

# Key endothelial cell angiogenic mechanisms are stimulated by the circulating milieu in sickle cell disease and attenuated by hydroxyurea

Flavia C. M. Lopes,<sup>1</sup> Fabiola Traina,<sup>1,2</sup> Camila B. Almeida,<sup>1</sup> Flavia C. Leonardo,<sup>1</sup> Carla F. Franco-Penteado,<sup>1</sup> Vanessa T. Garrido,<sup>1</sup> Marina P. Colella,<sup>1</sup> Raquel Soares,<sup>3</sup> Sara T. Olalla-Saad,<sup>1</sup> Fernando F. Costa,<sup>1</sup> and Nicola Conran<sup>1</sup>

<sup>1</sup>INCT de Sangue, Hematology Center, School of Medical Science, University of Campinas – UNICAMP, São Paulo, Brazil;

<sup>2</sup>Department of Internal Medicine, University of São Paulo at Ribeirão Preto Medical School, Ribeirão Preto, Brazil; and <sup>3</sup>Department of Biochemistry (I3S), Faculty of Medicine, University of Porto, Portugal

## ABSTRACT

As hypoxia-induced inflammatory angiogenesis may contribute to the manifestations of sickle cell disease, we compared the angiogenic molecular profiles of plasma from sickle cell disease individuals and correlated these with *in vitro* endothelial cell-mediated angiogenesis-stimulating activity and *in vivo* neovascularization. Bioplex demonstrated that plasma from patients with steady-state sickle cell anemia contained elevated concentrations of pro-angiogenic factors (angiopoietin-1, basic fibroblast growth factor, vascular endothelial growth factor, vascular endothelial growth factor-D and placental growth factor) and displayed potent pro-angiogenic activity, significantly increasing endothelial cell proliferation, migration and capillary-like structure formation. *In vivo* neovascularization of Matrigel plugs was significantly greater in sickle cell disease mice than in non-sickle cell disease mice, consistent with an up-regulation of angiogenesis in the disease. In plasma from patients with hemoglobin SC disease without proliferative retinopathy, anti-angiogenic endostatin and thrombospondin-2 were significantly elevated. In contrast, plasma from hemoglobin SC individuals with proliferative retinopathy had a pro-angiogenic profile and more significant effects on endothelial cell proliferation and capillary formation than plasma from patients without retinopathy. Hydroxyurea therapy was associated with significant reductions in plasma angiogenic factors and inhibition of endothelial cell-mediated angiogenic mechanisms and neovascularization. Thus, individuals with sickle cell anemia or hemoglobin SC disease with retinopathy present a highly angiogenic circulating milieu, capable of stimulating key endothelial cell-mediated angiogenic mechanisms. Combination anti-angiogenic therapy to prevent the progression of unregulated neovascularization and associated manifestations in sickle cell disease, such as pulmonary hypertension, may be indicated; furthermore, the benefits and drawbacks of the potent anti-angiogenic effects of hydroxyurea should be clarified.

## Introduction

Sickle cell disease (SCD) is caused by a mutation in the hemoglobin  $\beta$  chain, resulting in the production of an abnormal hemoglobin (HbS) in the erythrocyte. Under conditions of low oxygenation, HbS polymerizes leading the erythrocyte to adopt a sickle-shaped morphology. SCD is characterized by complex pathophysiological mechanisms that involve intravascular hemolysis and recurrent vaso-occlusion, in association with chronic vascular inflammation and endothelial activation,<sup>1,2</sup> leading to numerous clinical complications that include painful vaso-occlusive episodes, auto-infarction of the spleen, acute chest syndrome, stroke, pulmonary hypertension, renal damage and a shortened lifespan.<sup>3</sup>

Angiogenesis is the formation of new capillaries from pre-existing vessels and is essential for processes of development, reproduction and repair.<sup>4</sup> Alterations in angiogenesis have been associated with a number of pathological conditions, particularly in inflammatory diseases, and angiogenesis, chronic inflammation and cellular responses to changes in oxygen tension appear to be co-dependent.<sup>5-7</sup> The angiogenic process involves interactions of several cell types and mechanisms to establish a precise microenvironment appropriate for

capillary formation,<sup>8</sup> where endothelial cell (EC) proliferation and invasion occur in response to angiogenic mediators, followed by cell anastomosis to form lumen-containing capillaries.<sup>9,10</sup>

Although homozygous sickle cell anemia (HbSS; SCA) is often associated with a more severe phenotype, patients with HbSC disease (in which red cells produce both HbS and HbC) can have a similar or higher incidence of retinopathy and osteonecrosis, among other manifestations.<sup>11</sup> The levels of a number of angiogenic mediators have been reported to be elevated in SCD, including vascular endothelial growth factor (VEGF), placental growth factor (PlGF), angiopoietin-1 (Ang1), angiopoietin-2 (Ang2) and erythropoietin (EPO).<sup>12-16</sup> In addition, despite the heterogeneity of the SCD phenotype, several clinical manifestations associated with SCD, including proliferative retinopathy, pulmonary hypertension, leg ulcers and moyamoya syndrome, suggest an involvement of pathological angiogenic processes.<sup>17-24</sup> Although these observations indicate an angiogenic imbalance in SCD, the existence of pro-angiogenic mechanisms in SCD, and their consequences, remain unclear.

Hydroxyurea (or hydroxycarbamide) is currently the only drug approved by the Food and Drug Administration for use

©2015 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2014.119727

The online version of this article has a Supplementary Appendix.

Manuscript received on October 27, 2014. Manuscript accepted March 5, 2015.

Correspondence: conran@unicamp.br

as a therapy in SCD.<sup>25</sup> Hydroxyurea is a cytostatic drug that inhibits ribonucleotide reductase, arresting cell division in the S-phase; additionally, data suggest that hydroxyurea can generate nitric oxide following administration.<sup>26</sup> We recently reported that hydroxyurea exerts anti-angiogenic effects in VEGF-dependent EC angiogenic *in vitro* assays and inhibits VEGF-dependent neovascularization in Matrigel implants in C57BL/6 mice.<sup>27</sup> This anti-angiogenic activity appears to be mediated by the down-regulation of endothelial hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) expression, and consequent modulation of miRNA 221 expression, leading to the inhibition of EC proliferation and invasion/migration.<sup>27</sup>

To lend further support to the hypothesis of an unbalanced angiogenic state in SCD, we investigated the *in vitro* effects of plasma from patients with SCD on key steps of EC angiogenic behavior, namely, proliferative capability, invasive capacity and the formation of capillary-like EC structures. The plasma contents of angiogenic factors were associated with these data, as was the incidence of proliferative retinopathy in HbSC patients, a clinical manifestation thought to reflect augmented angiogenesis.<sup>28</sup> The angiogenic activity of plasma from patients on hydroxyurea therapy was also examined. Finally, to confirm that SCD pathophysiology is associated with exacerbated angiogenesis, *in vivo* neovascularization was examined in a SCD mouse model, compared with non-SCD mice, utilizing a Matrigel plug angiogenesis assay.

## Methods

### Patients

Patients with HbSS or HbSC disease (collectively termed SCD) were recruited, during steady-state, at the Hematology Center, UNICAMP (Table 1). Details on the diagnosis of HbSS/HbSC, our definition of steady-state and criteria for commencing hydroxyurea therapy (15–30 mg hydroxyurea/kg/day) are given in the

*Online Supplementary Information*. All patients had undergone ophthalmoscopy and fluorescein angiography for detection of proliferative retinopathy. A total of 29 HbSS patients, who did not have proliferative retinopathy, were recruited into the study. A total of 33 HbSC patients were recruited, of whom 16 did not have proliferative retinopathy (SC group) and 17 did (SCR group). One HbSC patient had been prescribed hydroxyurea following an ischemic stroke. Homozygous hemoglobin A (HbAA) healthy controls were age- and gender-matched, where possible. Informed written consent was obtained from all participants and the study was approved by the UNICAMP Ethics Committee, in accordance with national guidelines.

### Bioplex assay

The Fluorokine<sup>®</sup> MAP, Human Angiogenesis Custom Premix Kit A (R&D Systems, Minneapolis, USA) was used to quantify Ang1, basic fibroblast growth factor (bFGF), PlGF, platelet-derived growth factor (PDGF)-AA, PDGF-BB, VEGF, VEGF-D, endostatin and thrombospondin-2 (TSP-2) in plasma, according to the manufacturer's instructions. The preparation of the plasma for the assay is described in the *Online Supplementary Information*.

### In vitro culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC), obtained from the Global Bioresource Center (ATCC) were cultured, as described elsewhere.<sup>27</sup> Before all assays, cells (fourth to sixth passage) were incubated in low-serum-containing media (2% fetal bovine serum). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were employed to verify HUVEC viability.<sup>27</sup>

### Bromodeoxyuridine proliferation assay

HUVEC ( $1 \times 10^4$  cells/mL) were cultured in the absence or presence of 10% (v/v) plasma for 24 h, before quantifying cell proliferation by enzyme-linked immunosorbent assay using the Cell Proliferation bromodeoxyuridine (BrdU) kit (Roche, Penzberg, Germany), according to the manufacturer's instructions.

**Table 1.** Demographic, clinical and hematologic details of the patients participating in the study.

	SS	SSHU	SC	SCR	SCRHU
Male/female (n.)	4/13	9/3	4/12	9/7	0/1
Age (years)	38 (38; 21, 51)	39 (39; 23, 54)	37 (37; 16, 52)	46 (47; 33, 59)	67
Red blood cell count ( $10^{12}/L$ )	2.74 (2.72; 1.74, 3.97)	2.50 (2.48; 1.89, 3.63)	4.42 (4.50; 2.74, 5.56)	4.42 (4.47; 3.50, 5.53)	2.43
Hematocrit (%)	24.5 (24.1; 16.9, 32.9)	26.5 (26.4; 20.8, 37.4)	33.5 (33.2; 8.1, 14.3)	34.8 (35.0; 28.8, 40.4)	26.8
Hemoglobin (g/dL)	8.1 (8.1; 5.9, 11.0)	8.9 (8.3; 6.8, 12.9)	11.5 (11.1; 8.1, 14.3)	11.9 (12.0; 10.0, 14.1)	9.6
Mean corpuscular volume (fL)	90.5 (90.5; 75.4, 103.6)	107.3 (103.5; 84.9, 137.6)	76.7 (77.1; 56.3, 87.9)	79.1 (78.9; 71.1, 87.3)	110.1
Reticulocytes ( $\times 10^9/L$ )	422 (355; 172, 803)	272 (272; 151, 412)	187.2 (185; 73, 316)	377.5 (218; 81, 378)	94.1
White blood cells ( $\times 10^9/L$ )	10.5 (10.6; 6.3, 13.8)	7.7 (7.2; 4.0, 12.6)	8.0 (7.7; 4.1, 15.3)	13.4 (8.2; 3.54, 13.4)	4.4
HbF (%)	8.9 (7.0; 2.1, 25.9)	14.8 (14.2; 3.8, 25.1)	2.1 (1.15; 0.3, 8.0)	0.95 (0.7; 0.2, 2.6)	4.7
Platelets ( $\times 10^9/L$ )	465 (422; 111, 1029)	365 (364; 156, 1052)	289 (268; 70, 549)	303 (250; 89, 696)	231

SS: steady-state SCA patients; HU: hydroxyurea therapy (20–30 mg/kg/day for at least 12 months); SC: steady-state HbSC patients without retinopathy; SCR: steady-state HbSC patients with proliferative retinopathy. HbF: fetal hemoglobin. Data (except male/female value) are mean (median; minimum, maximum) values.

**Endothelial cell invasion assay**

HUVEC migration (during 22 h) in the absence/presence of 10% (v/v) plasma was quantified using a double-chamber assay (BD Biocoat™ Matrigel™ Invasion Chamber, BD-Biosciences, Franklin Lakes, USA), as described elsewhere.<sup>27</sup> Results represent the ratio of invading cells in plasma-treated cultures compared to control cultures for the same initial amount of cells seeded.

**Capillary-formation assay**

HUVEC ( $5 \times 10^4$  cells/mL) were cultured on growth factor-reduced Matrigel (BD-Biosciences)-coated plates for 17 h, in medium containing 10% plasma or Ham F12-K medium alone (control), as previously described.<sup>29</sup> A semi-quantitative measurement of cord formation in the entire Matrigel culture was performed and expressed as a tube-formation index.<sup>29</sup>

**Animals**

Five-month old C57BL/6, chimeric SCD mice and chimeric non-SCD mice were employed in the study. For details regarding animals, their maintenance and diet, refer to the *Online Supplementary Information*. Animal procedures were carried out in accordance with the National Institutes of Health revised guide for the care and use of laboratory animals and current Brazilian laws. This

study was approved by the Commission for Ethics in Animal Experimentation, UNICAMP (Protocol 3024-1, 2013).

**In vivo Matrigel plug neovascularization assay**

Matrigel was prepared with heparin in the absence or presence of recombinant VEGF (R&D Systems; 100 ng/mL, as previously standardized<sup>30</sup>) and 100  $\mu$ M hydroxyurea before subcutaneous inoculation into mice. Animals were euthanized after 7 days, and the Matrigel plugs were removed, weighed, photographed and hemoglobin content quantified, as previously described.<sup>30</sup>

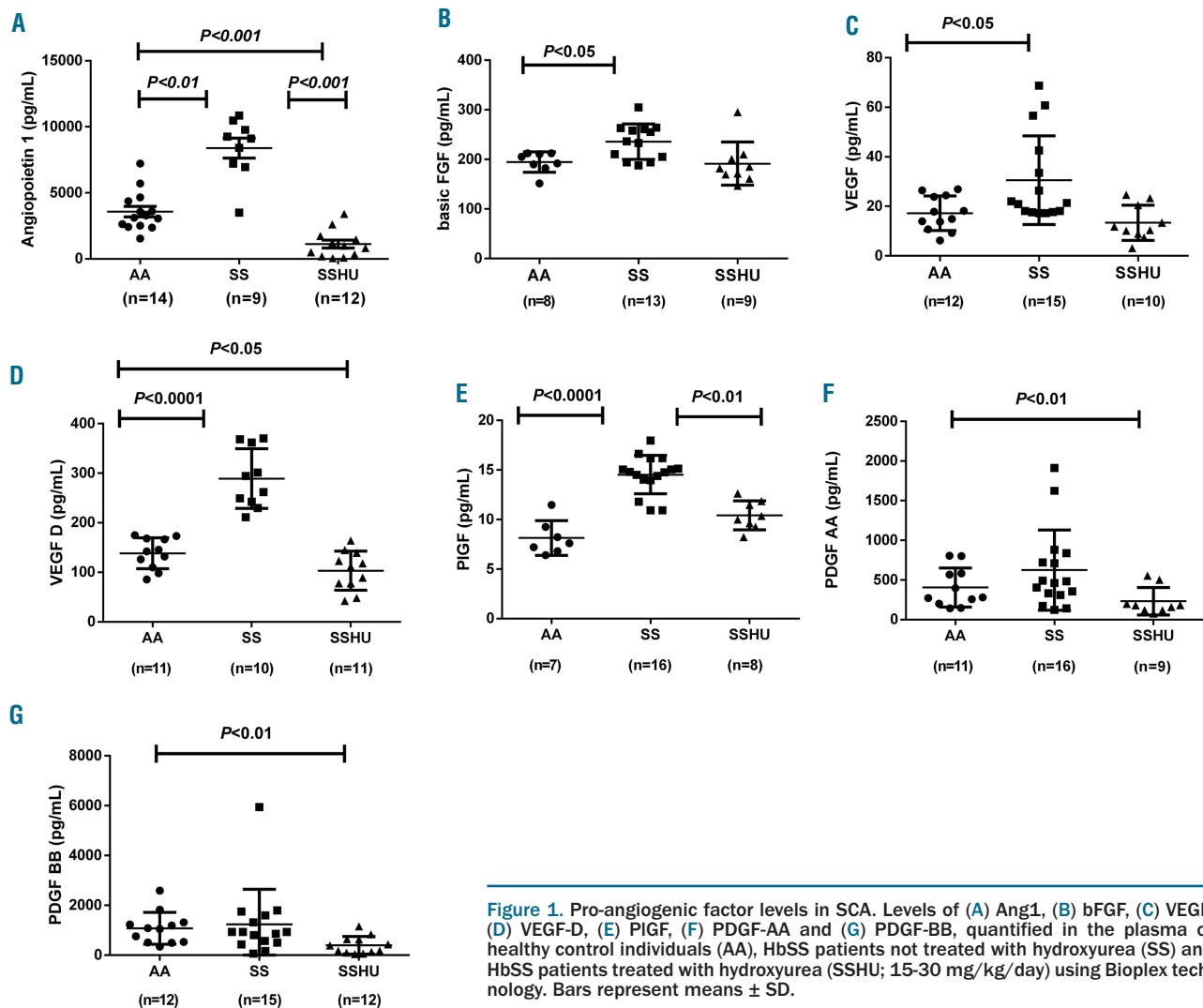
**Statistical analysis**

The statistical analyses are described in the *Online Supplementary Information*.

**Results**

**Circulating pro-angiogenic factor levels are significantly increased in steady-state sickle cell anemia**

Plasma was collected from control individuals (AA) and



**Figure 1.** Pro-angiogenic factor levels in SCA. Levels of (A) Ang1, (B) bFGF, (C) VEGF, (D) VEGF-D, (E) PlGF, (F) PDGF-AA and (G) PDGF-BB, quantified in the plasma of healthy control individuals (AA), HbSS patients not treated with hydroxyurea (SS) and HbSS patients treated with hydroxyurea (SSHU; 15-30 mg/kg/day) using Bioplex technology. Bars represent means  $\pm$  SD.

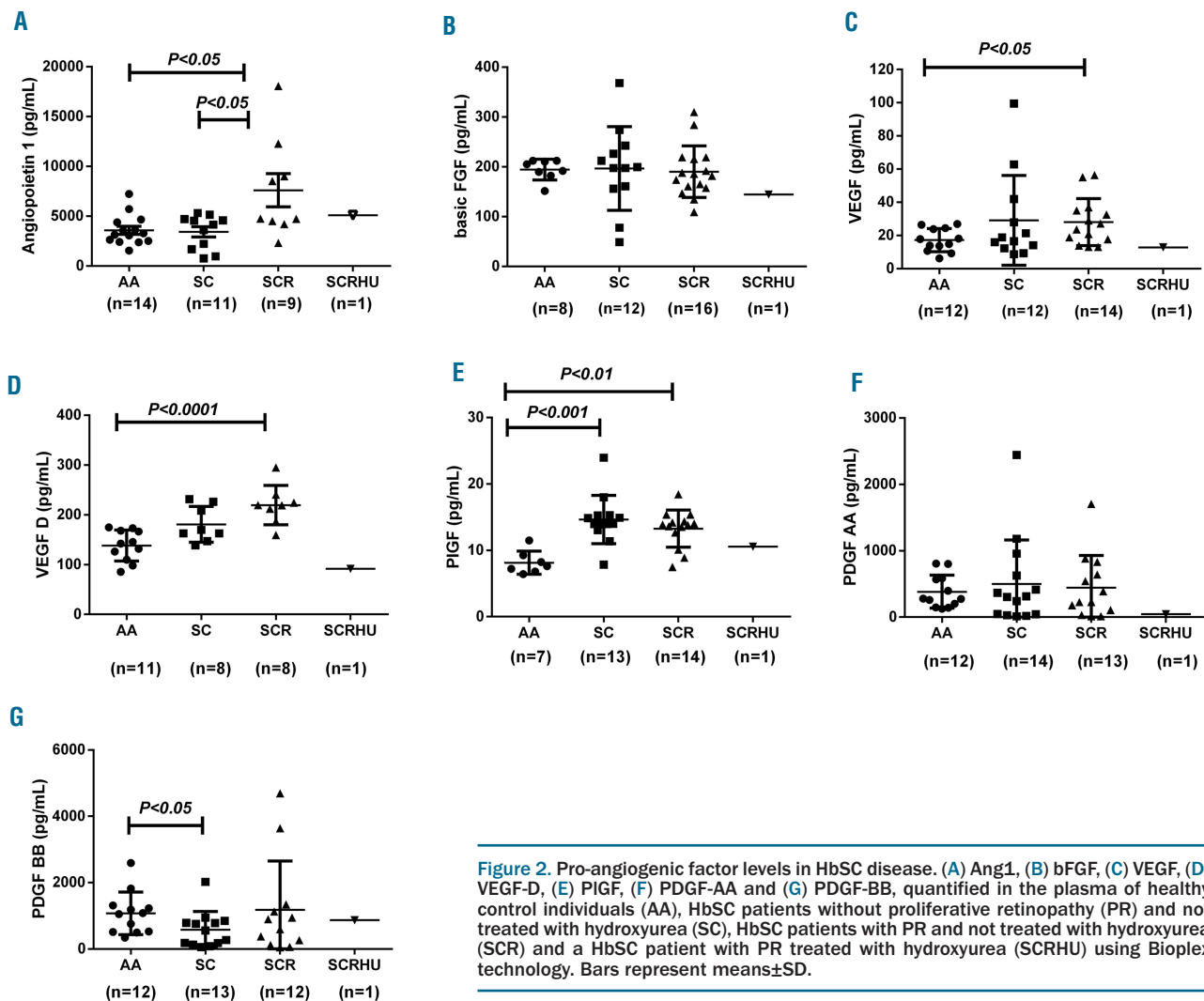
steady-state SCA individuals not on hydroxyurea therapy (SS). A Bioplex assay demonstrated that the levels of the pro-angiogenic factors Ang1, bFGF, VEGF, VEGF-D and PlGF were all significantly elevated ( $P < 0.05$ ), in the plasma of steady state SCA patients (not on hydroxyurea), compared to the levels in healthy control individuals (Figure 1). Levels of PDGF-AA and -BB were not significantly altered in SCA patients, compared to control individuals.

### Hydroxyurea therapy is associated with a reduction in circulating pro-angiogenic factors in sickle cell anemia

When levels of pro-angiogenic factors were compared in SCA patients on hydroxyurea (SSHU) with those not on hydroxyurea (SS) and control individuals (AA) (Figure 1), both bFGF and VEGF, which were significantly augmented in SS individuals, were unchanged compared to controls. Surprisingly, Ang1, VEGF-D, PDGF-AA and PDGF-BB were even lower in SSHU patients than in control (AA) individuals ( $P < 0.05$ ); PlGF was significantly increased in SSHU patients compared to AA individuals ( $P < 0.05$ ), but mean levels were lower than those of SS individuals ( $P < 0.01$ ).

### Circulating pro-angiogenic factor levels in steady-state HbSC disease

In a second group of analyses, pro-angiogenic factors were measured in the plasma of control (AA) individuals, steady-state HbSC individuals without retinopathy (SC), steady-state HbSC individuals with proliferative retinopathy (SCR) and in one SCR patient on hydroxyurea therapy (SCRHU) (Figure 2). PlGF, alone, was found to be elevated in SC plasma ( $P < 0.001$ ), compared to the level in control individuals. Interestingly, PDGF-BB was significantly decreased ( $P < 0.05$ ) in the SC group compared to AA controls. In those SC patients with retinopathy (SCR), a very different profile of pro-angiogenic factor expression was observed. Levels of Ang1, VEGF, VEGF-D and PlGF were all significantly elevated ( $P < 0.05$ ), compared to those in AA control subjects (Figure 2), while levels of PDGF-BB were similar to those of AA controls. No significant difference was observed in PDGF-AA levels, either relative to controls or to SC individuals. The SCRHU patient had increased Ang1 and PlGF levels and decreased bFGF, VEGF, VEGF-D, PDGF-AA and PDGF-BB levels, when compared to the control (AA) group.



**Figure 2.** Pro-angiogenic factor levels in HbSC disease. (A) Ang1, (B) bFGF, (C) VEGF, (D) VEGF-D, (E) PlGF, (F) PDGF-AA and (G) PDGF-BB, quantified in the plasma of healthy control individuals (AA), HbSC patients without proliferative retinopathy (PR) and not treated with hydroxyurea (SC), HbSC patients with PR and not treated with hydroxyurea (SCR) and a HbSC patient with PR treated with hydroxyurea (SCRHU) using Bioplex technology. Bars represent means  $\pm$  SD.

### Circulating anti-angiogenic factors are altered in HbSC disease and in those patients with sickle cell anemia on hydroxyurea therapy

Anti-angiogenic factors were assayed in the same groups of patients. Endostatin and TSP-2 levels were no different in SCA patients (SS) from those in healthy individuals (AA) (Figure 3A,B). Importantly, endostatin and TSP-2 levels were significantly higher ( $P<0.05$ ) in HbSC (SC) patients than in healthy controls (Figure 3C,D), but were not significantly different in those SC patients with proliferative retinopathy (SCR).

Hydroxyurea therapy was associated with significant increases in both endostatin and TSP-2 in SCA (Figure 3A,B;  $P<0.01$ ), while a relative elevation in both these factors was observed in the SCR patient on hydroxyurea (Figure 3C,D).

### Endothelial cell proliferation and invasion are enhanced in the presence of sickle cell disease plasma and inhibited by plasma from sickle cell disease patients treated with hydroxyurea

The effects of plasma from these individuals on key steps of the angiogenic process was studied, *in vitro*, using cultured HUVEC. Initially, possible cytotoxic effects of plasma (10-20% v/v) on HUVEC viability were assessed using an MTT assay (*data not shown*). Plasma had insignificant cytotoxicity for HUVEC, and a plasma concentration of 10% (v/v) was used for subsequent HUVEC assays.

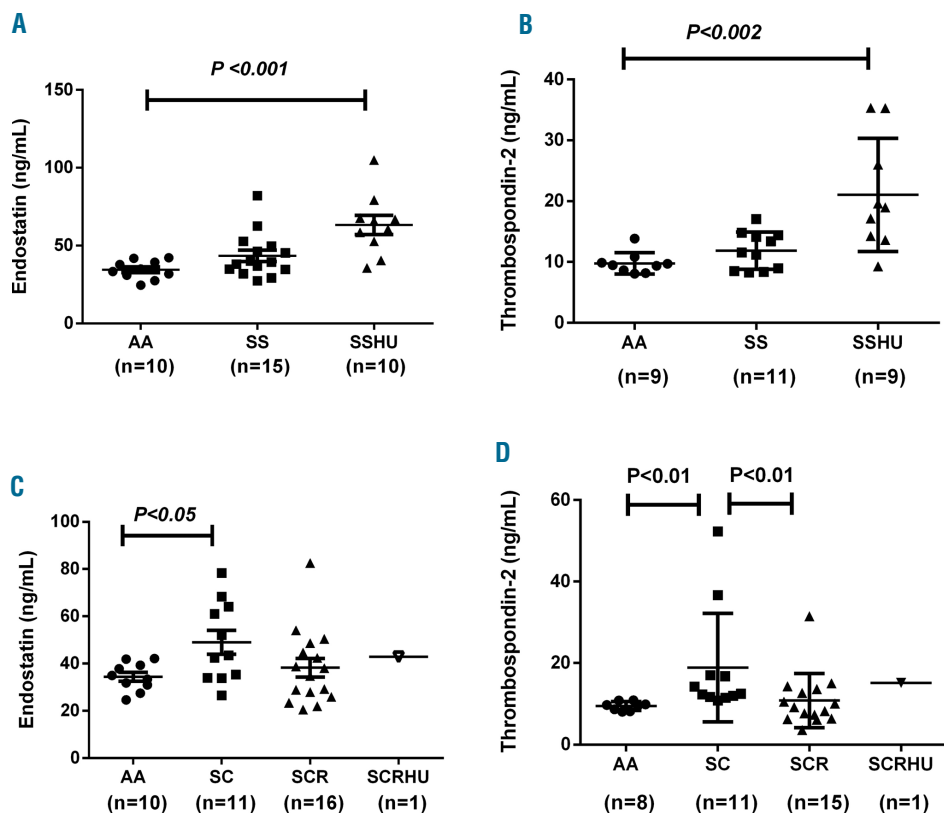
EC proliferation and migration/invasion are crucial for new vessel formation. Incubation of HUVEC with 10% plasma from SCA patients not treated with hydroxyurea (SS) for 24 h significantly increased cell proliferation

( $P<0.001$ ), as determined by the BrdU proliferation assay. In contrast, plasma from HbSS patients being treated with hydroxyurea (SSHU) reduced cell proliferation relative to that of controls ( $P<0.001$ ). Interestingly, 10% plasma from HbSC patients with or without proliferative retinopathy (SCR and SC, respectively) did not modify cell proliferation when compared to AA plasma, but plasma from an HbSC patient on hydroxyurea (SCRHU) reduced proliferation by about 13% (Figure 4A).

A double-chamber assay demonstrated a significantly increased cell invasion capacity for HUVEC when incubated with plasma from HbSS patients not treated with hydroxyurea (SS;  $P<0.01$ ) (Figure 4B). In contrast, plasma from SS patients on hydroxyurea (SSHU) significantly decreased cell invasion ( $P<0.01$ ). Plasma from HbSC patients without proliferative retinopathy (SC) increased cell invasion, although, plasma from HbSC patients with proliferative retinopathy (SCR) increased cell invasion significantly more ( $P<0.05$ ). Furthermore, plasma from an HbSC patient with proliferative retinopathy on hydroxyurea (SCRHU) decreased cell invasion by about 46% (Figure 4B).

### Plasma from sickle cell disease patients is highly pro-angiogenic, augmenting endothelial capillary-like structure formation, *in vitro*

We looked at the effects of plasma from healthy individuals and individuals with SCD on the ability of HUVEC to form capillary-like structures on Matrigel, as the proliferation and rearrangement of EC to assemble tube-like formations constitutes a major step in angiogenesis. In keeping with reports suggesting that human plasma, in the



**Figure 3.** Anti-angiogenic factor levels in SCA and HbSC disease. (A) Endostatin and (B) thrombospondin-2 were quantified in the plasma of healthy control individuals (AA), HbSS patients not treated with hydroxyurea (SS) and HbSS patients treated with hydroxyurea (SSHU). (C) Endostatin and (D) thrombospondin-2 were quantified in the plasma of HbSC patients without proliferative retinopathy (PR) and not treated with hydroxyurea (SC), HbSC patients with PR and not treated with hydroxyurea (SCR) and a HbSC patient with PR treated with hydroxyurea (SCRHU) using Bioplex technology. Bars represent means $\pm$ SD.

absence of diseases, is generally slightly anti-angiogenic, plasma from healthy individuals (AA; 10%) marginally decreased the formation of capillary-like structures by HUVEC ( $P>0.05$ ) compared to control cells (HUVEC in medium alone) after 17 h of incubation (Figure 5A). In addition, the morphology of the endothelial tubes was similar in the two groups (Figure 5B, C).

In contrast, capillary-like-structure formation increased significantly in the presence of plasma from SCA and HbSC patients, independently of the presence of proliferative retinopathy ( $P<0.05$ ), and SCR plasma induced significantly more capillary formation than did SC plasma (Figure 5A;  $P<0.05$ ). HUVEC cultured in the presence of SCD plasma formed branching and thick anastomosing capillaries on Matrigel (Figure 5D, F and G). Notably, HUVEC cultures incubated with plasma from patients treated with hydroxyurea (SSHU and SCRHU) showed considerably less angiogenic activity compared to control cultures, where tube formation indices decreased significantly in the presence of SSHU plasma ( $P<0.01$ ) (Figure 5A). Furthermore, capillaries formed in the presence of these samples of plasma displayed decreased branching and occasional single EC could be observed in cultures (Figure 5E and H).

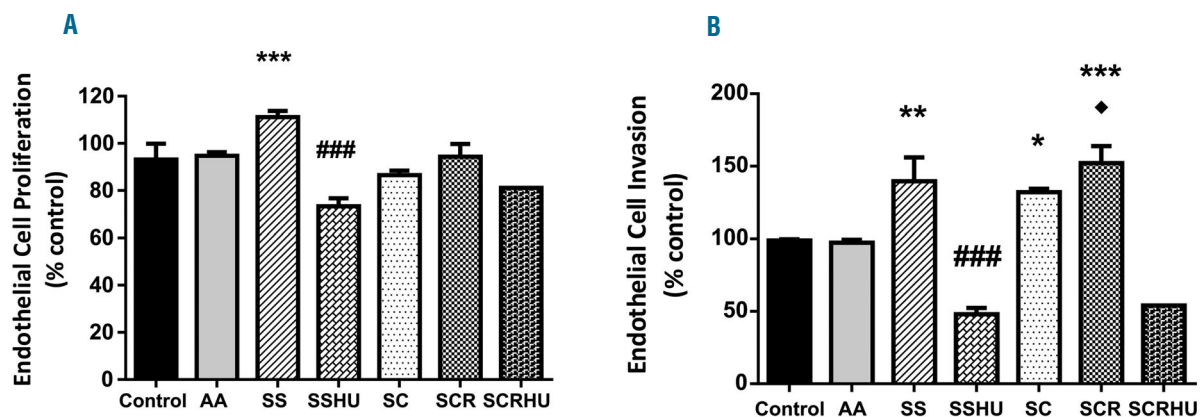
#### Pro-angiogenic factor concentration correlates with the angiogenic activity of sickle cell disease plasma

The angiogenic factor concentrations of each of the plasma samples from HbSS and HbSC individuals were correlated with the ability of each respective plasma sample to induce EC proliferation, invasion and capillary formation in HUVEC assays. Table 2 demonstrates that the pro-angiogenic factor content of each plasma sample correlated significantly with the capillary-formation and invasion-inducing activities of the HbSS plasma samples. Plasma Ang1, VEGF, VEGF-D and PDGF-AA concentrations corre-

lated positively and significantly with the ability of the respective plasma sample to induce cell invasion and capillary formation, while plasma bFGF and PlGF levels correlated only with the ability of the sample to induce capillary formation. For HbSC plasma, VEGF-D content correlated significantly with HUVEC capillary formation and migration, while Ang1, VEGF and VEGF-D concentrations correlated with EC capillary formation, proliferation and invasion when all HbSS/SC plasma samples were analyzed together. Levels of anti-angiogenic factors (endostatin and TSP-2) did not correlate significantly with the angiogenic activities of any of the SCD plasma samples (Table 2).

#### In vivo neovascularization is increased in sickle cell disease mice

To examine whether neovascularization, and therefore angiogenesis, is altered in SCD *in vivo*, wild-type C57BL/6 control mice and chimeric SCD mice were injected subcutaneously with Matrigel plugs to observe the formation of new blood vessels over a 7-day period. Matrigel plugs were supplemented, or not, with recombinant VEGF and/or 100  $\mu$ M hydroxyurea. At 7 days, the Matrigel plugs of C57BL/6 control mice that were not supplemented with VEGF had low hemoglobin contents and, therefore, low-level vascularization (Figure 6). In contrast, the basal neovascularization (VEGF-) observed in the Matrigel plugs of SCD mice was significantly higher than that of C57BL/6 control mice ( $P<0.05$ ; Figure 6A), in which levels of vascularization approached those of a positive control group in which the Matrigel was supplemented with VEGF (VEGF+). Increased vascularization can be visualized as the red color distributed in the whole plug (Figure 6B). Matrigel implants in SCD mice treated with both VEGF and 100  $\mu$ M hydroxyurea (VEGF+HU) demonstrated a strong inhibition of vascular development ( $P<0.05$ ). To

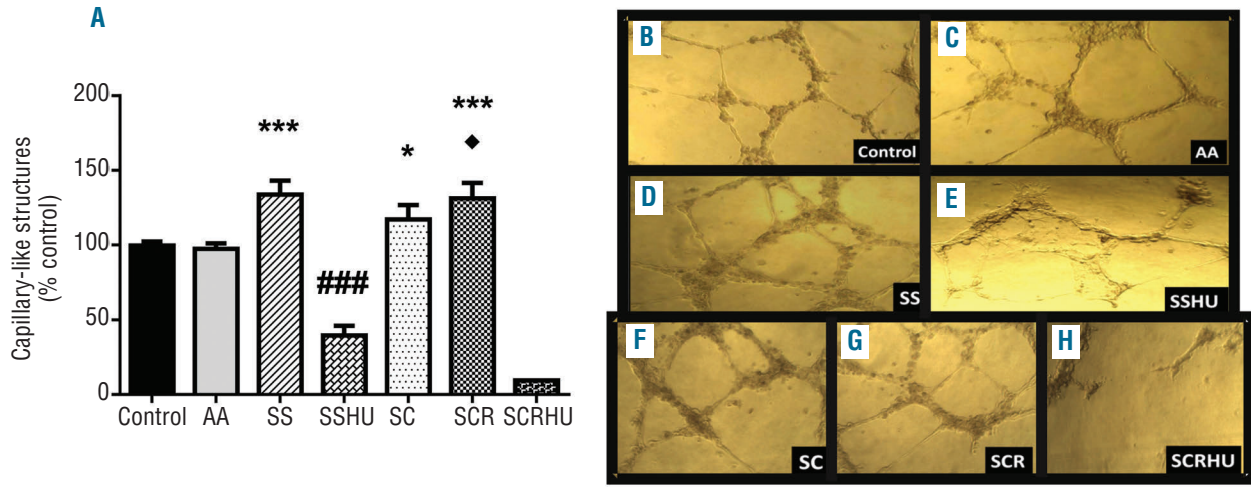


**Figure 4.** Effects of SCA and SCD plasma samples on endothelial cell proliferation and invasive capacity. HUVEC were incubated in the presence or absence of 10% plasma from healthy control individuals (AA), HbSS patients not treated with hydroxyurea (SS), HbSS patients treated with hydroxyurea (SSHU), HbSC patients without proliferative retinopathy (SC), HbSC patients with proliferative retinopathy (SCR) and an HbSC patient with proliferative retinopathy and treated with hydroxyurea (SCRHU) (17h, 37 °C). (A) Cell proliferation was evaluated by the BrdU assay. Bars represent the percentage of proliferating cells, in comparison with control cells (culture medium only). (B) Endothelial cell invasive capacity was evaluated using the double-chamber assay. Bars represent the percentage of invading cells relative to the initial amount of cells cultured. Results are expressed as percentage of control cells (cultured in the absence of plasma). Assays were performed in triplicate. Statistical differences between groups were evaluated by one-way analysis of variance (ANOVA test) (mean  $\pm$  SD; n=5/SCRHU=1; \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  vs. AA; ### $P<0.001$  vs. SS; \* $P<0.05$  vs. SC).

rule out any transplant-associated effect on neovascularization in chimeric SCD mice, we carried out similar Matrigel plug assays on chimeric non-SCD control mice (irradiated C57BL/6 mice that received C57BL/6 bone marrow) and found that both VEGF-stimulated and unstimulated vascularization of plugs was similar to that of C57BL/6 control mice (n=7;  $P>0.05$ , data not shown), indicating that the transplantation process, *per se*, has no significant effect upon neovascularization.

**Discussion**

While evidence supports the existence of an elevated production of pro-angiogenic factors in SCD,<sup>12-15,31,32</sup> few studies are available to establish a clear link between angiogenic factor production and pathological neovascularization in this disease. High levels of stromal-derived factor-1, augmented PlGF and discretely elevated PDGF-BB have been associated with pulmonary hypertension or



**Figure 5.** Effects of SCA and SCD plasma samples on endothelial capillary-like structure formation. (A) Semiquantification of tube-formation index in HUVEC cultures after incubation or not with 10% plasma from healthy control individuals (AA); HbSS patients not treated with hydroxyurea (SS); HbSS patients treated with hydroxyurea (SSHU); HbSC patients without proliferative retinopathy (SC); HbSC patients with proliferative retinopathy (SCR); and a HbSC patient with proliferative retinopathy and treated with hydroxyurea (SCRHU) (17h, 37 °C). Bars correspond to the tube formation index of HUVEC, compared to that of control cells (Control), incubated on Matrigel without plasma. Statistical differences between groups were evaluated by one-way analysis of variance (ANOVA test) (mean ± SD; n=5/SCRHU=1; \* $P<0.05$ , \*\*\* $P<0.001$  vs. AA; ### $P<0.001$  vs. SS; \* $P<0.05$  vs. SC). Photomicrographs (B-H) are representative images of capillary-like structure assembly of HUVEC cultures on Matrigel. Each culture was established in triplicate and visualized under a phase-contrast inverted microscope (50× magnification) (Zeiss Axiovert S100 - Moticam 2500).

**Table 2.** Correlations between levels of angiogenic factors and plasma angiogenic activity.

		Ang1	bFGF	VEGF	VEGF-D	PlGF	PDGF-AA	PDGF-BB	Endostatin	TSP-2
SS (N≥8)	Capillary formation	$r^s=-0.964$ <b><math>P=0.003</math></b>	$r^s=0.810$ <b><math>P=0.02</math></b>	$r^s=0.881$ <b><math>P=0.007</math></b>	$r^s=0.898$ <b><math>P=0.005</math></b>	$r^s=0.810$ <b><math>P=0.02</math></b>	$r^s=0.762$ <b><math>P=0.04</math></b>	$r^s=0.201$ $P=0.44$	$r^s=0.095$ $P=0.84$	$r^s=-0.107$ $P=0.84$
	Proliferation	$r^s=0.571$ $P=0.20$	$r^s=0.333$ $P=0.423$	$r^s=0.571$ $P=0.15$	$r^s=0.575$ $P=0.14$	$r^s=0.048$ $P=0.93$	$r^s=0.310$ $P=0.46$	$r^s=0.119$ $P=0.79$	$r^s=-0.357$ $P=0.39$	$r^s=-0.642$ $P=0.39$
	Invasion	$r^s=0.821$ $P=0.03$	$r^s=0.452$ $P=0.27$	$r^s=0.905$ <b><math>P=0.004</math></b>	$r^s=0.707$ <b><math>P=0.06</math></b>	$r^s=0.548$ $P=0.17$	$r^s=0.667$ <b><math>P=0.08</math></b>	$r^s=0.548$ $P=0.17$	$r^s=-0.405$ $P=0.33$	$r^s=-0.107$ $P=0.84$
SC (N≥9)	Capillary formation	$r^s=0.383$ $P=0.31$	$r^s=0.109$ $P=0.75$	$r^s=0.250$ $P=0.52$	$r^s=0.767$ $P=0.02$	$r^s=0.006$ $P=0.99$	$r^s=-0.018$ $P=0.97$	$r^s=0.017$ $P=0.98$	$r^s=-0.346$ $P=0.34$	$r^s=-0.350$ $P=0.36$
	Proliferation	$r^s=0.167$ $P=0.68$	$r^s=0.278$ $P=0.41$	$r^s=0.333$ $P=0.39$	$r^s=0.887$ $P=0.003$	$r^s=0.334$ $P=0.34$	$r^s=0.030$ $P=0.94$	$r^s=0.150$ $P=0.71$	$r^s=-0.191$ $P=0.56$	$r^s=-0.400$ $P=0.29$
	Invasion	$r^s=0.217$ $P=0.58$	$r^s=0.400$ $P=0.23$	$r^s=0.417$ $P=0.27$	$r^s=0.467$ $P=0.21$	$r^s=0.527$ $P=0.12$	$r^s=0.539$ $P=0.11$	$r^s=-0.067$ $P=0.88$	$r^s=0.063$ $P=0.86$	$r^s=-0.133$ $P=0.74$
SS/SC (N≥17)	Capillary formation	$r^s=0.700$ <b><math>P=0.003</math></b>	$r^s=0.469$ <b><math>P=0.04</math></b>	$r^s=0.657$ <b><math>P=0.005</math></b>	$r^s=0.889$ <b><math>P&lt;0.0001</math></b>	$r^s=0.490$ <b><math>P=0.04</math></b>	$r^s=0.348$ $P=0.16$	$r^s=0.201$ $P=0.44$	$r^s=-0.233$ $P=0.34$	$r^s=-0.212$ $P=0.43$
	Proliferation	$r^s=0.818$ <b><math>P=0.0002</math></b>	$r^s=0.421$ $P=0.07$	$r^s=0.576$ $P=0.02$	$r^s=0.881$ $P<0.0001$	$r^s=0.461$ $P=0.05$	$r^s=0.286$ $P=0.25$	$r^s=0.248$ $P=0.34$	$r^s=-0.252$ $P=0.30$	$r^s=-0.391$ $P=0.14$
	Invasion	$r^s=0.594$ $P=0.02$	$r^s=0.379$ $P=0.11$	$r^s=0.657$ <b><math>P=0.005</math></b>	$r^s=0.640$ <b><math>P=0.007</math></b>	$r^s=0.474$ <b><math>P=0.04</math></b>	$r^s=0.434$ $P=0.11$	$r^s=0.083$ $P=0.75$	$r^s=-0.283$ $P=0.24$	$r^s=-0.006$ $P=0.99$

SS: HbSS group (patients on and off hydroxyurea); SC: HbSC group (patients on and off hydroxyurea); Ang1: angiopoietin-1; bFGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor; PlGF: placental growth factor; PDGF: platelet-derived growth factor; TSP-2: thrombospondin-2. Spearman correlations were calculated between plasma angiogenic factors and the angiogenic activity of the plasma sample in endothelial angiogenesis assays. Statistically significant results are shown in bold.

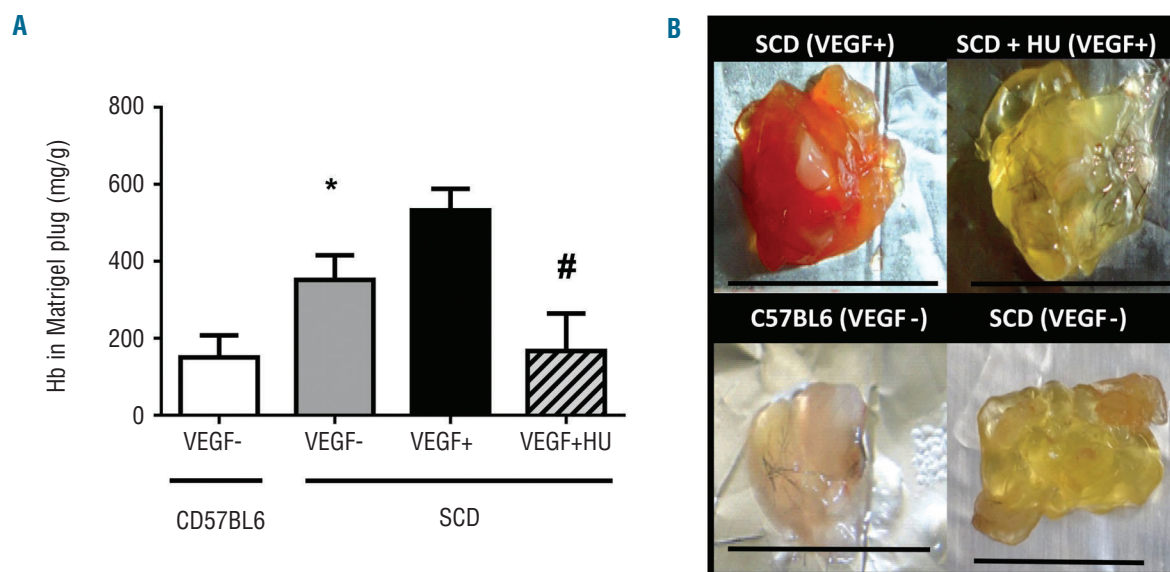
high tricuspid regurgitation velocity in SCD,<sup>13,16,19</sup> while increased pigment epithelium-derived factor has been related to SCD retinopathy.<sup>15</sup> Here we present data demonstrating that the profile of circulating angiogenic factors is significantly altered in SCD individuals, with corresponding effects on plasma angiogenic activity.

Plasma from a cohort of SCA (HbSS) patients contained higher concentrations of a number of pro-angiogenic factors (Ang1, bFGF, VEGF, VEGF-D and PlGF), compared with concentrations in control plasma. Ang1 induces vascular remodeling and enlargement via highly organized angiogenesis and tightening of EC junctions.<sup>33</sup> bFGF and VEGF play synergistic roles in angiogenesis,<sup>34</sup> in which bFGF (released from the extracellular matrix during wound healing) stimulates multiple cell types,<sup>35</sup> while VEGF is EC-selective and has potent vasopermeability activity, playing a primary role in angiogenesis by inducing EC proliferation, sprouting and tube formation.<sup>36,37</sup> VEGF-D is essential for hypoxia-driven vascular development,<sup>37</sup> and PlGF enhances VEGF-stimulated angiogenesis under pathological conditions.<sup>38</sup> Anti-angiogenic molecules, such as the matrix-derived peptide, endostatin, and the matrix-binding protein, TSP-2, are important for tissue repair and regeneration, since they prune superfluous vessels forming a stable, well-perfused vascular network;<sup>39</sup> however, neither endostatin nor TSP-2 concentrations were altered in SCA patients not on hydroxyurea. Vaso-occlusive events in SCD result in the occurrence of tissue ischemia, generating hypoxic conditions, EC activation, inflammatory events and the up-regulation of endothelial HIF-1,<sup>40</sup> known to induce the transcription of genes that drive angiogenesis, including VEGF.<sup>41</sup> Thus, it would seem likely that such events participate in the observed up-regulation of pro-angiogenic factors.

In addition to the increased concentrations of pro-angio-

genic factors observed in the circulation of SCA (HbSS) individuals, these plasma samples had potent pro-angiogenic activity, significantly accelerating the formation of capillary-like structures from EC on Matrigel and increasing the proliferation and invasion of these cells *in vitro*. The concentrations of a number of pro-angiogenic factors correlated significantly with the ability of the respective plasma samples to induce EC capillary-formation and invasive activity. As such, the elevated production of circulating pro-angiogenic factors in SCA appears to have physiologically relevant effects on major steps of EC-dependent angiogenesis. While multiple angiogenic factors may be involved in these effects, Ang1, VEGF and VEGF-D, whose concentrations correlated highly with EC invasion and vessel assembly, seem to contribute significantly to this activity, in keeping with the hypoxic nature of SCA. Moreover, compared to control mice, SCD mice demonstrated a significantly higher vascularization of Matrigel plugs over a 7-day period, confirming that this highly pro-angiogenic environment may stimulate neovascularization *in vivo*.

Although HbSC disease is associated with a clinically milder phenotype relative to SCA, the incidence of peripheral retinopathy is much higher in the former; hence HbSC patients from our cohort were divided into those without peripheral retinopathy (SC) and those with peripheral retinopathy (SCR).<sup>11</sup> We were careful to exclude any patients with peripheral retinopathy from the SCA (HbSS) group, in an attempt to identify specific differences in angiogenic profile between the HbSS and HbSC groups. Interestingly, HbSC individuals had a very different plasma angiogenic profile to that of HbSS individuals. Only PlGF levels were raised in HbSC plasma, together with a slight increment in VEGF-D. In contrast, plasma levels of the anti-angiogenic molecules, endostatin and TSP-2, were



**Figure 6.** *In vivo* angiogenesis is augmented in SCD chimeric mice and inhibited by hydroxyurea. A Matrigel plug neovascularization assay was employed to compare neovascularization in SCD chimeric (SS) and C57BL/6 mice and observe the effects of hydroxyurea on *in vivo* vessel formation. The following were injected subcutaneously into C57BL/6 and SS mice; a mixture of Matrigel and heparin without vascular endothelial growth factor (VEGF-, negative control); Matrigel, heparin and VEGF (VEGF+, positive control) or Matrigel, heparin, VEGF and 100  $\mu$ M hydroxyurea (VEGF+HU). Matrigel plugs were left in place for 7 days. (A) Quantification of hemoglobin (Hb) in the homogenized Matrigel plugs using Drabkin solution, results are expressed as Hb content in the Matrigel plug (mg/g) (mean $\pm$ SEM; C57BL/6, n=5; SS, n=4-6). (B) Representative photomicrographs of Matrigel plugs. Scale bars, 1 cm. \*  $P < 0.05$  vs. C57BL6; #  $P < 0.05$  vs. VEGF+HU.



significantly increased in HbSC disease in the absence of retinopathy. In accordance with the lower concentrations of pro-angiogenic factors in the circulation of HbSC patients, plasma from these individuals did not significantly alter EC proliferation and activated EC capillary formation and invasion to a lesser degree than retinopathic HbSC plasma. Plasma from HbSC patients with peripheral retinopathy was significantly more pro-angiogenic, demonstrating elevated concentrations of Ang1, VEGF, VEGF-D and PlGF, with no alterations in anti-angiogenic molecule content. Remarkably, this pro-angiogenic plasma induced similar alterations in EC invasion and capillary-formation abilities to those observed for HbSS plasma. Correlations between HbSC plasma factor content and angiogenic activity were found only between plasma VEGF-D content and EC capillary formation and proliferation in HbSC, implicating VEGF-D as a primordial factor in HbSC neovascularization.

Due to the large variability in the phenotype of SCA, and the size of the group of patients, plasma angiogenic activity was not correlated with any one clinical manifestation of the disease, but it may be speculated that such potent pro-angiogenic activity (potentially leading to uncontrolled neovascularization) may contribute to some clinical manifestations of the disease, including pulmonary hypertension, moyamoya vasculopathy and even other events such as nephropathy.<sup>42</sup> Importantly, while a clear difference between the angiogenic activities of retinopathic HbSC plasma and non-retinopathic HbSC plasma was observed, the angiogenic profile of retinopathic HbSC plasma was very similar to that of plasma from the SCA (HbSS) group of patients, none of whom demonstrated retinopathy. As such, it remains unclear as to why the highly pro-angiogenic environment of SCA (HbSS) rarely results in retinopathy, while pro-angiogenic factors appear to influence the incidence of peripheral retinopathy in HbSC. It seems probable that additional factors may participate in the development of peripheral retinopathy in HbSC; for instance, hemoglobin concentrations, and therefore blood viscosity, are higher in HbSC (see Table 1) and it may be that this viscosity, coupled with the production of angiogenic factors, incurs damage to the fragile retina. It has also been suggested that, in SCA, peripheral vessels are occluded much earlier on and more extensively, preventing further retinal damage and the formation of proliferative lesions;<sup>11</sup> however, more studies are required to provide support for these hypotheses.

We recently reported that hydroxyurea has anti-angiogenic effects both *in vitro* and *in vivo*, inhibiting EC HIF-1 $\alpha$  gene expression, capillary-structure formation, proliferation and invasion *in vitro*, as well as preventing *in vivo* neovascularization in C57BL/6 mice.<sup>27</sup> Importantly, hydroxyurea therapy also appears to affect the circulating angiogenic molecule profile in SCD. In SCA, hydroxyurea therapy was associated with lowering of the raised levels of Ang1, bFGF and VEGF and decreases in VEGF-D, PDGF-AA and PDGF-BB. Accordingly, in a retinopathic HbSC patient on hydroxyurea, pro-angiogenic factor concentra-

tions were not elevated when compared with concentrations in control plasma. Furthermore, hydroxyurea therapy was associated with significant increases in the productions of anti-angiogenic endostatin and TSP-2 in HbSS. While longitudinal studies are required to confirm whether hydroxyurea therapy has a direct effect on endothelial HIF-1 expression, known to be up-regulated in SCD mice<sup>40</sup>, the potent anti-angiogenic effects of hydroxyurea on EC,<sup>27</sup> together with the reduction in the incidence of hypoxic events in patients on hydroxyurea, may result in alterations in the production and secretion of these angiogenic factors. Concomitant inhibition of EC-angiogenic function was observed *in vitro* in the presence of plasma from SCD patients on hydroxyurea. Whether these alterations in EC-angiogenic mechanisms were inhibited by alterations in plasma angiogenic factor content or due to the presence of the hydroxyurea compound or its metabolites in the plasma (or a combination of these alterations) is not clear. The anti-angiogenic effects of hydroxyurea, including suppression of endothelial HIF-1 $\alpha$  gene expression,<sup>27</sup> and elevation of endostatin (which prevents retinal neovascularization and interferes in leg ulcer healing<sup>43,44</sup>) may have important consequences in SCD. While hydroxyurea therapy could be beneficial for clinical manifestations in which unregulated neovascularization participates, mechanisms for which angiogenesis may be important, such as wound healing and vascularization of infarcted tissues, may be hindered by this molecule. Although simultaneous improvements in vaso-occlusive mechanisms may outweigh the effects of hydroxyurea on angiogenic processes, there is some evidence to suggest that hydroxyurea therapy may complicate leg ulcer healing in SCD and myeloproliferative diseases<sup>45,46</sup> and further studies to explore the anti-angiogenic effects of hydroxyurea may be warranted.

In summary, the circulating vascular milieu encountered in SCA and retinopathic HbSC individuals is highly pro-angiogenic and capable of stimulating EC-mediated angiogenic mechanisms, in association with alterations in SCD neovascularization. While this pro-angiogenic state appears to participate in retinopathy in HbSC disease, the exact role that this unbalanced angiogenesis and unregulated neovascularization plays in the clinical manifestations of SCD needs to be clarified. Furthermore, the benefits and drawbacks of the apparent anti-angiogenic effects of hydroxyurea therapy in SCD require attention, as does the potential for the use of hydroxyurea as a therapeutic approach for HbSC retinopathy.

#### Acknowledgments

This study was financed by FAPESP, Brazil (grants 2008/57441-0 and 2009/16334-0), and CNPq (grant 565036/2010). We thank Prof. Dra. Cristina P. Vicente for assistance with microscopy.

#### Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

#### References

- Conran N, Costa FF. Hemoglobin disorders and endothelial cell interactions. *Clin Biochem.* 2009;42(18):1824-1838.
- Hebbel RP, Vercellotti G, Nath KA. A systems biology consideration of the vasculopathy of sickle cell anemia: the need for multi-modality chemo-prophylaxis. *Cardiovasc Hematol Disord Drug Targets.* 2009;9(4):271-292.
- Steinberg MH, Ohene-Frempong K, Heeney MM. Clinical and pathophysiological aspects of sickle cell anemia. In: Steinberg

- MH, Forget BG, Higgs DR, Weatherall DJ, ed. *Disorders of Hemoglobin*. 2nd ed. Cambridge: Cambridge University Press, 2009.
4. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med*. 1995;1(1):27-31.
  5. Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. The codependence of angiogenesis and chronic inflammation. *FASEB J*. 1997;11(6):457-465.
  6. Costa C, Incio J, Soares R. Angiogenesis and chronic inflammation: cause or consequence? *Angiogenesis*. 2007;10(3):149-166.
  7. Konisti S, Kiriakidis S, Paleolog EM. Hypoxia--a key regulator of angiogenesis and inflammation in rheumatoid arthritis. *Nat Rev Rheumatol*. 2012;8(3):153-162.
  8. Naldini A, Carraro F. Role of inflammatory mediators in angiogenesis. *Curr Drug Targets Inflamm Allergy*. 2005;4(1):3-8.
  9. Chidlow JH Jr, Shukla D, Grisham MB, Kevil CG. Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. *Am J Physiol Gastrointest Liver Physiol*. 2007;293(1):G5-G18.
  10. Niland S, Eble JA. Integrin-mediated cell-matrix interaction in physiological and pathological blood vessel formation. *J Oncol*. 2012;2012:125278.
  11. Nagel RL, Fabry ME, Steinberg MH. The paradox of hemoglobin SC disease. *Blood Rev*. 2003;17(3):167-178.
  12. Duits AJ, Rodriguez T, Schnog JJ, Group CS. Serum levels of angiogenic factors indicate a pro-angiogenic state in adults with sickle cell disease. *Br J Haematol*. 2006;134(1):116-119.
  13. Landburg PP, Nur E, Maria N, et al. Elevated circulating stromal-derived factor-1 levels in sickle cell disease. *Acta Haematol*. 2009;122(1):64-69.
  14. Brittain JE, Hulkower B, Jones SK, et al. Placenta growth factor in sickle cell disease: association with hemolysis and inflammation. *Blood*. 2010;115(10):2014-2020.
  15. Cruz PR, Lira RP, Pereira Filho SA, et al. Increased circulating PEDF and low sICAM-1 are associated with sickle cell retinopathy. *Blood Cells Mol Dis*. 2015;54(1):33-37.
  16. Niu X, Nouraie M, Campbell A, et al. Angiogenic and inflammatory markers of cardiopulmonary changes in children and adolescents with sickle cell disease. *PLoS one*. 2009;4(11):e7956.
  17. Dobson SR, Holden KR, Nietert PJ, et al. Moyamoya syndrome in childhood sickle cell disease: a predictive factor for recurrent cerebrovascular events. *Blood*. 2002;99(9):3144-3150.
  18. Kim SY, Mocanu C, McLeod DS, et al. Expression of pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in sickle cell retina and choroid. *Exp Eye Res*. 2003;77(4):433-445.
  19. Sundaram N, Tailor A, Mendelsohn L, et al. High levels of placenta growth factor in sickle cell disease promote pulmonary hypertension. *Blood*. 2010;116(1):109-112.
  20. Lee MT, Rosenzweig EB, Cairo MS. Pulmonary hypertension in sickle cell disease. *Clin Ad Hematol Oncol*. 2007;5(8):645-653.
  21. Shaikh S. Intravitreal bevacizumab (Avastin) for the treatment of proliferative sickle retinopathy. *Indian J Ophthalmol*. 2008;56(3):259.
  22. Minniti CP, Eckman J, Sebastiani P, Steinberg MH, Ballas SK. Leg ulcers in sickle cell disease. *Am J Hematol*. 2010;85(10):831-833.
  23. Minniti CP, Taylor J, Hildesheim M, et al. Laboratory and echocardiography markers in sickle cell patients with leg ulcers. *Am J Hematol*. 2011;86(8):705-708.
  24. Sapiha R, Hamel D, Shao Z, et al. Proliferative retinopathies: angiogenesis that blinds. *Int J Biochem Cell Biol*. 2010;42(1):5-12.
  25. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N Engl J Med*. 1995;332(20):1317-1322.
  26. Elfrod HL. Effect of hydroxyurea on ribonucleotide reductase. *Biochem Biophys Res Comm*. 1968;33(1):129-135.
  27. Lopes FC, Ferreira R, Albuquerque DM, et al. In vitro and in vivo anti-angiogenic effects of hydroxyurea. *Microvasc Res*. 2014;94:106-113.
  28. Kassim AA, DeBaun MR. Sickle cell disease, vasculopathy, and therapeutics. *Ann Rev Med*. 2013;64:451-466.
  29. Soares R, Balogh G, Guo S, Gartner F, Russo J, Schmitt F. Evidence for the notch signaling pathway on the role of estrogen in angiogenesis. *Mol Endocrin*. 2004;18(9):2333-2343.
  30. Carneiro A, Falcao M, Azevedo I, Falcao Reis F, Soares R. Multiple effects of bevacizumab in angiogenesis: implications for its use in age-related macular degeneration. *Acta Ophthalmol*. 2009;87(5):517-523.
  31. Patel N, Sundaram N, Yang M, Madigan C, Kalra VK, Malik P. Placenta growth factor (PlGF), a novel inducer of plasminogen activator inhibitor-1 (PAI-1) in sickle cell disease (SCD). *J Biol Chem*. 2010;285(22):16713-16722.
  32. Mohan JS, Lip PL, Blann AD, Bareford D, Lip GY. The angiopoietin/Tie-2 system in proliferative sickle retinopathy: relation to vascular endothelial growth factor, its soluble receptor Flt-1 and von Willebrand factor, and to the effects of laser treatment. *Br J Ophthalmol*. 2005;89(7):815-819.
  33. Koh GY. Orchestral actions of angiopoietin-1 in vascular regeneration. *Trends Mol Med*. 2013;19(1):31-39.
  34. Przybylski M. A review of the current research on the role of bFGF and VEGF in angiogenesis. *J Wound Care*. 2009;18(12):516-519.
  35. Hata Y, Rook SL, Aiello LP. Basic fibroblast growth factor induces expression of VEGF receptor KDR through a protein kinase C and p44/p42 mitogen-activated protein kinase-dependent pathway. *Diabetes*. 1999;48(5):1145-1155.
  36. Shibuya M. VEGF-VEGFR signals in health and disease. *Biomol Ther*. 2014;22(1):1-9.
  37. Otrrock ZK, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: review. *Blood Cells Mol Dis*. 2007;38(3):258-268.
  38. Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem*. 1994;269(41):25646-25654.
  39. Wietecha MS, Cerny WL, DiPietro LA. Mechanisms of vessel regression: toward an understanding of the resolution of angiogenesis. *Curr Top Microbiol Immunol*. 2013;367:3-32.
  40. Kaul DK, Fabry ME, Suzuka SM, Zhang X. Antisickling fetal hemoglobin reduces hypoxia-inducible factor-1 $\alpha$  expression in normoxic sickle mice: microvascular implications. *Am J Physiol Heart Circ Physiol*. 2013;304(1):H42-50.
  41. Vadlapatla RK, Vadlapudi AD, Mitra AK. Hypoxia-inducible factor-1 (HIF-1): a potential target for intervention in ocular neovascular diseases. *Curr Drug Targets*. 2013;14(8):919-935.
  42. Nakagawa T, Kosugi T, Haneda M, Rivard CJ, Long DA. Abnormal angiogenesis in diabetic nephropathy. *Diabetes*. 2009;58(7):1471-1478.
  43. Mori K, Ando A, Gehlbach P, et al. Inhibition of choroidal neovascularization by intravenous injection of adenoviral vectors expressing secreted endostatin. *Am J Pathol*. 2001;159(1):313-320.
  44. Smith E, Hoffman R. Multiple fragments related to angiostatin and endostatin in fluid from venous leg ulcers. *Wound Repair Regen*. 2005;13(2):148-157.
  45. Delaney KM, Axelrod KC, Buscetta A, et al. Leg ulcers in sickle cell disease: current patterns and practices. *Hemoglobin*. 2013;37(4):325-332.
  46. Kersgard C, Osswald MB. Hydroxyurea and sickle cell leg ulcers. *Am J Hematol*. 2001;68(3):215-216.