncol 139(7):1117–1127
20. Haglund C (1986) Tumour marker antigen CA125 in pancreatic cancer: a comparison with CA19-9 and CEA. Br J Cancer 54(6):897–901
21. Damaskos C, Garmpis N, Maratzas T et al (2014) Nuclear receptors in pancreatic tumor cells. Anticancer Res 34(12):6897–6911
22. Lunardi S, Jamieson NB, Lim SY et al (2014) IP-10/CXCL10 induction in human pancreatic cancer stroma influences lymphocytes recruitment and correlates with poor survival. Oncotarget 5(22):11064–11080
23. Liu J, Luo X, Xu Y et al (2016) Single-stranded DNA binding protein Ssbp3 induces differentiation of mouse embryonic stem cells into trophoblast-like cells. Stem Cell Res Ther 7(1):79
24. Inngjerdingen M, Damaj B, Maghazachi AA (2001) Expression and regulation of chemokine receptors in human natural killer cells. Blood 97(2):367–375
25. Patterson SJ, Pesenacker AM, Wang AY et al (2016) T regulatory cell chemokine production mediates pathogenic T cell attraction and suppression. J Clin Invest 126(3):1039–1051
26. Rosengren S, Corr M, Boyle DL (2010) Platelet-derived growth factor and transforming growth factor beta synergistically potentiate inflammatory mediator synthesis by fibroblast-like synoviocytes. Arthritis Res Ther 12(2):R65
27. Moon MY, Kim HJ, Kim JG et al (2013) Small GTPase Rap1 regulates cell migration through regulation of small GTPase RhoA activity in response to transforming growth factor-β1. J Cell Physiol 228(11):2119–2126
28. Lupu-Meiri M, Geras-Raaka E, Lupu R et al (2012) Knock-down of plasminogen-activator inhibitor-1 enhances expression of E-cadherin and promotes epithelial differentiation of human pancreatic adenocarcinoma cells. J Cell Physiol 227(11):3621–3628
29. Wu K, Hu G, He X et al (2013) MicroRNA-424-5p suppresses the expression of SOCS6 in pancreatic cancer. Pathol Oncol Res 19(4):739–748
30. Preis M, Gardner TB, Gordon SR et al (2011) MicroRNA-10b expression correlates with response to neoadjuvant therapy and survival in pancreatic ductal adenocarcinoma. Clin Cancer Res 17(17):5812–5821
31. Llobet-Navas D, Rodriguez-Barrueco R, de la Iglesia-Vicente J et al (2014) The microRNA 424/503 cluster reduces CDC25A expression during cell cycle arrest imposed by transforming growth factor β in mammary epithelial cells. Mol Cell Biol 34(23):4216–4231
32. Xiao X, Huang C, Zhao C et al (2015) Regulation of myofibroblast differentiation by miR-424 during epithelial-to-mesenchymal transition. Arch Biochem Biophys 566:49–57
33. Wei S, Li Q, Li Z, Wang L, Zhang L, Xu Z (2016) miR-424-5p promotes proliferation of gastric cancer by targeting Smad3 through TGF-β signaling pathway. Oncotarget 7(46):75185–75196
34. Ouyang H, Gore J, Deitz S, Korc M (2014) microRNA-10b enhances pancreatic cancer cell invasion by suppressing TIP30 expression and promoting EGF and TGF-β actions. Oncogene 33(38):4664–4674
35. Ma C, Wei F, Xia H et al (2017) MicroRNA-10b mediates TGF-β1-regulated glioblastoma proliferation, migration and epithelial-mesenchymal transition. Int J Oncol 50(5):1739–1748
36. Yu Q, Xu C, Yuan W et al (2017) Evaluation of plasma micrornas as diagnostic and prognostic biomarkers in pancreatic adenocarcinoma: mir-196a and mir-210 could be negative and positive prognostic markers, respectively. Biomed Res Int 2017:6495867
37. Abue M, Yokoyama M, Shibuya R et al (2015) Circulating miR-483-3p and miR-21 is highly expressed in plasma of pancreatic cancer. Int J Oncol 46(2):539–547
38. Wang P, Zhuang L, Zhang J et al (2013) The serum miR-21 level serves as a predictor for the chemosensitivity of advanced pancreatic cancer, and miR-21 expression confers chemoresistance by targeting FasL. Mol Oncol 7(3):334–345
39. Zhao G, Zhang JG, Liu Y et al (2013) miR-148b functions as a tumor suppressor in pancreatic cancer by targeting AMPKα1. Mol Cancer Ther 12(1):83–93
40. Greither T, Grochola LF, Udelnow A et al (2010) Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int J Cancer 126(1):73–80