

## A systems biology perspective on sVEGFR1: its biological function, pathogenic role and therapeutic use

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Received: June 10, 2009; Accepted: September 28, 2009

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### Abstract

Angiogenesis is the growth of new capillaries from pre-existent microvasculature. A wide range of pathological conditions, from atherosclerosis to cancer, can be attributed to either excessive or deficient angiogenesis. Central to the physiological regulation of angiogenesis is the vascular endothelial growth factor (VEGF) system – its ligands and receptors (VEGFRs) are thus prime molecular targets of pro-angiogenic and anti-angiogenic therapies. Of growing interest as a prognostic marker and therapeutic target in angiogenesis-dependent diseases is soluble VEGF receptor-1 (sVEGFR1, also known as sFlt-1) – a truncated version of the cell membrane-spanning VEGFR1. For instance, it is known that sVEGFR1 is involved in the endothelial dysfunction characterizing the pregnancy disorder of pre-eclampsia, and sVEGFR1's therapeutic potential as an anti-angiogenic agent is being evaluated in pre-clinical models of cancer. This mini review begins with an examination of the protein domain structure and biomolecular interactions of sVEGFR1 in relation to the full-length VEGFR1. A synopsis of known and inferred physiological and pathological roles of sVEGFR1 is then given, with emphasis on the utility of computational systems biology models in deciphering the molecular mechanisms by which sVEGFR1's purported biological functions occur. Finally, we present the need for a systems biology perspective in interpreting circulating VEGF and sVEGFR1 concentrations as surrogate markers of angiogenic status in angiogenesis-dependent diseases.

**Keywords:** angiogenesis • neovascularization • vascular endothelial growth factor (VEGF) • soluble fms-like tyrosine kinase 1 (sFlt-1) • molecular systems biology • systems pharmacology • computational modelling • multi-scale modelling

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## Introduction

### Angiogenesis in physiology and pathology

Angiogenesis or neovascularization – the growth of new capillaries from pre-existing microvasculature [1] – sustains tissue growth and repair in normal adult physiology: during wound healing, ovarian and endometrial cycling, as well as muscle adaptations to exercise training [2, 3]. Angiogenesis can occur through distinct pathways: elevated microvascular shear stress triggers *splitting angiogenesis*, the intraluminal splitting of a microvessel longitudinally into two vessels; while tissue hypoxia stimulates *sprouting angiogenesis*, the abluminal budding of a new capillary sprout laterally from an existing microvessel [3, 4]. Related to splitting angiogenesis is *intussusceptive angiogenesis* caused by the formation of transvascular tissue pillars dividing the existing microvessel [5]. Tight regulation of the dynamic equilibrium between pro-angiogenic (angiogenic) and anti-angiogenic (angiostatic) processes is critical to health, as an imbalance in either direction contributes to a myriad of pathological conditions. Diseases characterized by excessive and abnormally coordinated angiogenesis include cancer, retinopathy, choroidal neovascularization, arthritis, atherosclerosis, psoriasis and endometriosis, whereas heart, brain and peripheral ischemia, as well as diabetes, hypertension, pre-eclampsia and nephropathy are characterized by insufficient angiogenesis [6].

### Angiogenesis in current medicine

The wide range of ‘angiogenesis-dependent diseases’ offers the promise of angiogenesis as a therapeutic target. In clinical trials designed to manipulate the ‘angiogenic balance’ *in vivo* as a therapeutic strategy [2, 7, 8], anti-angiogenic therapy seeks to down-regulate angiogenesis stimulators and/or up-regulate angiogenesis inhibitors; while pro-angiogenic therapy aims to up-regulate angiogenesis stimulators and/or down-regulate angiogenesis inhibitors.

At the molecular level, common targets of these therapeutic angiogenesis inhibitors and stimulators are the ligands and receptors of the vascular endothelial growth factor (VEGF) system, in which the ligand VEGF transduces pro-angiogenic signals through receptor tyrosine kinases such as VEGFR2 (VEGF receptor-2), while the soluble receptor sVEGFR1 is inhibitory to angiogenic signalling, partially through VEGF sequestration.

In the category of anti-angiogenic therapy, a prime research focus has been on the development of angiogenesis inhibitors as anti-cancer drugs, including the FDA-approved bevacizumab (a humanized monoclonal antibody against VEGF), sorafenib and sunitinib (receptor tyrosine kinase inhibitors) [9]; many others still in clinical trials, including endostatin (a broad-spectrum endothelial cell inhibitor) [10, 11]; and recently, the gene/protein delivery of sVEGFR1 in pre-clinical studies [12–21]. Recent studies have demonstrated synergistic benefits

of combining specific anti-angiogenic therapy with conventional chemotherapy (the use of cytotoxic agents in targeting proliferating tumour cells) [9, 22].

In the category of pro-angiogenic therapy, also known as *therapeutic angiogenesis*, almost a dozen clinical trials have been conducted for the gene and protein delivery of VEGF to treat coronary artery disease (CAD) and peripheral arterial disease (PAD), which are atherosclerotic vascular diseases that result in muscle ischemia [23, 24]. These randomized, controlled clinical trials have not been able to reproduce the efficacy consistently observed in pre-clinical animal studies [23, 24]. One proposed reason is that patients with these vascular diseases may also suffer from ligand insensitivity due to impaired receptor signalling or increased expression of antagonists (*e.g.* sVEGFR1), rather than simply a deficiency in angiogenic growth factor expression [23, 25]. Another proposed reason is that the pharmacokinetics of VEGF administration are not optimal for localizing pro-angiogenic responses within ischemic tissue, and that systemic elevation of angiogenic growth factors in blood may contribute to: (i) side effects such as transient tissue oedema (VEGF strongly induces vascular permeability) and (ii) the counter-effect of further promoting angiogenesis at the vasa vasorum feeding the growth of primary atherosclerotic plaques [23, 26, 27].

### VEGF ligand and receptor system: where does sVEGFR1 fit?

#### *Ligands: the human VEGF family*

Human VEGF is a family of related proteins: VEGF-A, VEGF-B and PlGF (placental growth factor) primarily involved in the growth of blood vessels [28, 29]; and VEGF-C and VEGF-D primarily involved in lymphangiogenesis (growth of lymphatic vessels) [30]. VEGF is secreted in cysteine-linked dimeric form [28], primarily as anti-parallel homodimers (VEGF/VEGF) but possibly as heterodimers (*e.g.* PlGF/VEGF) [31].

In most adult tissues, VEGF-A (also commonly referred to as simply VEGF) is secreted primarily from mesenchymal, stromal and epithelial sources (*e.g.* myocytes in muscles; platelets in blood; tumour cells and stromal cells in tumours) to act on endothelial cells in a paracrine fashion [9, 32, 33]. The biological roles traditionally attributed to VEGF have thus been mostly vascular: promoting the survival, migration, and proliferation of endothelial cells and increasing vascular permeability of vessels [28, 33]. Both the sprouting and splitting modes of angiogenesis are dependent on VEGF-A [3, 4]. However, there is a growing list of non-vascular roles for VEGF (*e.g.* recruitment of inflammatory cells and endothelial precursor cells [33]; neuroprotection in central nervous system and retina [34]), as well as evidence for

autocrine and intracrine production and function of VEGF (*e.g.* in endothelial, tumour and haematopoietic stem cells [9, 35]).

Among the many splice variants of human VEGF-A (Fig. 1) are: the pro-angiogenic isoforms VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>183</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub>; and their anti-angiogenic counterparts VEGF<sub>xxx</sub>b most prevalent in non-angiogenic tissues, *e.g.* colon and skin [36, 37]. The VEGF-A exons conferring isoform-specific affinities with receptors and co-receptors are illustrated in Fig. 1. The relatively high endogenous expression of VEGF<sub>121</sub> and VEGF<sub>165</sub> have led to their testing as therapeutic agents in pro-angiogenic treatment for ischemic pathologies such as PAD and CAD [23, 24].

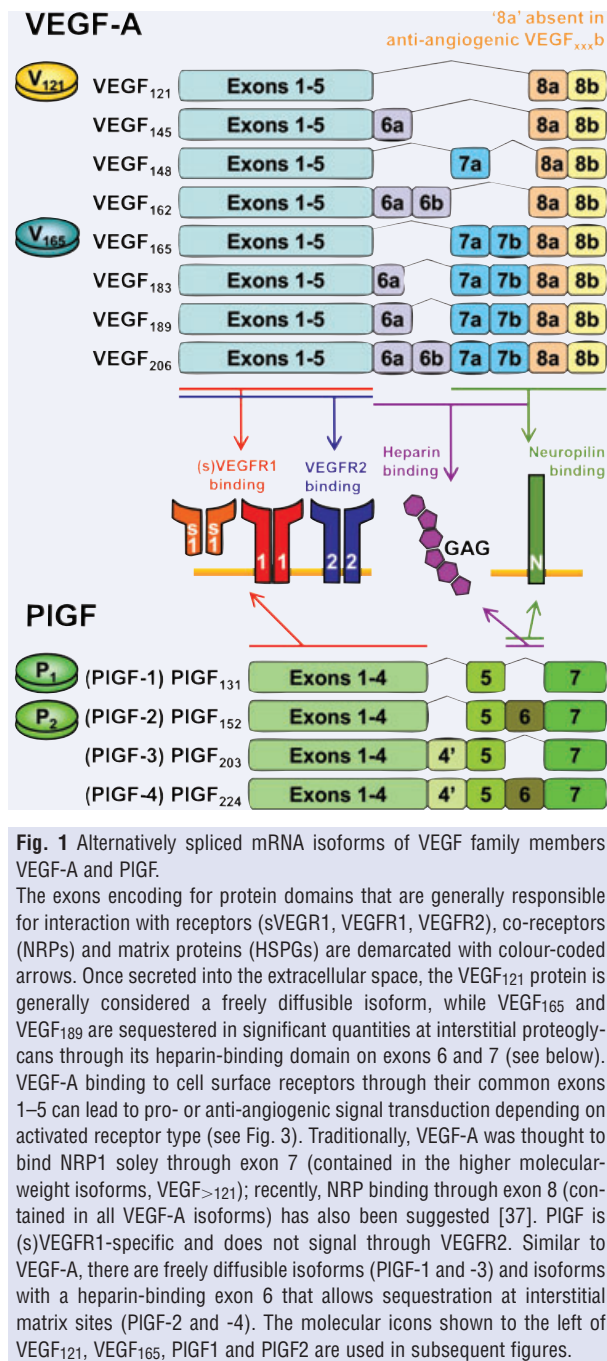
PIGF is also an important ligand for VEGFR1 and sVEGFR1, but not for VEGFR2 [28] (Fig. 1). The pathological disorder of pre-eclampsia sheds light on the critical interplay between PIGF and sVEGFR1. There is increasing evidence that hypoxia-induced placental production of sVEGFR1 plays a causal role in the pathogenesis of pre-eclampsia, a pregnancy disorder in which placental hypoxia and maternal endothelial dysfunction lead to multi-organ disease including hypertension and proteinuria [38–41]. In pre-eclampsia, increased circulating sVEGFR1 has been correlated with reduced free PIGF and reduced free VEGF in the blood compared to normal pregnancies [41–45].

#### Membrane-bound signalling receptors: VEGFRs

Full-length VEGF receptors-1, -2 and -3 (VEGFR1, VEGFR2, VEGFR3) are membrane-tethered receptor tyrosine kinases that are activated through homo/hetero-dimerization and ligand-induced trans-phosphorylation of intracellular tyrosine residues [29]. VEGFR3, as the receptor for VEGF-C and VEGF-D, largely mediates lymphangiogenic signalling [46] and will not be discussed here. VEGFR1 and VEGFR2 are expressed primarily on endothelial cells and they predominantly partake in the regulation of blood vessel angiogenesis (regulation of capillary sprouting and splitting), blood vessel maintenance, endothelial cell migration, endothelial cell proliferation, blood vessel permeability and dilation of blood vessels [28, 33]; but their expression on non-vascular cells (*e.g.* haematopoietic stem cells and megakaryocytes, the precursors of platelets; monocytic and dendritic immune cells; neural cells) further supports the aforementioned non-vascular biological roles of VEGF in normal adult physiology [34].

VEGFR2 (also known as KDR in human beings and Flk-1 in mice) is considered the predominant effector of pro-angiogenic signalling in sprouting angiogenesis and endothelial cell survival [9]. VEGFR2 is specific to VEGF-A and does not bind PIGF [29] (Fig. 1).

On the other hand, VEGFR1 (also known as FLT-1 in human beings and Flt-1 in mice) binds both VEGF-A and PIGF [29] (Fig. 1), and can mediate either anti- or pro-angiogenic signalling depending on the activating ligand type [46]. VEGF-A binding to VEGFR1 is generally considered anti-angiogenic – possibly due to sequestration of VEGF-A which lowers its availability for VEGFR2 activation, although direct intracellular signal transduction has not been ruled out [31, 46–48]. In contrast, PIGF binding to VEGFR1 leads



to pro-angiogenic signalling – either by direct intracellular activation of VEGFR1, by displacing VEGF-A which then binds and signals through VEGFR2, or by intracellular ‘crosstalk’ (intermolecular transphosphorylation) between the PIGF-activated VEGFR1 and VEGF-activated VEGFR2 [31, 46].

To our knowledge, the *in vivo* proportions of receptor homodimers (*e.g.* VEGFR1/VEGFR1, VEGFR2/VEGFR2) *versus*

heterodimers (*e.g.* VEGFR1/VEGFR2) have not been experimentally quantified. A computational study – through mathematical modelling of various ligand-induced and ligand-independent VEGFR dimerization schemes – has predicted that heterodimers may constitute 10–50% of actively signalling VEGF receptor complexes [48]. Receptor heterodimers may be activated by ligand homodimers (*e.g.* VEGF/VEGF [49]) or heterodimers (*e.g.* VEGF/PlGF [31]), and may transduce completely different signals (in sign or magnitude) compared to the homodimerized forms of its constituents [48].

### **Non-signalling co-receptors and matrix proteins: HSPGs and NRPs**

A variety of heparin-related proteins in the extracellular space have binding sites for the longer VEGF-A isoforms (VEGF<sub>>121</sub>). A significant source of interstitial VEGF-binding sites are the ‘SAS (N-sulphated/acetylated/sulphated) domains’ [50] on glycosaminoglycan chains of heparan sulphate proteoglycans (HSPGs) such as perlecan, agrin and collagen XVIII [51, 52]. A comparatively less prevalent diffusible source is the heparin-II binding domains of soluble fibronectins [53]. There are also HSPGs tethered on cell surfaces that facilitate surface VEGF-VEGFR binding and modulate the internalization rates of resultant complexes [46].

The cell-surface glycoproteins neuropilin-1 (NRP1) and neuropilin-2 (NRP2) were discovered originally as neuronal axon guidance receptors, but later found to be functionally significant in vascular formation as well [28, 54]. NRP1 directly binds VEGF<sub>165</sub>, PlGF-2 and possibly to a weaker degree, VEGF<sub>121</sub> [55]; NRP2 additionally binds VEGF<sub>145</sub> and VEGF-C, suggesting a functional bias for lymphangiogenesis [28, 46, 54].

NRPs are typically considered non-signalling receptors, but they serve as co-receptors for the VEGF family *via* complex formation with VEGFRs [46]. The presence of NRP1 exerts an overall pro-angiogenic effect on VEGF-signalling through two synergistic mechanisms [56]. Firstly, NRP1 can couple with VEGFR1 directly, with the resultant unligated complex NRP1-VEGFR1 permissive to subsequent binding by VEGF<sub>121</sub> but not VEGF<sub>165</sub> [56]. In other words, NRP1 makes VEGFR1 less available for VEGF<sub>165</sub> activation, thereby decreasing inhibitory or modulatory VEGFR1 signalling through the VEGF<sub>165</sub> isoform [56]. Secondly, NRP1 cannot couple VEGFR2 directly, but can still form a VEGFR2-VEGF<sub>165</sub>-NRP1 complex through non-overlapping VEGFR2-binding and NRP1-binding sites on the VEGF<sub>165</sub> ‘bridge’ [56]. In this manner, NRP1 augments VEGF<sub>165</sub> activation of VEGFR2 by both stabilizing VEGF<sub>165</sub>-VEGFR2 complexes and presenting NRP1-bound VEGF<sub>165</sub> to VEGFR2, thus increasing pro-angiogenic VEGFR2 signalling through the VEGF<sub>165</sub> isoform [56, 57]. An illustration and detailed discussion can be found in Mac Gabhann *et al.* [56].

### **Soluble receptors**

Soluble VEGF receptor-1 (sVEGFR1) was initially cloned in 1993 [58] and is a truncated ~110 kDa splice variant of the 180 kDa membrane-spanning VEGFR1 [28, 45, 59]. The natural occurrence

of sVEGFR1 – derived predominantly from alternative splicing [58], but possibly also from proteolytic cleavage of full-length VEGFR1 [16] – has been well documented, first in the pathophysiology of pre-eclampsia, and more recently also in numerous other physiological conditions [40, 41]. The molecular mechanisms of sVEGFR1’s purported anti-angiogenic effects have not been well elucidated, but are believed to include: (1) sequestration of VEGF ligands, much like VEGFR1 does, and effectively reducing VEGF-mediated activation of pro-angiogenic receptors and (2) heterodimerization with full-length VEGFR monomers to render the receptor dimer inactive, because sVEGFR1 lacks the intracellular tyrosine kinase domain needed to transphosphorylate its full-length partner [45, 60]. As discussed in detail below, there is growing interest in biomedical research to explore sVEGFR1 as a disease marker [42, 61–63] and a therapeutic vector for angiogenesis inhibition [12, 18, 64].

There is emerging evidence for soluble forms of other receptors as well. Soluble VEGFR2 is present in significant quantities in healthy human plasma (7–8 ng/ml) [65] and is up-regulated in acute myeloid leukaemia [66]. Soluble NRP1, a VEGF<sub>165</sub>-specific antagonist, has been documented in the kidney in human beings [67, 68]. However, this mini review will focus on sVEGFR1.

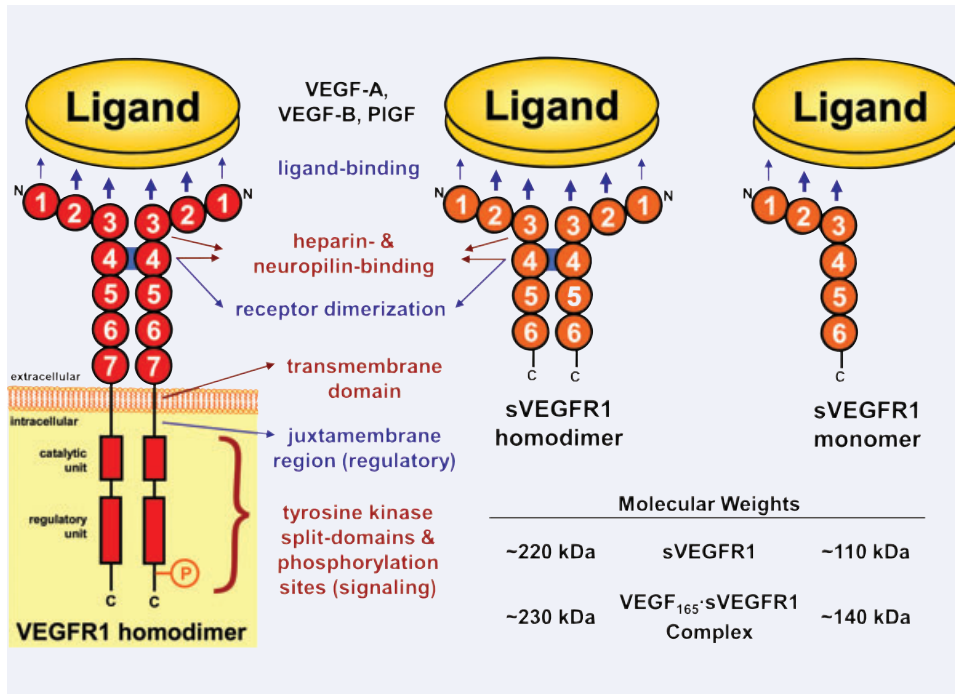
## **Molecular biology of sVEGFR1**

The molecular weight of sVEGFR1 appears to be both glycosylation- and species-dependent. The size of sVEGFR1 has been documented as 60 kDa in mice [69], 85–90 kDa when recombinantly expressed in transfected insect cells (Sf9) [58], 110 kDa when expressed by human umbilical vein endothelial cells (HUVEC) and primary human dermal microvascular endothelial cells (MVEC) [59, 60], 120–130 kDa when produced by melanoma cells (COLO-800) [59], 116 kDa in human placental tissue lysates [45] and 150 kDa (that deglycosylates to 115 kDa) in serum and amniotic fluid samples from pregnant women [70]. Of note, however, it is possible that these marked differences in molecular weight, particularly *in vivo*, may reflect proteolytic processing of either full-length VEGFR1 or sVEGFR1 (described further below), as there is no detectable difference in molecular weight when either mouse or human sVEGFR1 cDNA is expressed in various mammalian cells (CDK, BHA, unpublished observations).

sVEGFR1 has been found to bind VEGF in monomeric, dimeric, even multimeric forms *in vitro* and *ex vivo*, forming complexes weighing 115–145 kDa [58, 59], 220–230 kDa [58, 59] and 600–700 kDa [70], respectively, which suggests ligand-induced di/multi-merization, but the relative proportions of these various forms *in vivo* have yet to be quantified.

While sVEGFR1 has traditionally been considered a single protein, emerging evidence point to a family of at least four alternatively spliced soluble VEGFR1 proteins [71]. The mRNA transcripts of all known sVEGFR1 variants are common through to exon 13; thus invariably, the sVEGFR1 protein retains the first six N-terminal





**Fig. 2** Protein structures of soluble versus membrane-anchored VEGFR1. sVEGFR1 (110 kDa) is a soluble, truncated, alternatively spliced version of the full-length VEGFR1 protein (180 kDa) and has been found to bind VEGF in both dimeric and monomeric forms *in vitro*. Both share the first six immunoglobulin-like loops on their N-terminus; thus sVEGFR1 theoretically inherits VEGFR1's affinity for ligands (e.g. VEGF) and accessory molecules (e.g. interstitial heparan sulphate proteoglycans and neuropilins), as well as VEGFR1's dimerization properties. sVEGFR1 has a unique C-terminus and lacks the transmembrane region and intracellular tyrosine kinase signalling domains of VEGFR1.

immunoglobulin-like (Ig)-like extracellular motifs of VEGFR1, but lacks VEGFR1's seventh Ig-like domain as well as the membrane-anchoring region, regulatory (repressive) juxtamembrane domain and intracellular signalling tyrosine kinase domains [46, 58] (Fig. 2). The four known splice variants differ in their unique C-terminus as follows: (i) sFlt1\_v1, originally discovered in 1993 by Kendall *et al.* [58] and traditionally referred to as 'sFlt-1', ends with an extension of exon 13; (ii) sFlt1\_v2, identified by Thomas *et al.* as 'sFlt1-e15a' in 2007 [72] and Sela *et al.* as 'sFlt1-14' in 2008 [73], includes exon 14 and terminates with exon 15a sequences [73]; (iii) sFlt1\_v3, identified in 2009 by Heydarian *et al.* [71], includes exon 14 and terminates with exon 15b and (iv) sFlt1\_v4, also identified by Heydarian *et al.* in 2009 [71], includes exon 14 and terminates with an extension of exon 14. A detailed illustration of the structural differences between the 3' terminal exons of sVEGFR1 splice variants can be found in Heydarian *et al.* [71] Evidently, the splice variants of sVEGFR1 are subject to species-specific and cell-type-specific differential regulation at both transcriptional and translational levels, although the molecular details of which are still unclear [71, 73]. Hypoxia has been observed to up-regulate sVEGFR1 expression via HIF1- $\alpha$ , a process that likely contributes to the pathogenesis of pre-eclampsia [74].

Furthermore, an *in vitro* study documented in microvascular endothelial cells a  $\gamma$ -secretase-dependent intramembrane proteolysis of membrane-bound VEGFR1 and the intracellular release of an 80 kDa C-terminal fragment [16]. This finding prompted speculations of extracellular shedding of the remnant ~100 kDa N-terminal fragment, which may contribute to the intersti-

tial/plasma population of sVEGFR1 as a proteolytically cleaved counterpart to its alternatively spliced forms. More recently, ligand-induced proteolytic cleavage and ectodomain shedding of VEGFR1 was further documented in human leukaemia cancer cells [75]. Hereafter in this review, 'sVEGFR1' will refer to the entire family of alternatively spliced and cleaved variants of sVEGFR1.

Theoretically, the molecular interactions of sVEGFR1 with VEGF family ligands are expected to be similar to those of VEGFR1 due to preservation of the first six Ig-like domains: the first three domains, especially the second and third, are involved in the binding of competing ligand family members, including VEGF-A, VEGF-B and PlGF [76, 77]; the third and fourth domains are responsible for heparin-binding [77], suggesting possible sequestration at interstitial matrix sites (e.g. on heparan sulphate proteoglycans); NRP1-binding is mediated mostly through the third, but also the fourth, domain [77] and the fourth domain is responsible for ligand-induced receptor dimerization [76, 78], which presumably can facilitate both sVEGFR1-sVEGFR1 homodimerization and sVEGFR1-VEGFR heterodimerization.

## Physiological and pathophysiological roles of sVEGFR1

Endogenous sources of sVEGFR1 in human beings include vascular endothelial cells [40], vascular smooth muscle cells [73],

activated peripheral blood mononuclear monocytes [40], placental trophoblasts [40], corneal epithelial cells [79] and proximal tubular cells of the renal epithelia [80]. As alluded to, there is evidence for cell type-specific production of sVEGFR1 splice variants: human vascular smooth muscle cells were found to express sFlt1–14 while human endothelial cells expressed the traditional sFlt-1 [73]. This wide assortment of cell types capable of sVEGFR1 expression would suggest its active involvement in multiple physiological and pathological conditions.

Several biological functions of sVEGFR1 have been deduced from its capacity to neutralize VEGF: (1) anti-angiogenesis, by dampening angiogenic VEGF-VEGFR2 signalling [12, 46]; (2) anti-oedema, by interfering with VEGF-mediated vascular permeability through VEGFR1 or VEGFR2 [46, 81] and (3) anti-inflammation, by attenuating VEGF-VEGFR1-dependent monocyte and macrophage activation and migration [82]. The full extent to which the three potential actions of sVEGFR1 are involved in normal homeostasis is still uncertain. Nonetheless, exogenous administration of sVEGFR1 through gene and protein therapy has already demonstrated efficacy in controlling abnormal angiogenesis, oedema and inflammation in the 17 pre-clinical studies [12–15, 17–21, 64, 81–86] summarized in Table 1.

Of the three putative functions of sVEGFR1 listed above, a great deal of *in vivo* evidence supports an anti-angiogenic role. In normal human physiology, sVEGFR1 was shown to be critical for the maintenance of corneal avascularity needed for optical clarity. Among many anti-angiogenic molecules (angiostatin, endostatin, etc.) present in the cornea, only sVEGFR1 was necessary to suppress the pro-angiogenic effects of local VEGF-A, at least partially through ligand trapping [69, 79]. In the setting of human disease, there is increasing evidence that the hypoxia-induced placental production of sVEGFR1 plays a causal role in the pathogenesis of pre-eclampsia. Moreover, a recent animal study suggested a possible role for sVEGFR1 in the blunted angiogenic response in ischemic skeletal muscle in diabetic PAD [25], although this association has yet to be definitively proven in human PAD. While *in vivo* quantification of sVEGFR1 in human studies typically has not distinguished between the splice variants of sVEGFR1, a recent study suggested that they may be responsible for distinct physiological and pathological roles in human beings, *e.g.* that the sFlt1–14, an isoform expressed only in primates, is thought to play an important pathogenic role in human pre-eclampsia [73].

## **Molecular mechanism of sVEGFR1's anti-angiogenic potential**

The precise molecular mechanisms by which sVEGFR1 exerts inhibitory effects on VEGF-dependent signalling are unclear. Nevertheless, two mechanisms have been proposed: (1) direct ligand trapping of VEGF family members (including VEGF-A and

PlGF), *i.e.* lowering the effective concentrations of free VEGF available for receptor activation and (2) heterodimerization with surface VEGFRs to form dominant-negative complexes, *i.e.* lowering the effective density of unoccupied VEGFR available for ligand activation [60, 87]. More specifically, Figs 3 and 4 illustrate how the two mechanisms could affect angiogenic signalling at the endothelial cell surface and in the interstitial matrix, respectively. In this review, we deliberate the effects of sVEGFR1 within the context of sprouting angiogenesis specifically, but because VEGF-A is also involved in the splitting mode of angiogenesis, the inhibitory effects of sVEGFR1 on capillary sprouting will likely carry over to affect splitting angiogenesis as well.

Figure 3 considers the absolute intensity of VEGF signalling at capillary surfaces – relevant for activation of angiogenic sprouting – under the ligand system of PlGF-1, PlGF-2, VEGF<sub>121</sub> and VEGF<sub>165</sub>. In the absence of sVEGFR1, the membrane-tethered receptors (VEGFR1 and VEGFR2 homodimers; VEGFR1-VEGFR2 heterodimers) transduce ligand-specific signals, *e.g.* PlGF activation of VEGFR1 is pro-angiogenic while VEGF-A activation of VEGFR1 is weakly anti-angiogenic [46]. The introduction of sVEGFR1 presents new combinations of receptors that do not signal: diffusible sVEGFR1 acting as a VEGF sink (decoy receptor); and membrane-bound sVEGFR1-VEGFR heterodimers as dominant-negative complexes. Therefore, these non-signalling sVEGFR1 receptor complexes can concurrently exert anti-angiogenic effects at the cell surface by competing with the functional pro-angiogenic receptors for ligands, *e.g.* competing with VEGFR1 for PlGF and competing with VEGFR2 for VEGF-A (Fig 3). The intrinsic kinase and signalling activities of anti-angiogenic VEGF-VEGFR1 complexes are weaker than those of the pro-angiogenic VEGF-VEGFR2 complexes [46]; hence, the potential pro-angiogenic effects of sVEGFR1 arising from its competition with functional VEGFR1s (effectively reducing inhibitory signalling from VEGF-VEGFR1 complexes) may be much weaker than its anti-angiogenic effects described above. Also, it appears from these theoretical considerations that the extent of sVEGFR1's anti-angiogenic effects is ligand dependent. sVEGFR1 binding to PlGF is entirely anti-angiogenic, whereas binding to VEGF-A is only partially anti-angiogenic. *Ex vivo* evidence (sVEGFR1 from pooled amniotic fluids) supports sVEGFR1's capacity to act as a direct sink for VEGF and PlGF, but the relative contributions of this molecular mechanism and sVEGFR1 heterodimerization with transmembrane receptors to sVEGFR1's observed anti-angiogenic effects *in vivo* [59] is not known.

Figure 4 considers how the matrix-bound VEGF in the extracellular space – which shape the interstitial VEGF gradients that guide the migration and branching of sprouting vessels [46] – may additionally be attenuated by the presence of sVEGFR1. In the absence of sVEGFR1, the VEGF gradients sensed by endothelial tip cell filopodia may comprise freely diffusing VEGF, matrix-bound VEGF and active VEGF fragments (VEGF<sub>110</sub> and VEGF<sub>113</sub>) released from the matrix by plasmin or matrix metalloproteinase (MMP) cleavage of the larger isoforms of VEGF

**Table 1** Meta-analysis of 17 pre-clinical studies of the anti-angiogenic, anti-inflammatory and anti-oedema efficacies of sVEGFR1 (sFit-1) gene/protein therapy *in vivo*

Study	Therapy	Vector	Model	Disease	Route of administration	sFIT-1 expression	Effect on VEGF levels	Efficacy
				<b>Cancer</b>				
Kommareddy <i>et al.</i> [12]	Gene (sFit-1)	nanoparticle encapsulated plasmid DNA (non-viral)	orthotopic tumour xenograft in mice	human breast adeno-carcinoma	intravenous (systemic); tumour-specific targeting via EPR (enhanced permeability and retention) effect	liver and tumour-specific expression; no SKM expression	N/A	success: suppression of tumour growth; angiostatic
Kim <i>et al.</i> [117]	Gene (sFit-1)	pCMV-sFit-1 complexed to endothelial cell-targeted polymeric gene carrier (PEI-g-PEG-RGD)	subcutaneous tumour xenograft in mice	murine colon adeno-carcinoma	intravenous (systemic); angiogenic endothelium-targeting via integrin-binding RGD	N/A	N/A	success: inhibition of tumour growth; longer survival
Takei <i>et al.</i> [15]	Gene (sFit-1)	adeno-associated virus	subcutaneous and peritoneal tumour xenograft in mice	human ovarian serous adeno-carcinoma	intramuscular (hindlimb skeletal muscle)	high serum sFit-1 levels	lower serum VEGF levels; very high ascitic VEGF level	success: suppressed tumour growth and peritoneal dissemination; angiostatic
Mahendra <i>et al.</i> [18]	Gene (sFit-1)	adeno-associated virus	subcutaneous tumour xenograft in mice	human ovarian cancer	intramuscular (hindlimb quadriceps skeletal muscle)	systemic secretion (inferred?) and expression from muscle; no accumulation in liver	N/A	success: reduced tumour mass and increased tumour-free survival; no hepatotoxicity
Mahasrathi <i>et al.</i> [20]	Gene (sFit-1)	adenovirus	intrapitoneal tumour xenograft in mice	human ovarian carcinoma	intravenous (systemic)	~10x higher plasma sFit-1 levels than i.p. delivery (Mahasrathi); sFit-1 overexpression in liver	N/A	failure: reduced survival duration; sFit-1 related hepatotoxicity (hemorrhage and necrosis)
Mahasrathi <i>et al.</i> [21]	Gene (sFit-1)	adenovirus	subcutaneous tumour xenograft in mice	human ovarian carcinoma	cancer cells infected with Ad-sFit-1 <i>ex vivo</i> prior to inoculation	in situ (inferred) expression of sFit-1	N/A	success: suppressed tumour growth

Continued

Table 1 Continued

Study	Therapy	Vector	Model	Disease	Route of administration	sFlt-1 expression	Effect on VEGF levels	Efficacy
Mahasreshthi <i>et al.</i> [21]	Gene (sFlt-1)	adenovirus	intraperitoneal tumour xenograft in mice	human ovarian carcinoma	Intraperitoneal	no change in plasma sFlt-1 level <i>versus</i> untreated (Mahasreshthi)	N/A	success: increased survival duration
Liu <i>et al.</i> [14]	Gene (ECD <sub>1-3</sub> )	adenovirus	subcutaneous tumour xenograft in mice	human multiple myeloma	intravenous (systemic)	10x higher plasma sFlt-1 levels	N/A	success: angiostatic suppression of tumour growth
Gao <i>et al.</i> [13]	Gene (sFlt-1)	adenovirus	subcutaneous tumour xenograft in mice	human tongue carcinoma	Intratumoral	higher serum sFlt-1	N/A	success: reduced tumour growth and microvessel density
Zhang <i>et al.</i> [17]	Gene (ECD <sub>1-3</sub> )	adenovirus	subcutaneous tumour xenograft in mice	human colorectal cancer	Intratumoral	increased in tumour	N/A	success: reduced tumour volume and improved survival
Sako <i>et al.</i> [83]	Gene (sFlt-1)	adenovirus	intraperitoneal tumour xenograft in mice	human gastric cancer	intraperitoneal	high in peritoneum	N/A	success: suppressed peritoneal metastasis of gastric cancer
Ye <i>et al.</i> [19]	Cell-mediated Gene (ECD <sub>1-3</sub> )	embryonic kidney cells transduced with sFlt-1 retroviral vector	subcutaneous (dorsal flank) tumour xenograft in mice	human follicular thyroid carcinoma	subcutaneous injection of cells on other dorsal flank	N/A	N/A	success: suppressed tumour growth and intratumoral angiogenesis

Continued



Table 1 Continued

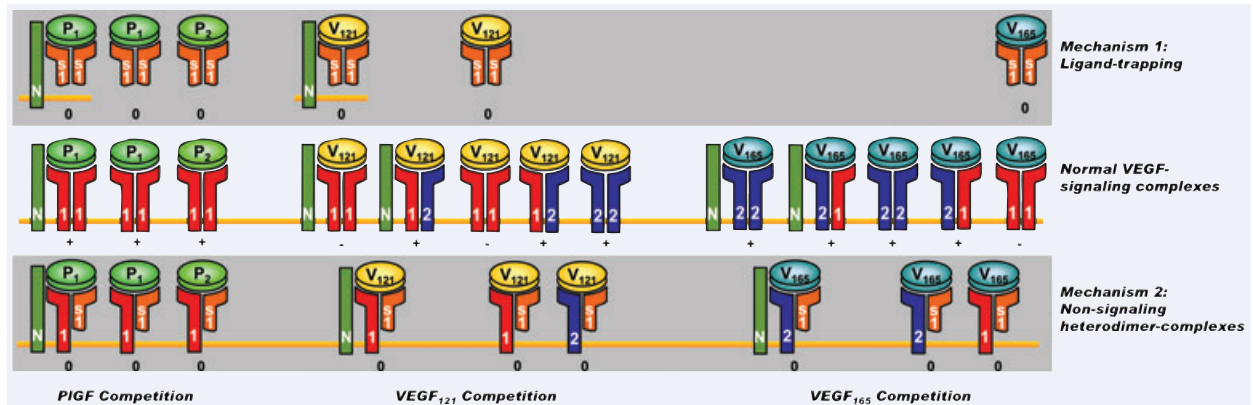
Study	Therapy	Vector	Model	Disease	Route of administration	sFlt-1 expression	Effect on VEGF levels	Efficacy
			<b>Ocular neovascularization</b>					
Rota <i>et al.</i> [84]	Gene (sFlt-1)	Adenovirus	ischemia-induced retinal neovascularization in rats	ischemic retinopathy	intravitreal (intraocular)	intravitreal expression	N/A	success: inhibition of retinal neovascularization
Gehlbach <i>et al.</i> [64]	Gene (sFlt-1)	Adenovirus	laser-induced choroidal neovascularization in mice	choroidal neovascularization	intravitreal (intraocular)	higher in choroid versus retina; none in plasma (Demetriades <i>et al.</i> )	N/A	success: suppressed choroidal neovascularization
Gehlbach <i>et al.</i> [64]	Gene (sFlt-1)	Adenovirus	laser-induced choroidal neovascularization in mice	choroidal neovascularization	periocular	higher in choroid versus retina; none in plasma (Demetriades <i>et al.</i> )	N/A	success: suppressed choroidal neovascularization
Gehlbach <i>et al.</i> [64]	Gene (sFlt-1)	Adenovirus	oxygen-induced retinal neovascularization in mice	ischemic retinopathy	periocular	low in retina (higher versus uninjected); none in plasma (Demetriades <i>et al.</i> )	N/A	success: reduced breakdown of blood-retinal barrier (vascular permeability) but not retinal neovascularization
			<b>Inflammation</b>					
Afuwape <i>et al.</i> [85]	Gene (sFlt-1)	Adenovirus	collagen-induced arthritis in mice	rheumatoid arthritis	intravenous (systemic)	high in liver, synovial tissue and serum	reduced VEGF in ankle joint lysates	success: suppressed arthritis severity and paw swelling, likely due to reduced synovial neovascularization
Tsao <i>et al.</i> [82]	Protein (sFlt-1-Fc fusion; R&D)	N/A	induced endotoxemia and sepsis in mice	Sepsis	intraperitoneal	N/A	attenuated endotoxemia-induced increase in plasma VEGF	success: anti-inflammatory

Continued

Table 1 Continued

Study	Therapy	Vector	Model	Disease	Route of administration	sFlt-1 expression	Effect on VEGF levels	Efficacy
Miotla <i>et al.</i> [86]	Protein (sFlt-1-PEG)	N/A	collagen-induced arthritis in mice	rheumatoid arthritis	intraperitoneal	N/A	N/A	success: reduced joint inflammation and bone/cartilage destruction
<b>Vascular permeability</b>								
Kumai <i>et al.</i> [81]	Gene (sFlt-1)	adenovirus	photochemically induced cerebral ischemia in rats	brain oedema (as a complication of brain ischemia)	intraventricular (lateral ventricle of brain)	sFlt-1 higher in periventricular area, cerebrospinal fluid, but not in serum	no change in cerebrospinal fluid	success: reduced infarct volume, brain oedema, blood-brain barrier permeability, monocyte/macrophage infiltration; but not angiostatic in ischemic region

Studies ordered by disease type, therapy type, then year. 'ECD1-3' = extracellular domains 1-3 of sFlt-1; 'PEG' = polyethylene glycol-linked.



**Fig. 3** How sVEGFR1 inhibits angiogenic signalling at the cell surface: two postulated mechanisms. The full set of possible ligand-receptor complexes in the absence of sVEGFR1 is shown in the middle row; those marked with '+' and '-' are thought to transduce pro- and anti-angiogenic signals, respectively. The presence of sVEGFR1 allows new combinations of complexes (top and bottom rows) that do not signal (marked by '0'); these non-signalling species exert anti-angiogenic effects by competing for ligands with pro-angiogenic species. Specifically, in mechanism 1 (top row), sVEGFR1 homodimers (or monomers, not shown) directly compete with surface VEGFRs for ligands (e.g. VEGF and PIGF), effectively lowering free ligand concentrations available to bind unoccupied surface VEGFRs. In mechanism 2 (bottom row), sVEGFR1 monomers dimerize with surface VEGFR monomers to form dominant-negative heterodimer complexes, effectively lowering the density of functional surface VEGFRs available to bind free ligands. These two mechanisms are not mutually exclusive and both are likely to occur *in vivo*, although their relative propensities are not known. 'P<sub>1</sub>' and 'P<sub>2</sub>' = placental growth factors-1 and -2; 'V<sub>121</sub>' and 'V<sub>165</sub>' = vascular endothelial growth factor-A (VEGF) isoforms 121 and 165; '1', '2', 'N' = surface receptors VEGFR1, VEGFR2, neuropilin-1; 's1' = soluble VEGFR1.

(VEGF<sub>>121</sub>). The possibility that sVEGFR1 might bind to matrix-bound VEGF has not been confirmed, but theoretically sVEGFR1 could exert anti-angiogenic effects on capillary sprouts simply by masking matrix-bound VEGF from direct filopodia sensing or by protecting matrix-bound VEGF from plasmin/MMP digestion and activation [87]. *In vitro* evidence supports a critical role for sVEGFR1 in vessel branching [88], but it remains unclear whether this is due to sVEGFR1-mediated attenuation of interstitial gradients or sVEGFR1 heterodimerization with surface VEGFRs.

Computational simulation of these biomolecular interactions provides an important means to decipher the relative contributions of each mechanism to sVEGFR1's anti-angiogenic effects [89, 90]. Experimentally, whether dominant-negative heterodimerization plays a significant role *in vivo* may be determined by quantifying the differential anti-angiogenic effects of sVEGFR1 *versus* ECD<sub>1-3</sub> (the first three extracellular Ig-like domains of VEGFR1), which may not be able to dimerize with surface VEGFR, as the fourth Ig-like domain appears to play a role in receptor dimerization [76, 78]. Several studies of ECD<sub>1-3</sub> gene delivery *in vivo* (Table 1) and *in vitro* (Table 2) have demonstrated angiostatic effects in tumour xenografts in mice as well as inhibitory effects on HUVEC proliferation, respectively. These data would suggest that VEGF sequestration alone is sufficient for sVEGFR1's anti-angiogenic effects. However, without a quantitative, controlled comparison of sVEGFR1 *versus* ECD<sub>1-3</sub> gene therapy, synergistic but redundant anti-angiogenic effects of the surface heterodimerization mechanism cannot be ruled out.

## sVEGFR1 as a clinical marker for disease

Since 2000, at least 20 studies [27, 42–44, 61–63, 66, 80, 91–101] have quantified sVEGFR1 levels in a diverse range of diseases associated with pathological changes of VEGF or PIGF levels, as summarized in Tables 3 to 5. Most measurements in Tables 3–5 were of circulating (plasma/serum) sVEGFR1, but a few measurements were from urine and tissue (brain) extracts as well.

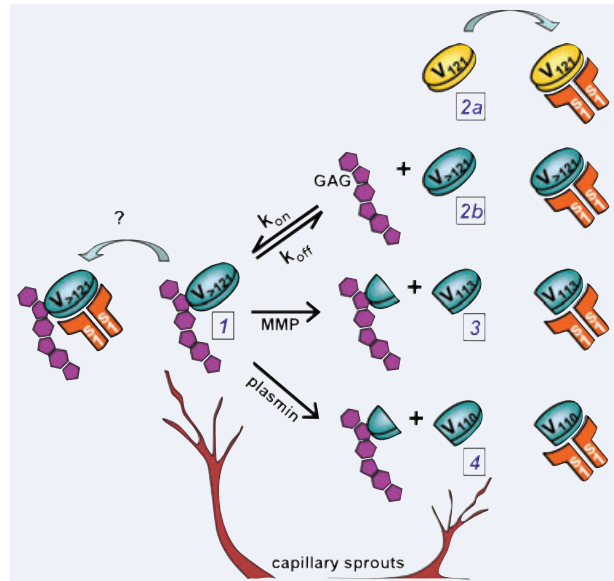
Researchers are increasingly finding clinical utility for sVEGFR1 as a diagnostic (disease identification) and/or prognostic (disease progression) marker in diverse medical conditions, either independently or in combination with VEGF or PIGF (Table 3). As noted, it is well established that increased circulating sVEGFR1 and reduced free PIGF and free VEGF in the blood are correlated with the pregnancy disorder pre-eclampsia (a disease of endothelial dysfunction and impaired angiogenesis) compared to normal pregnancies [41, 44, 45]. Quantitative metrics have shown predictive value for sVEGFR1 in differentiating pre-eclampsia among suspected pregnancies [42, 43]. Conversely, among cancers (diseases of unconstrained angiogenesis), including astrocytic gliomas, primary breast cancer, pancreatic cancer and acute myeloid leukaemia, low sVEGFR1 to VEGF ratios in tumour extracts, serum or plasma correlated with higher malignancy grades of tumours, poorer patient survival or weaker responsiveness to therapy [61, 66, 91, 93]. In liver cirrhosis (an inflammatory disease of pro-angiogenic status), increases in plasma VEGF

and sVEGFR1 were associated with a degree of hepatic insufficiency [62]. In suspected sepsis (systemic inflammation induced by infection), plasma sVEGFR1 and VEGF have shown potential for predicting illness severity or septic shock [63], where the changes in sVEGFR1 are thought to reflect a compensatory protective response to the VEGF-dependent inflammation [63, 102, 103]. In PAD – where skeletal muscle ischemia resulting from atherosclerotic occlusive arterial disease is accompanied by impaired arteriogenesis (the growth of arteriolar collaterals) around the primary occlusive site and impaired angiogenesis in the ischemic muscle downstream of the occlusion [23] – there is general consensus that plasma VEGF levels are increased relative to healthy controls [27, 27, 99, 101]. Although reported magnitudes of increased VEGF differ among studies, this may reflect differences in PAD severity among patient cohorts, as one study further correlated the plasma VEGF<sub>165</sub> concentrations with PAD severity (*i.e.* higher in patients with critical limb ischemia than in those with intermittent claudication) [99]. However, there is contradictory evidence regarding whether plasma sVEGFR1 levels are changed in PAD patients relative to healthy controls. Blann *et al.* and Belgore *et al.* documented significantly lower levels of sVEGFR1 [27, 101], while Findley *et al.* and Makin *et al.* observed no significant differences [99, 100]. Hence the potential use of plasma sVEGFR1 as a marker for disease severity in PAD remains controversial.

## Plasma VEGF and sVEGFR1: non-uniform predictors of angiogenic status across all diseases

While the studies described above have reported successful design of diagnostic/prognostic metrics for individual diseases or for severity of a given disease using combined measures of VEGF and sVEGFR1, a meta-analysis of 14 studies suggests that the plasma levels of these two angiogenic markers still do not adequately serve as reliable universal predictors of the overall angiogenic status across all angiogenesis-dependent diseases, as explained further below.

The 14 studies used in the meta-analysis (Fig. 5) included every study in Tables 3–5 that measured VEGF and/or sVEGFR1 specifically in plasma, *i.e.* excluding measurements from tissue homogenates, serum and urine for a consistent sampling source. The 14 studies are grouped according to disease type and angiogenic status: those marked by ‘+’ are diseases/conditions in which angiogenesis is up-regulated; ‘i’ further identifies angiogenic states that overlap with inflammatory states; those marked by ‘-’ are diseases of insufficient or impaired angiogenesis; those marked ‘±’ are atherosclerotic vascular diseases, in which angiogenesis fuels the growth of primary atherosclerotic plaques at obstructed blood vessels, but impaired angiogenesis (in addition to insufficient arteriogenesis) fails to provide sufficient oxygen delivery to the ischemic muscle tissues downstream of the obstructive site.



**Fig. 4** How sVEGFR1 attenuates extracellular VEGF gradients and hinders capillary sprout migration: postulated mechanisms. The interstitial VEGF gradients that endothelial vessel sprouts sense and respond to in migration may consist of: (1) matrix-bound VEGF, (2 a-b) free diffusible VEGF, (3) MMP-cleaved active fragment VEGF<sub>113</sub> and (4) plasmin-cleaved active fragment VEGF<sub>110</sub>. Theoretically, sVEGFR1 can attenuate VEGF gradients by binding any of these subpopulations: either masking them from endothelial receptor sensing, or protecting matrix-bound VEGF from dissociating or being cleaved into active diffusible forms. ‘V<sub>121</sub>’ = VEGF isoform 121; ‘V<sub>>121</sub>’ = VEGF isoforms 165, 189, 206, etc.; ‘MMP’ = matrix metalloproteinase; ‘GAG’ = glycosaminoglycan domains on heparin sulphate proteoglycans (interstitial VEGF-binding sites); ‘s1’ = soluble VEGFR1.

Firstly, plasma VEGF is elevated in almost all diseases/conditions (Fig. 5A), including the neoplastic diseases (acute myeloid leukaemia), diseases of abnormal ocular neovascularizations (retinopathy, glaucoma), inflammatory diseases (cirrhosis, sepsis) and diseases of cardiac and peripheral ischemia (CAD, PAD). Thus plasma VEGF alone cannot serve as a differential marker for angiogenic status. Secondly, there is not a consistent trend in plasma sVEGFR1 level changes within the diseases of supposedly similar angiogenic status (Fig. 5B). For example, among the diseases of aberrant ocular neovascularization, plasma sVEGFR1 decreased in the glaucomas but increased in proliferative retinopathy; among the atherosclerotic vascular diseases, plasma sVEGFR1 increased in atherosclerotic diabetes, but decreased in CAD and sometimes in PAD. Thirdly, neither can the pathological changes in plasma sVEGFR1:VEGF concentration ratios be used to differentiate between diseases of supposedly opposing angiogenic status (Fig. 5C). Thus, the simultaneous measurement of circulating VEGF and sVEGFR1 – a major VEGF neutralizing agent – in attempt to quantify the actual bioavailability of circulating VEGF, still does not uniformly predict the angiogenic status across the diseases examined here.

**Table 2** Meta-analysis of pre-clinical studies of the anti-angiogenic efficacy of sVEGFR1 (sFlt-1) gene/protein therapy *in vitro*

Study	Therapy	Vector	Model	Disease	Route of administration	sFlt-1 expression	Effect on VEGF levels	Efficacy
Liu <i>et al.</i> [14]	Gene (ECD <sub>1-3</sub> )	adenovirus	HUVEC (endothelial cells) and KM3 (multiple myeloma cells) proliferation assay	N/A	HUVEC: conditioned media from transfected KM3 cells; KM3: transfected with ADV-sFlt	high in conditioned media <i>versus</i> ADV-LacZ	N/A	success: inhibited HUVEC proliferation but not KM3 proliferation
Gao <i>et al.</i> [13]	Gene (sFlt-1)	adenovirus	HUVEC (endothelial cells) proliferation assay	N/A	conditioned media from transfected ovarian cancer cells	high in conditioned media <i>versus</i> AdGFP	N/A	success: inhibition of HUVEC proliferation
Mahendra <i>et al.</i> [18]	Gene (sFlt-1)	adeno-associated virus	HUVEC (endothelial cells) proliferation assay	N/A	conditioned media from transfected kidney cells	high in supernatant <i>versus</i> Ad-GFP	N/A	success: angiostatin-comparable inhibition of HUVEC proliferation
Mahasreshti <i>et al.</i> [21]	Gene (sFlt-1)	adenovirus	HUVEC (endothelial cells) proliferation assay	N/A	conditioned media from transfected ovarian cancer cells	high in supernatant <i>versus</i> mock-transduction	N/A	success: inhibition of HUVEC proliferation
Ye <i>et al.</i> [19]	Cell-mediated Gene (ECD <sub>1-3</sub> )	embryonic kidney cell line transduced with sFlt-1 retroviral vector	HUVEC (endothelial cells) proliferation assay	N/A	dual-chamber cell coculture Transwell system	N/A	N/A	success: inhibition of HUVEC proliferation

'ECD1-3' = extracellular domains 1-3 of sFlt-1.

## Systems biology perspective: unifying interpretation of plasma angiogenic markers

This section examines potential flaws and pitfalls in relying on just two markers in isolation – the plasma VEGF and sVEGFR1 levels – to encapsulate the angiogenic status of angiogenesis-dependent diseases. We also propose that a more integrative systems biology perspective [104, 105] can benefit the interpretation of these markers to aid prediction of the angiogenic status across a diversity of physiological and pathophysiological conditions.

### 1. Baseline heterogeneity in clinical measurements of healthy VEGF and sVEGFR1 levels in plasma

The quantitative variability in published measurements of circulating VEGF is well documented and can span up to three orders of

magnitude due to inter-study differences in assay protocols [106, 107]. Similarly, inter-study heterogeneity in measurements of circulating sVEGFR1 was reported in a meta-analysis of 10 studies examining normal pregnancies *versus* pre-eclampsia [44]. Having normalized the sample sources in Fig. 5 to consider only plasma measurements, healthy VEGF levels still varied over an order of magnitude from 14.3 pg/ml to 580 pg/ml, while healthy sVEGFR1 levels varied by three orders of magnitude from 22.5 pg/ml to 28 ng/ml. This striking data variability may be attributable to inter-study methodological differences, such as in the definition of inclusion/exclusion criteria of healthy cohorts, or in the sampling, preparation and analysis of blood protein concentrations. In particular, the healthy measurements of sVEGFR1 can be divided into two extreme ranges (Fig. 5), with one set well below 2 ng/ml and the other above 15 ng/ml; it is uncertain whether the systematically higher values reported in the latter dataset [27, 95–98, 101] could be accounted for by the in-house modified ELISA protocols commonly used in those studies (Table 3). Methodological disparities aside, natural variation of the physiological baseline could account for the wide intra-study ranges observed among individuals within control groups [27, 44]. Of particular concern



**Table 3** Meta-analysis of 20 studies of human VEGF and sVEGFR1 measurements in health and disease: diagnostic / prognostic value

Study					Proposed Marker Type		
Author	Year	Investigated disease / condition	Sample source	Assay	Marker	Association / indication	Potential tool
Lamszus <i>et al.</i> [91]	2003	malignancy of astrocytic gliomas ( <i>e.g.</i> glioblastoma = most severe grade)	homogenized tumour / brain extracts	ELISA (Bender MedSystems for sVEGFR1; R&D for VEGF)	lower sVEGFR1:VEGF	more malignant grade	Prognostic
Bando <i>et al.</i> [61]	2005	primary breast cancer	homogenized tumour extracts	ELISA (Bender MedSystems for sVEGFR1; R&D for free VEGF)	cut-off: sVEGFR1:(total VEGF) < 0.5	poor disease-free (P = 0.008) and overall (P = 0.0002) survival	Prognostic
Clavel <i>et al.</i> [92]	2007	arthritis (progressive study)	serum	ELISA (R&D; sandwich/free for both)	serum VEGF and sFlt-1	correlated with indices of inflammation and bone destruction	Prognostic
Chang <i>et al.</i> [93]	2008	pancreatic cancer	serum	ELISA (Quantikine, R&D; sandwich/free for both)	higher serum VEGF/sVEGFR1	poor survival	Prognostic
Woolcock <i>et al.</i> [42]	2008	pre-eclampsia	serum	ELISA (BD)	serum cut-off: sFlt-1 > 1.9 ng/ml	pre-eclampsia with 94% sensitivity, 78% specificity, 75% positive predictive value	Diagnostic
Diab <i>et al.</i> [43]	2008	pre-eclampsia	plasma	ELISA (R&D; capture/free sVEGFR1)	plasma cut-off: sFlt-1/PlGF > 3.92	pre-eclampsia and intrauterine growth restriction with 98% sensitivity, 95% specificity, 93% positive predictive value	Diagnostic
Widmer <i>et al.</i> [44]	2007	meta-analysis of pre-eclampsia studies (10 studies of sFlt-1; 14 studies of PlGF)			3rd-trimester increases in sFlt-1 and decreases in PlGF	pre-eclampsia	Diagnostic
Aref <i>et al.</i> [66]	2005	Acute myeloid leukaemia	plasma	ELISA (Quantikine, R&D)	higher plasma VEGF/sFlt-1	poor acute myeloid leukaemia outcome (therapy response / survival)	Prognostic
Bailey <i>et al.</i> [94]	2006	acute exercise	plasma	ELISA (R&D; free VEGF)	plasma: transient peak in sVEGFR1 and later dip in VEGF	hypoxia-induction of sVEGFR1?	unknown

Continued

**Table 3** Continued

Study					Proposed Marker Type		
Author	Year	Investigated disease / condition	Sample source	Assay	Marker	Association / indication	Potential tool
Jaroszewicz <i>et al.</i> [62]	2008	liver cirrhosis	plasma	ELISA (Chemikine, Chemicon for free VEGF <sub>165</sub> ; Quantikine, R&D for sFlt-1)	plasma VEGF and sVEGFR1	associated with indices of hepatic insufficiency	Prognostic
Kim <i>et al.</i> [80]	2005	stages of diabetic nephropathy	plasma	ELISA (Quantikine R&D for free VEGF; ReliaTech GmbH for free sFlt-1)			
Kim <i>et al.</i> [80]	2005		urinary	ELISA (Quantikine R&D for free VEGF; ReliaTech GmbH for free sFlt-1)	increased urinary VEGF and sFlt-1 excretion	diabetic microalbuminuria and proteinuria	unknown
Shapiro <i>et al.</i> [63]	2008	sepsis severity (prospective study)	plasma	ELISA (Quantikine, R&D; free VEGF and sVEGFR1)	plasma VEGF and sFlt-1	correlated with illness severity (noninfected versus infected without shock versus septic shock)	Diagnostic (prospective) / Prognostic
Felmeden <i>et al.</i> [95]	2003	hypertension	plasma	modified ELISA (R&D)	plasma: higher VEGF; lower sFlt-1	hypertensive ( <i>versus</i> control)	unknown
Blann <i>et al.</i> [27]	2002	diabetes with atherosclerosis (DM+A)	plasma	modified ELISA (R&D)	plasma: higher VEGF	DM+A ( <i>versus</i> control)	unknown
Chung <i>et al.</i> [96]	2003	CAD	plasma	modified ELISA (R&D)	plasma: higher VEGF; lower sFlt-1	CAD ( <i>versus</i> control)	unknown
Lip <i>et al.</i> [97]	2000	proliferative retinopathy	plasma	modified ELISA (R&D)	plasma VEGF	high in disease; low after successful laser treatment	Prognostic
Lip <i>et al.</i> [98]	2002	normal tension glaucoma (NTG)	plasma	modified ELISA (R&D)			
Lip <i>et al.</i> [98]	2002	primary open angle glaucoma (POAG)	plasma	modified ELISA (R&D)	plasma: higher VEGF; lower sFlt-1	glaucoma ( <i>versus</i> control)	unknown
Findley <i>et al.</i> [99]	2008	PAD severity (intermittent claudication, IC <i>versus</i> critical limb ischemia, CLI)	plasma	ELISA (R&D for VEGF <sub>165</sub> and sFlt-1)	plasma VEGF increases	correlated with PAD severity: highest in CLI <i>versus</i> (IC or healthy)	Prognostic

Continued

Table 3 Continued

Study					Proposed Marker Type		
Author	Year	Investigated disease / condition	Sample source	Assay	Marker	Association / indication	Potential tool
Makin <i>et al.</i> [100]	2003	PAD	plasma	ELISA (R&D)	plasma: higher VEGF	PAD ( <i>versus</i> control)	unknown
Blann <i>et al.</i> [27]	2002	PAD	plasma	modified ELISA (R&D)	plasma: higher VEGF; lower sFlt-1	PAD ( <i>versus</i> control)	unknown
Blann <i>et al.</i> [27]	2002	CAD	plasma	modified ELISA (R&D)	plasma: higher VEGF	CAD ( <i>versus</i> control)	unknown
Belgore <i>et al.</i> [101]	2001	cardiovascular disease (CVD+PAD)	plasma	modified ELISA (R&D)	plasma: higher VEGF; lower sFlt-1	PAD ( <i>versus</i> control)	unknown

Studies ordered by source and disease/condition, then by measured healthy values of plasma sVEGFR1.

in analysing levels of these proteins, in multiple instances the inter-study differences between 'healthy' baseline values often exceed in magnitude the intra-study differences between 'healthy' and 'disease/condition' values, especially for sVEGFR1 (Fig. 5). Thus, a standardization of assay protocols and calibrants for the clinical measurement of circulating VEGF and sVEGFR1 is imperative. If the variability of healthy baseline measurements does in fact reflect natural physiological variation, the significance and the diagnostic/prognostic utility of the pathological changes in plasma levels of VEGF and sVEGFR1 as shown in Fig. 5 would be questionable.

## 2. Effect of sVEGFR1 on VEGF bioavailability: VEGF-sVEGFR1 complexes

The rationale behind using VEGF:sVEGFR1 ratios as indicators of angiogenic status [61, 93] is based on an assumption that sVEGFR1 is the predominant regulator of circulating VEGF bioavailability, other than the intrinsic VEGF production rates. This assumption has yet to be validated, in light of mounting evidence of other potential blood carriers of VEGF: soluble VEGFR2 [65, 66], soluble NRP1 [67, 68], plasma fibronectin [53, 108–111] and platelets [112]. Furthermore, sVEGFR1 itself has additional ligand partners in addition to VEGF-A, as it also binds VEGF-B and placental growth factor (PIGF) [87, 113]. In order to assess how much of the bound/unavailable circulating VEGF is sVEGFR1-complexed and vice versa, simultaneous quantifications are necessary for free VEGF (capture/sandwich immunoassays [106]), free sVEGFR1, total VEGF (competitive immunoassays [106]), total

sVEGFR1 and VEGF-sVEGFR1 complexes (*e.g.* the specific modified assay developed by Belgore *et al.* [101]). In the 'Assay' column in Table 3, we note where free VEGF and sVEGFR1 were measured; in other cases the authors did not specify whether the measurement was for free or total species. In future experimental studies, this distinction should be carefully addressed in the design and reporting of experimental methodology. Crucially, experiments are needed to specifically determine whether a given ELISA will distinguish between bound and unbound proteins, *i.e.* whether individual antibodies detect epitopes that are masked after binding. In fact, differences in ELISA specificity may account for the observed marked differences in levels of circulating VEGF and sVEGFR1. Without direct quantifications of circulating VEGF-sVEGFR1 complexes, it would be nearly impossible to decipher, for example, how much of a pathological increase in available free VEGF is due to: increased VEGF production rate, increased VEGF-sVEGFR1 complex dissociation, or altered VEGF-binding to other carrier proteins.

## 3. Compartmental analysis: biotransport and biodistribution

VEGF and sVEGFR1 are soluble proteins subjected to pharmacokinetic transport between organ and tissue compartments (*e.g.* due to vascular permeability and lymphatic drainage). As such, their plasma concentrations do not necessarily reflect their interstitial concentrations within organs/tissues where the local angiogenic status is of interest with respect to the disease under consideration. This may be the case in PAD, in which two human

**Table 4** Meta-analysis of 20 studies of human VEGF and sVEGFR1 measurements in health and disease: healthy controls

Study		Healthy Control		VEGF				sVEGFR1			
Author	Year	n (# of patients)	Cohort description	Mean	Error		Units	Mean	Error		Units
Lamszus <i>et al.</i> [91]	2003	5	Healthy white matter	0.06	±S.D.	0.1	ng/mg	0	±S.D.	0	ng/mg
Bando <i>et al.</i> [61]	2005	N/A		N/A				N/A			
Clavel <i>et al.</i> [92]	2007	N/A		N/A				N/A			
Chang <i>et al.</i> [93]	2008	60	Healthy	187.63	±S.D.	393.32	pg/ml	0.01555	±S.D.	0.00198	ng/ml
Woolcock <i>et al.</i> [42]	2008	18	Normotensive women	N/A				0.47	IQR	0.11–0.89	ng/ml
Diab <i>et al.</i> [43]	2008	66	Normal pregnant women	N/A				0.5133	±S.D.	0.0726	ng/ml
Widmer <i>et al.</i> [44]	2007	N/A		N/A				N/A			
Aref <i>et al.</i> [66]	2005	10	Healthy	138	±S.E.M.	4.7	pg/ml	0.0225	±S.E.M.	0.0009	ng/ml
Bailey <i>et al.</i> [94]	2006	5	Healthy @ basal	37.3	±S.E.	7.7	pg/ml	0.0488	±S.E.	0.009	ng/ml
Jaroszewicz <i>et al.</i> [62]	2008	15	Healthy	46.8	±S.E.M.	4.1	pg/ml	0.1051	±S.E.M.	0.0059	ng/ml
Kim <i>et al.</i> [80]	2005	47	Healthy	14.3	IQR	48	pg/ml	0.11	IQR	0.25	ng/ml
Kim <i>et al.</i> [80]	2005	47	Healthy	27.8	IQR	49	pg/ mg creatinine	0.05	IQR	0.21	ng/ mg creatinine
Shapiro <i>et al.</i> [63]	2008	66	Noninfected	580	±S.D.	380	pg/ml	1.59	±S.D.	0.79	ng/ml
Felmeden <i>et al.</i> [95]	2003	60	Normotensive healthy	125	IQR	40–213	pg/ml	17	IQR	10–33	ng/ml
Blann <i>et al.</i> [27]	2002	14	Healthy	92.5	IQR	20–175	pg/ml	20	IQR	3–32	ng/ml
Chung <i>et al.</i> [96]	2003	34	Healthy	80	IQR	20–176	pg/ml	20	IQR	9–40	ng/ml
Lip <i>et al.</i> [97]	2000	18	Healthy	50	IQR	16–113	pg/ml	20	IQR	9.6–26	ng/ml

Continued

**Table 4** Continued

Study		Healthy Control		VEGF			sVEGFR1				
Author	Year	n (# of patients)	Cohort description	Mean	Error	Units	Mean	Error	Units		
Lip <i>et al.</i> [98]	2002	26	Healthy	83	IQR	13–125	pg/ml	28	IQR	18–39	ng/ml
Findley <i>et al.</i> [99]	2008	23	Healthy	50	±S.D.	30	pg/ml	0.9	±S.D.	0.4	ng/ml
Makin <i>et al.</i> [100]	2003	50	Healthy	78	IQR	69–100	pg/ml	0.9	IQR	0.2–2.9	ng/ml
Blann <i>et al.</i> [27]	2002	70	Healthy	77.5	IQR	20–149	pg/ml	22	IQR	14–37	ng/ml
Belgore <i>et al.</i> [101]	2001	40	Healthy	113	IQR	33–231	pg/ml	21	IQR	10–78	ng/ml

Studies ordered by source and disease/condition, then by measured healthy values of plasma sVEGFR1. S.D. = standard deviation, S.E.M. = standard error in the mean, IQR = interquartile range.

**Table 5** Meta-analysis of 20 studies of human VEGF and sVEGFR1 measurements in health and disease: disease/condition

Study		Disease/Condition		VEGF			sVEGFR1				
Author	Year	n (# of patients)	cohort description	mean	error	units	mean	error	Units		
Lamszus <i>et al.</i> [91]	2003	46	glioblastomas	11.9	±S.D.	20.74	ng/mg protein	3.26	±S.D.	4	ng/mg protein
Bando <i>et al.</i> [61]	2005	202	all primary breast cancers	0.532	95% CI	0.432–0.632	ng total VEGF/mg protein	0.949	95% CI	0.849–1.048	ng/mg protein
Clavel <i>et al.</i> [92]	2007	310	early arthritis @ baseline	465	±S.D.	270	pg/ml	0.035	±S.D.	0.03	ng/ml
Clavel <i>et al.</i> [92]	2007	310	early arthritis @ 1 yr	1212	±S.D.	1041	pg/ml	0.095	±S.D.	0.073	ng/ml
Chang <i>et al.</i> [93]	2008	92	pancreatic cancer	538.8	±S.D.	559.5	pg/ml	0.05094	±S.D.	0.05117	ng/ml
Woolcock <i>et al.</i> [42]	2008	18	pre-eclampsia	N/A				3.13	IQR	2.14–4.17	ng/ml
Diab <i>et al.</i> [43]	2008	8	early onset pre-eclampsia	N/A				2.562	±S.D.	1.611	ng/ml
Widmer <i>et al.</i> [44]	2007										
Aref <i>et al.</i> [66]	2005	43	Acute myeloid leukaemia	373.9	±S.E.M.	34	pg/ml	0.0497	±S.E.M.	0.0028	ng/ml
Bailey <i>et al.</i> [94]	2006	5	healthy @ peak change	17.5	±S.E.	2.5	pg/ml @ 2 hrs post-ex.	0.0729	±S.E.	0.0146	ng/ml @ 0.5 hrs post-ex.

Continued



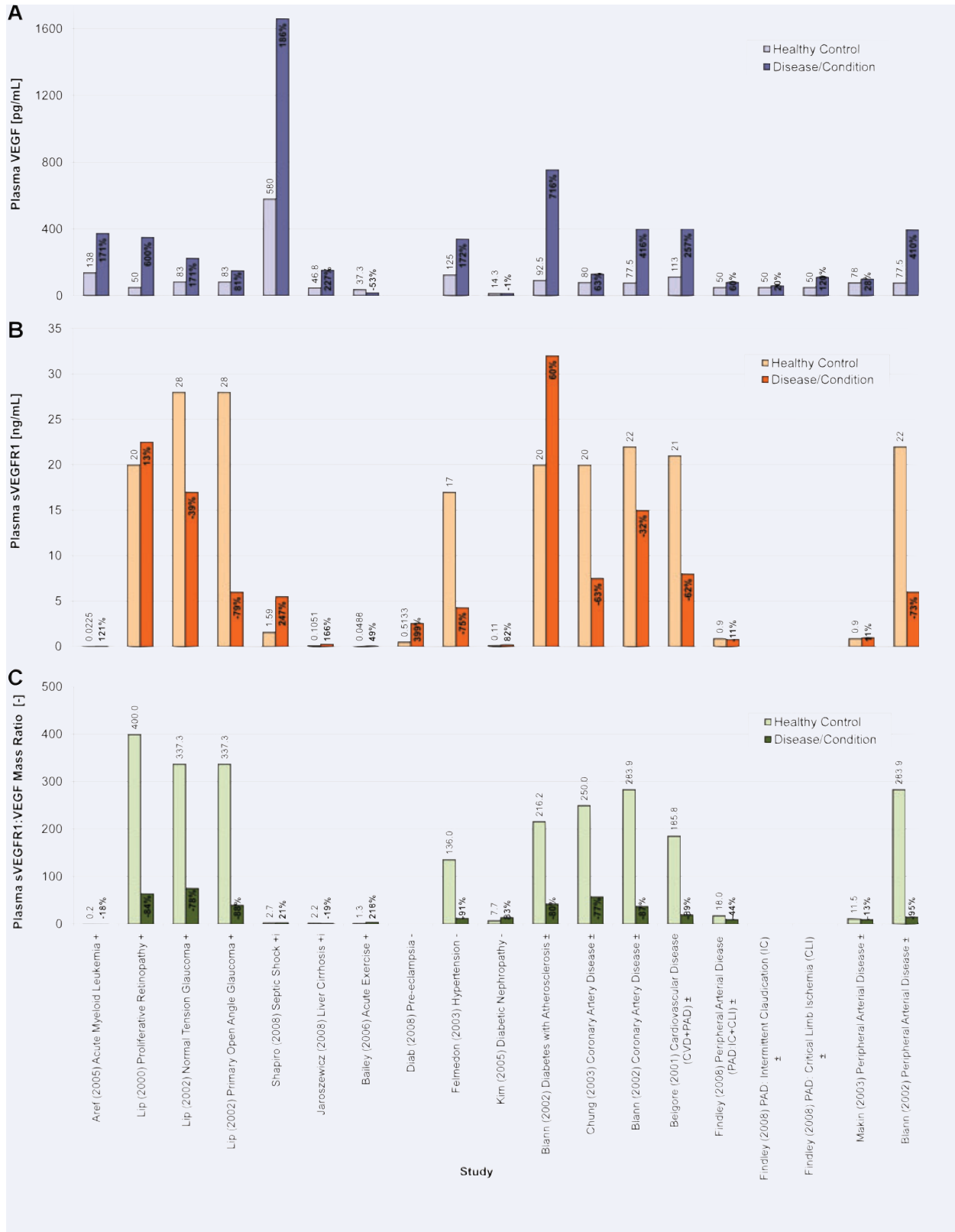
**Table 5** Continued

Study		Disease / Condition		VEGF			sVEGFR1		
Author	Year	n (# of patients)	cohort description	mean	error	units	mean	error	Units
Jaroszewicz <i>et al.</i> [62]	2008	78	liver cirrhosis of diff types	153.1	±S.E.M. 51.9	pg/ml	0.2798	±S.E.M. 0.0344	ng/ml
Kim <i>et al.</i> [80]	2005	33	proteinuria	14.2	IQR 23.3	pg/ml	0.2	IQR 0.24	ng/ml
Kim <i>et al.</i> [80]	2005	33	proteinuria	245	IQR 1048.4	pg/ mg creatinine	0.18	IQR 0.25	ng/ mg creatinine
Shapiro <i>et al.</i> [63]	2008	17	suspected infection + septic shock	1660	±S.D. 1710	pg/ml	5.51	±S.D. 3.71	ng/ml
Felmeden <i>et al.</i> [95]	2003	234	hypertensive	340	IQR 190–1300	pg/ml	4.3	IQR 1.5–14.0	ng/ml
Blann <i>et al.</i> [27]	2002	14	DM+A	755	IQR 100–1975	pg/ml	32	IQR 17–125	ng/ml
Chung <i>et al.</i> [96]	2003	111	CAD	130	IQR 100–250	pg/ml	7.5	IQR 1.9–19	ng/ml
Lip <i>et al.</i> [97]	2000	18	proliferative retinopathy	350	IQR 200–581	pg/ml	22.5	IQR 17.5–37.5	ng/ml
Lip <i>et al.</i> [98]	2002	26	NTG	225	IQR 110–500	pg/ml	17	IQR 6–60	ng/ml
Lip <i>et al.</i> [98]		24	POAG	150	IQR 118–235	pg/ml	6	IQR 2–19	ng/ml
Findley <i>et al.</i> [99]	2008	46	PAD (IC+CLI)	80	±S.D. 60	pg/ml	0.8	±S.D. 0.25	ng/ml
Findley <i>et al.</i> [99]		23	IC	60	±S.D. 30	pg/ml	N/A		
Findley <i>et al.</i> [99]		23	CLI	110	±S.D. 70	pg/ml	N/A		
Makin <i>et al.</i> [100]	2003	234	PAD	100	IQR 80–160	pg/ml	1	IQR 0.5–4.0	ng/ml
Blann <i>et al.</i> [27]	2002	70	PAD	395	IQR 187–912	pg/ml	6	IQR 1–26	ng/ml
Blann <i>et al.</i> [27]		70	CAD	400	IQR 139–1404	pg/ml	15	IQR 1–63	ng/ml
Belgore <i>et al.</i> [101]	2001	40	CVD (+PAD)	403	IQR 158–925	pg/ml	8	IQR 2–22	ng/ml

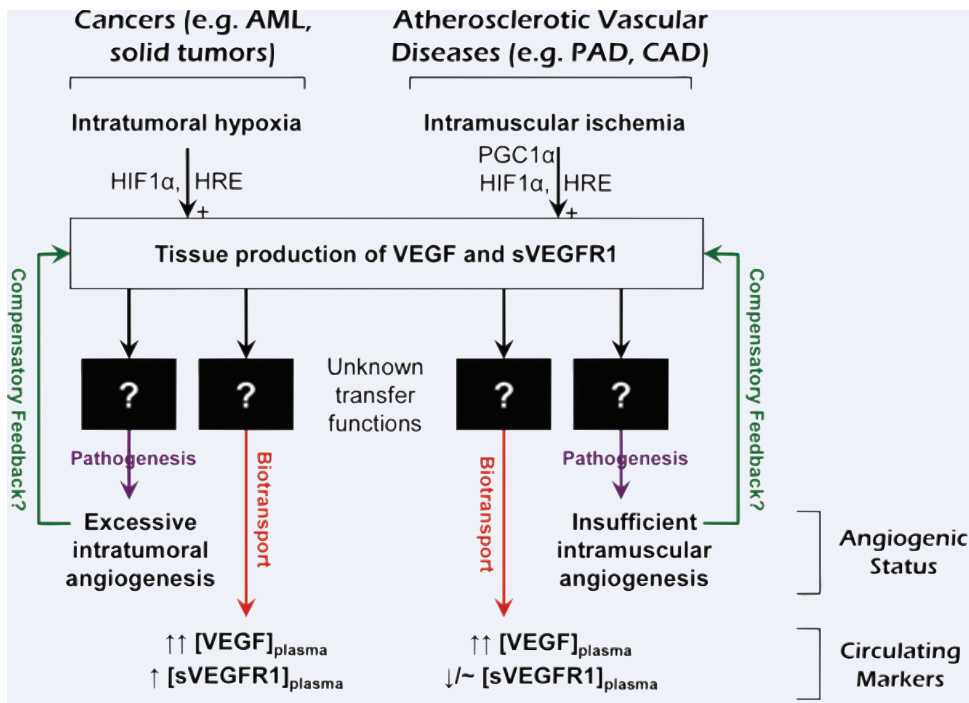
Studies ordered by source and disease/condition, then by measured healthy values of plasma sVEGFR1. S.D. = standard deviation, S.E.M. = standard error in the mean, IQR = interquartile range.

studies have documented increased sVEGFR1 in the plasma (Fig. 5), yet in a diabetic mouse model of PAD, muscle expression of sVEGFR1 increased after surgically induced hindlimb ischemia [25]. To the best of our knowledge, there are currently no available data assessing interstitial levels of sVEGFR1 in human beings. Interstitial VEGF has been measured by microdialysis [114, 115], but technical concerns have been raised regarding the accuracy in using high molecular-weight cut-off microdialysis

membranes to study macromolecules such as proteins, including unwanted ultrafiltration that could alter interstitial space compositions [116]. In the case that interstitial sVEGFR1 is truly elevated in PAD patients relative to healthy controls, in contrast to the unchanged/lowered sVEGFR1 levels observed in plasma, one explanation is that biotransport and biodistribution of sVEGFR1 are altered in PAD, *e.g.* lowered lymphatic drainage might lead to accumulation of sVEGFR1 within ischemic tissues.



**Fig. 5** Summary of 14 studies of plasma VEGF and sVEGFR1 measurements in health and disease. Studies are labelled as ' [First Author] (Year) [Disease/Condition of Study] [Status]', where status = {+, excessive angiogenesis; -, insufficient angiogenesis; i, inflammatory; ±, atherosclerotic vascular disease, with angiogenesis at primary atherosclerotic plaques, and insufficient angiogenesis in ischemic muscle). Data values are shown for healthy control bars; percentage changes relative to healthy controls are shown for disease/condition bars. In general, diseases of apparently opposing angiogenic status (e.g. ocular neovascularization *versus* cardiac ischemia) showed similar increases in plasma VEGF (A) and decreases in plasma concentration ratios of sVEGFR1:VEGF (C); while diseases of seemingly similar angiogenic status (e.g. glaucoma *versus* proliferative retinopathy) showed inconsistent changes in plasma sVEGFR1 (B). Error bars not shown due to inter-study variation in measures chosen (S.D. = standard deviation, S.E.M. = standard error in the mean, IQR = interquartile range). See Tables 4 and 5 for raw data.



**Fig. 6** Systems interpretation of circulating markers and angiogenic status. Both VEGF and sVEGFR1 have a HRE in their gene promoters, which is at least partly responsible for the up-regulated expression of both in response to hypoxia (e.g. in cancer) or ischemia (e.g. in atherosclerotic vascular diseases). It is uncertain how transfer functions (black boxes) transduce similar inputs into different outputs (angiogenic status; circulating markers) in different diseases. The transfer functions for the angiogenic status likely involve complex receptor expression/interactions and intracellular signalling processes. The transfer functions for the circulating markers likely include complex biotransport processes involv-

ing contributions from other tissue compartments. Whether circulating levels of VEGF and sVEGFR1 can serve as reliable surrogate markers of angiogenic status at the disease sites depends on the correlation between their respective transfer functions.

Therefore, in diseases in which the angiogenic markers in plasma seem to predict an angiogenic status other than that expected for the disease, the disparity may be corrected by examining the actual interstitial concentrations of these markers in the diseased compartments.

#### 4. Pathogenic phenomenon *versus* compensatory response

Even in the case that plasma levels of circulating VEGF and sVEGFR1 accurately reflect the interstitial levels within diseased tissues, it is difficult to discern whether static (single time-point) blood samples reflect pathogenic phenomena or compensatory

responses. For instance, in the case of cancer, elevated plasma VEGF (Fig. 5) typically represents a pathogenic process in which tumour hypoxia induces increased VEGF expression, thereby stimulating excessive angiogenesis in the diseased tissue. On the other hand, in atherosclerotic vascular diseases, the elevated plasma VEGF (Fig. 5) may be a result of compensatory VEGF up-regulation in response to ineffective angiogenesis at the ischemic muscle tissues or, as in tumours, a pathogenic increase in VEGF that stimulates the growth of the atherosclerotic plaque [26]. In fact, it is known that both VEGF and (s)VEGFR1 expression are hypoxia-inducible due to the presence of a hypoxia response element (HRE) in their gene promoter regions [87]; thus it is plausible that expression of VEGF and sVEGFR1 is up-regulated in both cancer and ischemic disease. In other words, what is different between

these diseases may be in the tissue responsiveness to ligand availability – excessive in cancer and deficient in ischemic diseases, as illustrated in Fig. 6. Therefore a more complex ‘systems biology’ interpretation of the plasma levels of VEGF and sVEGFR1 (the inputs) – one that considers downstream ligand-receptor interactions and signalling cascades (represented as black boxes in Fig. 6) – would be needed to predict the angiogenic status (the output) across these different disease states.

## Concluding remarks

Taken together, these necessary distinctions suggest that a systems biology model could provide important insights into the use of plasma VEGF and sVEGFR1 levels as surrogate markers of disease status for a variety of angiogenesis-dependent diseases. To generate such a mathematical model, further experimental data are needed to inform model extensions and model parameterizations: (1) standardization of methodological protocols and definition of healthy baseline ranges of circulating VEGF and sVEGFR1 under normal physiological fluctuations is needed to constrain their healthy ‘setpoints’ for model predictions; (2) quantification for the plasma concentrations and binding kinetics of all compet-

ing blood carriers, including sVEGFR1, is needed to accurately predict the bioavailability of circulating free VEGF; (3) real-time tracking of the biotransport processes (vascular permeability rates, lymphatic drainage rates, etc.) and systemic distributions (interstitial, intracellular and intravascular densities in various tissues) of VEGF and sVEGFR1 will help elucidate the relationship between protein measurements from varying body compartments; and (4) identification and model representation of all molecular components involved in the signal transduction networks of the VEGF system, including feedback loops (*e.g.* receptor expression levels as dependent on ligand activation and auto-phosphorylation), is crucial for discerning between pathogenic and compensatory responses in interpreting the correlation between concentrations of suspected protein markers and a particular disease status.

## Acknowledgements

We thank Dr. Amina A. Qutub, David Noren, Prakash Vempati and all members of the Popel Lab for their helpful discussions. We also thank Dr. Eli Keshet and Shay Sela for useful discussion of sFlt1-14. This work was supported by the NIH grants, R33 HL087351 and R01 HL079653. F.T.H.W. was supported by an NSERC PGS-M scholarship.

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