Angiogenesis-Related Genes in Endothelial Progenitor Cells May Be Involved in Sickle Cell Stroke

Mirta T. Ito, MS*; Sueli M. da Silva Costa, PhD*; Letícia C. Baptista, PhD; Gabriela Q. Carvalho-Siqueira, PhD; Dulcinéia M. Albuquerque, PhD; Vinicius M. Rios, BS; Stephanie Osypina-Prieto, PhD; Roberta C. Saez, MS; Karla P. Vieira, PhD; Fernando Cendes, MD, PhD; Margareth C. Ozelo, MD, PhD; Sara Teresinha O. Saad, MD, PhD; Fernando F. Costa, MD, PhD; Mónica B. Melo, PhD

Background—The clinical aspects of sickle cell anemia (SCA) are heterogeneous, and different patients may present significantly different clinical evolutions. Almost all organs can be affected, particularly the central nervous system. Transient ischemic events, infarcts, and cerebral hemorrhage can be observed and affect ≈25% of the patients with SCA. Differences in the expression of molecules produced by endothelial cells may be associated with the clinical heterogeneity of patients affected by vascular diseases. In this study, we investigated the differential expression of genes involved in endothelial cell biology in SCA patients with and without stroke.

Methods and Results—Endothelial progenitor cells from 4 SCA patients with stroke and 6 SCA patients without stroke were evaluated through the polymerase chain reaction array technique. The analysis of gene expression profiling identified 29 differentially expressed genes. Eleven of these genes were upregulated, and most were associated with angiogenesis (55%), inflammatory response (18%), and coagulation (18%) pathways. Downregulated expression was observed in 18 genes, with the majority associated with angiogenesis (28%), apoptosis (28%), and cell adhesion (22%) pathways. Remarkable overexpression of the MMP1 (matrix metalloproteinase 1) gene in the endothelial progenitor cells of all SCA patients with stroke (fold change: 204.64; \( P=0.0004 \)) was observed.

Conclusions—Our results strongly suggest that angiogenesis is an important process in sickle cell stroke, and differences in the gene expression profile of endothelial cell biology, especially MMP1, may be related to stroke in SCA patients. (J Am Heart Assoc. 2020;9:e014143. DOI: 10.1161/JAHA.119.014143.)

Key Words: gene expression • MMP1 • sickle cell anemia • stroke

Sickle cell anemia (SCA) is a monogenic disorder caused by the homozygous form of hemoglobin S (HbSS), which derives from a point mutation at the seventh codon of the β-globin gene \((HBB [hemoglobin subunit β])\), resulting in the substitution of glutamic acid for valine (p.Glu7Val) in the β-globin protein. SCA is a severe disease characterized by the polymerization of HbSS under hypoxic conditions, leading to the deformity and fragility of red blood cell membranes.1–3

Stroke is a catastrophic complication of SCA caused by stenosis and occlusion of large vessels.4 The abnormal interaction between sickle red blood cells and the vascular endothelium is a critical factor in its pathogenesis.5,6 Sickled red blood cells tend to adhere to the endothelium, resulting in endothelial cell activation in a hypercoagulable state, thrombus formation, and thus vascular occlusion. Three stroke syndromes may result from multifocal small vessel disease: clinical ischemic stroke (IS), hemorrhagic stroke, and clinically silent stroke (also referred as silent cerebral infarcts). IS is the classical stroke syndrome of SCA and is responsible for 20% of mortality in these patients.7,8 Risk factors include prior transient ischemic attack, frequent acute chest syndrome, and increased systolic blood pressure.9 IS affects ≈10% of pediatric patients with SCA, with the greatest prevalence during the first decade of life.10 The risk of stroke is considered to be 300-fold higher in children...
Gene Expression in SCA and Relation to Stroke  Ito et al

DOI: 10.1161/JAHA.119.014143

Methods
The data that support the findings of this study are available from the corresponding author on reasonable request.

A total of 10 HbSS patients (SCA) were recruited from the Hematology and Hemotherapy Center of the University of Campinas (São Paulo, Brazil). Four patients (aged >18 years) had history of clinical primary stroke events in combination with SCA than in other children without sickle cell disease (SCD). Notably, at 45 years of age, 1 in 4 adults with the disease may be affected. The standard method used to assess stroke risk in children with SCA is transcranial Doppler ultrasound. Children with elevated transcranial Doppler ultrasound velocity (defined as ≥200 cm/s) have a higher risk of developing primary stroke than those with normal transcranial Doppler ultrasound velocities (<170 cm/s). For these patients, intervention with blood transfusions reduces the risk of stroke by >90%.

Many studies have investigated genetic risk factors for stroke through gene association analysis and genome-wide association studies. This research has generated a list of putative polymorphisms in genes involved in different pathways, such as inflammatory and cellular adhesion, that may influence the risk of stroke. These methodologies, however, are dependent on large sample amounts. This difficulty, associated with differences in sample characteristics and experimental design, may explain much of the low reproducibility of results between different studies.

On the basis of animal studies, several authors have hypothesized that circulating endothelial progenitor cells (EPCs) might have prognostic value for acute IS. Recruitment of EPCs toward infarcted brain areas and molecular mechanisms of postischemic angiogenesis have been described. Endothelial colony-forming cells (ECFCs), adult EPCs that circulate in peripheral blood, have a high proliferative capacity and stable phenotype during in vitro culture and have been used as a model for the study of SCA endothelial function, as biomaterial in gene therapy, and as a pathophysiologic study tool for vascular disease.

In this study, our approach was based on the use of ECFCs from SCA patients with and without stroke followed by cDNA microarray analysis of genes possibly related with endothelial cell dysfunction. This approach, applied in patients with precisely defined phenotypes, may allow the identification of differences in endothelial gene expression associated with clinical heterogeneity among patients affected by vascular diseases.

| Parameters                   | With Stroke | Without Stroke |
|------------------------------|-------------|----------------|
| No. of patients              | 4           | 6              |
| Male/female                  | 1/3         | 1/5            |
| Age at blood collection, y   | 35 (26–44)  | 39.5 (29–55)   |
| Age at time of stroke, y     | 28.5 (11–41)| ...            |
| RBC count (10⁶/μL)           | 3.0 (2.8–3.6)| 2.8 (2.3–3.7) |
| Hemoglobin, g/dL             | 9.4 (8.5–11.4)| 8.3 (7.4–9.6)|
| Leukocytes, ×10⁹/L           | 9.6 (5.1–12.6)| 8.2 (6.1–11.1)|
| Platelets, ×10⁹/L            | 396 (304–473)| 352 (285–433)|
| Reticulocytes (absolute number)| 9.0 (6.2–14.5)| 11.9 (7.3–18.6)|
| Fetal hemoglobin (%)         | 3.3 (1.3–8.0)| 6.0 (2.6–14.8)|
| Hemoglobin S (%)             | 34.0 (14.4–56.8)| 74 (11.2–91.2)|
| Microalbuminuria, mg/g       | 39.4 (2.36–84.7)| 64 (4.0–277.2)|
| Acute chest syndrome         | 0           | 0              |
| Algal crisis                 | 0           | 01 (16.6%)     |
| Aseptic necrosis             | 0           | 1 (16.6%)      |
| Cholecystectomy              | 4 (100%)    | 3 (50%)        |
| Chronic kidney diseases      | 1 (25%)     | 1 (16.6%)      |
| Leg ulcer                    | 03 (75%)    | 0              |
| Hepatopathies                | 0           | 2 (33.3%)      |
| Systolic hypertension        | 01 (25%)    | 0              |
| Osteoporosis                 | 2 (50%)     | 02 (33.3%)     |
| Priapism                     | 0           | 0              |
| Pulmonary hypertension       | 01 (25%)    | 03 (50%)       |
| Retinopathy                  | 2 (50%)     | 3 (50%)        |
| Smoking                      | 1 (25%)     | 1 (16.6%)      |
| Alpha thalassemia            | 01 (25%)    | 02 (33.3%)     |
| Moyamoya disease             | 0           | 0              |
| RBC transfusion              | 04 (100%)   | 01 (16.6%)     |

RBC indicates red blood cells; SCA, sickle cell anemia.

Clinical Perspective

What Is New?

- Genes related to endothelial cell biology were associated with stroke in patients with sickle cell anemia.
- The MMP1 (matrix metalloproteinase 1) gene, which belongs to a family of proteins involved in inflammation, tissue reconstruction and repair, cell migration, and angiogenesis, presented remarkable overexpression in the endothelial colony-forming cells of patients with sickle cell anemia and stroke.

What Are the Clinical Implications?

- Overexpression of the MMP1 gene may serve as a marker of stroke or the overall process of vasculopathy in patients with sickle cell anemia.
with magnetic resonance imaging (MRI) evidence confirming IS. The other 6 patients (aged >20 years) had no previous history of clinical stroke or any evidence of silent infarcts, confirmed by MRI (control group). All patients underwent MRI, performed by a neurologist, at the Neuroimaging Laboratory of the Department of Neurology at the University of Campinas. In the control group, just after MRI, peripheral blood samples were collected and ECFC cultures were carried out immediately. Demographics and clinical data of SCA patients with and without stroke are shown in Table 1. Moreover, hematologic parameters, SCA-related complications, treatment, and MRI findings for each patient are shown in Tables 2 and 3.

Patients with MRI evidence of hemorrhagic stroke and patients with other hemoglobinopathies such as HbC, HbD, and β-thalassemia or hereditary persistence of fetal hemoglobin were not enrolled in this study. The only treatment for the patients in the stroke group was blood transfusion, aiming to keep the percentage of HbS <30%. Among the control group (patients without stroke), 1 patient was under regular blood transfusion because of low hemoglobin level and high frequency of vaso-occlusive crises (>3 crises per year). We emphasize that no patients were under hydroxychloroquine therapy. Written informed consent was obtained from all participants, and the study was approved by the university ethics committee, in accordance with national guidelines.

**MRI Acquisition Parameters**

MRIs were acquired in a 3-T Philips Intera Achieva scanner with the following sequences: (1) high-resolution volumetric T1-weighted image (180 slices, 1-mm thickness; angle flip, 8°; repetition time [TR], 7.1 ms; echo time [TE], 3.2 ms; matrix, 240×240; field of view, 240×240 mm); (2) 3-dimensional T2-weighted image (1.5-mm³ isotropic voxels; TR, 1800 ms; TE, 340 ms; field of view, 230×230×180 mm³); (3) 3-dimensional fluid-attenuated inversion recovery (voxel size, 1.2×1.2×0.6 mm³; field of view, 250×250×190 mm³; TE, shortest; TR, 4800 ms; inversion time [TI], 1650 ms); (4) 3-dimensional susceptibility-weighted imaging (SWI; 2-mm thickness; TR/TE, shortest); (5) diffusion-weighted images (thickness, 4 mm; TE, 70 ms; TR, 3500 ms); and (6) magnetic resonance angiography (TE, 3.5 ms; TR, 22 ms).

**MRI Evaluation for Signs of Stroke and Cerebral Small Vessel Disease**

All images were reviewed by a neurologist with experience in imaging and stroke (F.C.) in addition to routine radiological evaluation. All imaging sequences described earlier were used to evaluate signs of acute or remote stroke lesions and other major structural lesions. In addition, we evaluated signs of cerebral small vessel disease, also known as cerebral microangiopathy or microvascular disease, which results from damage to the cerebral microcirculation and mainly affects the blood supply and tissue of the deep white- and gray-matter areas of the brain. These signs include white-matter hyperintensities in the periventricular and deep white and gray matter, enlarged perivascular spaces, lacunes, microbleeds, and cerebral atrophy that were not related to a specific macroscopic focal injury such as trauma or infarction.

**Table 2. Hematologic Parameters of Patients With and Without Stroke**

| Patient | Sex | Age, y | Age (y) at Time of Stroke | Smoking | RBC (10⁶/μL) | Hemoglobin (g/dL) | WBC (×10⁹/L) | Reticulocytes (Absolute No.) | Platelets (×10⁹/L) | Fetal Hemoglobin (%) | Hemoglobin S (%) | α-Thalassemia |
|---------|-----|--------|--------------------------|---------|-------------|-----------------|-------------|---------------------------|----------------|----------------|----------------|-------------|
| With stroke |
| 1 | F | 28 | 11 | No | 3.6 | 11.4 | 9.4 | 14.5 | 436 | 2.8 | 43.3 | Normal |
| 2 | M | 44 | 39 | Yes | 2.7 | 7.6 | 7.5 | 18.7 | 296 | 2.6 | 91.2 | Normal |
| 3 | F | 42 | 41 | No | 2.7 | 7.4 | 8.6 | 11.2 | 420 | 3.6 | 88.0 | Heterozygous |
| 4 | F | 26 | 23 | No | 2.0 | 9.2 | 6.1 | 16.9 | 383 | 3.1 | 90.3 | Heterozygous |
| Without stroke |
| 5 | M | 30 | 30 | Yes | 3.0 | 9.6 | 11.1 | 9.5 | 285 | 9.5 | 84.5 | Normal |
| 6 | F | 52 | 39 | No | 2.5 | 8.1 | 7.8 | 17.3 | 299 | 2.8 | 11.2 | Normal |
| 7 | F | 56 | 41 | No | 2.3 | 8.4 | 7.5 | 12.4 | 433 | 14.8 | 79.2 | Normal |
| 8 | F | 29 | 29 | No | 2.3 | 9.2 | 6.1 | 16.9 | 383 | 3.1 | 90.3 | Heterozygous |

F indicates female; m, male; RBC, red blood cells; WBC, white blood cells.

*Age at sample collection.

DOI: 10.1161/JAHA.119.014143
ECFC Culture

Cultures of ECFCs were established from peripheral blood samples according to previously described methods. Peripheral blood (45 mL) was collected into sodium heparin (9 mL) from 10 HbSS patients. Briefly, the anticoagulated blood was diluted 1:2 with PBS, which was underlaid with an equivalent volume of Ficoll-Paque PLUS (GE Healthcare) before centrifugation (317g, 30 minutes, room temperature). Only mononuclear cells were isolated and resuspended in EBM-2 medium containing the EGM-2 BulletKit (Lonza), 10% additional fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Gibco, Life Technologies). The cells (7 x 10^6 cells/well) were then seeded onto 12-well tissue culture plates precoated with type 1 rat-tail collagen (Sigma-Aldrich) and cultured in a humidified incubator...
Gene Expression in SCA and Relation to Stroke

Ito et al

obtained and analyzed with the software RT 2 Pro from samples of each gene and the internal controls were combined with the RT2 qPCR Master Mix, according to the manufacturer’s instructions. The quantification and purity of the RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Only samples pure enough (A260/A230 ratio >1.8; A260/A280 ratio 1.8–2.0) and with concentration >100 ng/μL were used as templates for cDNA synthesis.

Synthesis of cDNA and Polymerase Chain Reaction Array for Human Endothelial Cell Biology

First-strand cDNA was synthesized from 1 μg of total RNA using the RT² First-Strand Kit (Qiagen). Subsequently, cDNA was combined with the RT² qPCR Master Mix, according to the manufacturer’s instructions, and added to the pathway-specific RT² Profiler PCR Array (PAHS-015Z; Qiagen). This array includes 84 genes associated with endothelial cell biology and 12 controls (5 housekeeping genes, 1 assay for detecting genomic DNA contamination, 3 reverse transcription controls, and 3 positive polymerase chain reaction [PCR] controls). The levels of gene expression were quantified at the ABI StepOnePlus Real-Time PCR (Applied Biosystems). The PCR conditions were 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 40 cycles of 60°C for 1 minute. The threshold cycle values from samples of each gene and the internal controls were obtained and analyzed with the software RT² Profiler PCR Array Data Analysis v3.5 (Qiagen; http://pcrdatanalysis.sabiosciences.com/pcr/arrayanalysis.php). This web portal calculates the P values using a Student t test, on the replicate 2^{ΔCt} values for each gene in the stroke group compared with the nonstroke group. The levels of gene expression were normalized in relation to the housekeeping genes Actin Beta (ACTB), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0). For each sample, the assays were performed in duplicate and the mean expression value of each gene was used for the analysis. Genes were considered to be differentially expressed if they showed a fold-change of at least 2-fold up or down in comparison to the gene-expression levels of the control group (SCA patients without stroke) or P<0.05.

Validation of PCR Array by Quantitative Reverse Transcription PCR

The results from the PCR array were validated by quantitative reverse transcription PCR (qRT-PCR) performed for selected genes that showed differential expression between SCA patients with versus without stroke. To select the genes, 2 variables were considered: fold change >2 in gene expression or P<0.05 (considered significant). Ten genes were selected, 5 upregulated (Fms Related Receptor Tyrosine Kinase 1 [FLT1]), Placental Growth Factor [PGF], Matrix Metalloproteinase 1 [MMP1], Protein C Receptor [PROCR] and Interleukin 6 [IL6]) and 5 downregulated (Intercellular Adhesion Molecule 1 [ICAM1], Selectin P Ligand [SELP/L], C-C Motif Chemokine Ligand 2 [CCL2], Kinase Insert Domain Receptor [KDR] and TNF Superfamily Member 10 [TNFSF10]) in patients with stroke. The same RNA samples from patients assessed through PCR array were used in validation experiments.

The RNA samples were treated with DNase I (Life Technologies), and cDNAs were synthesized from 1 μg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer’s protocol. The summary of the primer sequences, the size of the fragments obtained, and the primer concentrations are shown in Table 4. The qPCR reaction was performed in triplicate using ABI StepOnePlus Real Time PCR (Applied Biosystems). For PCR amplification, 6 μL of SYBR Green PCR Master Mix (Applied Biosystems), 3 μL (10 ng) of cDNA, and 3 μL of the specific primer were used. The cycling conditions were the same as those used in the PCR array. Amplification specificity was verified using a dissociation curve, and RNA expression was calculated relative to the expression of ACTB and GAPDH genes through the GeNorm program.

Confirmation of Differential Expression by Western Blot

To confirm the increased expression of the MMP1 gene detected by the PCR array and qRT-PCR, we performed Western blot analysis. Pelleted cells were resuspended in RIPA buffer (50 mmol Tris-HCl, pH 7.4, 150 mmol NaCl, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol sodium orthovanadate, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A) incubated for 45 minutes at 4°C and centrifuged for 20 minutes at 15 000g at 4°C. Protein concentrations were quantified by the Bio-Rad Protein Assay Kit (Sigma-Aldrich). Equal protein amounts were...
Gene Expression in SCA and Relation to Stroke

Ito et al.

Overnight with anti-MMP1 and fat-free dry milk (5%). Membranes were incubated in (10 mmol/L, pH7.4), NaCl (150 mmol/L), Tween 20 (0.1%).

DOI: 10.1161/JAHA.119.014143

System (Thermo Fisher).

Sequencing of the MMP1 Gene Promoter Region

To identify variants that could affect expression levels of the MMP1 gene in SCA patients, we analyzed the promoter region of the gene by direct Sanger sequencing. Total DNA was extracted from peripheral blood using the QIAamp DNA Blood Midi Kit, according to the manufacturer’s instructions. The quantification and purity of the DNA were measured using a NanoDrop 2000 spectrophotometer (260/280 absorbance).

Primers and PCR Amplification of the Promoter Region of the MMP1 Gene

The sequences covering the promoter region of the MMP1 gene were obtained from the database of the National Center for Biotechnology Information and the Ensembl Genome Browser. For PCR amplification, 3 pairs of primers were used to cover the promoter region (Table 5). PCR was prepared with 40 to 100 ng of DNA, 25 pmol of forward and reverse primers, 75 pmol of dNTP, 25 pmol of MgCl2, 250 pmol of enzyme buffer 1×, 0.1 U of Taq DNA polymerase (Invitrogen, Life Technologies), and ultrapure water to complete up to 25 μL. PCR was carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems–Applied Corp).

The amplification conditions were initial denaturation at 95°C for 5 minutes, 35 cycles at 95°C for 30 seconds, annealing for 30 seconds and extension at 72°C for 45 seconds, and finally extension at 72°C for 5 minutes. PCR products were analyzed by electrophoresis and compared with a 100-bp DNA ladder (Invitrogen) on an ethidium bromide–stained 1.5% agarose gel.

Sequencing Reaction

Sequencing reactions were performed with the same PCR primers. The reagents used for the sequencing reaction were the purified PCR product (40 ng); “Big Dye” 2× (ABI PRISM Big Dye Terminator v 3.1 Cycle Sequencing Kit; Applied Biosystems); 1× “Save Money” buffer (supplied by the same Big Dye

Table 4. Sequencing Primers Used in the qRT-PCR Assays

| Gene   | Primer Sequences                          | Fragment Size (bp) | Primer (concentration in nmol/L) |
|--------|-------------------------------------------|--------------------|----------------------------------|
| CCL2   | F: 5’-GCTCAAGCCGATGCACTAAAT-3’ R: 5’-CAGTTGCTGCTGATTTCC-3’ | 110                | 300                              |
| FLT1   | F: 5’-GAAAACGATACTCCTGCAAG-3’ R: 5’-CTATGGATTTGCTGATGGT-3’ | 150                | 300                              |
| ICAM1  | F: 5’-GAAATCTGAAATGAGGGCCAC-3’ R: 5’-ACAACATGCTGATTGGGCCAC-3’ | 81                 | 150                              |
| IL6    | F: 5’-CAGAGGAGCCCGTATGAGA-3’ R: 5’-CCAGGGAGAAAGCAAGCTG-3’ | 64                 | 70                               |
| KDR    | F: 5’-TGAAGAGGAGGCAACCACCAC-3’ R: 5’-AGAAATCCACACATCACATG-3’ | 147                | 150                              |
| MMP1   | F: 5’-CAAGGACATATGAACCTTCG-3’ R: 5’-GCAATGAGAATGTCGTCTTC-3’ | 143                | 300                              |
| PEG    | F: 5’-GAATTGAGGAACTGTTGACG-3’ R: 5’-GATTCTTATGACGCTGGCTG-3’ | 126                | 70                               |
| PROCR  | F: 5’-GAAAACGAGAAAGGAGGCA-3’ R: 5’-CTAGACGACACACAGAAGATG-3’ | 94                 | 70                               |
| SELPLG | F: 5’-GCTGGTGGCATGCTGCTTG-3’ R: 5’-GGCTTTGCGGTGCTTCCTG-3’ | 102                | 150                              |
| TNFSF10| F: 5’-TGAAGAGGAGGCAAGCAGGACT-3’ R: 5’-CTCTCTTTCAACGCGTGAGCC-3’ | 147                | 300                              |
| ACTB   | F: 5’-TGAAGCAGACATCGTCTGTGGAC-3’ R: 5’-CAGAGGCTGACAGGACTGAGCA-3’ | 81                 | 150                              |
| GAPDH  | F: 5’-AGATGACAAGCTGCTCTCTCGTC-3’ R: 5’-GGGTATGAGTTCTTCCCCACGAT-3’ | 96                 | 150                              |

bp indicates base pair; F, forward; qRT-PCR, quantitative reverse transcription polymerase chain reaction; R, reverse. CCL2 (C-C Motif Chemokine Ligand 2); FLT1 (Fms-Related Receptor Tyrosine Kinase 1); ICAM1 (Intercellular Adhesion Molecule 1); IL6 (Interleukin 6); KDR (Kinase Insert Domain Receptor); MMP1 (Matrix Metalloproteinase 1); PEG (Placental Growth Factor); PROCR (Protein C Receptor); SELPLG (Selectin P Ligand); TNFSF10 (TNF Superfamily Member 10); ACTB (Actin Beta); GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase).

loaded onto 10% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose membrane. Nonspecific sites were blocked by incubation with a buffer containing Tris (10 mmol/L, pH7.4), NaCl (150 mmol/L), Tween 20 (0.1%), and fat-free dry milk (5%). Membranes were incubated overnight with anti-MMP1–specific primary antibody (Abcam) at 4°C, followed by horseradish peroxidase–conjugated secondary antibody (KPL conjugate peroxidase, goat antimouse IgG; Santa Cruz Biotechnology) at room temperature for 1 hour. GAPDH antibody (Santa Cruz Biotechnology) was used as a loading control. Immunoreactivities were visualized by the ECL Chemiluminescent Western Blotting Analysis System (Thermo Fisher).

Statistical analysis and graph construction for qRT-PCR and Western blot were performed with the GraphPad Prism 4 program (GraphPad Software). The Fisher-Plattman permutation test was used for the qRT-PCR analyses; P<0.05 was considered statistically significant after Benjamini-Hochberg adjustment for multiple tests.

Table 5. Primers Used for MMP1 Gene Promoter Sequencing

| Fragment | Primer Sequences                          | Fragment Size (bp) |
|----------|-------------------------------------------|--------------------|
| 1        | F: 5’-AGTCAGGCTCGCTCCTTCACG-3’ R: 5’-GAGAACACACTCTCGTGTTGAAACA-3’ | 856                |
| 2        | F: 5’-TACATGGATTTGCTGATGGT-3’ R: 5’-TTCATGCCTGACTCACTGAGA-3’ | 711                |
| 3        | F: 5’-CTCAGCCCACCTCCTCTCTC-3’ R: 5’-TGTCATGACGTTCGTCTTC-3’ | 611                |

bp indicates base pair; F, forward; R, reverse.
Genes expression in SCA and relation to stroke

Results

In this study, we used the human endothelial cell biology RT² Profiler PCR Array to examine the differential expression of 84 genes in ECFCs from SCA patients with and without stroke. Twenty-nine differentially expressed genes were identified. Among these, 11 were upregulated and 18 were downregulated (Table 6) in patients with stroke compared with patients without stroke.

Validation Studies

Based on the PCR array results, comparing patients with and without stroke, 5 upregulated genes (FLT1, PGF, MMP1, PROCR and IL6) and 5 downregulated genes (ICAM1, SELPLG, CCL2, KDR and TNFSF10) were chosen for validation by qRT-PCR (considering fold change >2 or P<0.05). ACTB and GAPDH were used as housekeeping genes because the expression of these genes did not differ among patients. Individual RNA samples from each studied patient were assessed.

The qRT-PCR analysis demonstrated that all SCA patients with stroke (n=4) had threshold cycle values ranging between 18.0 and 19.3 cycles. Among the 6 SCA patients without stroke, 4 had threshold cycle values ranging between 27.0 and 30.6 cycles and 2 had threshold cycle values close to those of the group with stroke, at 19.8 and 21.7 cycles. Results obtained for the PCR array and for qRT-PCR are shown in Table 7. Graphs comparing both groups were generated using the program GraphPad Prism 4 (Figure 1).

Table 6. Genes That Showed Altered Expression Comparing SCA Patients With and Without Stroke by PCR Array

| Gene       | Fold Change | P Value | Pathway Involved                        |
|------------|-------------|---------|-----------------------------------------|
| PF4        | 5.02        | 0.0680  | Angiogenesis                            |
| KIT        | 3.72        | 0.8006  | Angiogenesis                            |
| FLT1       | 3.49        | 0.0218* | Angiogenesis                            |
| PGF        | 2.93        | 0.0150* | Angiogenesis                            |
| PLA1       | 2.52        | 0.8081  | Angiogenesis                            |
| THBD       | 2.08        | 0.1683  | Platelet activation                     |
| MMP1       | 204.64      | 0.0004† | Angiogenesis/coagulation                |
| PLAT       | 3.85        | 0.3670  | Coagulation                             |
| PROCR      | 1.64        | 0.0479* | Coagulation                             |
| IL6        | 3.05        | 0.4405  | Inflammatory response                   |
| IL1B       | 2.20        | 0.2231  | Inflammatory response                   |
| SELE       | –6.79       | 0.2093  | Cell adhesion                           |
| ICAM1      | –2.83       | 0.0531  | Cell adhesion                           |
| SELPLG     | –2.29       | 0.0090† | Cell adhesion                           |
| SELL       | –2.27       | 0.2139  | Cell adhesion                           |
| CCL2       | –4.01       | 0.0799  | Angiogenesis                            |
| MMP9       | –2.67       | 0.1141  | Angiogenesis                            |
| CCL5       | –2.64       | 0.3832  | Angiogenesis                            |
| TYMP       | –2.50       | 0.0860  | Angiogenesis                            |
| KDR        | –1.62       | 0.0360* | Angiogenesis                            |
| I7         | –42.71      | 0.2101  | Apoptosis                               |
| TNFSF10    | –13.34      | 0.0253* | Apoptosis                               |
| TNF        | –5.00       | 0.3930  | Apoptosis                               |
| OCLN       | –2.16       | 0.2451  | Apoptosis                               |
| EDNRA      | –2.12       | 0.2611  | Apoptosis                               |
| TFPI       | –2.33       | 0.1797  | Coagulation                             |
| APOE       | –3.06       | 0.2442  | Inflammatory response                   |
| CX3CL1     | –5.36       | 0.1323  | Vasoconstriction and vasodilation       |
| ACE        | –3.41       | 0.1797  | Vasoconstriction and vasodilation       |

PCR indicates polymerase chain reaction; SCA, sickle cell anemia.

DOI: 10.1161/JAHA.119.014143
Gene Expression in SCA and Relation to Stroke  Ito et al

**Table 7. Comparison of Results Obtained for the PCR Array and for qRT-PCR**

| Gene       | PCR Array | qRT-PCR |
|------------|-----------|---------|
|            | Fold Change | P Value | Fold Change | Adjusted P Value |
| FLT1       | 3.49       | 0.0218* | 2.17        | 0.2440 |
| PGF        | 2.93       | 0.0150* | 3.54        | 0.0238* |
| MMP1       | 204.64     | 0.0004* | 25.21       | 0.0238* |
| PROCR      | 1.64       | 0.0479* | 2.04        | 0.2440 |
| IL6        | 3.05       | 0.4405  | 1.74        | 0.7354 |
| ICAM1      | −2.83      | 0.0531  | −6.45       | 0.1666 |
| SELPLG     | −2.29      | 0.00901 | 1.28        | 0.7381 |
| CCL2       | −4.01      | 0.0799  | −2.18       | 0.2440 |
| KDR        | −1.62      | 0.0360* | −5.02       | 0.2440 |
| TNFSF10    | −13.34     | 0.0253* | −4.64       | 0.1666 |

PCR indicates polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

*P<0.05.

Flt1 (Fms-Related Receptor Tyrosine Kinase 1); PGF (Placental Growth Factor); MMP1 (Matrix Metalloproteinase 1); PROCR (Protein C Receptor); IL6 (Interleukin 6); ICAM1 (Intercellular Adhesion Molecule 1); SELPLG (Selectin P Ligand); CCL2 (C-C Motif Chemokine Ligand 2); KDR (Kinase Insert Domain Receptor); TNFSF10 (TNF Superfamily Member 10).

CCL2, KDR and TNFSF10 genes. However, SELPLG showed a divergent result, presenting an opposing expression profile (Table 7). Statistically significant differences (P<0.05) with both technologies were observed for the PGF and MMP1 genes (Figure 1).

MMP1 expression was significantly increased both in the PCR array and qRT-PCR. Levels of expressed MMP1 proteins were analyzed in 3 SCA patients with stroke and 6 SCA patients without stroke by Western blot (Figure 2A). We did not succeed in culturing cryopreserved aliquots of ECFCs from 1 patient with stroke. As shown in Figure 2B, MMP1 was expressed in 3 patients with stroke and 2 patients without stroke. Figure 2C shows levels of MMP1 in patients with and without stroke. The MMP1 levels were undetectable in another 4 patients without stroke.

Western blot confirmed higher protein expression of MMP1 in ECFCs from SCA patients with stroke, in accordance with increased gene expression detected by qRT-PCR. The primary antibody used was ab38923, which binds to reduced and native MMP1 but does not cross-react with the other MMP family members (MMP2, MMP3, MMP9, etc.). This antibody reacts with the “pro” and active forms of MMP1, generating 2 bands of 62 and 54 kDa, respectively. The P value calculated by the Mann-Whitney U test was 0.2619. We emphasize that high MMP1 expression levels were also detected in 2 SCA patients without stroke by RT-PCR, and the presence of the protein was confirmed by Western blot. Curiously, these 2 patients had other complications that are known to be associated with increased levels of MMP1. If these samples were excluded from the Western blot analysis, because they could interfere with the comparison between the groups with and without stroke, the calculated P value would be 0.0571. It is possible to observe a tendency for greater protein level in the stroke group. However, because of the great variability of band intensity in the stroke group and the small number of patients, the statistical significance was not observed.

Analysis of the Promoter Region of the MMP1 Gene

To investigate a possible association between differential expression and single-nucleotide variants, direct DNA sequencing of the MMP1 promoter region was carried out in DNA samples from the studied patients. Single-nucleotide variants at the MMP1 promoter region associated with stroke or vascular disease, such as rs1799750, rs498186, rs475007, rs514921, and rs494379, have been described previously.24–27 The results showed no statistically significant differences in the distributions of single-nucleotide variants between the studied groups (P≥0.05).

Discussion

In this study, we correlate—for the first time—the differential expression of genes involved in endothelial cell biology with a specific clinical complication of SCA. Stroke is a common and potentially devastating manifestation of SCA that can affect children and adults. The analysis of gene expression profiles identified 29 differentially expressed genes; 11 were upregulated, with most being associated with angiogenesis (55%), inflammatory response (18%), and coagulation (18%) pathways. Downregulated expression was observed in 18 genes, most of which were associated with angiogenesis (28%), apoptosis (28%), and cell adhesion (22%) pathways. This finding is consistent with current hypotheses regarding the context of the pathophysiology of SCA. Angiogenesis contributes to neurorepair processes, including neurogenesis and synaptogenesis.28–32 Angiogenesis requires proteolysis and remodeling of the extracellular matrix to allow endothelial cells to migrate and invade the surrounding tissue33; therefore, the action of MMPs in this process is crucial.

Interestingly, our results showed remarkable overexpression of the MMP1 gene in the ECFCs of all SCA patients with stroke. In addition, Western blot analysis confirmed these data. MMPs are a large group of zinc-dependent proteases that degrade all extracellular matrix proteins and are involved in inflammatory processes, tissue reconstruction and repair,
Figure 1. Relative mRNA levels in arbitrary units (AU) of hemoglobin S (HbSS) patients with stroke and without stroke by quantitative reverse transcription polymerase chain reaction. *P<0.05 and **P<0.01. A, Expression levels of upregulated genes Placental Growth Factor (PGF); Protein C Receptor (PROCR); Fms-Related Receptor Tyrosine Kinase 1 (FLT1); Matrix Metalloproteinase 1 (MMP1) and Interleukin 6 (IL6). B, Expression levels of downregulated genes TNF Superfamily Member 10 (TNFSF10); C-C Motif Chemokine Ligand 2 (CCL2); Intercellular Adhesion Molecule 1 (ICAM1); Kinase Insert Domain Receptor (KDR) and Selectin P Ligand (SELPLG).
cell migration, and angiogenesis. Their activities are regulated under physiologic conditions at the levels of transcription, zymogen activation, and inhibition by its interaction with the tissue inhibitor of MMPs. MMPs play a role in the structure of blood vessels in the brain, and unregulated activity of these enzymes can contribute to neurologic disorders and stroke. In acute stroke, the first MMP activated during hypoxia is MMP2, followed by activation of MMP9 by MMP3 and by free radicals generated soon after vessel occlusion. Active MMPs degrade the basal lamina and disrupt the tight junction proteins located in the clefts that join the endothelial cells, opening the blood–brain barrier and leading to vasogenic edema, hemorrhage, leukocyte infiltration, and progressive inflammatory reactions.

MMP1 plays a role in the degradation of interstitial collagen types I, II, and III, major structural components of the fibrous plaque that are associated with the mechanism of atherosclerotic stroke. MMP1 protein has been shown to be expressed by macrophages, smooth muscle cells, and microvascular endothelial cells both at the boundary between the fibrous cap and the lipid core and in the shoulder region of the plaque. The expression of this protease by vascular cells seems to contribute to plaque instability. Currently, no studies indicate the clinical significance of MMP1 in stroke in SCA patients. However, a single guanine (1G to 2G) variant located at the MMP1 promoter region −1607 1G/2G (rs1799750) has been associated with IS risk. Experiments demonstrated that the promoter comprising the 2G alleles creates an E-26 virus transcription site and increases transcription capacity. MMP1/−1607 1G/2G seems to display association with IS risk in the Chinese population, but no relation was identified in Tunisian patients. In the present study, the association of this variant with increased MMP1 gene expression was not found. This observation raises the possibility that other factors may be regulating the expression of MMP1 in the patients studied and/or that the effect of the variant can be associated with genetic ethnic background. The precise mechanism by which the overexpression of the MMP1 gene may affect the risk of sickle cell stroke has yet to be elucidated. Curiously, high MMP1 expression levels were also detected in 2 SCA patients without stroke who had liver disease. Hepatic dysfunction in patients with SCA is predominantly caused by vascular occlusion by sickled red blood cells with acute ischemia, cholestasis, hepatic sequestration crisis, transfusional iron overload, hepatitis C virus infection, and chronic hemolysis,
leading to the development of pigment stones.\textsuperscript{42} MMPs play an important role in all stages of progressive liver injury. Hepatic ischemia and reperfusion injury results in MMP-dependent and independent release of reactive oxygen species, cytokines, and other proinflammatory mediators and consequently activates innate immune cells, leading to upregulation of the expression of liver vascular adhesion molecules. Proinflammatory factors, such as TNF-\(\alpha\) (tumor necrosis factor \(\alpha\)) and inducible NOS (nitric oxide synthase)–derived NO, can stimulate the expression and activation of MMPs, which modulate the activity of some of these factors through proteolytic cleavage, thus providing a possible feedback loop that would amplify and sustain the inflammatory environment.\textsuperscript{43}

Interestingly, MMP1 seems to be a candidate for an antifibrotic role because it has been associated with the regression of liver fibrosis in rodents, through cleavage of the fibrillar extracellular matrix and promotion of apoptosis among the activated hepatic stellate cells.\textsuperscript{44} In humans, MMP1 is expressed predominantly in monocytes and Kupffer cells in the early stage of nonalcoholic steatohepatitis and in hepatic progenitor cells and proliferating capillary endothelial cells during the advanced stage of the disease, contributing to the repair and regeneration of fibrotic liver.\textsuperscript{45}

Distinct subphenotypes of clinical complications of SCD have been proposed: a vasculopathy subphenotype, comprising pulmonary hypertension, priapism, leg ulceration, and stroke, and a viscosity–vaso-occlusive subphenotype involving vaso-occlusive pain crisis, acute chest syndrome, and osteonecrosis.\textsuperscript{13} Despite the small number of patients, it is interesting to note that 3 of 4 patients (75\%) in the stroke group also had leg ulcer, which is well known to be secondary to vasculopathy.\textsuperscript{46} In contrast, no one in the group without stroke has leg ulcer. We investigated other complications, such as pulmonary hypertension and albuminuria, and did not find any difference between groups.

Moreover, the analysis of the gene profiles of the ECFCs from SCA patients showed that the \textit{PGF} gene was upregulated in patients with stroke compared with patients without stroke; a statistically significant difference was observed for both technologies used. PGF is a member of the VEGF family. In mammals, the VEGF family consists of 5 members: VEGFA, VEGFB, VEGFC, and VEGFD, and PGF. The biological effects of VEGFs are mediated by tyrosine kinase receptor VEGF receptors VEGFR1, VEGFR2, and VEGFR3, also known as FLT1 (Fms-related tyrosine kinase 1), KDR, and FLT4, respectively. PGF is a pleiotropic cytokine that stimulates the growth and migration of endothelial cells, angiogenesis, leukocyte infiltration, tumor growth, and revascularization of ischemic tissues.\textsuperscript{41} In patients with SCD, increased serum levels of PGF have been described, leading to studies that suggest a possible association between inflammation and angiogenesis in SCD pathogenesis and with SCD complications.\textsuperscript{42,43} Interestingly, Gaál and collaborators carried out a systematic evaluation of VEGFs regarding their angiogenic potential and their shortcomings in the murine central nervous system.\textsuperscript{47} They showed that PGF was the most efficient and safe angiogenic factor, as the blood–brain barrier remained intact, and had minimal adverse effects, making it a potential candidate for therapeutic central nervous system revascularization.\textsuperscript{44}

Among differentially expressed genes validated by qRT-PCR, we also highlight \textit{ICAM1}. Endothelial \textit{ICAM1} is essential for the activation and migration of leukocytes to sites of inflammation. SCD is an inflammatory state, and circulating endothelial cells from these patients display increased expression of adhesion molecules.\textsuperscript{48} In contrast, downregulation of \textit{ICAM1} expression has been observed in SCA patients with stroke, in comparison to patients without stroke. The analysis by PCR array showed a clear tendency toward a statistically significant difference (fold change: \(-2.83; P=0.053\)) that was not confirmed by qRT-PCR analysis (fold change: \(-6.45; P=0.1666\)). We performed \textit{ICAM1} gene expression analysis of total RNA isolated from peripheral blood leukocytes. However, a statistically significant difference between the 2 groups was not observed (\(P>0.05\)).

We emphasize that a mainstay of our approach is the use of cultured ECFCs, which are not endothelial cells that reside naturally in the affected organs and thus are free of tissue specification phenotypes and acquired influences. However, studies have shown that after stroke, ischemic penumbral tissue releases angiogenic factors that induce proliferation of endothelial cells and that EPCs present in the systemic circulation are able to migrate and differentiate into mature endothelial cells in the ischemic area and promote neovessel formation.\textsuperscript{49,50} In addition, emerging evidence suggests an important role of EPCs in liver angiogenesis and fibrosis. In a previous study, it was shown that circulating EPCs are significantly increased in patients with cirrhosis. EPCs stimulate angiogenesis by resident liver sinusoidal endothelial cells in cirrhosis through paracrine factors.\textsuperscript{51} Recently, the contribution of EPCs to alcoholic liver injury via paracrine secretion of inflammatory and angiogenic mediators has been also reported.\textsuperscript{52}

Furthermore, regarding treatment, all patients in the stroke group and only 1 patient in the control group (without stroke) were under regular blood transfusion. All stroke patients (n=4) had some vasculopathy including leg ulcers (n=3), retinopathy (n=2), pulmonary hypertension (n=1), and microalbuminuria (n=1) before their stroke events, indicating severe disease progression. Frequent red cell transfusions reduce vascular endothelial activation, inflammation, and the risk of recurrent or silent strokes.\textsuperscript{53} Peripheral blood
samples for isolation of ECFCs were collected immediately before transfusion therapy. Moreover, in this study, we used ECFCs from passages 3 to 5. Chang Milbauer et al.19 stimulated Blood Outgrowth Endothelial Cells from 5 donors with IL-1β and TNF-α and monitored gene expression. In response, 122 genes significantly changed expression levels; however, all of them returned to baseline by 1 subsequent passage. Thus, we hypothesized that the effects of transfusion treatment may have been reduced after all these passages and no longer influenced the gene expression level and that the observed expression profile reflected only cell culture conditions and the genetics of the subjects.19 We underscore that 2 patients from the control group and 1 from the stroke group were heterozygous for α-thalassemia (carriers of a 3.7 kb deletion in the α-globin gene). However, given the small number of the patients, it is not possible to evaluate whether the presence of α-thalassemia could influence our results.

Our results suggest statistically significant differences in gene expression of ECFCs between SCA patients with and without stroke. The migratory features of these cells in SCA support the association of these cells with sickle cell stroke. The present study showed, for the first time, that phenotypic heterogeneity (in this case, SCA with versus without stroke) can have a genetic endothelial basis and most likely involves overexpression of the MMP1 gene. Although our sample size was small, our analysis strongly suggest that angiogenesis is an important process in sickle cell stroke, worthy of further studies.

Although this study has some limitations, such as the small number of patients in each group and the lack of data before stroke, our preliminary results provide novel insights that can guide future interesting studies. Further research using transcriptomes in ECFCs shortly after stroke and in individuals at high risk of stroke and a study of the role of MMP1 in ECFC functions could contribute to better understanding of MMP1 involvement in the pathophysiology of vasculopathy and stroke in SCA.

Sources of Funding
This work was supported by the São Paulo Research Foundation (grant 2014/00984-3) and Coordination for the Improvement of Higher Education Personnel (grant 4570-2018).

Disclosures
None.

References
1.  Frenette PS, Atweh GF. Sickle cell disease: old discoveries, new concepts, and future promise. J Clin Invest. 2007;117:850–858.
2.  Adewoyin AS. Management of sickle cell disease: a review for physician education in Nigeria (sub-saharan Africa). Anemia. 2015; 2015:791498.
3.  Driss A, Asare KO, Hibbert JM, Gee BE, Adamkiewicz TV, Stiles JK. Sickle cell disease in the post geno Bader-Meunier mc era: a monogenic disease with a polygenic phenotype. Genomics Insights. 2009;2009:23–48.
4.  Pegelow CH, Colangelo L, Steinberg M, Wright EC, Smith J, Phillips G, Vichinsky E. Natural history of blood pressure in sickle cell disease: risks for stroke and death associated with relative hypertension in sickle cell anemia. Am J Med. 1997;102:171–177.
5.  Switzer JA, Hess DC, Nichols FT, Adams RJ. Pathophysiology and treatment of stroke in sickle-cell disease: present and future. Lancet Neurol. 2006;5:501–512.
6.  Hebbel RP, Osarogiagbon R, Kaul D. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. Microcirculation. 2004;11: 129–151.
7.  Balkaran B, Char G, Morris JS, Thomas PW, Serjeant BE, Serjeant GR. Stroke in a cohort of patients with homozygous sickle cell disease. J Pediatr. 1992; 120:360–366.
8.  Ohene-Frempong K, Weiner SJ, Sleeper LA, Miller ST, Embury S, Mooker TH, Wethers DL, Pegelow CH, Gill FM. Cerebrovascular accidents in sickle cell disease: rates and risk factors. Blood. 1998;11:289–294.
9.  Ikram MA, Seshadri S, DeStefano AL, Dichgans M, Rosand J, Meschia JF, Stefansson K, Dichgans M, Markus HS; International Stroke Genetics Consortium. Genetic risk factors for ischaemic stroke and its subtypes (the METASTROKE collaboration): a meta-analysis of genome-wide association studies. Lancet Neurol. 2012;11:951–962.
10.  Kato GJ, Gladwin MT, Steinberg MH. Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev. 2007;21:137–47.
11.  Flanagan JM, Frohlich DM, Howard TA, Schultz WH, Driscoll C, Nagasubramania N, Mortier NA, Kimble AC, Aygun B, Adams RJ, Helms RW, Ware RE. Genetic predictors for stroke in children with sickle cell anemia. Blood. 2011;117:6681–6684.
12.  Zhang ZG, Zhang L, Jiang Q, Chopp M. Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. Circ Res. 2002;90:284–288.
13.  Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. Stroke. 2002;33:1362–1368.
14.  Beck H, Voswinckel R, Wagner S, Ziegelhoeffer T, Heil M, Helisch A, Schaper W, Acker T, Hatsukami TS, Higashida RT, Johnston SC, Kidwell CS, Lutsep HL, Miller E, Costa FF. Increased adhesive and inflammatory properties in blood outgrowth endothelial cells from sickle cell anemia patients. Microvasc Res. 2013;90:173–179.
15.  Chang Milbauer L, Wei P, Enenstein J, Jang A, Hillyer CA, Scott JP, Nelson SC, Bodempudi V, Topper JN, Yang RB, Bircs HC, Fan W, Hebbel RP. Genetic endothelial systems biology of sickle stroke risk. Blood. 2008;111:3872–3879.
16.  Lin Y, Weisborg DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest. 2000;105:71–77.
17.  Easton JD, Saver JL, Albers GW, Alberts MJ, Chaturvedi S, Feldmann E, Hatsukami TS, Higashida RT, Johnston SC, Kidwell CS, Lutsep HL, Miller E, 2012.
Sacco RL; American Heart Association, American Stroke Association Stroke Council, Council on Cardiovascular Surgery and Anesthesia, Council on Cardiovascular Radiology and Intervention, Council on Cardiovascular Nursing, Interdisciplinary Council on Peripheral Vascular Disease. Definition and evaluation of transient ischemic attack: a scientific statement for healthcare professionals from the American Heart Association/American Stroke Association Stroke Council; Council on Cardiovascular Surgery and Anesthesia; Council on Cardiovascular Radiology and Intervention; Council on Cardiovascular Nursing; and the Interdisciplinary Council on Peripheral Vascular Disease. The American Academy of Neurology affirms the value of this statement as an educational tool for neurologists. Stroke. 2009;40:2276–2293.

22. Blair GW, Hernandez MV, Thrippletion MJ, Doublal FN, Wardlaw JM. Advanced neuroimaging of cerebral small vessel disease. Curr Treat Options Cardiovasc Med. 2017;19:56.

23. Wardlaw JM, Smith EE, Biessels GJ, Cordonnier C, Fazekas F, Frayne R, Huang X-Y, Han L-Y, Huang X-D, Guan C-H, Mao X-L, Ye Z-S. Association of matrix metalloproteinase-1 and matrix metalloproteinase-3 gene variants with ischemic stroke and its subtype. J Stroke Cerebrovas Dis. 2017;26:140–152.

24. Qintao C, Yan L, Changhong D, Xiaoliang G, Xiaochen L. Genetic polymorphism of matrix metalloproteinase-1 and coronary artery disease susceptibility: a case-control study in a Han Chinese population. Genet Test Mol Biomarkers. 2014;18:826–831.

25. Zhang G, Li W, Guo Y, Li D, Liu Y, Xu S. MMP gene polymorphisms, MMP-1 -1607 G/C, MMP-12 -82 A/G, and ischemic stroke: a meta-analysis. Stroke Cerebrovas Dis. 2018;27:140–152.

26. Chehaibi K, Hrira MY, Nouira S, Maatouk F, Ben Hamda K, Slimane MN. Matrix metalloproteinase-1 and matrix metalloproteinase-3 gene variants with ischemic stroke in a Tunisian population. J Neurol Sci. 2014;342:107–113.

27. Brea D, Sobrino T, Ramos-Cabrer P, Castillo J. Reorganisation of the cerebral vasculature following ischaemia. Rev Neurol. 2009;49:645–654.

28. Hermann DM, Zechariah A. Implications of vascular endothelial growth factor for postischemic neurovascular remodeling. J Cereb Blood Flow Metab. 2009;29:1620–1633.

29. Nakagomi N, Nakagomi T, Kudo S, Nakano-Doi A, Saino O, Takata M, Yoshikawa H, Stern DM, Mutsumaya T, Taguchi A. Endothelial cells support survival, proliferation, and neuronal differentiation of transplanted adult ischemia-induced neural stem/progenitor cells after cerebral infarction. Stem Cells. 2009;27:1285–1295.

30. Snaypy M, Lemasson M, Brill MS, Blais M, Massouh M, Ninkovic J, Gravel C, Berthod F, Gôtz M, Barker PA, Parent A, Saghatelayan A. Vasculature guides migrating neuronal precursors in the adult mammalian forebrain via brain-derived neurotrophic factor signaling. J Neurosci. 2009;29:4172–4188.

31. Teng H, Zhang ZG, Wang L, Zhang RL, Zhang L, Morris D, Gregg SR, Wu Z, Jiang A, Lu M, Zlokovic BV, Chopp M. Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. J Cereb Blood Flow Metab. 2008;28:764–771.

32. Rundhaug JE. Matrix metalloproteinases and angiogenesis. J Cell Mol Med. 2005;9:267–285.

33. Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev. 2000;14:2123–2133.

34. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 2003;92:827–839.

35. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim Biophys Acta. 2010;1803:55–71.

36. Yang Y, Rosenberg GA. Matrix metalloproteinases as therapeutic targets for stroke. Brain Res. 2015;1623:30–38.

37. Wasserman JK, Schlichter LC. Neuron death and inflammation in a rat model of intracerebral hemorrhage: effects of delayed minocycline treatment. Brain Res. 2007;1136:208–218.

38. Nikkari ST, O’Brien KD, Ferguson M, Hatsukami T, Weigus HG, Alpers CE, Cloves AW. Interstitial collagenase (MMP-1) expression in human colonic atherosclerosis. Circulation. 1995;92:1393–1398.

39. Rutter JL, Mitchell TI, Buttice G, Meyers J, Gusella JF, Ozelius LJ, Brinckerhoff CE. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. Cancer Res. 1998;58:5321–5325.

40. Chang JJ, Stanfill A, Pourmotabbed T. The role of matrix metalloproteinase polymorphisms in ischemic stroke. Int J Mol Sci. 2016;17:E1323. DOI: 10.3390/ijms17081323.

41. Shah R, Taborda C, Chawla S. Acute and chronic hepatobiliary manifestations of sickle cell disease: a review. World J Gastrointest Pathophysiol. 2017;8:108–116.

42. Duarte S, Baber J, Fuji T, Coito AJ. Matrix metalloproteinases in liver injury, repair and fibrosis. Matrix Biol. 2015;44:146–156.

43. Irumo Y, Nishio T, Morimoto T, Nitta T, Stefanovic B, Choi SK, Brenner DA, Yamazaki Y. Delivery of matrix metalloproteinase-1 attenuates established liver fibrosis in the rat. Gastroenterology. 2003;124:445–458.

44. Yokomori H, Inagaki Y, Ando W, Hara M, Komiya T, Kojima S, Oda M, Kuroda H, Suzuki Y, Okazaki I. Spatiotemporal expression of matrix metalloproteinase-1 in progression of nonalcoholic steatohepatitis. J Gastroenterol Hepatol. 2016;31:11–20.

45. Minniti CP, Delaney K-MH, Gorbach AM, Xu D, Lee C- CR, Malik N, Korouakis J, Antalek M, Maivelett J, Peters-Lawrence M, Novelli EM, Lanzkron SM, Axelrod KC, Kato GJ. Vasculopathy, inflammation, and blood flow in leg ulcers of patients with sickle cell anemia. Am J Hematol. 2014;89:1–6.

46. Gali E, Tammela T, Anisimov A, Marbacher S, Honkanen P, Zarkada G, Korouakis A, Antalek M, Maivelett J, Peters-Lawrence M, Novelli EM, Lanzkron SM, Axelrod KC, Kato GJ. Vasculopathy, inflammation, and blood flow in leg ulcers of patients with sickle cell anemia. Am J Hematol. 2014;89:1–6.

47. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. N Engl J Med. 1997;337:1584–1590.

48. Zhang SJ, Zhang H, Wei YJ, Su WJ, Liao ZK, Hou M, Zhou JY, Hu SS. Adult endothelial progenitor cells from human peripheral blood maintain monocyte/macrophage function throughout in vitro culture. Cell Res. 2006;16:577–584.

49. Mikroiva NA, Jackson JA, Hunninghake R, Kenyon J, Chan KWH, Swindlehurst G, Brinckerhoff CE, Brinckerhoff CE, Brinckerhoff CE. The role of matrix metalloproteinase activity in the murine brain reveals placentation growth factor as prime candidate for CNS revascularization. Blood. 2013;122:658–666.

50. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. N Engl J Med. 1997;337:1584–1590.

51. Kaur S, Tripathi D, Dongre K, Garg V, Rooge S, Mukpadhayy A, Sakhuja P, Sarin SK. Increased number and function of endothelial progenitor cells stimulate angiogenesis by resident liver sinusoidal endothelial cells (SECs) in cirrhosis through paracrine factors. J Hepatol. 2012;57:1193–1198.

52. Kaur S, Sehgal R, Shastry SM, McCaughan G, McGuire HM, Fazeekas St de Groth B, Sarin S, Trehanapati N, Seth D. Circulating endothelial progenitor cells present an inflammatory phenotype and function in patients with alcoholic liver cirrhosis. Front Physiol. 2018;9:556.

53. Hyacinthi H, Adams RI, Voeks JH, Hibbert JM, Gee BE. Frequent red cell transfusions reduced vascular endothelial activation and thrombogenicity in children with sickle cell anemia and high stroke risk. Am J Hematol. 2014;89:47–51.