Title: METHODS AND COMPOSITIONS FOR TREATING ATHEROSCLEROSIS

Abstract: The current disclosure provides methods and compositions for treating atherosclerosis, and is based on inhibiting anti-angiogenesis in vivo. Aspects relate to a method for treating atherosclerosis in a subject comprising: administering a surgical treatment to a subject determined to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, and/or increased levels of angiostatin.

(57)
METHODS AND COMPOSITIONS FOR TREATING ATHEROSCLEROSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. Provisional Patent Application No. 62/295,749, filed February 16, 2016, which is hereby incorporated by reference in its entirety.

BACKGROUND

1. Field of the Invention

[0002] The present invention is generally related to the field of medicine and methods and compositions for treating atherosclerosis.

2. Description of Related Art

[0003] Atherosclerosis is a systemic disease process in which fatty deposits, inflammation, cells, and scar tissue build up within the walls of arteries, is the underlying cause of the majority of clinical cardiovascular events. Atherosclerosis can develop in large and small arteries supplying a variety of end-organs, including the heart, brain, kidneys, and extremities. The current disclosure further relates to methods and compositions for treating or ameliorating atherosclerosis or AD in a subject.

[0004] Cardiovascular disease is the leading global cause of death, accounting for 17.3 million deaths per year, a number that is expected to grow to more than 23.6 million by 2030. Nearly 787,000 people in the U.S. died from heart disease, stroke and other cardiovascular diseases in 2011. That's about one of every three deaths in America. About 2,150 Americans die each day from these diseases, one every 40 seconds. About 85.6 million Americans are living with some form of cardiovascular disease or the after-effects of stroke. Heart disease is the No. 1 cause of death in the world and the leading cause of death in the United States, killing over 375,000 Americans a year. Heart disease accounts for 1 in 7 deaths in the U.S.  

[0005] Intra-cranial atherosclerosis (ICAS) is the most common cause of stroke worldwide. It accounts for at least 10% of all strokes in the United States and as much as 33% to 67% of stroke in countries with predominantly Asian, Hispanic, and Black populations. ICAS carries a worse prognosis than other stroke etiologies with an annual rate of recurrent stroke and death of 15% despite intensive medical management, and as high as 25% in certain populations.
Therefore, there is a continuing need for methods and compositions for treating, ameliorating or preventing atherosclerotic disease and stroke.

**SUMMARY OF THE INVENTION**

The current disclosure provides methods and compositions for treating atherosclerosis, and is based on inhibiting anti-angiogenesis *in vivo*. Aspects relate to a method for treating atherosclerosis in a subject comprising: administering a surgical treatment to a subject determined to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2. In some embodiments, the method further comprises administering a non-surgical therapeutic to a subject determined to not have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

In some embodiments, the subject is determined to have or is one that has all of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and increased levels of TSP-2. In some embodiments, the subject is determined to have or is one that has all of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, and increased levels of angiostatin. In some embodiments, the subject is determined to have or is one that has all of: increased levels of HGF, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin and increased levels of TSP-2. In some embodiments, the subject is determined to have at least 1, 2, 3, 4, 5, or 6 of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

In some embodiments, the atherosclerosis is intracranial atherosclerosis. Atherosclerosis is a medical condition characterized by the hardening and narrowing of the arteries.

In some embodiments, the increased HGF comprises an increase in an HGF polypeptide wherein the polypeptide is a fragment of the HGF protein. In some embodiments, the HGF fragment is an anti-angiogenic factor. In some embodiments, the fragment is a NK1, NK2, NK3, or NK4 fragment. In some embodiments, the fragment is a NK1 fragment. In some embodiments, the fragment is a NK2 fragment. In some
embodiments, the fragment is a NK3 fragment. In some embodiments, the fragment is a NK4 fragment.

[0011] In some embodiments, the non-surgical treatment comprises one or more of an ACE inhibitor, an angiotension II receptor blocker, an antiarrhythmic, an antiplatelet, aspirin, a beta blocker, a calcium channel blocker, a clot buster, digoxin, a diuretic, a nitrate, or a blood thinner. In some embodiments, the surgical treatment comprises encephaloduroarteriosynangiosis, stent placement, angioplasty, heart bypass, valvuloplasty, enhanced external counterpulsation, ablation, pacemaker placement, implantation of a cardioverter defibrillator, lead extraction, left ventricular assist device placement, or heart transplant.

[0012] In some embodiments, the method further comprises determining the levels of at least two angiogenic factors in the subject. In some embodiments, the determining is done by reviewing or receiving a report of results of angiogenic factor levels in a patient sample. In some embodiments, the determining is done by measuring the angiogenic factor level in a patient's sample. The measuring may be done by a method known in the art or described herein. In some embodiments, the patient is one that has had angiogenic factors measured in a sample from the patient by a method known in the art or described herein. In some embodiments, the patient's sample has been tested according to a method described herein or according to an assay described herein. In some embodiments, the angiogenic factor are two or more of vascular endothelial growth factor (VEGF) isoforms 165a (VEGF165a), VEGF165b, VEGF-D, VEGF receptor (VEGFR)1, VEGFR2, VEGFR3, platelet-derived growth factor (PDGF)-AA, PDGFF-BB, fibroblast growth factor (FGF) b, hepatocyte growth factor (HGF), NK1, NK2, NK3, NK4, heparin-binding epidermal growth factor (HB-EGF), transforming growth factor (TGF) β1, TGF β2, bone morphogenic protein (BMP)-2, BMP-9, stromal cell derived factor (SDF)-1, interleukin (IL) 4, thrombospondin (TSP)-1, TSP-2, endostatin, and angiostatin. In some embodiments, at least, at most, or exactly 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 (or any derivable range therein) angiogenic factor levels are determined.

[0013] In some embodiments, the method further comprises comparing the level of the angiogenic factor to a control. In some embodiments, the control is the level of an angiogenic factor in a normal patient. In some embodiments, the control is the level of an angiogenic factor in a diseased patient (i.e. one that has atherosclerosis). In some embodiments, the control is a cut-off value. The cut-off value may be at least, at most, or exactly 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 12, 130, 140, 150, 160, 170, 180, 190,
In some embodiments, the method further comprises performing a statistical analysis of the levels of the angiogenic factors. In some embodiments, the statistical analysis is principal component analysis. In some embodiments, the increase or decrease in levels are assessed by a principal component analysis. In some embodiments, the method further comprises comparing the level to a cut-off value. In some embodiments, an increased level is above a cut-off value and a decreased level is below a cut-off value.

In some embodiments, the subject is a human patient. The subject may be a mammal, such as a human, or a common laboratory experimental animal, such as a rat, mouse, cat, dog, rabbit, donkey, horse, or pig.

In some embodiments, the method further comprises administration of an anti-angiogenic blocking agent. In some embodiments, the anti-angiogenic blocking agent is administered prior to surgery. The anti-angiogenic blocking agent may be administered at least, at most, or exactly, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 days or hours (or any range derivable therein) before or after surgery. In some embodiments, the anti-angiogenic blocking agent is administered locally to an atherosclerotic region during surgery.

In some embodiments of the methods and compositions disclosed throughout this disclosure, the anti-angiogenic blocking agent is GMI-1070, a proteinase activated receptor (PAR) - 1 agonist, meizothrombin, granzyme A, activated protein C receptor - endothelial protein C receptor (APC-EPCR), kalikrein (KLK) 4, KLK5, KLK6, matrix metalloproteinase (MMP1), proatherocytin, pen C13; PAR-4 antagonists, tcY-NH₂ ; heparan sulfate proteoglycans, analogues of heparin sulfate proteoglycans, aprotinin, or combinations or derivatives thereof.

In some embodiments, the angiogenic factor is a circulating blood factor. In some embodiments, the levels of the at least two angiogenic factors are or were determined from a blood sample from the subject. The sample may be a blood sample or other patient sample. In some embodiments, the patient sample is a urine, fecal, DNA, RNA, tissue, serum, whole blood, or plasma sample.
Further aspects of the disclosure relate to a method for treating atherosclerosis in a subject comprising administering an anti-angiogenic blocking agent to the subject. In some embodiments, the anti-angiogenic blocking agent is GMI-1070, aprotininase activated receptor (PAR) - I agonist, meizothrombin, granzyme A, activated protein C receptor - endothelial protein C receptor (APC-EPCR), kalikrein (KLK) 4, KLK5, KLK6, matrix metalloproteinase (MMP1), proatherocytin, pen C13; PAR-4 antagonists, tεY-NH₂; heparan sulfate proteoglycans, analogues of heparin sulfate proteoglycans, aprotinin, or combinations thereof.

In some embodiments, the subject has undergone previous treatment for atherosclerosis. In further embodiments, the subject has not yet undergone a treatment for atherosclerosis. In some embodiments, the subject was a poor or non-responder to the previous treatment. In some embodiments, the previous treatment was a non-surgical treatment described herein.

In some embodiments, the atherosclerosis is intracranial. In some embodiments, the method further comprises administering a surgical treatment to the subject. The agent may be administered in a time-frame described previously or in the description that follows.

Further aspects relate to an assay system comprising agents for detecting proteins or protein levels, wherein the proteins comprise one or more of VEGF165a, VEGF165b, VEGF-D, VEGFR1, VEGFR2, VEGFR3, PDGF-AA, PDGF-BB, FGFb, HGF, NK1, NK2, NK3, NK4, HB-EGF, TGF β1, TGF β2, BMP-2, BMP-9, SDF-1, IL 4, TSP-1, TSP-2, endostatin, and angiostatin. In some embodiments, the assay system consists of agents for detecting VEGF165a, VEGF165b, VEGF-D, VEGFR1, VEGFR2, VEGFR3, PDGF-AA, PDGF-BB, FGFb, HGF, NK1, NK2, NK3, NK4, HB-EGF, TGF β1, TGF β2, BMP-2, BMP-9, SDF-1, IL 4, TSP-1, TSP-2, endostatin, and angiostatin. In some embodiments, the assay system consists of agents for detecting VEGF165a, VEGF165b, VEGF-D, VEGFR1, VEGFR2, VEGFR3, PDGF-AA, PDGF-BB, FGFb, HGF, NK1, NK2, NK3, NK4, HB-EGF, TGF β1, TGF β2, BMP-2, BMP-9, SDF-1, IL 4, TSP-1, TSP-2, endostatin, and angiostatin, and a control. The control may be one or multiple detecting agents to determine a normalized or base-line expression level. In some embodiments, the proteins consist of HGF, VEGF165a, VEGFRI, endostatin, and angiostatin. In some embodiments, the proteins consist of HGF, VEGF165a, VEGFRI, endostatin, angiostatin, and a control. In some embodiments, the proteins consist of HGF, VEGF165a, VEGFRI, endostatin, angiostatin, and TSP-2. In some embodiments, the proteins consist of HGF, VEGF165a, VEGFRI, endostatin, angiostatin, TSP-2 and a control. In some embodiments, the proteins consist of...
HGF, VEGFRI, endostatin, angiostatin, and TSP-2. In some embodiments, the proteins consist of HGF, VEGFRI, endostatin, angiostatin, TSP-2 and a control.

[0023] In some embodiments, the agent for detecting HGF comprises an agent that detects a fragment of the HGF protein. In some embodiments, the fragment is NK1, NK2, NK3, or NK4 fragment. In some embodiments, the assay system further comprises one or more of a buffer, a detectable label, or a solid support. In some embodiments, the assay system comprises one or more elements described throughout the disclosure.

[0024] In some embodiments, the agent for detecting the angiogenic factors is a polypeptide. In some embodiments, the polypeptide is an antibody. In some embodiments, the agent is labeled with a detectable label.

[0025] A further aspect relates to a method for predicting a subject's response to atherosclerosis medical treatment comprising: determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using an assay system described herein; and predicting that the subject will respond poorly if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

[0026] A further aspect relates to a method for predicting a subject's response to atherosclerosis medical treatment comprising: determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using an assay system described herein; and predicting that the subject will respond poorly if the subject is determined to have one or more of increased levels of HGF, increased levels of VEGFRI, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

[0027] A further aspect relates to a method for predicting a subject's response to atherosclerosis medical treatment comprising: determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using an assay system described herein; and predicting that the subject will respond poorly if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, and/or increased levels of angiostatin.

[0028] Further aspects relate to a method for treating atherosclerosis in a subject comprising: determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using an assay system described herein; administering a
surgical treatment if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2. In some embodiments, the method further comprises administering a non-surgical therapeutic if the subject does not have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

[0029] Further aspects relate to a method for treating atherosclerosis in a subject comprising: determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using an assay system described herein; administering a surgical treatment if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, and/or increased levels of angiostatin. In some embodiments, the method further comprises administering a non-surgical therapeutic if the subject does not have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, and/or increased levels of angiostatin.

[0030] Further aspects relate to a method for treating atherosclerosis in a subject comprising: determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using an assay system described herein; administering a surgical treatment if the subject is determined to have one or more of increased levels of HGF, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2. In some embodiments, the method further comprises administering a non-surgical therapeutic if the subject does not have one or more of increased levels of HGF, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

[0031] Further aspects relate to a method for predicting a subject's response to atherosclerosis medical treatment comprising: determining the levels of at least two angiogenic factors in the subject; predicting that the subject will respond poorly if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2. In some embodiments, the subject is predicted to respond poorly when the subject is determined to have: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin and increased levels of TSP-2. In some embodiments, the subject is
predicted to respond poorly when the subject is determined to have: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, and increased levels of angioatin. In some embodiments, the subject is predicted to respond poorly when the subject is determined to have: increased levels of HGF, increased levels of VEGFRI, increased levels of endostatin, increased levels of angioatin and increased levels of TSP-2. In some embodiments, the subject has been diagnosed with atherosclerosis. In some embodiments, the subject has not received any treatment for atherosclerosis. In some embodiments, the subject has not responded poorly to any medical treatment, such as a non-surgical medical treatment. In some embodiments, the atherosclerosis is intracranial atherosclerosis. In some embodiments, increased HGF comprises an increase in an HGF polypeptide and wherein the polypeptide is a fragment of the HGF protein. In some embodiments, the fragment is NK1, NK2, NK3, or NK4 factor. In some embodiments, the medical treatment is non-surgical. In some embodiments, the method further comprises administration of surgical treatment if the subject is predicted to respond poorly to medical treatment.

In some embodiments, the surgical treatment comprises encephaloduroarteriosynangiosis, stent placement, angioplasty, heart bypass, valvuloplasty, enhanced external counterpulsation, ablation, pacemaker placement, implantation of a cardioverter defibrillator, lead extraction, left ventricular assist device placement, or heart transplant. In some embodiments, the subject is predicted to respond to non-surgical medical treatment when the subject does not have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, and/or increased levels of angioatin. In some embodiments, the subject is predicted to respond to non-surgical medical treatment when the subject does not have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, increased levels of angioatin, and/or increased levels of TSP-2. In some embodiments, the subject is predicted to respond to non-surgical medical treatment when the subject does not have one or more of increased levels of HGF, increased levels of VEGFRI, increased levels of endostatin, increased levels of angioatin, and/or increased levels of TSP-2. In some embodiments, the method further comprises administration of a non-surgical treatment.

In some embodiments, the non-surgical treatment comprises one or more of an ACE inhibitor, an angiotension II receptor blocker, an antiarrhythmic, an antiplatelet, aspirin, a beta blocker, a calcium channel blocker, a clot buster, digoxin, a diuretic, a nitrate, or a blood thinner. In some embodiments, the at least two angiogenic factors comprise two or more of VEGF165a, VEGF165b, VEGF-D, VEGFRI, VEGFRII, VEGFRIII, PDGF-AA,
PDGFF-BB, FGFb, HGF, NK1, NK2, NK3, NK4, HB-EGF, TGF β1, TGF β2, BMP-2, BMP-9, SDF-1, IL 4, TSP-1, TSP-2, endostatin, and angiotatin.

[0032] Further aspects relate to the use of a surgical treatment for the treatment of atherosclerosis in a subject determined to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, and/or increased levels of angiotatin.

[0033] Further aspects relate to the use of a surgical treatment for the treatment of atherosclerosis in a subject determined to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, increased levels of angiotatin, and/or increased levels of TSP-2. Further aspects relate to the use of a surgical treatment for the treatment of atherosclerosis in a subject determined to have one or more of: increased levels of HGF, increased levels of VEGFRI, increased levels of endostatin, increased levels of angiotatin, and/or increased levels of TSP-2.

[0034] Further aspects relate to the use of a non-surgical treatment for the treatment of atherosclerosis in a subject determined not to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, and/or increased levels of angiotatin. Further aspects relate to the use of a non-surgical treatment for the treatment of atherosclerosis in a subject determined not to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, increased levels of angiotatin, and/or increased levels of TSP-2.

[0035] Further aspects relate to the use of an anti-angiogenic blocking agent for treating atherosclerosis in a subject in need thereof. Further aspects relate to the use of an anti-angiogenic blocking agent in the preparation of a medicament for treating atherosclerosis.

[0036] Further method aspects relate to a method of assessing level of response of a subject to a clinical management regimen of Intracranial atherosclerotic stroke (ICAS) or stroke, comprising: measuring circulating blood level of pro-angiogenic factor (PAGF) in the subject prior to the start of the clinical management regimen (PAGF L₀), measuring circulating blood level of anti-angiogenic factor (AAGF) in the subject prior to the start of the clinical management regimen (AAGF L₀), measuring PAGF circulating blood level in the subject at a time point of or after a clinical management regimen (PAGF Lt), measuring
AAGF circulating blood level in the subject at a time point of clinical management regimen (AAGF L_t), comparing PAGF L_0 with AAGF L_0 and AAGF L_t with AAGF L_0, and designating the subject as responding to the clinical management regimen if the PAGF L_t is higher than PAGF L_0 or as lacking response to the clinical management regimen if the PAGF L_0 is higher than PAGF L_t; or designating the subject as not responding to the clinical management regimen if the AAGF L_t is higher than AAGF L_0 or as responding to the clinical management regimen if AAGF L_0 is higher than AAGF L_t.

In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the PAGF is selected from the group consisting of BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, IL4, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD and SDF-1-alpha, and the AAGF is selected from the group consisting of endostatin, angiostatin, sVEGFR1, thrombospondin-1 and thrombospondin-2.

In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the subject is a human patient.

In another aspect, it is provided a method of treating or ameliorating Intracranial atherosclerotic stroke (ICAS) or stroke in a subject, comprising (a) subjecting the subject to an encephaloduroarteriosynangiosis (EDAS) procedure, (b) administering to the subject a neovascularization inducer, or a combination of (a) or (b).

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises an angiogenic factor.

In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer is selected from the group consisting of BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, IL4, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD, SDF-1 alpha, or a combination thereof.

In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises monocytes.

In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer are monocytes that are obtained from blood of the subject and cultured in a culture medium prior to use.

In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the method comprises (a) subjecting the subject to the encephaloduroarteriosynangiosis (EDAS) procedure, and (b) administering to the subject
the neovascularization inducer locally at the site of the EDAS, wherein the neovascularization inducer comprises BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, IL4, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD, SDF-1 alpha, or a combination thereof.

[0045] In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the administering comprises administering to the subject a DNA construct encoding the neovascularization inducer agent.

[0046] In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the subject is a human being.

[0047] In a further aspect, it is provided a composition, which composition comprising a neovascularization inducer in an effective amount to induce cerebrovascularization in a subject having Intracranial atherosclerotic stroke (ICAS) or stroke. In some embodiments of the composition, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, IL4, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD, SDF-1 alpha, or a combination thereof.

[0048] In some embodiments of the composition, optionally in combination with any or all of the various embodiments disclosed herein, the composition further comprises a pharmaceutically acceptable carrier. In some embodiments of the composition, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises monocytes.

[0049] In some embodiments of the composition, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer are monocytes that are obtained from blood of the subject and cultured in a culture medium prior to use.

[0050] In a further aspect, it is provided a method of fabricating a composition, comprising providing a neovascularization inducer in an effective amount to induce cerebrovascularization in a subject having Intracranial atherosclerotic stroke (ICAS) or stroke, and forming the composition.

[0051] In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer is BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, IL4, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD, SDF-1 alpha, or a combination thereof.
In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the composition further comprises a pharmaceutically acceptable carrier.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises monocytes.

In some embodiments of the method, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer are monocytes that are obtained from blood of the subject and cultured in a culture medium prior to use.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 shows Graphic representation of Angiogenic Profiles (AP) selected by Principal Component Analysis (PCA) AP-4 was significantly associated to failure of medical management and poor mRS at 6 months follow up independently of age and baseline mRS. HGF and several antiangiogenic factors are the dominant components of AP-4.

FIG. 2A-B shows a simulation using the ordinal logistic model predictor profiler for 5,000 runs with uniform random values, depicting the probability of mRS 0 to 2 (a) and 3 to 5 (b) with a range of possible AP4s. The shadow areas represent the confidence of prediction.

FIG. 3 shows the relative levels of angiogenic factors.

FIG. 4 shows the VEGF-165A Ratios in ICAS treatment. The histogram showing mean VEGFA-165a/b ratios over time for the IMM and EDAS group. While an initial elevation of the ratio in week 1 immediately after surgery (red arrow) could be attributed to the operative insult, elevations in the IMM group in months 3 and 6 (green arrow) may indicate a protective effect.

**DETAILED DESCRIPTION**

1. **Definitions**

The term "clinical management" of intracranial arterial stenosis (ICAS) (also referred to as intracranial arterial stenosis) refers to the treatment of this condition by medical (pharmacological, surgical or endovascular - e.g., intravascular manipulations or implants) means. As used herein, the term clinical management is used interchangeably with the term "medical management." ICAS can be produced by multiple etiologies including intracranial atherosclerosis, inflammatory process, degenerative diseases, moyamoya disease or undefined arteriopaties.
The term "neovascularization" refers to the formation of functional microvascular networks with red blood cell perfusion. Neovascularization differs from angiogenesis in that angiogenesis is only a component of the neovascularization, mainly characterized by the protrusion and outgrowth of capillary buds and sprouts from pre-existing blood vessels. See, e.g., Martin Rucker, et al., Elsevier: Biomaterials 27(2006) pp 5027-5038. As used herein, the term "neovascularization" is used interchangeably with the term "cerebrovascularization" or "revascularization", which refers to neovascularization within the intracranial space or area. In some embodiments, the terms "neovascularization," "cerebrovascularization," and "vascularization" are used interchangeably. The term "neovascularization inducing agent" refers any agent that is effective to stimulate or induce or otherwise promote cerebrovascularization or revascularization. In some embodiments, the term neovascularization is also referred to as "collateral formation."

As used herein, the term "agent" refers to neovascularization inducing agent in an effective amount for promoting neovascularization (e.g., cerebrovascularization). An example of the agent is a VEGF such as VEGF-D. In some embodiments, the term also encompasses a PEGylated agent bearing a short alkyl chain, a short polymer chain, a short poly(amino acid) chain, or acyl group such as methyl or ethyl or acetyl, for example. More detailed discussion of neovascularization inducing agent is described below. In some embodiments, the term "agent" is also referred to from time to time as "bioactive agent", "compound", "chemical", "chemical compound", peptide, polypeptide, or protein.

The term pro-angiogenic growth factor (PAGF) refers to an agent that promotes angiogenesis or neovascularization. Sometimes, PAGF is used interchangeably with the term angiogenic growth factor. Conversely, as used here, the term anti-angiogenic growth factor or agent (AAGF) refers to an agent that inhibits or otherwise slows angiogenesis or neovascularization. Examples of such PAGF or AAGF are described above and below.

The term "effective" is also referred to as "therapeutically effective" refers to a neovascularization inducing agent inducing statistically significant result of neovascularization under clinical conditions.

The term "therapeutically effective amount", as used herein, is an amount of an agent that is sufficient to produce a statistically significant, measurable change of a condition using the agent disclosed herein as compared with the condition in the repaired tissue without using the agent. Such effective amounts can be gauged in clinical trials as well as animal studies.
Such a statistically significant, measurable, and positive change of a condition in repaired tissue using the agent disclosed herein as compared with the condition without using the agent is referred to as being an "improved condition."

As used herein, the term "safe and effective amount" refers to the quantity of a component which is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this disclosure. The specific safe and effective amount or therapeutically effective amount will vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal or animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

As used herein, the term "significantly" or "significant" shall mean statistically significant.

The term "derivative" as used herein refers to neovascularization inducer agent which have been chemically modified, for example by ubiquitination, labeling, pegylation (derivatization with polyethylene glycol) or addition of other molecules. A molecule is also a "derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half-life, etc. The moieties can alternatively decrease the toxicity of the molecule, or eliminate or attenuate an undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., MackPubl., Easton, Pa. (1990). As such, a "derivative" polypeptide or peptide is one that is modified, for example, by glycosylation, pegylation, phosphorylation, sulfation, reduction/alkylation, acylation, chemical coupling, or mild formalin treatment. A derivative may also be modified to contain a detectable label, either directly or indirectly, including, but not limited to, a radioisotope, fluorescent, and enzyme label.

The term "functional" when used in conjunction with "derivative" or "variant" refers to a compound or agent which possess a biological activity that is substantially similar to a biological activity of the neovascularization inducer compound or agent of which it is a derivative or variant. By "substantially similar" in this context is meant that at least 50% of the relevant or desired biological activity of a corresponding neovascularization inducer compound or agent is retained, e.g., preferably the variant retains at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 100% or even higher (i.e., the variant or derivative has greater activity than the original neovascularization inducer compound or
agent), e.g., at least 110%, at least 120%, or more compared to a measurable activity of the neovascularization inducer compound or agent.

[0070] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

[0071] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[0072] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0073] The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1%> of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term "about" meaning within an acceptable error range for the particular value should be assumed.

[0074] The term "active fragment or variant" is meant a fragment that is 100% identical to a contiguous portion of the peptide, polypeptide or protein, or a variant that is at least 90%, preferably 95% identical to a fragment up to and including the full length peptide, polypeptide or protein. A variant, for example, may include conservative amino acid substitutions, as defined in the art, or nonconservative substitutions, providing that at least e.g. 10%, 25%, 50%, 75% or 90% of the activity of the original peptide, polypeptide or protein is retained.
Unless otherwise indicated, the terms "peptide", "polypeptide" or "protein" are used interchangeably herein, although typically they refer to peptide sequences of varying sizes.

The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs) or single base mutations in which the polynucleotide sequence varies by one base. The presence of SNPs may be indicative of, for example, a certain population with a propensity for a disease state, that is susceptibility versus resistance.

Derivative polynucleotides include nucleic acids subjected to chemical modification, for example, replacement of hydrogen by an alkyl, acyl, or amino group. Derivatives, e.g., derivative oligonucleotides, may comprise non-naturally-occurring portions, such as altered sugar moieties or inter-sugar linkages. Exemplary among these are phosphorothioate and other sulfur containing species which are known in the art. Derivative nucleic acids may also contain labels, including radionucleotides, enzymes, fluorescent agents, chemiluminescent agents, chromogenic agents, substrates, cofactors, inhibitors, magnetic particles, and the like.

The term "expression vector" as used herein refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules, siRNA, ribozymes, and the like. Expression vectors can contain a variety of control sequences, which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operatively linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well.

By "encoding" or "encoded", "encodes", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code.
As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

"Sample" is used herein in its broadest sense. A sample comprising polynucleotides, polypeptides, peptides, antibodies and the like may comprise a bodily fluid; a soluble fraction of a cell preparation, or media in which cells were grown; a chromosome, an organelle, or membrane isolated or extracted from a cell; genomic DNA, RNA, or cDNA, polypeptides, or peptides in solution or bound to a substrate; a cell; a tissue; a tissue print; a fingerprint, skin or hair; and the like.

The terms "patient", "subject" or "individual" are used interchangeably herein, and refers to a mammalian subject to be treated. In some embodiments, the subject is a human, some cases, the methods of the disclosure find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

"Treatment" is an intervention performed with the intention of preventing the development or altering the pathology or symptoms of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. As used herein, "ameliorated" or "treatment" refers to a symptom which is approaches a normalized value (for example a value obtained in a healthy patient or individual), e.g., is less than 50% different from a normalized value, preferably is less than about 25% different from a normalized value, more preferably, is less than 10% different from a normalized value, and still more preferably, is not significantly different from a normalized value as determined using routine statistical tests. For example the term "treat" or "treating" with respect to tumor cells refers to stopping the progression of said cells, slowing down growth, inducing regression, or amelioration of symptoms associated with the presence of said cells. Treatment of an individual suffering from an infectious disease organism refers to a decrease and elimination of the disease organism from an individual. For example, a
decrease of viral particles as measured by plaque forming units or other automated diagnostic methods such as ELISA etc.

II. Angiogenic Factors

[0085] Angiogenic factors may be pro- or anti-angiogenic factors, unless specified otherwise. The description of certain angiogenic factors is provided below.

A. Endostatin

[0086] Endostatin is a 20-kDa C-terminal fragment derived from the proteolytic cleavage of collagen XVIII. Collagen XVIII is quite a ubiquitous molecule. Along with perlecans and agrin, is one of the major proteoglycans of basal membranes and the adjacent connective tissue. Endostatin's antiangiogenic function involves inhibition of endothelial cell proliferation and migration. This is achieved by multiple mechanisms that include binding with the integrin α5β1 to activate the Src-kinase pathway, which downregulates the activity of RhoA GTPase and inhibits signaling pathways mediated by small kinases of the Ras and Raf families. Endostatin also inhibits angiogenesis by inhibiting the actions of VEGF and FGF by competing for binding to either glypican or their high-affinity receptors. Endostatin potently inhibits endothelial cell migration via down regulation of c-myc expression and inhibition of MAPK (p38 and INK). Several reports have also indicated that endostatin induces endothelial cell apoptosis by abrogation of cyclin D1 expression, which produces arrest of the cell cycle in G1, and by induction of autophagy, by modulating Beclin1 and β-catenin levels. Previous studies have shown in a mouse model of arterial injury that elevated serum levels of endostatin resulted in reduced reendothelialization after arterial damage, mechanism that could conceivably participate in the progression of ICAS and failure to medical management that we have observed. The arrest of the endothelial cell cycle and consequent endothelial apoptosis induced by the high endostatin levels could also explain the reduced ability of patients undergoing indirect revascularization to form new connections to the intracranial circulation and the resulting lower neovascularization indexes.

B. Angiostatin

[0087] Angiostatin is a 38-kDa fragment derived from the proteolytic cleavage of plasmin that contains the first four kringle domains of plasminogen. Among these individual kringles, K1 to K3 are the most potent inhibitors of endothelial cell growth. The complete mechanisms by which angiostatin produces antiangiogenesis are not totally understood.
However, in general the preservation of the kringle structure is essential for the maintenance of the antiangiogenic effects. There are reports of possible receptors of angiostatin (ATPase, angiomiobietin), as well as indications that angiostatin may arrest the endothelial cell cycle at G2. Endothelial apoptosis has also been linked to the antiangiogenic activity of angiostatin. Due to the nonoverlapping nature of the inhibitory pathways, endostatin and angiostatin may act synergistically. This ability has been confirmed in vitro by isobolographic analysis of treatment of endothelial cells with a combination of angiostatin and endostatin, which resulted in synergistic inhibition. Applicant’s study demonstrated that endostatin and angiostatin vary together in ICAS patients and they were important components of the antiangiogenic profile associated with failure of medical management and poor functional status. In addition, angiostatin and endostatin constituted the main components of the resolved angiogenic profile for angiographic neovascularization, which has a negative correlation with the formation of new vessels after surgery.

C. VEGFR-1

The soluble VEGFR-1 is an approximately 100-kDa molecule encoded by the sFlt-1 (VEGFR-1) gene on chromosome 13q12. VEGFR-1 is produced by different human cells including endothelium, vascular smooth muscle, monocytes, placental trophoblasts, corneal epithelial cells, and proximal tubular cells of the renal epithelium. The soluble VEGFR-1 has antiangiogenic effects by either ligating circulating members of the VEGF family (VEGFA 165a, VEGF-D, VEGF-C, PDGFAA, PDGFBb) or by forming heterodimers with monomer VEGFRs in the cell surface, creating decoy surface receptors for these molecules. Several studies have measured plasma concentrations of VEGFR-1. While it is well established that high levels of VEGFR-1 are associated with preeclampsia, its role in atherosclerotic conditions such as coronary artery disease and peripheral arterial disease remains controversial. In the examples described herein, the VEGFR-1 displayed high covariance with endostatin and angiostatin, and the VEGFR-1 PAC coefficient was comparable to the endostatin and higher than the one for angiostatin in the anti-angiogenic profile, AP4. However, Applicants did not find a significant association between VEGFR-1 levels and the degree of neovascularization after surgery.

D. HGF

HGF is a heterodimer with two subunits (α: 69KDa and β: 34KDa) encoded by the HGF gene on chromosome 7q21.1. HGF is secreted by cells of mesenchymal origin as a pro-
HGF inactive precursor that is bound to heparin-like proteoglycans within the extracellular matrix of most tissues. Pro-HGF is cleaved into the mature αβ heterodimer by different extracellular proteases including HGF activator, urokinase-type plasminogen activator, Factors XI and XII, matriptase and hepsin. HGF by activation of its Met receptor (RTK receptor tyrosine kinase) acts as a pleiotropic factor that stimulates multiple biological processes, such as cell proliferation, survival, motility, differentiation and morphogenesis during embryogenesis, organ regeneration, and tumor invasiveness. The α subunit of the HGF is constituted by a N-terminal hairpin and subsequent four kringle domains. Previous studies have prepared by elastase-digestion of HGF a fragment called NK4, identical to the HGF a subunit (N-terminal hairpin and 4 kringle domains) except for the absence of the C-terminal 16 amino acids (i.e., Asn479 to Arg494), which has demonstrated to be a potent antiangiogenic factor by inhibition of endothelial proliferation not only HGF-mediated, but also induced by FGF and VEGF. Naturally occurring fragments of HGF (NK1and NK2) have also shown antiangiogenic effects, although not as potent as NK4. Interestingly, the association between elevated circulating HGF and stroke has been previously observed. Other in the field conducted a nested case-control study from the population of The Women's Health Initiative Observational Study in 972 patients with new strokes and matched controls to evaluate the relationship between HGF levels and risk of stroke. They found that the risk of stroke was significantly greater among women in the highest quartile levels of baseline HGF (OR: 1.46, 95%CI: 1.1-1.9) in a conditional logistic regression model that adjusted for body mass index. It is not clear what is the mechanism that explains this association, but a role for angiogenesis in large arteries atherosclerotic plaque has been advocated. However, the actual role of proangiogenesis in the small intracranial arteries has been questioned. It may be possible that increases in HGF may result in increased concentrations of the antiangiogenic fragments NK1 and NK2, which would coincide with our observation that increases in antiangiogenic molecules predict poorer outcomes.

III. Methods of Treating Atherosclerosis

A. Cerebral collateral circulation

[0090] Cerebral collateral circulation is the auxiliary network of vessels that can provide cerebral blood flow when principal conduits fail as reported in the following studies:
WASID: Severe ICAS (>70%) presence of poor collaterals increased the risk of stroke by 6 fold in the compromised vascular territory (none or poor vs good collaterals: 30% vs 5%, HR 6.05; 95%CI 1.41-25.92; log rank p=0.0056); SAMMPRIS: the presence of good collaterals seemed to influence the stroke risk: 0/66 (0%) in the medical arm and 0/51 (0%) in the stent arm had vessel territory stroke when collaterals were complete (Liebeskind DS. Stroke. 2003;34:2279-2284; Wei L, et al. Stroke. 2001;32:2179-2184; and Liebeskind et al. ISC 2012; February 103 New Orleans).

B. Arteriogenesis

[0091] Arteriogenesis is the outgrowth of a mature collateral arterioles upon stenosis or occlusion of a major conductance artery driven by hemodynamic factors such as an increase in stretch and/or fluid shear stress (FSS) on endothelial cells. With an arterial occlusion, a pressure gradient develops over interconnecting collaterals between the pre- (high pressure areal) and post-occlusive (low pressure areal) regions. Flow through these collateral vessels deforms ECs, raising FSS on the endothelium, which functionally and morphologically activates it.

C. Encephaloduroarteriosynangiosis

[0092] Encephaloduroarteriosynangiosis (EDAS) is a form of indirect revascularization initially employed for the treatment of pediatric moyamoya disease.” In this procedure, branches of the external carotid artery (ECA), in particular the superficial temporal artery (STA) and middle meningeal artery (MMA) are re-routed intracranially and placed in intimate contact with middle cerebral artery branches at the brain surface. Prior trials by our clinical laboratory have demonstrated that EDAS produces vascular connections between the ECA branches and the intracranial circulation in adults with moyamoya and more recently that EDAS also induces formation of new vessels in patients with ICAS averting stroke. In these patients, EDAS has successfully reduced transient ischemic attacks, prevented stroke, and demonstrated angiographic neovascularization with improvement in cerebral perfusion.

[0093] Figure 1 shows baseline and 5 months post EDAS surgical angiogram of the external carotid artery. Notice the enlargement of the STA (white arrow) and the MMA (black arrow), as well as multiple anastomosis between these vessels and the middle cerebral artery branches (arrowheads) and cerebral cortical blush.

[0094] The evidence (Figure 1) in adults for post-EDAS neovascularization from vessels that do not have innate connections to the intracranial arteries represents a new phenomenon
that has not been previously studied. Encouraged by this finding, and the fact that vascular collaterals are of crucial importance to forestalling stroke we have evaluated the role of circulating angiogenic factors and angio-regulatory circulating cells in response to medical management and EDAS, seeking for mechanistic explanations for the outcomes of the procedure and candidates for therapeutic interventions. Several of our findings can have impact on the management of symptomatic patients with ICAS by identifying neovascularization inducers, potential pharmacological mediators, and modified surgical strategies targeting collateral blood flow sources. Of additional relevance, the long-term significance of this application is the possibility to regulate vessel formation, which can provide essential key steps for overcoming barriers in the application of stem cell therapy in stroke through the development of suitable vascular scaffolds that could support reparative stem-cell treatments.

D. Mechanisms of vessel formation


E. Indirect revascularization

[0097] Indirect revascularization techniques, such as encephaloduroarteriosynangiosis (EDAS), were initially used for the treatment of moyamoya disease in children. This technique offers several advantages: (1) it obviates the need for temporary occlusion of cerebral vessels, which is inherent to bypass, (2) it avoids hyperperfusion since a direct anastomosis with immediate increase in blood flow is not established, and (3) it is technically less demanding than bypass surgery. Applicants have demonstrated that in adults with
moyamoya disease, EDAS produces robust revascularization with a quantifiable increase in collateral circulation that develops gradually, avoiding the potential problems of abrupt flow restoration with direct bypass and stenting. In response to the failure of other interventions in the treatment of ICAS, Applicants introduced the use of EDAS for its treatment.

5 F. Discoveries of Angiogenesis in ICAS and EDAS

[0098] The evidence of neovascularization in adult patients undergoing EDAS for ICAS represents an exceptional phenomenon. Native collaterals tend to fail in patients with stroke and the endothelial cells of the cerebral vasculature seem to stall in the process of forming new vessels. This failure has been attributed to evolutionary mechanisms intended to prevent the development of vascular malformations given the specialized nature of brain endothelial cells and the pleiotropic effects of the angiogenic factors produced by them. When extracranial vessels (STA and MMA) are rerouted by EDAS surgery, their cells do not appear to suffer the inhibition of the cerebral endothelium and new vessels connecting to the intracranial arteries grow and mature to reach sizes observable on angiography (at least 200 pm in diameter). This unprecedented neovascularization challenges the traditional understanding of vessel formation by the classic mechanisms of vasculogenesis (exclusively in the embryologic period), arteriogenesis (led by pressure gradients and sheer stress in pre-existing vessels), and angiogenesis mediated by hypoxia). Based on clinical observations, this phenomenon sometimes called synangiosis (Greek syn; together, angio: vessel, and osis: action or process) seems to be triggered by the hypoperfusion and resulting hypoxic environment. It results in the generation of mature vessels, which are large enough to be seen on angiography and to provide flow to cerebral areas in need.

G. Additional Surgical Treatment Methods

[0099] Embodiments of methods described herein comprise administration of a surgical procedure. The surgical procedure may be, for example, a surgical procedure known in the art or described herein or selected from encephaloduroarteriosynangiosis, stent placement, angioplasty, heart bypass, valvuloplasty, enhanced external counterpulsation, ablation, pacemaker placement, implantation of a cardioverter defibrillator, lead extraction, left ventricular assist device placement, or heart transplant.
IV. Additional Methods

[0100] In one aspect of the disclosure, it is provided a method of assessing level of response of a subject to a clinical management regimen of Intracranial atherosclerotic stroke (ICAS) or stroke, comprising: measuring circulating blood level of pro-angiogenic factor (PAGF) in the subject prior to the start of the clinical management regimen (PAGF L₀), measuring circulating blood level of anti-angiogenic factor (AAGF) in the subject prior to the start of the clinical management regimen (AAGF L₀), measuring PAGF circulating blood level in the subject at a time point of or after a clinical management regimen (PAGF L₄), measuring AAGF circulating blood level in the subject at a time point of clinical management regimen (AAGF Lt), comparing PAGF L₄ with PAGF L₀ and AAGF L₄ with AAGF L₀, and designating the subject as responding to the clinical management regimen if the PAGF L₄ is higher than PAGF L₀ or as lacking response to the clinical management regimen if the PAGF L₀ is higher than PAGF L₄; or designating the subject as NOT responding to the clinical management regimen if the AAGF Lt is higher than AAGF L₀ or as responding to the clinical management regimen if AAGF L₀ is higher than AAGF Lt.

[0101] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the PAGF is selected from the group consisting of BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, JLA, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD and SDF-1 alpha, and the AAGF is selected from the group consisting of endostatin, angiostatin, sVEGFR1, thrombospondin-1 and thrombospondin-2.

[0102] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the subject is a human patient.

[0103] In another aspect of the disclosure, it is provided a method of treating or ameliorating Intracranial atherosclerotic stroke (ICAS) or stroke in a subject, comprising (a) subjecting the subject to an encephaloduroarteriosynangiosis (EDAS) procedure, (b) administering to the subject a neovascularization inducer, or a combination of (a) or (b).

[0104] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises an angiogenic factor.

[0105] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer is selected from the group consisting of BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HBEGF, IL4, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD, SDF-1 alpha, or a combination thereof.
In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises monocytes.

In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer are monocytes that are obtained from blood of the subject and cultured in a culture medium prior to use.

In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the method comprises (a) subjecting the subject to the encephaloduroarteriosynangiosis (EDAS) procedure, and (b) administering to the subject the neovascularization inducer locally at the site of the EDAS, wherein the neovascularization inducer comprises BMP2, BMP9, PDGF-AA, PDGF- BB, HGF, FGF2, KGF, HB-EGF, IL4, TPO, TGF-beta 1, TGF-beta 2 , VEGF, VEGFD, SDF- lalpha, or a combination thereof.

In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the administering comprises administering to the subject a DNA construct encoding the neovascularization inducer agent.

In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the subject is a human being.

V. Assay Systems

Embodiments of the disclosure relate to assays and products for the diagnosis and treatment of atherosclerosis. In some embodiments, the levels of angiogenic factors can be determined by measuring the levels of polypeptides encoded by these genes in a patient sample. Methods suitable for this purpose include, but are not limited to, immunoassays such as ELISA, RIA, FACS, dot blot, Western Blot, immunohistochemistry, and antibody-based radioimaging. Protocols for carrying out these immunoassays are well known in the art. Other methods such as 2-dimensional SDS-polyacrylamide gel electrophoresis can also be used. These procedures may be used to recognize any of the polypeptides encoded by the angiogenic factors described herein.

One example of a method suitable for detecting the levels of target proteins in samples is an immunoassay such as an ELISA. In an exemplifying immunoassay, antibodies or other molecules capable of specifically binding to the target proteins (i.e. angiogenic factors) are immobilized onto a selected surface exhibiting protein affinity, such as wells in a polystyrene or polyvinylchloride microtiter plate. Then, patient samples to be tested are
added to the wells. After binding and washing to remove non-specifically bound immunocomplexes, the bound antigen(s) can be detected. Detection can be achieved by the addition of a second antibody which is specific for the target proteins and is linked to a detectable label. Detection may also be achieved by the addition of a second antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label. Before being added to the microtiter plate, cells in the peripheral blood samples can be lysed using various methods known in the art. Proper extraction procedures can be used to separate the target proteins from potentially interfering substances.

[0113] In another immunoassay embodiment, protein-containing samples from the patient are immobilized onto the well surface and then contacted with the antibodies. After binding and washing to remove non-specifically bound immunocomplexes, the bound antigen is detected. Where the initial antibodies are linked to a detectable label, the immunocomplexes can be detected directly. The immunocomplexes can also be detected using a second antibody that has binding affinity for the first antibody, with the second antibody being linked to a detectable label.

[0114] Another typical immunoassay involves the use of antibody competition in the detection. In this immunoassay, the target proteins are immobilized on the well surface. The labeled antibodies are added to the well, allowed to bind to the target proteins, and detected by means of their labels. The amount of the target proteins in an unknown sample is then determined by mixing the sample with the labeled antibodies before or during incubation with coated wells. The presence of the target proteins in the unknown sample acts to reduce the amount of antibody available for binding to the well and thus reduces the ultimate signal.

[0115] Different assay formats can have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immunocomplexes. For instance, in coating a plate with either antigen or antibody, the wells of the plate can be incubated with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate are then washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test samples. Examples of these nonspecific proteins include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.
In immunoassays, a secondary or tertiary detection means can also be used. After binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control and/or clinical or biological sample to be tested under conditions effective to allow immunocomplex (antigen/antibody) formation. These conditions may include, for example, diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween and incubating the antibodies and antigens at room temperature for about 1 to 4 hours or at 49°C overnight. Detection of the immunocomplex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

After all of the incubation steps in an immunoassay, the contacted surface can be washed so as to remove non-complexed material. For instance, the surface may be washed with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immunocomplexes between the test sample and the originally bound material, and subsequent washing, the occurrence of the amount of immunocomplexes can be determined.

To provide a detecting means, the second or third antibody can have an associated label to allow detection. In one embodiment, the label is an enzyme that generates color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one may contact and incubate the first or second immunocomplex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immunocomplex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, e.g., by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azido-di-(3-ethyl)-benzhiazoline-6-sulfonic acid (ABTS) and hydrogen peroxide, in the case of peroxidase as the enzyme label. Quantitation can be achieved by measuring the degree of color generation, e.g., using a spectrophotometer.

Suitable labels may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. The labels can be suitable for small scale detection or more suitable for high-throughput screening. As such, suitable labels include,
but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes, and proteins, including enzymes. The label may be simply detected or it may be quantified. A response that is simply detected generally comprises a response whose existence merely is confirmed, whereas a response that is quantified generally comprises a response having a quantifiable (e.g., numerically reportable) value such as an intensity, polarization, and/or other property. In luminescence or fluorescence assays, the detectable response may be generated directly using a luminophore or fluorophore associated with an assay component actually involved in binding, or indirectly using a luminophore or fluorophore associated with another (e.g., reporter or indicator) component.

Examples of luminescent labels that produce signals include, but are not limited to bioluminescence and chemiluminescence. Detectable luminescence response generally comprises a change in, or an occurrence of, a luminescence signal. Suitable methods and luminophores for luminescently labeling assay components are known in the art and described for example in Haugland, Richard P. (1996) Handbook of Fluorescent Probes and Research Chemicals (6.sup.th ed.). Examples of luminescent probes include, but are not limited to, aequorin and luciferases.

Examples of suitable fluorescent labels include, but are not limited to, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue(TM), and Texas Red. Other suitable optical dyes are described in the Haugland, Richard P. (1996) Handbook of Fluorescent Probes and Research Chemicals (6.sup.th ed.).

In another aspect, the fluorescent label is functionalized to facilitate covalent attachment to a cellular component present in or on the surface of the cell or tissue such as a cell surface marker. Suitable functional groups, including, but not are limited to, isothiocyanate groups, amino groups, haloacetyl groups, maleimides, succinimidyl esters, and sulfonyl halides, all of which may be used to attach the fluorescent label to a second molecule. The choice of the functional group of the fluorescent label will depend on the site of attachment to either a linker, the agent, the marker, or the second labeling agent.

Attachment of the fluorescent label may be either directly to the cellular component or compound or alternatively, can by via a linker. Suitable binding pairs for use in indirectly linking the fluorescent label to the intermediate include, but are not limited to, antigens/antibodies, e.g., rhodamine/anti-rhodamine, biotin/avidin and biotin/strepaavidin.

Another suitable method is RIA (radioimmunoassay). An example of RIA is based on the competition between radiolabeled-polypeptides and unlabeled polypeptides for
binding to a limited quantity of antibodies. Suitable radiolabels include, but are not limited to, \(^{125}\text{I}\). In one embodiment, a fixed concentration of \(^{125}\text{I}\)-labeled polypeptide is incubated with a series of dilution of an antibody specific to the polypeptide. When the unlabeled polypeptide is added to the system, the amount of the \(^{125}\text{I}\)-polypeptide that binds to the antibody is decreased. A standard curve can therefore be constructed to represent the amount of antibody-bound \(^{125}\text{I}\)-polypeptide as a function of the concentration of the unlabeled polypeptide. From this standard curve, the concentration of the polypeptide in unknown samples can be determined. Various protocols for conducting RIA to measure the levels of polypeptides in breast cancer cell samples are well known in the art.

Suitable antibodies as binding agents in the methods described herein can include, but are not limited to, polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, single chain antibodies, Fab fragments, and fragments produced by a Fab expression library.

Antibodies can be labeled with one or more detectable moieties to allow for detection of antibody-antigen complexes. The detectable moieties can include compositions detectable by spectroscopic, enzymatic, photochemical, biochemical, bioelectronic, immunochemical, electrical, optical or chemical means. The detectable moieties include, but are not limited to, radioisotopes, chemiluminescent compounds, labeled binding proteins, heavy metal atoms, spectroscopic markers such as fluorescent markers and dyes, magnetic labels, linked enzymes, mass spectrometry tags, spin labels, electron transfer donors and acceptors, and the like.

Protein array technology is discussed in detail in Pandey and Mann (2000) and MacBeath and Schreiber (2000), each of which is herein specifically incorporated by reference. These arrays typically contain thousands of different proteins or antibodies spotted onto glass slides or immobilized in tiny wells and allow one to examine the biochemical activities and binding profiles of a large number of proteins at once. To examine protein interactions with such an array, a labeled protein is incubated with each of the target proteins immobilized on the slide, and then one determines which of the many proteins the labeled molecule binds. In certain embodiments such technology can be used to quantitate a number of proteins in a sample, such as a breast cancer biomarker proteins.

The basic construction of protein chips has some similarities to DNA chips, such as the use of a glass or plastic surface dotted with an array of molecules. These molecules can be DNA or antibodies that are designed to capture proteins. Defined quantities of proteins are immobilized on each spot, while retaining some activity of the protein. With
fluorescent markers or other methods of detection revealing the spots that have captured these proteins, protein microarrays are being used as powerful tools in high-throughput proteomics and drug discovery.

[0130] The earliest and best-known protein chip is the ProteinChip by Ciphergen Biosystems Inc. (Fremont, Calif). The ProteinChip is based on the surface-enhanced laser desorption and ionization (SELDI) process. Known proteins are analyzed using functional assays that are on the chip. For example, chip surfaces can contain enzymes, receptor proteins, or antibodies that enable researchers to conduct protein-protein interaction studies, ligand binding studies, or immunoassays. With state-of-the-art ion optic and laser optic technologies, the ProteinChip system detects proteins ranging from small peptides of less than 1000 Da up to proteins of 300 kDa and calculates the mass based on time-of-flight (TOF).

[0131] The ProteinChip biomarker system is the first protein biochip-based system that enables biomarker pattern recognition analysis to be done. This system allows researchers to address important clinical questions by investigating the proteome from a range of crude clinical samples (i.e., laser capture microdissected cells, biopsies, tissue, urine, and serum). The system also utilizes biomarker pattern software that automates pattern recognition-based statistical analysis methods to correlate protein expression patterns from clinical samples with disease phenotypes.

[0132] In other aspects, the levels of polypeptides in samples can be determined by detecting the biological activities associated with the polypeptides. If a biological function/activity of a polypeptide is known, suitable in vitro bioassays can be designed to evaluate the biological function/activity, thereby determining the amount of the polypeptide in the sample.

VI. Compositions

[0133] In a further aspect of the disclosure, it is provided a composition, which composition comprising a neovascularization inducer in an effective amount to induce cerebrovascularization in a subject having Intracranial atherosclerotic stroke (ICAS) or stroke.

[0134] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, IL4, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD, SDF-1 alpha, or a combination thereof.
In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the composition further comprises a pharmaceutically acceptable carrier.

In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises monocytes.

In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer are monocytes that are obtained from blood of the subject and cultured in a culture medium prior to use.

The pharmaceutical composition described herein may be administered to a subject in need of treatment by a variety of routes of administration, including orally and parenterally, (e.g., intravenously, subcutaneously or intramedullary), intranasally, as a suppository or using a "flash" formulation, i.e., allowing the medication to dissolve in the mouth without the need to use water, topically, intradermally, subcutaneously and/or administration via mucosal routes in liquid or solid form. The pharmaceutical composition can be formulated into a variety of dosage forms, e.g., extract, pills, tablets, microparticles, capsules, oral liquid. The term "compound" is used interchangeably with "agent" or "molecule."

There may also be included as part of the pharmaceutical composition pharmaceutically compatible binding agents, and/or adjuvant materials. The active materials can also be mixed with other active materials including antibiotics, antifungals, other virucidal and immunostimulants which do not impair the desired action and/or supplement the desired action.

In one embodiment, the mode of administration of the pharmaceutical composition described herein is oral. Oral compositions generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the aforesaid compounds or agents may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like. Some variation in dosage will necessarily occur, however, depending on the condition of the subject being treated. These preparations should produce a serum concentration of active ingredient of from about 0.01 nM to 1,000,000 nM, e.g., from about 0.2 to 40 .mu.M. A preferred concentration range is from 0.2 to 20 .mu.M and most preferably about 1 to 10 .mu.M. However, the concentration of active ingredient in
the drug composition itself depends on bioavailability of the drug and other factors known to those of skill in the art.

[0141] In another embodiment, the mode of administration of the pharmaceutical compositions described herein is topical or mucosal administration. A specifically preferred mode of mucosal administration is administration via female genital tract. Another preferred mode of mucosal administration is rectal administration.

[0142] Various polymeric and/or non-polymeric materials can be used as adjuvants for enhancing mucoadhesiveness of the pharmaceutical composition disclosed herein. The polymeric material suitable as adjuvants can be natural or synthetic polymers. Representative natural polymers include, for example, starch, chitosan, collagen, sugar, gelatin, pectin, alginate, karya gum, methylcellulose, carboxymethylcellulose, methylethyl cellulose, and hydroxypropylcellulose. Representative synthetic polymers include, for example, poly(acrylic acid), tragacanth, poly(methylvinylether-co-maleic anhydride), poly(ethylene oxide), carbopol, poly(vinyl pyrrolidone), poly(ethylene glycol), poly(vinyl alcohol), poly(hydroxyethylmethacrylate), and polycarbophil. Other bioadhesive materials available in the art of drug formulation can also be used (see, for example, Bioadhesion—Possibilities and Future Trends, Gurny and Junginger, eds., 1990).

[0143] It is to be noted that dosage values also varies with the specific severity of the disease condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted to the individual need and the professional judgment of the person administering or supervising the administration of the aforesaid compositions. It is to be further understood that the concentration ranges set forth herein are exemplary only and they do not limit the scope or practice of the disclosure. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

[0144] The formulation may contain the following ingredients: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, corn starch and the like; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; and a sweetening agent such as sucrose or saccharin or flavoring agent such as peppermint, methyl salicylate, or orange flavoring may be added. When the dosage unit form is a capsule, it may contain, in addition to material of the above type, a liquid carrier such as a fatty oil. Other dosage unit forms may contain other various materials which modify the physical form of the dosage unit, for example, as coatings. Thus tablets or pills may be coated with sugar, shellac,
or other enteric coating agents. Materials used in preparing these various compositions should be pharmaceutically pure and non-toxic in the amounts used.

[0145] The solutions or suspensions may also include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methylparabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0146] The pharmaceutical compositions can be prepared as formulations with pharmaceutically acceptable carriers. Preferred are those carriers that will protect the active compound or agent against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as polyanhydrides, polyglycolic acid, collagen, and polylactic acid. Methods for preparation of such formulations can be readily performed by one skilled in the art.

[0147] Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) are also preferred as pharmaceutically acceptable carriers. Methods for encapsulation or incorporation of compounds into liposomes are described by Cozzani, I.; Jori, G.; Bertoloni, G.; Milanesi, C.; Sicuro, T. Chem. Biol. Interact. 53, 131-143 (1985) and by Jori, G.; Tomio, L.; Reddi, E.; Rossi, E. Br. J. Cancer 48, 307-309 (1983), for example. These may also be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811 (which is incorporated herein by reference in its entirety). For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

[0148] Other methods for encapsulating compounds within liposomes and targeting areas of the body are described by Sicuro, T.; Scarcelli, V.; Vigna, M. F.; Cozzani, I. Med. Biol.

[0149] The pharmaceutical composition described herein may be administered in single (e.g., once daily) or multiple doses or via constant infusion. The compounds may also be administered alone or in combination with pharmaceutically acceptable carriers, vehicles or diluents, in either single or multiple doses. Suitable pharmaceutical carriers, vehicles and diluents include inert solid diluents or fillers, sterile aqueous solutions and various organic solvents. The pharmaceutical compositions formed by combining the compounds of this disclosure and the pharmaceutically acceptable carriers, vehicles or diluents are then readily administered in a variety of dosage forms such as tablets, powders, lozenges, syrups, injectable solutions and the like. These pharmaceutical compositions can, if desired, contain additional ingredients such as flavorings, binders, excipients and the like according to a specific dosage form.

[0150] Thus, for example, for purposes of oral administration, tablets containing various excipients such as sodium citrate, calcium carbonate and/or calcium phosphate may be employed along with various disintegrants such as starch, alginic acid and/or certain complex silicates, together with binding agents such as polyvinylpyrrolidone, sucrose, gelatin and/or acacia. Additionally, lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc are often useful for tableting purposes. Solid compositions of a similar type may also be employed as fillers in soft and hard filled gelatin capsules. Preferred materials for this include lactose or milk sugar and high molecular weight polyethylene glycols. When aqueous suspensions or elixirs are desired for oral administration, the active pharmaceutical agent therein may be combined with various sweetening or flavoring agents, coloring matter or dyes and, if desired, emulsifying or suspending agents, together with diluents such as water, ethanol, propylene glycol, glycerin and/or combinations thereof.

[0151] For parenteral administration, solutions of the compounds of this disclosure in sesame or peanut oil, aqueous propylene glycol, or in sterile aqueous solutions may be employed.

[0152] Such aqueous solutions should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, the sterile aqueous media employed are all readily available by standard techniques known to those skilled in the art.
For intranasal administration or administration by inhalation, the compounds of the
disclosure are conveniently delivered in the form of a solution or suspension from a pump
spray container that is squeezed or pumped by the patient or as an aerosol spray presentation
from a pressurized container or a nebulizer, with the use of a suitable propellant, e.g.,
dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide
or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined
by providing a valve to deliver a metered amount. The pressurized container or nebulizer may
contain a solution or suspension of a compound of this disclosure. Capsules and cartridges
(made, for example, from gelatin) for use in an inhaler or insufflator may be formulated
containing a powder mix of a compound or compounds of the disclosure and a suitable
powder base such as lactose or starch.

The pharmaceutical composition provided herein can also be used with another
pharmacologically active agent effective for a disease such as neurodisorders, cardiovascular
disorders, tumors, AIDS, depression, and/or type-1 and type-2 diabetes. Such additional
agents can be, for example, antiviral agent, antibiotics, anti-depression agent, anti-cancer
agents, immunosuppressant, anti-fungal, and a combination thereof.

The pharmaceutical composition described herein can be formulated alone or
together with the other agent in a single dosage form or in a separate dosage form. Methods
of preparing various pharmaceutical formulations with a certain amount of active ingredient
are known, or will be apparent in light of this disclosure, to those skilled in this art. For
examples of methods of preparing pharmaceutical formulations, see Remington's

In one embodiment, if desirable, the composition of disclosure can be formulated
into a scaffold. Such a scaffold can include a carrier, which can be biodegradable, such as
degradable by enzymatic or hydrolytic mechanisms. Examples of carriers include, but are not
limited to synthetic absorbable polymers such as such as but not limited to poly(-alpha-
hydroxy acids) such as poly (L-lactide) (PLLA), poly (D, L-lactide) (PDLLA), polyglycolide
(PGA), poly (lactide-co-glycolide (PLGA), poly (-caprolactone), poly (trimethylene
carbonate), poly (p-dioxanone), poly (-caprolactone-co-glycolide), poly (glycolide-co-
trimethylene carbonate) poly (D, L-lactide-co-trimethylene carbonate), polyarylates,
polyhydroxybutyrate (PUB), polyanhydrides, poly (anhydride-co-imide), propylene-co-
fumarates, poly lactones, polyesters, polycarbonates, polyanionic polymers, polyanhydrides,
polyester-amides, poly(amo-no-acids), homopolypeptides, poly(phosphazenes), poly
(glaxanone), polysaccharides, and poly(orthoesters), polyglactin, polyglactic acid,
polyaldonic acid, polyacrylic acids, polyalkanoates; copolymers and admixtures thereof, and any derivatives and modifications. See for example, U.S. Pat. No. 4,563,489, and PCT Int. Appl. # WO/03024316, herein incorporated by reference. Other examples of carriers include cellulosic polymers such as, but not limited to alkylcellulose, hydroxyalkylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropyl-methylcellulose, carboxymethylcellulose, and their cationic salts. Other examples of carriers include synthetic and natural bioceramics such as, but not limited to calcium carbonates, calcium phosphates, apatites, bioactive glass materials, and coral-derived apatites.

[0157] In one embodiment, the carrier may further be coated by compositions, including bioglass and or apatites derived from sol-gel techniques, or from immersion techniques such as, but not limited to simulated body fluids with calcium and phosphate concentrations ranging from about 1.5 to 7-fold the natural serum concentration and adjusted by various means to solutions with pH range of about 2.8-7.8 at temperature from about 15-65 degrees C. Other examples of carriers include collagen (e.g. Collastat, Helistat collagen sponges), hyaluronan, fibrin, chitosan, alginate, and gelatin, or a mixture thereof.

[0158] In one embodiment, the carrier may include heparin-binding agents; including but not limited to heparin-like polymers e.g. dextran sulfate, chondroitin sulfate, heparin sulfate, fucan, alginate, or their derivatives; and peptide fragments with amino acid modifications to increase heparin affinity. See for example, Journal of Biological Chemistry (2003), 278(44), p. 43229-43235, the teachings of which are incorporated herein by reference.

[0159] In one embodiment, the scaffold may be in the form of a liquid, solid or gel.

[0160] In one embodiment, the scaffold can be a carrier that is in the form of a flowable gel. The gel may be selected so as to be injectable, such as via a syringe at the site where bone formation is desired. The gel may be a chemical gel which may be a chemical gel formed by primary bonds, and controlled by pH, ionic groups, and/or solvent concentration. The gel may also be a physical gel which may be formed by secondary bonds and controlled by temperature and viscosity. Examples of gels include, but are not limited to, pluronics, gelatin, hyaluronan, collagen, polylactide-polyethylene glycol solutions and conjugates, chitosan, chitosan & b-glycerophosphate (BST-gel), alginates, agarose, hydroxypropyl cellulose, methyl cellulose, polyethylene oxide, polylactides/glycolides in N-methyl-2-pyrrolidone. See for example, Anatomical Record (2001), 263(4), 342-349, the teachings of which are incorporated herein by reference.
In one embodiment of the scaffold, the carrier may be photopolymerizable, such as by electromagnetic radiation with wavelength of at least about 250 nm. Example of photopolymerizable polymers include polyethylene (PEG) acrylate derivatives, PEG methacrylate derivatives, propylene fumarate-co-ethylene glycol, polyvinyl alcohol derivatives, PEG-co-poly(-hydroxy acid) diacrylate macromers, and modified polysaccharides such as hyaluronic acid derivatives and dextran methacrylate.

In one embodiment, the scaffold may include a carrier that is temperature sensitive. Examples include carriers made from N-isopropylacrylamide (NiPAM), or modified NiPAM with lowered lower critical solution temperature (LCST) and enhanced peptide binding by incorporation of ethyl methacrylate and N-acryloxsuccinimide; or alkyl methacrylates such as butylmethacrylate, hexylmethacrylate and dodecylmethacrylate (PCT Int. Appl. WO/2001070288; U.S. Pat. No. 5,124,151, the teachings of which are incorporated herein by reference).

In one embodiment of the scaffold, where the carrier may have a surface that is decorated and/or immobilized with cell adhesion molecules, adhesion peptides, and adhesion peptide analogs which may promote cell-matrix attachment via receptor mediated mechanisms, and/or molecular moieties which may promote adhesion via non-receptor mediated mechanisms binding such as, but not limited to polycationic polyamino-acid-peptides (e.g. poly-lysine), polyanionic polyamino-acid-peptides, Mefp-class adhesive molecules and other DOPA-rich peptides (e.g. poly-lysine-DOPA), polysaccharides, and proteoglycans. See for example, PCT Int. Appl. WO/2004005421; WO/2003008376; WO/9734016, the teachings of which are incorporated herein by reference.

In one embodiment of the scaffold, the carrier may be comprised of sequestering agents such as, but not limited to, collagen, gelatin, hyaluronic acid, alginate, poly(ethylene glycol), alkylcellulose (including hydroxyalkylcellulose), including methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, and carboxymethylcellulose, blood, fibrin, polyoxyethylene oxide, calcium sulfate hemihydrate, apatites, carboxyvinyl polymer, and poly(vinyl alcohol). See for example, U.S. Pat. No. 6,620,406, herein incorporated by reference.

In one embodiment of the scaffold, the carrier may include buffering agents such as, but not limited to glycine, glutamic acid hydrochloride, sodium chloride, guanidine, heparin, glutamic acid hydrochloride, acetic acid, succinic acid, polysorbate, dextran sulfate, sucrose, and amino acids. See for example, U.S. Pat. No. 5,385,887, herein incorporated by reference. In one embodiment, the carrier may include a combination of materials such as...
those listed above. By way of example, the carrier may be a PLGA/collagen carrier membrane.

[0168] In one embodiment, the scaffold can be an implant of the various embodiments described herein.

[0169] In one embodiment, the composition according to this disclosure may be contained within a time release tablet. A bioactive agent described herein can be formulated with an acceptable carrier to form a pharmacological composition. Acceptable carriers can contain a physiologically acceptable compound that acts, for example, to stabilize the composition or to increase or decrease the absorption of the agent. Physiologically acceptable compounds can include, for example, carbohydrates, such as glucose, sucrose, or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins, compositions that reduce the clearance or hydrolysis of the anti-mitotic agents, or excipients or other stabilizers and/or buffers. Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, for example, phenol and ascorbic acid. One skilled in the art would appreciate that the choice of a carrier, including a physiologically acceptable compound depends, for example, on the route of administration.

[0170] The composition can have a dosage of about 1 ng to about 500 mg, for example, about 10 ng, 20 ng, 50 ng, 100 ng, 200 ng, 500 ng, 1 micro gram, about 10 micro gram, about 50 micro gram, about 100 micro gram, about 200 micro gram, about 500 micro gram, or about 1 mg (or any range derivable therein).

[0171] Embodiments of the composition can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable may include powder, tablets, pills, capsules.

VII. Method of Making

[0172] In a further aspect of the present disclosure, it is provided a method of fabricating a composition, comprising providing an anti-angiogenic agent in a therapeutically effective amount and forming the composition.

[0173] In some embodiments, the anti-angiogenic agent is GMI-1070, aproteinase activated receptor (PAR) - 1 agonist, meziothrombin, granzyme A, activated protein C receptor - endothelial protein C receptor (APC-EPCR), kalikrein (KLK) 4, KLK5, KLK6, matrix metalloproteinase (MMP1), proatherocytin, pen C13; PAR-4 antagonists, tcY-NH₂ ;
heparan sulfate proteoglycans, analogues of heparin sulfate proteoglycans, aprotinin, or combinations thereof.

[0174] In a further aspect of the present disclosure, it is provided a method of fabricating a composition, comprising providing a neovascularization inducer in an effective amount to induce cerebrovascularization in a subject having Intracranial atherosclerotic stroke (ICAS) or stroke, and forming the composition.

[0175] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer is BMP2, BMP9, PDGF-AA, PDGF-BB, HGF, FGF2, KGF, HB-EGF, JLV, TPO, TGF-beta 1, TGF-beta 2, VEGF, VEGFD, SDF-1 alpha, or a combination thereof.

[0176] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the composition further comprises a pharmaceutically acceptable carrier.

[0177] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer comprises monocytes.

[0178] In some embodiments of the disclosure, optionally in combination with any or all of the various embodiments disclosed herein, the neovascularization inducer are monocytes that are obtained from blood of the subject and cultured in a culture medium prior to use.

[0179] Before the peptide synthesis starts, the amine terminus of the amino acid (starting material) can protected with FMOC (9-fluoromethyl carbamate) or other protective groups, and a solid support such as a Merrifield resin (free amines) is used as an initiator. Then, step (1) through step (3) reactions are performed and repeated until the desired peptide is obtained: (1) a free-amine is reacted with carboxyl terminus using carbodiimide chemistry, (2) the amino acid sequence is purified, and (3) the protecting group, e.g., the FMOC protecting group, is removed under mildly acidic conditions to yield a free amine. The peptide can then be cleaved from the resin to yield a free standing peptide or peptide mimetics.

VIII. Combination Therapy

[0180] Any of the disclosed therapies described herein may be used in any particular combination. The compositions and related methods may also be used in combination with the administration of traditional therapies.
[0181] The therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agents and/or a proteins or polynucleotides are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the therapeutic composition would still be able to exert an advantageously combined effect on the subject. In such instances, it is contemplated that one may administer both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for administration significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

[0182] Various combinations of therapy may be employed, for example if one therapy is labeled "A" and another therapy is labeled "B":

\[ \text{A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B} \]

\[ \text{B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A} \]

[0183] Administration of the compositions of the disclosure to a patient/subject will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the composition. It is expected that the treatment cycles would be repeated as necessary. It is also contemplated that various standard therapies, such as hydration, may be applied in combination with the described therapy.

IX. Examples

[0184] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.
EXAMPLE 1

[0185] Intracranial atherosclerosis (ICAS) is one of the most common causes of stroke worldwide. It accounts for at least 10% of all strokes in the United States and as much as 67% in countries with predominantly Asians, Hispanics, and Blacks, which constitute the majority of the world population. ICAS carries a worse prognosis than other stroke etiologies, with an annual rate of recurrent stroke and death of 15% despite intensive medical management, and as high as 35% in certain populations. Recent randomized controlled clinical trials have shown that angioplasty, stenting, and bypass surgery fail to improve outcomes in ICAS. The prevalence, poor prognosis, and lack of treatment options for ICAS point to a pressing need to identify new targets for developing improved therapeutic strategies. Some of the promising initial works of Arenillas and Navarro-Sobrino have identified angiogenesis as a potential contributor to the pathophysiology of ICAS. Encephaloduroarteriosynangiosis (EDAS), an alternative treatment currently under evaluation, is a form of indirect revascularization, which utilizes the ability of donor arteries to form new vessels. However, a comprehensive investigation of angiogenic factors in the response to intensive medical management and operative treatments, like EDAS, has not been conducted.

[0186] Applicants conducted a prospective cohort study in order to evaluate the role of circulating angiogenic factors in patients with ICAS. The hypotheses tested were (1) outcome phenotypes of failure of medical management in patients with ICAS are associated with endogenous antiangiogenic profiles, and (2) among individuals failing medical management, favorable angiogenic profiles are associated to quantitative evidence of angiographic neovascularization. The specific objectives of this study were to determine the association between circulating angiogenic profiles in patients with ICAS and the response to medical management in terms of stroke, transient ischemic attack (TIA), and functional clinical status at 6 months; and to surgical management in terms of angiographic neovascularization after EDAS surgery.

A. MATERIALS AND METHODS

1. Population and study design

[0187] This is a prospective longitudinal cohort study of patients diagnosed with ICAS in 4 centers in the Los Angeles Area: UCLA Ronald Reagan Hospital, Greater Los Angeles VA Hospital, Olive View UCLA County Hospital and Harbor UCLA Hospital. Patients aged ≥25
and <85 years with a diagnosis of severe ICAS (>70% stenosis) of any intracranial artery as measured by catheter, CT, or MR angiography between 2012 and 2014 were considered for inclusion. Independent neurologists of the stroke services determined patients' eligibility for the study, based on the inclusion and exclusion criteria summarized in Table 1. The study received approval from the local Institutional Review Boards.

[0188] Upon enrollment, medical management was optimized for all patients as detailed below, and they were followed to determine treatment success. A patient's phenotype was defined as "non-responder" to medical management if they had any signs of stroke or TIA events, or "responder" if the patient was asymptomatic. Demographic data, cardiovascular risk-factor history, smoking history, physical activity status, medications, detailed neurological examination, and modified Rankin Score (mRS) were recorded. Baseline blood chemistry, lipid panel, and HbAlc were obtained.

[0189] Patients with recurrent symptoms despite adequate medical management were considered candidates for surgical revascularization and, if eligible, they followed the management protocols of the "EDAS Revascularization for Symptomatic Intracranial Arterial Stenosis Study" (ClinicalTrials.gov identifier: NCT01819597).

2. Interventions

[0190] Medical management: Immediately upon enrollment, all patients underwent intensive medical treatment, with management of the primary risks factors (systolic blood pressure and low density lipoprotein [LDL]), secondary risk factors (diabetes, non-high-density lipoprotein [non-HDL], smoking, weight, and exercise) and antiplatelet medications, following the principles of the SAMMPRIS trial medical regimen.13

[0191] Surgical management: The EDAS technique has been described in detail in prior publications.14 Patients eligible for surgery continued intensive medical management with no interruption. The EDAS surgeries were performed at the Ronald Reagan Hospital at UCLA.

[0192] Follow up: All patients were initially managed medically and underwent follow ups at 2 weeks, one, three, and six months after optimization, or as needed if they presented TIAs or stroke. Once patients failed medical management, they were considered for surgery. Surgical patients received follow ups at one, three, and six months, or as needed if they presented TIAs or stroke, and had catheter angiograms at 6 months. Neurologists from the UCLA Stroke Center (not involved in the analytical evaluation of the angiogenic factors) defined the clinical endpoints. They also determined the modified Rankin scores for each patient at every evaluation point. Patient adherence to the scheduled visits was monitored by
the research coordination center and transport services were provided to encourage patient adherence to the follow up protocol.

[0193] **Angiogenic factor measurements:** Blood samples were collected at each visit. Blood was extracted in EDTA tubes, serum was immediately separated by centrifugation at 3500rpm for 15 minutes at 4°C, and stored in 2 cc cryovials at -80°C. Quantitative measurement of human angiogenic factors was performed by using enzyme-linked immunosorbent assay (ELISA Quantikine®, R&D Systems, MN; RayBio®, Raybiotech, GA; and Ciraplex® Aushon Biosystems, MA, USA) to detect plasma level of vascular endothelial growth factor (VEGF) isoforms 165a and 165b, VEGF-D, soluble VEGF receptor (VEGFR)1, VEGFR2, VEGFR3, platelet-derived growth factor (PDGF)-AA, PDGFF-BB, fibroblast growth factor (FGF) b, hepatocyte growth factor (HGF), heparin-binding epidermal growth factor (FIB-EGF), transforming growth factor (TGF) β1, TGF β2, bone morphogenic protein (BMP)-2, BMP-9, stromal cell derived factor (SDF)-1, interleukin (IL) 4, thrombospondin (TSP)-1, TSP-2, endostatin, and angiostatin. Each sample was analyzed twice, with the mean of the two values reported as the factor level. The mean intra-assay coefficient of variation (CV) was established with a threshold of less than 20% CV to consider a measurement valid.

3. **Statistical considerations**

[0194] Descriptive statistics were computed to summarize demographic and angiogenic factor data. A logarithmic transformation was used for correction to the angiogenic factors data skewness (Transformed angiogenic factor [Tr Angiogenic Factor]= (\(^\wedge\)n Angiogenic factor +1)). Principal component analysis (PCA) was applied to the angiogenic factors at each time point to reduce data dimensionality and to derive the equations for composite indexes that were denominated as angiogenic profiles (APs). As no p values are computed during the creation of the APs, this methodology does not increase the false discover|r rate, while capturing as much of the variability in the original variables as possible. APs with eigenvalues higher or equal to 1.0 were included in the inferential analysis. This threshold was selected as the components with eigenvalues above 1 accounted for 75% of the total variance.

[0195] To evaluate the association of the APs with the phenotypic outcome, first a stepwise logistic regression model was built using the APs as predictor variables. Then, the significantly associated APs were incorporated in a multivariable logistic regression including the relevant clinical predictor variables: age, gender, previous stroke, hypertension,
hypercholesterolemia, diabetes, and obesity (defined as a BMI ≥ 30). The fitness of each model was evaluated using R square and AUCs of the corresponding ROCs. Odds ratios were calculated for each variable from the regression parameters with its corresponding 95% confidence intervals.

[0196] The association of APs to the clinical functional outcome at 6 months was evaluated with an ordinal regression model for the outcome variable modified Rankin Score (mRS) including the following as predictor variables: age, gender, baseline mRS and the APs. In individuals failing medical management that underwent EDAS surgery, a "neovascularization index" was defined as the Perren grades plus an additional point if evidence of collaterals in the ASITN grades were observed in cerebral angiography at 6 months after surgery. A linear regression model was built using stepwise regression with minimum BIC stopping rule and backward direction for the outcome variable "neovascularization index," including as predictor variables: age, diabetes, smoking history, and the baseline APs. If more than one AP was associated with a specific response, the coefficients of the regression were used to resolve the final angiogenic profile associated with the outcome variable.

B. RESULTS

[0197] Eighty patients were considered eligible for the study in the period between July 2012 and December 2014. Six patients declined to participate. Seventy-four patients were enrolled in the study. Table 2 presents details of the demographic and risk factor characteristics of the patients enrolled. Mean age was 61.8 (SD=12.3, Range 28.6 to 83.3) years. There were 39 females (52.7%). The majority of the enrolled patients were non-Hispanic Whites. The most common location of the qualifying lesion was the middle cerebral artery (MCA). At presentation the most common mRS was 1 for both groups.

[0198] There were 45 patients (60.9%) that responded to medical management and 29 (39.1%) that did not. There were no large differences in age, gender, race, ethnicity, location of the qualifying lesion, or general cardiovascular risk factors between the "non-responder" and "responder" groups. Of the 29 non-responding cases, 3 had only TIAs, 17 had new strokes without TIAs, and 9 had both TIAs and strokes. Samples for patients non-responding to medical management were collected within 30 days of their symptoms in all cases. The mean time between symptoms and sample collection was 7.6 days (SD=7.2 Range 0 to 27 days).
Table 3 depicts the medians and interquartile range (IQR) for the baseline angiogenic factor measurements for each group. Transformed angiogenic factor values were used to conduct the principal component analysis. There were 7 APs with eigenvalues higher than 1. They were named consecutively API to AP7 in order of decreasing eigenvalue. The details of the coefficients for each transformed angiogenic factor value are provided in the Table 4 and a graphic representation of the composition of the APs is depicted in Figure 1.

1. **APs association to response to medical management**

A logistic regression model was built to evaluate the association of the APs with the phenotypic outcomes "responder" and "non-responder." The whole model reached statistical significance with a $p=0.001$, $RSquare = 0.24$, $AUC = 0.81$ with 68.9% sensitivity and 80% specificity. As shown in table 5, the only APs significantly associated to the outcome "non-Responder" was AP4 ($p=0.02$), odds of "non-responder" 2.1 (95% CI= 1.2-4.1). AP4 was then evaluated in a multivariable logistic regression including the relevant clinical predictor variables: age, gender, previous stroke, hypertension, hypercholesterolemia, diabetes, and obesity. The multivariable logistic regression model parameter results and odds ratios are described in table 6. The model reached statistical significance with a $p<0.0001$, $RSquare = 0.62$, misclassification rate = 0.09, $AUC = 0.96$ with 93.1% sensitivity and 89% specificity. In the multivariable model, AP4 was the only significant predictor parameter with a $p=0.003$, odds of "non-responder" 7.2 (95% CI=2.4-34.4). The AP4 is characterized by high levels of HGF, lower levels of the proangiogenic isoform VEGF165a, and high levels of the antiangiogenic factors VEGFR1, Endostatin and Angiostatin.

2. **APs association to functional outcomes at 6 months**

Applicants built a multivariable ordinal logistic regression model to evaluate the association of the APs to functional outcomes at 6 months from enrollment in terms of modified Rankin Score (mRS). The model, described in table 7, reached significance with a $p=0.01$, $RSquare = 0.23$ and a minimum $AUC = 0.82$. In the model, there was a significant association of AP4 at baseline to the 6-months mRS ($p=0.002$), independently of age, gender, and baseline mRS. A prediction profiler was constructed using the ordinal model with 5,000 simulation runs of random factor settings to facilitate the interpretation of the association between AP4 and the outcome 6-months mRS. Figure 2 shows the graphical query of the factor AP4 in the multivariate model as predictor variable for the calculated probabilities of the 6-months mRS. The slopes of the linear fits demonstrate negative parameters for mRS=0
(-0.1) and mRS=1 (-0.02), and positive parameters for the remaining mRS values from +0.01 for mRS=2 to +0.09 for mRS=5.

3. APs association to angiographic neovascularization after EDAS at 6 months

Among the 29 patients that failed medical management, 15 underwent EDAS revascularization. They were 5 males and 10 females, with a mean age 60.7 (SD=12.7 Range 32.1 to 83.3) years. Eleven patients completed angiographic follow up at 6 months after surgery. A "neovascularization index" was calculated by determining the Perren grades and then adding a point if the angiogram demonstrated new collaterals in the ASITN grades. Stepwise regression with minimum Bayesian information criterion (BIC) stopping rule and backward direction was used to build a regression model for the outcome variable "neovascularization index." This model, described in table 8, reached statistical significance with a p=0.02 and RSquare=0.93. Significant predictors of the degree of revascularization included age, diabetes and the APs 1 to 6 at baseline. The solution of the regression formula for the significant AP components revealed a strong negative association of the antiangiogenic factors endostatin and angiostatin. Figure 3 illustrates the coefficients of the resolved formula for all significant APs in association with the neovascularization index.

C. DISCUSSION

Angiogenesis has been extensively studied in peripheral vascular disease and myocardial ischemia. However, limited investigations have been conducted evaluating the role of angiogenesis in stroke and intracranial atherosclerosis. The pioneering works of Arenillas and Navarro-Sobrino have shown that angiogenesis and the balance of pro and antiangiogenic factors may have an influence in acute stroke and in the evolution of intracranial atherosclerosis. Arenillas et al. performed a study on 40 patients with ICAS with a single time measurement of VEGF and endostatin. They found that the ratio endostatin/VEGF was positively correlated to the extent of ICAS, and that endostatin was an independent predictor of new ischemic strokes and TIAs (Hazard ratio 7.2; CI: 1.6-33.8, p=0.01). Navarro-Sobrino et al. conducted a study in 108 patients with acute stroke of various etiologies (60% cardioembolic, 20% atherothrombotic, and 20% cryptogenic). They measured 11 promoters and 4 antiangiogenic factors in patients who presented with acute ischemic strokes affecting the middle cerebral artery and that were treated with IV rTPA.
They showed that an acute anti-angiogenesis status determined by high endostatin plasma level was associated to worse outcomes in terms of mRS at 3 months.\textsuperscript{18} 

[0204] Applicants conducted a prospective cohort study in a population of 72 patients with confirmed ICAS of at least 70\% of intracranial vessels who underwent strict medical management following the SAMMPRIS protocol recommendations and followed them for a period of 6 months. Applicants measured 13 proangiogenic and 8 antiangiogenic factors and evaluated their association to failure of medical management, functional status at 6 months and, in those patients failing medical management and undergoing EDAS surgery, angiographic neovascularization. Our results show a strong association between circulating antiangiogenic profile AP4 and failure to medical management, poor functional status, and reduced neovascularization. The antiangiogenic profile was a stronger predictor of failure than other clinical factors, including a history of previous stroke, diabetes, and age. The antiangiogenic profile was also a predictor of poor functional status independent of baseline mRS and age. Lastly, digital subtraction angiographic evaluation of patients that received an indirect revascularization with EDAS showed a negative correlation between neovascularization and the antiangiogenic factors endostatin and angiostatin. Altogether the results of this study strikingly point to the importance of antiangiogenesis as a component of ICAS.

[0205] Applicants used PCA to define the angiogenic profiles, which allows for reduction of data dimensionality in factors expected to be highly correlated, as those involved in angiogenesis, while minimizing loss of information. In addition, PCA provides insights into patterns and relationships in the data.\textsuperscript{20} This approach allowed us to overcome, to some extent, the current limitations in the biological understanding of all the interactions and roles of the proangiogenic and antiangiogenic factors in response to cerebral ischemia in ICAS. Amongst the angiogenic profiles defined to explain the variance between angiogenic factors, AP4 was the most antiangiogenic, based on its PCA coefficients. AP4 was found to be statistically significant with regards to failure to medical management and outcomes at 6 months. The statistical approach revealed high correlations among different inhibitors of angiogenesis that constituted the AP4: endostatin, angiostatin and VEGFRI, which vary together. It also showed that when more than one AP was significantly associated to an outcome, as in this study occurred with the outcome neovascularization index, the solution of the model revealed a predominant correlation between endostatin and angiostatin and a strong negative association with neovascularization. Our study confirms the previously suggested
association of endostatin with progression of the disease and reveals that ICAS has a complex antiangiogenic profile dependence involving also angiotatin and VEGFR1.

[0206] In summary, this prospective cohort study of patients with severe ICAS identified a circulating angiogenic profile, characterized by a high correlation between factors HGF, endostatin, angiotatin, and VEGFR-1, which was independently and significantly associated with failure to medical management and poor functional status at 6 months. It also showed a profile dominated by endostatin and angiotatin as significant negative predictor of neovascularization after EDAS surgery. This angiogenic profile exhibited the highest contribution of antiangiogenic factors compared to the other profiles that describe the dataset.

Altogether, these findings identify antiangiogenesis as a key component of ICAS failure to medical management, demarcating a novel research direction with the possibility of defining a new pharmaceutical field centered on drugs which decrease endogenous antiangiogenesis in order to protect against stroke and other cardiovascular conditions.

D. Tables

15 STROBE Statement—Checklist of items that should be included in reports of cohort studies

<table>
<thead>
<tr>
<th>Item No</th>
<th>Recommendation</th>
</tr>
</thead>
</table>
| Title and abstract | (a) Indicate the study's design with a commonly used term in the title or the abstract  
(b) Provide in the abstract an informative and balanced summary of what was done and what was found |
| Introduction |  
Background/rationale | Explain the scientific background and rationale for the investigation being reported  
Objectives | State specific objectives, including any prespecified hypotheses  
Methods |  
Study design | Present key elements of study design early in the paper  
Setting | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection  
Participants | (a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up  
(b) For matched studies, give matching criteria and number of exposed and unexposed  
Variables | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable  
Data sources/measurement | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe |
comparability of assessment methods if there is more than
one group

<table>
<thead>
<tr>
<th>Bias</th>
<th>9</th>
<th>Describe any efforts to address potential sources of bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study size</td>
<td>10</td>
<td>Explain how the study size was arrived at</td>
</tr>
<tr>
<td>Quantitative variables</td>
<td>11</td>
<td>Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why</td>
</tr>
</tbody>
</table>
| Statistical methods | 12| (a) Describe all statistical methods, including those used to control for confounding  
(b) Describe any methods used to examine subgroups and interactions  
(c) Explain how missing data were addressed  
(d) If applicable, explain how loss to follow-up was addressed  
(g) Describe any sensitivity analyses |
| Results       | |
| Participants  | 13*| (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed  
(b) Give reasons for non-participation at each stage  
(c) Consider use of a flow diagram |
| Descriptive data | 14*| (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders  
(b) Indicate number of participants with missing data for each variable of interest  
(c) Summarise follow-up time (eg, average and total amount) |
| Outcome data  | 15*| Report numbers of outcome events or summary measures over time |
| Main results  | 16| (a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included  
(b) Report category boundaries when continuous variables were categorized  
(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period |
| Other analyses| 17| Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses |
| Discussion    | |
| Key results   | 18| Summarise key results with reference to study objectives |
| Limitations   | 19| Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias |
| Interpretation| 20| Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from |
similar studies, and other relevant evidence

Generalisability 21 Discuss the generalisability (external validity) of the study results

Other information
Funding 22 Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting.

Table 1

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
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<tr>
<td>1. 70% to 99% ICAS of a major intracranial artery diagnosed by angio, TCD, MRA, or CTA.</td>
<td>1. Intracranial tumor or vascular malformation.</td>
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<td>2. Patient is willing and able to return for all follow-up visits required by the protocol.</td>
<td>2. Any hemorrhagic infarct within 14 days before enrollment or any other intracranial hemorrhage (subarachnoid, subdural, or epidural) within 30 days.</td>
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<td>3. Patient understands the purpose and requirements of the study, can make him- or herself understood, and has provided consent.</td>
<td>3. Intracranial arterial stenosis related to arterial dissection, moyamoya disease, or any known infectious or vasculitic disease.</td>
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<td>4. Presence of any unequivocal cardiac sources of embolism.</td>
<td>5. Major surgery (including open femoral, aortic, or carotid surgery) within previous 30 days or planned in the next 180 days after enrollment.</td>
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<td>6. Severe neurologic deficit that renders the patient not independent.</td>
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Table 2

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<th>Responder</th>
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<td>SD</td>
<td>Mean</td>
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<tr>
<td>VEGFA 165a (pg/ml)</td>
<td>Median (IQR)</td>
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<td>1016.1 (703.5)</td>
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<td>Median (IQR)</td>
<td>84.0 (149.9)</td>
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<td>HBEGF (pg/ml)</td>
<td>Median (IQR)</td>
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<td>1.4 (1.5)</td>
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<td>Median (IQR)</td>
<td>122.9 (36.5)</td>
<td>110.9 (26.1)</td>
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<td>Median (IQR)</td>
<td>29.3 (29.5)</td>
<td>30.9 (40.3)</td>
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<td>SDF1 (pg/ml)</td>
<td>Median (IQR)</td>
<td>3890.4 (4210.0)</td>
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<td>IL4 (pg/ml)</td>
<td>Median (IQR)</td>
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<td>0.0 (0.2)</td>
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<td>VEGFR1 (pg/ml)</td>
<td>Median (IQR)</td>
<td>178.1 (156.1)</td>
<td>114.4 (71.5)</td>
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<td>VEGFR2 (pg/ml)</td>
<td>Median (IQR)</td>
<td>13334.4 (3724.0)</td>
<td>14594.8 (4727.9)</td>
</tr>
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</table>

Table 3
Table 4 (Can be supplementary table 1)

### Composite Angiogenic Profiles (APs) Constructed by Principal Component Analysis

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<thead>
<tr>
<th></th>
<th>AP-1</th>
<th>AP-2</th>
<th>AP-3</th>
<th>AP-4</th>
<th>AP-5</th>
<th>AP-6</th>
<th>AP-7</th>
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<tbody>
<tr>
<td>Tr VEGFA165a</td>
<td>0.051</td>
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<td>-0.243</td>
<td>0.019</td>
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Table 8
EXAMPLE 2

A. Introduction

[0207] Ischemic stroke is the leading cause of adult disability and the second cause of all death worldwide. One of the most common underlying pathologies is intracranial atherosclerosis (ICAS). Recently, a surgical indirect revascularization procedure to supplement blood flow to hypoperfused areas, Encephaloduro-arteriosynangiosis (EDAS), has been proposed for the treatment of IAS. Encephalomyosynangiosis (EMS), a similar form of indirect revascularization, uses the temporals muscle rather than the superficial temporal artery. While EDAS is promising as a treatment for ICAS, the angiogenic mechanisms, which cause vessel growth are poorly understood. In this Example, Applicants present the development of a reproducible animal model of ischemia and synangiosis, and evaluated the development of neovascularization on the surgical side.

B. Methods

[0208] Applicants used a modification of the MCA ischemia model developed by Tamura et al. Healthy 12-24 week old male (average weight 28 g) C57/BL6J mice were employed. After induction of general anesthesia in a custom-made rotating stereotactic frame, a midline incision was performed measuring 15 mm in length. The periosteum was elevated and the temporalis muscle was incised at the temporals line level. The muscle was elevated in block until the zygomatic arch was visualized. Then a high speed micro-drill was used to perform a craniectomy on the squama of the temporals bone. A distal branch of the MCA is consistently found at this level and accessible for surgical ligature. This allows for the generation of a local area of ischemia that is usually not larger than 8% of the hemispheric volume. Then, the temporalis muscle is used as donor for the external carotid artery vessels.

[0209] The muscle is reattached to the bone with 2 10-O nylon sutures ensuring close approximation of the surfaces of the muscle and the brain. The field is then irrigated and the wound is closed with interrupted stitches. All animals postoperative care and euthanasia were performed according to the Guidelines of the NIH and the Chancellors Animal Research Committee at UCLA. After euthanasia mice were perfused transcardially with PBS followed by 4% paraformaldehyde. Tissues were paraffin-embedded after decalcification and section in sequential coronal \( \Omega \mu \pi \) slides. Specimens underwent H&E staining along with immunofluorescence studies for markers of cellular proliferation (Ki67), endothelial cell (CD-31) and nuclei (DAPI). For immunofluorescence sections were deparaffinezed,
incubated in citrate at 100°C for 20 minutes, administered a blocking agent, then incubated with primary antibodies at 1:100 dilution at 4°C over night. The secondary antibodies used are Alexa Fluor 567 and 488 at 1:200 dilution.

Sections were visualized with stereo and confocal microscopy. Quantitative analysis of fluorescence intensity was done using Image J (version 1.48 NTH). Colocalization was performed using BioImageXD (version 1.0) Normalized indexes for cell proliferation and endothelial cell density were created with cell proliferation represented by Ki67/DAPI intensity and endothelial cell density by CD31/DAPI intensity. Indexes were compared using two sample t-test while colocalization statistical analysis was performed using the Manders' Coefficient Method.

C. Results

A total of 12 mice were operated. One subject died after surgery (attrition rate 8%). All other subjects displayed no deficits. The number of DAPI+ cells/image was no different between the synangiosis and the control side. The index CD31/DAPI was significantly higher in the synangiosis side (mean 0.90 ± 1.13 vs. controls: 0.60 ± 0.65, p<0.001). The proliferation index Ki67/DAPI was significantly higher in the synangiosis side (mean 0.21 ± 0.08 vs. controls: 0.12 ± 0.05, p=0.03). Colocalization of endothelial cell marker CD-31 and nuclear proliferation marker Ki67 showed significant difference between the surgical and the control side (mean percentage 73.0 +/-8.3 vs. 33.6 +/-10.9, p=0.04).

D. Conclusion

Our model of synangiosis in mice was well tolerated by the animals with a low attrition rate, and produced quantifiable neovascularization in the brain which was significantly higher in the synangiosis side. This model provides a reliable quantitative tool for future studies of neovascularization and collateral formation in individuals with ischemia.

EXAMPLE 3

A. Introduction

Recent research in the isoforms of the VEGF A has shown an important role for the balance of the pro-angiogenic VEGF-A (isoform 165a) and the anti-angiogenic VEGF-A (isoform 165b) in atherosclerotic disease of the peripheral vessels. The anti-angiogenic isoform of VEGF-A (165b) is a powerful competitive innate inhibitor of the angiogenic effects of VEGF-A. This isoform is only found in complex mammal species, and is one of the
multiple mechanisms controlling angiogenesis that appear as individuals become more complex in the evolutionary phylogenetic tree. Intracranial atherosclerosis (ICAS) is one of the most common causes of stroke worldwide. Despite intensive medical management, the current therapeutic strategies focus in stabilization of the risk factors associated with its development, i.e. anti platelets, blood pressure control and statins. A deeper understanding of alternative paths that may contribute to the development of the disease or collaterals to prevent stroke could open new avenues for treatment. The objective of this study was to evaluate the effects of IMM and EDAS in the VEGFA165a/b ratio in patients with ICAS.

B. Methods

This is a prospective observational study of VEGFA165a and VEGFA165b in patients with stenosis greater than 70% due to ICAS. All patients received Intensive Medical Management (IMM) following the model of medical management of the SAMMPRIS Trial. Patients with persistent symptoms underwent EDAS while maintaining FMM. Serum samples were collected at baseline, 1 week, 1, 3, and 6 months. VEGFA isoforms were quantified using multiplex sandwich ELISA. All samples were run in duplicate and accepted as valid if the intersample variability was less than 20%. A mixed model was built for the outcome variable VEGFA165a/b ratio using the predictor variables timepoint, treatment, and the interaction of time and treatment. The restricted maximum likelihood method was used to fit the model with random effects to account for the repeated measurements and inter-subject variability.

C. Results

Seventy-two patients with ICAS were enrolled. There were 58 that received IMM alone and 14 that failed IMM and had revascularizations with EDAS, while maintaining IMM.

Mean age was 61.8 ± 12.3, and 53% were females. The regression model demonstrated that there were no significant differences in the VEGFA165a/b ratio at baseline and 1 month after enrollment. Significant differences in VEGFA165a/b ratio were found at one week with higher levels in the surgical group (EDAS: 0.46 ± 0.22, IMM: 0.24 ± 0.07, p=0.03) and at 3 and 6 months with higher levels in the IMM group (3m: EDAS: 0.29 ± 0.14, IMM:0.45 ± 0.20, p=0.03, 6m: EDAS:0.19 ± 0.11, IMM 0.37 ± 0.19 p=0.01).

The mixed model analysis showed significant negative associations between baseline and VEGF-A 165 a/b ratio (p=0.05) and the interaction of being in the medical...
group at 1 week (p=0.0006) Table 2. There is a significant positive association between the interaction of medical management and 3 months after optimization of treatment and the levels of the VEGF-A 165 a/b ratio (p=0.006).

D. Conclusion

While the surgical event may well explain the early elevation of the VEGFA165a/b ratio one week after surgery, the elevation of a proangiogenic profile by the 3rd and 6th month in the IMM group is relevant. None of the patients in the IMM or EDAS groups had strokes in the last 6 months follow-up, and the peak (early for EDAS and at 3 and 6 months for IMM) of the VEGFA165a/b ratio may indicate a protective effect, averting stroke.

EXAMPLE 4

A. Introduction

Intracranial atherosclerosis (ICAS) is one of the most common causes of stroke worldwide. It accounts for at least 10% of all strokes in the United States and as much as 67%, in countries with predominantly Asians, Hispanics, and Blacks, which constitute the majority of the world population.

ICAS carries a worse prognosis than other stroke etiologies, with an annual rate of recurrent stroke and death of >15% despite intensive medical management, and as high as 35% in certain populations. The objective of this study was to identify circulating angiogenic factor profiles associated to success or failure of intensive medical treatment in ICAS patients.

B. Methods

This is a prospective observational study of circulating angiogenic factors in 72 patients with ICAS treated medically, strictly following the model of medical management of the SAMMPRIS Trial. Twenty-one pro and antiangiogenic factors were isolated from plasma in patients with intracranial arterial stenosis greater than 70% due to ICAS.

The following factors were analyzed: vascular endothelial growth factor (VEGF) isoforms 165a and 165b, VEGF-D, soluble VEGF receptor (VEGFR)1, VEGFR2, VEGFR3, platelet-derived growth factor (PDGF)-AA, PDGFF-BB, fibroblast growth factor (FGF) b, hepatocyte growth factor (HGF), heparin-binding epidermal growth factor (HB-EGF), transforming growth factor (TGF) β1, TGF β2, bone morphogenic protein (BMP)-2, BMP-9, stromal cell derived factor (SDF)-1, interleukin (IL) 4, thrombospondin (TSP)-1, TSP-2,
endostatin, and angiostatin. Levels of angiogenic factors were determined by plasma multiplex sandwich ELISA.

[0223] Samples were run in duplicate with variation coefficient lower than 20%. Patients were followed for 6 months and mRS were collected. Failure to medical management was defined as presence of TIA or stroke within 30 days of presentation. Angiogenic profiles (APs) were defined using Principal Component Analysis (PCA) Figure 1. Seven profiles were selected (Eigenvalue greater than 1.0). A logistic regression model was built for the outcome variable medical management failure/success using the 7 APs as predictors. An ordinal logistic regression model was built to predict the outcome variable mRS at 6 months using age, gender, baseline mRS and APs as predictors.

C. Results

[0224] Seventy-two patients with ICAS were included. Mean age was 61.8 (SD 12.3)yrs. There were 53% females. The predominant region of stenosis was the MCA. Of the 7 APs defined by PCA, the regression model identified AP-4 as significantly associated to failure of medical management (P=0.02).

[0225] AP-4 was characterized by high levels of HGF and the anti-angiogenic factors VEGFR1, Endostatin, Angiostatin, VEGFR3, and TSP2. At 6 months follow up, AP-4 was also positively associated with mRS, independently of baseline mRS and age (P=0.002).

D. Conclusion

[0226] In this extensive prospective study of angiogenic factors in patients with ICAS, a profile characterized by elevation of HGF and several circulating anti-angiogenic factors was strongly associated with failure to medical management and poor outcomes at 6 months. This finding supports the concept that neutralization of antiangiogenic factors may serve as a new pharmacological target to treat cerebral ischemia in patients with ICAS.

[0227] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar
substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. All references cited in this application are specifically incorporated by reference for all purposes.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.


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- 63 -


41. Rubin JS, Bottaro DP, Aaronson SA. Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product. *Biochimica et Biophysica Acta.* 1993;1155:357-371


44. Kuba K, Matsumoto K, Date K, Shimura H, Tanaka M, Nakamura T. Hgf/nk4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. *Cancer Res.* 2000;60:6737-6743


CLAIMS

1. A method for treating atherosclerosis in a subject comprising:
   administering a surgical treatment to a subject determined to have one or more of:
   increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI,
   increased levels of endostatin, increased levels of angioatin and/or increased levels of TSP-2.

2. The method of claim 1, wherein the method further comprises administering a non-
   surgical therapeutic to a subject determined to not have one or more of increased levels of
   HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of
   endostatin, increased levels of angioatin and/or increased levels of TSP2.

3. The method of claim 1 or 2, wherein the subject is determined to have all of:
   increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI,
   increased levels of endostatin, increased levels of angioatin and increased levels of TSP2.

4. The method of claim 1 or 2, wherein the subject is determined to have all of:
   increased levels of FIGF, decreased levels of VEGF165a, increased levels of VEGFRI,
   increased levels of endostatin, and increased levels of angioatin.

5. The method of claim 1 or 2, wherein the subject is determined to have all of:
   increased levels of HGF, increased levels of VEGFRI, increased levels of endostatin,
   increased levels of angioatin and increased levels of TSP2.

6. The method of any one of claims 1-2, wherein the atherosclerosis is intracranial
   atherosclerosis.

7. The method of any one of claims 1-6, wherein increased HGF comprises an increase
   in an FIGF polypeptide wherein the polypeptide is a fragment of the HGF protein.

8. The method of claim 7, wherein the fragment is NK1, NK2, NK3, or NK4 fragment.

9. The method of any one of claims 1-8, wherein the non-surgical treatment comprises
   one or more of an ACE inhibitor, an angiotension II receptor blocker, an antiarrhythmic, an
   antiplatelet, aspirin, a beta blocker, a calcium channel blocker, a clot buster, digoxin, a
   diuretic, a nitrate, or a blood thinner.
10. The method of any one of claims 1-8, wherein the surgical treatment comprises encephaloduroarteriosynangiosis, stent placement, angioplasty, heart bypass, valvuloplasty, enhanced external counterpulsation, ablation, pacemaker placement, implantation of a cardioverter defibrillator, lead extraction, left ventricular assist device placement, or heart transplant.

11. The method of any one of claims 1-10, wherein the angiogenic factor is two or more of vascular endothelial growth factor (VEGF) isoforms 165a (VEGF165a), VEGF165b, VEGF-D, VEGF receptor (VEGFR)1, VEGFR2, VEGFR3, platelet-derived growth factor (PDGF)-AA, PDGF-BB, fibroblast growth factor (FGF) b, hepatocyte growth factor (HGF), NK1, NK2, NK3, NK4, heparin-binding epidermal growth factor (FIB-EGF), transforming growth factor (TGF) β1, TGF β2, bone morphogenic protein (BMP)-2, BMP-9, stromal cell derived factor (SDF)-1, interleukin (IL) 4, thrombospondin (TSP)-1, TSP-2, endostatin, and angiostatin.

12. The method of any one of claims 1-11, wherein the method further comprises determining the levels of at least two angiogenic factors in the subject;

13. The method of claim 12, wherein the method further comprises comparing the level of the angiogenic factor to a control.

14. The method of claim 12, wherein the level of the angiogenic factor is compared to a control.

15. The method of any one of claims 1-14, wherein the method further comprises performing a statistical analysis of the levels, wherein the analysis is principal component analysis.

16. The method of any one of claims 1-15, wherein the increase or decrease in levels are assessed by a principal component analysis.

17. The method of any one of claims 1-16, wherein the method further comprises comparing the level to a cut-off value.

18. The method of claim 17, wherein an increased level is above a cut-off value and a decreased level is below a cut-off value.
19. The method of any one of claims 1-18, wherein the subject is a human patient.

20. The method of any one of claims 1-19, wherein the method further comprises administration of an anti-angiogenic blocking agent.

21. The method of claim 20, wherein the anti-angiogenic blocking agent is administered prior to surgery.

22. The method of claim 20, wherein the anti-angiogenic blocking agent is administered locally to an atherosclerotic region during surgery.

23. The method of any one of claims 20-22, wherein the anti-angiogenic blocking agent is GMI-1070, aproteinase activated receptor (PAR) - 1 agonist, mezirothrombin, granzyme A, activated protein C receptor - endothelial protein C receptor (APC-EPCR), kalikrein (KLK) 4, KLK5, KLK6, matrix metalloproteinase (MMP1), proatherocytin, pen C13; PAR-4 antagonists, tcY-NH₂; heparan sulfate proteoglycans, analogues of heparin sulfate proteoglycans, aprotinin, or combinations thereof.

24. The method of any one of claims 1-23, wherein the angiogenic factor is a circulating blood factor.

25. The method of any one of claims 1-24, wherein the levels of the at least two angiogenic factors are or were determined from a blood sample from the subject.


27. The method of claim 26, wherein the anti-angiogenic blocking agent is GMI-1070, aproteinase activated receptor (PAR) - 1 agonist, mezirothrombin, granzyme A, activated protein C receptor - endothelial protein C receptor (APC-EPCR), kalikrein (KLK) 4, KLK5, KLK6, matrix metalloproteinase (MMP1), proatherocytin, pen C13; PAR-4 antagonists, tcY-NH₂; heparan sulfate proteoglycans, analogues of heparin sulfate proteoglycans, aprotinin, or combinations thereof.

28. The method of claim 26, wherein the subject has undergone previous treatment for atherosclerosis.
29. The method of claim 28, wherein the subject was a poor or non-responder to the previous treatment.

30. The method of any one of claims 26-29, wherein the atherosclerosis is intracranial.

31. The method of any one of claims 26-30, wherein the method further comprises administering a surgical treatment to the subject.

32. The method of claim 31, wherein the anti-angiogenic blocking agent is administered prior to surgery.

33. The method of claim 31, wherein the anti-angiogenic blocking agent is concurrently to surgery.

34. The method of claim 33, wherein the anti-angiogenic blocking agent is administered locally to an atherosclerotic region during surgery.

35. An assay system comprising agents for detecting proteins, wherein the proteins comprise one or more of VEGF165a, VEGF165b, VEGF-D, VEGFR1, VEGFR2, VEGFR3, PDGF-AA, PDGFF-BB, FGFb, HGF, NK1, NK2, NK3, NK4, HB-EGF, TGF β1, TGF β2, BMP-2, BMP-9, SDF-1, IL 4, TSP-1, TSP-2, endostatin, and angiotatin.

36. The assay system of claim 35, wherein the proteins consist of HGF, VEGF165a, VEGFR1, endostatin, and angiotatin.

37. The assay system of claim 35, wherein the proteins consist of HGF, VEGFR1, endostatin, angiotatin, and TSP-2.

38. The assay system of claim 35, wherein the proteins consist of HGF, VEGF165a, VEGFR1, endostatin, angiotatin, and TSP-2.

39. The assay system of any one of claims 36-38, wherein the agent for detecting HGF comprises an agent that detects a fragment of the HGF protein.

40. The assay system of claim 39, wherein the fragment is NK1, NK2, NK3, or NK4 fragment.

41. The assay system of any one of claims 35-40, wherein the assay system further comprises one or more of a buffer, a detectable label, or a solid support.
42. The assay system of any one of claims 35-41, wherein the agent is an antibody.

43. The assay system of any one of claims 35-41, wherein the agent is labeled with a detectable label.

44. A method for predicting a subject's response to atherosclerosis medical treatment comprising:
   determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using the assay system of any one of claims 35-43; and
   predicting that the subject will respond poorly if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

45. A method for treating atherosclerosis in a subject comprising:
   determining the levels of at least two angiogenic factors in the subject; wherein the levels were or are determined using the assay system of any one of claims 35-44;
   administering a surgical treatment if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2; or
   administering a non-surgical therapeutic if the subject does not have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

46. A method for predicting a subject's response to atherosclerosis medical treatment comprising:
   determining the levels of at least two angiogenic factors in the subject; wherein the angiogenic factors comprise HGF, VEGF165a, VEGFR1, endostatin, angiostatin, and TSP-2; and
   predicting that the subject will respond poorly if the subject is determined to have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.
47. The method of claim 46, wherein the subject is predicted to respond poorly when the subject is determined to have: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of eiidostatin, increased levels of angiostatin, and increased levels of TSP-2.

48. The method of claim 46, wherein the subject is predicted to respond poorly when the subject is determined to have: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of eiidostatin, and increased levels of angiostatin.

49. The method of claim 46, wherein the subject is predicted to respond poorly when the subject is determined to have: increased levels of HGF, increased levels of VEGFRI, increased levels of eiidostatin, increased levels of angiostatin, and increased levels of TSP-2.

50. The method of any one of claims 46-49, wherein the subject has been diagnosed with atherosclerosis.

51. The method of claim 50, wherein the atherosclerosis is intracranial atherosclerosis.

52. The method of any one of claims 46-51, wherein the subject has not received any treatment for atherosclerosis.

53. The method of any one of claims 46-52, wherein increased HGF comprises an increase in an HGF polypeptide and wherein the polypeptide is a fragment of the HGF protein.

54. The method of claim 53, wherein the fragment is NK1, NK2, NK3, or NK4 factor.

55. The method of any one of claims 46-53, wherein the medical treatment is non-surgical.

56. The method of any one of claims 46-55, wherein the method further comprises administration of surgical treatment if the subject is predicted to respond poorly to medical treatment.

57. The method of claim 56, wherein the surgical treatment comprises encephaloduroarteriosynangiosis, stent placement, angioplasty, heart bypass, valvuloplasty, enhanced external counterpulsation, ablation, pacemaker placement, implantation of a
cardioverter defibrillator, lead extraction, left ventricular assist device placement, or heart transplant

58. The method of any one of claims 46-55, wherein the subject is predicted to respond to non-surgical medical treatment when the subject does not have one or more of increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiostatin, and/or TSP-2.

59. The method of claim 58, wherein the method further comprises administration of a non-surgical treatment.

60. The method of claim 59, wherein the non-surgical treatment comprises one or more of an ACE inhibitor, an angiotension II receptor blocker, an antiarrhythmic, an antiplatelet, aspirin, a beta blocker, a calcium channel blocker, a clot buster, digoxin, a diuretic, a nitrate, or a blood thinner.

61. The method of any one of claims 46-60, wherein the at least two angiogenic factors comprise two or more of VEGF165a, VEGF165b, VEGF-D, VEGFR1, VEGFR2, VEGFR3, PDGF-AA, PDGFF-BB, FGFb, HGF, NK1, NK2, NK3, NK4, HB-EGF, TGF β1, TGF β2, BMP-2, BMP-9, SDF-1, IL4, TSP-1, TSP-2, endostatin, and angiostatin.

62. The method of any one of claims 46-61, wherein the level of the angiogenic factor is compared to a control.

63. The method of any one of claims 46-62, wherein the method further comprises performing a statistical analysis of the levels, wherein the analysis is principal component analysis.

64. The method of any one of claims 46-63, wherein the increase or decrease in levels are assessed by a principal component analysis.

65. The method of any one of claims 46-64, wherein the method further comprises comparing the level to a cut-off value.

66. The method of claim 65, wherein an increased level is above a cut-off value and a decreased level is below a cut-off value.
67. The method of any one of claims 46-66, wherein the method further comprises treatment of the atherosclerosis with administration of an anti-angiogenic blocking agent.

68. The method of claim 67, wherein the anti-angiogenic blocking agent is administered prior to surgery.

69. The method of claim 67, wherein the anti-angiogenic blocking agent is administered locally to an atherosclerotic region during surgery.

70. The method of any one of claims 67-69, wherein the anti-angiogenic blocking agent is GMI-1070, a proteinase activated receptor (PAR) -1 agonist, meziothrombin, granzyme A, activated protein C receptor - endothelial protein C receptor (APC-EPCR), kalikrein (KLK) 4,KLK5, KLK6, matrix metalloproteinase (MMP1), proatherocytin, pen C13; PAR-4 antagonists, tcY-NH₂; heparan sulfate proteoglycans, analogues of heparin sulfate proteoglycans, aprotinin, or combinations thereof.

71. The method of any one of claims 46-70, wherein the angiogenic factor is a circulating blood factor.

72. The method of any one of claims 46-71, wherein the levels of the at least two angiogenic factors are or were determined from a blood sample from the subject.

73. Use of a surgical treatment for the treatment of atherosclerosis in a subject determined to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

74. Use of a non-surgical treatment for the treatment of atherosclerosis in a subject determined not to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFRI, increased levels of endostatin, increased levels of angiostatin, and/or increased levels of TSP-2.

75. Use of an anti-angiogenic blocking agent for treating atherosclerosis in a subject in need thereof.

76. Use of an anti-angiogenic blocking agent in the preparation of a medicament for treating atherosclerosis.
Y (Probability(mRS 6-months=0)) = 0.3022 - 0.1126X
Y (Probability(mRS 6-months=1)) = 0.2403 - 0.02233X
Y (Probability(mRS 6-months=2)) = 0.1271 + 0.01109X

FIG. 2A
Y (Probability(mRS 6-months=3)) = 0.09365 + 0.01911X
Y (Probability(mRS 6-months=4)) = 0.03485 + 0.009871X
Y (Probability(mRS 6-months=5)) = 0.2018 + 0.09502X

FIG. 2B
INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 17/50876

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, G01 N 33/50, G01 N 33/53, G01 N 33/68, A61 P 9/1 0 (201 7.01)
CPC - C12Q 2600/106, G01 N 2800/52, G01 N 33/5091, C12Q 1/68, G01 N 2800/60, G01 N 33/48, G01 N 33/68, G01 N 33/6893

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>&quot;GONZALEZ et al., Intracranial arterial stenoses: current viewpoints, novel approaches, and surgical perspectives. Neurosurg Rev. April 2013, Vol 36, No 2, pp 175-185 (author manuscript pp 1-15). Especially p 2, para 3; p 4, para 3; para p 5, para 4; p 6, para 2&quot;</td>
<td>1-2, 6, 73, 74</td>
</tr>
<tr>
<td>Y</td>
<td>&quot;US 2009/0175791 A1 (KAVILE et al.) 9 July 2009 (09.07.2009); para [0347], [0769], [0821], [0943]&quot;</td>
<td>3-5, 46-51</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. | See patent family annex.

- Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier application or patent but published on or after the international filing date
  - "U" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or which is of special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

- Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- Document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- Document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- Document member of the same patent family

Date of the actual completion of the international search: 6 July 2017
Date of mailing of the international search report: 27 Jul 2017

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-272-8300

Authorized officer: Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-6, 46-51, 73, 74, drawn to methods and uses comprising administering a surgical treatment to treat atherosclerosis to a subject determined to have one or more of: increased levels of HGF, decreased levels of VEGF165a, increased levels of VEGFR1, increased levels of endostatin, increased levels of angiotatin and/or increased levels of TSP-2

Group II: Claims 26-30, 75, 76, drawn to methods and uses comprising administering antiangiogenic blocking agent to treat atherosclerosis

Group III: Claims 35-40, drawn to an assay system comprising agents for detecting proteins

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-6, 46-51, 73, 74

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)
International application No.
PCT/IB 17/50876

Box No. Ill (Observations where unity of invention is lacking):

The inventions listed as Groups I, II, and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I requires surgical treatment of atherosclerosis, not required by Groups II and III.

Group II requires antiangiogenic blocking agent treatment of atherosclerosis, not required by Groups I and III.

Group III requires assay system comprising agents for detecting proteins, not required by Groups I and II.

Common Technical Features

The feature shared by Groups I and II is the treatment of atherosclerosis.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by the article entitled “Drug-Eluting Stents for the Treatment of Intracranial Atherosclerosis” by Abou-Chebl et al. (hereinafter ‘Abou-Chebl’).

Abou-Chebl teaches a method for treating atherosclerosis in a subject (Abstract “We present our experience with the feasibility and safety of using DES (drug-eluting stents) for patients with symptomatic intracranial atherosclerosis … Elective intracranial stenting with DES appears to be feasible and safe”).

As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

The feature shared by Groups I and III are the proteins HGF, VEGF165a, VEGFR1, endostatin, angiostatin, and TSP-2.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by US 2009/0175791 A1 to Kavile et al. (hereinafter ‘Kavile’).

Kavile teaches VEGFR1, endostatin, angiostatin, TSP-2 as anti-angiogenic agents (para [0821] “exemplary anti-angiogenic agents that are useful in connection with combined therapy are listed in Table D”); Table D lists “VEGFR1”, “Angiostatin”, “Endostatin”, “TSP-2”).

Kavile also teaches HGF as a ligand of a vasculature cell surface receptor (para [0347] “a ligand or growth factor that binds to an intratumoral vasculature cell surface receptor. . . . scatter factor/hepatocyte growth factor (HGF)”).

Although Kavile does not specifically teach VEGF165a, Kavile does teaches VEGF165 for the stimulation of human dermal microvascular endothelial cell lines (para [0943] “Human dermal microvascular endothelial cell lines . . . . The cells were then serum starved for 24 hours and stimulated through the addition of 50 ng/ml VEGF165”), thus VEGF165a which is an isofrom of VEGF165, would have been obvious to one of ordinary skill in the art in view of Kavile.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Groups I, II, and III therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Claims 7-25, 31-34, 41-45, 52-72 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).