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Combining bevacizumab and chemoradiation in rectal cancer. Translational results of the AXEBEam trial

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Background: This study characterises molecular effect of bevacizumab, and explores the relation of molecular and genetic markers with response to bevacizumab combined with chemoradiotherapy (CRT).

Methods: From a subset of 59 patients of 84 rectal cancer patients included in a phase II study combining bevacizumab with CRT, tumour and blood samples were collected before and during treatment, offering the possibility to evaluate changes induced by one dose of bevacizumab. We performed cDNA microarrays, stains for CD31/CD34 combined with α -SMA and CA-IX, as well as enzyme-linked immunosorbent assay (ELISA) for circulating angiogenic proteins. Markers were related with the pathological response of patients.

Results: One dose of bevacizumab changed the expression of 14 genes and led to a significant decrease in microvessel density and in the proportion of pericyte-covered blood vessels, and a small but nonsignificant increase in hypoxia. Alterations in angiogenic processes after bevacizumab delivery were only detected in responding tumours. Lower *PDGFA* expression and PDGF-BB levels, less pericyte-covered blood vessels and higher CA-IX expression were found after bevacizumab treatment only in patients with pathological complete response.

Conclusions: We could not support the 'normalization hypothesis' and suggest a role for *PDGFA*, PDGF-BB, CA-IX and α -SMA. Validation in larger patient groups is needed.

The standard treatment for patients with locally advanced rectal cancer (LARC) is chemoradiotherapy (CRT) followed by total mesorectal excision surgery. With this treatment still ~30% of the patients will die within 5 years of completion of treatment due to the development of local and distant recurrences (Aklilu and Eng, 2011). To overcome this, an intensification of the preoperative treatment is urgently needed. One of the major factors of resistance to radiation therapy is hypoxia. Hypoxia in tumours is caused by

an increase in diffusion distances with tumour expansion resulting in an inadequate supply of oxygen to cells at increasing distance from blood vessels (diffusion-related chronic hypoxia) or by chaotic vasculature and high interstitial pressures within tumours resulting in inadequate blood flow throughout the tumour (perfusion-related acute hypoxia). Anti-angiogenic treatments have been proposed to transiently normalise the tumour vasculature by pruning the immature vessels and by decreasing interstitial

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pressure, resulting in a transient increase in oxygenation and a window of opportunity for enhanced radioresponse and drug delivery (Wildiers *et al*, 2003; Tong *et al*, 2004; Winkler *et al*, 2004; Jain, 2005). Such combination of an anti-angiogenic agent and CRT could consequently be superior to individual treatment alone. Direct evidence was obtained in rectal cancer patients for the antivasular effect of bevacizumab (Avastin), a humanised monoclonal antibody to vascular endothelial growth factor (VEGF), which was the first angiogenesis inhibitor to be approved for cancer treatment (Willett *et al*, 2004).

Many phase I/II trials reported promising response rates and safety results for adding bevacizumab prior and concurrent to 5-fluorouracil (5-FU)-based CRT in the preoperative treatment of LARC (Verstraete *et al*, 2012). However, the pathological complete response (pCR) rate between similar studies varied between 13 and 40%, stressing the importance of a good selection of patients for this combination treatment. Some trials reported pCR rates below 20% (Czito *et al*, 2007; Willett *et al*, 2010; Kennecke *et al*, 2012; Dellas *et al*, 2013; Landry *et al*, 2013), which is not better than trials reported with 5-FU-based CRT alone. However, others reported higher response rates (Willett *et al*, 2005; Crane *et al*, 2010; Koukourakis *et al*, 2011; Nogue *et al*, 2011; Resch *et al*, 2012; Spigel *et al*, 2012), indicating that the addition of bevacizumab possibly offers higher efficacy to a subgroup of patients. Unfortunately, there are no prospectively validated biomarkers of response, toxicity or resistance to anti-angiogenic therapy available (Lambrechts *et al*, 2013). Moreover, the exact effect of bevacizumab on rectal tumours has not been fully elucidated yet. Only the group of Willett *et al* (2004, 2005, 2009) analysed extensively the impact of bevacizumab monotherapy.

Accordingly, better insights in the effect of bevacizumab alone and in the molecular interaction between anti-angiogenic agents and CRT are necessary to optimise this combination treatment for LARC and to allow understanding why some subgroups respond better than others.

In our study, rectal cancer patients were treated with a combination of bevacizumab, chemotherapy (capecitabine +/- oxaliplatin) and radiotherapy (AXEBeam study). The addition of oxaliplatin to fluoropyrimidine-based CRT was investigated before in several phase III trials. Apart from one report, in which the treatment schedule in the control groups might be suboptimal (Rodel *et al*, 2012), no improvement in pCR rate was detected when oxaliplatin was added (Gerard *et al*, 2010; Aschele *et al*, 2011; Roh *et al*, 2011). Therefore, we included in most of our analysis both patients that were treated with and without oxaliplatin, assuming that they would have similar treatment responses.

The main aim was to get more insight into the molecular effects of bevacizumab by studying bevacizumab-induced changes on gene expression level and different markers in blood and tissue samples in 59 included patients. In addition, we performed a subordinate exploratory analysis to identify possible key factors in the combined treatment of bevacizumab with CRT, and assessed their prognostic potential. However, the setup of this study only allows us to address relationships descriptively; no causal associations can be defined.

MATERIALS AND METHODS

Patient selection, treatment and outcome. In a Belgian investigator-driven multicentric randomised phase II study (AXEBeam, NCT00828672), 84 patients with advanced rectal cancer with bad prognostic factors (T2-3Nx with tight or threatened resection margins, T4Nx) have been treated with bevacizumab (5 mg kg⁻¹) in combination with capecitabine (1650 mg m⁻² per day) and radiotherapy (45 Gy, 1.8 Gy per day) with oxaliplatin (50 mg m⁻²,

arm A) or without oxaliplatin (arm B; Supplementary Figure e1). The study was approved by a central Independent Ethics Committee and 59 patients gave informed consent to use samples for translational research. Table 1 lists the patient characteristics and clinical outcomes of those 59 patients. For data analysis, the pathological response was used to differentiate responders (pCR) from non-responders (no pCR). In addition, tumour regression was graded by using the Dworak regression grade.

Sample collections and storage. Tumour tissue and blood samples were collected before treatment (T0) and after the first loading dose of bevacizumab but before CRT (T1). Extra blood samples were taken after two (T2) doses of bevacizumab (during CRT). Biopsies of healthy mucosa (M) were taken at baseline (Supplementary Figure e1).

Biopsies for RNA extraction were fixed in RNA later (Qiagen GmbH, Hilden, Germany) and stored at -80 °C. Biopsies for tissue analysis were fixed in 4% formalin for paraffin embedding. The number of samples used for each analysis is indicated in Figure 1 with a detailed overview in Supplementary Table e1.

ELISA assays. Enzyme-linked immunosorbent assays (ELISA) to measure circulating levels of PDGF-AA, PDGF-BB, thrombospondin-1, IL-8, angiopoietin-2 and CYR61 were performed on blood samples of all 59 patients at T0, T1 and T2 (Figure 1). Quantikine R&D System kits (R&D Systems, Inc., Minneapolis, MN, USA) were used and the individual manufacturer's instructions of the kits were followed. The optical density was determined using the microplate reader Multiscan FC (Thermo Scientific, Waltham, MA, USA) set to 450 nm, with a wavelength correction set to 540 nm. Patient samples were tested in duplicate and the mean value was used for analysis. Results of all levels were expressed as pg ml⁻¹ or ng ml⁻¹ and calculated as median values ± s.d. Extra ELISA and luminex analysis performed on a subset of the patient group can be found in Supplementary Materials and Methods.

Immunohistochemistry and scoring. The paraffin-embedded tissues of 59 patients taken at T0 and T1 were stained for haematoxylin-eosin to identify tumour. They were analysed by an

Table 1. Patient characteristics and pathological response.

	Arm A (n = 31), no. of patients (53%)	Arm B (n = 28), no. of patients (47%)
Age		
Mean	59	60
Range	26–77	45–78
Gender		
Male	9 (29%)	8 (29%)
Female	22 (71%)	20 (71%)
Tumour stage		
cT2	2 (6%)	1 (4%)
cT3	22 (71%)	20 (71%)
cT4	7 (23%)	7 (25%)
Nodal stage^a		
cN0	5 (16%)	2 (7%)
cN1	11 (36%)	9 (32%)
cN2	14 (45%)	16 (57%)
pCR	11 (36%) ^b	3 (11%)
Dworak		
0	1 (3%)	1 (4%)
1	3 (10%)	5 (18%)
2	4 (13%)	10 (36%)
3	11 (36%)	9 (32%)
4	11 (36%) ^b	3 (11%)

Abbreviation: pCR = pathological complete response.
^aNodal stage of two patients not known.
^bOne patient lost to follow-up.

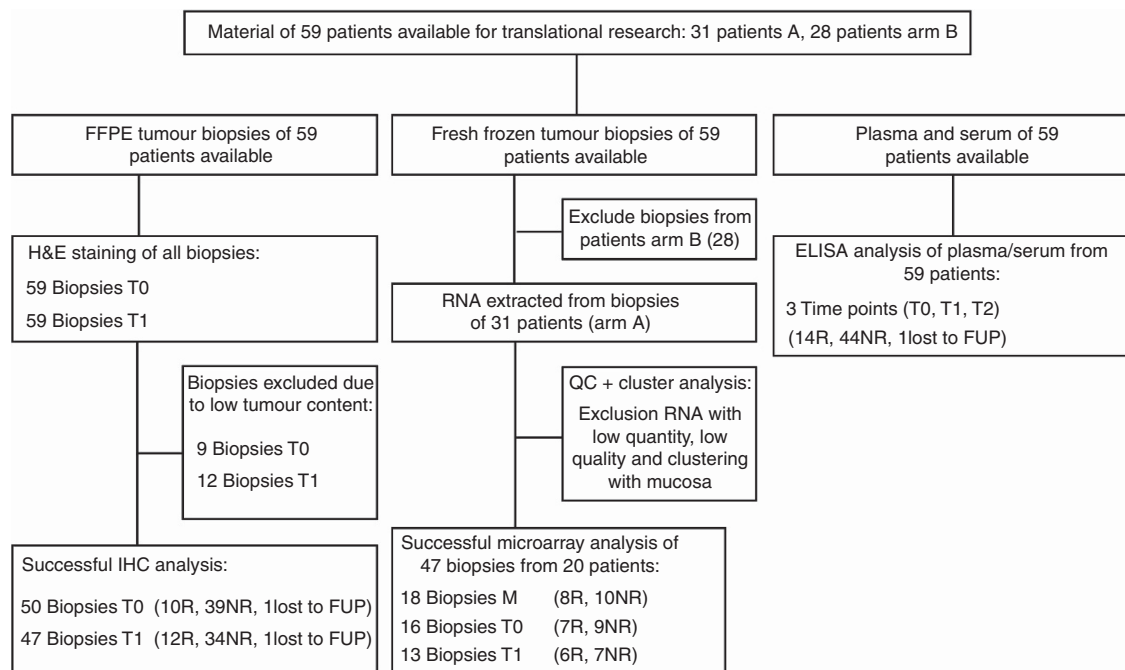


Figure 1. Inclusion and selection process of the samples used for translational research. After clinical and technical selection of the 59 patients included, 50 and 47 patients, respectively, at T0 and T1 were evaluated for tissue markers, 20 patients for gene expression levels and 59 patients for blood markers. Abbreviations: FFPE = formalin-fixed paraffin embedded; FUP = follow-up; H&E = haematoxylin–eosin; M = healthy mucosa; NR = non-responder; QC = quality control; R = responder; T0 = tumour taken at baseline; T1 = tumour taken after one dose bevacizumab.

experienced pathologist, and biopsies without tumour were excluded from the analysis (T0 $n = 9$; T1 $n = 12$). Immunohistochemical (IHC) analysis was performed on biopsies from 50 and 47 patients taken, respectively, at T0 and T1 (Figure 1).

Sections were stained for CD31/34 combined with α -SMA as a measure for blood vessel maturation. CA-IX was used as marker of hypoxia. Endogenous peroxidase activity was blocked followed by antigen retrieval and a blocking step. Samples were incubated with primary antibodies and stainings were visualised using secondary antibodies followed by DAB or Vector Vip incubation. Detailed specifications on the IHC staining are listed in Supplementary Table e2.

Quantitative analysis of the immunostained histological sections for microvessel density (MVD) and CA-IX expression was performed as described earlier (Verstraete *et al*, 2013). CD31/34-stained blood vessels surrounded by α -SMA positivity were counted in 10 high density fields to calculate the percentage of pericyte-covered blood vessels. Several slides were reviewed by an independent observer, and no disagreement in score was found. Median values are described in the Results section.

Details about the Ki67 and cleaved PARP staining can be found in Supplementary Table e5 and Supplementary Materials and Methods.

RNA extraction and cDNA synthesis. Gene expression levels were analysed on tissue of patients from treatment arm A only in order to assess a homogenous cohort. Tissue included mucosal biopsies ($n = 18$) and tumour taken at T0 ($n = 16$), as well as T1 ($n = 13$; Figure 1).

Tissue was broken down by combination of physical (Dounce homogeniser) and chemical homogenisation (Trizol, Invitrogen, Life Technologies Europe B.V.) and RNA was isolated by means of RNeasy Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. RNA quality and quantity was determined by NanoDrop spectrophotometer (NanoDrop Technologies, Centreville, DE, USA) and by means of the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA, USA). Only RNA showing no

signs of degradation or impurities (260/280 and 260/230 nm ratios, > 1.8) was considered suitable for microarray analysis and used for labelling. cDNA was synthesised from 2.5 μ g of RNA using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen, Life Technologies Europe B.V., Gent, Belgium).

Microarray and data processing. Microarray hybridisation procedure was performed on Affymetrix Primeview arrays according to the manufacturer's instructions (Affymetrix Inc., Santa Clara, CA, USA). Analysis of the microarray data was performed in the R programming environment in conjunction with the packages developed within the Bioconductor project (Gentleman *et al*, 2004). Differential expression was assessed via the moderated t -statistic described by Smyth (2004) and implemented in the limma package (version 3.12.1; Gentleman *et al*, 2004). To control the false-discovery rate, multiple testing correction was performed by the method of Benjamini and Hochberg (1995). Data are publically available through National Center for Biotechnology Information (GSE60331). Cluster analysis distinguished the tumour biopsies (T0 and T1) from the normal mucosal biopsies (M). Tumour biopsies that clustered together with the mucosal biopsies were excluded from the analysis.

Pathway analysis. Gene enrichment and pathway analysis was performed with the web-based DAVID Bioinformatics Resource 6.7 programme suite (<http://david.abcc.ncifcrf.gov/>) (Huang *et al*, 2009). Analysis was performed on the list of upregulated and downregulated genes with a fold change of ≥ 1 or ≤ -1 (and a corrected $P < 0.001$ for the comparison between tumour and mucosa samples). The Kyoto Encyclopedia of Genes and Genomes pathways and the Gene Ontology (GO) terms of biological processes in DAVID were used to categorise enriched biological themes (significance level after Bonferonni correction $P < 0.05$). To explore functional interactions or partnership between the genes we loaded them into the STRING 9.1 programme (<http://string-db.org/>; Franceschini *et al*, 2013).

Statistical analysis. The Mann–Whitney test or Kruskal–Wallis test was applied when appropriate to compare the expression of the blood and tissue markers at the different time points to evaluate the on-treatment changes. The comparison of the circulating and tissue expression levels between the responders and non-responders, as classified according to the pathological response, was done by the Mann–Whitney *U*-test and for the Dworak regression grade using the Kruskal–Wallis test. A *t*-test was used to compare treatment-induced changes (T1/T0 and T2/T0) of gene and protein expression levels between patients with and without a pCR. Median reported in Results section. A significance level of $P=0.05$ was used in all cases. Statistical analysis was done using Statistica software 12 (StatSoft Inc., Tulsa, OK, USA) and the R statistical language.

RESULTS

Impact of bevacizumab on gene expression, blood markers and tissue markers. To evaluate biomarker responses to bevacizumab treatment and to unravel its mechanism of action, we profiled gene expression changes by characterising the molecular consequences in response to one or two doses of bevacizumab. To achieve this, tumour biopsies obtained at baseline (T0) and after a single dose of bevacizumab (T1) were compared using microarray analysis and validated with ELISA and IHC at T0, T1 and T2 (Supplementary Figure e1 and Supplementary Table e1).

Changes in gene expression in response to bevacizumab. The microarray data revealed that one dose of bevacizumab changed the expression of only 14 genes in all patients (fold change of >1 or <-1 ; (Supplementary Table e3). Considering separate outcomes, 71 and 31 genes showed altered expression in patients with a pCR and without a pCR respectively. A functional annotation analysis of the gene changes in the responders showed significant enrichment of angiogenesis-related GO biological processes derived from nine altered genes (Table 2). No significant enriched biological processes could be found for the non-responders.

Among those nine genes (*FLT1*, *IL-8*, *PDGFA*, *CTGF*, *ROBO4*, *ENPEP*, *THBS1*, *ANGPT2* and *CYR61*), only for *PDGFA*, a relevant difference was found in bevacizumab-induced changes in expression between patients with and without a pCR ($P=0.1$). As shown in Supplementary Figure e2, *PDGFA* expression decreased in responders with almost 10% after bevacizumab treatment (T1/T0 = (0.8–0.9)), whereas almost no bevacizumab-induced decrease was observed in the non-responders (T1/T0 = (0.88–1.1)).

Changes in circulating marker levels in response to bevacizumab. Six circulating markers (PDGF-AA, PDGF-BB, thrombospondin-1, IL-8, angiotensin-2 and CYR61), selected based on the microarray results and literature, were quantified by ELISA. None of these markers showed significant changes in circulating levels between T0 and T1. However, small but significant treatment-induced changes between T0 and T2 were observed for PDGF-AA,

PDGF-BB, thrombospondin-1, angiotensin-2 and IL-8. The median level and range for each biomarker at T0, T1 and T2 is shown in Supplementary Table e4.

When comparing treatment-induced changes in patients with and without a pCR, most proteins showed similar declines in both patient groups (data not shown). Only for PDGF-AA and PDGF-BB, modest differences could be observed between T0 and T2. Responding patients showed a larger decrease (from 575.9 to 331.5 pg ml^{-1} ; $P=0.11$) in PDGF-AA levels between T0 and T2 compared with the non-responders (from 704.1 to 539.7 pg ml^{-1} ; $P=0.06$, $P=0.06$; Figures 2A and B). For PDGF-BB, the treatment-induced changes were significantly larger in responding compared with non-responding patients ($P=0.002$) with a significant decrease in responders (from 430.5 to 206.2 pg ml^{-1} ; $P=0.03$) and almost no alteration in non-responders (from 452.5 to 343.1 pg ml^{-1} ; $P=0.18$; Figures 2C and D). The changes of PDGF-AA and PDGF-BB on individual patient level are shown in Supplementary Figure e3.

Changes in tissue markers in response to bevacizumab. Histological data on all patients demonstrated that a single dose of bevacizumab induced a significant but small decrease in the MVD, more specifically in the proportion of vessels covered by α -SMA-positive pericytes. The MVD shifted from 74 blood vessels per mm^2 (36–126 blood vessels per mm^2) to 62 blood vessels per mm^2 (22–151 blood vessels per mm^2 ; $P=0.02$) and the proportion of mature vessels declined from 87% (20–98%) before treatment to 73% (35–91%) afterwards (T1; $P<0.0001$). The decrease in pericyte-covered blood vessels was similar in both groups (Figures 3A and B). However, the effect on the MVD was more pronounced in the responders (from 71 to 53 blood vessels per mm^2 ; $P=0.11$) than in the non-responders (from 74 to 64 blood vessels per mm^2 ; $P=0.20$) although not significantly ($P=0.91$; Figures 3C and D).

Hypoxia, measured by CA-IX expression, showed a small but nonsignificant increase during bevacizumab treatment from 10 to 20% ($P=0.08$). This increase was more pronounced, although not significant ($P=0.18$), in the responders (from 13 to 38%; $P=0.15$) in contrast to the non-responders (from 10 to 11%; $P=0.41$; Figures 3E and F). The changes in tissue markers on individual patient level are shown in Supplementary Figure e4.

Exploration of prognostic biomarkers. In order to explore prognostic biomarkers, the genes and proteins affected by bevacizumab were correlated with the pathological response of the patients and the Dworak regression grade. However, as the number of patients in some Dworak-response groups was too small (Table 1), no relevant statistical data could be obtained for this response parameter. We evaluated the expression at baseline (for selection of patients before start of treatment) and the expression after one (T1) or two (T2) loading doses of bevacizumab.

Biomarkers at baseline. When comparing gene expression between baseline tumour and mucosa samples (T0 vs M), 1585 and 1542 genes were found differentially expressed in responders and non-responders, respectively (fold change of ≥ 1 or ≤ -1 and $P<0.001$). Using these genes, 41 and 30 GO biological processes were enriched in responders and non-responders, respectively. The unique biological processes for each patient group are presented in Table 3. Significant GO processes in the responding patients include migratory pathways such as wound healing, cell motion and motility, and pathways involved in cell proliferation such as regulation of mitosis, nuclear division and spindle organisation, which might reflect a good response to oxaliplatin. Also processes related to angiogenesis such as vasculature development, blood vessel development and blood vessel morphogenesis were enriched at baseline in responding patients but not in the non-responding patients. This could suggest

Table 2. Alterations in biological processes in response to bevacizumab in responding patients

Term	Genes	P-value
Angiogenesis		0.0002
Blood vessel morphogenesis	<i>FLT1</i> , <i>IL-8</i> , <i>PDGFA</i> , <i>CTGF</i> , <i>ROBO4</i> , <i>ENPEP</i> , <i>TSP-1</i> , <i>ANGPT2</i> , <i>CYR61</i>	0.0028
Blood vessel development		0.0083
vasculature development		0.0099

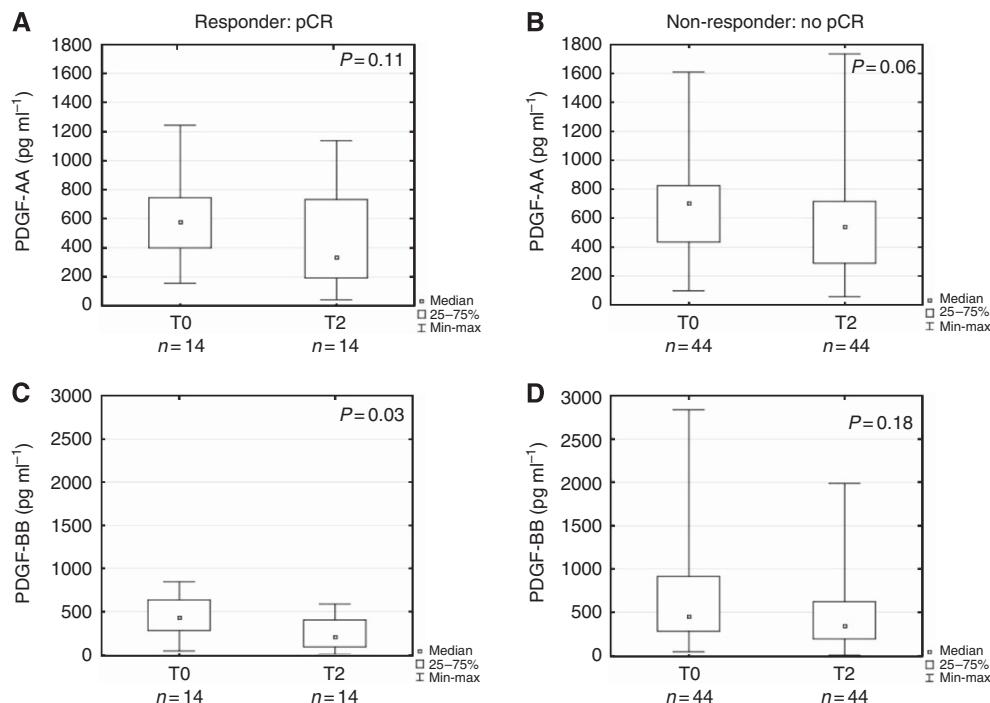


Figure 2. Changes in circulating markers during bevacizumab treatment. Box plots of circulating levels at T0 and T2 measured by ELISA in responding vs non-responding patients. (A, B) Changes in PDGF-AA and (C, D) PDGF-BB levels. Each plot represents the 25th and 75th percentile. The square inside the box indicates the median, and the whiskers indicate the minimum and maximum values. *P*-values: Mann–Whitney *U*-test. Abbreviations: pCR = pathological complete response; T0 = baseline; T2 = after two doses bevacizumab.

that these processes show an intrinsic sensitive profile for bevacizumab treatment.

Biomarkers during bevacizumab treatment. After one loading dose of bevacizumab, patients obtaining a pCR had significant lower tumoral *PDGFA* expression compared with the ones without pCR (6.19 vs 6.93; *P* = 0.04), less pericyte-covered blood vessels (64% vs 77%; *P* = 0.02) and a higher CA-IX expression (38% vs 11%; *P* = 0.004; Figures 4A–C).

After two doses of bevacizumab, responders had significant lower levels of PDGF-BB (206.2 pg ml⁻¹) compared with the non-responders (343.1 pg ml⁻¹; *P* = 0.04; Figure 4D).

These data indicate that *PDGFA*, PDGF-BB, CA-IX and pericyte-covered blood vessels might be used as prognostic markers after validation.

DISCUSSION

Investigating the effect of anti-VEGF therapy, radiotherapy and chemotherapy in clinical trials has often been hampered by the inability to perform serial tumour biopsies in patients, thereby preventing direct analysis of molecular and genetic changes after anti-angiogenic therapies. With the design of this study, we had the unique opportunity to investigate the molecular effects of a single loading dose of bevacizumab and examine potential pathways underlying the interaction of VEGF inhibition and CRT.

Changes induced by bevacizumab in AXEBeam trial vs Willett *et al*. Until now it is still not completely clear which effect bevacizumab has on tumours from rectal cancer patients. Willett *et al* (2004, 2005) measured molecular, cellular and physiological markers before treatment and 2 weeks after one dose of bevacizumab (5 mg kg⁻¹) on six patients from their phase I trial and on 32 patients from their phase II trial (Willett *et al*, 2009), in accordance with the setup of the AXEBeam trial. In Table 4, the analysed parameters are listed, and alterations observed after

bevacizumab monotherapy are compared between the trials from Willett *et al* (2004, 2009) and the AXEBeam trial.

Data from Willett *et al* (2004, 2009) demonstrated antivasular and vascular normalising effects. The antivasular effects, illustrated by a decrease in blood flow, blood volume and MVD, were confirmed by our study with a decrease in MVD. The normalisation effects, which are suggested by a decrease in IFP, less angiopoietin-2-positive blood vessels, higher tumour cell proliferation and an increase in α -SMA coverage, were not observed in the AXEBeam trial. In contrast, we observed a decrease in α -SMA coverage, a slight increase in hypoxia and a decrease in tumour cell proliferation. Bevacizumab was found to stimulate an increase of total circulating VEGF in all studies. However, it is believed that no reliable measurement can be performed once patients have been dosed with bevacizumab, as the majority of VEGF molecules are antibody bound and thus inactive (Loupakis *et al*, 2007; Brostjan *et al*, 2008). In conclusion, the effects of bevacizumab do not seem as straightforward as presented by Willett *et al* (2004, 2009) and the normalisation hypothesis. To fully unravel the working mechanism of bevacizumab, further molecular research is needed.

Angiogenic gene profiling as biomarker. Given the low power of the study, we performed a gene group analysis that might give us more information about changes in response to treatment compared with an analysis at individual gene level. Exploring differentially expressed genes within a biological context defined by GO processes between responding and non-responding patients might give clues about relevant processes for prognosis that would be difficult to identify when evaluating differential expression of individual genes. The multiple comparison error is more pronounced for the individual gene analysis because there are much more individual genes than GO themes. Nevertheless, we have to be aware of limitations inherent to this technique (Khatri and Draghici, 2005).

Already before start of treatment, our gene expression analysis of the tumours revealed distinct intrinsic characteristics between

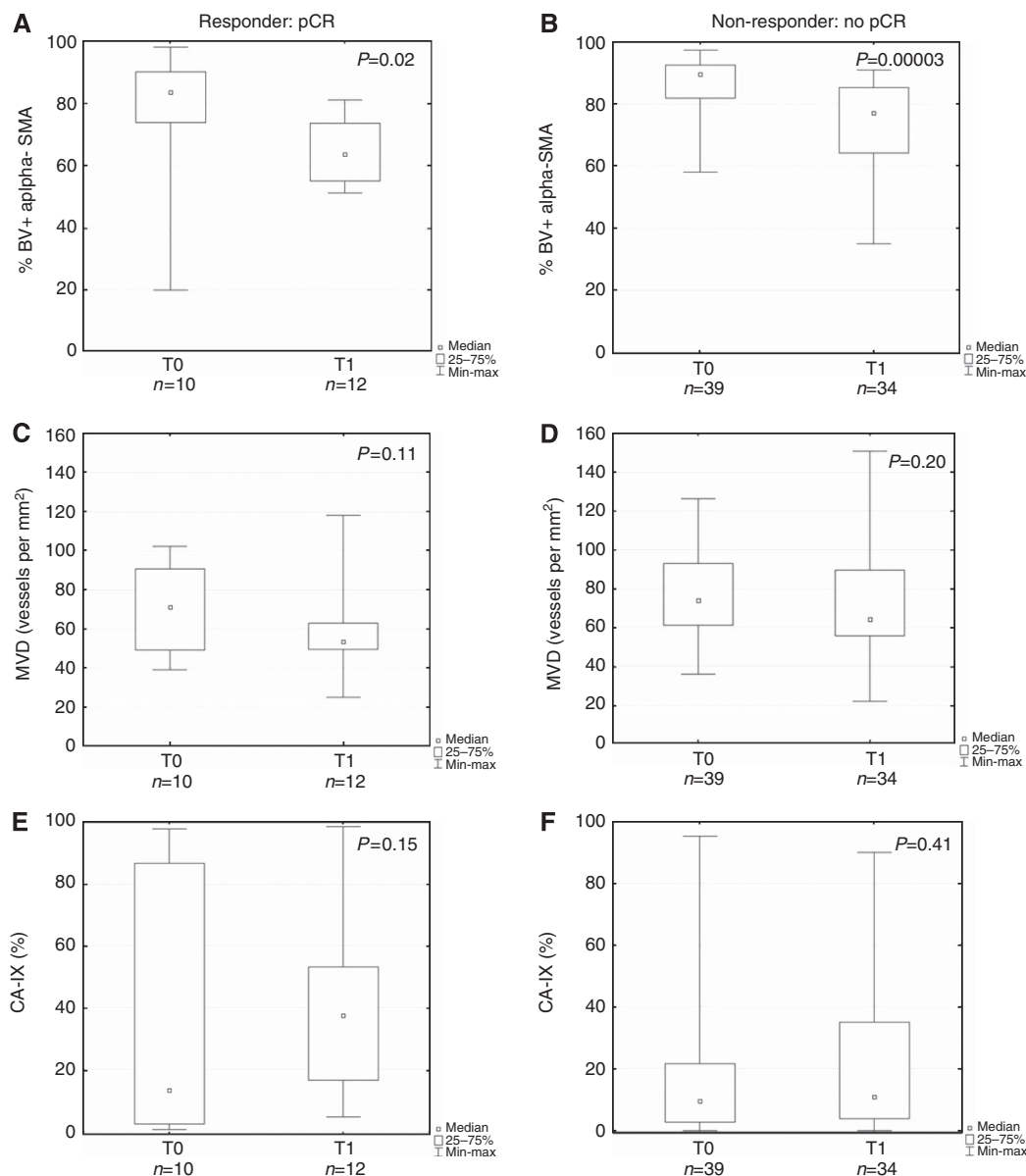


Figure 3. Changes in tissue markers during bevacizumab treatment. Box plots of expression levels at T0 and T1 measured by IHC in responding vs non-responding patients. **(A, B)** Changes in percentage of pericyte-covered blood vessels (BV), **(C, D)** MVD and **(E, F)** tumour hypoxia (CA-IX). Each plot represents the 25th and 75th percentile. The square inside the box indicates the median, and the whiskers indicate the minimum and maximum values. Indicated *P*-values by Mann–Whitney *U*-test. Abbreviations: pCR = pathological complete response; T0 = baseline; T1 = after one dose bevacizumab.

patients which after treatment showed a pCR or no pCR. First, tumours from responding patients showed to be more highly correlated to angiogenesis, hypothesising the notion that those tumours may be upfront more susceptible to anti-angiogenic treatment than tumours without an angiogenic profile. This is supported by earlier data that showed that vatalanib only has anti-angiogenic effect when the VEGFR pathway is activated (Giatromanolaki *et al*, 2012). Different groups have also shown that response to VEGFR inhibitors can be predicted based on a set of angiogenesis-related genes (Watanabe *et al*, 2011; Wilson *et al*, 2012; Brauer *et al*, 2013).

Moreover, only in those responding tumours with an angiogenic phenotype, an alteration was observed after one bevacizumab dosage in angiogenesis-related GO processes, derived from nine altered genes. These angiogenic-related changes possibly indicate an effect of bevacizumab, as no biological processes were found in non-responding tumours to be influenced by one dose of

bevacizumab. Consistently, Brauer *et al* (2013) performed microarray analysis on tumours from anti-VEGF-treated mice, revealing a decreased expression of known endothelial-specific genes and genes implicated in blood vessel development in response to anti-VEGF treatment. Koukourakis *et al* (2009) found a direct effect of bevacizumab on specific cancer cell pathways but not on hypoxia target genes or endothelial markers.

Among all nine genes responsible for the altered angiogenic processes, only *PDGFA* seems to be prognostic at the individual gene level. *PDGFA* expression decreased in response to one dose of bevacizumab in responders but not in non-responders. This makes it a possible prognostic genetic marker, with responding patients having lower *PDGFA* levels after one dose of bevacizumab compared with the non-responders.

Next to the angiogenic processes, gene expression analysis at baseline also revealed pathways in responder patients that might be attributed to the effect of oxaliplatin. It is known that this platinum

compound exerts its effect through inhibition of DNA synthesis by preventing DNA replication and transcription. But it might also affect cell cycle progression (Volland *et al*, 2006), migration of cells

(Gaur *et al*, 2014) and spindle formation during mitosis (Sohn *et al*, 2010). As in our study, patients receiving oxaliplatin in their chemotherapy regimen (arm A) show a higher pCR rate, those enriched biological processes in responding patients might reflect a higher sensitivity to oxaliplatin, rather than to bevacizumab. The GO category for spindle organisation was reported before to separate responders from non-responders at baseline in patients with breast cancer treated with bevacizumab and chemotherapy, but was hypothesised to be more likely relevant to the response to docetaxel (Yang *et al*, 2008).

Table 3. Unique biological processes in patients with and without a pathological complete response at baseline

Term		Gene count	P-value
Responders			
GO:0001944	Vasculature development	61	0.00016
GO:0006928	Cell motion	96	0.00034
GO:0001568	Blood vessel development	59	0.00039
GO:0016477	Cell migration	63	0.00107
GO:0051325	Interphase	32	0.00353
GO:0006259	DNA metabolic process	97	0.00372
GO:0007626	Locomotory behaviour	61	0.00412
GO:0008285	Negative regulation of cell proliferation	74	0.00708
GO:0051783	Regulation of nuclear division	21	0.01059
GO:0007088	Regulation of mitosis	21	0.01059
GO:0051674	Localisation of cell	65	0.01078
GO:0048870	Cell motility	65	0.01078
GO:0048514	Blood vessel morphogenesis	49	0.01621
GO:0051329	Interphase of mitotic cell cycle	30	0.01822
GO:0007051	Spindle organisation	18	0.02463
GO:0042060	Wound healing	45	0.02858
GO:0001501	Skeletal system development	65	0.03977
Non-responders			
GO:0046942	Carboxylic acid transport	39	0.00325
GO:0015849	Organic acid transport	39	0.00389
GO:0060348	Bone development	34	0.00695
GO:0007586	Digestion	28	0.00741
GO:0031667	Response to nutrient levels	46	0.01340
GO:0007584	Response to nutrient	36	0.01966

Role of tissue biomarkers. Consistent with the gene expression data, bevacizumab modified certain angiogenic parameters in tumoral tissue that were more explicit in responders. Both the decrease in MVD and increase in hypoxia were more pronounced in responding patients compared with non-responders.

When looking for associations, patients with a pCR had less pericyte-covered blood vessels and more hypoxia within the tumour after one dose of bevacizumab treatment compared with patients who did not have a pCR, indicating that CA-IX and pericyte-covered blood vessels might be prognostic markers.

Apart from the decrease in MVD (Willett *et al*, 2004), our observations are in contrast with the literature in which according to the normalisation hypothesis (Jain, 2005), one dose of bevacizumab increased the number of pericyte-covered blood vessels (Willett *et al*, 2004) and decreased hypoxia (Jain, 2001; Winkler *et al*, 2004) leading to an enhanced radiation response and better outcome. Also, lower CA-IX expression was reported to be associated with better clinical outcome in patients treated with bevacizumab-based chemotherapy (Sathornsumetee *et al*, 2008; Hong *et al*, 2009). According to other reports pericyte content of CRC was not linked to treatment outcome in metastatic CRC patients under bevacizumab-containing therapy (Goede *et al*, 2010).

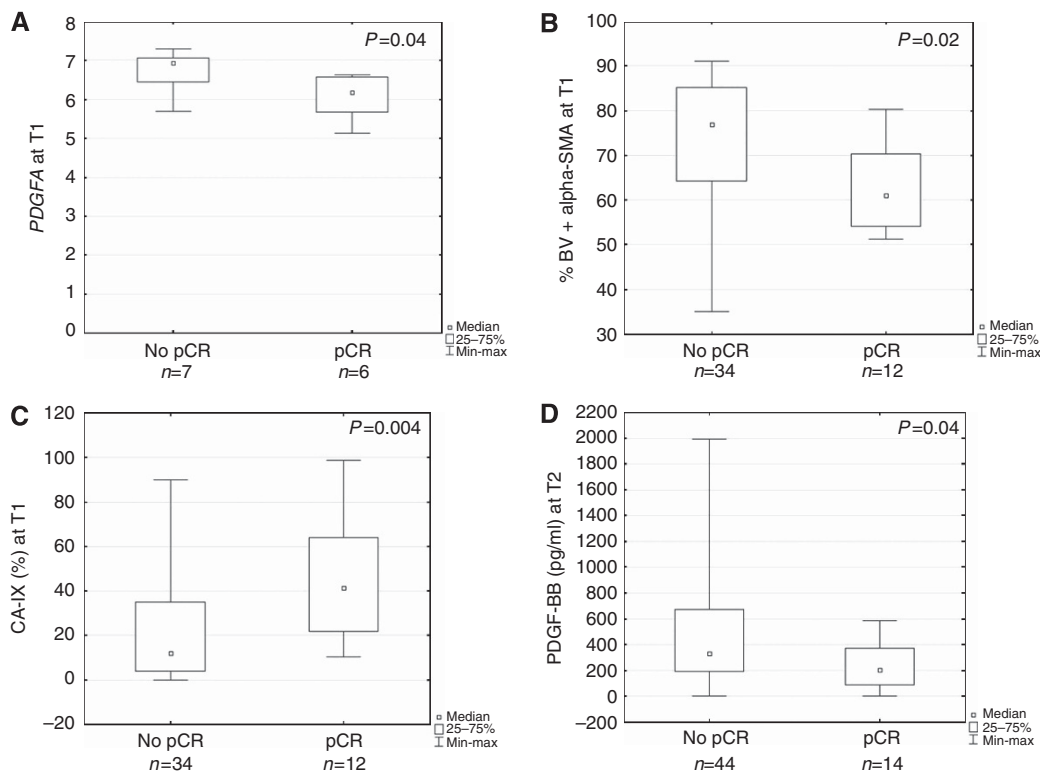


Figure 4. Correlation of markers with treatment response. Box plots of expression levels for patients with and without pCR. (A) Tumoral PDGFA expression, (B) pericyte coverage of blood vessels (BV) and (C) CA-IX expression after one dose of bevacizumab (T1). (D) Circulating PDGF-BB after two doses of bevacizumab (T2). Each plot represents the 25th and 75th percentile. The square inside the box indicates the median and the whiskers indicate the minimum and maximum values. P-values: Mann–Whitney U-test. Abbreviation: pCR = pathological complete response.

Table 4. Alterations in response to one dose of bevacizumab

Parameter	Willett phase I	Willett phase II	AXEBeam study	Willett vs AXEBeam
Blood flow	Decrease ^a	Decrease ^a	NA	?
Blood volume	Decrease ^a	Decrease	NA	?
PS product	No effect	Decrease ^a	NA	?
FDG uptake	No effect	No effect	NA	?
IFP	Decrease ^a	Decrease ^a	NA	?
MVD	Decrease ^a	NA	Decrease ^a	=
% α -SMA coverage	Increase	NA	Decrease ^a	≠
% Ang-2-positive BV	Decrease ^a	NA	NA	?
Tumour cell proliferation	Increase	NA	Decrease ^a	≠
Tumour cell apoptosis	Increase ^a	NA	No effect	≠
CA-IX expression	NA	NA	Increase	?
CECs	Decrease ^a	No effect	NA	?
CPCs	Decrease ^a	No effect	NA	?
Circulating VEGF	Increase ^a	Increase ^a	Increase ^{a,b}	=
Circulating PIGF	Increase ^a	Increase ^a	No effect ^c	≠
Circulating sVEGFR1	NA	No effect	No effect ^c	=
Circulating sVEGFR2	NA	No effect	No effect ^c	=
Circulating IL-6	NA	Increase ^a	No effect ^c	≠
Circulating IL-8	NA	No effect	Decrease	≠
Circulating IL-1 β	NA	No effect	NA	?
Circulating PDGF-AA	NA	NA	No effect	?
Circulating PDGF-BB	NA	NA	No effect	?
Circulating TSP-1	NA	NA	No effect	?
Circulating TSP-2	NA	NA	No effect ^b	?
Circulating Ang-2	NA	NA	Decrease	?
Circulating Ang-1	NA	NA	No effect ^b	?
Circulating Cyr61	NA	NA	No effect	?
Circulating FGFa	NA	NA	No effect ^c	?
Circulating FGFb	NA	No effect	No effect ^c	=
Circulating osteopontin	NA	NA	No effect ^c	?
Circulating sICAM	NA	NA	No effect ^c	?
Circulating VEGF-D	NA	NA	No effect ^b	?
Circulating SDF1 α	NA	No effect	NA	?
Circulating TNF- α	NA	No effect	NA	?
Circulating GM-CSF	NA	No effect	NA	?
Circulating CEA	NA	No effect	NA	?
Gene expression	NA	NA	cfr. Table e3	?
Tumour regression	1/6 Patients	No effect	NA	?

Abbreviations: Ang = angiopoietin; BV = blood vessels; CEA = carcinoembryonic antigen; CECs = circulating endothelial cells; CPCs = circulating progenitor cells; FGF = fibroblast growth factor; IFP = interstitial fluid pressure; GM-CSF = granulocyte macrophage colony-stimulating factor; IL = interleukin; MVD = microvessel density; NA = Not analysed; PDGF = platelet-derived growth factor; PIGF = placental growth factor; PS = permeability surface area product; SDF = stromal cell-derived factor; TNF = tumour necrosis factor; TSP = tromboposponin; VEGF = vascular endothelial growth factor.

^aSignificant change $P < 0.05$.

^b32 Patients were analysed.

^c14 Patients were analysed.

Contradicting results between our data and literature, however, could be related to small differences in the protocol and moment of sample collection, as normalisation is reported to be transient and context dependent. But then again, as VEGF facilitates the process of pericyte recruitment to endothelial cells (Benjamin *et al*, 1998), one could also hypothesise that blocking VEGF can result in less pericyte recruitment and coverage. When following this reasoning, the observed decrease in pericyte coverage after bevacizumab treatment in our study seems logic in contrast to the normalisation hypothesis where an increase in pericyte coverage is expected.

The complex influence of all angiogenic regulators is believed to be summarised by the MVD. Although MVD has been used before as a surrogate marker of angiogenesis in CRC and has been associated with prognosis (Frank *et al*, 1995; Zheng *et al*, 2003; Galizia *et al*, 2004), the significance of MVD in evaluating the response to anti-angiogenic agents remains unclear. Although some reports found a positive correlation between MVD and benefit from bevacizumab plus CRT (Foerzler *et al*, 2010; Gasparini *et al*, 2012), we and others did not (Jubb *et al*, 2006). Nevertheless, although we could not find a correlation between MVD and response, it is remarkable that MVD

decreased, although not significantly, after bevacizumab delivery in responding patients but not in non-responders. As mentioned before, the fraction of immature vessels provided additional information in our study, illustrating that assessing pericyte recruitment can probably more realistically indicate the angiogenic status of tumour vasculature compared with MVD counting that reflects only the presence of blood vessels.

Although we identified in responders several significant GO processes related to cell proliferation that are most likely relevant to the response to oxaliplatin, no differences were observed in Ki67 and cleaved PARP levels between responders and non-responders (data not shown).

Angiogenic circulating markers as biomarkers. Circulating PDGF-AA showed a more pronounced decrease in responders after two doses of bevacizumab (T2), which was also reflected in tumoral *PDGFA* expression levels. Bevacizumab treatment caused also a large decrease between T0 and T2 in circulating PDGF-BB levels in patients with a pCR, which was not observed in non-responders. As a result, responders displayed, after two doses of bevacizumab, lower PDGF-BB levels in their blood compared with the non-responders, indicating that this protein might be used as a prognostic marker.

However, combined chemoradiation with or without multi-tyrosine kinase inhibitors against VEGF has also been shown to decrease circulating levels of PDGF-AA or PDGF-BB (Loven *et al*, 2008; Inanc *et al*, 2012; Shin *et al*, 2012). Therefore, as in our study the first cycle of CRT was already started at T2, we have to be aware that the observed decline might be partly due to the CRT.

The other four markers tested (thrombospondin-1, angiopoietin-2, IL-8 and CYR61) displayed no differences between the two response subgroups upon bevacizumab delivery, and were not found to be associated with pathological response before or after bevacizumab treatment. However, in literature, data indicate a role for some of them in response to bevacizumab (Goede *et al*, 2010; Huang *et al*, 2010; Kopetz *et al*, 2010; Loupakis *et al*, 2011; Abajo *et al*, 2012; Liu *et al*, 2013).

Hypothesis. Putting all our data together, we hypothesise that bevacizumab in this clinical trial fulfils a role as inhibitor of new blood vessel formation as it blocks VEGF as facilitator of pericyte recruitment (Benjamin *et al*, 1998). Our data suggest that patients respond better when the tumour has less pericyte-covered blood vessels, leading to destabilisation of the vessels that makes them more susceptible to bevacizumab, resulting in a better response. Pericytes may express VEGF as survival factor for their neighbouring endothelial cells (Darland *et al*, 2003; Franco *et al*, 2011) and provide endothelial cell survival signals. As a consequence, more mature vessels may acquire independence from tumour-secreted VEGF resulting possibly in insensitivity to VEGF withdrawal (Evensen *et al*, 2009; Sitehy *et al*, 2011), which may show resistance to bevacizumab (Helfrich *et al*, 2010; Sitehy *et al*, 2011; Weisshardt *et al*, 2012) and might support tumour growth. Correspondingly, more hypoxia detected in our responding patients is consistent with a less-functional vasculature and might enhance the cell kill caused by chemotherapy through hypoxia-induced apoptosis (Selvakumaran *et al*, 2008). Higher PDGF-BB levels in the non-responding patients are also rational as they provide mitogenic signals for pericyte recruitment and maturation (Lindahl *et al*, 1997; Abramsson *et al*, 2003), and were reported to be detected in blood of progressing patients and around vessels that developed resistance to anti-VEGF therapy (Huang *et al*, 2004; Kopetz *et al*, 2010).

This hypothesis, by which pericytes protect endothelial cells from anti-angiogenic therapy, promotes the combination of VEGF and PDGF inhibitors. Blocking the survival factor VEGF together with blockage of pericyte recruitment and maturation could prevent endothelial survival directly and indirectly (Shaheen

et al, 2001; Bergers and Benjamin, 2003; Erber *et al*, 2004; Helfrich *et al*, 2010; Franco *et al*, 2011).

The current study should be considered as a pilot study looking for hypothesis-generating hints with several limitations. The trial did not allow us to compare results between the bevacizumab-containing regimen and CRT alone, as no arm was included without bevacizumab. Moreover, many treatments are involved in producing the observed phenotype of response, masking possible interactions and confounders. Therefore, it is impossible to speculate on a prognostic or predictive effect of the biomarkers studied, and it is not clear whether the correlations with response we observed are driven by bevacizumab, by the chemotherapy, by the radiotherapy or a combination of those treatments. One cannot exclude the possibility that the hypothesis we put forward may also be the result of mechanisms next to bevacizumab activity in this setting. Owing to a small sample size, we have to be aware of limited statistical power when interpreting our data. It should be noted that several markers identified in our study as potential biomarkers show a considerable overlap between responders and non-responders, limiting their clinical applicability. It is indeed unlikely that a single biomarker will allow differentiating between responders and non-responders. However, before any of these markers can be considered a biomarker, either on its own or in a panel of different markers, these markers should be prospectively validated in independent randomised cohorts to fully assess their prognostic or predictive value.

CONCLUSION

In summary, we could not support the 'normalization hypothesis' that states that bevacizumab results in more mature blood vessels and less hypoxia. Analysis of the microarray data showed an intrinsic angiogenic phenotype displayed in tumours from patients with a pCR. Moreover, angiogenic pathways are influenced by bevacizumab only in those tumours in contrast to non-responding tumours. Our exploratory data point towards a possible prognostic role for *PDGFA*, PDGF-BB, CA-IX and pericyte-covered blood vessels. Our findings suggest a role for pericyte recruitment and vessel maturation for the susceptibility of the tumour vasculature to bevacizumab treatment. Further exploration of our mechanistic hypothesis and validation of the candidate markers is demanded in large, well-powered randomised studies in order to contribute to a more rational therapeutic approach of anti-angiogenic agents in combinations with CRT.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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