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Summary

Bevacizumab is the first anti-angiogenic agent approved for the treatment of metastatic colorectal cancer (mCRC). The need for patient selection before initiating therapy necessitates the study of various proteins expressed in mCRC tissue as candidate predictive markers. Immunohistochemistry is a valuable, commonly available and cost-effective method to assess predictive biomarkers. However, it is subject to variations and therefore requires rigorous protocol standardizations. Furthermore, validated quantification methodologies to study these angiogenic elements have to be applied. Based on their function in tumor angiogenesis and their relation to the mechanism of action of bevacizumab, protein markers were divided in four groups: I) vascular endothelial growth factor A-signaling proteins, II) other relevant angiogenesis factors, III) factors regarding the tumor microenvironment and IV) tumor intrinsic markers. Conceivably, nimbly selecting a small but relevant group of therapy guided patients by the appropriate combination of predictive biomarkers may confer great value to this angiogenic inhibitor.

Keywords

List of abbreviations

5-FU	5-fluorouracil
αSMA	alpha smooth muscle actin
ABL1	c-abl oncogene 1
ACTA2	aorta smooth muscle actin-alpha 2
AKT1	v-akt murine thymoma viral oncogene homolog 1
ANGPT1	angiopoietin 1
ANGPT2	angiopoietin 2
BRAF	v-raf murine sarcoma viral oncogene homolog B
CA9	carbonic anhydrase IX
CRC	colorectal cancer
CSF1R	colony stimulating factor 1 receptor
CXCL12	chemokine (C-X-C motif) ligand 12
CXCR4	chemokine (C-X-C motif) receptor 4
DDR2	discoidin domain receptor tyrosine kinase 2
EC	endothelial cell
EFNB2	ephrin-B2
EGF	epidermal growth factor
EGFL7	EGF-like-domain 7
EGFR	EGF receptor
EMA	European Medicines Agency
ENG	endoglin
EPHA2	EPH receptor A2
EPHB4	EPH receptor B4
ERBB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGFR1	fibroblast growth factor receptor 1
FGFR2	fibroblast growth factor receptor 2
FIGF	c-fos induced growth factor
FLT1	fms-related tyrosine kinase 1
FLT3	fms-related tyrosine kinase 3
FLT4	fms-related tyrosine kinase 4
FOLFIRI	folinic acid – fluorouracil (infusion) – irinotecan
FRK	fyn-related kinase
HER2	human epidermal growth factor receptor 2
IFL	irinotecan – fluorouracil (bolus) – leucovorin
IHC	immunohistochemistry
KDR	kinase insert domain receptor
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAP1LC3	microtubule-associated protein 1 light chain 3
mCRC	metastatic colorectal cancer
MKI67	marker of proliferation Ki-67
MVD	microvessel density
NRP1	neuropilin 1

ORR	overall response rate
OS	overall survival
PDGF	platelet-derived growth factor
PDGFA	platelet-derived growth factor-alpha
PDGFRB	platelet-derived growth factor receptor-beta
PECAM1	platelet/endothelial cell adhesion molecule 1
PFS	progression-free survival
PGF	placental growth factor
PTEN	phosphatase and tensin homolog
RET	ret proto-oncogene
SCID	severe combined immunodeficiency
SDF1	stromal cell-derived factor 1
TAMs	tumor-associated macrophages
TEK	endothelial TEK tyrosine kinase
THBS1	thrombospondin 1
TIE	tyrosine kinase with immunoglobulin-like and EGF-like domains
TIMP3	TIMP metallopeptidase inhibitor 3
TP53	tumor protein p53
TRNAK2	transfer RNA lysine 2
TYMS	thymidylate synthetase
VEGF	vascular endothelial growth factor
VEGFA	vascular endothelial growth factor A
VEGFB	vascular endothelial growth factor B
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2
VEGFR3	vascular endothelial growth factor receptor 3

Introduction

Colorectal cancer (CRC) is a malignant epithelial tumor originating from the large bowel represents about 10% of all new cancers [1,2]. Five-year survival rates of more than 90% are observed for localized CRC (stage I) but this number is reduced to about 10% for metastatic CRC (mCRC) (stage IV) [3]. Tumor angiogenesis represents one of the targets for treatment for mCRC [4]. Angiogenesis is a complex process encompassing tumor factors, cytokines derived from the extracellular matrix and host factors that ultimately result in the growth of new blood vessels from the preexisting vasculature [4]. As the efficient diffusion of oxygen to mammalian cells is limited to approximately 100 μm, angiogenesis is indispensable for normal animal growth and development [4]. The expansion of a tumor beyond a volume of around 1 to 2 mm³ is possible only if the neoplastic cells start to secrete several proangiogenic factors that stimulate endothelial cell (EC) division and migration [4]. Intratumoral hypoxia is sensed by hypoxia-inducible factor and the von Hippel-Lindau tumor suppressor gene pathway. In addition, mutated or highly expressed oncogenes also increase the expression of angiogenic factors by tumor cells [4]. Accordingly, the angiogenic balance is altered, and the release of proangiogenic molecules exceeds that of anti-angiogenic ones, triggering the so-called 'angiogenic switch' [4]. These angiogenic proteins include the vascular endothelial growth factors (VEGF), the platelet-derived growth factors (PDGF), the fibroblast growth factors (FGF), and the angiopoietin (ANGPT) family of ligands [4]. These ligands interact extracellularly with their respective receptors – VEGF-, PDGF-, FGF- and tyrosine kinases with immunoglobulin-like and epidermal growth factor (EGF)-like domains (TIE) receptors – causing receptor dimerization, phosphorylation, and activation of downstream pathways that will lead to EC proliferation, differentiation, and migration, as well as altered capillary permeability [4]. Various synthetic anti-angiogenic agents with

different mechanisms of action have been developed, including monoclonal antibodies exclusively directed against VEGFA (e.g. bevacizumab), against multiple factors (e.g. ziv-aflibercept) or against VEGF receptors (e.g. ramucirumab). Besides monoclonal antibodies, small molecule inhibitors (axitinib, pazopanib, regorafenib, sorafenib, sunitinib, and vandetanib) target multiple receptors (Tables

Table 1) [5]. Bevacizumab (Avastin®, Genentech, Inc.) binds only VEGFA and neutralizes all human VEGFA isoforms and bioactive proteolytic fragments [6]. Anti-angiogenic compounds are able to block the formation of new blood vessels, reduce vascular permeability, promote capillary regression, stimulate vascular normalization, and restore dendritic cell function [4]. In addition, the inhibition of angiogenesis may also increase the efficacy of chemotherapy, either by decreasing the elevated interstitial pressure in tumors and improving the delivery of cytotoxic agents [4] or by enhancing the sensitivity of tumor ECs to the effects of anti-neoplastic therapy [4]. FOLFIRI is the preferred irinotecan-based chemotherapy regimen to be combined with bevacizumab for previously untreated patients with mCRC [4]. The regimen includes irinotecan plus folinic acid and a bolus of fluorouracil (5-FU) followed by 5-FU infusion. The combination of oxaliplatin-based regimens (FOLFOX) and bevacizumab has also been evaluated in both first-line and second-line treatment of mCRC [4]. The addition of bevacizumab to chemotherapy results in a significant improvement in survival and response, although at the expense of increased toxicity [4,7,8]. A common side-effect of the treatment is hypertension, but this is considered manageable [4].

The response to an anti-angiogenic treatment regimen varies significantly – a consequence of the multiplicity of angiogenic mechanisms and the intrinsic heterogeneity of tumor biology [9–13]. As such, the ability of a biomarker combination to predict efficacy to a

therapeutic combination under such complexity is clearly of the utmost importance [14], sparing patients unnecessary toxicity and decreasing costs. A number of potentially predictive biomarkers for anti-angiogenesis therapies have emerged from pre-clinical and phase I-III studies (Figure 1, Table 2, Table 3) [14–17]. They include circulating cytokines, tissue factors, genetic markers and functional imaging. The focus of this review will be on candidate protein biomarkers for bevacizumab-containing therapy in mCRC, expressed in tumor tissue and detectable by immunohistochemistry. Further, we subdivide candidate biomarkers into four groups based on their function in tumor angiogenesis and their relationship to the working mechanism of bevacizumab. Finally, we provide an overview of the pre-clinical and clinical study results of each subgroup and also of their response to bevacizumab therapy.

VEGFA-signaling proteins

VEGFA is part of the primary angiogenic pathway in tumors and has many isoforms and receptors. Tumor cells excrete VEFGA, which interacts with the receptors of nearby endothelial cells, resulting in an angiogenic response towards the tumor cells. Because bevacizumab binds VEGFA, it is expected that expression of the latter in the tumor is needed for effective treatment. Furthermore, endogenic VEGFA-binding proteins imitate the action of bevacizumab (i.e. by capturing VEGFA). In effect, these proteins deplete the tumor environment of VEGFA. Overexpression of these endogenous proteins may train the tumor to survive the VEGFA deprivation by switching to a hypoxic pathway, in effect creating resistance to bevacizumab-containing therapy. As a result, clones of different cell populations (e.g. tumor cells, immune cells) that do not rely on VEGFA-signaling can be selected by evolutionary pressure and become the dominant tumor clone, driving further growth and spread.

- ***Vascular endothelial growth factor A (VEGFA)***

Tumor cells and tumor-associated stroma secrete a variety of pro-angiogenic factors that activate ECs in nearby blood vessels, of which VEGFA (Figure 2E,F) is the most prominent [18]. A paracrine/autocrine VEGFA loop that affects tumor cells is most likely non-existent [19]. Because bevacizumab binds directly to VEGFA, the correlation of VEGFA tumor expression levels to response is a plausible hypothesis. However, despite the logic of this hypotheses and its dominant role in the angiogenic cascade, the predictive or prognostic potential of VEGFA as a biomarker remains contradictory in the scientific literature [20]. Presumably, a host of factors such as clinico-pathological differences between cohorts, methodological diversity, and the differences between primary tumor tissue and metastatic lesions introduce such variations. The location of the metastasis also seems relevant.

Moreover, two anti-angiogenic isoforms of VEGFA have been identified [21,22]. It is plausible that the immunohistochemistry (IHC) antibodies against VEGFA detect both the angiogenic and the anti-angiogenic isoforms. Finally, as described previously, up-regulation of pro-angiogenic factors is a common mechanism by which tumors escape angiogenesis inhibition; thus explaining resistance to bevacizumab-containing therapies.

The independence of VEGFA expression from clinical benefit is demonstrated in several studies. In one study, VEGFA expression in tumor cells was assessed in mCRC tissue arrays and whole sections [23]. This method could not, however, distinguish subjects who are likely to benefit from bevacizumab. In addition, disease control rate, progression-free survival (PFS) and overall survival (OS), were similar in patients with low VEGFA expression as compared to those with high VEGFA expression in another study [24].

Nevertheless, several studies encourage further exploration of potential correlation. For example, the understanding that VEGFA-mediated angiogenesis might be most essential during the early stages of tumor progression and therefore, bevacizumab treatment is likely more effective during the early stage of tumor progression (Table 4**Fout! Verwijzingsbron niet gevonden.**), provides context for which such patients with high levels of VEGFA expression may benefit from bevacizumab-directed treatment [25]. In another study, despite the lack of correlation between treatment response and VEGFA expression, patients with partial remission following six months of treatment often exhibit higher VEGFA expression compared to patients with stable disease [26]. In another study, the authors did demonstrate that VEGFA expression by IHC in mCRC patients is significantly high in responders than in non-responders [27].

Potentially, the level of VEGFA is important only in a select group of tumors. For example, tumors that have activated an angiogenic switch and rely almost exclusively on VEGFA for

angiogenesis might be the most susceptible to VEGFA blockade. Therefore, VEGFA dependency might be related to different molecular subtypes of mCRC [2]. Evidently, more evidence is required for a potential role of VEGFA, its isoforms and stage-dependent expression in mCRC.

- ***Vascular endothelial growth factor A splice isoforms (VEGFA$_{121}$b, VEGFA$_{165}$b)***

The VEGFA gene encodes for multiple isoforms, identified by their length and c-terminal sequence [28]. Two families of proteins are formed by alternative splicing of the terminal exon: pro-angiogenic (VEGFA$_{XXX}$) and anti-angiogenic (VEGFA$_{XXX}$b) isoforms [28]. In many tissues, including normal colon, the anti-angiogenic isoforms form a substantial portion of total VEGFA [28]. VEGFA$_{165}$b is the most common inhibitory isoform and is downregulated in colon cancer, although to a variable extent among different patients [28]. Furthermore, bevacizumab binds VEGFA$_{165}$b with the same affinity as normal VEGFA$_{165}$ [28]. Overexpression of VEGFA$_{165}$b in human CRCs grown in mice confers resistance to bevacizumab treatment [28]. Therefore, bevacizumab may be less effective against tumors with high VEGF$_{165}$b levels [28]. It is conceivable that in patients with a high VEGFA$_{165}$b level, the effective dose of bevacizumab left for inhibiting VEGFA that targets the tumor is decreased [28]. Conversely, patients with lower VEGFA$_{165}$b levels may have a more angiogenic tumor, making it more susceptible to bevacizumab [28]. The binding of VEGFA$_{165}$b by bevacizumab might even stop a more dominant anti-angiogenic effect than can be achieved by binding the pro-angiogenic effect of VEGFA$_{165}$ potentially worsening the condition [28]. Another anti-angiogenic isoform in mCRC is VEGFA$_{121}$b [22]. It has similar inhibitory properties on angiogenesis as VEGFA$_{165}$b [22]. The VEGFA$_{XXX}$b isoforms can act as a limiter on excessive angiogenesis during conditions of uncontrolled growth. However, our

knowledge about these isoforms is limited, and measuring VEGFA$_{165}$b is not straightforward [29].

- *c-fos induced growth factor (FIGF, VEGFD)*

Bevacizumab targets only VEGFA and given the redundancy within biologic family members, overexpression of other VEGF-family ligands could constitute a mechanism of resistance [30]. Several studies have shown that growth factors which are implicated in lymphangiogenesis [30], such as c-fos induced growth factor (FIGF, also known as VEGFD) and its correspondent receptor fms-related tyrosine kinase 4 (FLT4) (also known as VEGF receptor 3 (VEGFR3)) can also participate in angiogenesis [31,32]. The N- and C-terminal propeptides of FIGF can be proteolytically cleaved, and the mature forms can bind to kinase insert domain receptor (KDR, also known as VEGF receptor 2 (VEGFR2)) [33]. As VEGFA is blocked by bevacizumab therapy, mCRC cells that produce other angiogenic factors keep on growing. FIGF could be one of these factors and tumor cells that produce it seem to be resistant to VEGFA blockade. Indeed, low expression of FIGF in mCRC has been associated with greater bevacizumab benefits on PFS and OS as shown by IHC using tissue arrays [30]. Only a limited number of studies report on FIGF and its relation to bevacizumab response in mCRC.

- *fms-related tyrosine kinase 1 (FLT1, VEGFR1)*

fms-related tyrosine kinase 1 (FLT1, also known as VEGF receptor 1 (VEGFR1)) (Figure 3A,B) acts as a negative regulator of VEGFA-mediated angiogenesis in its membrane-bound form during development. However, it is a stimulator of angiogenesis when activated by its specific ligands (placental growth factor (PGF) and vascular endothelial growth factor B (VEGFB)) [34]. VEGFA has also a high affinity to FLT1 in addition to PGF and VEGFB, but its tyrosine kinase activity when binding VEGFA is comparatively weak and the downstream

signaling, if present, is poorly understood [34]. The study of FLT1 by IHC is challenging, because it is expressed in ECs [34], monocytes [35], some hematopoietic cells [36], pericytes [33], and tumor cells [37]. In one study, tumor samples from 230 patients were tested for FLT1 expression in blood vessels and PFS was chosen as the primary endpoint [25]. Endothelial FLT1 staining was lower than endothelial KDR staining in mCRC. Patients with a low ratio FLT1 to platelet/EC adhesion molecule 1 (PECAM1) endothelial staining showed increased benefit from bevacizumab treatment, though this effect was not statistically significant [25]. Through stimulation of vascular endothelial growth factor receptors on tumor cells, VEGFA may have an additional role in cancer. An autocrine loop might be responsible for increased tumorigenesis [19,38]. Tumor samples from patients in the MAX-study (mitomycin C, Avastin® and Xeloda®) were tested for FLT1 expression. Lower expression of FLT1 was associated with greater bevacizumab benefit for OS, but not for PFS [30]. We presume that bevacizumab mimics (soluble) FLT1, because of FLT1 has high affinity for VEGFA, but holds weak activation potential. Therefore, tumor vessels and cells with high membrane bound or soluble FLT1 levels are most likely more resistant to bevacizumab therapy [39].

- ***Kinase insert domain receptor (KDR, VEGFR2)***

VEGFA signaling through KDR on ECs is the major pathway that activates angiogenesis by inducing the proliferation, survival, sprouting and migration of ECs, and also by increasing endothelial permeability [33]. Endothelial KDR expression has been studied in mCRC samples but no relationship to KDR expression on ECs was observed [25]. It has been reported that KDR can also be present on tumor cells themselves [40], but in another study KDR expression was not detectable by IHC in cancer cells [41]. Human cancers contain several distinct blood vessel types. The sensitivity of each of the vessel types to anti-VEGF therapy with ziv-

aflibercept has been studied [42]. Late-forming vascular malformations, feeding arteries, and draining veins that expressed low levels of KDR are largely resistant to blockade by ziv-aflibercept. In contrast, early-forming mother vessels and glomeruloid microvascular proliferations, which express high levels of KDR, are highly susceptible, showing the importance of the maturity status of vessels in tumors. Whether KDR is an appropriate marker to assess the functional status needs to be investigated. Up till now no predictive value for KDR expression in endothelial or tumor cells in mCRC has been reported. Nevertheless, because KDR is the most important receptor for VEGFA allowing angiogenesis to occur, we speculate that KDR expression in ECs correlates with the sensitivity to VEGFA and thus bevacizumab therapy. Moreover, KDR expression is induced in immature vessels through hypoxia sensing pathways [43]. Thus, it is conceivable that vessels with pronounced KDR expression will be more sensitive to VEGFA/VEGFB blockade [44].

- *fms-related tyrosine kinase 4 (FLT4, VEGFR3)*

FLT4 is a receptor that has mostly been implicated in lymphangiogenesis, but there is evidence for its expression in blood vessels of solid tumors [32,45]. FLT4 can heterodimerize with KDR, leading to differential phosphorylation and a potentially different signal transduction. Although FLT4 expression is upregulated in angiogenic blood vessels of tumors [46], it has been reported that FLT4 has no clinically applicable prognostic significance in mCRC patients [47].

- *Neuropilin 1 (NRP1)*

The trans-membrane co-receptor neuropilin 1 (NRP1) (Figure 3C,D) binds VEGFA and KDR, resulting in increased affinity of VEGFA for the extracellular domain of KDR [19,48]. Co-expression of NRP1 and KDR on endothelial and tumor cells promotes angiogenesis and vasculature development [48]. NRP1 also acts as a co-receptor for semaphorins, a number of

growth factors and the inactive latent form bound to latency-associated peptide [48]. It is thought that endothelial and tumor cells that highly express (soluble) NRP1 are resistant to bevacizumab because these cells can cope with low VEGFA levels and thus additional blockade of VEGFA by bevacizumab will be ineffective [39]. On the other hand, mCRC patients with low NRP1 levels in endothelial or tumor cells tend to have an increased benefit from bevacizumab [25]. In cell culture, NRP1 increases during bevacizumab treatment and combined blockade of NRP1 and VEGFA inhibits tumor growth additively [41].

NRP1 is also up-regulated in tumor-associated macrophages (TAMs) of the alternatively activated M2 phenotype. In the tumor microenvironment, TAMs may adopt a trophic role that promotes angiogenesis, matrix proteolysis, and tumor progression. NRP1 expression in TAMs is significantly increased after bevacizumab treatment [41], indicating that an immunomodulating tumor environment is present. The TAMs turn off immune system activation by producing anti-inflammatory cytokines and promote angiogenesis [49]. Up till now, the cells of the immune system have not been extensively studied for a link with bevacizumab response in mCRC.

Other relevant angiogenesis factors

Angiogenesis is a redundant process with multiple pathways resulting in the formation of new blood vessels. Besides VEGFA, other angiogenic proteins can maintain a vascular supply to the tumor. When VEGFA is inhibited by bevacizumab, tumors that inherently use different angiogenic proteins for their vascular network can be selected and maintain the tumor's growth and progression.

- *Pericyte coverage: aorta smooth muscle actin-alpha 2 (ACTA2, αSMA); platelet-derived growth factor receptor-beta (PDGFRB)*

During angiogenesis in embryos and adults, platelet-derived growth factor subunit B is expressed by sprouting ECs, whereas its receptor, platelet-derived growth factor receptor-beta (PDGFRB), is localized on pericytes [50]. Pericyte coverage of vascular sprouts is needed to stabilize the newly grown vascular walls [50]. The level of vessel maturation is most likely involved in the response to anti-angiogenic therapies [44]. Varying degrees of pericyte recruitment to the tumor microvasculature occur in different tumor types [51]. Pericytes are known to produce VEGFA, which is a survival factor for ECs [52]. It seems reasonable to speculate that pericytes serve as a 'private' source of VEGFA for the adjacent ECs. If the pericytes were absent or could not produce VEGFA, the endothelium would become vulnerable to VEGFA blockade [9]. Furthermore, tyrosine kinase inhibitors, which block multiple kinases including PDGFRB, enhance the effect of VEGFA inhibitors. Pericyte coverage as a predictive marker for bevacizumab-containing therapy has been studied by staining mCRC tumor vessels with aorta smooth muscle actin-alpha 2 (ACTA2, also known as alpha smooth muscle actin (αSMA)) (Figure 2G,H) [53]. However, pericyte coverage was not able to discriminate between responders and non-responders. In another study, 80 patients with a primary CRC resection were treated with bevacizumab. Tumors were stained using

dual immunofluorescence staining for coagulation factor VIII (an EC marker) and ACTA2. Multiple fields of view were scored. One half of the patients were metastatic at diagnosis, the others subsequently developed metastases. There was no difference between the levels of immature and mature vessels in tumors of early-stage patients and metastatic patients. Patients with higher levels of immature blood vessels, and hence lower levels of mature blood vessels, experienced longer survival following treatment [54]. ACTA2 staining can also be used to study the tumor stromal architecture [55]. TMAs consisting of surgical tumor samples from 56 patients with mCRC were stained for CD31 and ACTA2 followed by scoring two phenotypes: tumor vessel and stromal vessel [55]. RECIST response information for FOLFIRI and bevacizumab treatment as first- or second-line therapy post-surgery was available for each patient. The 'stromal vessel' phenotype group had a poorer response than the 'tumor vessel' phenotype group (P=0.05) [55]. We presume that mature vessels with pericytes are resistant to VEGFA-blockade because pericytes can deliver VEGFA to the ECs with which they are in contact [39].

- ***Angiopoietin 2 (ANGPT2)***

The angiopoietins, a family of vascular regulatory molecules binding to the endothelial tyrosine receptor (TEK), play an important role in neovascularization. One of them, ANGPT2 (Figure 3G,H), is a molecule that promotes the destabilization of blood vessels by inhibiting the recruitment of pericytes to blood vessels [56]. For instance, in several tumor types, upregulated ANGPT2 levels correlate with metastasis [57]. VEGFA blockade reduces the expression of ANGPT2 in ECs in rectal tumors [41]. Moreover, at day 12 after bevacizumab, ANGPT2 expression decreases proportionally with microvessel density (MVD), but the percentage of ANGPT2-positive blood vessels remains high (90% to 100%) [58]. Low ANGPT2 expression may lead to more stable blood vessels through pericyte coverage. Accordingly,

we assume that low ANGPT2-expressing tumors are more resistant to VEGFA blockade than tumors with high ANGPT2 expression.

Factors regarding the tumor microenvironment

Some proteins influence the delivery and/or working mechanisms of bevacizumab combined with the chemotherapy to the tumor microenvironment. As bevacizumab is given as a combination therapy, it is important that the tumor is responsive to the chemotherapy as well. There are also proteins in the microenvironment that can reinforce or inhibit the action of bevacizumab and can tip the balance. For example, when synergetic proteins are abundantly present in the tumor and bevacizumab is being introduced, angiogenesis can be inhibited and the tumor size stabilized. Multiple types of angiogenic proteins can be used by the tumor to increase angiogenesis in its environment. Non-responsive tumors can be those tumors that do not have inhibitors expressed for the alternative angiogenic proteins. Accordingly, inhibition of VEGFA is not sufficient.

- *Thrombospondin 1 (THBS1)*

Thrombospondin 1 (THBS1) is a major negative regulator of angiogenesis compromising EC survival, migration, and responses to VEGFA [59]. The regulation of THBS1 is a complex and controversial phenomenon [59]. In some cases, the tumor microenvironment might override the effects of the THBS1 regulator tumor protein p53 (TP53) [59]. For instance, hypoxia can reduce THBS1 levels and induce VEGFA expression, irrespective of TP53 status [59]. Furthermore, the chemotherapy backbone, 5-FU, can induce THBS1 expression [60]. In THBS1-positive mCRC treated with bevacizumab and chemotherapy, the overall response rate (ORR) was 0% versus 30% in the THBS1-negative group [47]. Because treatment efficacy according to the expression of THBS1 was non-significant, THBS1 did not seem to have clinically applicable prognostic significance [47]. The role of THBS1 as a predictive biomarker needs to be more clearly established.

- *TIMP metallopeptidase inhibitor 3 (TIMP3)*

TIMP metallopeptidase inhibitor 3 (TIMP3) is a member of a family of endogenous inhibitors of matrix metalloproteinases and a potent inhibitor of angiogenesis and tumor growth. TIMP3 expression in mCRC patients suggests a host response to restrict the extent of local tissue degradation, tumor invasion, and angiogenesis [27]. TIMP3 is present in both tumor and stromal cells and is more highly expressed in responders than in non-responders to bevacizumab [27]; this may explain the synergetic function of bevacizumab and TIMP3 to inhibit angiogenesis in mCRC.

- ***Chemokine (C-X-C motif) ligand 12 (CXCL12, SDF1) and chemokine (C-X-C motif) receptor 4 (CXCR4)***

Chemokine (C-X-C motif) ligand 12 (CXCL12, also known as stromal cell-derived factor 1 (SDF1)) is one of the key stimuli involved in signaling interactions between tumor cells and their microenvironment [61]. It promotes tumor angiogenesis by recruiting circulating endothelial progenitor cells to the tumor stroma [61]. VEGFA blockade by bevacizumab upregulates the expression of chemokine (C-X-C motif) receptor 4 (CXCR4) and CXCL12 in rectal cancer cells as shown by IHC in tumor biopsies before and after bevacizumab treatment [41]. The CXCL12–CXCR4 pathway may be a relevant resistance mechanism when anti-VEGFA agents are used, but the predictive value in mCRC has not yet been studied.

- ***Endothelial cell markers: platelet/EC adhesion molecule 1 (PECAM1, CD31) and CD34***

PECAM1 (also known as CD31) (Figure 2G,H) is believed to be a highly specific marker for ECs, even though expression of PECAM1 has also been reported in macrophages and dendritic cells. Besides PECAM1, CD34 is expressed in ECs, but also in reactive fibroblasts and some types of benign and malignant mesenchymal neoplasms. CD34 staining can help to distinguish between subtypes of blood vessel, i.e. vascular channels and sinuses. Typically, the expression of these proteins is used to evaluate the number of blood vessels in the

tumor, the so-called MVD, a surrogate marker of tumor angiogenesis [62]. Retrospective analyses have been performed to evaluate MVD as a prognostic factor and/or predictor of benefit for bevacizumab in mCRC. In one study, the estimated hazard ratios (HRs) for risk of death for bevacizumab-treated patients were smaller than one regardless of the level of MVD, suggesting that the predictive value of MVD is low [23]. However, high MVD levels are associated with an increased benefit from bevacizumab, when PFS was the primary endpoint [25,53]. Moreover, PFS is significantly shorter in patients with an MVD greater than the median value [63]. In a small study with 15 mCRC patients who underwent irinotecan plus folinic acid and a bolus of 5-FU (IFL) combined with bevacizumab, primary tumor samples stained for PECAM1 and CD34 were analyzed by a computerized image analysis program to calculate the intratumoral MVD. The treatment response was evaluated by computed tomography scanning. Two types of blood vessel, undifferentiated (PECAM1$^+$/CD34$^-$) and differentiated (CD34$^+$), were identified. No significant correlation between tumor shrinkage and MVD was found [64]. However, the percentage of tissue samples consisting of metastatic tumor instead of primary tumor could have biased the study results [39,58]. Moreover, it is known that inter-observer variability for vessel counting is high [62] and therefore the results from multiple studies are difficult to compare. In addition, it has been reported that MVD is not a measurement of functionality of the vessel network in a tumor [43]. Accordingly, the vessel maturity or EC proliferation should be assessed. We propose to develop more robust methods for vessel counting and to include functional markers such as ACTA2.

- ***EGF-like-domain 7 (EGFL7)***

EGF-like-domain 7 (EGFL7) is important for the development of the vascular system and is postnatally expressed in highly vascularized tissues. It is also upregulated at sites of

pathological angiogenesis and acts as a chemo-attractant in EC recruitment. The presence of EGFL7 is necessary for the tubulogenesis of blood vessels [65]. High levels of EGFL7 could decrease the efficiency of the delivery of chemotherapy because they induce immature and leaky sprouting vessels during neoangiogenesis near the tumor cells. When only anti-VEGFA is used to treat mCRC (e.g. during the chemotherapy-free maintenance period), this might actually benefit the tumors that strongly express EGFL7 [65].

- ***EPH receptor B4 (EPHB4)***

Migrating ECs express receptors for axon-guidance cues, including ephrin receptors [66]. Ephrin-B2 (EFNB2) and its receptor, EPH receptor B4 (EPHB4), regulate vessel morphogenesis by several mechanisms [66]. Endothelial EPHB4 activation of EFNB2 induces mural angiopoietin 1 (ANGPT1) expression and increases TEK activation. As a result, pericyte investiture is enhanced and tumor blood vessel leakiness declines [67]. Patients with high EPHB4 expression are more resistant to VEGFA blocking and patients with low EPHB4 mRNA levels are better responders to bevacizumab than those with high levels [68]. This is another protein which indicates that the vessel maturation status or pericyte coverage of the tumor blood vessels can predict bevacizumab response in mCRC. Direct study of the pericyte coverage is possible as explained in the previous section.

- ***Endoglin (ENG, CD105)***

Endoglin (ENG, also known as CD105) seems to be specific to activated/proliferating ECs [47]. It is preferentially expressed in the activated EC participating in neoangiogenesis, especially in tumors, and is undetectable or weakly expressed in vessels of normal tissues. However, expression of ENG in patients treated with bevacizumab does not show clinically applicable prognostic significance in mCRC patients [47].

Tumor intrinsic markers

Tumor condition proteins are indicative of the functional status of the tumor cells, which could be relevant to angiogenesis. They cause indirect effects that could explain resistance or responsiveness to bevacizumab treatment.

- *Thymidylate synthetase (TYMS)*

Thymidylate synthetase (TYMS) is the target enzyme for 5-FU. TYMS protein and mRNA have been shown to predict the response to 5-FU-based chemotherapy [27]. In a retrospective analysis of tumor samples, TYMS expression has been quantified using a visual grading system. In accordance with the results obtained by real-time reverse-transcriptase polymerase chain reaction, IHC analysis showed that expression of TYMS was significantly lower in responders with mCRC than in non-responders to bevacizumab treatment [27]. However, conflicting results were reported concerning the predictive and prognostic value of TYMS for 5-FU-based therapy in mCRC [27], therefore more research in this area is needed.

- *Carbonic anhydrase IX (CA9)*

The uncontrolled growth of a tumor combined with anti-angiogenic therapy induces hypoxia and an increased expression of hypoxia-regulated genes. One of these genes gives rise to carbonic anhydrase IX (CA9) (Figure 2A,B), which is a transmembrane protein that plays a major role in the adaptation and proliferation of cells in hypoxic and acidic conditions [24]. Tumor hypoxia, which is one of the driving forces of tumor angiogenesis, is known to be associated with treatment failure in several malignancies. CA9 expression correlates to poor prognosis in most tumor types and with worse outcome in bevacizumab-treated patients with mCRC [24]. The correlation between the expression of CA9 and the efficacy of bevacizumab in mCRC patients has been evaluated via IHC staining of CA9. Patients with a low CA9 score had an improved PFS as compared to those with a high score [24]. Low CA9

expression was also associated with longer OS after bevacizumab treatment [24]. CA9 is linked to prognosis, but the predictive value of CA9 for bevacizumab could not be fully assessed in this study because a patient group that did not receive bevacizumab was not included. Furthermore, in cell culture and xenografts, CA9 expression is associated with increased tumor growth, as well as necrosis and apoptosis [69]. Moreover, CA9 knockdown enhances the effect of bevacizumab treatment, reducing tumor growth rate *in vivo* [69].

▪ ***Epidermal growth factor receptor (EGFR)***

The EGF receptor (EGFR) signaling pathway (Figure 2C,D) plays a key role in the development and growth of several tumors [70]. In mCRC, mutations in Kirsten rat sarcoma viral oncogene homolog (KRAS), which is the signaling molecule downstream of EGFR, are predictive of ineffective EGFR targeted therapy. There is also a clear connection between EGFR signaling and angiogenesis. The inhibition of EGFR can decrease VEGFA expression in upper gastrointestinal tract cancer and EGFR signaling induces VEGFA expression [70]. However, in one study a difference between EGFR subgroups has not been reported in mCRC because all the patients gained a benefit from bevacizumab treatment regardless of the EGFR IHC expression level [25].

▪ ***v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2, HER2)***

v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2, also known as human epidermal growth factor receptor 2 (HER2)) encodes a transmembrane tyrosine kinase receptor, homologous to EGFR. This receptor is involved in the growth and progression of malignant cells. ERBB2 participates in and correlates to VEGFA expression. VEGFA is higher in ERBB2-positive mCRC tumor specimens than in those that are VEGFA-negative [71]. In one study, patients with ERBB2 expression showed a decreased benefit [25], but in another no statistically significant differences were found [20].

- ***Tumor protein p53 (TP53)***

TP53 (Figure 3E,F) is inactivated in the majority of human cancers. Tumor cells deficient in TP53 display a diminished rate of apoptosis under hypoxic conditions, which might reduce their reliance on vascular supply, and hence their responsiveness to anti-angiogenic therapy. In contrast, several studies have indicated that tumor angiogenesis may be enhanced by TP53 overexpression. Increased expression of TP53 is correlated with increased microvessel counts in cancers of the lung, colon and stomach [72]. Five randomized, controlled trials investigated the association of TP53 expression with the outcomes of mCRC patients, but there was no consensus on the predictive value or prognostic impact of nuclear TP53 overexpression. Furthermore, the results from studies that examined mCRC and *p53* status were inconsistent [20,73]. Mice bearing tumors derived from TP53$^{-/-}$ HCT116 human CRC cells were less responsive to anti-angiogenic combination therapy than mice bearing isogenic TP53$^{+/+}$ tumors. Although it is thought that anti-angiogenic therapy targets genetically stable ECs in the tumor vasculature, genetic alterations that decrease the vascular dependence of tumor cells can affect the therapeutic response of tumors to this therapy.

- ***Phosphatase and tensin homolog (PTEN)***

Phosphatase and tensin homolog (PTEN) is a tumor-suppressing protein that regulates the activity of the phosphatidylinositol (4,5)-bisphosphate 3-kinase (i.e. v-akt murine thymoma viral oncogene homolog 1 (AKT1)) by converting phosphatidylinositol (3,4,5)-triphosphate back to phosphatidylinositol (4,5)-bisphosphate. PTEN loss leads to AKT1-mediated hyperphosphorylation that protects cells from apoptosis. Together with other proteins, PTEN loss is also responsible for tumor angiogenesis [20]. PTEN expression was compared with the

bevacizumab response in 34 retrospectively collected mCRC tumor samples but statistically significant differences were not found [20].

- *Microtubule-associated protein 1 light chain 3 (MAP1LC3)*

Autophagy is a survival pathway for cancer cells under conditions of cell stress. As a consequence of anti-angiogenic therapy, solid tumors encounter hypoxia and imbalances in nutrient supply [74]. One essential protein for autophagosome formation is microtubule-associated protein 1 light chain 3 (MAP1LC3) [75]. Tumors from mice treated with bevacizumab, oxaliplatin, or both were processed for immunostaining of MAP1LC3. Increased staining of this protein in bevacizumab- and oxaliplatin-treated tumors was observed. Tumors were also stained for blood vessels and cell viability, using PECAM1 and marker of proliferation Ki-67 (MKI67) antibodies. MKI67 staining showed a lower number of proliferating cells in cell culture when treated with a combination of bevacizumab, oxaliplatin and chloroquine (an autophagy inhibitor) than without chloroquine [74]. The hypoxic effects induced by bevacizumab in tumors *in vivo* might be critical to its therapeutic action and autophagy might attenuate it. The response to DNA-damaging drugs consists of the induction of autophagy as well, and its inhibition could sensitize colon tumors to oxaliplatin [74].

Five-year view and expert commentary

We propose that the immunohistochemical detection of proteins in cancer tissue offers the most potential for developing biomarkers. Nonetheless, exploiting protein expression is not straightforward as it is influenced by many laboratory processes and therefore requires forethought and diligent handling. We recommend a fit-for-purpose assay to manage the variations inherent to an immunohistochemical assay for a complicated target and to allow for comparisons between studies. The development of such an assay should consider the following factors. Firstly, antibody specificity should be determined and established using appropriate cell or tissue controls. Most proteins have multiple isoforms, which can differ in functionality and the differentiation by immunohistochemistry is non-trivial [21,22]. Secondly, stabilizing the proteins throughout the specimen processing should also be carefully considered [76–78]. New systems that track fixation time and temperature during sample processing can provide improvement. Thirdly, the interpretation and scoring of the immunohistochemical stain also results in several more decisions. Different cell types may express the same protein, but in different quantities and locations, making it a valuable practice to record the cell type and location in addition to the amount expressed [34,37]. Tumor localization also plays a role [79]. Primary tumors have a more complex molecular network driving angiogenesis as compared to metastatic tumors. Even the timing of the sampling in relation to metastasis (synchronous versus metachronous) can show a difference [79]. These variations may be contributing factors to the current lack of a definite predictive biomarker for bevacizumab therapy in mCRC.

Many candidate biomarkers belong to the group of tumor intrinsic markers (group IV), which are indirectly linked to angiogenesis and the response to bevacizumab therapy. More research on the proteins of the other groups (I - III) is warranted. The expression of these

proteins frequently correlates with the therapeutic response and they often have a direct link with angiogenesis and the mechanisms that confer resistance to bevacizumab therapy. In addition, post-translational modifications need to be taken into account, e.g. the EC surface glycome can activate VEGFA-like signaling by soluble galactoside-binding lectin 1 [80]. We feel that the most promising candidate biomarkers are FIGF, FLT1, NRP1, ACTA2, and CA9.

The emerging applications of automatic image analysis in histopathology and multiplex technologies, such as quantum dots [81] and hyperspectral imaging [82], may help to overcome the immunohistochemical assay challenges mentioned above. Furthermore, these advanced techniques can be used to assess different combinations of markers that can adequately characterize the type of tumor responses *in situ*, as a single marker is unlikely to reveal the full complexity of angiogenesis in a tumor. The combination of several markers on a single slide allows a 'high content' but also 'compact' analysis of tumor regions. This high-content approach will preserve scarce tissue while assessing and relating multiple targets in the same tumor region. Currently, immune modulating therapies are emerging in cancer treatment. Since the immune system has a dynamic relationship with angiogenesis and blood vessel functioning [80], immune-based therapies may advantageously affect the efficiency of anti-angiogenesis therapies. The dual and interconnected effects of vascular and immune compartments complicate the hunt for biomarkers that forecast efficacy for these combination strategies.

In conclusion, while some well-performed immunohistochemistry-based studies exist, there is a clear need to develop reliable new assays that can encompass the complexities of the angiogenic pathway. The portfolio of immunohistochemical biomarkers that predict for

response or resistance to angiogenic inhibitors such as bevacizumab will be required to leverage the recent advances of image analysis and high-content methodologies.

Key issues

- Current biomarkers can be categorized in the following groups:

    I) VEGFA-signaling proteins. When VEGFA-binding proteins are over-expressed in a tumor before therapy, the tumor is probably already adapted to cope with the deprivation of VEGFA and will survive bevacizumab therapy; however, over-expression of VEGFA, the target of bevacizumab, does not have predictive value, in part because the different isoforms may not be detected.

    II) Other relevant angiogenesis factors. When these are over-expressed, the tumor cell may redirect its reliance from VEGFA to these factors for vascular supply.

    III) Factors regarding the tumor microenvironment. The tumor may over-express these factors that interfere with the delivery of chemotherapy and/or bevacizumab. They can also have a synergistic effect.

    IV) Tumor intrinsic markers. These may phenotypically and functionally describe the tumor cells and demonstrate indirect links to angiogenesis.

- A meticulously developed, fit-for-purpose immunohistochemistry assay will enable longitudinal assessment and comparisons across large patient sets.

- The specimen origin (primary versus metastatic) is important as well as the timing of the sampling relative to the metastasis (synchronous versus metachronous presentation).

- Study of protein markers requires knowledge of the potentially occurring isoforms and post-translational modifications.

- Because tumor angiogenesis is a redundant process, multiple markers will be required to predict patient survival after bevacizumab-combining therapy.

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Reference Annotations

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    These two papers highlight the importance of the heterogeneous character of human cancers. CRC is classified based on clinico-pathological features and five molecular markers, or on a genome-scale analysis.

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- *** of considerable interest:*

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These two reviews contain the essential information to start exploring new candidate predictive biomarkers.

- Bates DO, Catalano PJ, Symonds KE, *et al.* Association between VEGF splice isoforms and progression-free survival in metastatic colorectal cancer patients treated with bevacizumab. *Clin. Cancer Res.* 18(22), 6384–91 (2012).

  This paper shows the importance of the often forgotten isoforms of a candidate protein biomarker such as VEGFA. More knowledge about their existence and functionality is needed, but also the measurement of the different isoforms must be improved.

- Lambrechts D, Lenz H-J, de Haas S, Carmeliet P, Scherer SJ. Markers of Response for the Antiangiogenic Agent Bevacizumab. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 31(9) (2013).

  This review reports the results of candidate biomarkers that have been explored in large clinical trials. One of the few studies in which sufficient clinical samples have been investigated with standardized assays.

- Croci DO, Cerliani JP, Dalotto-Moreno T, *et al.* Glycosylation-Dependent Lectin-Receptor Interactions Preserve Angiogenesis in Anti-VEGF Refractory Tumors. *Cell*. 156(4), 744–58 (2014).

  This research paper analyzes the glycome of the endothelial cell surface and highlights the importance of post-translational protein modifications and their impact on biological function.

Acknowledgements and financial disclosure

Tables

Table 1: Different VEGFA pathway inhibitors and their targets based on the full prescribing information as published by the Food and Drug Administration (FDA) and the European public assessment reports as published by the European Medicines Agency (EMA).

Drug / Targets	axitinib (Inlyta®)	bevacizumab (Avastin®)	pazopanib (Votrient®)	regorafenib (Stivarga®)	sorafenib (Nexavar®)	sunitinib (Sutent®)	vandetanib (Caprelsa®)	ziv-aflibercept (Zaltrap®)
ABL1				X				
BRAF				X	X			
CSF1R						X		
DDR2				X				
EGFR							X	
EPHA2				X				
FGFR1/2				X				
FRK				X				
KIT			X	X	X	X		
MAPK11				X				
PDGFR			X	X	X	X		
PGF								X
RET				X		X	X	
TEK				X				
TRNAK2				X				
VEGFA		X						X
VEGFB								X
FLT1	X		X	X		X		
KDR	X		X	X	X	X	X	
FLT3					X	X		
FLT4	X		X	X	X	X	X	

ABL1: c-abl oncogene 1; BRAF: v-raf murine sarcoma viral oncogene homolog B; CSF1R: colony stimulating factor 1 receptor; DDR2: discoidin domain receptor tyrosine kinase 2; EGFR: epidermal growth factor receptor; EPHA2: EPH receptor A2; FGFR1/2: fibroblast growth factor receptor 1/2; FRK: fyn-related kinase; KIT: v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; MAPK11: mitogen-activated protein kinase 11; PDGFR: platelet-derived growth factor receptor; PGF: placental growth factor; RET: ret proto-oncogene; TEK: endothelial TEK tyrosine kinase; TRNAK2: transfer RNA lysine 2; VEGFA/B: vascular endothelial growth factor A/B; FLT1/3/4: fms-related tyrosine kinase 1/3/4; KDR: kinase insert domain receptor

Table 2: Overview of biomarkers studied in human tissue and their predictive value

Biomarker	Predictive Value	median PFS diff	median OS diff	PFS HR (95% CI)	OS HR (95% CI)	Number of patients	Ref.
PECAM1/CD31, CD34	no difference	NA	NA	NA	1.00 (0.87 - 1.15)	278	[23]
	high MVD: PFS↑	NA	NA	0.38 (0.19 - 0.78)	NA	242	[25]
	high MVD: PFS↓	16m vs. 10m (P=0.02)	NA	NA	NA	19	[63]
	no correlation	NA	NA	NA	NA	15	[64]
	high MVD: OS↑PFS↑	14m vs. 10m (P=0.70)	NA	NA	NA	17	[53]
NRP1	low NRP1: PFS↑	NA	NA	0.46 (0.22 - 0.93)	NA	244	[25]
TP53	low TP53: OS↑	NA	25.1m vs. 16.3m (P=NA)	NA	0.32 (0.15 - 0.70)	266	[73]
	no difference	16.2m vs. 13.9m (P=0.39)	NA	NA	NA	34	[20]
FIGF/VEGFD	low VEGFD: OS↑ PFS↑	NA	NA	0.21 (0.08 - 0.55)	0.35 (0.13 - 0.90)	268	[30]
FLT1/VEGFR1	lower FLT1: OS↑	NA	NA	NA	NA	268	[30]
	lower FLT1/PECAM1: PFS↑	NA	NA	0.62 (0.34 - 1.12)	NA	230	[25]
CA9	low CA9: OS↑ PFS↑	4.7m vs. 2.4m (P=0.03)	24.1m vs. 10.2m (P=0.03)	NA	NA	31	[24]
TIMP3	responders had higher expression	NA	NA	NA	NA	22	[27]
TYMS	responders had lower expression	NA	NA	NA	NA	22	[27]
VEGFA$_{165}$b	low VEGF$_{165}$b:VEGF$_{total}$ ratio: OS↑ PFS↑	8m vs. 5.2m (P<0.02)	13.6m vs. 10.6m (P>0.05)	0.49 (0.26 – 0.93)	0.68	97	[28]
EPHB4	low EPHB4: OS↑	NA	48m vs. 16m	NA	5.95 (1.18 – 29.96)	13	[68]
EGFL7	responders had lower expression	NA	NA	NA	NA	122	[65]
CXCL12, CXCR4	upregulation after VEGFA blockade	NA	NA	NA	NA	12	[41]
ANGPT2	downregulation after VEGFA blockade	NA	NA	NA	NA	12	[41]
THBS1	high THBS1: ORR↑	6m vs. 6m (P>0.05)	16m vs. 15m (P>0.05)	NA	NA	42	[47]

Marker	Result					N	Ref
pericyte coverage	no difference	13m vs. 10.5m (P=0.64)	NA	NA	NA	17	[53]
	high ACTA2: OS↑	NA	NA (P=0.03)	NA	NA	80	[54]
	tumor vessel phenotype (low ACTA2): reponse↑	NA	NA	NA	NA	56	[55]
VEGFA	no difference	NA	27.7m vs. 19.7m, NR vs. 16.26m	NA	0.13, 0.49	278	[23]
	no difference	NA	NA	0.91, 0.74, 0.57	NA	241	[25]
	no difference	16.4m vs. 14.3m (P=0.53)	NA	NA	NA	34	[20]
	no difference	3.9m vs. 3.9m (P=0.25)	9.1m vs. 11.5m (P=0.68)	1.54 (0.72 - 3.30)	1.66 (0.48 - 5.77)	31	[24]
	responders had higher expression	NA	NA	NA	NA	22	[27]
	no difference; PR had higher expression than SD	NA	NA	NA	NA	12	[26]
KDR/VEGFR2	no difference	NA	NA	0.82, 0.61, 0.68	NA	240	[25]
FLT4/VEGFR3	low VEGFR3: ORR↑	6m vs. 6m (P>0.05)	16m vs. 15m (P>0.05)	NA	NA	42	[47]
EGFR	no difference	NA	NA	0.64, 0.67, 0.72	NA	240	[25]
ERBB2/HER2	no difference	NA	NA	0.60, 0.90	NA	237	[25]
	no difference	14m vs. 15m (P=0.53)	NA	NA	NA	34	[20]
ENG	low ENG: ORR↑	6m vs. 6m (P>0.05)	16m vs 15m (P>0.05)	NA	NA	42	[47]
PTEN	no difference	14m vs 15m (P=0.39)	NA	NA	NA	34	[20]

PECAM1: platelet/EC adhesion molecule; MVD: microvessel density; NRP1: neuropilin 1; TP53: tumor protein p53; FIGF: c-fos induced growth factor; VEGFA/D: vascular endothelial growth factor A/D; FLT1/4: fms-related tyrosine kinase 1/4; VEGFR1/2/3: VEGF receptor 1/2/3; CA9: carbonic anhydrase IX; TIMP3: TIMP metallopeptidase inhibitor 3; TYMS: thymidylate synthetase; EPHB4: EPH receptor B4; EGFL7: EGF-like-domain 7; CXCL12: chemokine (C-X-C motif) ligand 12; CXCR4: chemokine (C-X-C motif) receptor 4; ANGPT2: angiopoietin 2; THBS1: thrombospondin 1; ACTA2: aorta smooth muscle actin-alpha 2; KDR: kinase insert domain receptor; EGFR: epidermal growth factor receptor; ERBB2: v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; HER2: human epidermal growth factor

receptor 2; ENG: endoglin; PTEN: phosphatase and tensin homolog; NA: not available; PD: progressive disease; SD: stable disease; HR: hazard ratio; ORR: overall response rate; OS: overall survival; PFS: progression-free survival; diff: difference

Table 3: Overview of biomarkers studied in mice and their predictive value

Biomarker	Predictive Value	Cell line	Model	Number of animals	Ref.
VEGFA$_{121}$b	high VEGFA$_{121}$b: inhibition of angiogenesis	LS174t	lumbar region of nude mice	12	[22]
CA9	increased expression of CA9 in response to bevacizumab treatment	HT29	subcutaneous in female mice	10	[69]
KDR/VEGFR2	early-forming vessels strongly expressing KDR are highly susceptible to VEGFA blockade	Ad-VEGFA$_{164}$	injected into ears and flank skin of female athymic nude mice	60	[42]
MAP1LC3	high MAP1LC3: resistance to bevacizumab	HT29	injected into flank skin of C.B.17 SCID female mice	48	[74]
α2-6-linked sialic acid; LGALS1	high α2-6-linked sialic acid, low LGALS1: sensitive to bevacizumab	CT26	implanted into syngeneic mice	NA	[80]

VEGFA: vascular endothelial growth factor A; CA9: carbonic anhydrase IX; KDR: kinase insert domain receptor; VEGFR2: VEGF receptor 2; MAP1LC3: microtubule-associated protein 1 light chain 3; LGALS1: soluble galactoside-binding lectin 1; NA: not available; SCID: severe combined immunodeficiency

Table 4: Molecular profile of fast- and slow-progressing tumors

	Slow-progressing tumor	Fast-progressing tumor
early	VEGFA	VEGFA
progression	VEGFA, FGF2	VEGFA
advanced	VEGFA, FGF2, PDGF, IL-8	VEGFA
final	VEGFA, FGF2, PDGF, IL-8, PGF	VEGFA

VEGFA: vascular endothelial growth factor A; FGF2: fibroblast growth factor 2 (basic); PDGF: platelet-

derived growth factor; IL-8: interleukin 8; PGF: placental growth factor

Figure legends

Figure 1: Overview of the potential predictive biomarkers for bevacizumab studied in human tissue. The different tissue compartments harbor different markers: vascular endothelial growth factor (VEGF)-signaling proteins (orange), other relevant angiogenesis factors (red), factors regarding the tumor microenvironment (green) and tumor intrinsic markers (purple) showing the relevance of scoring methodology. Neuropilin 1 (NRP1) can be expressed in macrophages but also in endothelial cells and tumor cells capable of binding VEGFA (solid arrow). NRP1- and fms-related tyrosine kinase 4 (FLT4) can form complexes with kinase insert domain receptor (KDR) (dotted arrow). Angiopoietin 1 and 2 (ANGPT1/2) are predominantly detected in the endothelial cells and the extracellular matrix. A tissue inhibitor of metalloproteinase 3 (TIMP3), thrombospondin 1 (THBS1) and chemokine (C-X-C motif) ligand 12 (CXCL12) is found in the extracellular matrix. Pan-endothelial markers such as platelet/EC adhesion molecule 1 (PECAM1) and CD34 are used for vessel morphology assessments. EPH receptor B4 (EPHB4), endoglin (ENG), FLT4 and EGF-like-domain 7 (EGFL7) are also endothelial cell specific. Pericytes covering vessels can be recognized by staining with cytoplasmic aorta smooth muscle actin-alpha 2 (ACTA2) or membrane platelet-derived growth factor receptor-beta (PDGFRB). Tumor cells share the endothelial cell candidate biomarkers NRP1, FLT1 and KDR. They also express epidermal growth factor receptor (EGFR), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2), carbonic anhydrase IX (CA9), phosphatase and tensin homolog (PTEN), tumor protein p53 (TP53), chemokine (C-X-C motif) receptor 4 (CXCR4), microtubule-associated protein 1 light chain 3 (MAP1LC3) and thymidylate synthetase (TYMS). In addition, they express and secrete (dashed arrow) VEGFA and VEGFD, placental growth factor (PGF), platelet-derived growth factor A (PDGFA) and fibroblast growth factor (FGF).

Figure 2: Selected representative images of the immunohistochemistry assays studied and discussed. Images were taken in areas adjacent to the tumor area (A, C, E, G) and in tumor areas (B, D, F, H) of human metastatic colorectal cancer tissue sections. In the tumor area, the staining for carbonic anhydrase IX (CA9) can be strong in the membrane of the tumor cells in the tumor area (tm) (B) in contrast to the epithelial cells in the crypts (cp) in tissue adjacent to the tumor (A). Vessel-associated cells (arrow) in the stroma (sm) are negative. The epithelial growth factor receptor (EGFR) has a weak staining in the membrane of the cells of plexi myenterici (Auerbach's plexi) (pm) located between the muscle layers in tissue adjacent to the tumor (C). In the tumor area, the staining for EGFR can be weak to moderate in the cytoplasm and membrane of tumor cells (D). Vessel-associated cells (arrow) in the stroma (sm) are negative. A moderate to strong granular staining for vascular endothelial growth factor A (VEGFA) can be observed in the cytoplasm of tumor cells in the tumor area (F), but there is also weak staining near the vessel-associated cells. In the tissue adjacent to the tumor, weak to moderate VEGFA staining can be observed in the cytoplasm of and near epithelial cells and vessel-associated cells (E). The fluorescent staining pattern for the combination CD31 and CD34 (red) and aorta smooth muscle actin-alpha 2 (ACTA2) (cyan) highlights the high frequency of mature vessels (black asterisk) in adjacent tumor tissue (G). In the tumor area more immature vessels (red asterisk), lacking ACTA2 expression, can be observed (H). Scale bars represent 50 µm for the chromogenic stainings and 20 µm for the fluorescent stainings.

Figure 3: Selected representative images of the immunohistochemistry assays studied and discussed in this review. Images were taken in areas adjacent to the tumor area (A, C, E, G) and in tumor areas (B, D, F, H) of human metastatic colorectal cancer tissue sections. The staining for fms-related tyrosine kinase 1 (FLT1) is weak to moderate in vessel-associated cells (arrow) of tissue adjacent to tumor (A), but vessel-associated cells in the stroma (sm) as well as the membrane of tumor cells in tumor areas (tm) show strong staining (B). Vessel-associated cells in the mucosa of tissue adjacent to tumor are moderately stained for neuropilin 1 (NRP1) (C). No staining can be observed in the epithelial cells of the crypts (cp). However, in the tumor area moderate to strong staining can be observed in the cytoplasm of tumor cells (D). Aspecific staining in the nucleus of tumor cells is also present. Vessel-associated cells show stronger staining. In tissue adjacent to the tumor area, the staining of tumor protein p53 (TP53) is weak in the nucleus of some epithelial cells (E). However, in the tumor area a strong staining can be observed in the nucleus of tumor cells (F). Vessel-associated cells are negative. Angiopoietin 2 (ANGPT2) expression is restricted to vessel-associated cells. In the tumor area, a strong staining can be observed (H); this is in contrast to the tissue adjacent to tumor (G). Scale bars represent 50 µm.

	Adjacent to Tumor	Tumor
CA9	cp	sm, tm, tm
EGFR	pm	tm, sm, tm
VEGFA	cp	sm, tm, tm, sm
CD31/CD34 - ACTA2	cp	tm, sm

	Adjacent to Tumor	Tumor
FLT1 (VEGFR1)	A	B — tm, sm, tm, tm
NRP1	C — cp	D — tm, sm
TP53	E — cp	F — tm
ANGPT2	G — cp	H — tm, sm, tm