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Evaluation of efficacy and safety markers in a phase II study of metastatic colorectal cancer treated with aflibercept in the first-line setting

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Background: Aflibercept (ziv-aflibercept) is an anti-angiogenic agent recently approved in combination with FOLFIRI for the treatment of metastatic colorectal cancer (mCRC) patients previously treated with oxaliplatin. Despite heterogeneity in response to aflibercept, no biomarkers for efficacy or adverse effects have been identified. Here we present biomarker data from the randomised phase II AFFIRM trial assessing aflibercept in combination with mFOLFOX6 first line in mCRC.

Methods: Ninety-six somatic mutations in key oncogenic drivers of mCRC and 133 common single-nucleotide polymorphisms (SNPs) in vascular endothelial growth factor (VEGF) pathway genes were analysed, and 27 plasma markers measured at baseline, during and after treatment. We assessed correlations of these three classes of biomarkers with progression-free survival (PFS) and adverse events (AEs).

Results: Somatic mutations identified in *KRAS*, *BRAF*, *NRAS*, *PIK3CA* and *PIK3R1* did not significantly correlate with PFS (multiple testing-adjusted false discovery rate (FDR) or multiple testing-adjusted FDR > 0.3). None of the individual SNPs correlated with PFS (multiple testing-adjusted FDR > 0.22), but at the gene level variability in *VEGFB* significantly correlated with PFS (multiple testing-adjusted FDR = 0.0423). Although none of the plasma markers measured at baseline significantly correlated with PFS, high levels of circulating IL8 at baseline together with increased levels of IL8 during treatment were significantly associated with reduced PFS (multiple testing-adjusted FDR = 0.0478). No association was found between biomarkers and AEs.

Conclusions: This represents the first biomarker study in mCRC treated with aflibercept. High IL8 plasma levels at baseline and subsequent increases in IL8 were associated with worse PFS, suggesting that IL8 may act as a potentially predictive biomarker of aflibercept treatment outcome.

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Angiogenesis inhibitors have been established over the past decade as valuable tools to inhibit tumour growth and improve tumour response to chemotherapy. These include the humanised monoclonal antibody against vascular endothelial growth factor (VEGFA), bevacizumab (Avastin; Bennouna *et al*, 2013), and the soluble humanised chimeric VEGF receptor fusion protein, aflibercept (ziv-aflibercept; Van Cutsem *et al*, 2012; Chiron *et al*, 2014). Despite the significantly prolonged survival observed with these angiogenesis inhibitors, patients still succumb to their disease, suggesting that inhibition of a single-angiogenic growth factor alone is insufficient to induce vessel normalisation and stop tumour growth. Indeed, subsets of patients treated with anti-angiogenic therapies do not respond, gradually develop resistance or present with therapy-related adverse events (AEs) (Jubb and Harris, 2010). The identification of potential biomarkers that predict response to anti-angiogenic treatment (Lambrechts *et al*, 2013) or the occurrence of these AEs is thus warranted, as it will enable better patient selection for anti-angiogenic therapy (Lambrechts *et al*, 2014; Schneider *et al*, 2014).

Aflibercept is an anti-angiogenic agent, which has recently been approved in combination with FOLFIRI for the treatment of patients with metastatic colorectal cancer (mCRC) who were previously treated with oxaliplatin. The approval was based on data obtained from the VELOUR trial—a multicenter, randomised, placebo-controlled phase III trial, which compared the efficacy of aflibercept *vs* placebo in combination with the FOLFIRI regimen as a second-line treatment for patients with mCRC previously treated with oxaliplatin (Van Cutsem *et al*, 2012; Joulain *et al*, 2013). AFFIRM, an open-label, non-comparative, phase II study, was conducted to assess the combination of aflibercept and modified FOLFOX6 (mFOLFOX6) given as first-line therapy in patients with mCRC (Pericay *et al*, 2012). The primary end point was 12-month progression-free survival (PFS), whereas exploration of biomarkers was among the secondary objectives.

In an attempt to understand the key factors associated with aflibercept efficacy and safety, we conducted an investigation assessing biomarkers for aflibercept treatment in prospectively collected tumour tissues and serially sampled plasma from patients participating in the AFFIRM study. Plasma proteins and genetic variants, representing either single-nucleotide polymorphisms (SNPs) in VEGF pathway genes or somatic mutations in key oncogenic drivers of mCRC, were analysed to assess if they could predict response to aflibercept with respect to PFS. Subsequently, we also assessed whether any of these markers correlated with anti-angiogenic drug-induced AEs, such as gastrointestinal perforation, thrombosis, hypertension and proteinuria.

MATERIALS AND METHODS

Patients. The intent-to-treat population comprised 236 randomised patients with mCRC randomised to receive either mFOLFOX6 plus 4 mg kg^{-1} IV aflibercept every 2 weeks ($N=119$) or mFOLFOX6 alone ($N=117$). Patient eligibility criteria have been previously described (Pericay *et al*, 2012). The primary end point of the study was to estimate PFS at 12 months. Secondary objectives included the objective overall response rate, PFS, the overall survival (OS) time and safety profiling (Ciombor *et al*, 2013). Response was recorded based on Response Evaluation Criteria in Solid Tumors (RECIST, version 1.0). Patients with AEs were defined as experiencing the event at least once during the study treatment period, and all AEs were graded using Common Terminology Criteria for Adverse Events (CTCAE, version 3.0). The trial protocol and genetic biomarker studies were approved by the institutional review board at each site and were in accordance with the Declaration of Helsinki, US Food and Drug Administration Good Clinical Practices, and local ethics and legal requirements. All patients included in this study provided written informed consent for genetic biomarker testing. ClinicalTrials.gov NCT00851084.

Somatic mutation selection and profiling. Ninety-six hotspot mutations in eight genes frequently mutated in mCRC were selected from the COSMIC database (Supplementary Table S1). After the tumour area was marked by a pathologist, tumour DNA was extracted from three formalin-fixed paraffin-embedded tumour slides per patient collected at the time of diagnosis using the Qiagen DNAeasy kit (Qiagen, Antwerp, Belgium). Mutation profiling was carried out using Sequenom MassARRAY (Sequenom, San Diego, CA, USA) as previously described (Lambrechts *et al*, 2012; Zhao *et al*, 2014). Mutations were grouped by gene for the prediction of efficacy or safety.

SNP selection and genotyping. One hundred and eighty-three tagging SNPs in VEGF pathway genes (Lambrechts *et al*, 2012) and four SNPs known to be associated with an increased susceptibility to hypertension and thrombosis were selected (Supplementary Table S2). Germline DNA was extracted from peripheral blood using the Qiagen DNAeasy kit (Qiagen, Belgium) and genotyped using Sequenom MassARRAY as previously described (Zhao *et al*, 2014).

Plasma profiling. The plasma concentration of 27 cytokines, growth factors or soluble receptors was determined by enzyme-linked immunosorbent assays (ELISA) using two Fluorokine MAP kits (the human angiogenesis panel A and the human high sensitivity cytokine panel; R&D Systems, Abingdon, UK). Competition experiments were conducted to test interference of aflibercept with the detection of VEGFA, VEGFD and placental growth factor (PlGF). Angiopoietin-2 (ANGPT2), SDF1-α, HGF, VEGFC, soluble VEGF receptor 3 (sVEGFR3) and sVEGFR2 were assessed by single ELISA (R&D Systems). Plasma markers were analysed at baseline, at 30 and 60 days after the first study treatment infusion and 30 days after the last aflibercept infusion.

Statistical analysis. Differences between patients with evaluable biomarkers and patients without evaluable biomarkers were assessed using a two-sided Fisher's exact test for categorical variables and ANOVA for continuous variables. Biomarkers were analysed as quantitative variables, by coding the absence or presence of a somatic mutation as 0 or 1, and SNP genotypes as 0, 1 or 2 depending on the number of minor alleles present. The linear effects of baseline biomarkers on PFS were assessed using a Cox proportional hazard model with the following co-variates: Eastern Cooperative Oncology Group performance status (0–1 *vs* 2), liver-only metastases (yes/no), and the number of distant metastasis organs (1 *vs* >1), a treatment effect, a biomarker effect and a biomarker-treatment interaction effect. The significance of the latter two effects was jointly tested by a two-degrees-of-freedom Wald test. Extended statistical methods are described in Supplementary Methods.

RESULTS

Of the 236 patients in the ITT population of the AFFIRM trial, 227 (96%) were evaluable for PFS. Of these, 130 (57%) provided at least one biological sample, 60 (46%) and 70 (54%) of which participated in the mFOLFOX6 and mFOLFOX6 plus aflibercept arms, respectively. There was no difference at a $P<0.05$ between patients who provided a biological sample and those who did not in terms of patient biometrics, ethnicity and disease characteristics at baseline, or at efficacy and safety end points (Table 1). Of those who provided at least one biological sample, 51 (39%) provided samples for each of the three biomarker types, with 88 (68%) and 97 (74%) patients providing samples for two or one of the biomarker types,

respectively. Each biomarker type was analysed separately, to avoid patient groups that were too small for sub-analyses.

Somatic mutations and efficacy. Somatic mutation profiling was performed on tumour DNA obtained from 93 out of 227 (41%) patients evaluable for response, 47 (51%) of whom had been treated with aflibercept. The median mutation call rate per patient was 96.4% and 89 (96%) patients had a call rate >80%. Mutations were detected in *KRAS* ($n=31$), *PIK3CA* ($n=10$), *PIK3R1* ($n=5$), *BRAF* ($n=3$) and *NRAS* ($n=3$); 45 patients carried only wild-type (wt) alleles, 44 patients carried a single mutation and four carried a mutation both in *PIK3CA* and *KRAS*. We assessed the effect of the gene mutation status on PFS, while allowing for interaction with both treatment arms. *P*-values were lowest, but not significant for *KRAS* (unadjusted *P*-value = 0.0601; multiple testing-adjusted false discovery rate (FDR) = 0.30; Supplementary Table S3). Although patients carrying mutant *KRAS* tumours exhibited a slightly worse median PFS when treated with mFOLFOX6 compared with patients treated with mFOLFOX6 plus aflibercept (7.7 *vs* 10.1 months), this did not reach significance (Table 2). There was also

Table 1. Summary of baseline disease characteristics in patients from the biomarker evaluable or non-evaluable population

	Non-evaluable population		Evaluable population	
	mFOLFOX6 (*N* = 57)	Aflibercept plus mFOLFOX6 (*N* = 49)	mFOLFOX6 (*N* = 60)	Aflibercept plus mFOLFOX6 (*N* = 70)
Gender: male/female	32/25	33/16	36/24	43/27
Age: median (min : max)	66 (44 : 87)	62 (29 : 75)	62 (37 : 81)	63 (41 : 79)
Age: <65/⩾65 years	27/30	28/21	38/22	42/25
Race: Caucasian/Asian/Black/other	41/16/0/0	37/11/0/1	49/11/0/0	60/9/1/0
Weight (kg): median (min : max)	68 (40 : 107)	70 (40 : 115)	73 (48 : 134)	71 (40 : 117)
BSA (m^2): median (min : max)	1.8 (1 : 2)	1.8 (1 : 2)	1.8 (1 : 2)	1.8 (1 : 2)
Prior adjuvant chemotherapy: yes/no	50/7	45/4	55/5	63/7
Prior surgery: yes/no	35/22	27/22	26/34	29/41
Prior radiotherapy: yes/no	53/4	47/2	57/3	62/8
ECOG performance status: 0/1/2	55/2	46/3	60/0	69/1
Number of metastatic organs involved at baseline: 0/1/>1	0/16/41	0/15/34	1/15/44	0/12/58
Liver-only metastases: yes/no	15/42	20/29	18/42	14/56

Abbreviation: ECOG = Eastern Cooperative Oncology Group.

Table 2. Kaplan–Meier estimates of effect of biomarkers on months of PFS

	mFOLFOX6 median (99% CI)	Aflibercept plus mFOLFOX6 median (99% CI)	Hazard ratio *vs* mFOLFOX6 (99% CI)
Somatic mutations			
All patients	10.9 (8.80–14.19)	8.5 (7.72–11.63)	1.439 (0.739–2.801)
KRAS wt	11.2 (7.62–12.48)	10.1 (7.95–12.78)	0.971 (0.424–2.221)
KRAS mt	10.1 (3.71–NC)	7.7 (5.82–12.85)	2.571 (0.785–8.423)
RAS wt	10.0 (7.62–12.19)	10.1 (7.95–12.88)	0.888 (0.362–2.176)
RAS mt	10.9 (3.71–15.64)	7.9 (6.67–10.48)	2.340 (0.855–6.404)
Germline SNPs			
All patients	9.3 (8.11–11.33)	9.0 (7.46–10.91)	1.304 (0.7–2.432)
rs3741403			
C/C	8.9 (6.28–9.46)	6.7 (3.81–9.99)	1.322 (0.459–3.806)
C/T or T/T	10.9 (7.10–14.23)	9.3 (8.08–11.70)	1.412 (0.667–2.989)
rs2346176			
C/C	11.8 (9.23–15.57)	7.7 (4.14–9.20)	3.449 (0.934–12.73)
C/T or T/T	8.8 (5.59–10.91)	10.0 (7.92–11.10)	0.974 (0.479–1.979)
rs4953344			
T/T	9.2 (5.62–10.91)	9.3 (7.98–11.63)	0.87 (0.426–1.778)
C/C or C/T	11.8 (8.11–15.64)	7.5 (3.81–10.91)	2.912 (0.919–9.226)
rs2881324			
T/T	11.8 (5.09–15.64)	8.1 (4.63–9.99)	2.159 (0.679–6.863)
G/G or G/T	9.2 (7.10–10.91)	9.3 (7.46–11.10)	0.888 (0.432–1.822)
Plasma protein markers, baseline			
All patients	8.8 (6.57–10.02)	8.5 (6.67–10.05)	0.979 (0.505–1.897)
IL8 $\leq 19\,pg\,ml^{-1}$	8.8 (5.62–10.91)	9.3 (7.52–11.10)	0.764 (0.363–1.607)
IL8 $>19\,pg\,ml^{-1}$	8.8 (5.09–15.64)	4.1 (2.33–8.54)	2.71 (0.735–9.984)

Abbreviations: CI = confidence interval; mt = mutant; PFS = progression-free survival; SNP = single-nucleotide polymorphism; wt = wild-type. Detailed *P*-values for each of these estimates are highlighted in the Supplementary Tables. Multiple testing corrected FDR values are, respectively, 0.3005 and 0.1803 for KRAS and RAS, 0.2207 for each of the SNPs, and 0.5962 for baseline IL8.

no effect of the *RAS* (*KRAS*, *BRAF* and *NRAS*) mutation status on PFS (Table 2).

Germline SNPs and efficacy. Germline DNA was available for 97 randomised patients, 53 of whom had been treated with aflibercept, and was genotyped for 133 SNPs.

Principle component analysis showed that genotype variance was partly explained by race (Supplementary Figure S1). As the proportion of Asian patients in AFFIRM was quite large (16 Asian *vs* 81 non-Asian patients), we first analysed SNP data without Asian patients, and subsequently carried out a sensitivity analysis by including them while stratifying for race. Among the 133 SNPs tested, there were four SNPs with a $P<0.05$ on PFS and a multiple testing-adjusted FDR of 0.22. One SNP was in *VEGFB*, which is inhibited by aflibercept (rs3741403, $P=0.0021$), whereas three other SNPs were located in *EPAS1,* (rs2346176, $P=0.0051$; rs4953344, $P=0.0065$; and rs2881324, $P=0.0066$; Supplementary Table S4), which codes for HIF-2alpha, a mediator of cellular response to hypoxia. These SNPs also exhibited the lowest *P*-values when including Asian patients (Supplementary Table S4).

The combined effects of individual SNPs in a given gene were explored in a gene-wise association analysis. Of the 17 genes tested, we observed a significant effect for *VEGFB*, ($P=0.0025$; multiple testing-adjusted FDR = 0.0423; Supplementary Table S5), in which three SNPs were genotyped, whereas *EPAS* ranked second (multiple testing-adjusted FDR = 0.1205). To assess which combination of SNPs accounted for the effect on PFS, we estimated haplotype frequencies consisting of rs3741403, rs1058735 and rs594942 (Supplementary Table S6). The most frequent haplotype in *VEGFB*, that is, the TCC haplotype, was selected in a stepwise Cox model. The hazard ratio for the TCC haplotype was 0.214, but the interaction with treatment arm was not selected, suggesting that it had a prognostic effect.

Profiling of plasma markers for efficacy. Plasma levels of 27 markers were measured at different time points (i.e., at baseline (87 patients); 30 and 60 days after start of treatment (82 and 73 patients); and 30 days after the last treatment (56 patients; Supplementary Table S7)). We assessed the association of each plasma marker at baseline with PFS, while allowing for an interaction with treatment (Supplementary Table S8). The lowest *P*-value was obtained for IL8 ($P=0.0221$; multiple testing-adjusted FDR = 0.596 and $P=0.0218$ for interaction) with a possible threshold effect at 19 pg ml^{-1} IL8 (Table 1; Supplementary Methods).

We also analysed whether treatment-related changes in plasma markers could predict aflibercept treatment outcome. The Cox model included the effect of baseline plasma levels and the difference in expression measured at baseline and the last time point before discontinuation, disease progression or death (i.e., at 30 days or 60 days of treatment), while allowing for interaction with the treatment arm. IL8 was the only marker with a significant effect of change from baseline on PFS ($P=0.0018$; multiple testing-adjusted FDR = 0.0478; Table 3). In particular, high baseline or post-baseline increased IL8 levels corresponded to a higher probability of disease progression at 12 months (Figure 1). A multivariate Cox model with lasso penalisation was carried out to check if additional markers in combination with IL8 marker would improve prediction of PFS. A maximum of likelihood was not reached by the cross-validation procedure (R Package penalised), which was interpreted as a failure of additional biomarkers to better predict PFS.

Interestingly, some cytokines showed significant expression changes after the start of treatment. Expression levels at day 30 after treatment start were compared to baseline, and at day 60 to day 30 after treatment start. The following significant changes were detected after FDR adjustment in the aflibercept/mFOLFOX6 arm (Table 4): a decrease in soluble VEGFR3, an increase in PlGF and a decrease in ANGPT2 (Figure 2; Supplementary Figure S2).

Table 3. Effect of plasma marker changes from baseline on PFS

	P-values			
Protein	Joint effect	Joint effect (multiple testing-adjusted FDR)	Change from baseline effect	Treatment by Change from baseline effect
IL8	0.0018	0.0478	0.0006	0.2028
IL10	0.0342	0.4525	0.5214	0.8204
VEGFA	0.0619	0.4525	0.0189	0.0704
CXCL12	0.0670	0.4525	0.1714	0.0318
CSF2	0.0855	0.4619	0.0266	0.1682
VEGFC	0.1127	0.5072	0.0742	0.9355
IL5	0.1886	0.7275	0.0684	0.1657
Endostatin	0.2418	0.7882	0.9988	0.2668
PDGFA	0.3092	0.7882	0.1508	0.9922
TNF	0.3209	0.7882	0.1770	0.1554
IL4	0.3211	0.7882	0.1938	0.7631
FGF2	0.3851	0.8191	0.6468	0.3957
sVEGFR3	0.3944	0.8191	0.2533	0.1729
THBS2	0.4677	0.8426	0.2387	0.6683
FGF1	0.4770	0.8426	0.4669	0.2242
PlGF	0.4993	0.8426	0.2501	0.2755
ANGPT2	0.5638	0.8924	0.3373	0.8394
IL1B	0.6261	0.8924	0.3345	0.5414
PDGFB	0.6583	0.8924	0.3730	0.6009
IL12	0.6611	0.8924	0.7934	0.7744
IL2	0.7984	0.9482	0.9352	0.6027
sVEGFR2	0.8375	0.9482	0.6009	0.5613
HGF	0.8383	0.9482	0.5804	0.5665
IFNG	0.8559	0.9482	0.6923	0.9101
ANGPT1	0.8814	0.9482	0.6269	0.8538
VEGFD	0.9131	0.9482	0.6838	0.7528
IL6	0.9963	0.9963	0.9893	0.9594

Abbreviations: FDR = false discovery rate; PFS = progression-free survival. *P*-values (and FDR) of the joint effects of plasma marker and treatment by plasma marker interactions are shown. *P*-values for the change from baseline and the interaction are also presented.

Plasma markers correlated with safety end points. Finally, we also assessed which of these biomarkers correlated with any of the following binary safety end points (occurrence of an AE or not): hypertension, proteinuria grade $\geqslant$ 2 (either derived from laboratory data or when nephrotic syndrome was reported as an AE), haemorrhage or venous thromboembolism. Patients in the biomarker evaluable population were exposed slightly longer to treatment than patients in the population without evaluable biomarkers (median number of cycles: 12 *vs* 9 or 10), but there was no difference in exposure between both treatment arms in the biomarker evaluable population (Supplementary Table S9). The frequency of these safety end points did not differ significantly between the populations with and without evaluable biomarkers (Supplementary Table S10). No effect of any type of biomarker (gene mutation status, SNP genotypes, baseline plasma biomarker levels) on any AE was observed at a multiple testing-adjusted FDR level below 0.5 (Supplementary Table S11). For each plasma marker, we also defined the cutoff levels that maximise the interaction with treatment. The optimal cutoff was obtained for HGF, at a level of 1.43 pg ml^{-1} (71th percentile). Although hypertension was strongly associated with aflibercept treatment

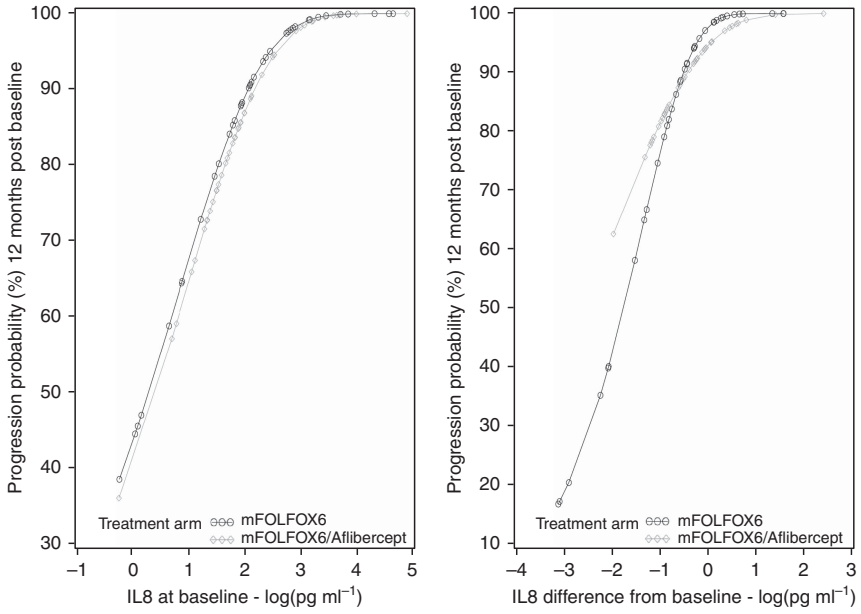

Figure 1. Relation between IL8 levels and probability of disease progression. Depicted is the probability of disease progression after 12 months in relation (left) to IL8 plasma levels at baseline and (right) to the difference between IL8 plasma levels at baseline and at the last measurement point before disease progression.

Table 4. Expression changes of plasma markers in response to treatment

Plasma biomarker	Time–treatment	Slope estimate (month^{-1})	*P*-value	Multiple testing-adjusted FDR
sVEGFR3	T1-aflibercept/mFOLFOX6	−1.3619	<0.0001	<0.0001
PlGF	T1-aflibercept/mFOLFOX6	0.2081	<0.0001	<0.0001
HGF	T2-mFOLFOX6	0.1864	0.0004	0.0137
ANGPT2	T1-aflibercept/mFOLFOX6	−0.2722	0.0016	0.0443

Abbreviation: FDR = false discovery rate. Table of slope estimates for expression changes of plasma markers (change from baseline) that are significantly different from zero. Slopes are estimated between baseline and day 30 (time T1), and between day 30 and day 60 (time T2) for each treatment arm.

(OR = 50.4), only 20.0% of patients with high levels of plasma HGF (> 1.43 ng ml^{-1}) developed aflibercept-induced hypertension in the aflibercept/mFolfox6 treatment arm, compared with 75.8% of patients with low HGF levels (< 1.43 ng ml^{-1}; Supplementary Table S12).

DISCUSSION

AFFIRM was an open-label, non-comparative, phase II study conducted to assess the combination of aflibercept and mFOLFOX6 given as first-line therapy in patients with mCRC. Patients who received aflibercept in combination with mFOLFOX6 or mFOLFOX6 alone had 12-month PFS rates of 25.8% (95% CI: 17.2%–34.4%) and 21.2% (95% CI: 12.2%–30.3%), respectively (Pericay *et al*, 2012). In contrast to bevacizumab, which prolongs PFS when combined with mFOLFOX6, aflibercept did not have a significant effect on PFS in this study. It should be noted, however, that in AFFIRM mFOLFOX6 arm only served as an internal benchmark, since the sample size of the study did not allow for an adequately powered statistical comparison between the two treatment groups. Nevertheless, this study provided an opportunity to conduct the first comprehensive biomarker analysis of aflibercept using blood and archived tumour tissue collected from participating patients. The most relevant finding is that treatment outcomes with aflibercept appear to be independent of *(K)RAS* mutation status. From a clinical standpoint, this observation is important as aflibercept is considered as a second-line treatment option for patients with mCRC previously treated with oxaliplatin (Bennouna *et al*, 2013). In first line, many patients may have been treated with the angiogenesis inhibitor bevacizumab combined with oxaliplatin (Van Cutsem *et al*, 2009). The OS and PFS outcomes of bevacizumab in the treatment of patients with mCRC are generally considered to be independent of *KRAS* gene status (Petrelli *et al*, 2013; Sastre *et al*, 2013). If the therapeutic effects of aflibercept had been limited to either *KRAS* wt or mutant tumours, this would have important consequences for second-line treatment options, as anti-EGFR treatments—which are generally used as a second- or third-line treatment after bevacizumab—are only recommended in *KRAS* wt tumours (Brodowicz *et al*, 2013). Our observations should be interpreted cautiously, however, as AFFIRM was a non-comparative study. Nevertheless, evaluation of our finding in further studies is warranted.

Studies suggest that chemokines and their receptors serve as important regulators of various metastatic and advanced cancers (Coussens and Werb, 2002; Miles *et al*, 2013). Interestingly, we identified that high IL8 levels at baseline correlated with shorter survival times, and patients with increasing levels of IL8 during treatment were more likely to progress. This suggests that patients with high IL8 levels, at baseline or during treatment, are at increased risk of disease progression during aflibercept therapy.

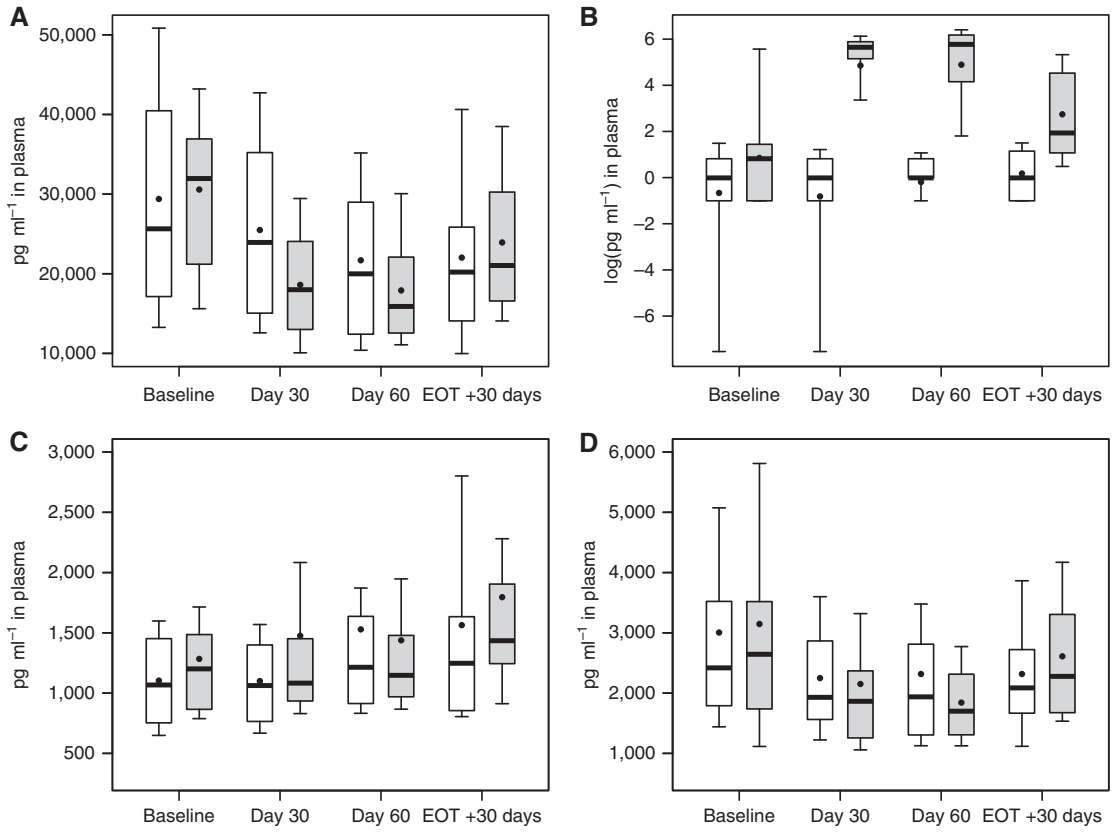

Figure 2. Plasma levels of sVEGFR3 (**A**), PlGF (**B**), HGF (**C**) and ANGPT2 (**D**). Concentrations are displayed for mFOLFOX6-treated (White) and mFOLFOX6/Aflibercept-treated (grey) patients at baseline, 30 days after the first study treatment infusion, 60 days after first infusion and 30 days after last infusion. Plasma levels for PlGF are log-transformed to allow visualisation of low levels of PlGF.

The role of IL8 and its receptor CXCR1 and -2 in tumour development and progression is well documented (Bennouna *et al*, 2013). Expression and secretion of IL8 by tumour cells enhances proliferation and survival through autocrine activation, and promotes angiogenesis and neutrophil infiltration into the tumour (Bennouna *et al*, 2013). IL8 also preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells (Mizukami *et al*, 2005). Interestingly, in a phase II trial of 43 hepatocellular carcinoma patients receiving bevacizumab (Boige *et al*, 2012), and another phase II trial of 42 mCRC patients on bevacizumab (Kopetz *et al*, 2010), elevated baseline IL8 also predicted shorter PFS times. Likewise, lower baseline IL8 levels were observed by Abajo *et al* (2012) in mCRC patients responding to a bevacizumab-containing therapy, whereas Hayashi *et al* (2014) observed increased baseline IL8 serum levels in non-responding mCRC patients participating to a phase 2 trial involving FOLFIRI chemotherapy plus bevacizumab. Similarly, IL8 levels correlated with OS in plasma samples from mCRC patients receiving capecitabine, oxaliplatin and bevacizumab (Liu *et al*, 2013). Another study in advanced hepatocellular carcinoma found that patients high levels of IL8 were also associated with poor outcome (Zhu *et al*, 2009), whereas in a phase 2 study in urotehlial cancer increases in IL8 after 4 weeks of pazopanib treatment were associated with a lower response probability (Necchi *et al*, 2014). SNPs in *IL8* and its receptors have also been correlated with bevacizumab treatment outcome, thus further highlighting the potential role of IL8 in predicting response to anti-angiogenic therapies (Schultheis *et al*, 2008; Lambrechts *et al*, 2013).

In addition, we searched for relative changes in plasma markers induced by aflibercept treatment. We observed a decrease in sVEGFR3 and ANGPT2 levels, as well as an increase in PlGF levels. For PlGF, the increase most likely reflects an upregulation to compensate that most of the circulating PlGF is bound by aflibercept. We did not observe such compensatory upregulation for VEGFA. In this respect, aflibercept differs with respect to the consistent upregulation in VEGFA observed in most bevacizumab trials (Zhu *et al*, 2013). However, absence of a compensatory upregulation of VEGFA might be due to interference of aflibercept in the ELISA assay. Indeed, competition ELISA experiments for VEGFA, PlGF and VEGFD with aflibercept confirmed that aflibercept strongly interferes with VEGFA, but not PlGF or VEGFD antibodies used in the MAP kits (Supplementary Figure S3). In contrast to several other biomarker studies in mCRC involving bevacizumab treatment, we failed to see a change in sVEGFR2 following aflibercept treatment.

Finally, we also observed that ANGPT2 levels decrease following aflibercept delivery. ANGPT2 is a secreted factor that binds the endothelial cell-specific receptor tyrosine kinase TIE2 and has a complex role during angiogenesis. ANGPT2 is highly expressed by tumour endothelial cells, inhibits Tie2 activity and destabilises blood vessels, thereby facilitating VEGF-dependent vessel growth (Daly *et al*, 2013). Yet tumour xenografts treated with the combination of an ANGPT2-specific antibody (REGN910) and aflibercept demonstrated reduced tumour vascularity and tumour perfusion that was more pronounced than with either single agent, resulting in more extensive tumour cell death and more potent inhibition of tumour growth (Daly *et al*, 2013). Likewise, a human monoclonal anti-ANGPT2 antibody has a broader antitumor activity when combined with VEGF inhibitors (Brown *et al*, 2010). This indicates that ANGPT2 might indeed have a protective role in tumour endothelial cells by activating TIE2, thereby limiting the anti-vascular effects of VEGF inhibition. Interestingly, Goede *et al* (2010) reported that amongst 34 mCRC patients receiving bevacizumab, low pre-therapeutic serum ANGPT2 levels were

associated with a significant better response rate, prolonged median PFS and reduction of 91% in the risk of death. On the other hand, in tumour biopsies collected before and 12 days after bevacizumab monotherapy in rectal cancer patients, ANGPT1 and ANGPT2 were both downregulated in cancer cells. Our data therefore add to the emerging role of ANGPT2 as a potential mediator of response to anti-angiogenic therapies.

In conclusion, we here present the very first data investigating biomarkers for aflibercept treatment. Our observations for IL8, PlGF and ANGPT2 suggest that circulating cytokine levels before therapy, as well as their changes during aflibercept therapy, are consistent with those seen for other anti-VEGF agents. Pending validation in other aflibercept-containing regimens, these exploratory biomarker data could be useful to guide patient selection in future studies with aflibercept.

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CONFLICT OF INTEREST

DL and EVC have served on advisory boards for Sanofi and received research funding from Sanofi to conduct this study. GF has received honoraria for participation in *ad hoc* advisory boards and for lectures from Sanofi. JZ has received travel and research support and honoraria for participation in advisory boards from Sanofi. VT, CZ, EM and MC are employees of Sanofi. BT, MM, XS, CP and GP declare no conflict of interest. The clinical trial, collection of tissue for biomarker evaluation and the evaluation of biomarkers were sponsored and funded by Sanofi-Aventis, Paris, France. BT holds a postdoctoral fellowship of the Fund for Scientific Research Flanders (FWO-F).

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