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(54) Title: PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF ENDOTHELIAL DYSFUNCTION

(57) Abstract: Endothelial dysfunction is characterized by altered vasoactive properties especially through an impaired nitric oxide (NO) production, leading to a reduced lumen diameter consecutive to excessive vasoconstriction. In particular, endothelial dysfunction is a hallmark of peripheral arterial disease which is defined as vascular occlusion below the level of the inguinal ligament, and which is one of the most severe complications of diabetes. The inventors identify endothelial cell (EC)-derived Dhh as a critical regulator of vascular integrity downstream of Klf2 and as a new mediator of inflammation-induced endothelial dysfunction. Additionally, the inventors used the Hedgehog agonist SAG which is useful for preventing endothelial dysfunction and found that SAG administration decreased inflammation and edema in a model of critical limb ischemia. Accordingly, hedgehog agonists are suitable for the treatment of endothelial dysfunction and in particular for the treatment of peripheral arterial diseases.



PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF ENDOTHELIAL DYSFUNCTION

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of endothelial dysfunction.

BACKGROUND OF THE INVENTION:

Endothelial dysfunction is characterized by altered vasoactive properties especially through an impaired nitric oxide (NO) production, leading to a reduced lumen diameter consecutive to excessive vasoconstriction. Also, endothelial dysfunction leads to abnormal vascular leakage mainly because of an alteration of endothelial intercellular junctions. In addition, dysfunctional endothelial cells (ECs) express increased levels of adhesion and prothrombotic molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) acquiring pro-inflammatory and pro-thrombotic phenotypes. Endothelial dysfunction is known to be consecutive to cardiovascular risk factors including age, diabetes, obesity and hypertension. More specifically it is induced by disturbed blood flow, pro-inflammatory cytokines or high glucose levels.

Endothelial dysfunction is a hallmark of peripheral arterial disease. Peripheral arterial disease (PAD), defined as vascular occlusion below the level of the inguinal ligament, is one of the most severe complications of diabetes and is increasingly present in daily practice with 1,000/1,000,000 new cases every year.(Norgren et al., 2007). The most common presentations are intermittent claudication and its most severe form, critical limb ischemia (CLI). The prognosis of CLI remains poor, with a 25% rate of primary major amputation, a chronic pain rate of 20% and a mortality rate of 25% at one year.(Norgren et al., 2007) Treatment usually involves surgical revascularization, either by open bypass surgery or by percutaneous transluminal angioplasty.(Jaff et al., 2015) However, up to 30% of patients remain ineligible for any effective revascularization strategy,(Dormandy and Rutherford, 2000) especially in case of long-segment occlusions and distal disease which is characteristic for diabetic patients.(Collinson and Donnelly, 2004). Alternatives, such a lumbar sympathectomy as well as infusions of vasodilators (prostaglandins),(Altstaedt et al., 1993),(Petronella et al., 2004) have been proposed, to improve blood flow distally. However, their effects remain poor, mainly because they can only help improve blood-flow in the remaining patent and functional capillaries. In addition, several pro-angiogenic factors (including vascular endothelial growth

factor [VEGF] and fibroblast growth factor [FGF]) have been tested in clinical trials on humans as a supplement or alternative to surgical revascularization. However, their effects in humans are limited in comparison to animal models, as most studies showed no significant difference between placebo and active groups for any outcomes.(Roncalli et al., 2008) Therefore, new therapeutic strategies for these “no-option” patients are urgently needed.

Accumulating evidences demonstrate that Hedgehog signaling is essential for micro-vessel integrity. For instance Hh signaling was shown to promote blood brain barrier integrity and immune quiescence both in the setting of multiple sclerosis (Alvarez, 2011) and in the setting of stroke (Xia, 2013). Conversely, activation of Hedgehog signaling by Smoothened (Smo) agonists was shown to protect from blood brain barrier disruption in the setting of stroke (Chechneva, 2014) and HIV-infection (Singh, 2016). Recently it was shown that Dhh constitutive KO of endothelial specific Smo KO leads to blood nerve barrier breakdown in adult mice (Renault 2016). Nevertheless, the role of EC-derived Dhh has never been investigated.

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of endothelial dysfunction. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors identify endothelial cell (EC)-derived Dhh as a critical regulator of vascular integrity downstream of Klf2 and as a new mediator of inflammation-induced EC dysfunction. Additionally, the inventors used the Hedgehog agonist SAG which is useful for preventing EC dysfunction and found that SAG administration decreased inflammation and edema in a model of critical limb ischemia.

Accordingly, the first object of the present invention relates to a method of treating endothelial dysfunction in a patient in need thereof comprising administering to the patient a therapeutically effective amount of a hedgehog agonist.

As used herein, the term “endothelial dysfunction” has its general meaning in the art and refers to a condition in which the endothelium loses its physiologic properties and shifts toward a vasoconstrictor, prothrombotic, and proinflammatory state. Endothelial dysfunction has been associated with a variety of processes, including hypertension, atherosclerosis, aging, heart and renal failure, coronary syndrome, obesity, vasculitis, infections, sepsis, rheumatoid arthritis, thrombosis, smoking as well as with type 1 and type 2 diabetes.

In some embodiments, endothelial dysfunction is associated with diseases resulting from ischemia and/or reperfusion injury of organs and/or of parts of the body selected from the

group comprising heart, brain, peripheral limb, kidney, liver, spleen and lung, and/or wherein the endothelial dysfunction is associated with diseases selected from a group comprising infarctions such as myocardial infarction and critical limb ischemia, and/or wherein the endothelial dysfunction is associated with diseases selected from the group comprising
5 ischemic diseases such as peripheral arterial occlusive disease, e.g. critical leg ischemia, myocardial infarction and ischemic diseases of organs, e.g. of the kidney, spleen, brain and lung.

In some embodiments, the method of the present invention is particularly suitable for the treatment of endothelial dysfunction in a patient suffering from a systemic inflammatory
10 response syndrome or sepsis. As used herein, the term "systemic inflammatory response syndrome" (or "SIRS") is in accordance with its normal meaning, to refer to an inflammatory state of the whole body without a source of infection. There are four major diagnostic symptoms of SIRS, although any two of these are enough for a diagnosis (see e.g. Nystrom (1998) Journal of Antimicrobial Chemotherapy, 41, Suppl A, 1-7). As used herein, the term "sepsis" refers to
15 a form of SIRS which is caused by a suspected or proven infection (see e.g. Nystrom (1998) Journal of Antimicrobial Chemotherapy, 41, Suppl. A, 1-7). An infection that leads to sepsis may be caused by e.g. a virus, a fungus, a protozoan or a bacterium.

In some embodiments, the method of the present invention is particularly suitable for the treatment of endothelial dysfunction in a patient suffering from diabetes mellitus. As used
20 herein, the term "diabetes mellitus" refers to a disease caused by a relative or absolute lack of insulin leading to uncontrolled carbohydrate metabolism, commonly simplified to "diabetes," though diabetes mellitus should not be confused with diabetes insipidus. As used herein, "diabetes" refers to diabetes mellitus, unless otherwise indicated. A "diabetic condition" includes pre-diabetes and diabetes. Type 1 diabetes (sometimes referred to as "insulin-
25 dependent diabetes" or "juvenile-onset diabetes") is an auto-immune disease characterized by destruction of the pancreatic β cells that leads to a total or near total lack of insulin. In type 2 diabetes (T2DM; sometimes referred to as "non-insulin-dependent diabetes" or "adult-onset diabetes"), the body does not respond to insulin, though it is present.

Accordingly, in some embodiments, the present invention is particularly suitable for the
30 treatment of diabetic micro-and/or macroangiopathy. In some embodiments, the method of the present invention is particularly suitable for the treatment of diabetic nephropathy, diabetic dermopathy, diabetic retinopathy and diabetic neuropathy.

In some embodiments, the method of the present invention is particularly suitable for the treatment of a peripheral arterial disease. As used herein, the term "peripheral arterial

disease” or “PAD” refers to acute and chronic critical limb ischemia, Buerger's disease and critical limb ischemia in diabetes. As used herein, the term "critical limb ischemia" or "CLI" generally refers to a condition characterized by restriction in blood or oxygen supply to the extremities (e.g., hands, feet, legs) of an individual that may result in damage or dysfunction of a tissue in the extremities. Critical limb ischemia may cause severe pain, skin ulcers, or sores, among other symptoms, and in some cases leads to amputation. Critical limb ischemia may be characterized by vasoconstriction, thrombosis, or embolism in one or more extremities. Any tissue in an extremity that normally receives a blood supply can experience critical limb ischemia. In particular, the Hh agonist of the present invention is particularly suitable for promoting muscle perfusion and for preventing myopathy in the setting of critical limb ischemia.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a patient having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a patient beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or

treatment upon achievement of a particular predetermined criteria [e.g., pain, disease manifestation, etc.]).

As used herein, the term "hedgehog agonist" refers to an agent that upon administration leads to the activation of Hedgehog signalling pathway. Thus, the agonist is typically a Desert Hedgehog polypeptide, a Smoothed (Smo) agonist Smo or a ligand of Ptch1, Ptch2, Cdon, Boc, Gas1, Hhip, or Lrp2. Screening assays for hedgehog agonists are well known in the art and typically include those described in US 20050282231.

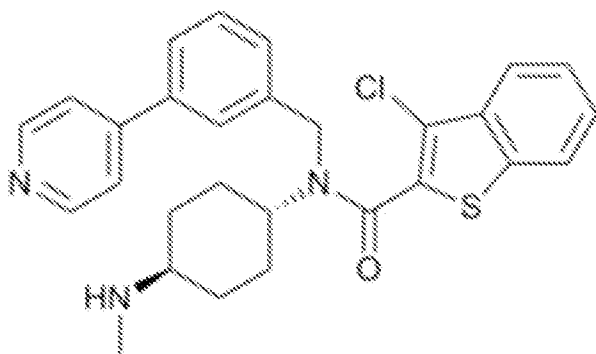
In some embodiments, the hedgehog agonist of the present invention is a Desert hedgehog (Dhh) polypeptide. The term "desert hedgehog" has its general meaning in the art and refers to the desert hedgehog protein encoded by the DHH gene (Gene ID: 50846). The protein is predicted to be made as a precursor that is autocatalytically cleaved; the N-terminal portion is soluble and contains the signalling activity while the C-terminal portion is involved in precursor processing. More importantly, the C-terminal product covalently attaches a cholesterol moiety to the N-terminal product, restricting the N-terminal product to the cell surface and preventing it from freely diffusing throughout the organism. The sequence of desert hedgehog protein and nucleic acids for encoding such proteins are well known to those of skill in the art. For example, Genbank Acc. Nos. NP_066382.1 (SEQ ID NO:1) provides the complete amino acid sequence of Homo sapiens desert hedgehog. However, it should be understood that, as those of skill in the art are aware of the sequence of these molecules, any desert hedgehog protein or gene sequence variant may be used as long as it has the properties of a desert hedgehog. According to the invention the term encompasses desert hedgehog itself or fragments thereof providing that the polypeptide is capable of activating Hedgehog signalling pathway. In some embodiments, the desert hedgehog polypeptide is fused to a Fc portion of an antibody. As used herein, "Fc portion" encompasses domains derived from the constant region of an immunoglobulin, preferably a human immunoglobulin, including a fragment, analog, variant, mutant or derivative of the constant region. Suitable immunoglobulins include IgG1, IgG2, IgG3, IgG4, and other classes. According to the invention, the desert hedgehog polypeptides may be produced by conventional automated peptide synthesis methods or by recombinant expression. General principles for designing and making proteins are well known to those of skill in the art.

SEQ ID NO:1

malltnllpl cclallalpa qscgpgrgpv grrryarkql vpillykqfvp gvpertlgas gpaegrvarg
serfrdlvpn ynpdiifkde ensgadrlmt erckervnal aiavmmmwpg vrlrvtegwd edghhaqdsl
hyegraldit tsdrdrnkyg llarlaveag fdwvyyesrn hvhsvskadn slavraggcg pgnatvrlws

gerkgrelh rgdwvlaada sgrvvptpvl lfdrdlqrr asfvavetew pprkllltpw hlvfaargpa
 papgdfapvf arrragdsv lapggdalrp arvarvaree avgvfapla hgtllvndvl ascyavlesh
 qwahrafapl rllhalgall pggavqptgm hwysrllrlyl aeellg

In some embodiments, the hedgehog agonist of the present invention is a small organic
 5 molecule. Exemplary Hh agonists include but are not limited to, e.g., benzothiophene
 smoothened agonists (a family of biaryl substituted 1,4-diaminocyclohexanamides of 3-
 chlorobenzothiophene-2-carboxylic acids), SAG (Hh-Ag1.3) agonists (e.g., 3-chloro-N-
 [(1r,4r)-4-(methylamino)cyclohexyl]-N-[3-(pyridin-4-yl)benzyl]benzo[b]thiophene-2-
 carboxamide, including SAG21k; see, e.g., Bragina et al Neurosci. Lett.2010 482: 81–5, Heine
 10 et al, Sci Transl Med 2011 3 (105); Das et al Sci Transl Med 52013: 201ra120), oxysterols (see,
 e.g., Nachtergaele Nat Chem Biol. 2012 Jan 8;8(2):211-20, US20150118277 and US
 20150140059) purmorphamine (Sinha Nature Chemical Biology 2, 29 - 30 (2006) , those agents
 and methods of derivation described in, e.g., Brunton et al. (2009) Bioorg Med Chem Lett.
 19(15):4308–4311; Chen et al. (2002) PNAS. 99(22):14071-14076; Frank-Kamenetsky, et al.
 15 (2002) J Biol.1(2):10; Paladini et al. (2005) J Invest Dermatol.125(4):638-46; Nakamura et al.
 (2014) J Cell. Physiol. ePub; U.S. Patent Nos: 8,852,937; 7,479,539; 7,115,653; 6,683,192;
 6,683,108; 6,639,051; 6,613,798, U.S. Patent Application Pub. Nos: 20130085096;
 20120238500; 20120148549; 20100183560; 20080207740; 20080171328; 20070110698;
 20050112125; 20050070578; 20050054568; 20050014796; 20030139457; 20030022819;
 20 20020198236, and the like; the disclosures of which are incorporated by reference herein in
 their entirety. In some embodiments, the hedgehog agonist of the present invention is SAG (3-
 chloro-N-[trans-4-(methylamino)cyclohexyl]-N-[[3-(4-pyridinyl)phenyl]methyl]-
 benzo[b]thiophene-2-carboxamide) which has the following formula:



25 By a "therapeutically effective amount" of the Hedgehog agonist of the invention as
 above described is meant a sufficient amount of the compound. It will be understood, however,
 that the total daily usage of the compounds and compositions of the present invention will be
 decided by the attending physician within the scope of sound medical judgment. The specific

therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

The Hedgehog agonist of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms. Galenic adaptations may be done for specific delivery in the small intestine or colon. Preferably, the pharmaceutical compositions contain vehicles

which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol ; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists.

It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Solutions comprising Hedgehog agonists of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils.

Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The Hedgehog agonist of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifusoluble agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate

solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed. For parenteral administration in an aqueous solution, for example, the solution 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, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. The Hedgehog agonist of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered. In addition to the Hedgehog agonists of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: *Dhh* downregulation mediates TNF α -induced EC dysfunction (A-B)
HUVECs were treated or not with 10 ng/mL TNF α , in the presence or not of 100 nM SAG. (A) -catenin interaction with Cdh5 was evaluated by co-immunoprecipitation assay. The

experiment was repeated 3 times. (B) VCAM-1 mRNA expression were quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. (C) HUVECs were transfected with Klf2 or control siRNAs and then treated or not with 100 nM SAG. VCAM-1 mRNA expression was quantified via RT-qPCR. (D) HUVECs were treated or not with 10 ng/mL TNF α , in the presence HeLa conditioned medium containing or not FL-Dhh. VCAM-1 mRNA expression was quantified via RT-qPCR. (E) HUVECs were transfected with Klf2 or control siRNAs and then treated with HeLa conditioned medium containing or not FL-Dhh. VCAM-1 mRNA expression was quantified via RT-qPCR. qPCR experiments were repeated 3 times, each experiment included triplicates. **: $p \leq 0.01$; NS: not significant.

Figure 2: SAG limits LPS-induced disruption of EC intercellular junction but not LPS-induced lung inflammation (EC activation). C57BL/6 mice were sacrificed 6 hours after they were administered or not with 10 mg/kg LPS together or not with 5mg/kg SAG. n= 8 mice/group. (A) Dhh mRNA in lung extracts was quantified via RTqPCR. (B) Aortas were immuno-stained with anti-Cdh5 antibodies (in red). Nuclei were stained with DAPI (in blue). En face staining is shown. (C) Average junction thickness was quantified as the % of Cdh5+ surface area and normalized to the number of nuclei/HPF (n=5 aorta/group). (D) β -catenin interaction with Cdh5 was evaluated by co-immunoprecipitation assay in lung protein extracts (#: LPS-treated vs Ctrl, $p \leq 0.05$, \$: LPS+SAG vs LPS, $p \leq 0.05$). (E) VCAM-1 mRNA in lungs were quantified via RT-qPCR (n=5 mice/group). (F) VCAM-1 proteins in lungs were quantified by western blot (n=5 aorta/group) (#: LPS-treated vs Ctrl, $p \leq 0.05$; \$: LPS+SAG vs LPS, not significant). (G) Lung cross sections were immuno-stained with anti-GR1 antibodies to identify neutrophils. (H) Neutrophil infiltration was quantified as the number of GR1+ cells/mm² (n=5 aorta/group). **: $p \leq 0.01$; ***: $p \leq 0.001$, NS: not significant.

Figure 3. SAG prevents high glucose induced EC dysfunction. A. Dhh mRNA expression was quantified via qRTPCR in ECs isolated from the ischemic muscle of diabetic mice (n=4) and from the muscle of healthy CTRL mice (free of ischemia, n = 4). B-D. HUVECs were grown in standard medium, Glucose-enriched medium or Glucose enriched medium with SAG therapy. B. HUVECs were immunostained with anti-Cdh5 antibodies (red) and nuclei were stained with DAPI (blue). The experiment was repeated 3 times. C. beta catenin interaction with Cdh5 was evaluated by co-immunoprecipitation assay. The experiment was repeated 7 times. D. VCAM-1 and E. SOD2 mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. F. Femoral arteries were harvested from CTRL mice (black), Streptozotocin-induced Diabetic mice (red) and Streptozotocin-induced Diabetic mice treated with SAG therapy (blue) and submitted to

vasomotricity tests. The cumulative concentration-relaxation curve to ACh (on the left) and nitroprusside sodium (SNP), which is a NO donor (on the right) are shown. The response is expressed as the percentage of decrease of phenylephrine-induced pre-contraction. Values were normalized to an initial 80 mM KCl-induced contraction. G. Micropellets containing the dilution polymer (CTRL), VEGF (positive-CTRL) or SAG therapy were implanted in mice eyes. Flat whole-mount of mice cornea were immunostained with anti-CD31 antibodies (white) (x80) 10 days after implantation and quantified as the surface of CD31 positive elements. *: $p \leq 0.05$, **: $p \leq 0.01$, NS: not significant. Mann Whitney test

Figure 4. SAG therapy improved EC function without increasing capillary density.

Diabetic mice submitted to sequential femoral and iliac artery ligation were treated or not with SAG (n=12 mice/group). A. Mice skeletal muscle cross sections were immunostained with anti-CD31 antibodies to identify ECs (brown) (x260) quantified as the number of CD31 positive elements B. Mice skeletal muscle cross sections were submitted to immuno-fluorescence labeling with anti-albumin antibodies (white) (x260) quantified as positive albumin areas C. VCAM-1, D. SOD2 mRNA expression was quantified via RT-qPCR. E. Mice skeletal muscle cross sections were immunostained with anti-CD68 immuno-staining to identify macrophages (brown) (x260) quantified as the number of CD68 positive elements. *: $p \leq 0.05$, **: $p \leq 0.01$, NS: not significant. Mann Whitney test

Figure 5. SAG therapy improved microvessel perfusion and reduced myopathic features. Diabetic mice submitted to sequential femoral and iliac artery ligation were treated or not with SAG (n=12 mice/group). A. Mice skeletal muscle cross sections were submitted to Fluorescence labeling (x260) of Isolectin-B4 to identify perfused vessels (green) and anti-CD31 antibodies (red) to identify ECs and vessel perfusion was assessed with quantitative analysis of the Ratio of Isolectin-B4 positive elements over CD31 positive elements B. Perfusion ratio of ischemic limb over control limb on laser Doppler perfusion imaging C. Mice skeletal muscle cross sections were immunostained with anti-desmin antibodies to identify myocytes (brown) (x260) quantified as desmin negative areas D. MyoG mRNA expression was quantified via RT-qPCR. E. Mice skeletal muscle cross sections were immunostained with anti-BrdU antibodies to identify cell proliferation (brown) (x260) quantified as the number of BrdU positive elements *: $p \leq 0.05$, **: $p \leq 0.01$, NS: not significant. Mann Whitney test

EXAMPLE 1: Desert Hedgehog is a critical regulator of endothelium integrity downstream of Klf2

Material & methods:

Human tissue samples

Human muscle biopsies were obtained via a protocol approved by the *Comité de protection des personnes* (CPP) Sud-Ouest et Outre Mer III in accordance with the guidelines of the declaration of Helsinki. All patients gave informed consent.

Mice

5 C57BL/6J mice were obtained from Charles River Laboratories and bred in our animal facility.

Dhh Floxed mice were generated at the “Institut Clinique de la Souris” (Strasbourg, France) through the International Mouse Phenotyping Consortium (IMPC) from a vector generated by the European conditional mice mutagenesis program, EUCOMM. Smo Floxed
10 (Smo^{Flox}) mice¹⁵ and Rosa26^{mTmG} mice¹⁶, were obtained from the Jackson Laboratories. Pdgfb-Cre^{ERT2} mice¹⁷ and Cdh5-Cre^{ERT2} mice (Azzoni et al., 2014) were kindly given by M. Fruttiger and RH. Adams respectively.

Animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific
15 purposes and approved by the local Animal Care and Use Committee of Bordeaux University.

Cre recombinase of Pdgfb-Cre^{ERT2} mice was activated by intraperitoneal injection of 1 mg tamoxifen for 5 consecutive days. Mice were phenotyped 4 weeks later. Successful and specific activation of the Cre recombinase was verified in both Pdgfb-Cre^{ERT2}; Rosa26^{mTmG} mice and Cdh5-Cre^{ERT2}; Rosa26^{mTmG} mice.

20 ***Brain vessel enrichment***

Mouse brains were mechanically homogenized using a Potter in HBSS containing 1% HEPES. After a 10 min centrifugation at 2000g, the tissue pellet was re-suspended in HBSS containing 1% HEPES and 17,5% dextran. Vessels were extracted from brain tissue by strong agitations followed by a 15 minute centrifugation at 4400g. Brain vessels were then re-
25 suspended in HBSS containing 1% HEPES and 1% BSA and washed with the same buffer first using a 100 µm cell strainer and then glass beads for 45 minutes on ice. Finally, vessels were centrifugated 10 min at 4000g and re-suspended in RIPA for protein preparation.

EC isolation from mouse tissue

Mouse brain lung or heart tissue was dissociated in 2 mg/mL type 4 collagenase for 1
30 hour at 37°C and subsequently filtrated on 70 µm and 40 µm cell strainer. ECs were labelled using rat anti-CD31 antibodies (BD Pharmingen Inc) and rat anti-Endoglin antibodies (Santa Cruz Biotechnology) and purified using anti-rat IgG MicroBeads (Miltenyi biotec) and MACS® Cell Separation Columns (Miltenyi biotec).

In vivo permeability assessment

To quantify vascular permeability, mice were injected with 50 μ L 25 mg/mL 70 kDa FITC-Dextran in the tail vein, 30 minutes before they were sacrificed. Microscopic visualization of FITC-dextran extravasation was done on paraffin-embedded tissue sections.

Lung edema assessment

5 Lung edema was assessed by measuring the wet/dry weight ratio. The wet weight was measured immediately after the lung was harvested while the dry weight was measured after the lung was incubated for 72 hours at 85°C.

LPS induced-systemic inflammation

10 Systemic inflammation was induced by intraperitoneal injection of 10 mg/kg LPS. Mice were sacrificed 6 hours later.

Cell culture

In vitro experiments were performed with human umbilical vein endothelial cells (HUVECs) (Lonza) or Human Dermal Microvascular Endothelial Cells (HMVEC-D) (Lonza). HUVECs were cultured in endothelial basal medium-2 (EBM-2) supplemented with EGMTM-2 BulletKitsTM (Lonza) while HMVEC-D were cultured in EBM-2 medium supplemented with EGMTM-2MV BulletKitTM (Lonza). HeLa ATCC®CCL-2TM were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% fetal bovine serum. Before any treatment cells were serum starved in 0.5% fetal bovine serum containing culture medium for 24 hours.

siRNA/Transfection

HUVECs were transfected with human Dhh siRNA, human Klf2 siRNA or universal scrambled negative control siRNA duplex (Origen) using JetPRIMETM transfection reagent (Polyplus Transfection), according to the manufacturer's instructions.

Plasmids/Transfection

25 The N-Dhh expressing vector (e.i. pIRES-NDhh) was previously described¹⁸. Full length (FL)-Dhh was PCR amplified from a HUVEC cDNA library and cloned into the EcoRI-BamHI sites of pcDNA3.1 vector (Invitrogen). The WT Smo expressing vector pRK-WT Smo¹⁹ was kindly given by B. Wang. The -2617 to +152, 621 to +152, -371 to +152, -194 to +152, -124 to +152 and -54 to +152 fragments of the human Dhh promoter were amplified by PCR and cloned into the *PvuII-NheI* sites of pGL3 basic vector (Promega). The Klf-2 encoding vector pcDNA3-hKlf2²⁰ was kindly provided by H. Ohguchi.

30 HUVECs and HeLa were transfected using JetPRIMETM transfection reagent (Polyplus Transfection), according to the manufacturer's instructions.

For gene reporter assays, Cells were transfected with pGL3 basic vector (Promega) alone or containing fragments of Dhh promoter together with a transcription factor expressing vector, and pHook-LacZ (Invitrogen). Cell lysis was performed using Passive Lysis Buffer (Promega), after 48 hours. Luciferase and β -galactosidase activity were measured using
5 Luciferase assay reagent (Promega) according to the manufacturer's instructions and the ONPG spectrophotometric method, respectively.

Immunostaining

Prior immunostainings, cells were fixed with 100% methanol for 10 minutes. Thoracic aortas were fixed with 10% formaline for 10 minutes immediately after they were harvested,
10 washed with PBS, cleaned and opened longitudinally. Heart, brain, and lung tissues were methanol fixed paraffin embedded and cut into 7 μ m thick sections.

ECs were identified with rat anti-CD31 antibodies (BD Pharmingen Inc), Neutrophils were identified with rat anti-Ly6G (GR1) antibodies (BD Pharmingen Inc). Dhh and Smo were identified using rabbit anti-C terminal Dhh (Santa Cruz Biotechnology, Inc), rabbit anti-Smo
15 antibodies (Abcam) respectively. Cell-cell junction were stained using goat anti- Cadherin-5 antibodies (Santa Cruz Biotechnology, Inc), rabbit anti-ZO-1 antibodies (Invitrogen), rabbit anti-Claudin-5 antibodies (Invitrogen), mouse anti JAM-A antibodies (Santa Cruz Biotechnology, Inc). VCAM-1 was identified using rabbit anti VCAM-1 antibodies (Abcam)

For immunofluorescence analyses, primary antibodies were resolved with Alexa-Fluor-
20 conjugated secondary antibodies (Invitrogen) and nuclei were counterstained with DAPI (1/5000). For immunohistochemical analyses, primary antibodies were sequentially stained with biotin-conjugated secondary antibodies (Vector Laboratories) and streptavidin-HRP complex (Amersham), then the stain was developed with a DAB substrate kit (Vector Laboratories); tissues were counterstained with hematoxylin.

Quantitative RT-PCR

25 RNAs were isolated by using Tri Reagent® (Molecular Research Center Inc) as instructed by the manufacturer from cells or mouse tissue that had been snap-frozen in liquid nitrogen and homogenized. For quantitative RT-PCR analyses, total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) and amplification was performed on
30 a DNA Engine Opticon®2 (MJ Research Inc) using B-R SYBER® Green SuperMix (Quanta Biosciences). Primer sequences are reported in Supplemental table 1.

The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to β -actin mRNA expression.

Immunoprecipitation/Western blot analysis

Prior to western blot analysis, Dhh was immunoprecipitated with rabbit anti-Hh antibodies (Santa Cruz Biotechnology) or rabbit anti-C terminal Dhh antibodies (Santa Cruz Biotechnology) while Cdh5 was immunoprecipitated with goat anti-Cdh5 antibodies (Santa Cruz Biotechnology)

5 Expression of Dhh, β -catenin, Cdh5, Claudin-5, JAM-A and Connexin 43 was evaluated by SDS PAGE using mouse anti-Dhh antibodies (Sigma), rabbit anti- β -catenin antibodies (Sigma), goat anti-Cdh5 antibodies (Santa Cruz Biotechnology), rabbit anti-Claudin-5 antibodies (Millipore), mouse anti JAM-A antibodies (Santa Cruz Biotechnology, Inc) and rabbit anti-Connexin 43 antibodies (Sigma)

10 Equal protein loading was confirmed using monoclonal anti- α -tubulin antibodies (Sigma-Aldrich).

Statistics

Results are reported as mean $\pm$ SEM. Comparisons between groups were analyzed for significance with the non parametric Mann-Whitney test. Differences between groups were considered to be significant when $p \leq 0.05$. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Results:

Dhh is expressed by ECs in adults

In addition of being found to be expressed by Schwann cells and Sertoli cells, developmental studies revealed Dhh expression in the ECs of the major blood vessels of the mouse embryo ⁸. We verified whether Dhh was also expressed in adult ECs and found that Dhh mRNA is enriched in the CD31+ fractions of adult mouse brain, heart and lungs (data not shown). In addition, we detected Dhh protein by immunostaining in the ECs in the human adult skeletal muscle (data not shown). We then compared the expression levels of Dhh with the one of Shh and Ihh in ECs. While we detected some Ihh mRNAs which is consistent with previous literature ²¹, Shh mRNA expression was barely detectable (data not shown). Finally, we transfected HUVECs with a full-length Dhh encoding vector and performed a western blot analysis of Dhh expression. Accordingly to a previous investigation ²², we found that Dhh is poorly processed and mainly exist as a 45 kDa full-length protein. Nevertheless we found that full length Dhh is significantly secreted (data not shown).

30 Altogether this first set of data revealed for the first time that Dhh is expressed in adult EC. It is the main Hh ligand expressed by ECs and it is mainly expressed as a full length unprocessed protein.

EC-derived Dhh promotes EC intercellular junction integrity

To investigate the role of EC-derived Dhh we first performed cell culture assays in which Dhh expression was knocked down using siRNAs. When Dhh expression was downregulated Cdh5 dependent junctions were altered and displayed a “zig zag” phenotype (data not shown). Accordingly we found that Cdh5 interaction with β -catenin was significantly reduced ECs in which Dhh expression was downregulated (data not shown). We then verified the role of Dhh in maintaining adherens junction *in vivo*, in mice in which Dhh expression had been specifically invalidated in ECs: i.e. *Pdgfb-cre*^{ERT2}; *Dhh*^{Flox/Flox} mice (*Dhh*^{ECKO}). We performed Cdh5 staining of “en face” aortas and found that Cdh5 staining was significantly thicker in *Dhh*^{ECKO} mice compared to their control littermates (data not shown).

Besides, because Hh signaling in EC has previously been involved in maintaining tight junction integrity^{4,12}, we examined the impact of Dhh KD both *in vitro* and *in vivo* on ZO-1, Cldn5 and JAM-A expression. Dhh deficient ECs exhibited a discontinuous and zig zag staining for ZO-1 (data not shown) which was confirmed in *Dhh*^{ECKO} aorta (Supplemental Fig 3B) and brain (data not shown). Similarly Cldn5 and JAM-A staining's were weaker and discontinuous both in cultured ECs (data not shown) and in *Dhh*^{ECKO} aorta (data not shown). We quantified ZO-1 and JAM-1 protein expression in brain vessels of *Dhh*^{ECKO} mice and their control littermates and found that ZO-1 and JAM-A were significantly downregulated (data not shown) in the absence of endothelial Dhh. Altogether, these results demonstrate the essential role of EC-derived Dhh for tight junctions integrity.

Finally, to measure the consequences of EC-EC junction alterations observed in the absence of endothelial Dhh, both *Dhh*^{ECKO} mice and their control littermates were administered with FITC-labelled 70 kDa dextran. We observed abnormal vessel leakage both in the brain and in the heart of *Dhh*^{ECKO} mice. Moreover, *Dhh*^{ECKO} mice displayed lung edema (data not shown).

In conclusion, this second set of data demonstrates, for the first time, the essential role of EC-derived Dhh in maintaining endothelial intercellular junction integrity.

EC-derived Dhh prevents EC activation.

To test whether EC-derived Dhh is necessary to prevent EC activation, we first performed cell culture assays and found that Dhh KD promoted both the expression of adhesion molecules such as VCAM-1 and ICAM-1 (data not shown) and the expression of inflammatory cytokines including Interleukin-6 (Il-6) and Chemokine ligand 2 (Ccl2) (data not shown). In addition, the increased VCAM-1 expression in *Dhh*^{ECKO} vessels was confirmed *in vivo* in lung sections (data not shown). We measured the consequences of Dhh KD-induced EC activation on neutrophil infiltration in the lungs after mice were administered with LPS. The density of

neutrophils was significantly higher in LPS-treated Dhh^{ECKO} mice compared to LPS treated control mice confirming and demonstrating the essential role of Dhh in preventing EC from acquiring a pro-inflammatory phenotype.

Dhh is a direct transcriptional target of Klf-2.

5 Next we moved on and investigated molecular mechanisms controlling Dhh expression in ECs. Notably, we previously reported that Dhh mRNA is significantly downregulated in the ischemic skeletal muscle of aged mice compared to the one of young mice¹³ and in the sciatic nerve of obese diabetic mice¹². We found that, in ECs, Dhh was more specifically downregulated by inflammatory cytokines including TNF α and Il-1 β and by oxidative stress
10 (H₂O₂) (data not shown). In order to investigate molecular mechanism involved in TNF α -induced Dhh downregulation we cloned the human Dhh promoter and performed gene reporter assays. We found that the Dhh promoter region regulated by TNF α is included between base pair -124 and base pair -54 of Dhh promoter (data not shown). Interestingly, within this promoter region, MatInspector software (Genomatix) identified 2 GC-rich regions potentially recognized
15 by Klf2. We then verified that, according to the literature²³, Klf2 was significantly downregulated in TNF α -treated ECs (data not shown) and that Klf2 was able to activate Dhh promoter. Indeed, Klf2 activated all Dhh promoter constructs, except for the -54 Dhh promoter plasmid which did not include the 2 GC-rich regions potentially bound by Klf2 (data not shown). To confirm the role of these 2 GC-rich regions, respectively located at base pair -74
20 (GC rich 1) and base pair -59 (GC rich 2) in mediating Klf2-induced Dhh promoter activation, we mutated each of these cis-regulating sequences (data not shown). Klf2 was not able to activate the -124 Dhh promoter plasmid when GC-rich 1 and/or GC-rich 2 sequences were mutated. Finally, to confirm the essential role of Klf2 in promoting Dhh expression in ECs, we downregulated Klf2 expression in HUVECs through siRNA transfection and found that Klf2
25 KD significantly inhibited Dhh expression in ECs (data not shown).

Altogether those results demonstrate that Dhh is a direct transcriptional target of Klf2 in ECs and identified 2 Klf2 cis-regulating sequences on the human Dhh promoter.

Dhh is a downstream effector of Klf2

We then hypothesized that Dhh could be a downstream effector of Klf2. Klf2 was
30 notably shown to inhibit TNF α -induced VCAM-1 expression^{2, 23}. We first tested whether rescuing Hh signaling with the Hh agonist SAG in TNF α -treated ECs or in Klf2 deficient ECs would restore Cdh5 dependent junction integrity and prevent VCAM-1 overexpression. As shown **Fig 1A** SAG restored Cdh5 interaction with β -catenin in TNF α -treated ECs while it did not prevent VCAM-1 overexpression (**Fig 1B**). Consistently SAG did not modulate VCAM-1

expression in ECs transfected with Klf2 siRNAs (**Fig 1C**). Nevertheless, when we used conditioned medium of FL-Dhh-overexpressing HeLa to restore Hh signaling, we found that, on the contrary to SAG, FL-Dhh significantly reduced both TNF α - and Klf2 siRNA-induced VCAM-1 expression (**Fig 1D and 1E**)

5 We next tested the role of Dhh, *in vivo*, in LPS-induced endothelial dysfunction. Dhh was significantly downregulated in the lung of mice 6 hours after they were administered with LPS (**Fig 2A**). Consistently with *in vitro* data, SAG administration prevented LPS-induced Cdh5 staining thickening of “en face” aortas (**Fig 2B and 2C**) and LPS-induced impaired Cdh5 interaction with β -catenin in lung extracts (**Fig 2D**). On the contrary, SAG did not modulate
10 LPS-induced VCAM-1 expression (**Fig 2E and 2F**) or LPS-induced neutrophil infiltration in the lung (**Fig 2G and 2H**).

In conclusion, this set of data demonstrates that Dhh downregulation, secondary to Klf2 downregulation promotes EC dysfunction induced by acute inflammatory stress. Importantly, these results demonstrate for the first time that Dhh signaling agonists can be used to prevent
15 TNF α or LPS-induced EC dysfunction.

In order to understand why FL-Dhh but not SAG was able to downregulate TNF α or Klf2 siRNA-induced VCAM-1 expression, we decided on investigating the role of Smo in ECs.

Smo is necessary for EC-EC junction integrity.

First, we performed Smo immunostaining of HUVECs transfected with a Smo
20 expressing vector and found that Smo is located at the intercellular junctions (data not shown). We then used the Smo inhibitor GDC-0449 to inhibit Smo in cultured ECs and found that GDC-0449 induced both altered zig zag Cdh5 and ZO-1 staining of junctions (data not shown). The effect of GDC-0449 on adherens junction was confirmed by measuring Cdh5 co-immunoprecipitation with β -catenin (data not shown). By the means time, we verified that SAG
25 was able to restore Cdh5 interaction with β -catenin in ECs transfected with Dhh siRNAs (data not shown). After we demonstrated that Smo was necessary for EC-EC intercellular junction *in vitro*, we verified this result *in vivo*, and found that Cdh5 staining of “en face” aortas of Smo^{ECKO} mice and their control littermates revealed significantly thicker junctions in the absence of endothelial Smo (data not shown). Finally the consequences of altered EC intercellular junction
30 in Smo^{ECKO} mice was measured after mice were administered with FITC-labelled 70 kDa dextran. Smo ECKO mice displayed abnormal vessel leakage in the brain confirming previous investigation ⁴ and demonstrating the essential role of Smo in mediating Dhh-induced maintenance of EC intercellular junctions.

Dhh prevents EC activation independently on Smo.

Finally, we investigated the role of Smo in the regulation of VCAM-1 expression and found first *in vitro* that Smo inhibition by GDC-0449 did not modify VCAM-1 expression level (data not shown). Moreover, SAG did not prevent Dhh KD-induced VCAM-1 expression in cultured ECs (data not shown). Consistently, neutrophil density in the lung of LPS treated Smo^{ECKO} mice was not different from the one of LPS treated control mice (data not shown).

Altogether this last set of data demonstrates that endothelial Smo does modulate EC inflammatory phenotype. This result indicates that Dhh prevents EC activation independently on Smo which explains why the Smo agonist SAG did not prevent Klf2 siRNA, TNF α or LPS-induced EC activation.

Discussion:

The present paper highlights for the first time the critical role of EC-derived Dhh by using endothelial specific Dhh KO mice. More specifically, it reveals that Dhh is essential for endothelial integrity in adult by maintaining both adherens and tight junctions and by preventing EC activation. Dhh expression in ECs has been first reported by developmental studies⁸, nevertheless its role in ECs had never been identified before.

Because Dhh is downregulated by several cardiovascular risk factors including age¹³, diabetes¹² and inflammatory chemokines. The present work implies that the loss of Dhh expression in such conditions may be one of the molecular events triggering endothelial dysfunction. Such paradigm is supported by the fact that SAG therapy was able to prevent TNF α or LPS-induced disruption of adherens junctions. In addition, it is consistent with several data from the literature. Indeed, Smo agonist therapy was previously shown to prevent blood brain barrier disruption in the setting of stroke⁶ or HIV infection⁷. We have demonstrated that SAG therapy restore Cldn5 expression and prevented endoneurial capillary leakage in diabetic mice¹².

At molecular level, we found that Dhh is a direct transcriptional target of Klf2. Consistently, Dhh was found to be modulated by Klf2 in two microarray analysis, the first one compared mRNA expression profile of HUVEC transduced with a Klf2 expressing lentivirus with the one of control HUVECs² and the second one compared gene expression in double Klf2/Klf4 KO ECs and WT ECs²⁴. When we know that Klf2 has been proposed as a “*molecular switch*” regulating endothelial function in health and disease²⁵ by regulating endothelial barrier function¹ and by preventing EC activation², the present work identifies Dhh as a downstream effector of Klf2. Accordingly, we found that restoring Hh signaling with FL-Dhh containing culture medium decreased VCAM-1 expression in ECs transfected with Klf2

siRNAs. Notably, on the contrary to Klf2 which is a transcription factor, Dhh is a secreted molecule for which agonist can be generated. This is already the case of Smo agonists.

The role of Hedgehog signaling in vascular biology in adults has been first described about 15 years ago by JM Isner's group²⁶. More specifically, Shh has been shown to be strongly overexpressed in ischemic tissues^{27, 28} and proposed to promote revascularization of ischemic tissues, since when administered ectopically; Shh is proangiogenic²⁶. Even though Ptch1 expression was detected in ECs²⁶, ECs were for a long time believed not to respond to Hh ligands. Shh was indeed proposed to promote angiogenesis indirectly by increasing VEGFA expression in fibroblasts²⁶. Recombinant Hh protein (Shh, Ihh and Dhh) were first reported to modulate EC function directly, *in vitro*, through Hh non canonical signaling^{29, 30}. Finally, the role of Hh signaling in ECs *in vivo* has been first reported in 2011 using endothelial deficient Smo mice⁴. Hh signaling in ECs has not been involved in angiogenesis but has been reported to promote blood brain barrier integrity by promoting tight junction protein expression including Cldn5 and JAM-A. The present work confirms the critical role of endothelial Hh signaling for endothelial integrity, identifies EC-derived Dhh as one of its main actor and extends it "outside" of the nervous system. Accordingly, so far, Hh signaling has been exclusively involved in maintaining endothelial tight junctions^{4, 5, 12, 31}, while the present study reveals for the first time to our knowledge that Hh signaling is also necessary for adherens junction integrity. Finally, it identifies Dhh as the activator of Hh signaling in adults ECs though an autocrine action. Shh which is produced by astrocytes was proposed to regulate Hh signaling in brain ECs⁷, nevertheless, on the contrary to Dhh, Shh expression inversely correlate with abnormal vascular leakage, Indeed, Shh is most often found to be upregulated in pathologic conditions such as multiple sclerosis⁴ or subarachnoid hemorrhage³¹.

Hh signaling especially in ECs is still poorly understood, as mentioned above, recombinant Hh proteins were proposed to act exclusively through Hh non canonical signaling in cultured ECs since they were not able to active Gli1 transcription^{29, 30}. The present studies demonstrate that Dhh-induces both Smo dependent and independent effect in ECs. Indeed Dhh promotes adherens junction integrity via Smo while it prevents VCAM-1 expression independently on Smo (Fig 7). We have tested whether Dhh inhibition in ECs leads to a downregulation of Gli1 or Gli2, which was not the case (Supplemental Fig 6), suggesting again that Dhh do not act through Hh canonical signaling. Further investigations are necessary to fully describe Dhh-induced signaling in ECs to promote EC intercellular junction integrity and VCAM-1 downregulation. In the same vein, while Shh processing has been well described³², Dhh was proposed not to undergo substantial autoprocessing or secretion, and not to function

in paracrine signalling. Rather, Dhh was proposed to functions as a juxtacrine signaling ligand to activate a cell contact-mediated Hh signaling response²². We indeed found that Dhh is poorly processed and mainly exist as a full length 45 kDa protein. Nevertheless, on the contrary to Pettigrew et al.'s data, we found that the 45 kDa Dhh protein is substantially secreted. While the cholesterol-modified aminoterminal cleavage product is believed to be responsible for all Hh-dependent signaling events, interestingly one study reported that unprocessed Shh is also active³³.

Collectively, our findings establish endothelial Dhh as a master regulator of endothelial biology and vascular integrity. Most importantly the present work further proves that Hh signaling in ECs may be targeted to ameliorate EC function in diseased conditions.

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EXAMPLE 2: Correcting endothelial cell dysfunction is a working strategy to improve critical limb ischemia.

Methods:

Human tissue samples

Human muscle tissues were retrieved from a library of tissues collected for a hospital clinical research program entitled "Critical ischemia of inferior limbs: metabolic, morphologic an immunohistochemical characterization of the involved tissues". (N°RCB : 2009-A00889-48). The local ethics committee of each center approved the study methodology and design and written informed consent was given by all patients. The inclusion criteria were man or women in the need of a surgical intervention on the lower limb, i.e Saphenous stripping for control patients, which had to be free from any history or clinical sign of PAD, and amputation or bypass for patients with PAD. Claudication status was determined by a walking distance <200m due to a cramp-like pain in the leg on Treadmill testing (Rutherford class 3). Presence of critical limb ischemia was assessed by clinical evaluation (Rutherford class 4 to 6)³³ associated with an ankle or toe systolic blood pressure <50 or 30 mmHg, respectively.

The inclusions were performed between August 2011 and April 2013 in three major vascular surgery departments in south-west France. In the absence of contra-indication or technical impossibility, three levels of biopsies were performed for each CLI patient, from the most ischemic one to the edge of amputation. The biopsies were immediately fixed in methanol and sent to Inserm UMR1034 (core lab) where the morphology and immunohistochemistry studies of the ischemic tissues were performed.

Hence, 25 muscles fixed in methanol and included in paraffin of Rutherford class 5 and 6 patients were finally selected and compared to 10 control (CTRL) patients, free from any history or clinical signs of PAD, undergoing saphenous stripping.

Mice

C57BL/6 mice were obtained from Charles River Laboratories and bred in our animal facility. Smo Floxed (^{Flox}) mice¹³ were obtained from the Jackson Laboratories and bred together with Cdh-Cre^{ERT2} mice³⁴ (provided by RH. Adams) to obtain Cdh5-Cre^{ERT2}; Smo^{Flox/Flox} mice and Smo^{Flox/Flox} CTRL mice. Animal experiments were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the local Animal Care and Use Committee of Bordeaux University. Smo^{Flox} mice were genotyped. Cre recombinase of Cdh-Cre^{ERT2} mice was activated by intraperitoneal injection of 1 mg tamoxifen (Sigma-Aldrich) for 5 consecutive days.

Streptozotocin treatment

Adult male C57BL/6 mice, 6 weeks of age, were injected intraperitoneally with 55 mg/kg/day of streptozotocin (Sigma Chemical, St Louis, MO, USA) dissolved in sterile citrate buffer (0.05 mol/L sodium citrate, pH 4.5, 45 mg/kg) after 4 hours of fasting. Either streptozotocin or citrate buffer (CTRL) was administered for 5 days consecutively during the first week of the study. Blood samples were collected from the vena caudalis and whole blood glucose levels were measured using a glucose analyzer [One Touch Ultra Mini Blood Glucose Monitoring System; Johnson & Johnson (China) Co.]. Mice with a blood glucose level >280 mg/dL were considered diabetic and used for experiments.³⁵ At the same time, a weight measurement was performed. The surgery to induce HLI was performed 14 weeks after induction of diabetes.

Behavioral testing of neuropathy

Mice were placed in an individual clear acrylic box with a wire mesh floor and acclimated for 20 min. Tactile responses were evaluated by quantifying the withdrawal threshold of the hind paw in response to stimulation with flexible Von Frey filaments. The

mechanical withdrawal threshold was measured by applying a series of six calibrated Von Frey filaments (Bioseb) to the mid-plantar surface of the foot.

Mice were placed in an individual clear acrylic box on a glass plate and acclimated for 20 min. The hind paw withdrawal latency in response to infra-red heat stimulus was determined with Plantar test (Bioseb). The baseline was set between 3 and 7 sec by adjusting the heat source intensity (90 one-digit steps), and an automatic 15 sec cut-off time was used to prevent tissue damage.

Electromyography

After isoflurane anesthesia, recording and stimulation electrodes were placed subcutaneously on the hindlimbs and tail to measure the action potentials and assess the presence of motor or sensitive neuropathy. The evaluation of hindlimb motor potentials was performed by stimulating the nerve with a successive discharge of 3 V at the level of the ischial notch and the ankle. The latency between the appearances of two action potentials corresponds to the conduction time and the nerve conduction velocity was evaluated by calculating the ratio of the distance between the stimulation points and conduction time. In the tail, the same principle was used, with a stimulation of 5 V. The evaluation of the sensory potentials of the paw was performed by stimulating the nerve by a discharge of 10 V at the level of the foot arch and recording the response at the level of the ankle. The signals were recovered using a signal amplifier (AD instruments) and processed by Labchart software (AD instruments).³⁶

Hindlimb ischemia models and assessments

Chronic HLI was induced by sequential ligation of the femoral and iliac arteries (Lig. seq.) at day 0 and day 4, as previously described.²³ More precisely, surgery was performed under general anesthesia. Induction was ensured by spontaneous ventilation through a mask delivering a mixture of 4% isoflurane (Aerrane; Baxter Healthcare, Maurepas, France) and air and maintained with a mixture of 2% isoflurane and air. Mice were aseptically prepared and a 5mm incision was made in the left thigh region and re-used on day 4. On day 0, ligation of the left femoral artery was performed midway between the superficial epigastric artery and the bifurcation of the popliteal and saphenous arteries by a nod of **7.0 Prolene® (Ethicon, Issy les Moulineaux, France)**, three collateral vessels were also ligatured. Four days later, ligation of the right common iliac artery was performed by a retroperitoneal approach 0.5 cm distal to its origin, after visualization of the origin of internal iliac artery. The connective tissues were closed with 6.0 absorbable running sutures. In this unilateral ischemia model, the contralateral limb can be considered as a control.³⁷ The duration of ischemia was counted from the first

operation onwards. To minimize pain caused by the surgery, mice were administered intraperitoneally with 0.05 mg/kg buprenorphin 30 min before and 8 hrs after surgery.

Alternatively, Hind limb ischemia was induced by ligation and resection of the femoral artery (Lig. Ex. model) as previously described.¹⁸ Briefly, HLI was induced by two ligations, one at the proximal end of the femoral artery and the other at the distal portion of the saphenous vein, and the femoral artery; all side-branches were dissected and excised as previously described.

For assessment of proliferation, mice were injected intraperitoneally with 50 mg/kg BrdU (Sigma) 24 hours before they were sacrificed. For assessment of muscle hypoxia, mice were administered intraperitoneally with 60 mg/kg pimonidazole HCL (Hypoxypore Inc.) 1 hour before they were sacrificed. For assessment of capillary perfusion, mice were administered intravenously with 50 µL FITC-labelled BS1-Lectin (Vector) 30 minutes before they were sacrificed.

Mice were sacrificed by cervical dislocation 28 days after surgery. For histological assessment and gene expression analysis, the tibialis anterior muscle was harvested and cut in half. The lower half was fixed in methanol, paraffin-embedded, and cut into 6µm sections, and the upper half was snap frozen in liquid nitrogen. Each group included at least 6 animals. Only mice, in which there was histologic evidence of ischemia in the tibialis anterior muscle, as assessed by hematoxylin and eosin staining, were included in the study.

Laser Doppler perfusion imaging (LDPI)

LDPI was performed with a MoorLDI2-IR apparatus to study foot perfusion after mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), to avoid hemodynamic alterations at the time of each study. Animals were placed on a heating pad to keep them warm and to normalize the temperature from animal to animal. The results were expressed as the ratio of the ischemic to non-ischemic hindlimb.

SAG therapy

Mice submitted to the sequential ligation model of chronic HLI were treated with SAG at 5mg/kg/day or vehicle (NaCl 9‰) by intraperitoneal injections from day 4 until sacrifice.

EC isolation from mouse tissue

Ischemic tibialis anterior muscle or healthy muscle was dissociated in 2 mg/mL type 4 collagenase for 1 hour at 37°C and subsequently filtrated on 70 µm and 40 µm cell strainer. Endothelial cells were labelled using rat anti-CD31 antibodies (BD Pharmingen Inc) and rat anti-Endoglin antibodies (Santa Cruz Biotechnology) and purified using anti-rat IgG MicroBeads (Miltenyi biotec) and MACS® Cell Separation Columns (Miltenyi biotec).

Corneal angiogenesis

Pellets were prepared as previously described.³⁸ Briefly, VEGFA pellets were prepared by mixing 5 µg of VEGFA protein (Shenandoah) diluted in 10 µL sterile H₂O containing 0.1% BSA with 2.5 mg sucrose octasulfate-aluminum complex (Sigma-Aldrich Co.). SAG-containing pellets were prepared from 25 nmol SAG (Sigma-Aldrich Co.). Six µL of 12% hydron in ethanol was added, and the suspension was deposited on a 400 µM nylon mesh (Sefar America Inc., Depew NY, USA), then both sides of the mesh were covered with a thin layer of hydron and allowed to dry.

Mice were anesthetized by intraperitoneal injection of an anesthetic mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and a micropocket was created in the eye to place the "micropellet". The mice were sacrificed at day 10 and the corneas were harvested and stained for the expression of CD31 with a flat whole-mount for fluorescent imaging to assess the angiogenic response.

Immunostaining

Capillary and macrophage density were evaluated in sections stained for the expression of CD31 (rat anti-mouse CD31 antibodies, BD Pharmingen Inc /mouse anti-human CD31 antibodies, Dako) and CD68 (rat anti-CD68 antibodies, Biolegend) respectively. Muscle hypoxia was identified using the Hypoxyprobe™-1 Plus kit following the manufacturer's instructions. BrdU-positive cells were identified using rat anti-BrdU antibodies (Oxford Biotechnology). Fibrosis was assessed after Masson trichrome staining of muscle sections and quantified as the percentage of light green-positive area. Edema was assessed after albumin staining of muscle sections using sheep anti-albumin antibodies (Abcam). For each muscle section, CD31-, Lectin-, CD68-, hypoxyprobe-, BrdU-, light green- or albumin-positive areas were quantified in 10 pictures randomly taken under x260 magnification. Myogenesis was assessed after desmin staining (rabbit polyclonal anti-desmin antibodies, Millipore) of muscle sections and quantified as the percentage of remaining desmin-negative area using a single picture of the entire muscle under x20 magnification. Immunohistochemistry was performed on the Ventana Benchmark auto-staining system for reproducibility. All pictures were taken on an Axiozoom V16 (Zeiss) and all quantifications were done using ImageJ/Fiji v2.0.0-rc-59 software (National Institute of Health, USA).

For immunohistochemical analyses, primary antibodies were sequentially stained with biotin-conjugated secondary antibodies (anti-rat antibodies, Jackson immunoresearch, anti-rabbit antibodies, Amersham) and streptavidin-HRP complex (Amersham), then the stain was

developed with a DAB substrate kit (Vector Laboratories); tissues were counterstained with hematoxylin.

For immunofluorescence analyses, primary antibodies were resolved with Alexa-Fluor-conjugated secondary polyclonal antibodies (Invitrogen) and nuclei were counterstained with DAPI (1/5000).

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

RNAs were isolated by using Tri Reagent® (Molecular Research Center Inc) as instructed by the manufacturer from $3 \cdot 10^5$ cells or from skeletal muscle that had been snap-frozen in liquid nitrogen and homogenized. For quantitative RT-PCR analyses, total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) and amplification was performed on a DNA Engine Opticon®2 (MJ Research Inc) using B-R SYBER® Green SuperMix (Quanta Biosciences). The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to beta-actin mRNA expression.

Immunoprecipitation/Western blot analysis

Prior to western blot analysis, Cdh5 was immunoprecipitated with goat anti-Cdh5 antibodies (Santa Cruz Biotechnology).

Expression of desmin, β -catenin, Cdh5, was evaluated by SDS PAGE, using mouse anti-desmin antibodies (Clinisciences), rabbit anti- β -catenin antibodies (Sigma), goat anti-Cdh5 antibodies (Santa Cruz Biotechnology) respectively. Equal protein loading was controlled using monoclonal anti- α -tubulin antibodies (Sigma). The signal was scanned using an Odyssey Infrared imager (Li-cor).

Wire myography

Femoral arteries, dissected and segmented (1.8-2.0 mm length) were mounted using two tungsten threads in a Mulvany myograph (Danish Myo Technology, Aarhus, Denmark) as previously described.³⁹ Arteries were bathed in Krebs buffer (119 mM NaCl, 4.7 mM KCl, 1.5 mM CaCl_2 , 1.17 mM MgSO_4 , 1.18 mM KH_2PO_4 , 25 mM NaHCO_3 and 5.5 mM glucose) and maintained at 37°C. The arteries were studied under a resting tension corresponding to equivalent transmural pressures of 100 mmHg (13.3kPa), which was previously demonstrated to be the optimal resting tension in a passive length-tension relationship. The arteries functionality was checked by adding 80 mM KCl and no difference was observed between the three groups. The arteries contraction was evaluated with phenylephrine (α 1-adrenergic receptor agonist). The relaxant responses to acetylcholine and sodium nitroprusside were determined after precontraction with phenylephrine ($3 \cdot 10^{-7}\text{M}$, which corresponds approximately to 90% of the maximal contraction) by adding increasing concentrations

(ranging from 10^{-9} to 10^{-4} M) of the vasodilating agent and establishing a concentration/relaxation curve.

Statistics

Results are reported as mean $\pm$ SEM. Comparisons between groups were analyzed for significance with the non-parametric Mann-Whitney test or the Kruskal-Wallis test (for more than two groups) using **GraphPad Prism v7.0 (GraphPad Inc, San Diego, Calif)**. Differences between groups were considered to be significant when $p \leq 0.05$ (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$).

Results:

Critical limb ischemia is associated with clear signs of microvascular endothelial dysfunction

We have designed a clinical research program in order to finely phenotype the ischemic muscle of CLI patients. We accumulated muscle biopsies from 25 CLI patients (Rutherford 5-6), 6 patients with IC (Rutherford 3) and 10 control (CTRL) patients and performed a series of immuno-histological analysis. We first evaluated capillary density and observed no significant difference in capillary density between the muscle of patients with CLI or IC and the muscle of CTRL patients (data not shown). Nevertheless, CLI patients showed a significant increase in albumin extravasation, indicating abnormal vascular leakage (data not shown). The phenotype of patients with IC was intermediate. On the contrary, patients with CLI showed clear signs of inflammation, with significantly higher densities of macrophages, while macrophage density in IC patients was not different from that of control patients (data not shown). Besides, we found that the muscle of CLI patients, contrary to the one of IC patients, presented myopathic features with the presence of immature muscle fibers and a significant increase in desmin negative areas (data not shown) associated with increased fibrosis areas (data not shown).

This first set of data demonstrates for the first time that the human phenotype of CLI is characterized by a “normal” capillary density associated with signs of endothelial dysfunction including increased vascular leakage and increased local inflammation. This microvascular dysfunction is associated with significant muscle damage.

Diabetic mice submitted to sequential femoral and iliac artery ligation display a dramatically decreased foot perfusion with “normal” capillary density

In order to study the role of EC dysfunction in CLI and to test therapeutic options, we searched for an experimental model of CLI. One of the main limitations of the most commonly used mouse models of hindlimb ischemia (HLI, single proximal ligation of the iliac or femoral artery or complete excision of the femoral artery) is that they are models of acute HLI, which

are not representative of CLI, especially because mice rapidly form collaterals, which results in quick blood flow recovery without any treatment. Complete blood flow restoration may indeed occur as soon as 7 days after surgery depending on the model.²² Recently, a new mouse model of “chronic” limb ischemia induced by the sequential ligation of femoral and iliac arteries has been described.²³

With the aim to use a mouse model of HLI that recapitulated most of the features observed in the phenotype of CLI patients, we compared the phenotype of mice submitted to femoral artery ligation followed by its excision (lig.ex. surgery),¹⁸ mice submitted to sequential ligation of femoral and iliac arteries (seq.lig. surgery) and diabetic mice submitted to seq.lig. surgery. Diabetes, which is one of the main risk factor of CLI, was induced by streptozotocin administrations 12 weeks before seq.lig. surgery was performed. Diabetic mice had significantly reduced body weights and elevated blood plasma glucose levels (data not shown). The presence of vascular complications of diabetes was verified by assessing neuropathy by nerve velocity assessments, which revealed significant alterations of both motor and sensory nerve conduction velocities (data not shown) and behavioral tests, showing significant allodynia to Von Frey's monofilament and hypoalgesia on Plantar test (data not shown).

Twenty-eight days after HLI was induced, blood flow recovery was the best in mice submitted to Lig.ex. surgery. It was significantly diminished in mice submitted to Seq.lig. surgery and even worse in diabetic mice submitted to Seq.lig. surgery (data not shown). Accordingly, the clinical score of necrosis was inversely correlated with blood flow recovery. It was the worst in diabetic mice submitted to Seq.lig. surgery and worse in mice submitted to Seq.lig surgery compared to mice submitted to Lig.ex surgery (data not shown).

Besides, we compared capillary density in the 3 models 28 days after HLI surgery to the one of a healthy skeletal muscle. Surprisingly, we found no significant difference in capillary density between healthy skeletal muscles and ischemic muscles of mice (diabetic or not) submitted to Seq.lig. surgery, on the contrary to mice submitted to Lig.ex. surgery, which displayed significantly higher capillary density (data not shown).

Hence, diabetic mice, 28 days after being submitted to seq.lig. surgery, show extensive chronic limb ischemia, with very low blood flow and normal capillary density. To verify whether this model recapitulates the phenotype of human CLI, we further characterized both the phenotype of myocytes to assess muscle damage and the phenotype of capillaries to examine EC function in diabetic mice submitted to Seq.lig surgery and compared it to the one of healthy skeletal muscle.

The muscle of diabetic mice submitted to sequential femoral and iliac artery ligation display myopathic features

We first identified myocytes after desmin immunostaining and found a significant increase in desmin-negative areas in the muscle of diabetic mice submitted to Seq.lig. surgery compared to healthy muscle (data not shown). In addition, desmin staining of the ischemic muscle revealed "myopathic features", such as a heterogeneous size of muscle fibers, rounded shapes and central nuclei. Consistently, fibrosis, identified after Masson Trichrome staining, was significantly increased in the ischemic muscle (data not shown). Muscle damage was confirmed by western blot analysis of desmin expression and qPCR analysis of Myogenin (MyoG), early markers of muscle differentiation, which revealed significantly increased levels of both desmin and MyoG in the setting of ischemia (data not shown). This result, together with a significantly increased cell proliferation (data not shown), testifies myocyte immaturity and myopathy.

Altogether, these results demonstrate that the ischemic muscle of diabetic mice submitted to Seq.lig surgery displays characteristics approaching the human phenotype with "normal" capillary density associated with evident myopathy.

The muscle of diabetic mice submitted to sequential femoral and iliac artery ligations displays clear signs of endothelial dysfunction

We then explored the phenotype of small vessels to identify EC dysfunction. Twenty-eight days after HLI was induced, both interstitial albumin positive surface area and macrophage density were significantly increased in the muscle of diabetic mice submitted to Seq.lig surgery as compared to healthy skeletal muscle (data not shown) suggesting abnormal vascular leakage and increased EC activation respectively.

EC activation was confirmed by significantly higher expression of VCAM-1 and ICAM-1 (data not shown). Moreover, we detected decreased antioxidant capacities as shown by a significant decrease in Superoxide dismutase-2 (SOD2) (data not shown) and PGC-1 β mRNA expressions (data not shown) in the ischemic muscle of diabetic mice submitted to Seq.lig surgery. Finally, to assess the functional perfusion consequences of EC dysfunction, mice were intravenously administered FITC-labeled BS1-lectin, and the ratio of Lectin-positive over CD31-positive elements was quantified. We found a decreased percentage of perfused capillaries in the ischemic muscle of diabetic mice submitted to Seq.lig surgery as compared to healthy skeletal muscle (data not shown). Consistently, we detected a significant increase in hypoxypromote-positive areas in these mice (data not shown).

This set of data, together with data described in the above paragraph, reveal that the ischemic muscle of diabetic mice submitted to Seq.lig surgery shows features comparable to the human phenotype of CLI, with evident myopathy and EC dysfunction. Accordingly, diabetic mice submitted to Seq.lig. surgery can then be considered as a suitable model of CLI.

Most importantly, both human and mouse data obtained so far, highlight for the first time an evident altered EC function within the ischemic muscle in the setting of CLI. With the aim to measure the contribution of EC dysfunction in the pathophysiology of CLI, we tested whether ameliorating EC function would improve capillary perfusion and muscle repair.

Dhh is significantly reduced in ECs isolated from the ischemic muscle

As mentioned in the introduction, EC dysfunction in the setting of cardiovascular risk factors (including diabetes) is triggered, at least in part, by the loss of Dhh expression.²⁰ We then tested whether Dhh is consistently downregulated in the ischemic muscle of diabetic mice submitted to Seq.lig. surgery.

More specifically, we measured Dhh mRNA expression in ECs isolated from the ischemic muscle of diabetic mice submitted to Seq.lig surgery and compared it to the one of ECs isolated from healthy muscles. Dhh expression was significantly lower in the CD31-positive fraction of ischemic muscles (**Figure 3A**), suggesting that Dhh agonists may be used to improve EC function in this model.

SAG is not pro-angiogenic but restores diabetes-induced EC dysfunction.

Before testing whether restoring Dhh signaling would ameliorate EC function, and the overall muscle recovery, we tested the properties of the Hh signaling pathway agonist, SAG, on EC function both in vivo and in vitro.

First, we tested whether SAG is able to restore Cdh5-dependent junctions in glucose-treated ECs and found that, while Cdh5 staining of glucose treated ECs displayed a zig-zag phenotype testifying altered junction integrity, SAG treated ECs had more linear junctions (**Figure 3B**). Adherens junction integrity was further quantified by measuring Cdh5 interaction with β -catenin. Consistently, while glucose significantly reduced Cdh5 interaction with β -catenin, SAG treatment significantly promoted Cdh5 interaction with β -catenin in glucose treated-ECs (**Figure 3C**) confirming the protective effect of SAG on EC adherens junctions.

Then, we tested whether SAG would prevent EC activation and found that SAG significantly inhibited glucose-induced VCAM-1 overexpression (**Figure 3D**). The effect of SAG on oxidative stress was evaluated in vitro by measuring SOD2 expression. As shown in **Figure 3E**, SAG increased the expression of SOD2 in the presence of high glucose levels. The effect of SAG on eNOS activity was evaluated in vasomotricity tests ex vivo. We found a significant

impairment of acetylcholine-dependent femoral artery relaxation in diabetic mice in comparison to control mice ($p=0.002$), which was normalized in diabetic mice treated with SAG, despite increased median effective concentration (EC_{50}) of acetylcholine. We then used nitroprusside sodium (NO donor) to verify that impaired femoral artery relaxation was due to
5 deficient NO-secretion by ECs. Accordingly, we did not find a significant difference between the three groups, thus confirming that the relaxation defect was in fact due to endothelial dysfunction and that SAG acted by rescuing NO production by ECs (**Figure 3F**).

Finally, because Hh ligands, when administered ectopically, were found to be pro-angiogenic,^{24,18,25} we tested whether SAG is pro-angiogenic in the mouse model of corneal
10 angiogenesis. Quantitative analysis of the surface of CD31-positive elements showed the absence of angiogenic response after implantation of corneal pellets impregnated with SAG (**Figure 3G**). Altogether, these results demonstrate that SAG can be used to improve EC function, at least in the setting of diabetes. Moreover, we found that SAG is not pro-angiogenic which makes it the perfect molecule to test whether ameliorating EC function without
15 increasing capillary density is a suitable strategy to improve ischemic muscle perfusion.

SAG therapy improved EC function and ameliorated microvessel perfusion without increasing capillary density.

Finally, to test (1) whether SAG treatment ameliorates EC function in the ischemic muscle and (2) whether ameliorating EC function subsequently improves ischemic muscle
20 perfusion and repair, diabetic mice were randomly assigned to be treated with NaCl or SAG, right after the second HLI surgery was performed (i.e. ligation of the iliac artery). More specifically, mice were administered intraperitoneally with 5mg/kg/days SAG or with 200 μ L NaCl 9‰ until sacrifice (i.e. for 24 days).

As expected, SAG therapy did not modify capillary density within the ischemic skeletal
25 muscle (**Figure 4A**). However, SAG therapy significantly reduced interstitial albumin positive surface area (**Figure 4B**), VCAM-1 expression (**Figure 4C**) together with macrophage infiltration (**Figure 4D**) and increased SOD2 mRNA expression (**Figure 4E**). In conclusion, SAG successfully decreased vascular leakage, EC activation and restored anti-oxidant capacities of ECs.

We then tested whether amelioration of EC function did improve capillary perfusion.
30 We first measured capillary perfusion after mice were I.V. administered with FITC-labeled BS1-Lectin and found that the number of Lectin-positive over CD31-positive elements was significantly increased in SAG treated-mice (**Figure 5A**). In addition, we measured the overall

blood flow recovery in the ischemic foot and found that the blood flow ratio in the ischemic leg/control tended to increase (by almost 140%) in SAG-treated mice (**Figure 5B**).

Moreover, we measured myocyte phenotype and found that SAG significantly decreased desmin-negative areas, which was associated with improved myocyte differentiation: myocytes acquired a more regular and squared shape (**Figure 5C**). In addition, MyoG (**Figure 5D**) expression level was significantly diminished in SAG-treated muscles. Accordingly, cell proliferation was significantly decreased after SAG therapy (**Figure 5E**).

Altogether, this set of data demonstrates for the first time that SAG therapy, which significantly ameliorated EC function within the ischemic skeletal muscle, successfully improved muscle perfusion and ischemic muscle recovery.

SAG ameliorated EC function and ischemic muscle repair by targeting ECs

Lastly, to verify that ECs are effectively the cells responding to SAG and to prove that myopathy was prevented by treating EC dysfunction, diabetic, endothelial deficient Smo (Smo^{ECKO}) mice were submitted to Seq. lig. surgery and treated or not with SAG for 24 days.

Consistently with WT mice, SAG therapy did not induce any significant difference in the number of CD31-positive elements within the ischemic muscle of Smo^{ECKO} mice (data not shown). However, on the contrary to WT mice, the percentage of albumin positive surface area was comparable in Smo^{ECKO} mice treated or not with SAG (data not shown). Macrophage density also remained unchanged in SAG-treated Smo^{ECKO} mice (data not shown). Accordingly, SAG therapy did not increase foot perfusion in Smo^{ECKO} mice as the blood flow ratio in the ischemic foot/control was not different in Smo^{ECKO} mice treated or not with SAG (data not shown). Finally, SAG did not lead to decreased desmin-negative areas and was not associated with improved myocyte differentiation since MyoG expression was not different in Smo^{ECKO} treated or not with SAG (data not shown).

This last set of data proves that the effects of SAG therapy observed in the present study are specifically due to the action of SAG on ECs. Consequently, ameliorating EC function is a working strategy to improve muscle perfusion and recovery in the setting of CLI.

Discussion:

Collectively, our findings establish for the first time that the ischemic muscle of CLI patients presents a myopathic phenotype associated with normal capillary density but evident signs of microvessel dysfunction. In addition, the present study reveals that specifically targeting EC dysfunction significantly improves muscle perfusion and prevents muscle damage without increasing the number of microvessels. Altogether, the present findings open the way to a new class of therapies for CLI.

Notably, as mentioned in the introduction, previous studies disagree establishing whether capillary density within the ischemic muscle is increased, diminished or not modified; nevertheless two studies reported an altered capillary structure with a significant basement membrane thickening.^{9,14} The present human study reveals that capillary density within the ischemic muscle of IC and CLI patients is equivalent to that of a healthy skeletal muscle in control subjects. Moreover, three levels of biopsies were performed for each CLI patient, from the most ischemic one to the edge of amputation, and capillary density did not differ significantly between the 3 levels (data not shown).

Moreover, it further demonstrates an evident microangiopathy characterized by increased capillary permeability together with muscle inflammation, which highlights EC activation. Microangiopathy was consistently found in our mouse model and further confirmed by significantly increased VCAM-1 and ICAM-1 expression together with decreased antioxidant properties.

Based on the assumption that making more blood vessels would improve tissue perfusion and lead to limb salvage, several pro-angiogenic therapies have been tested, first in animal models of HLI and then in human clinical trials. The first results obtained in animals were very promising.^{26,27} Consistently, phase I clinical trials successfully demonstrated increased collateral vessel formation after arterial gene transfer of VEGFA encoding plasmids in CLI patients.²⁸ However, larger randomized clinical trials of angiogenic therapies for IC or CLI have been negative.²⁹ The measured end points were amputation rates, decrease in pain and peak walking time. Considering that capillary density is not diminished in the muscle of CLI patients, and the negative results obtained with pro-angiogenic therapies, it is reasonable to hypothesize that making more capillaries may not be what is necessary to improve ischemic muscle perfusion and recovery and to achieve limb salvage in CLI patients. Accordingly, we decided to go further in exploring the role of EC dysfunction in the pathophysiology of CLI. The present study demonstrates that ameliorating EC function and capillary functionality is a functioning strategy to ameliorate ischemic muscle perfusion and to prevent myopathy.

Besides, the present study further highlights the therapeutic potential of Hh signaling pathway agonists to ameliorate EC function. Indeed, it has recently been pointed out that the Hh signaling pathway is essential to microvascular integrity.^{20,30} More precisely, specific inactivation of Dhh in ECs leads to the loss of adherent junctions and an overexpression of interleukin (Il)-6, VCAM-1 and ICAM-1 (Hollier et al. unpublished data) signaling EC activation. Consistently, re-establishment of Dhh activity with the Smoothened agonist SAG was shown to prevent diabetes-induced vascular leakage in the sciatic nerve of mice.²⁰ In the

present study, we further characterized the effect of SAG on EC function, and showed that SAG prevents diabetes-induced impaired vasomotricity, glucose-induced loss of adherens junction and glucose-induced VCAM-1 expression. However, on the contrary to Hh ligands, it is not pro-angiogenic. Notably, therapies currently available to improve EC function, like Angiotensin-converting enzyme (ACE) inhibitors, Angiotensin type-1 receptor antagonists and statins, prevent some EC dysfunction features (including oxidative stress, pro-inflammatory phenotype and eNOS uncoupling).³¹ SAG appears then as one of the best therapies for EC dysfunction especially because of its wide spectrum of action.

Finally, and most importantly, the present study reveals for the first time that ameliorating EC function is a working strategy to improve ischemic muscle perfusion and to prevent myopathy. More specifically, we demonstrated that SAG therapy decreased EC activation, vascular leakage, and restored SOD2 expression. This leads to an improved capillary perfusion together with an improved myocyte organization i.e. a decreased desmin and MyoG expression and an increased myofiber cross-sectional area. Notably, desmin expression level and myofiber roundness were shown to be inversely correlated with calf muscle strength in patients with PAD.^{15,32}

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REFERENCES:

20 Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

CLAIMS:

1. A method of treating endothelial dysfunction in a patient in need thereof comprising administering to the patient a therapeutically effective amount of a hedgehog agonist.
2. The method of claim 1 wherein the patient suffers from a systemic inflammatory
5 response syndrome or sepsis.
3. The method of claim 1 wherein the patient suffers diabetes mellitus
4. The method of claim 1 for the treatment of diabetic micro-and/or macroangiopathy.
5. The method of claim 4 for the treatment of diabetic nephropathy, diabetic dermopathy, diabetic retinopathy and diabetic neuropathy.
- 10 6. The method of claim 4 for the treatment of a peripheral arterial disease.
7. The method of claim 6 wherein the peripheral arterial disease is selected from the group consisting of acute and chronic critical limb ischemia, Buerger's disease and critical limb ischemia in diabetes.
8. The method of claim 1 wherein the hedgehog agonist is a Desert hedgehog (Dhh)
15 polypeptide.
9. The method of claim 1 wherein the hedgehog agonist is SAG (3-chloro-N-[trans-4-(methylamino)cyclohexyl]-N-[[3-(4-pyridinyl)phenyl]methyl]-benzo[b]thiophene-2-carboxamide).

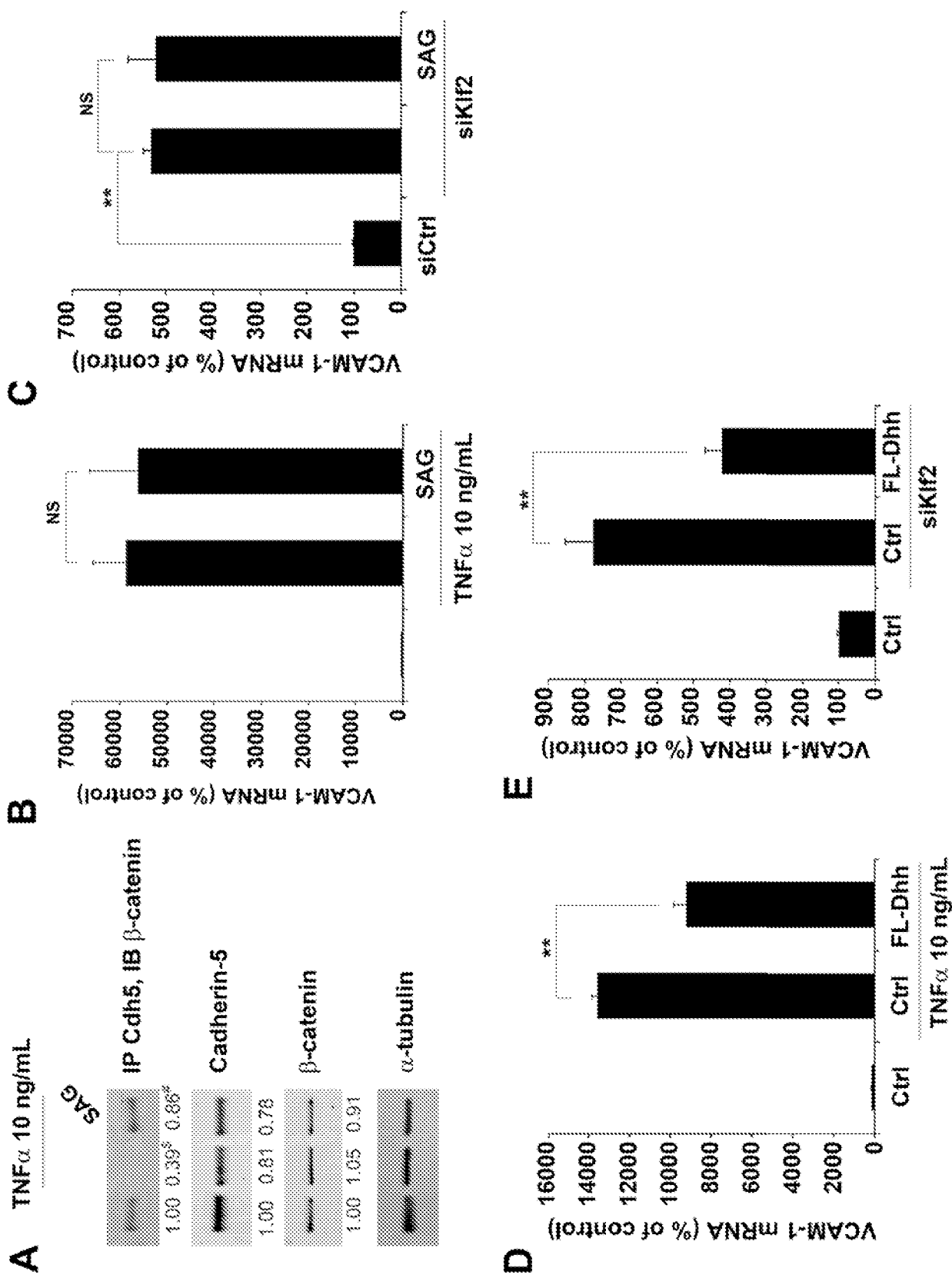


Figure 1

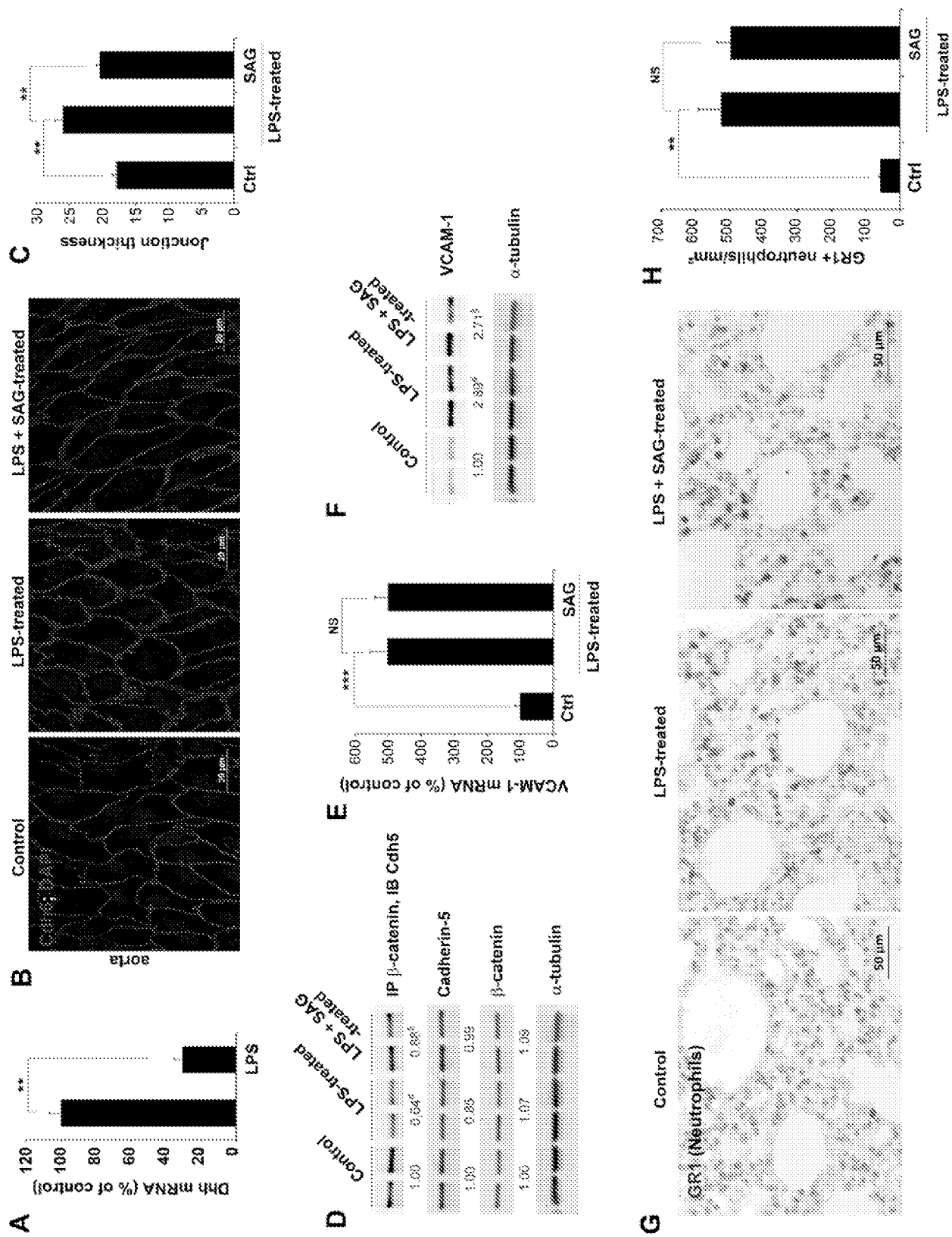


Figure 2

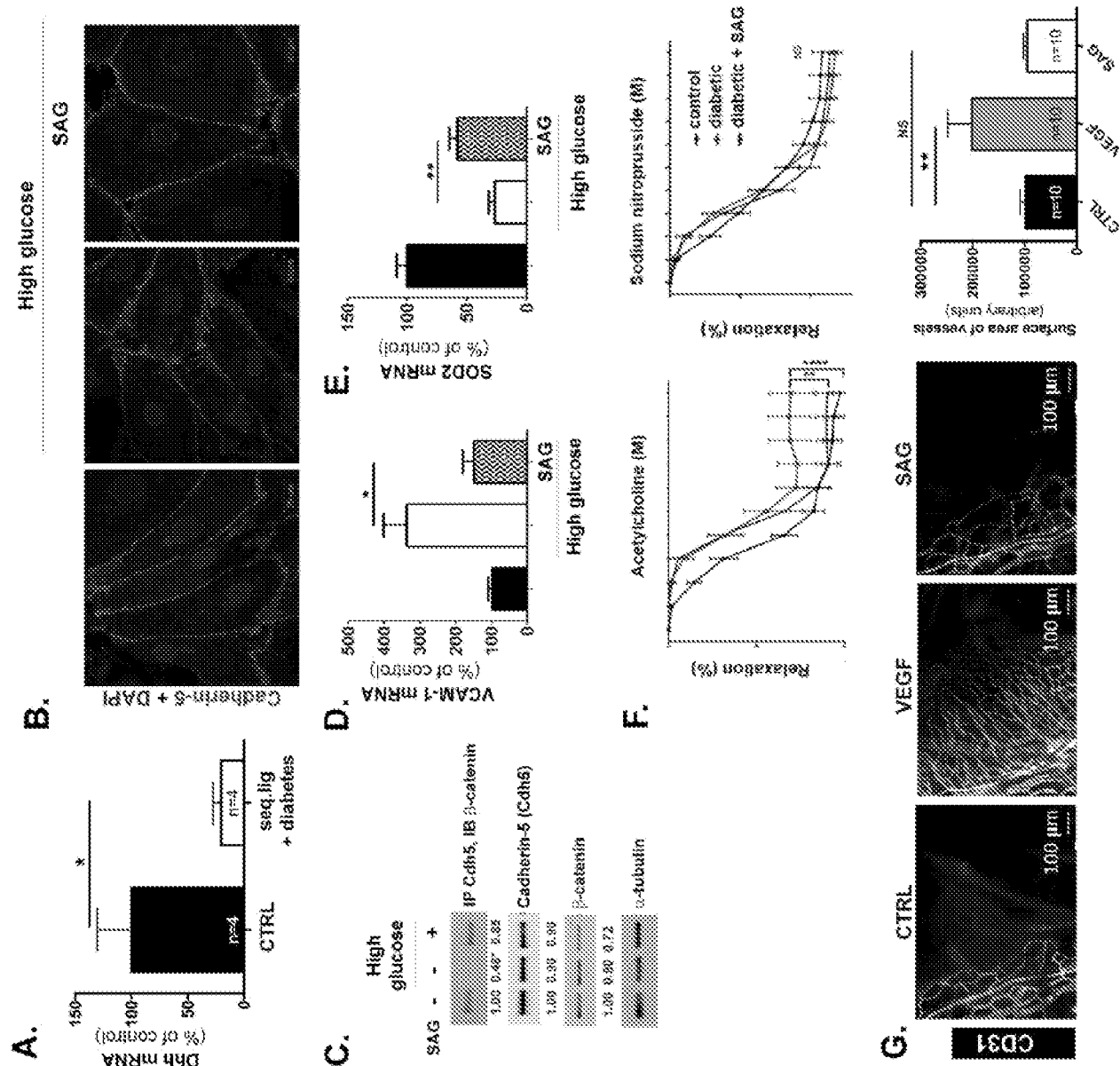


Figure 3

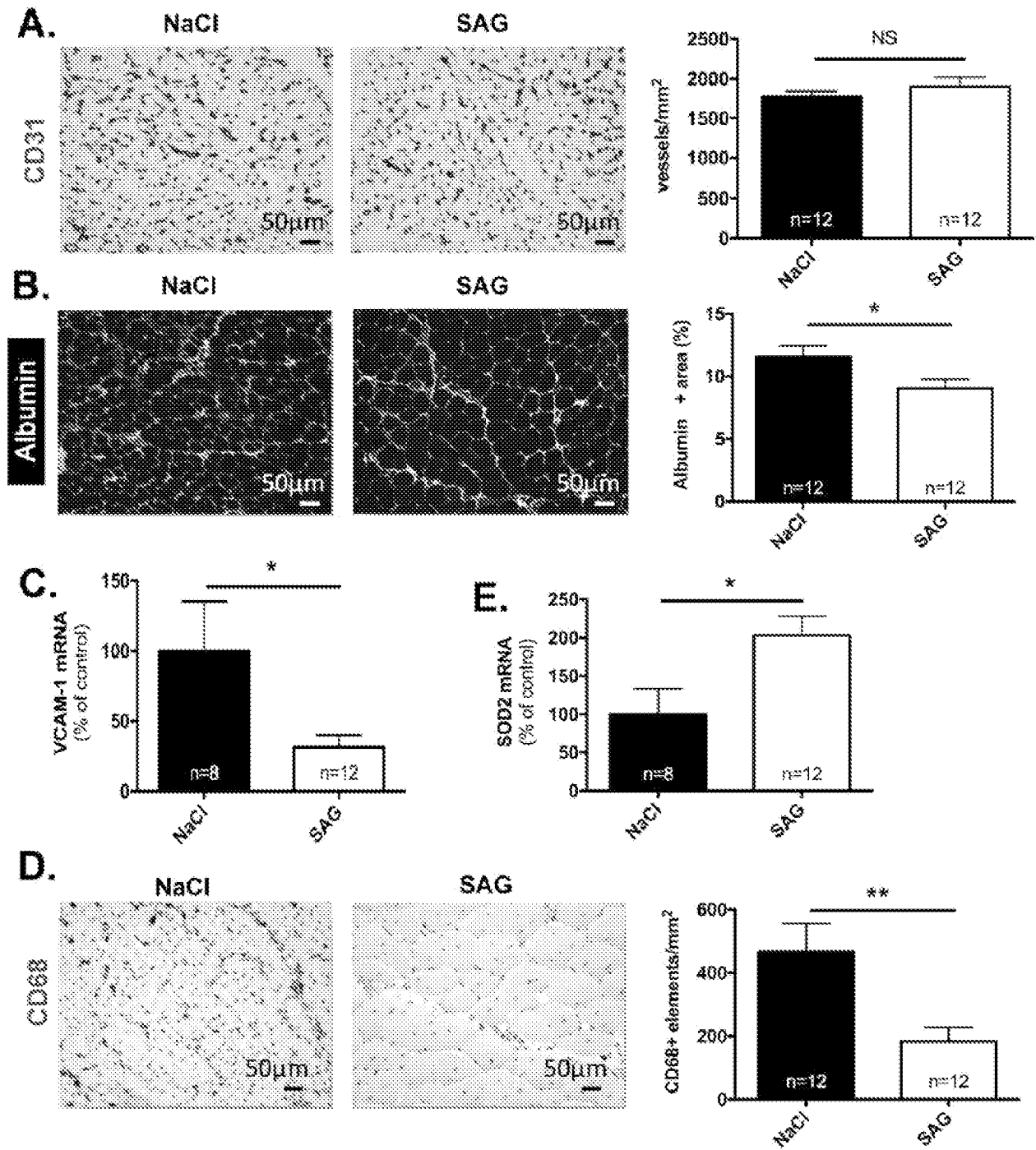


Figure 4

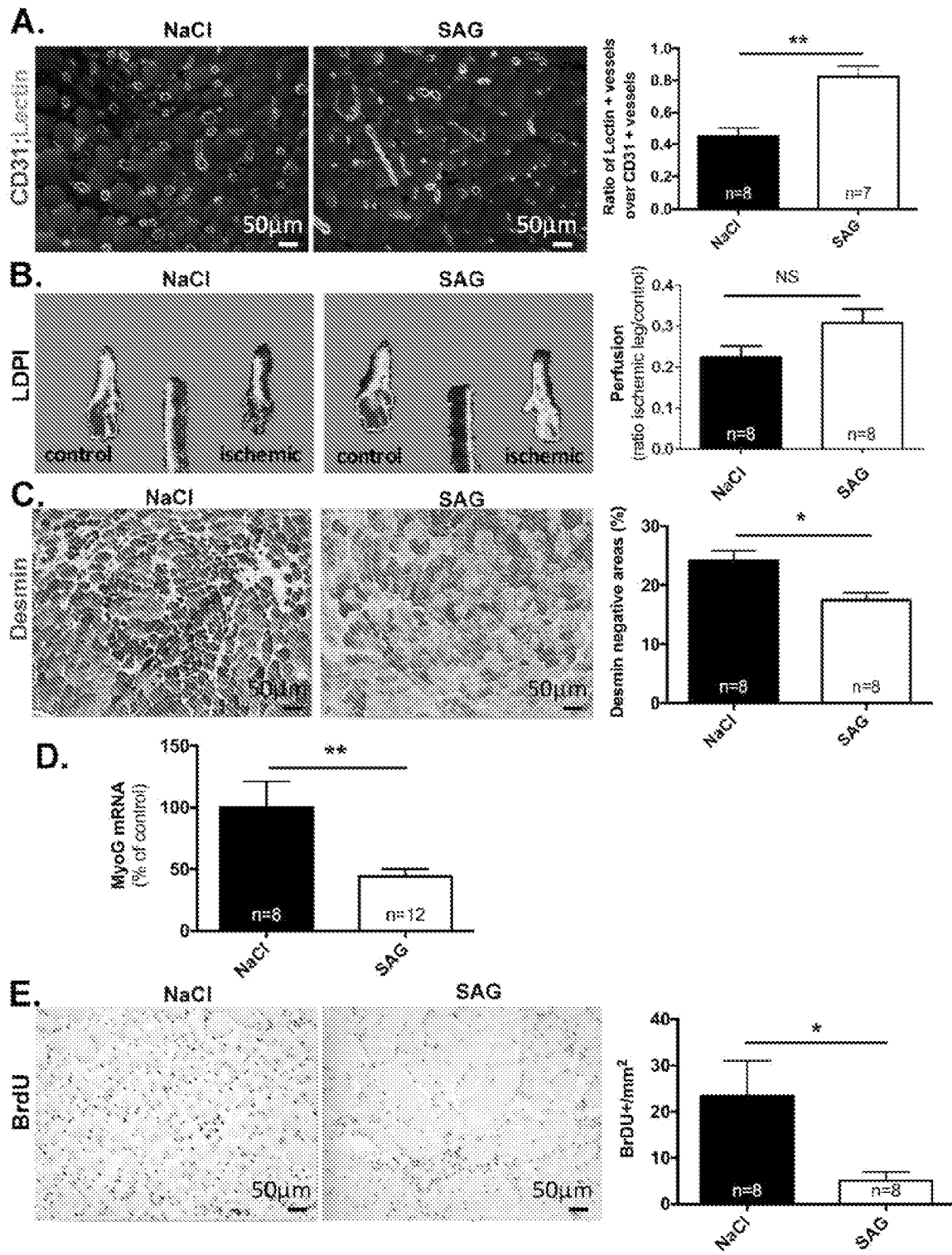


Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/001161

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/4436 A61K31/00 A61P9/08 A61P9/10
ADD.

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)

A61K A61P

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

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

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CARADU C ET AL: "Endothelial cell dysfunction is a new potential therapeutic target for the treatment of critical limb ischemia", ARCHIVES OF CARDIOVASCULAR DISEASES SUPPLEMENTS, vol. 9, no. 2, 6 April 2017 (2017-04-06), page 163, XP085135682, ISSN: 1878-6480, DOI: 10.1016/S1878-6480(17)30404-4 the whole document ----- -/--	1-9

☒ 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

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" 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

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Date of the actual completion of the international search

18 April 2018

Date of mailing of the international search report

25/04/2018

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Hornich-Paraf, E

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/001161

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CARADU C ET AL: "Dhh-induced autocrine signaling in endothelial cells is critical for endothelium integrity", ARCHIVES OF CARDIOVASCULAR DISEASES SUPPLEMENTS, vol. 9, no. 2, 7 April 2017 (2017-04-07), pages 195-196, XP085135762, ISSN: 1878-6480, DOI: 10.1016/S1878-6480(17)30486-X the whole document -----	1-9
X	CANDICE CHAPOULY ET AL: "Impaired Hedgehog signalling-induced endothelial dysfunction is sufficient to induce neuropathy: implication in diabetes", CARDIOVASCULAR RESEARCH, vol. 109, no. 2, 8 December 2015 (2015-12-08), pages 217-227, XP055466785, GB ISSN: 0008-6363, DOI: 10.1093/cvr/cvv263 the abstract 2. Methods 3. Results 4. Discussion -----	1-9
X	QIN YUAN ET AL: "Sonic hedgehog improves ischemia-induced neovascularization by enhancing endothelial progenitor cell function in type 1 diabetes", MOLECULAR AND CELLULAR ENDOCRINOLOGY, ELSEVIER IRELAND LTD, IE, vol. 423, 7 January 2016 (2016-01-07), pages 30-39, XP029412412, ISSN: 0303-7207, DOI: 10.1016/J.MCE.2016.01.005 the whole document, in particular the abstract 3. Results 2. Materials and methods 4. Discussion -----	1-9
A	M.-A. RENAULT ET AL: "Desert Hedgehog Promotes Ischemia-Induced Angiogenesis by Ensuring Peripheral Nerve Survival", CIRCULATION RESEARCH., vol. 112, no. 5, 23 January 2013 (2013-01-23), pages 762-770, XP055466794, US ISSN: 0009-7330, DOI: 10.1161/CIRCRESAHA.113.300871 in particular the abstract the discussion ----- -/--	1-9

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2017/001161

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SEIFERT KATRIN ET AL: "Potent small molecule Hedgehog agonists induce VEGF expression in vitro", BIOORGANIC & MEDICINAL CHEMISTRY, vol. 20, no. 21, 1 November 2012 (2012-11-01), pages 6465-6481, XP028945250, ISSN: 0968-0896, DOI: 10.1016/J.BMC.2012.08.026 the whole document -----	1-9
A	DÍAZ-CORÁNGUEZ MÓNICA ET AL: "Cell autonomous sonic hedgehog signaling contributes to maintenance of retinal endothelial tight junctions", EXPERIMENTAL EYE RESEARCH, ACADEMIC PRESS LTD, LONDON, vol. 164, 22 July 2017 (2017-07-22), pages 82-89, XP085205957, ISSN: 0014-4835, DOI: 10.1016/J.EXER.2017.07.014 the whole document, in particular 1. Introduction 4. Discussion -----	1-9