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Analysis of plasma cytokines and angiogenic factors in patients with pretreated urothelial cancer receiving Pazopanib: the role of circulating interleukin-8 to enhance the prognostic accuracy

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Background: Pazopanib achieved the end point of clinical activity in pretreated patients with urothelial cancer in a single-group, phase 2 trial. The objective was to identify biological predictors of clinical benefit to pazopanib in these patients.

Methods: EDTA blood samples were collected at baseline (T0) and after 4 weeks (T1) of treatment, together with radiological imaging in all 41 patients to analyse plasma circulating angiogenic factor levels by multiplex ELISA plates. Changes from T0 to T1 in marker levels were matched with response with the covariance analysis. Univariable and multivariable analyses evaluated the association with overall survival (OS), adjusted for prespecified clinical variables. Net reclassification improvement (NRI) tested the performance of the recognised Cox model.

Results: Increasing IL8^{T1} level associated with lower response probability at covariance analysis ($P=0.010$). Both IL8^{T0} ($P=0.019$) and IL8^{T1} ($P=0.004$) associated with OS and the prognostic model, including clinical variables and IL8^{T1} best-predicted OS after backward selection. The NRI for this model was 39%. When analysed as a time-varying covariate, IL8^{T1} level <80 pg ml⁻¹ portended significantly greater response (~80%) and 6-month OS (~60%) probability than level ≥80.

Conclusion: IL8-level changes during pazopanib allowed for a prognostic improvement and were associated with response probability.

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Despite a remarkable incidence rate (fourth most common malignancy in men in United States), the progress in the therapeutic paradigm of urothelial cancer (UC), particularly in advanced stages, was stagnant in the last few decades (Gallagher *et al*, 2008). A paradigm shift is needed to advance the field. For patients with advanced disease and who have failed chemotherapy regimens, a variety of single-agent or combination therapies have yielded modest response rates and poor survival estimates. Although vinflunine is approved by the European Medicines Agency for progressive disease after platinum-based therapy, the US Food and Drug Administration has no approved agents (Sonpavde *et al*, 2010). A plethora of unsuccessful phase 2 trials of targeted compounds, either alone or combined with chemotherapy, was tested in UC at different clinical stages. With regards to the antiangiogenic setting, a compelling preclinical rationale fostered clinical research in the field, and results were reported with the use of compounds targeting the vascular-endothelial growth factor (VEGF) receptor axis, including sorafenib, aflibercept, sunitinib, everolimus, and bevacizumab (Dreicer *et al*, 2009; Gallagher *et al*, 2010; Twardowski *et al*, 2010; Bellmunt *et al*, 2011; Hahn *et al*, 2011; Choueiri *et al*, 2012; Seront *et al*, 2012; Balar *et al*, 2013; Galsky *et al*, 2013; Milowsky *et al*, 2013). Despite the negative results achieved in a small study sponsored by the National Cancer Institute in United States (Pili *et al*, 2013), pazopanib was active in our single-group, phase 2 study, whereby an objective response rate of 17.1% was achieved in heavily pretreated patients (Necchi *et al*, 2012). Taking together the results of these trials, an invariably uniform scenario can be drawn consisting of a rather low response rate ranging from 5 to 15%, and a small impact on expected progression-free (PFS) and overall survival (OS). Despite this, a small cohort of extreme responders could be identified by obtaining an incredibly long-term clinical benefit from antiangiogenic compounds. Paradigmatic examples are those observed in the sunitinib trial (one partial response (PR) lasting 24 months; Gallagher *et al*, 2010) and in the everolimus trial (one response duration of 26 months; Iyer *et al*, 2012; Milowsky *et al*, 2013).

This is the reason why further investigations on targeted agents should aim at identifying this class of long-term survivors for whom an antiangiogenic approach might have sense. In the absence of available tissue- and blood-based predictors, we aimed at evaluating circulating angiogenic factors (CAFs) over time in our phase 2 trial of pazopanib. Yet, the role of interleukin-8 (IL8) was anticipated (Necchi *et al*, 2012), and herein we present the full results of circulating biomarker analyses, matched with contextual computed tomography (CT)/positron emission tomography (PET) results. Refining the prognostic ability of recognised clinical factors could facilitate the proper selection of patients for conducting confirmatory trials with pazopanib in this disease as well as the interpretation of retrospective data from phase 2 studies with similar compounds.

PATIENTS AND METHODS

Forty-one patients having failed at least one platinum-based chemotherapy regimen were enrolled in a single-group, phase 2 trial of Pazopanib 800 mg orally daily until disease progression/unacceptable toxicity. Ten millilitres of EDTA plasma samples were collected at baseline and every 4 weeks until drug discontinuation, together with CT and PET/CT restaging. Samples were centrifuged for 20 min at 2200 r.c.f./4 °C and immediately stored at ≤ -20 °C. The amount of cCAFs, such as VEGF, serum VEGF receptor (VEGFR)-1 and -2, stem-cell factor (cKIT), IL6, IL8, and IL12, hepatocyte growth factor, and transforming growth factor- β (TGF β), was determined at baseline (T0) and after 4 weeks of treatment (T1) using commercially available ELISA kits

(R&D Systems Inc., Minneapolis, MN, USA), according to manufacturer's protocols. Samples and standards were added to the wells of a microtitre plate and the different CAFs were captured by the specific antibodies immobilised to the wells of the plate. Successively, a horseradish peroxidase-conjugated detection antibody was added to detect the bound protein. After incubation, the wells were washed and the antigen complex bound to the well was detected by addition of tetramethylbenzidine substrate solution, and a blue colour developed in proportion to the amount of the biomarker present in the sample. Colour development was then stopped, turning the colour in the wells to yellow. The absorbance of the colour was measured at 450 nm, producing a signal that is proportional to the amount of the biomarker bound. Response Evaluation Criteria for Solid Tumors (RECIST) version 1.1 were used to evaluate objective response – primary end point of the trial (Eisenhauer *et al*, 2009). Patients were categorised as responders (complete response (CR), PR, including stable disease (SD)) or non-responders for study purposes. Metabolic responses were based on the European Organisation for Research and Treatment of Cancer criteria (Young *et al*, 1999). The PET CR was defined by the fluorodeoxyglucose (FDG) uptake disappearance in all lesions detected at baseline, whereas PR was defined as a decrease of standard uptake value (SUV) $_{\max} \geq 25\%$. Non-responders were considered as those patients with either a SUV $_{\max}$ decrease $< 25\%$ or any increase of FDG uptake, and the appearance of new focal FDG uptake(s) with anatomical confirmation. The metabolic response evaluation was assessed per patient by a blinded, referral nuclear medicine physician as the sum of SUV $_{\max}$ of the target lesions. Clinical protocol and the informed consents relative to clinical and biological study participation were approved by the Institutional Review Board of the Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. All patients provided written informed consent before study entry.

Statistical methods. Statistical analyses focused on the investigation of CAFs as possible biomarkers. The association structure between biomarkers was investigated by estimating a Pearson's partial correlation matrix, whereby correlations between pairs of biomarkers were adjusted for their associations with others.

Changes in CAF levels between T0 (baseline) and T1 (after 4 weeks of treatment) were tested by means of paired Student's *t*-tests. To investigate whether T1 concentrations were associated with tumour response (either RECIST or PET), covariance analyses were adopted, which are known as the most efficient approach for the analysis of pre-post designs.

The prognostic effect of singly taken CAFs on OS was investigated using Cox proportional hazard regression models, with and without adjustment for clinical variables. For the sake of parsimony, such an adjustment was obtained by using the score of a separate Cox model including the following covariates, chosen on the basis of prior knowledge and previously used (Necchi *et al*, 2012): Eastern Cooperative Oncology Group performance status (0 versus ≥ 1), presence of liver metastases, site of tumour primary (bladder versus upper tract), haemoglobin level at baseline (< 10 versus ≥ 10 g dl $^{-1}$), and number of disease sites (1–2 versus > 2). As regards the biomarkers, T0 levels for all CAFs and T1 levels only for those factors that changed significantly from baseline were modelled in the above analyses. Furthermore, for investigating CAF joint prognostic effect, the factors that achieved a Wald's test *P*-value ≤ 0.10 in either unadjusted or adjusted analyses were entered into a multivariable Cox model. A backward elimination procedure was then used to identify the strongest prognostic biomarkers. Net reclassification improvement (NRI) was calculated to measure the improvement in 6-month prediction yielded by selected CAFs when added to clinical variables in multivariable Cox models. This index is a novel measure of model predictive performance that has been specifically recommended for the

assessment of biomarkers (Pencina *et al*, 2012; Rapsomaniki *et al*, 2012). The NRI is calculated distinctly for event and non-event patients, and the two estimates are then averaged. For completeness, we report all these figures, although the average is more meaningful for clinical interpretation: the closer to one, the better the prognostic improvement towards a reference model, which, in our case, relies on information provided by clinical variables only.

The analyses were carried out using the SAS (SAS Institute Inc., version 9.2) and R 2.15.2 software (<http://www.r-project.org/>, last access September 30th, 2013). Statistical significance was set at the conventional two-sided 5% level.

RESULTS

Updated clinical outcomes. Forty-one patients with UC and treated with at least one dose of pazopanib were enrolled in the study between February 2010 and July 2011. The majority of patients (51%) entered beyond the second line, 17.1% had a PR, and 51.2% had a clinical benefit. Median PFS and OS were 2.6 (95% CI, 1.7–3.7) and 4.7 months (95% CI, 4.2–7.3 months), respectively (Necchi *et al*, 2012).

There were two very long-term responders. The first patient (ID 04) had a PR lasting 32 months and an OS of 37.9 months, in spite of having poor prognostic features, namely, an upper tract UC in origin, third-line treatment for progressive bulky retroperitoneal disease, and the presence of isolated liver metastasis. The second patient (ID 12) had a bladder primary and was treated in

second-line setting for disseminated nodal disease progressing after four cycles of cisplatin and gemcitabine. He obtained a durable SD of 19 months and the OS was 35.9 months.

Circulating biomarker assessment. Although some significant results were achieved when investigating the association structure between CAFs (Supplementary Table 1), no strong correlations (as quantified by a coefficient $\rho > 0.80$) were detected. In particular, the highest levels of correlation were observed between VEGF and VEGFR2 ($\rho = -0.48$), VEGFR1 and IL8 ($\rho = 0.45$), and VEGFR2 and TGF β ($\rho = 0.48$).

Table 1 and box plots of Figure 1 provide a descriptive analysis of pre–post pazopanib treatment biomarker levels. Significant T1–T0 modulation was observed for VEGF ($P < 0.001$), VEGFR2 ($P < 0.001$), cKIT ($P < 0.001$), and IL8 ($P = 0.008$). A 4-week increase was reported for VEGF and IL8 concentrations, whereas VEGFR2 and cKIT levels decreased. A separate description of T0–T1 CAFs changes in the two long-term responders is provided in Supplementary Table 2.

A significant association was detected between tumour RECIST response and IL8^{T1} levels ($P = 0.010$ at covariance analysis). In detail, median IL8^{T0} levels were comparable between responders (67 pg ml⁻¹) and non-responders (67.5 pg ml⁻¹), whereas IL8^{T1} levels differed significantly between the two groups, being 69.8 pg ml⁻¹ in responders (Figure 2A) and 97.6 pg ml⁻¹ in non-responders (Figure 2B). As regards associations with PET response compared with baseline, significant results were achieved for IL12^{T0} levels ($P = 0.039$ from covariance analysis) in contrast to IL8^{T0} and IL8^{T1} levels ($P = 0.111$ each).

Table 1. Median (IQR) CAF circulating levels at T0, T1, and Δ

	T0	T1	Δ^a	P-value ^b
VEGF				
Median (IQR)	124.3 (79.3, 156.7)	183.3 (132.3, 250.7)	61.4 (18.6, 148.7)	<0.001
VEGFR1				
Median (IQR)	136.6 (123.1, 168.1)	133.1 (114.9, 172.9)	-3.2 (-25.5, 28.2)	0.971
VEGFR2				
Median (IQR)	5489.3 (5059.3, 6190.3)	4234.8 (3488.8, 4564.3)	-1385.0 (-1789.5, -955.0)	<0.001
cKIT				
Median (IQR)	13.8 (9.2, 17.6)	10.7 (7.0, 13.1)	-2.8 (-4.9, -1.1)	<0.001
IL6				
Median (IQR)	12.8 (4.4–26.9)	16.7 (8.3–29.7)	3.8 (-4.7, 15.0)	0.067
IL8				
Median (IQR)	67.0 (49.8 – 84.0)	87.3 (55.3 – 102.8)	10.5 (-6.0, 31.5)	0.008
IL12				
Median (IQR)	44.2 (21.9–66.9)	37.1 (25.1–56.5)	-4.4 (-11.4, 5.4)	0.193
HGF				
Median (IQR)	481.5 (364.0 – 671.5)	532.0 (415.5 – 688.5)	82.0 (-38, 151)	0.257
TGFβ				
Median (IQR)	29.0 (26.4 – 35.0)	29.0 (22.9 – 33.5)	-2.5 (-5.8, 2.5)	0.070

Abbreviations: CAF = circulating angiogenic factor; HGF = hepatocyte growth factor; IL = interleukin; IQR = interquartile range; KIT = stem-cell factor; TGF β = transforming growth factor- β ; VEGF(R) = vascular-endothelial growth factor (receptor).

^aT1–T0, values in pg ml⁻¹ (VEGF, VEGFR1, VEGFR2, IL6, IL8, IL12, HGF and TGF β) or ng ml⁻¹ (cKIT).

^bPaired Student's t-test, P-value.

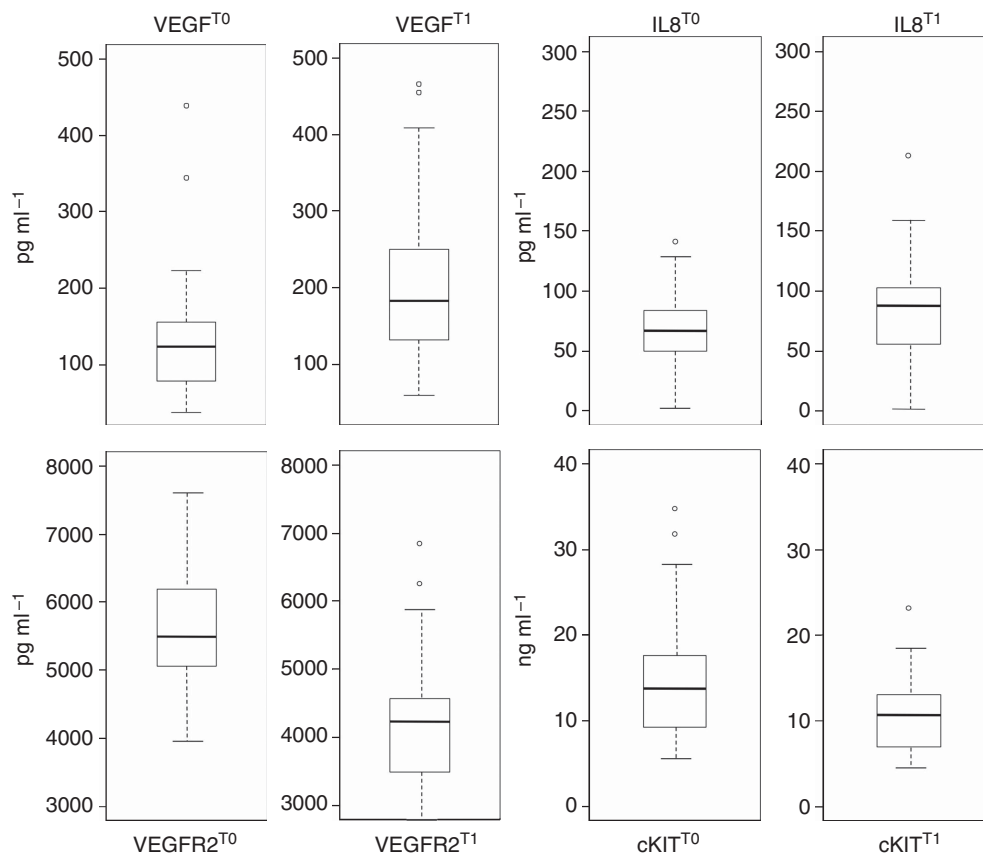


Figure 1. Box plots showing biomarker levels. Box plots showing biomarker levels at baseline (T0) and after 4 weeks of pazopanib (T1). Only those biomarkers that showed a significant change from baseline level are plotted.

Table 2 shows the results of Cox model analyses focusing on the prognostic effects of singly taken CAFs on OS. Significant results were obtained for $IL8^{T0}$ ($P=0.015$), $IL8^{T1}$ ($P=0.012$), $VEGF^{T1}$ ($P=0.007$), and $TGF\beta^{T0}$ ($P=0.038$). After adjustment for the clinical variables, only $IL8^{T0}$ ($P=0.019$) and $IL8^{T1}$ ($P=0.004$) remained significant.

By jointly modelling $IL8^{T1}$, $IL8^{T0}$, $VEGF^{T1}$, $cKIT^{T1}$, and $TGF\beta^{T0}$, which are the biomarkers selected for the multivariable analysis, the average prognostic improvement over clinical variables (as quantified by NRI) was 60% (Table 3, model 1). Nevertheless, by applying a backward variable selection procedure, only $IL8^{T1}$ retained its statistical significance. In this case, the average improvement in prediction was 39%.

By plotting the response probability and 6-month survival (Figure 3A and B) according to $IL8^{T1}$ (and by adjusting for the fixed – median – value of $IL8^{T0}$ at 67 pg ml^{-1}), it turned out that a threshold of 80 pg ml^{-1} might be a reasonable cut-off value for prognostic discrimination; in particular, patients below the threshold show a relatively favourable prognosis for both outcomes.

DISCUSSION

Our study reports a prognostic improvement by adding a biological variable to clinical parameters in the context of an antiangiogenic treatment.

There are a number of preclinical evidences supporting a role for angiogenesis in UC. Angiogenesis and VEGF possess key roles in UC initiation, progression, and invasion. Moreover, investigators have demonstrated an association between VEGF expression

and prognosis of UC, as well as an improved tumour control with platinum-based chemotherapy plus antiangiogenic therapy in preclinical models (Dickinson *et al*, 1994; Jaeger *et al*, 1995; Wu *et al*, 2003). The present proof-of-principle trial showed a consistent pattern of cytokine reaction in UC patients treated with pazopanib and provided circumstantial evidences for the role of microenvironment as a framework of druggable targets in this disease.

To the best of our knowledge, this is the first time that the role of IL8 evaluated as dynamically in relation to response and outcome was obtained in the clinic. The most clinically sound observation was that patients with high baseline and 4-week levels of IL8, and, most importantly, those with rising serum levels of IL8 encompassing 80 pg ml^{-1} at 4 weeks of treatment, had a significantly lower chance of responding and 6-month survival probability. Interleukin-8 level at 4 weeks were then an independent prognostic factor for survival, together with recognised clinical variables.

Results should be taken with caution based on the limitations of the small sample size and the absence of a control arm. Moreover, when looking at the individual patient levels, it turned out that one of the two long-term survivors who achieved a prolonged SD with pazopanib had T1 levels rising to 100.0 pg ml^{-1} (Supplementary Table 2).

Rising levels of IL8 have been already associated with the development of resistance to antiangiogenic agent sunitinib in preclinical models of renal cell carcinoma, but this mechanistic association is hard to be unravelled based on present results (Huang *et al*, 2010). In fact, IL8 is produced by tumour cells of different histologies and raising serum concentrations of this chemokine were associated with tumour burden and increasing stage in a variety of solid neoplasms (Mian *et al*, 2003; Waugh and

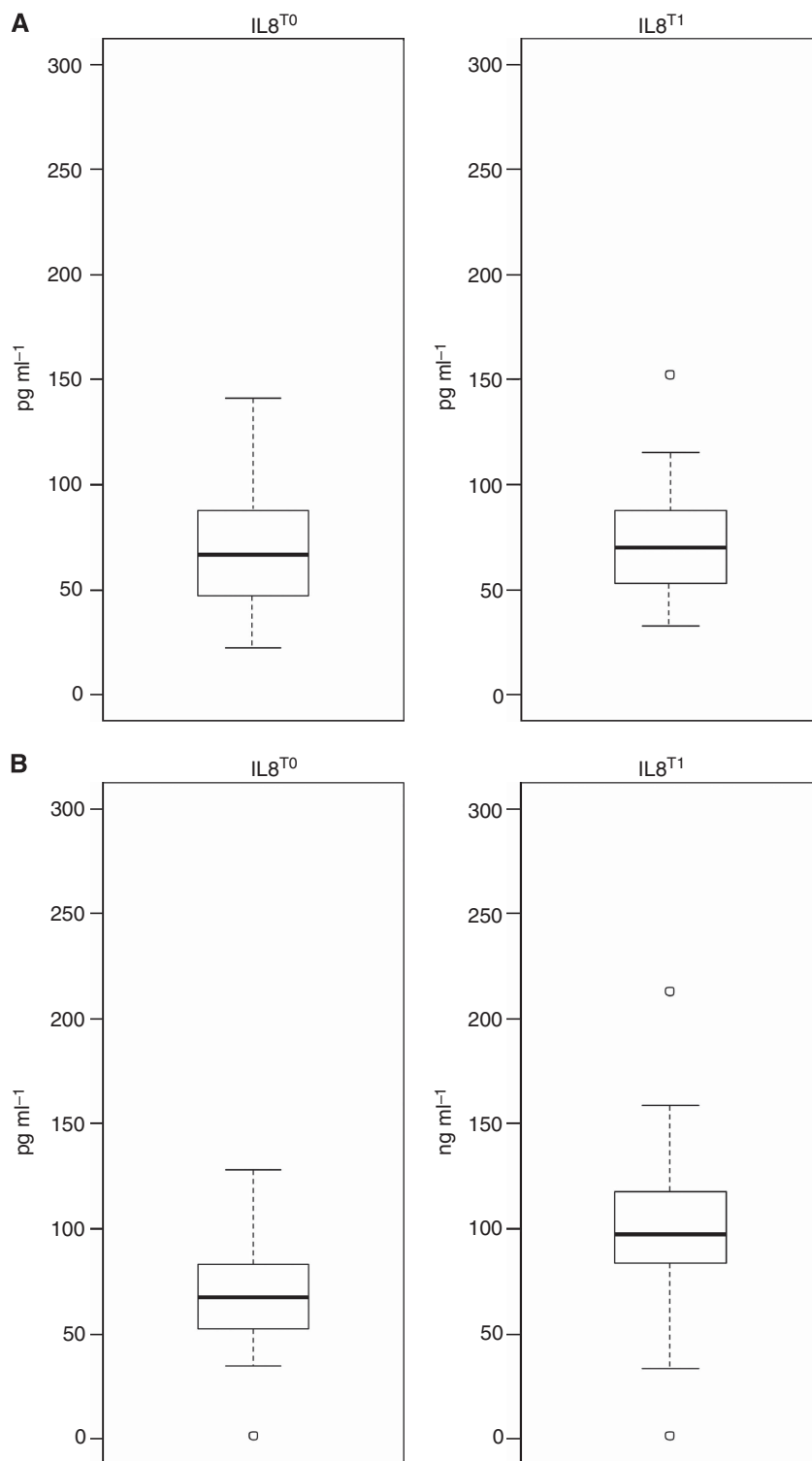


Figure 2. Box plots showing IL^{T0} and IL^{T1}. Box plots showing IL^{T0} and IL^{T1} for both responders (A) and non-responders (B). Responses were defined by RECIST v1.1 criteria after 4 weeks of treatment.

Wilson 2008; Britschgi *et al*, 2012; Lippitz, 2013). Hence, the question whether IL8 levels and their change over time might be solely a drug and tumour-induced epiphenomenon or rather a signal to allow selecting patients who are most likely to respond/survive remains unanswered. On the other hand, IL8 is a recognised mediator of tumour growth and metastatisation potential, and its role as a predictor of clinical benefit has been already reported in bladder cancer and renal cell carcinoma patients receiving sunitinib and pazopanib, respectively (Bellmunt

et al, 2011; Tran *et al*, 2012). Furthermore, interleukin-8, as well as IL6, represents an activation of the immunostimulatory system and has been associated with a worse prognosis in cancer, independent of the tumour heterogeneity (Lippitz, 2013). Taken together, present observations and available knowledge might provide a rationale for the therapeutic role of agents targeting IL8 in UC, in combination or a sequence with a TKI. Among the available drugs, although the activity of the fully human anti-IL8 antibody ABX-IL8 was provided only in preclinical models (Mian *et al*, 2003), another

Table 2. Cox model analysis

Marker ^a	Unadjusted			Adjusted		
	HR ^b	95% CI ^c	P-value ^c	HR ^b	95% CI ^c	P-value ^c
VEGF ^{T0}	1.21	0.84–1.74	0.311	1.01	0.70–1.46	0.943
VEGF ^{T1}	1.69	1.16–2.48	0.007	1.47	0.97–2.25	0.071
VEGFR1 ^{T0}	1.13	0.80–1.60	0.480	0.94	0.66–1.35	0.750
VEGFR2 ^{T0}	0.71	0.40–1.24	0.227	0.87	0.48–1.58	0.650
VEGFR2 ^{T1}	0.78	0.49–1.23	0.280	0.86	0.54–1.37	0.532
cKIT ^{T0}	1.18	0.81–1.73	0.395	1.32	0.92–1.87	0.128
cKIT ^{T1}	1.14	0.64–2.02	0.652	1.56	0.92–2.65	0.101
IL6 ^{T0}	1.07	0.89–1.29	0.482	1.05	0.83–1.33	0.680
IL8 ^{T0}	1.72	1.11–2.67	0.015	1.73	1.09–2.73	0.019
IL8 ^{T1}	1.87	1.15–3.05	0.012	2.09	1.27–3.43	0.004
IL12 ^{T0}	1.65	0.91–2.97	0.097	1.51	0.84–2.73	0.170
HGF ^{T0}	1.00	0.95–1.06	0.857	0.98	0.93–1.04	0.535
TGFβ ^{T0}	1.28	1.01–1.63	0.038	1.10	0.85–1.43	0.471

Abbreviations: 95% CI = 95% confidence interval; HGF = hepatocyte growth factor; IL = interleukin; KIT = stem-cell factor; TGFβ = transforming growth factor-β; VEGF(R) = vascular-endothelial growth factor (receptor); HR = hazard ratio.
^aT1 values only for markers with significant modulation at 5% level.
^bHR for interquartile range
^cP = Wald's test P-value.

Table 3. NRI of multivariable Cox models incorporating CAFs information

	NRI		
	Event	Non-event	Average
Model 1 ^a	0.44	0.75	0.60
Model 2 ^b	0.52	0.25	0.39

Abbreviations: NRI = Net reclassification improvement; CAF = circulating angiogenic factor; IL = interleukin; KIT = stem-cell factor; TGFβ = transforming growth factor-β; VEGF = vascular-endothelial growth factor.
^aModel including IL8^{T1}, IL8^{T0}, VEGF^{T1}, cKIT^{T1}, TGFβ^{T0}.
^bModel including IL8^{T1} only, after applying a backward variable selection procedure.

anti-IL8 compound, reparixin (Dompè s.p.a.), is currently in phase 2 development in early breast cancer (ClinicalTrials.gov number NCT01861054).

Investigation on the prognostic contribution of IL8 should be pursued further in this disease, particularly in trials with antiangiogenic TKIs, to validate a potential tool for a patient-enrichment design. This could apply to two ongoing phase 2 trials of pazopanib combined with paclitaxel and gemcitabine, respectively, in UC (registered with ClinicalTrials.gov, number NCT01108055 and NCT01622660, respectively).

Although aberrations resulting in sensitivity to VEGFR-directed TKI might exist in the microenvironment rather than the tumour itself, this theory has yet to be proven. Yet, another signal corroborating the role of microenvironment in this setting was relative to the association of baseline levels of IL12 and metabolic response at 4 weeks (lower levels associated with PET response). Again, this observation should be cautiously unravelled, but a possible explanation may be that IL12 is an essential pro-inflammatory cytokine that is decreased in several cancer types, particularly in later stages; hence, it might be in relation to

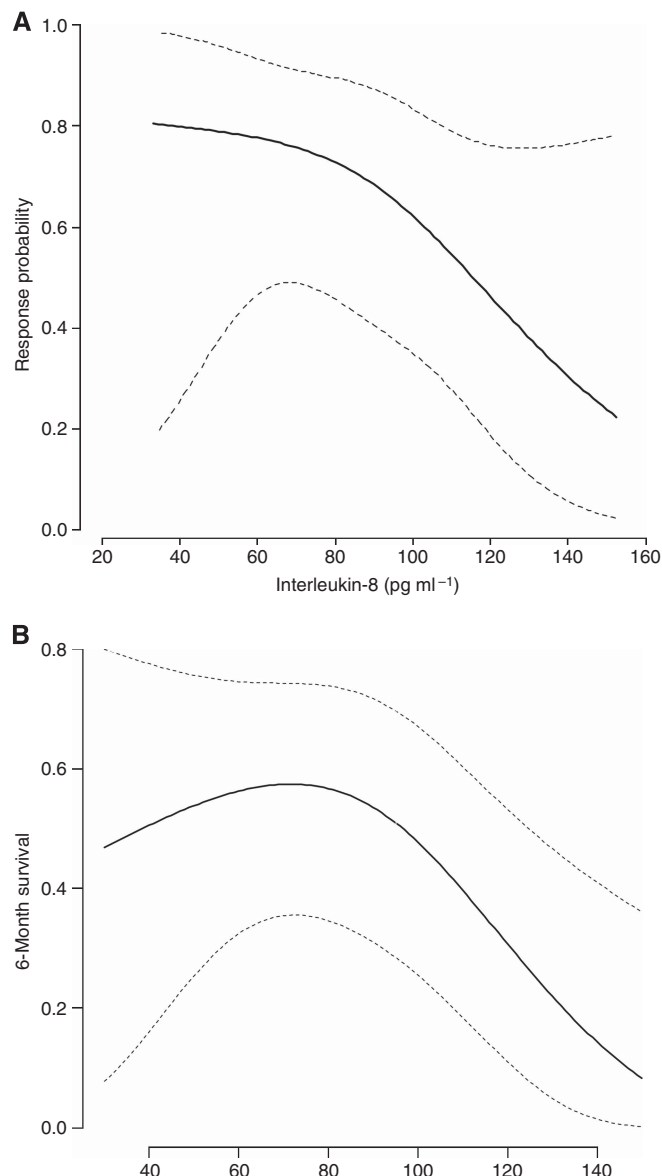


Figure 3. Results by analysing IL8^{T1} level. Results by analysing IL8^{T1} level as a continuous time-varying covariate in association with response probability by the logistic regression model (A) and with 6-month survival probability by the Cox model (B). The model was adjusted for a fixed IL8^{T0} value of 67 pg ml⁻¹ (median value).

inflammatory and FDG-avid peri-tumoural tissue, the first to be dampened by an active targeted compound (Del Vecchio *et al*, 2007). Baseline TGFβ provided signals of prognostic effect. TGFβ is one of the principal immune-suppressive factors secreted by tumour cells and it possesses a huge spectrum of activity depending on the type of activated receptor (Bierie and Moses, 2006). A phase 2 trial is ongoing at our centre with the fully human monoclonal antibody directed against TGFβ receptor ALK1, PF03446962 (Pfizer Inc, La Jolla, CA, USA), a compound endowed with distinct antivascular activity, as second-line therapy in UC (ClinicalTrials.gov, number NCT01620970). Combined results from our group and from other clinical trials worldwide underscored the clinical meaning of targeting angiogenesis thus far, but an improvement in trial design based on patient selection/enrichment is desperately needed. Thus far, the sobering realisation of clinical trials with this class of agents was that of a small activity followed by resistance developing in a few months. Observations are hampered by the class activity of

these drugs, not corresponding to tumour shrinkage for the majority of cases. A discrepancy was usually observed between an overall modest survival improvement and the existence of small subset of patients achieving an incredibly long-term response-stabilisation or even CR, far beyond what could be reasonably expected *a priori*. Going forward, an international cooperation to validate the present findings is required. The design of a multicentre data set, including CAFs from multiple cohorts of patients receiving anti-VEGF(R) compounds in phase 2 trials has the potential to render these results broadly applicable to antiangiogenic drugs in future clinical trials.

In conclusion, a caveat of present series is that tumour biology is suboptimally captured by clinical and laboratory features, such as those evaluated and the discovery of molecular predictors linked to an aggressive phenotype, and treatment resistance still needs a paradigm change. This is the reason why we are now moving towards a genomic profiling that yields a number of theoretical advantages over the former approach to guide informed clinical trials (Iyer *et al*, 2012). Extensive genomic profiling of tumour samples, particularly of defined subsets of patients who achieve extreme responses to antiangiogenic drugs, such as pazopanib, may allow for the identification of a landscape of novel druggable biomarkers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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