TARGETING ANGIOGENIC PATHWAYS IN COLORECTAL CANCER: COMPLEXITIES, CHALLENGES AND FUTURE DIRECTIONS

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Targeting angiogenic pathways in colorectal cancer: complexities, challenges and future direction

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Abstract:

Colorectal cancer (CRC) is one of the commonest cancers in the world. During the last decade, the development of targeted therapies has given cancer treatment a novel direction in management of metastatic CRC (mCRC) and has enriched the therapeutic armamentarium in the management of this disease. In mCRC, targeting angiogenesis via the vascular endothelial growth factor (VEGF) pathway has been of particular interest based on the favourable survival benefit demonstrated by bevacizumab in clinical trials. More recently, large phase III studies have shown clinical efficacy for the new anti-angiogenic agents aflibercept and regorafenib. However, the results of pre-clinical and clinical studies of other anti-angiogenic agents have been disappointing.

Furthermore, the benefits from angiogenic inhibitors (AIs) in an unselected patient population are modest. Research into predictive biomarkers is therefore essential, but has, to date, been unsuccessful. Nevertheless, aflibercept and regorafenib have been shown to benefit both bevacizumab naive and refractory patients, suggesting that acquired resistance to AIs can be potentially reversed. This review describes the most recent advances in development of AIs in mCRC with particular focus on aflibercept and regorafenib, the existing challenges for the evaluation of these agents in clinical practice and potential strategies in designing clinical trials that could lead to the discovery of clinically meaningful biomarkers.

1. Introduction:

Colorectal cancer (CRC) is one of the fourth commonest cancers in the world and is associated with a high morbidity and mortality. In United States, over 1.4 million cases of CRC were diagnosed in 2013 and the projected mortality in 2013 was estimated to be more than 50,000[1]. CRC accounted for 9% and 8% of all cancer-mortality in males and females respectively in 2013[2]. Similarly, in Europe, CRC is the second commonest cancer and the second commonest cause of cancer-related mortality in both genders [3]. Despite recent improvements in screening and better insights into molecular biology of CRC, the 5–year survival rates for patients diagnosed with metastatic colorectal cancer (mCRC) remain poor[4]. These data reflect that CRC is a significant global health problem and that it requires further attention in terms of improving the screening procedures, gaining more insight into better selection of patients for existing therapies and the development of new therapies.

For patients with unresectable mCRC, the treatment goal in majority of the patients remains achieving disease control, prolonging survival, and providing palliation of symptoms. A small proportion of patients with liver-only metastatic disease may still achieve cure after presenting with colorectal liver metastases (CLM)[5]; however, the majority of them relapse with unresectable metastatic disease[6, 7].Chemotherapy has been the mainstay of treatment in the management of mCRC; from fluorouracil (5-FU) therapy to current combination treatment options, we have made some significant improvements. The median overall survival (OS) has therefore improved from 12 months with 5-FU monotherapy to 30-33 months with current regimens and the estimated median OS has improved from 12 months to 30-33 months with the modern regimens [8-13].This improvement in survival outcomes can be partly attributed to the incorporation of anti-angiogenic agents into the current therapeutic armamentarium. Bevacizumab was one of the first targeted agents to have received regulatory approval for use in mCRC [14]; following that the other angiogenesis-inhibitors (AIs) aflibercept and regorafenib have received regulatory approvals for use in the second and third line mCRC setting respectively [15, 16].

In this review, we will discuss the role of angiogenesis in mCRC, the impact of AIs in this disease, challenges in selection of patients for AIs and future trial designs that can potentially help us overcome these challenges.

2. The role of angiogenesis in mCRC:

Angiogenesis is a complex and tightly regulated physiological process comprising of sprouting, splitting, and remodelling of existing vessels. It is regulated by a balance

between various pro-angiogenic factors (e.g. growth factors, chemokines, enzymes, adhesion molecules, and endothelial specific factors) and anti-angiogenic factors (e.g. angiostatin, endostatin, thrombospondin, canastatin, and pigment epithelium-derived factor) [17, 18]. An imbalance between the pro-angiogenic and anti-angiogenic factors has been well-recognised as one of the hallmarks of cancer[19]. Under normal physiological conditions, angiogenesis occurs during wound repair, tissue remodelling or inflammatory processes; however, this process is deregulated and chaotic in neoplasms, which results in leaky, tortuous and inefficient vessels [20, 21] that have structural and functional abnormalities.

One of the most recognised proangiogenic pathways which have potential therapeutic implications is the vascular endothelial growth factor (VEGF)/ vascular growth factor receptor (VEGFR) signalling pathway. **Figure 1** shows the complexity and cross talks associated with this pathway. It comprises of a family of homodimeric glycoprotein ligands including: VEGF-A, -B, -C, -D, -E and placental growth factor (PIGF) [22, 23]. VEGF-A (also commonly referred to as VEGF) which is located on chromosome 6, is the most widely known gene in this pathway which undergoes alternative splicing to yield mature isoforms of 121, 145, 165, 183, 189 and 206 amino acids[24-26]; of those 121, 145 and 165 have been shown to be related to angiogenesis in many in vivo experiments[27]. Many growth factors and cytokines have been implicated in regulation of VEGF pathway; however, the main factor that regulates its expression is hypoxia[28]. As a consequence of hypoxic conditions, which most tumours acquire as their growth outstrips the blood supply, transcription factor hypoxia-inducible factor-1 (HIF-1) and HIF-2 are activated, which leads to increased expression of VEGF[29]. Additionally, hypoxia also causes up-regulation of VEGF by increasing the stability of mRNA51 and by hypoxic translation of the VEGF mRNA [30, 31]. VEGF activation then leads to cascade of downstream signals, mainly through VEGFR-2, which is normally expressed by endothelial cells [32]. VEGF-B and placental growth factor (PIGF) bind to VEGFR-1, whereas VEGF-C and –D bind to VEGFR-2 and -3 receptors respectively [32]. The activation of VEGFR-1 by PIGF has been shown to be associated with recruitment of monocytes, which may be a means for tumour escape from angiogenic signals [33]. In CRC, VEGFR-1 activation also plays a leading role in cell survival, tumour progression and development of metastases [34]. VEGF-C and –

D play an important role in lymphangiogenesis and in development of metastases in many cancers including CRC by activation of VEGFR-3[35, 36].

The VEGF pathway is not the only pathway known to be associated with angiogenesis. More recently, the human angiopoietin family proteins, Ang-1 and Ang-2 have emerged as other important regulators of angiogenesis[37]. Ang-1 and Ang-2 are primarily over-expressed in perivascular cells and weibel-palade bodies of endothelial cells respectively [38, 39]. Ang-1 stimulates auto-phosphorylation of tyrosine kinase receptor (Tie-2), which is expressed by endothelial cells. This leads to activation of intracellular signalling pathways, which promote survival, and maintenance of endothelial cells under normal physiological conditions [40]. In contrast, Ang-2 is an inhibitory ligand for the Tie-2 receptor that disrupts the integrity of normal vasculature by competing with Ang-1, thus counteracting vascular normalisation [41-43]. Ang-2 can be over-expressed in multiple tumour types, including CRC; high Ang-2 levels have also been shown to promote metastatic growth and poorer survival outcomes in CRC [44-46]. Moreover, higher expression of Ang-2 expression has been associated with lymph node metastasis, venous invasion and high microvascular density in CRC [47]. Ang-2 ligand secretion is also known to be VEGF-dependent and can be down regulated in response and efficacy to anti-VEGF therapy [48, 49]. Blockade of the Ang-2/Tie-2 axis has however proved to be difficult in comparison to the VEGF pathway due to agonistic and antagonistic properties of Ang-1 and Ang-2 ligands respectively. However, potential treatment options blocking this pathway are being evaluated in clinical trials (NCT02141295).

Fibroblast growth factor (FGF)/FGF receptor (FGFR) signalling comprises of multiple cellular processes that can result in a cascade of downstream signals leading to cell growth, survival and angiogenesis. The FGF family includes 22 ligands (FGF-1 to -22) and 5 receptors (FGFR-1 to -5) [50, 51]. FGF and VEGF pathways can cross talk with each other, therefore promoting angiogenesis; indeed FGF-2 is know to induce the expression of VEGF in vascular endothelial cells and it has been shown that blockade of VEGF can reduce FGF-2 levels, highlighting the cross talk between the two pathways[52]. Likewise the platelet derived growth factor (PDGF) pathway contains five PDGF dimeric ligands and two receptors (PDGFR α and β) and is integral to regulation of angiogenesis. Activation of the PDGF pathway can lead to intracellular

signalling cascades which overlap with the VEGF signalling system and in turn promote vascular integrity, development and stabilisation[53, 54].

The epidermal growth factor (EGF) pathway is closely related to the VEGF pathway in terms of cross talks leading to resistance mechanisms, thereby affecting the efficacy of existing treatment options. Previous in vitro studies have demonstrated that increased VEGFR-1 expression can be associated with resistance to anti-EGFR therapies like cetuximab and gefitinib (a selective inhibitor of the EGFR tyrosine kinase domain), the former of which is licensed for use in mCRC [55]. Moreover, HIFs can be up regulated under hypoxic conditions, which then can increase VEGF expression and a cascade of downstream events [56]. Furthermore, VEGF production by tumour cells may be stimulated by activation of EGFR, which may result in increased migration and proliferation of endothelial cells, promoting angiogenesis [57, 58]. Based on close crosstalk between the two pathways, and some pre-clinical evidence [59-63], it was hypothesised that the dual blockade of VEGF and EGF pathways together may yield clinical benefit, however, the studies combining anti-VEGF and anti-EGFR antibodies didn't show any improvement in efficacy [64, 65]. Although several explanations, including overlapping toxicities, altered tumour vascularity[66] and downstream signalling interactions[65] have been proposed to be potential reasons for lack of efficacy when combing the blockade of two pathways, no robust evidence explaining the scientific rationale for the failure of dual blockade currently exists.

3. AIs in metastatic colorectal cancer in the clinic:

As noted before, the management of mCRC has evolved over last decade or so, with the median OS improving from 12 months with fluorouracil based single agent therapy to 30-33 months with new regimens[8, 11, 13, 67]. Regardless of the sequence of the available chemotherapy regimens, the outcome may be optimised in patients who are able to receive all the available drugs alone or in combination, including 5-FU, irinotecan, oxaliplatin, bevacizumab, aflibercept, regorafenib and cetuximab/panitumumab (in RAS wild type patients) during the course of their treatment. The currently available drugs, which are approved for treatment of mCRC, include three AIs, which highlight the importance of targeting angiogenesis in mCRC. In this section we will briefly discuss the available clinical data on approved AIs in mCRC.

3.1. Bevacizumab in mCRC:

Bevacizumab is a humanised monoclonal antibody, which binds to VEGF-A **(Figure 1)**, thereby inhibiting its interaction with VEGFR-1 and VEGFR-2 [68]. Bevacizumab comprises of human framework regions with 7% murine protein sequence, forming an antibody that inhibits all isoforms of human VEGF [69]. Numerous clinical studies have demonstrated the efficacy of bevacizumab in mCRC in various combinations, sequencing and duration [70]. In the landmark clinical trial comparing a bolus Irinotecan, 5-FU and Leucovorin (IFL) regimen with or without bevacizumab, a significant progression-free survival (PFS) (10.6 vs. 6.2 months, Hazard ratio [HR] =0.54, P<0.001), OS (20.3 vs. 15.6 months; HR =0.66, P<0.001) and objective response rate (ORR) (44.8% vs. 34.8%, P=0.004) advantage was observed in favour of the bevacizumab arm [71]. This led to US Food and Drug Administration (FDA) approval for this drug in mCRC. Subsequently the superiority of infusional versus bolus 5-fluorouracil (5-FU)-based regimens in combination with bevacizumab was established in the 430 patients in the BICC-C study. In this study patients were randomised to IFL, FOLFIRI (5-FU with irinotecan), or CAPIRI (capecitabine with irinotecan). The trial was later amended to include addition of bevacizumab to the treatment arms, which demonstrated an OS advantage in favour of the FOLFIRI plus bevacizumab arm, compared with IFL (28.0 vs. 19.2 months)**(Table 1)**[72]. Other studies combining bevacizumab with oxaliplatin have shown conflicting data. In the phase III NO16966 trial, a 2x2 factorial design was utilised to evaluate addition of bevacizumab or placebo to XELOX (capecitabine and oxaliplatin) or FOLFOX4 (5-FU,

leucovorin and oxaliplatin). This study demonstrated a minor improvement in PFS (9.4 vs. 8 months) in favour of bevacizumab containing regimens; however, no significant improvement in OS was observed (21.3 vs. 19.9 months, P=0.77) [14]. However, in this study a large number of patients were not treated until progression and early withdrawal was noted in high proportion of patients and- the results of the study should therefore be interpreted with caution. In the TREE study, modified FOLFOX6 with bolus 5-FU or CAPOX (capecitabine and oxaliplatin) were compared to addition of bevacizumab with or without the two chemotherapy regimens. A median OS of 23.7 months for the groups receiving bevacizumab was observed compared to 18.2 months for the non-bevacizumab groups[73]. It is however noteworthy that the addition of bevacizumab was not conducted in randomised fashion; therefore the results of this study also need to be interpreted with caution.

Based on the favourable results obtained from some of the above studies, bevacizumab has been often used as first-line treatment in mCRC; however, the choice of first-line therapy in RAS wild type tumours remains controversial and should be determined based on clinical features and preference of the patients [74]. In view of relatively common use of bevacizumab in first line setting, one of the important questions that arose is in clinical practice is the use of bevacizumab beyond progression. The Bevacizumab Regimens: Investigation of the Treatment Effects and Safety (BRiTE) study (600/1953 patients treated beyond progression) was an observational study that demonstrated that continuation of bevacizumab beyond progression was a single independent factor associated with better OS [75]. The concept of maintenance bevacizumab has also been explored in other clinical studies. **(Table 1)**[76, 77]. The CAIRO-3 study investigated the efficacy of maintenance capecitabine with bevacizumab versus observation in patients with stable disease or response after the induction treatment of CAPOX and Bevacizumab (CAPOX-B). At the time of initial progression (PFS1), patients were treated with CAPOX-B until second progression in both arms (this time point was referred to as PFS2). PFS2 was chosen as the primary endpoint of the study. While the final results of the study are yet to be published, this study met the primary endpoint with PFS2 of 11.7 vs. 8.5 months in favour of CAPOX-B arm[76]. CAIRO-3 was a well-conducted study, which took into account the common bias of improving PFS in the maintenance studies as PFS2 was chosen as the primary endpoint. Based on the results of this study, patients

with good response to bevacizumab may be considered for treatment until progression; however, clinical factors like extent to response to the treatment, patient preference and impact of treatment on quality of life should be carefully considered.

3.2. Aflibercept in mCRC:

Aflibercept is a recombinant VEGFR-antibody protein that is generated by fusion of the second immunoglobulin (Ig) domain of VEGFR-1 and the third Ig domain of VEGFR-2 to the Fc domain of human IgG1 [78]. Aflibercept traps the different isoforms of VEGF-A with 1000-fold higher intensity compared to bevacizumab, in addition to binding to PIGF and VEGF-B [79]. Aflibercept has been found to be active with a broad pharmacological index in number of pre-clinical solid tumour and mouse model studies [80-82]. Animal studies showed that aflibercept-VEGF complexes have no platelet-activating potential, suggesting that pro-thrombotic events were less likely to be associated with aflibercept. Phase I studies showed effective pharmacokinetic (PK) and pharmacodynamic (PD) effects indicative of effective VEGF blockade and a dose of 4mg/kg every 2 weeks was identified as the maximum tolerated dose (MTD) [83]. Based on the strong pre-clinical data, demonstration of effective VEGF blockade in early phase studies and the success of bevacizumab in mCRC; aflibercept was evaluated in mCRC. The phase II AFFIRM study recruited 236 patients with treatment naive mCRC to FOLFOX6 with or without aflibercept; however, this study failed to demonstrate any survival advantage in favour of aflibercept arm[84]. In the EFC10262-VELOUR trial, the addition of aflibercept to the standard FOLFIRI regimen was evaluated in 1226 patients with mCRC after failure of an oxaliplatin-based chemotherapy [15].The addition of aflibercept to FOLFIRI significantly improved OS relative to placebo plus FOLFIRI (hazard ratio [HR], 0.817; 95.34% CI, 0.713 to 0.937; P = .0032) with median OS of 13.50 versus 12.06 months, respectively. Aflibercept also significantly improved PFS; HR, 0.758; 95% CI, 0.661 to 0.869; P < .0001), with median PFS times of 6.90 versus 4.67 months, respectively. The effects on OS and PFS showed a consistent trend across all pre-specified subgroups, including bevacizumab pre-treated patients. ORR was 19.8% (95% CI, 16.4% to 23.2%) with aflibercept plus FOLFIRI compared with 11.1% (95% CI, 8.5% to 13.8%) with placebo plus FOLFIRI (P = .0001). These data led to the approval of aflibercept in combination with FOLFIRI in patients who have refractory disease to first-line oxaliplatin based treatment.

3.3. Regorafenib in mCRC:

Regorafenib (BAY73-4506) is an oral multi-kinase inhibitor (MKI), which has anti-angiogenic (VEGFR-1, -2, -3, PDGF, Tie-2, and FGFR) [85], anti-oncogenesis (KIT, RET, RAF1, BRAF) and anti-stromal (PDGF and FGFR) [86] properties. Pre-clinical studies confirmed a broad spectrum of anti-tumour activities [86, 87]. In a phase 1b study, 38 patients with heavily pre-treated (median 4 lines of prior treatment) mCRC were enrolled (dose escalation n=15, dose expansion n=23). Of the 27 evaluable patients, 1 (4%), 19 (70%) and 7 (26%) had a partial response (PR), stable disease (SD) and progressive disease (PD) respectively. The median PFS was 107 days and there was no statistically significant difference found in the PFS of KRAS mutant or wild type patients [88]. Following this, regorafenib was evaluated in a large phase III study. This study included 760 patients, nearly all of who were refractory to oxaliplatin, irinotecan and fluoropyrimidines, who had progressed within three months of the last therapy. Patients were randomised in 2:1 fashion to regorafenib (n=500) or placebo (n=253) respectively. This study demonstrated a statistically significant difference in OS and PFS in favour of regorafenib; with a median OS of 6.4 months versus 5.0 months (HR =0.77, 95% confidence interval [CI] =0.64-0.94, P=0.0052); and median PFS of 1.9 versus 1.7 months (HR=0.49M 95% CI=0.64-0.94, P=<0.000001)[16]. These data led to the FDA and EMA approvals of this product in the refractory mCRC setting. However, regorafenib is currently not commonly used due to the modest benefit in OS and the current lack of a validated biomarker. There are a number of clinical trials being designed or currently recruiting which are investigating the efficacy of regorafenib in other indications for mCRC.

3.4. Ramucirumab in mCRC:

Ramucirumab (IMC-1121B) is a fully humanised monoclonal antibody that potentially blocks the binding of the VEGF ligand to VEGFR-2 receptors by binding to the extracellular domain of VEGFR-2 receptors with high affinity [89, 90]. PD data from pre-clinical studies confirmed that ramucirumab effects the VEGF/CEGFR-2 interaction, VEGF-stimulated VEGFR-2 activation, proliferation of human endothelial cells and VEGF-induced phosphorylation of VEGFR-2 in both human umbilical vein endothelial cells and porcine aortic endothelial cells over-expressing VEGFR-2 [91]. Ramucirumab has demonstrated strong anti-tumour activity as single agent and in

combination with other therapies in a range of malignancies in animal models [89]. It has also been evaluated in mCRC; although the results from a large phase III study in second-line mCRC are currently awaited, an initial press release from the drug company suggests that the drug may have shown efficacy in this setting. Considering the success of AIs (aflibercept and regorafenib) after failing on bevacizumab coupled with the activity demonstrated by ramucirumab in gastric cancer in treatment-refractory patients [92], it will not be surprising if the drug shows efficacy in refractory mCRC. This will further open the window of opportunity for clinical trials in various lines of mCRC and will potentially add treatment options that are available for this disease.

4. Current research challenges:

In recent years, there has been more emphasis on the development of targeted therapies that are selected based on the understanding of patient's tumour biology

and molecular characteristics. The search for biomarkers that can predict response or resistance to specific therapeutic interventions has therefore become increasingly important in order to ensure that the right treatment can be selected for the right patients. The clinical observation that a significant proportion of patients who receive AIs may have improvement in PFS, which may not translate into an OS advantage, suggests that there are acquired mechanisms of resistance to AIs, which are incompletely understood. Furthermore, unlike cytotoxic chemotherapy, AIs may cause cytostatic effects, therefore the reliance upon Response Evaluation Criteria In Solid Tumours (RECIST) criteria may not provide useful insights into real clinical benefit from these agents. More precise information may therefore be gained from the use of biomarkers in determining the optimal dose and duration of AIs [93]. Here we discuss the issues surrounding the success of antiangiogenic therapies in mCRC and need for clinical trials prospectively validating this approach so that rigorously validated biomarkers are available for clinical use in order to optimise benefits from anti-angiogenic therapies.

4.1. Resistance to AIs:

Since Judith Folkman's initial hypothesis that angiogenesis could be explored as a therapeutic target [94], significant progress has been made in the last four decades with the development of a number of AIs. However, despite the encouraging pre-clinical results, AIs in most solid malignancies have failed to demonstrate a sustained anti-tumour activity and the magnitude of benefit across most tumour types has been rather modest [95]. As noted before, a number of clinical trials in mCRC and other cancers demonstrated significant improvements in PFS, which didn't translate into a improvements in OS; suggesting that both intrinsic and acquired mechanisms of resistance to AIs exist [96]. One of the important clinical observations made through several clinical trials is that AIs may show efficacy in three different ways: a) tumours show vascular response and associated significant shrinkage in the size of the tumour (cytotoxic effect) or b) tumours show vascular response but no accompanying tumour shrinkage (cytostatic effect) or c) tumours show minimal vascular response and stabilisation of the disease [97]. This suggests that tumours perhaps have adaptive vascular and extra-vascular mechanism to overcome treatment with AIs. It is therefore of pivotal importance to develop a good understanding about these response and

resistance mechanisms so that strategies can be developed to effectively overcome such challenges. Here we discuss important proposed mechanisms of resistance to anti-angiogenic therapies.

4.1.1. Blood vessels heterogeneity and alternative tumour vascularisation:

The tumour vessels are heterogeneous which may influence the response or resistance to AIs. Pre-clinical studies have demonstrated that anti-VEGF therapy suppresses the newly formed vessels more effectively compared to mature vessels [98, 99]. This may be due to dependence of nascent vessels on VEGF and on maturation, the sensitivity to anti-VEGF agents may be lost [97]. As pericyte recruitment into the vessels mediated by PDGF is one of the important aspects of vessel maturation, the clinical effects seen by use of MKIs (e.g., sorafenib, sunitinib and regorafenib) may be due to targeting of pericytes [100, 101]. Moreover, despite the common belief that the VEGF pathway is primarily responsible for sprouting angiogenesis, it is increasingly recognised that tumour vascularisation may be dependent on various diverse mechanisms [102, 103]. These alternative mechanisms of tumour vascularisation are well described in the literature in various human malignancies, and in other reviews [97] in more detail; we will briefly describe “vessel co-option” model here which may be more closely related to CLM [104]. Vessel co-option occurs when tumours invade the surrounding tissue and recruit existing blood vessels. Vessel co-option has been a well described phenomenon in various malignancies including glioblastoma [105], melanoma [106], adenocarcinoma of the lung[107] and liver metastases from breast and colorectal cancers [104, 108]. The clinical implication of this could be exploiting the role of histological growth patterns and response to chemotherapy in patients undergoing chemotherapy combinations with AIs. It is of particular interest in patients with CLM, as up to 40% of such patients may undergo tumour resection after having initial chemotherapy; this may provide an opportunity for high quality tissue collection and forming an association with radiological responses in a prospective fashion.

In addition to heterogeneity of tumour vasculature, alternative pro-angiogenic pathways may stimulate angiogenesis by activating blood vessel formation when the VEGF pathway is blocked. Numerous pre-clinical studies have identified alternative candidates, which may become activated on blockade of VEGF pathways; these

include angiopoietins, FGF1 and FGF2[109], PDGF-C[110], PIGF[111], hepatocyte growth factor (HGF) [112], interleukin (IL)-8 [113], delta-like ligand 4 (DLL-4)-Notch pathway [114], and EGF [96]. Although a strong body of pre-clinical evidence supports the notion that alternative angiogenic pathways may be important adaptive mechanism of resistance when treating patients with AIs, the clinical validation of such findings are currently lacking. Rational clinical designs facilitating tissue collection at various time points may be extremely helpful in validating such findings (as discussed below).

4.1.2. Stromal infiltration:

Stroma comprises of a heterogeneous cell population including fibroblasts, immune cells (lymphocytes, monocytes and granulocytes), myeloid cells (CD11b+ Gr+) and vessel forming cells (endothelial cells and pericytes), which are required by solid tumours for their growth and proliferation [115]. Evidence to the fact that stromal cells may play an important role in tumour progression [116, 117], and that they can lead to resistance to various therapies including AIs [118, 119], is well recognised. Several pre-clinical studies have demonstrated that invasion of tumours by various stromal cells can mediate resistance to AIs [120, 121]. Although the exact mechanism of resistance mediated by stromal cells to AIs is incompletely understood, several proposed mechanisms include release of pro-angiogenic factors and averting immune surveillance [122]. Indeed immature myeloid cells and endothelial progenitor cells (EPCs) may induce resistance by directly invading the tumour vasculature [97, 123] or through secretion of angiogenic factors like BV-8[124] or PDF-C [110]. Moreover, dual blockade of granulocyte colony stimulating factor (G-CSF), which is a key regulator of BV-8 expression, and the VEGF pathway has been shown to be associated with enhanced anti-tumour activity [125]. Although dual blockade of angiogenesis and stromal cells appears promising on review of pre-clinical data, it may be extremely challenging to block stromal cells in clinical practice. This is because stromal cells are genetically stable compared to cancer cells which carry genetic aberrations; however, they are constantly instructed by cancer cells in the tumour micro-environment. It is therefore possible that the stromal cells have different molecular and cellular characteristics based on the tumour type. Furthermore the intra-tumour heterogeneity exhibited by tumour cells within the same patient may make it even more challenging to targeting stroma with a “one size fits all” approach.

4.2. Lack of validated biomarkers

The search for predictive biomarkers that can determine response to anti-cancer therapies is one of the biggest challenges for the application of precision medicine in the clinic. Predictive biomarkers have allowed the rational use of targeted oncology therapies in patients with some solid cancers like breast cancer, gastrointestinal stromal tumours (GIST), non-small cell lung cancer and metastatic melanoma [126]. The only validated predictive biomarker in mCRC thus far remains mutation in RAS genes, which are a negative predictive marker of response to anti-EGFR therapies. The search for biomarkers to anti-angiogenesis therapies has proven to be challenging due to complexity of tumour-host interactions and the complexity of the VEGF pathway. There are number of potential biomarkers that have been examined in various clinical studies, however, none of them have been validated vigorously in prospective clinical trials in order for them to be used regularly in the clinic.

4.2.1. Circulating biomarkers:

VEGF-A has been investigated as a predictive biomarker in clinical trials of different malignancies including mCRC [127]. Although alterations in circulating VEGF levels have not been shown to be associated with treatment outcomes with anti-VEGF therapy in the clinic, they may serve as important PD biomarkers [93]. A phase I/II study of combination of chemotherapy and bevacizumab in rectal cancer patients showed that plasma VEGF and PIGF levels were significantly raised in patients receiving anti-VEGF therapy, suggesting that these could be utilised as PD markers [128].There is evidence from preclinical models that adaptive exposure of bevacizumab to CRC cells results in increased invasive capacity and migration after one week of exposure; increased expression of VEGFR-1, PIGF and VEGF-B was also noted in the cells which were chronically exposed to bevacizumab [129]. Loupakis et al. found an association between resistance to bevacizumab and low active VEGF concentrations; however, the VEGF levels remained low at the time of progression, contrary to PIGF and VEGFR-2 levels which were increased at the time of disease progression [130]. The AVAGAST [131] (Avastin in advanced gastric cancer trial) also showed that low VEGA levels were associated with improved outcomes, however, these findings were not reproduced by similar studies in CRC [132]. Other studies showed that VEGFR-2 and VEGFR-3 levels were associated with response to

bevacizumab in breast cancer and renal cell carcinoma [133, 134]. Validation of these findings by further prospective studies is however lacking at this stage, which restricts use of these potential biomarkers in the clinic.

Circulating endothelial progenitor cells (CEPCs) or circulating endothelial cells (CECs), which are released from the bone marrow to the blood have also been investigated as potential biomarkers to anti-angiogenic therapies [127]. Some trials have shown correlation between the lower number of CECs and response to anti-angiogenic therapy; while others have shown that higher numbers of CECs can be associated with progression of disease[135, 136]. Serum carcinoembryonic antigen (CEA), a commonly used biomarker in mCRC, has also been shown to potentially increase the sensitivity of VEGF in patients treated with bevacizumab [137]. DNA excision repair protein (ERCC-1) and thymidylate synthase (TS) genes expression have also been shown to be associated with improved OS in patients with mCRC that were treated with FOLFOX4 and PTK/ZK (a selective inhibitor of VEGF-mediated Flt-1 and KDR receptors) in the first and second line settings respectively [138]. Another study examining the gene expression profiles of cancer cells and tumour-associated macrophages in tumour biopsies before and on day 12 of bevacizumab monotherapy in patients with rectal cancer identified up-regulation of stromal cell-derived factor 1 alpha (SDF1-α) and its receptors CXCR4, and CXCL6. In addition higher SDF-1α levels during bevacizumab treatment was associated with higher rates of distant metastases at 3 years [139]. These findings suggest that the SDF1/CXCR4 pathway could represent a candidate pathway for validation in clinical studies in order to inform mechanism of response or resistance to AIs. The expression of other secreted proteins linked to angiogenesis and detected in circulation in various studies, includes VEGF-B, VEGF-C, VEGF-D, VEGFR2, FGF1, FGF2, PDGF-A, PDGF-C, HGF, Ang1, Ang2, interleukins including: IL-6, IL-8, IL-10, IL-12, MCP-1, tumour necrosis factor (TNF)-alpha, matrix metallopeptidase (MMP-9), tissue inhibitor of metalloproteinases (TIMP-1), soluble vascular cell adhesion molecule (sVCAM-1), soluble intercellular adhesion molecule (sICAM1), E-selectin and osteopontin.

The above discussion summarises the number of potential biomarkers which may have a role to play in future management of patients being treated with anti-angiogenic therapies in mCRC; however, none of them have been validated in prospective clinical trials and so are unavailable in the clinic. Additionally, there are number of challenges

in quantification of circulating cytokines as biomarkers of response to anti-angiogenic therapies; e.g. circulating VEGF may not provide accurate value as improper handling of platelets may release VEGF in to the circulation [122]. Moreover, anti-VEGF antibodies like bevacizumab or aflibercept may form complexes with circulating VEGF that is still measured as total VEGF, providing a false estimate of circulating VEGF in the plasma [140]. Despite all these limitations, circulating biomarkers may have an important role to play in future management of mCRC. As noted before there are at least three anti-angiogenic therapies now approved for management of mCRC. Exploration of biomarkers in clinical trials will help improve our knowledge about these biomarkers and therefore facilitate incorporation of them into future clinical practice.

4.2.2. Imaging biomarkers:

Imaging modalities have been employed to establish tumour response to anti-angiogenic therapies, as they permit inspection of tumour morphology and blood flow, which are important parameters in determining response or resistance to anti-vascular therapies. There is some evidence that dynamic contrast enhanced MRI (DCE-MRI) can be helpful in evaluating the tumour vascular heterogeneity and anti-angiogenic effects [141]. The effects of anti-angiogenic treatment can be detected by using DCE-MRI as early as 48 hours after initiating treatment [142]. One of the caveats however is that the vast majority of mCRC patients present with or develop CLM and in the presence of CLM, response to anti-angiogenic treatment may be modified by the predominant vascular pattern associated with the CLM [5]. Nevertheless, DCE-MRI has been recognised as a new imaging biomarker of anti-angiogenic activity and its parameters (Ktrans) have been correlated with microvessel density and in some tumours with degree of VEGF expression [143]. Moreover, hypervascular metastases were found to be an independent predictor of disease progression in a study of metastatic breast cancer patients [144]. It is well established that CLM demonstrate variable degrees of hypervascular rim enhancement, which may correspond with a peri-tumoural desmoplastic reaction, peritumoural inflammation, and vascular proliferation at histopathological examination [145]. It is thus possible that the thickness of the enhancing rim and the quantitative MR vascular parameters associated with the peri-tumoural rim of tumour metastases may reflect the degree of neo-angiogenesis. DEC-MRI therefore can be potentially used as a useful tool to assess response to anti-angiogenic therapies in mCRC. Diffusion weighted magnetic

resonance imaging (DW-MRI) is another imaging modality, which offers useful information about the tumour cellularity and its parameter of Apparent Diffusion Coefficient (ADC) is known to be an emerging biomarker of anti-tumour response; with an increase in ADC associated with tumour necrosis [146]. Anti-angiogenic therapies may cause cellular swelling due to reduction in the blood flow to the tumour area, leading to reduction in the ADC; DW-MRI therefore may provide useful information in determining response to anti-angiogenic therapies [146, 147]. Finally, positron emission tomography (PET) has been utilised as imaging biomarker for AIs in some studies; Zr-ranibizumab, a VEGF-labelled compound, showed promising results as an imaging biomarker in a xeno-patient study, during and after treatment with sunitinib[148]. However, more vigorous data are required before PET can be used as a routine imaging biomarker in the clinic [122].

Although validation of imaging biomarkers is required, the fact that they are becoming increasingly available in many research centres means that imaging biomarkers are likely to be available and validated for use in the clinic in the near future.

4.2.3. Surrogate biomarkers:

Blockade of VEGF can lead to reduction in nitric oxide (NO) synthesis, which can lead to vasoconstriction and development of hypertension [149]. Hypertension (HTN) has thus been considered as another biomarker of response to angiogenesis inhibitors. Studies of bevacizumab and systemic chemotherapy combinations used in the treatment of mCRC and metastatic breast cancer showed that grade 2-4 HTN was associated with significantly better survival and response rates in both cancers respectively [150, 151]. Moreover, Schneider et al. showed that VEGF-2578AA (a single nucleotide polymorphism [SNP]) in patients with grade 3-4 HTN, was associated with a better OS and ORR compared to VEGF-2578CA and VEGF-2578CC genotypes [151]. Of note, there are other studies examining the relevance of various SNPs in patients receiving anti-angiogenic therapies, however a detailed description of them is beyond the scope of this review and has been previously discussed extensively in other reviews[152].

As noted above, HTN can only be used as a surrogate biomarker, as a number of other patho-physiological factors may contribute to the development of HTN and treatment with anti-angiogenic therapies may not always lead to rise in blood pressure.

Nevertheless, HTN can be a useful clinical indicator of response or resistance to anti-angiogenic therapies when coupled with tumour markers like CEA and CA19-9 in the clinic.

4.3. Clinical trial designs to facilitate biomarker discovery:

One of the lessons learnt from number of seminal studies evaluating the role of RAS and other mutations as predictive biomarkers of response to anti-EGFR therapy is that the retrospective analysis of the banked tissue may have some limitations. These include the quality and quantity of the banked tissue, lack of availability of fresh tissue limiting some sequencing analyses and the statistical issues like the results not reflecting the intention to treat population. It is therefore imperative that prospective tissue collection studies with adaptive designs are designed to overcome such challenges. Contemporary prospective biomarker studies will benefit from a better understanding of tumour heterogeneity, access to novel sampling techniques like liquid biopsies and more advanced sequencing technologies.

One of the issues with biomarker-driven clinical studies is the uncertainty about the appropriate statistical models and endpoints. Some of the earliest biomarker-driven studies used an enrichment trial design, which means that only the positively screened patients for a specific biomarker were included in the randomised cohorts [153]. The issue with enrichment designs are however two-fold; a) this design may significantly reduce the number of subjects in the study, which are required for biomarker validation and b) this design will only be useful when there is compelling evidence to believe that the preliminary data are robust enough to preclude other patients participating in the studies modelled on such designs. Enrichment trial deigns are therefore not likely to be of clinical utility in establishing biomarkers for anti-angiogenic therapies as firstly, there are no robust preliminary data for biomarkers for angiogenesis and secondly, anti-angiogenic therapies effect the tumour stroma and thus may not have been selected merely based on the tumour's molecular characteristics. Another approach might be to use hybrid designs, where a sub-group of patients are randomised to targeted therapies based on a biomarker, and another group is randomised to the standard of care treatment. Advantages of hybrid designs compared to enrichment designs are that they allow larger groups of patients to be analysed and potential biomarkers can be thus assessed in all randomised patients across both groups[154].

However, when considering biomarkers for anti-angiogenic therapies, hybrid designs will be faced with similar limitations due to the lack of any compelling evidence to single out a biomarker. Adaptive trial designs that evaluate the success of biomarkers based on an ongoing basis may well be therefore required to meet challenges associated with anti-angiogenic therapies. We have started a number of tissue collection studies in our institute which incorporate prospective tissue collection at various time points. This will allow us the opportunity to overcome the issues associated with tumour heterogeneity. In addition, these hypothesis-generating studies may provide useful insights into issues like concordance between tissue and liquid biopsies. Finally, where possible, biomarker studies should be performed in monotherapy studies so that contamination due to changes in tumour molecular characteristics resulting from other therapies can be minimised **(Figure 2).**

5. Future directions:

Clinical efficacy demonstrated by AIs in mCRC provides the proof-of-principle that attacking angiogenesis is a valid treatment strategy in this disease. It is however clear from both pre-clinical and clinical data that some cancer cells start closer to the point of commitment to angiogenesis than others, and they are more likely to be sensitive to AIs; others demonstrate intrinsic/acquired resistance to these agents. The spiralling cost and complexity of developing AIs, and their impact on a proportion of patients warrants that the key issues in their development are addressed so that the full potential of this treatment strategy can be ensured in the clinic. Some of the important considerations are outlined below:

5.1.1. Understanding the biology of cancers:

It is now well established that tumours exhibit both intra-tumour and inter-tumour heterogeneity which complicates the process of precision medicine and biomarker discovery [155]. Most of the information available to date is restricted to the macro-heterogeneity level; however, much of the uncertainty surrounds the origin of micro-metastatic disease and clonal evolution of cancer cells at the micro-heterogeneity levels. Some studies have demonstrated that in patients with multiple metastases, variable response to AIs was observed with some metastatic lesions responding to the treatment while others were progressing [156]. This observation suggests that understanding the role of cancer evolution is critically important in establishing the novel therapeutic approaches for AIs and for other targeted therapies so that clinical outcomes can be improved.

Additionally, as noted earlier, tumours may vascularise by different mechanisms than VEGF-driven angiogenesis. Furthermore, previous clinical studies indicated that whilst bevacizumab was effective in metastatic setting, it showed little clinical efficacy in the adjuvant clinical trials in mCRC [157, 158].However, post-hoc analysis of one of the recent trials showed that patients with mismatch repair deficiency (dMMR) derived statistically significant survival benefit from the addition of bevacizumab when compared to those with MMR proficient tumours [159]. These observations may suggest that a) VEGF-driven angiogenesis is likely to play an important role only in established macro-metastatic disease [97] and/or b) AIs may have a role in only a proportion of patients with early commitment to angiogenesis, i.e. at the time of developing micro-metastatic disease. There are currently a number of clinical trials examining the role of AIs in various solid malignancies in the adjuvant setting; however it is concerning that they may not demonstrate efficacy unless biomarker driven trials with sound scientific rationale are conducted.

In order to ensure that better results from the ongoing clinical trials are achieved, it is imperative that we improve our understanding of tumour heterogeneity and tumour vasculature heterogeneity of both primary and metastatic sites. As noted before, one such window of opportunity lies in the management of CLM due to the unique opportunity of obtaining viable tissue in the patients undergoing resection and the interesting biological behaviour of these cancer as they represent both macro and

micro-metastatic disease[5]; in such patients valuable information can be gained about the role of neo-adjuvant chemotherapy and the histological growth patterns of the resected liver metastatic disease. Moreover, understanding the various histological growth patterns and their respective response to AIs in this setting will help in understanding the impact of vascular heterogeneity in these cancers. Finally, the scientific information gained from such studies could have broader applications in establishing biomarkers of response and resistance to AIs.

5.1.2. Biomarker-driven trials and collaborations:

Tumour endothelial cells tend to be genetically stable compared to tumour cells which may have mutations, deletions or amplifications; the response of the tumour vasculature to AIs therefore can be a host-mediated response that can be influenced by the genetic variability of the host[132]. Several studies have examined the predictive role of SNPs in candidate genes in trials where bevacizumab or other AIs were used [151, 160-164]; however, in almost all of these studies only limited numbers of SNPs were used based on candidate gene approaches. This has resulted in heterogeneous data which has not been validated and replicated and therefore is not commonly used in clinical practice. This brief discussion reflects upon the urgent and un-met need of developing biomarkers that can be validated in larger prospective clinical trials. Moreover, there is a growing need to examine the novel biomarkers in both plasma and solid tissue; and candidate biomarkers should be examined against the advanced imaging techniques like DCE-MRI or DW-MRI so that a homogenous set of biomarkers with broader clinical implications become available. Besides the scientific challenges in establishing such studies, other pertinent issues include the inclusion of biomarker studies in current health-care models and the financial implications which need to be met in order to set up these studies. Global collaborations are therefore required between clinicians, academics and funding bodies to build such research ecosystems. One such example includes the initiative taken by European Innovative Medicines Initiative (IMI) - the IMI consortium includes 21 European sites who are working together to obtain ethically approved metastatic tissue from paired biopsies from patients with mCRC [126]. The ultimate aim of this consortium will be to apply major sequencing techniques on the collected tissue in order to obtain valuable information on the genomics, proteomics, transcriptomics and epigenetics of these paired samples. Finally, by incorporating statistical and system

biology approaches, and by forming collaborations between industry and clinicians/scientists, we will get closer to our aim of achieving precision medicine for our patients with mCRC.

6. Conclusion:

In conclusion the pivotal role of angiogenesis in cancer evolution and progression is well-established. Moreover, in mCRC AIs have demonstrated efficacy in various clinical settings, supporting the hypothesis that angiogenesis is central to the survival of cancer cells. Further work on determining the exact structure of tumour vasculature, and interaction with alternative signalling pathways will provide a sound platform for developing bio-marker driven studies, which will allow us to overcome the challenges of tumour heterogeneity, and acquired tumour resistance. Validated biomarkers are essential to identify patients most likely to benefit from treatment with AIs and therefore optimise their clinical utility. Finally, close collaborations between scientists, clinicians

and industry will help guide clinical decisions for patients with mCRC, according to the desired principles of precision medicine.

List of Abbreviations

Colorectal cancer (CRC), metastatic CRC (mCRC), vascular endothelial growth factor (VEGF), vascular growth factor receptor (VEGFR), angiogenic inhibitors (AIs), colorectal liver metastases (CLM), overall survival (OS), fluorouracil (5-FU), angiogenesis-inhibitors (AIs), hypoxia-inducible factor-1 (HIF-1), hypoxia-inducible factor-2 (HIF-2), placental growth factor (PIGF), angiopoietin family proteins (Ang-1 and Ang-2), Fibroblast growth factor (FGF)/FGF receptor (FGFR), platelet derived growth factor (PDGF), epidermal growth factor (EGF), bolus Irinotecan, 5-FU and Leucovorin (IFL), progression-free survival (PFS), objective response rate (ORR), US

Food and Drug Administration (FDA), FOLFIRI (5-FU with irinotecan), CAPIRI (capecitabine with irinotecan), XELOX or CAPOX (capecitabine and oxaliplatin), FOLFOX4 (5-FU, leucovorin and oxaliplatin), Bevacizumab Regimens: Investigation of the Treatment Effects and Safety (BRiTE), immunoglobulin (Ig), pharmacokinetic (PK), pharmacodynamic (PD), maximum tolerated dose (MTD), hazard ratio (HR), multi-kinase inhibitor (MKI), confidence interval (CI), European Medical Association (EMA), Response Evaluation Criteria In Solid Tumours (RECIST), hepatocyte growth factor (HGF), interleukin (IL), delta-like ligand 4 (DLL-4), endothelial progenitor cells (EPCs), granulocyte colony stimulating factor (G-CSF), gastrointestinal stromal tumours (GIST), . Circulating endothelial progenitor cells (CEPCs), circulating endothelial cells (CECs), carcinoembryonic antigen (CEA), excision repair protein (ERCC-1), thymidylate synthase (TS), stromal cell-derived factor 1 alpha (SDF1-α), tumour necrosis factor (TNF), matrix metallopeptidase (MMP), tissue inhibitor of metalloproteinases (TIMP), soluble vascular cell adhesion molecule (sVCAM), soluble intercellular adhesion molecule (sICAM1), dynamic contrast enhanced MRI (DCE-MRI), Diffusion weighted magnetic resonance imaging (DW-MRI), Apparent Diffusion Coefficient (ADC), positron emission tomography (PET), nitric oxide (NO), Hypertension (HTN), single nucleotide polymorphism (SNP), European Innovative Medicines Initiative (IMI).

Conflicts of interest:

The authors have no conflicts of interest to declare

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Figure 1

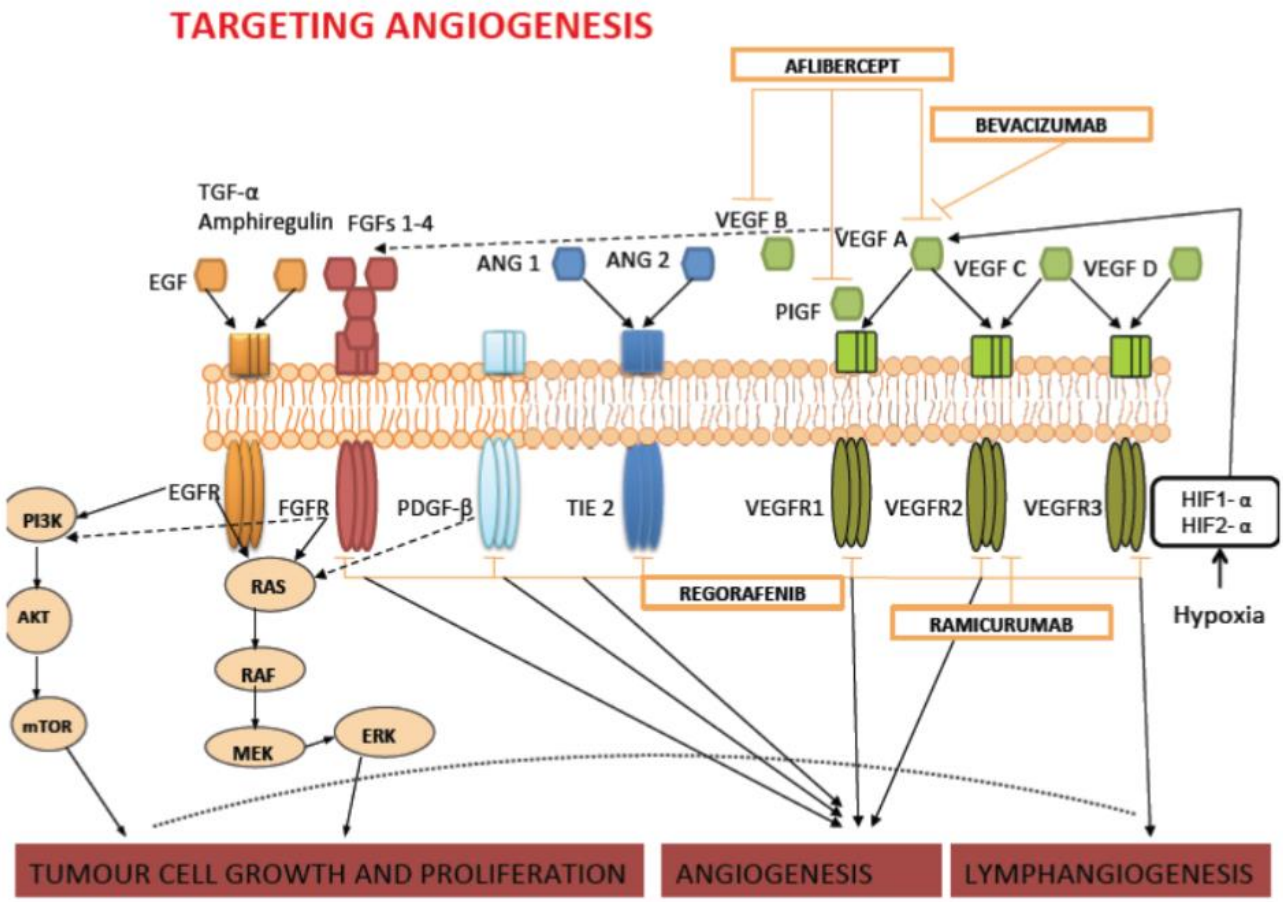

Fig. (1). Schematic diagram of main angiogenesis pathways and the interactions with angiogenesis inhibitors in mCRC: VEGF: Vascular endothelial growth factor; HIF: Hypoxia-inducible factor; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; PIGF: Placental growth factor.

Figure 2

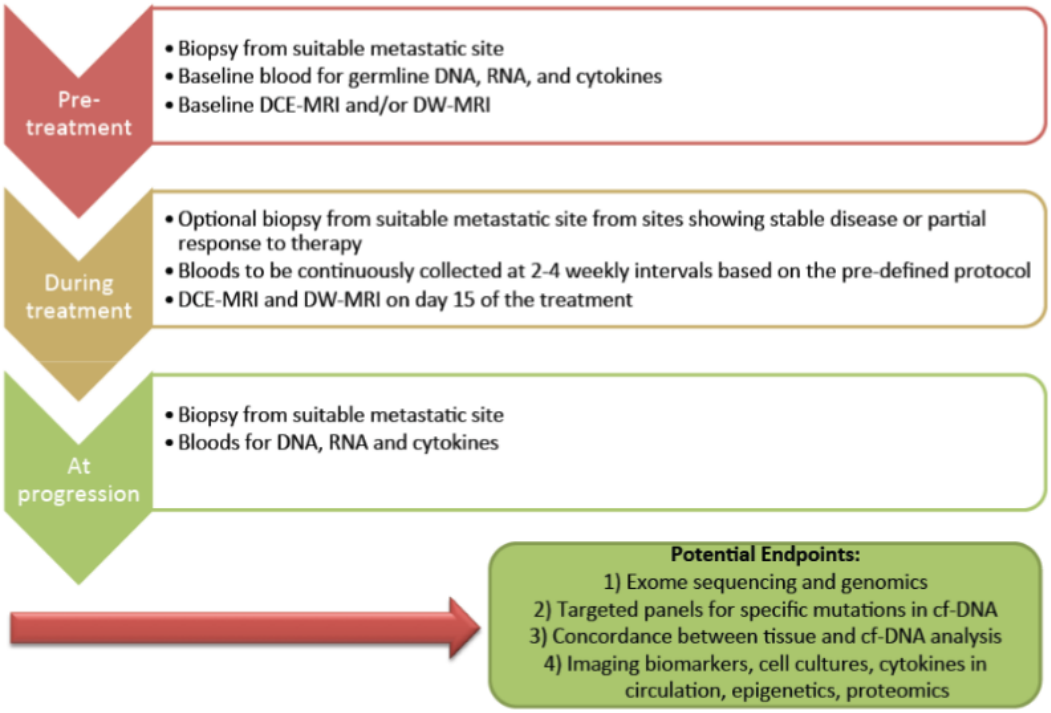

Fig. (2). Proposed clinical trial designs to facilitate hypothesis-generating biomarker discovery. DCE-MRI= Dynamic contrast-enhance magnetic resonance imaging, DW-MRI= Diffusion weighted magnetic resonance imaging and cf-DNA= cell free DNA.

Table 1: Landmark clinical trials of angiogenesis inhibitors in mCRC:

First author	No. of pts.	Line of therapy	Median PFS (in months)	Median OS(in months)	Median ORR (%)	Chemotherapy combination	Comments
Bevacizumab in mCRC							
Hurwitz[71]	813	First	10.6 vs. 6.2 P<0.001)	20.3 vs. 15.6 P<0.001)	44.8% vs. 34.8%, P=0.004	IFL+Bev vs. IFL	Lack of randomised data for FOLFIRI; IFL not a standard regimen these days
Fuchs [72, 165]	117	First		28.0 vs. 19.2 (P=0.037)		FOLFIRI+Bev vs. mIFL+Bev	
Stathopoulos[166]	222	First		25.0 vs. 22.0 (P=0.1391)	36.8% vs. 35.2%	FOLFIRI+Bev vs. FOLFIRI	
Hochster[73]	223	First	FOLFOX6= 9.9 vs. 8.7 bFOL= 8.3 vs. 6.9 CAPOX= 10.3 vs. 5.9	FOLFOX6= 26.1 vs.19.2 bFOL= 20.4 vs.17.9 CAPOX= 24.6 vs.17.2	FOLFOX6= 52% vs. 41% bFOL= 39% vs. 20% CAPOX = 46% vs. 27%	FOLFOX6, bFOL, CAPOX with or without Bev	
Saltz[14]	1401	First	9.4 vs. 8.0 (P=0.023)	21.3 vs. 19.9 (P=0.077)	38% vs. 38%	FOLFOX or XELOX with out without Bev	Drug dosage lower than usual
Bennouna, J [77, 167]	820	First/Second, beyond progression	5.7 vs. 4.1 (P=<0.001)	11.2 vs. 9.8 (P=<0.01)	5% vs. 3%	Irinotecan or oxaliplatin based chemotherapy with or without Bev	Results independent of the KRAS status
Giantonio[168]	829	Second or beyond	7.3 vs. 4.7(*P* < .0001)	12.9 vs. 10.8 (P=0.0011)	22.7% vs. 8.6% (P=< .0001)	FOLFOX4 with or without Bev or Bev alone	
Aflibercept in mCRC							
Pericay[84]	236	First	8.4 vs. 8.7		49.1% vs. 45.9%	FOLFOX6 with or without aflibercept	Paper not published so far but aflibercept only licensed for use with FOLFIRI
Van Cutsem[15]	614	Second	6.9 vs. 4.6 (P=0.0001)	13.5 vs. 12.06 (P=0.0032)	19.8% vs. 11.1%	FOLFIRI with or without aflibercept	Led to licensing of aflibercept in second line mCRC

Regorafenib in mCRC							
Grothey [16]	760	Conventional treatment refractory	1.9 vs. 1.7 (P=<0.000001)	6.4 vs. 5.0 (P=0.0052)		Regorafenib vs. placebo	Led to licensing of regorafenib in treatment refractory mCRC

No=number, pts= Patients, Bev=Bevacizumab, OS=Overall survival, ORR=Overall response rate, mCRC=Metastatic colorectal cancer, IFL=Irinotecan, 5-FU and leucovorin, FOLFIRI=5-FU and Irinotecan, FOLFOX6= 5-FU and oxaliplatin, CAPOX or XELOX=Capecitabine and oxaliplatin.

Table 2: Ongoing clinical trials with Angiogenesis inhibitors with a biomarker component

Drug	Trial	Phase	Treatment arms	Line of therapy	Primary endpoint
Bevacizumab	NCT01640405 (VISNU-1)	III	FOLFOX + Bev Versus FOLFOXIRI + Bev as First Line Treatment of Patients With mCRC Not Previously Treated and With Three or More Circulating Tumoral Cells (CTCs)	First	PFS OS CTCs
	NCT01937715	I/II	A Study Of PF-05212384 Plus FOLFIRI Versus Bev Plus FOLFIRI In mCRC		DLT PFS PI3K phosphorylation
	NCT01640444 (VISNU-2)	II	Influence of BRAF and PIK3K Status on the Efficacy of FOLFIRI Plus Bev or Cetuximab in Patients With RAS Wild-type mCRC and < 3 CTCs	First	OS CTCs
Aflibercept	NCT02079220	II	A Phase II Study of Ziv-aflibercept in Combination with XELOX Chemotherapy in the Front-Line Treatment of Patients With mCRC	First	PFS
	NCT02045030	II	Study to Identify Biomarkers of Clinical Response to Aflibercept in Patients With mCRC		Biomarker
	NCT01661972 (X-TRAP)	I/II	Phase I/II Study of Capecitabine Plus Aflibercept to Treat mCRC	Treatment refractory or unfit	R2PD
	NCT01661270 (AFLAME)	III	A Study of Aflibercept Versus Placebo With FOLFIRI in Patients With mCRC Previously Treated With an Oxaliplatin Chemotherapy	Second	PFS
	NCT02173990 (PULSAR)	II	mCRC Treated With First-line Aflibercept-based Treatment	First	PFS DCE-US evaluation (biomarker)
	NCT02129257 (AMALTHEA)	II	Clinical Trial of Combination Chemotherapy With Aflibercept in Patients With Advanced Colorectal Cancer	First	PFS at 1 year Biomarkers
	NCT01652196	II	Aflibercept and FOLFOX6 Treatment for Previously Untreated Stage IV Colorectal Cancer	First	PFS
Regorafenib	NCT01298570	II	Regorafenib+FOLFIRI Versus Placebo+FOLFIRI as 2nd Line treatment in mCRC	Second	PFS
	NCT01949194	II	Study to Determine the Efficacy of Regorafenib in mCRC Patients and to Discover Biomarkers	Second	Biomarkers

	NCT01875380 (REFRAME)	II	Regorafenib in Frail and/or Unfit for Chemotherapy Patients With mCRC	First	PFS at 6 months
	NCT02175654 (PREVIUM)	II	Regorafenib as Single Agent in Patients With mCRC With Any RAS or BRAF Mutation Previously Treated With FOLFOXIRI Plus Bevacizumab	Second	PFS at 6 months
	NCT01996969	Exploratory	Identification of Predictive Biomarker of Regorafenib in Refractory Colorectal Cancer	Third or beyond	Biomarkers
	NCT02175095	Exploratory	18F]FLT-PET as a Predictive Imaging Biomarker of Treatment Responses to Regorafenib	Third or beyond	Biomarkers

FOLFOX=5-FU and oxaliplatin, Bev=Bevacizumab, mCRC=Metastatic colorectal cancer, CTCs=Circulating tumour cells, PFS=Progression free survival, OS=Overall survival, FOLFIRI=5-FU and irinotecan, DLT=Dose limiting toxicity, R2PD= Recommended phase II dose, DCE-US= Dynamic contrast enhanced ultrasound scan, FOLFOXIRI=5-FU, oxaliplatin and irinotecan, FLT-PET= Positron emission tomography (PET) tracer 3'-deoxy-3'[(18)F]-fluorothymidine, [(18)F]-FLT