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The Combination of Circulating Ang1 and Tie2 Levels Predicts Progression-Free Survival Advantage in Bevacizumab-Treated Patients with Ovarian Cancer

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Abstract

Purpose: Randomized ovarian cancer trials, including ICON7, have reported improved progression-free survival (PFS) when bevacizumab was added to conventional cytotoxic therapy. The improvement was modest prompting the search for predictive biomarkers for bevacizumab.

Experimental Design: Pretreatment training ($n = 91$) and validation ($n = 114$) blood samples were provided by ICON7 patients. Plasma concentrations of 15 angio-associated factors were determined using validated multiplex ELISAs. Our statistical approach adopted PFS as the primary outcome measure and involved (i) searching for biomarkers with prognostic relevance or which related to between-individual variation in bevacizumab effect; (ii) unbiased determination of cutoffs for putative biomarker values; (iii) investigation of biologically meaningfully predictive combinations of putative biomarkers; and (iv) replicating the analysis on candidate biomarkers in the validation dataset.

Results: The combined values of circulating Ang1 (angiopoietin 1) and Tie2 (Tunica internal endothelial cell kinase 2) concentrations predicted improved PFS in bevacizumab-treated patients in the training set. Using median concentrations as cutoffs, high Ang1/low Tie2 values were associated with significantly improved PFS for bevacizumab-treated patients in both datasets (median, 23.0 months vs. 16.2; $P = 0.003$) for the interaction of Ang1–Tie2 treatment in Cox regression analysis. The prognostic indices derived from the training set also distinguished high and low probability for progression in the validation set ($P = 0.008$), generating similar values for HR (0.21 vs. 0.27) between treatment and control arms for patients with high Ang1 and low Tie2 values.

Conclusions: The combined values of Ang1 and Tie2 are predictive biomarkers for improved PFS in bevacizumab-treated patients with ovarian cancer. These findings need to be validated in larger trials due to the limitation of sample size in this study. *Clin Cancer Res; 20(17); 4549–58. ©2014 AACR.*

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Introduction

Ovarian cancer is the fourth commonest cause of female cancer-related death, accounting for thousands of lives each year. For several decades the standard of care has been surgery and platinum-based cytotoxic chemotherapy. Despite attempts to optimize these modalities (1, 2), progression-free survival (PFS) and overall survival (OS) remained stable prompting the investigation of new treatment strategies, including those that target tumor vasculature (3–6).

Angiogenesis, the formation of new blood vessels, has been validated as a target for cancer treatment in multiple randomized clinical trials that evaluated the benefit of adding vascular endothelial growth factor (VEGF) pathway inhibitors to conventional therapy (7–11). The approach has revealed improvements in PFS and/or OS that were statistically significant but clinically relatively modest (12); observations that also pertain to two recent trials in ovarian cancer in which patients were randomized to receive carboplatin and paclitaxel ± the anti-VEGF antibody, bevacizumab [GOG218(3) and ICON7(4)].

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Translational Relevance

Bevacizumab has been evaluated in several randomized trials in ovarian cancer. In all trials a statistically significant improvement in progression-free survival was reported but the benefit was modest and there remains a critical need to identify predictive biomarkers for all vascular endothelial growth factor (VEGF) pathway inhibitors, including bevacizumab. Here, we report the analysis of blood samples, taken before treatment in the ICON7 clinical trial, using a validated multiplex ELISA that was capable of measuring the plasma concentrations of multiple angio–related factors. The data show that angiopoietin 1 and Tie2 concentrations identify a subgroup of patients who benefit from bevacizumab. The data are important because a recent trial has reported that angiopoietins are a valid target in ovarian cancer. Together these data highlight the possibility of differential regulation of ovarian cancer angiogenesis through VEGF or angiopoietin.

The modest improvement in survival in trials of antiangiogenic agents in solid tumors triggered a search for predictive biomarkers to allow selection of patients most likely to benefit from this class of drugs to optimize efficacy while reducing toxicity and expense. Recent data highlighted the potential predictive value of soluble, low molecular weight VEGFA in pretreatment plasma taken from patients with pancreatic, stomach, and breast cancer but not in colorectal, lung, and renal cancers (13). This biomarker may also hold the predictive value for OS at the highest quartile of plasma concentrations in patients treated within the GOG218 ovarian cancer trial (14).

Here, we present an analysis of the international blood sample collection taken before treatment in the ICON7 (4) clinical trial, which recruited 1,528 new patients with high-risk, early-stage ovarian cancer, and FIGO stage III/IV disease. Patients were randomized to receive six cycles of conventional dose carboplatin and paclitaxel ± bevacizumab 7.5 mg/kg every 3 weeks for up to 12 months. The trial reported a 1.5-month improvement in PFS [HR, 0.81; 95% confidence interval (CI), 0.70–0.94; $P = 0.004$ log-rank test] in the experimental arm. In the advanced disease subset of the experimental arm, improvement in PFS was 3.6 months and early analysis of OS showed a 7.8-month benefit. Having previously developed and validated to Good Clinical Practice for Laboratories (GCPL) standards multiplex angiogenesis–related ELISAs (15), we applied this technology to determine the predictive significance of pretreatment plasma concentrations of 15 angiogenesis–related factors implicated in VEGF biology (VEGFA, -C, and -D; and VEGF receptors, VEGFR1, and VEGFR2; refs. 16, 17), angiogenic factors in ovarian cancer (fibroblast growth factor, FGF2; interleukin, IL8; angiopoietin, Ang1 and Ang2; and Tunica internal endothelial cell kinase 2, Tie2; refs. 18, 19), or potential mediators of resistance to VEGF [placental growth factor, PlGF (refs. 20, 21); FGF2 (ref. 22); platelet-derived growth factor, PDGFbb (ref. 23); granulocyte colony–stimulating factor, GCSF (ref. 24); or hepatocyte growth factor, HGF (ref. 25)].

Materials and Methods

ICON7, sample processing, and patient selection

We report our study in accordance with REMARK guidelines (26). There were 1,528 patients randomized in ICON7, of whom most (81.5%) had FIGO stage III/IV disease. Samples were obtained from all participating Gynecologic Cancer Intergroup (GCIG) groups except for the Arbeitsgemeinschaft Gynäkologische Onkologie (AGO).

Plasma (EDTA) samples were collected at each trial center using Standard Operating Procedures. Samples were separated into aliquots, frozen at −80°C, shipped to the Biobank (University of Leeds, Leeds, UK), and stored anonymously at −80°C. Projects using ICON7 translational research samples underwent peer review, were approved by the Trial Management and Steering Committees and the Ethics Committee in charge of the trial. To enrich the predictive performance of the chosen biomarkers, we selected samples, equally from the experimental or control arms, from patients where there was a measurable tumor response, determined according to RECIST (27) or, in the case of nonmeasureable but evaluable disease, according to GCIG criteria (28). This focused the discovery arm on angio-related biomarkers rather than toxicity biomarkers. We used a training set of 91 women; and a validation set of 114 women from whom pretreatment plasma was available.

Multiplex ELISA

Assay measurements were performed in the Clinical and Experimental Pharmacology GCPL laboratories, Cancer Research UK Manchester Institute (Paterson Building; Manchester, UK). ELISAs were performed using the SearchLight Plus charged couple device imaging system (Aushon BioSystems). Angiogenesis-associated protein concentrations were assessed using two six-plex ELISAs of Ang2, FGFb, HGF, PDGFbb, VEGFA and VEGFC and GCSF, IL8, KGF, PlGF, VEGFR1, and VEGFR2, respectively, a duplex of Ang1 and Tie2 and a single plex of VEGFD.

All ELISA plexes were subjected to in-house validation as previously described (15). As an example of part of the validation process, Supplementary Fig. S1 demonstrates that Ang1 and Tie2 (on the same plex) are measured independently of each other. Serum CA125 concentrations were determined at each clinical site.

Outcome measures

The primary outcome was PFS; the secondary outcome was tumor response. PFS was calculated from the date of randomization to the date of disease progression or death,

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whichever occurred first. Patients who were alive without disease progression at the end of the study were censored at the date of their last assessment. Disease progression was defined clinically or by RECIST (27, 29) criteria. Asymptomatic progression on the basis of CA125 levels was insufficient to define progression. Tumor response was dichotomized as responder (complete or partial response) or nonresponder (stable or progressive disease). Best response was assessed in patients with measurable disease at baseline who received at least one cycle of protocol treatment and was defined as the best confirmed response recorded in the interval between the start of treatment through to 70 days after the last cycle of cytotoxic treatment.

Data analysis

The prognostic importance of each individual candidate biomarker (clinical variable or putative protein biomarker) was assessed by including it (continuously and dichotomized at the distribution median) as a sole covariate in a proportional hazards model for progression and by testing for the corresponding null hypothesis of no effect via a Wald test. A plot of the Martingale residuals from each marker-specific analysis was examined for evidence of nonlinearity in the biomarker–hazard relationship. Transformations such as $\log_2$ transformation were applied if evidence of nonlinearity was found.

To identify variables of predictive importance with respect to treatment effect, we fitted a Cox model of PFS for each distinct combination of a putative biomarker and treatment. Each of these fitted models included the treatment $\times$ biomarker interaction term. Where there was evidence of an interaction (i.e., the effect of treatment changes with the value of the biomarker), this was taken to suggest the possible predictive potential of the biomarker with respect to patient benefit from bevacizumab. A Kaplan–Meier estimator was used for the visualization of the PFS for each of the identified putative biomarker. It should be noted that this is an observational analysis and the difference in PFS did not taken confounders, for example, prognostic factors, into account at this stage. Therefore, we refrain from reporting *P* values in this analysis.

Following REMARK guidelines, we also explored the use of multivariable fractional polynomial interaction (MFPI; ref. 30) models to avoid restricting assumptions to linearity. This approach also allowed us to assess for nonpredefined cutoffs and avoid data-driven definition of cutoffs.

A parsimonious subset of biomarkers highlighted by the above analysis provided the basis for a further stage of the study, in which we looked into the prognostic and predictive value of combinations of biomarkers. Here, we hypothesized that combinations of biomarkers offer added discrimination in our prediction models, and sought to explore specific combinations of biomarkers based on our initial screening and biologically driven hypothesized combinations.

To prevent the estimate from being unduly influenced by a few patients with extreme biomarker measurements, Cox regression models for each putative biomarker were validated using a bootstrap resampling technique. For each model, 1,000 bootstrap samples were generated and the relative frequency with which each specific candidate biomarker resulted as significant was recorded. The analyses were carried out using R software (version 2.7.1; The R development Core Team, R Foundation for Statistical Computing) and SPSS (version 19, SPSS Inc.).

Correction for multiple testing

The proteins investigated in this study were selected because of their biologic relevance to angiogenesis. Because of this role in angiogenesis, it is perhaps not surprising that the concentrations of many cytokines are highly correlated (Supplementary Table S1). Methodologies for the correction of multiple testing, such as Bonferroni correction, assume independence in test statistics and are, therefore, inappropriate. In this study, no correction was carried out for multiple testing during the screening of each individual candidate biomarker, so that more candidates were shortlisted for further investigation. When the optimum model was derived using the combination of putative biomarkers, a single-step maxT approach was applied to determine the appropriate cutoff for *P* values under multiple testing. Bootstrap resampling technique was also used to estimate the complicated dependency among the putative biomarkers.

Validation set

The model, including all the identified biomarkers, was further validated using an additional dataset of 114 validation patients. As suggested by the REMARK guidelines, the validation was firstly carried out by applying the identical Cox model from the analysis of the training data to the validation sample with the same estimated parameters, by generating a prognostic index for each patient and by subsequently classifying all validation group patients into high- and low-risk groups according to their prognostic indices. Kaplan–Meier curves were plotted for each risk group to compare with actual survival probabilities and model-based estimates. The model parameters were then reoptimized using the validation dataset and the results were compared with the model derived using the training dataset. Finally, a model comprising both training and validation datasets was developed as a more generalized survival model.

Results

Patient characteristics

The characteristics of the 91 participating patients in the training dataset are shown in Table 1. Forty-four were in the standard arm and 47 in the experimental arm. Demographic characteristics, histologic types, and surgery for the TRICON7 patients were similar to those of the patients in the main trial. However, the median age was

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Table 1. Patient characteristics of the training dataset versus main trial

	Training set		Validation set		ICON7, main trial[a]	
	Standard (*N* = 44)	Bevacizumab (*N* = 47)	Standard (*N* = 49)	Bevacizumab (*N* = 65)	Standard (*N* = 764)	Bevacizumab (*N* = 764)
Age, y						
Median (range)	60 (38–75)	53 (31–71)[b]	58 (38–79)	55 (34–76)	57 (18–81)	57 (24–82)
Race, *n* (%)						
White	41 (93)	42 (89)	48 (98)	60 (92)	737 (96)	730 (96)
Asian/Black/Other	3 (7)	5 (11)	1 (2)	5 (8)	27 (4)	34 (4)
ECOG PS, *n* (%)[c]						
0	16 (37)	22 (47)	19 (39)	29 (45)	358 (47)	334 (45)
1	27 (63)	23 (49)	26 (53)	34 (52)	354 (47)	366 (49)
2	0	2 (4)	4 (8)	2 (3)	43 (8)	45 (6)
Origin of cancer, *n* (%)						
Ovary epithelial	42 (95)	37 (79)	43 (88)	59 (91)	667 (87)	673 (88)
Fallopian tube	1	2	1	1	29 (4)	27 (4)
Primary peritoneal	1	8 (17)	5 (10)	5 (8)	56 (7)	50 (6)
Multiple sites	0	0	0	0	12 (2)	14 (2)
Histology, *n* (%)						
Serous	32 (73)	40 (83)	35 (71)	42 (64)	529 (69)	525 (69)
Mucinous	1	0	1	0	15 (2)	19 (2)
Endometrioid	5 (11)	3	4 (8)	0	57 (7)	60 (8)
Clear cell	5 (11)	3	5 (10)	14 (22)	60 (8)	67 (9)
Mixed	0	0	2	9 (14)	48 (6)	40 (5)
Other	1	1	2	0	55 (7)	53 (7)
FIGO stage, *n* (%)						
I/IIA	4 (9)	5 (11)	5 (10)	5 (8)	75 (10)	67 (9)
IIB/IIC	5 (11)	2	7 (14)	10 (15)	70 (9)	70 (9)
III	0	1	1	3	14 (2)	18 (2)
IIIA	3	1	3	1	32 (4)	22 (3)
IIIB	2	8 (17)	3	1	44 (6)	45 (6)
IIIC	26 (59)	25 (53)	26 (53)	36 (55)	432 (57)	438 (57)
IV	4 (9)	5 (11)	4 (8)	9 (14)	97 (12)	104 (13)
Grade, *n* (%)						
Grade 1, well	1	2	2	5 (8)	56 (7)	41 (5)
Grade 2, moderately	9 (20)	5 (11)	8 (16)	9 (14)	142 (19)	175 (23)
Grade 3, poorly	34 (77)	40 (84)	36 (74)	49 (75)	556 (74)	538 (71)
Unknown	0	0	3	2	10	10
Debulking surgery, *n* (%)						
No (inoperable)[d]	0	0	0	0	17 (2)	13 (2)
Yes	44 (100)	47 (100)	49 (100)	65 (100)	747 (98)	751 (98)
>1-cm residual disease	16 (36)	15 (32)	20 (41)	33 (51)	195 (26)	192 (26)
≤1-cm residual disease	28 (64)	32 (68)	29 (59)	32 (49)	552 (74)	559 (74)
Intent to start chemotherapy following surgery, *n* (%)						
≤4 weeks	13 (30)	22 (47)	[e]	[e]	328 (43)	326 (43)
>4 weeks	31 (70)	25 (53)			436 (57)	438 (57)

[a]Data from appendix to ref. (4).
[b]The Kruskal–Wallis test, $P = 0.030$
[c]Missing data on PS in one patient.
[d]Inoperable cases were excluded in this analysis.
[e]Data not available.

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significantly lower in the patients treated with bevacizumab (Kruskal–Wallis test, $P = 0.030$), and, accordingly, we investigated the prognostic and predictive role of age in our modeling. The majority of patients had high-grade serous FIGO stage IIIc disease that had undergone cytoreductive surgery with less than 1-cm residual disease and these were similarly distributed between the allocated treatment arms.

Protein biomarker levels by treatment

Concentrations of the 15 angiogenesis-associated proteins were measured in the plasma of patients before they received cytotoxic therapy ± bevacizumab. Supplementary Fig. S2 demonstrates that most of these proteins were present at the lower end of the detectable concentration range in keeping with the hypothesis that the patients had undergone effective cytoreductive surgery leaving little cytokine-producing tumor.

The biomarker concentration frequency distributions for the angiogenesis-associated proteins are shown in Supplementary Fig. S2. Most had a skewed distribution (skewness > 1.5; Supplementary Table S2), whereas IL8, Tie2, and VEGFR2 had reasonably normal distributions. There were no differences in median pretreatment concentrations of biomarkers between the treatment arms, although CA-125 was greater in the experimental arm (median 163 vs. 74, Kruskal–Wallis test, $P = 0.0177$; Supplementary Table S3). We assessed the impact of timing of sample collection (and the effect of physiologic angiogenesis) and, in general, found no relationship between the interval from surgery to chemotherapy and plasma concentrations of the angiogenesis-associated proteins (Supplementary Table S4). The exceptions were borderline higher median levels of Tie2 ($P = 0.020$) and HGF ($P = 0.042$) in women commencing chemotherapy greater than 4 weeks, compared with less than 4 weeks, after surgery.

Biomarker screening and candidate selection

As expected FIGO stage and residual disease (both $P < 0.0001$) were associated with PFS and were included in the Cox model. Tie2 was the only protein showing borderline prognostic significance ($P = 0.041$; Table 2). All candidate biomarkers were assessed for their ability to identify patients likely to benefit from addition of bevacizumab. Table 2 shows that treatment interacts with Ang1 ($P = 0.032$) on a linear continuous scale. The results were validated using bootstrap resampling technique (Supplementary Table S5).

To test for possible nonlinear relationships between angiogenesis-related biomarkers and treatment interaction, we performed MFPI models separately for each biomarker. Two striking observations emerged: (i) for Ang1, as a linear model on the nontransformed continuous scale, there was a clear suggestion of an interaction, shown graphically in Fig. 1, (left); and (ii) for Tie2, although there was no significant interaction term, there was clear evidence of heterogeneity in the relation between high levels of Tie 2 and treatment (Fig. 1, right).

Table 2. Screening for prognostic and predictive biomarkers of PFS in the training dataset ($N = 91$)

	Prognostic modeling[a]	Treatment prediction modeling[b]
Biomarkers	***P***	***P***
Ang1	0.576	<u>0.032</u>
Tie2	<u>0.041</u>	0.745
Ang2	0.669	0.666
FGFb	0.691	0.520
GCSF	0.403	0.445
HGF	0.763	0.596
IL8	0.881	0.692
KGF	0.467	0.700
PDGFbb	0.636	0.301
PlGF	0.360	0.342
VEGFA	0.845	0.928
VEGFC	0.566	0.658
VEGFD	0.695	0.678
VEGFR1	0.463	0.582
VEGFR2	0.550	0.683

NOTE: The underlined values indicate that the *P* values are statistically significant.

[a]All models were performed inserting one biomarker at a time, i.e., univariate Cox models.

[b]All models were performed inserting one biomarker at a time, including the biomarker and the biomarker × treatment interaction term. The reported *P* value is for the interaction term.

In both these models, the changes in relations between biomarker and treatment approximated to the medians of each biomarker distributions. Therefore, we explored further the relationships of Ang1 and Tie2 and binary variables via dichotomization around the median concentration.

The Kaplan–Meier survival estimator was used to visualize the PFS of patient groups defined as having supra- or infra-median concentrations of Ang1 and Tie2, in the two treatment arms, as shown in Supplementary Fig. S3A–S3C. As mentioned in Materials and Methods section, this is an observational analysis and no *P* values were reported. For Ang1 (Supplementary Fig. S3A), there was no difference in median PFS, in the standard arm, in women defined by high (>2,978 pg/mL) or low (≤2,978 pg/mL) values (16.9 vs. 17.8 months); but in the bevacizumab arm, patients with high Ang1 had a shorter median PFS than those with low Ang1 (15.3 vs. 19.3 months). For Tie2, there was no difference in median PFS in the standard arm in women defined by high (>18,822 pg/mL) or low (≤18,822 pg/mL) values (17.4 vs. 17.0 months); but in the bevacizumab arm, patients with high Tie2 have a shorter median PFS than those with low Tie2 (15.3 vs. 23.0 months;

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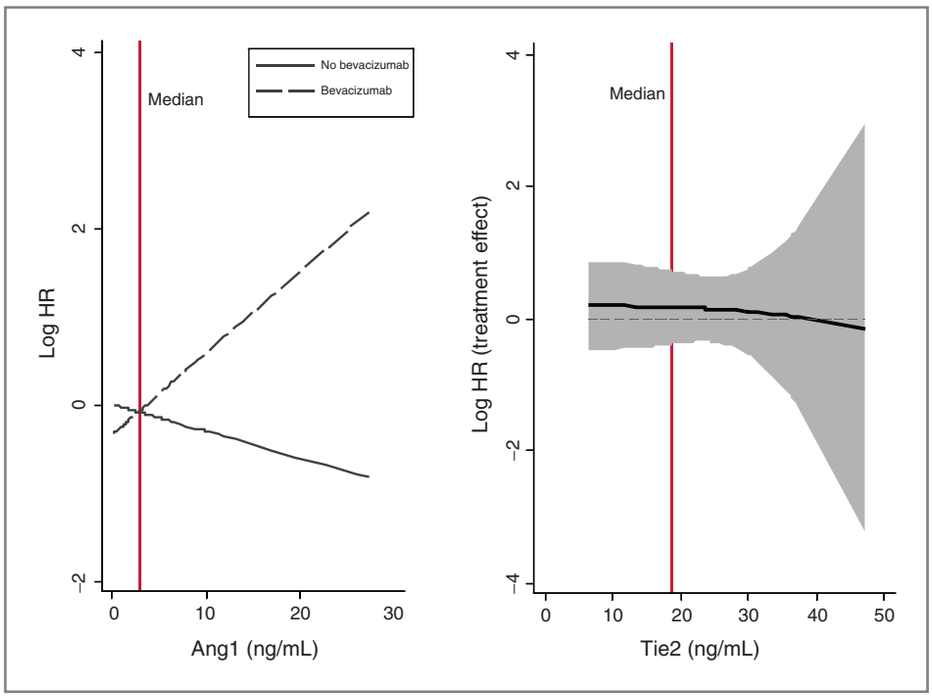

Figure 1. Multivariate fractional polynomial analysis for interactions of treatment × Ang1 (left) and treatment × Tie2 (right) interactions keeping the biomarkers continuous, fitted by FP1 functions with power 1.0 (i.e., linear). Functions were estimated within multivariable models adjusting for age. In the left-hand plot: solid line, estimated effect of biomarker in patients not treated with bevacizumab; dashed line, estimated effect of biomarker in patients treated with bevacizumab; In the right-hand plot: effect of bevacizumab by biomarker status, with 95% point-wise CI. Horizontal dashed lines denote zero and the main effect of bevacizumab in the absence of an interaction. The vertical red lines denote the medians of the respective biomarker distributions (p50: 50th percentile).

Supplementary Fig. S3B). The interaction term was borderline significant ($P = 0.052$). Together the data suggest that Ang1 and Tie2 individually provide predictive information about the expected efficacy of bevacizumab.

Predictive advantage of combined Ang1 and Tie2

At a tissue level, Ang1 is a ligand for the Tie2 receptor, a putative regulator of VEGF inhibitor resistance (31); yet at a plasma level, the correlation between Ang1 and Tie2 levels was weak (Spearman coefficient = 0.269). This background motivated our subsequent investigation of a possible three-way interaction between the effects of treatment, Ang1 and Tie2 on PFS. FIGO stage and size of residual disease were included as prognostic factors because of their significant *P* values in the univariate analysis (Table 2) as well as their known clinical relevance with PFS. Table 3 summarizes the results of a Cox regression analysis of the dependence of hazard on PFS of Ang1, Tie2, and treatment simultaneously, allowing for high-order interactions between the corresponding three effects, and shows a significant evidence of a three-way interaction ($P = 0.003$). Here, the *P* value is 0.015, after correction for multiple testing. The three-way Ang1–Tie2–bevacizumab interaction was validated in a subsequent bootstrap analysis (bootstrap frequency = 84.3%).

The clinical relevance of combining Ang1 and Tie2 is shown in Supplementary Fig. S3C. In women treated by bevacizumab, high Ang1/low Tie2 values (median PFS, 23 months) were associated with an improved median PFS of up to 6.8 months compared with the following: other Ang1/Tie2 category combinations (median PFS, 17.9 months), women receiving standard treatment with high Ang1/low Tie2 values (median PFS, 16.2 months) or other Ang1/Tie2 category combinations (median PFS, 17.4 months). For women with high Ang1/low Tie2 values, treatment with bevacizumab had an expected HR of 0.21 for PFS when compared with standard treatment.

Clinical trial decision tree

As a framework to a future clinical trial, the relationship between Ang1–Tie2 and treatment is pictorially conveyed in Fig. 2, as a decision tree. Patients with high Ang1 and low Tie2 gain a significant benefit from bevacizumab (median PFS, 23.0 months for the bevacizumab arm vs. 16.2 months for the standard arm; log-rank test $P = 0.006$). In contrast, in the high-Ang1 and high-Tie2 groups, the median PFS for the bevacizumab arm (12.8 months) is significantly (log-rank $P = 0.007$; expected HR, 3.60) lower than the median PFS for the standard treatment arm (28.5 months). There were no significant differences in PFS associated with treatment for women with low Ang1 values irrespective of Tie2 concentrations. We will discuss these observations further in the following sections.

Tumor response

Similar patterns emerged; among women with high Ang1 and low Tie2 plasma concentrations, there was a greater chance of tumor response (OR, 1.76) after bevacizumab treatment compared with standard treatment. However, these associations were not statistically significant (Supplementary Table S6).

Validation set

Patient and tumor characteristics for the validation set are shown in Table 1. There were 49 patients from the

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Table 3. Cox proportional hazard models exploring Ang1 and Tie2 as a joint biomarker in the training dataset ($N = 91$)

Covariates	HR (95% CI)	*P*	Bootstrap frequency (%)
FIGO stage			
FIGO stage I	1.000 (referent)		
FIGO stage II	0.978 (0.203–4.694)	0.978	
FIGO stage III	2.827 (0.838–9.535)	0.094	
FIGO stage IV	14.775 (3.478–62.760)	< 0.001	
Size of residual disease			
≤1-cm residual disease	1.000 (referent)		
>1-cm residual disease	1.363 (0.774–2.402)	0.283	
Treatment			
Standard arm	1.000 (referent)		
Bevacizumab arm	0.626 (0.228–1.722)	0.477	9.9
Individual biomarkers			
Ang1			
<median	1.000 (referent)		
≥median	0.413 (0.146–1.168)	0.096	61.9
Tie2			
≥median	1.000 (referent)		
<median	0.463 (0.138–1.546)	0.210	23.3
Interaction terms[a]			
Ang1 × Treatment	5.677 (1.387–23.241)	0.016	51.7
Tie2 × Treatment	2.369 (0.539–10.422)	0.254	12.5
Ang1 × Tie2	8.132 (1.684–39.255)	0.009	65.7
Ang1 × Tie2 × Treatment	0.038 (0.004–0.139)	0.003	84.3

NOTE: There were no missing data.

[a]In all models, standard arm, 0; bevacizumab arm, 1; ang1 < median, 0; ang2 ≥ median, 1; tie2 < median, 1; tie2 ≥ median, 0. All interaction terms are multiplicative.

standard arm and 65 from the bevacizumab arm. The characteristics were broadly similar to those from the main trial. However, the validation set included a larger proportion of patients with residual disease greater than 1 cm when compared with the main trial (46% vs. 26%; χ^2 test, $P < 0.001$). In addition, in the validation dataset more patients in the control arm had greater than a centimeter of residual disease than the treatment arm (62% vs. 38%; χ^2 test, $P = 0.016$).

The prognostic indices derived from the training set differentiated classes of high and low probability for progression in the validation set. The two risk groups showed significant differences in PFS (log-rank $P = 0.008$) as shown in Fig. 3, indicating that the survival model developed using the training dataset was valid on the validation dataset.

The Cox model derived using the training patients was reoptimized using the validation patients, including the same prognostic and predictive biomarkers (Supplementary Table S7). In the model, size of residual disease was a significant prognostic factor ($P < 0.001$ vs. $P = 0.283$ in the training set). In keeping with the model based on the training dataset, a significant interaction of order three among the dichotomized values of Ang1 and Tie2 and treatment ($P = 0.025$) was found. Improved treatment effects from bevacizumab were confirmed in the patients with high Ang1 and low Tie2, demonstrating an HR of 0.27 against the control arm, which was consistent with the HR of 0.21 observed in the training dataset. It is noteworthy in this cohort with a high proportion of residual disease, that bevacizumab treatment was associated with a beneficial effect, consistent with the main ICON7 trial findings (4).

Discussion

Main findings

We report some of the first translational research to emerge from the ICON7; a trial that showed that patients with advanced-stage/high-risk ovarian cancer benefitted from bevacizumab in combination with carboplatin and paclitaxel (4). We measured the plasma concentrations of a range of angiogenesis-associated proteins identifying two predictive biomarkers that emerged from 15 screening biomarkers; Ang1 and Tie2. Specifically, the effective predictor of a beneficial effect of bevacizumab was the combination of a supra-median concentration of Ang1 and an infra-median concentration of Tie2. Each of the

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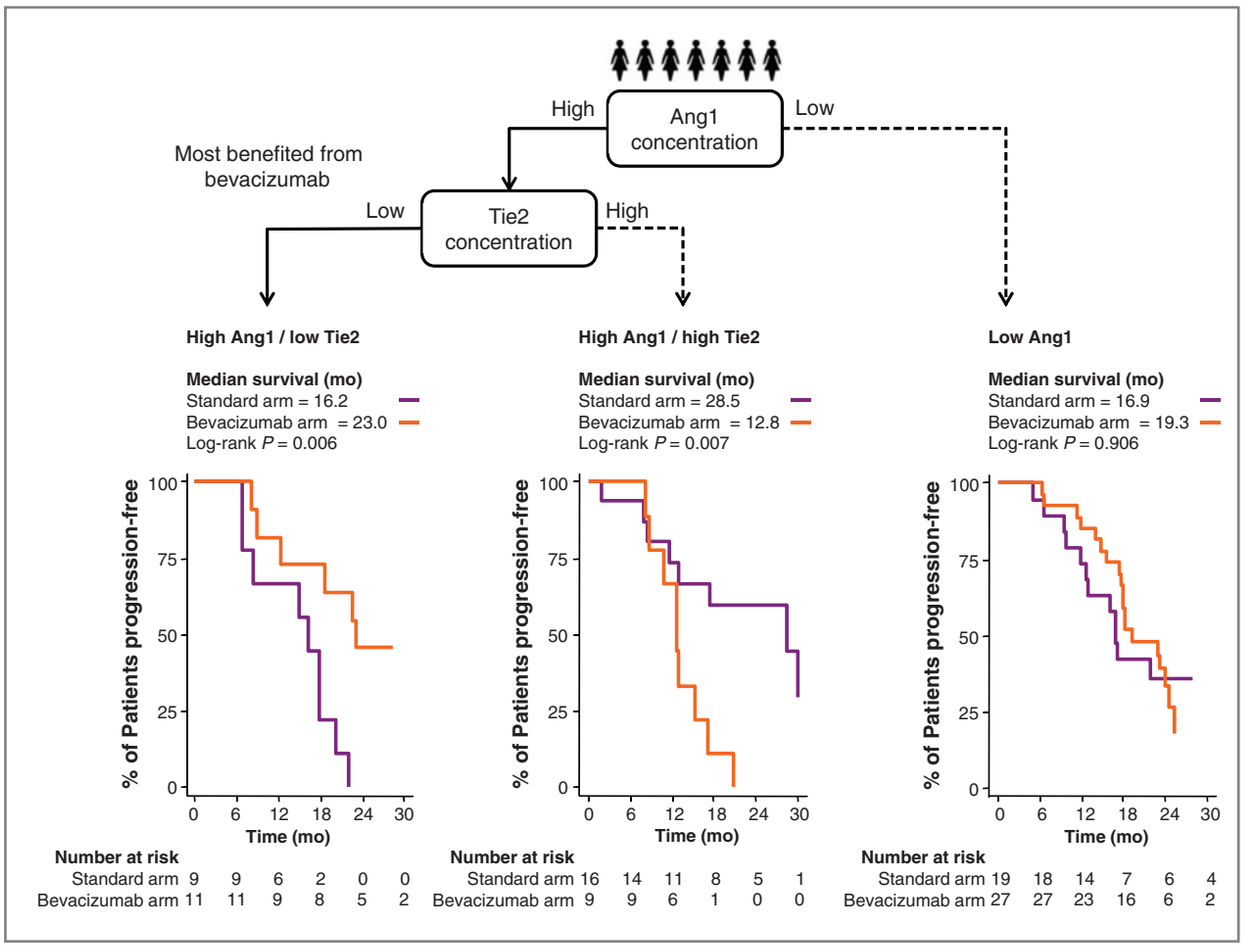

Figure 2. Flow Diagram showing predictive effects of different Ang1 and Tie2 concentrations. Patients are classified according to whether they had pretreatment plasma concentrations of Ang1. There was no predictive value in patients who had low Ang1 (right-hand graph). Those with Ang1 concentrations above the median are then categorized according to their plasma Tie2 concentrations before treatment. Benefit from bevacizumab was observed in the arm with high Ang1 and low Tie2 (left graph). Those with high Ang1 and high Tie2 do not benefit from bevacizumab (middle graph).

putative biomarkers involved in the analysis is supported by previous independent biologic evidence. We validated the significance of this finding in an independent validation dataset. The biomarker combination provides predictive information, over and beyond, that provided by the two markers individually. Supra-median concentrations for both Ang1 and Tie2 were associated with no benefit from bevacizumab. The biologic implication of these finding is that the Ang1–Tie2 axis may play a pivotal role in mediating resistance to VEGF pathway inhibitors.

Biologic plausibility

It is perhaps not surprising that the angiopoietin family has been identified as mediating resistance to VEGF pathway inhibitors in ovarian cancer. Recent data have confirmed the activity of broad-spectrum angiopoietin inhibitors in the disease (19), suggesting that ovarian cancer is a disease that is highly dependent on angiogenesis and that there may be some exclusivity between different angiogenic factors in the disease. However, this remains unproven.

The data show that patients with high Ang1 and low Tie2 plasma concentrations before treatment are most likely to benefit from bevacizumab. This observation raises some important mechanistic issues. As Ang1 is a key mediator of vascular maturity (32), raised perivascular concentrations of Ang1 are more likely to be associated with increased pericyte coverage (33), and hence resistance to VEGF inhibitors (34, 35). Thus, there is a critical need for detailed histologic studies to test the hypothesis that clinical benefit from bevacizumab in ovarian cancer is associated with reduced pericyte coverage and Ang1 expression. In the training dataset, we also found that the combination of high Ang1 and high Tie2 were associated with a particularly poor outcome, but this was not supported by the validation dataset.

We have shown that Tie2 concentrations are associated with several important clinical parameters in ovarian cancer, including FIGO stage, cytoreductive surgery, and

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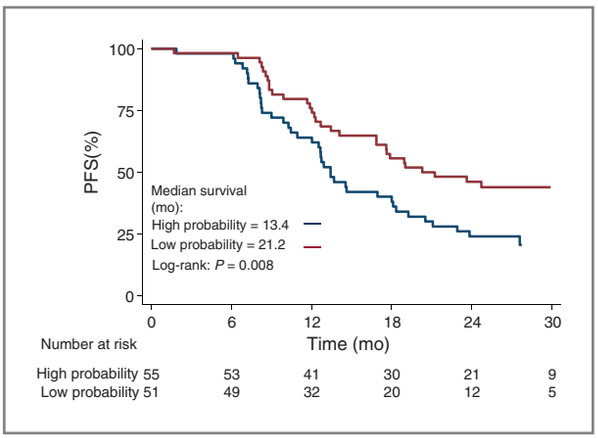

Figure 3. Kaplan–Meier analysis of the risk groups defined for the validation patients using the Cox model, including all prognostic and predictive biomarkers. The Cox survival model of the training set, including all prognostic and predictive biomarkers, was validated by assigning prognostic indices to the additional validation patients and subsequently divided them into two risk groups: "high probability" and "low probability" for disease progression. Kaplan–Meier analysis was carried out for the risk groups showing that they are significantly different. Not possible to calculate probability in 8 patients.

CA125. These findings suggest that the molecule is released from tumor vessels in proportion to the amount of tumor present. Although this remains the most likely explanation, an alternative would be that Tie2 is released from infiltrating Tie2-expressing monocytes, which are known to mediate resistance to VEGF inhibitors (36).

Limitations and strengths

It is conceivable that the significant results might reflect multiple testing. This is theoretically true if our analyses were random but on the contrary, the statistical analyses were targeted and based on biologically driven questions. Our key interaction term, the *P* value was 0.003; a value that remains significant after correction for multiple testing. Second, the "power" of our study is indicated by the confidence intervals around our effect estimates and repeated with significant effects in the independent training and validation sets. Nevertheless, the absolute numbers involved in the study highlight the need to validate the findings in further trials.

There are several strengths in this study. This was a secondary analysis within a protocol-driven trial, allowing examination of a true predictive treatment effect of biomarkers independent of treatment selection bias. Second, we also measured biomarkers to clinical decision quality standards. Third, our analytical design included biologically driven targeted analysis; unbiased determination of cutoffs for putative biomarker values; testing of linear and nonlinear assumptions; training and validation datasets; and internal validation with bootstrapping.

Future research

We conducted an analysis of the pre-chemotherapy plasma concentrations of angiogenic factors in patients participating in ICON7. Our data show that patients with ovarian cancer who have raised plasma concentrations of Ang1 and low Tie2 are those that benefit most from bevacizumab, when concurrently treated with carboplatin and paclitaxel. Our data suggest that ovarian cancer, which is an angiogenesis-dependent disease, might be categorized into VEGF or Ang inhibitor–sensitive disease; the question remains of whether treatment with one inhibitor leads to escape through the alternate mechanism. These findings and hypotheses will be addressed in further prospective investigations.

Disclosure of Potential Conflicts of Interest

A.R. Clamp reports receiving speakers bureau honoraria from Roche and is a consultant/advisory board member for GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Backen, A.R. Clamp, A. Oza, S. Bannoo, R.E. Banks, C. Dive, G.C. Jayson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Backen, A.G. Renehan, A.R. Clamp, C. Berzuini, C. Zhou, A. Oza, S.J. Scherer, G.C. Jayson
Writing, review, and/or revision of the manuscript: A. Backen, A.G. Renehan, A.R. Clamp, C. Berzuini, C. Zhou, A. Oza, S.J. Scherer, R.E. Banks, G.C. Jayson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Bannoo, G.C. Jayson
Study supervision: A. Oza, C. Dive, G.C. Jayson

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References

1. Vergote I, Trope CG, Amant F, Kristensen GB, Ehlen T, Johnson N, et al. Neoadjuvant chemotherapy or primary surgery in stage IIIC or IV ovarian cancer. N Engl J Med 2010;363:943–53.
2. Bookman MA, Brady MF, McGuire WP, Harper PG, Alberts DS, Friedlander M, et al. Evaluation of new platinum-based treatment regimens in advanced-stage ovarian cancer: a phase III trial of the gynecologic cancer inter group. J Clin Oncol 2009;27:1419–25.
3. Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. N Engl J Med 2011;365:2473–83.

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4. Perren TJ, Swart AM, Pfisterer J, Ledermann JA, Pujade-Lauraine E, Kristensen G, et al. A phase 3 trial of bevacizumab in ovarian cancer. N Engl J Med 2011;365:2484–96.
5. Aghajanian C, Blank SV, Goff BA, Judson PL, Teneriello MG, Husain A, et al. OCEANS: a randomized, double-blind, placebo-controlled phase III trial of chemotherapy with or without bevacizumab in patients with platinum-sensitive recurrent epithelial ovarian, primary peritoneal, or fallopian tube cancer. J Clin Oncol 2012;30:2039–45.
6. Pujade-Lauraine E, Hilpert F, Weber B, Reuss A, Poveda A, Kristensen G, et al. AURELIA: a randomized phase III trial evaluating bevacizumab (BEV) plus chemotherapy (CT) for platinum (PT)-resistant recurrent ovarian cancer (OC). J Clin Oncol 2012. 30(Suppl. Abstracts): LBA5002.
7. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–90.
8. Raymond E, Dahan L, Raoul JL, Bang Y-J, Borbath I, Lombard-Bohas C, et al. Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. New Engl J Med 2011;364:501–13.
9. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–42.
10. Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med 2007;357:2666–76.
11. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, et al. Paclitaxel-carboplatin alone or with bevacizumab for non–small cell lung cancer. N Engl J Med 2006;355:2542–50.
12. Jayson GC, Hicklin DJ, Ellis LM. Antiangiogenic therapy-evolving view based on clinical trial results. Nat Rev Clin Oncol 2012;9:297–303.
13. Jayson GC, de Haas S, Delmar P, Miles D, Shah MA, Van Cutsem E, et al. Evaluation of plasma VEGFA (pVEGFA) as a pan-tumour biomarker (BM) for the treatment of patients (pts) with bevacizumab (bev). ESMO/ECCO. Sweden 2011.
14. Birrer MJ. Biomarker (BM) results from GOG-0218, a phase 3 trial of front-line bevacizumab (BV) and chemotherapy (CT) for ovarian cancer (OC) ESMO. Vienna, Austria 2012. p. 198 P.
15. Backen AC, Cummings J, Mitchell C, Jayson G, Ward TH, Dive C. 'Fit-for-purpose' validation of SearchLight multiplex ELISAs of angiogenesis for clinical trial use. J Immunol Methods 2009;342:106–14.
16. Jayson GC, Zweit J, Jackson A, Mulatero C, Julyan P, Ranson M, et al. Molecular imaging and biological evaluation of HuMV833 anti-VEGF antibody: implications for trial design of antiangiogenic antibodies. J Natl Cancer Inst 2002;94:1484–93.
17. Drevs J, Siegert P, Medinger M, Mross K, Strecker R, Zirrgiebel U, et al. Phase I clinical study of AZD2171, an oral vascular endothelial growth factor signaling inhibitor, in patients with advanced solid tumors. J Clin Oncol 2007;25:3045–54.
18. Bast RC, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. Nat Rev Cancer 2009;9:415–28.
19. Karlan BY, Oza AM, Richardson GE, Provencher DM, Hansen VL, Buck M, et al. Randomized, double-blind, placebo-controlled phase II study of AMG 386 combined with weekly paclitaxel in patients with Recurrent ovarian cancer. J Clin Oncol 2012;30:362–71.
20. Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell 2007;131:463–75.
21. Van de Veire S, Stalmans I, Heindryckx F, Oura H, Tijeras-Raballand A, Schmidt T, et al. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. Cell 2010;141:178–90.
22. Batchelor TT, Sorensen AG, di Tomaso E, Zhang WT, Duda DG, Cohen KS, et al. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell 2007;11:83–95.
23. Apte SM, Fan D, Killion JJ, Fidler IJ. Targeting the platelet-derived growth factor receptor in antivascular therapy for human ovarian carcinoma. Clin Cancer Res 2004;10:897–908.
24. Shojaei F, Wu X, Qu X, Kowanetz M, Yu L, Tan M, et al. G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. Proc Nat Acad Sci U S A 2009;106:6742–7.
25. Lu KV, Chang JP, Parachoniak CA, Pandika MM, Aghi MK, Meyronet D, et al. VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. Cancer Cell 2012;22:21–35.
26. Altman DG, McShane LM, Sauerbrei W, Taube SE. Reporting recommendations for tumor marker prognostic studies (REMARK): explanation and elaboration. PLoS Med 2012;9:e1001216.
27. RECIST. 2010; Available from: http://www.recist.com/index.html
28. GCIG. Available from: http://gcig.igcs.org/CA-125.html
29. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.
30. Royston P, Sauerbrei W. A new approach to modelling interactions between treatment and continuous covariates in clinical trials by using fractional polynomials. Stat Med 2004;23:2509–25.
31. Huang H, Bhat A, Woodnutt G, Lappe R. Targeting the ANGPT-TIE2 pathway in malignancy. Nat Rev Cancer 2010;10:575–85.
32. Fagiani E, Christofori G. Angiopoietins in angiogenesis. Cancer Lett 2013;328:18–26.
33. Sundberg C, Kowanetz M, Brown LF, Detmar M, Dvorak HF. Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis *in vivo*. Lab Invest 2002;82:387–401.
34. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 2003;111:1287–95.
35. Greenberg JI, Shields DJ, Barillas SG, Acevedo LM, Murphy E, Huang J, et al. A role for VEGF as a negative regulator of pericyte function and vessel maturation. Nature 2008;456:809–13.
36. Lewis CE, De Palma M, Naldini L. Tie2-expressing monocytes and tumor angiogenesis: regulation by hypoxia and angiopoietin-2. Cancer Res 2007;67:8429–32.

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