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(54) Title: INHIBITORS FOR TREATING OR PREVENTING A PULMONARY ARTERIAL HYPERTENSION IN SYSTEMIC SCLEROSIS PATIENTS AND METHOD FOR DIAGNOSING SAID DISEASE

(57) Abstract: The present invention relates to inhibitors of pro-MMP10 expression and/or MMP10 activity for treating or preventing PAH in SSc patients.The present invention further relates to an *in vitro* method for diagnosing a pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient from a biological sample by measuring pro-MMP expression in said sample and comparing it to one reference value.

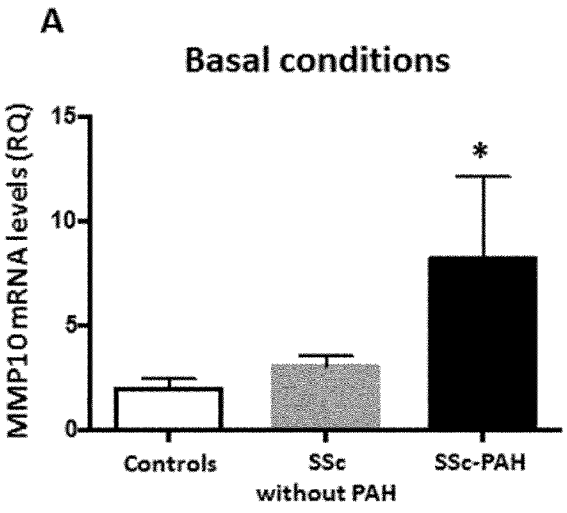


Figure 1

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INHIBITORS FOR TREATING OR PREVENTING A PULMONARY ARTERIAL HYPERTENSION IN SYSTEMIC SCLEROSIS PATIENTS AND METHOD FOR DIAGNOSING SAID DISEASE

FIELD OF THE INVENTION

The present application relates to an inhibitor of pro-MMP10 expression and/or MMP10 activity, for use in the treatment or prevention of PAH in SSc patients. The present invention further relates to an in vitro method for diagnosing pulmonary arterial hypertension (PAH) in systemic sclerosis (SSc) patients from a biological sample of said patient by measuring the expression level of the matrix metalloproteinase-10 (MMP10) and comparing it with reference value.

BACKGROUND OF THE INVENTION

Systemic sclerosis (SSc) is a severe connective tissue disorder of unknown origin that affects the skin and internal organs. Microvascular alterations are key features of the disease, with outcome depending on the extent and severity of vascular lesions. Studies with animal models have shown that endothelial cell apoptosis could be a primary event in the pathogenesis of SSc. Endothelial cell damage and apoptosis result in disorganization of the capillary architecture and loss of capillaries, which are constant at all stages of the disease. This decreased capillary density results in insufficient blood flow and reduces the supply of oxygen, which leads to tissue hypoxia (Avouac et *al.* 2011).

Pulmonary arterial hypertension (PAH) is a devastating condition related to SSc vasculopathy, it is characterized by a pulmonary vascular remodeling that leads to an increase in pulmonary vascular resistance, right ventricular hypertrophy, uncompensated right ventricular failure, and ultimately death (McLaughlin et *al.* 2011). PAH can be classified into five main categories with shared clinical and pathological characteristics, including idiopathic PAH and PAH associated with other diseases and conditions (Simonneau et *al.* 2013). Although PAH is rare in the general population (idiopathic PAH having an incidence of 1-2/million/year, Ling et *al.* 2012), it is more common in several associated conditions, most noticeably connective tissue diseases.

Recently, there has been increasing interest in PAH related to systemic sclerosis (SSc), which is the connective tissue disease most often associated with PAH (Badesch et *al.* 2004). SSc-PAH has a prevalence of about 9% according to a recent meta-analysis, and

an estimated incidence of 0.61 patient-years (Avouac et *al.*2010). PAH is one of the leading causes of death in SSc and the prognosis for patients with SSc-PAH is worse compared to other PAH subgroups. Indeed, the 1-year mortality rate in patients with idiopathic PAH is approximately 15% vs. 30% in patients with SSc-PAH prospectively followed in the European Scleroderma Research and Trials (EUSTAR) cohort (Humbert et al, 2010 and Tyndall et al. 2010). Moreover, current therapeutic approaches in SSc-PAH largely provide symptomatic relief while the prognosis remains poor due to the lack of specific molecular targets and the involvement of several factors in the development of PAH and even more in SSc-PAH.

There is thus a need for new treatments of SSc-PAH. Indeed, this subset exhibits the worse prognosis of all PAH. New treatments that would not only provide symptomatic relief but that would instead target underlying mechanisms of SSc-PAH are highly warranted. In addition, since SSc-PAH is a very serious condition, methods for early detection of PAH in SSc patients, permitting prevention or early treatment of PAH in SSc patients are also needed.

A recent study by Hoffman et *al.* (2015) was performed using human lung tissue with the aim to elucidate the expression profile of collagens and their processing enzymes in healthy donors and in idiopathic pulmonary arterial hypertension (iPAH) patients. It was found that some collagens, a disintegrin and some metalloproteases, for example MMP19 and ADAM33, are significantly elevated only in intima and media lung vessels of iPAH patients compared with healthy donors vessels, whereas MMP10, ADAM17 and TIMP1 revealed significantly decreased expression in intima and media lung vessels of iPAH patients compared with healthy donors vessels.

Thus, there are several investigations in the field of the idiopathic pulmonary arterial hypertension (iPAH) but there a few studies of mechanism of action of pulmonary arterial hypertension related to systemic sclerosis. Consequently, there are no efficient therapeutics targets allowing treating this disease or biomarkers allowing its good diagnosis or prognosis. SSc-PAH is a poor prognosis diseases Life expectancy of patients suffering from this disease is very low (about 4 years). So, it is necessary to find out new efficient therapeutic target molecules and method for diagnosing this disease easily and effectively.

SUMMARY OF THE INVENTION

The inventors have found that a specific metalloproteinase, MMP10, is overexpressed in endothelial cells derived from progenitors obtained from patient suffering from SSc-PAH compared to SSc patients without PAH. The inventors have also

showed that the expression of MMP10 is increased in the EPCs obtained from patients suffering from SSc without PAH compared to healthy subjects.

These findings were unexpected since it has been shown in Hoffman et *al.* (2015) that the expression of MMP10 was significantly decreased in intima and media of vessels obtained from patients suffering from idiopathic pulmonary arterial hypertension (iPAH). Hence, this highlight a potential key molecule of SSc-PAH as compare to other PAH forms.

Moreover, based on a relevant animal model of SSc-PAH, the inventors demonstrated that the overexpression of MMP10 and its precursor pro-MMP10 is directly involved in the biological mechanism of PAH development in SSc patients, and that inhibiting pro-MMP10 expression and/or MMP10 activity permits to inhibit or reverse PAH development in SSc patients, by decreasing vascular remodeling.

In a first aspect, the present invention thus relates to an inhibitor of pro-MMP10 expression and/or MMP10 activity, for use in the treatment or prevention of PAH in a SSc patient. Specific inhibitors are cited in the detailed description bellow.

Further, the inventors demonstrated that overexpression of MMP10 precursor pro-MMP10 in serum of SSc patients may be used for diagnosing PAH in SSc patients.

In a second aspect, the present invention thus relates to an *in vitro* method for diagnosing a pulmonary arterial hypertension (PAH) in systemic sclerosis (SSc) patients from a biological sample of said patient, comprising the following steps:

a) measuring *in vitro* the expression level of the precursor of matrix metalloproteinase-10 (pro-MMP10) in said sample;

b) comparing the expression level of pro-MMP10 measured in step a) to the expression level of pro-MMP10 in at least one reference sample, and

c) diagnosing PAH from said comparison.

This method allows determining if a SSc patient is or not suspected to suffer from PAH and should thus be investigated by right heart catheterism and, if diagnosis of PAH is confirmed, further treated in order to improve its life expectancy. Since investigation of PAH by right heart catheterism is quite invasive, the method according to the invention is advantageous since it permits to resort to this invasive method only when presence of PAH is already suspected based on the non-invasive diagnosis method according to the invention. This method could also allow:

- determining a risk stratification among patients, in which case the invasive investigation of PAH by right heart catheterism may be applied for instance only to patients in the high risk classes,
- monitoring a SSc-PAH patient treated for PAH until PAH is over,

- determining if a SSc-PAH patient has a high risk to develop advanced PAH and further treat the patient in order to prevent or to delay advanced PAH development, or
- determining the presence or absence of a specific type of PAH, in particular group 3 of PAH as defined by WHO.

DESCRIPTION OF THE FIGURES

Figure 1A-D: Increased MMP10 mRNA levels in circulating endothelial progenitor cell (EPC)-derived endothelial cells from patients with pulmonary arterial hypertension (PAH) associated with systemic sclerosis (SSc). A-B, Increased mRNA levels by qPCR in SSc-PAH EPC-derived endothelial cells in basal conditions (A) and after hypoxic exposure (B) compared to cells derived from SSc patients without PAH and healthy controls. Bars represent mean±SEM, n=10 controls, n=30 SSc patients without PAH and n=6 SSc patients with PAH. Statistical analyses: Kruskal-Wallis test; * $P<0.05$. **C-D** Comparison of microarray and qPCR measurements in individual SSc-PAH, SSc without PAH and control cells for MMP10 in basal conditions (C) and after hypoxic exposure (D). Statistical analyses: Spearman's rank correlation test.

Figure 2 A-C Increased MMP10 protein levels in circulating endothelial progenitor cell (EPC)-derived endothelial cells from patients with pulmonary arterial hypertension (PAH) associated with systemic sclerosis (SSc). A, Representative images of immunoblot analysis of MMP10 protein in EPC-derived endothelial cell lysates issued from patients with SSc-PAH (n=2) compared to SSc patients free of PAH (n=2) and healthy controls (n=2). **B**, Quantitative analysis of immunoblots assessed by the ImageJ software showing increased MMP10 protein levels in SSc-PAH EPC-derived endothelial cells. Bars represent mean±SEM, n=4 controls, n=4 SSc patients without PAH and n=4 SSc patients with PAH. Statistical analyses: Kruskal-Wallis test; ** $P<0.01$. **C**. Quantitative analysis of immunofluorescence assessed by the ImageJ software showing increased MMP10 immunostaining intensity in SSc-PAH EPC-derived endothelial cells. Bars represent mean±SEM, n=4 controls, n=4 SSc patients without PAH and n=4 SSc patients with PAH. Statistical analyses: Kruskal-Wallis test; ** $P<0.01$.

Figure 3A-D: Increased MMP10/TIMP1 mRNA ratio in circulating endothelial progenitor cell (EPC)-derived endothelial cells from patients with pulmonary arterial hypertension (PAH) associated with systemic sclerosis (SSc). A-B, Positive correlation between MMP10 and TIMP1 mRNA levels in individual SSc-PAH, SSc without PAH and control cells in basal conditions (A) and after hypoxic exposure (B). Statistical analyses: Spearman's rank correlation test. **C-D**, Increased MMP10/TIMP1 mRNA ratio assessed by

qPCR in SSc-PAH EPC-derived endothelial cells in basal conditions (C) and after hypoxic exposure (D) compared to cells derived from SSc patients without PAH and healthy controls. Bars represent mean±SEM, n=10 controls, n=30 SSc patients without PAH and n=6 SSc patients with PAH. Statistical analyses: Kruskal–Wallis test; * $P<0.05$.

Figure 4A-C: Serum proMMP10 levels are elevated in patients with pulmonary hypertension associated with systemic sclerosis (SSc-PAH). Levels of circulating proMMP10 protein were markedly increased in the serum of patients with pulmonary arterial hypertension (PAH) associated with systemic sclerosis (SSc) compared with those from SSc patients without PAH and control subjects. In addition, amongst SSC-PAH patients, proMMP10 concentrations were substantially increased in the subset of SSc patients with PAH associated with interstitial lung disease (SSc-PAH3, WHO clinical classification system Dana Point 2008 - group 3) compared to the subset of SSc patients with PAH (SSc-PAH1, WHO group 1). Similar results were obtained from A, the discovery cohort; B, the replication cohort; C, the combined cohort. Values are means±SEM. Statistical analyses: Student's t-test; **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

Figure 5: Serum proMMP10 levels. Comparison of MMP10 mRNA levels in EPC-derived endothelial cells and pro-MMP10 serum concentrations in SSc-PAH, SSc without PAH and healthy controls. Statistical analyses: Spearman's rank correlation test.

Figure 6A-B: Expression of MMP10 protein by immunohistochemistry in lungs of three patients with pulmonary arterial hypertension (PAH) associated with systemic sclerosis (SSc). Representative photomicrographs of distal pulmonary arteries in lung sections from patients with SSc-PAH. **A**, typical onion-skin lesion corresponding to laminar and concentric fibrosis of the intimal layer. **A** staining of smooth muscle cells, fibroblasts/myofibroblasts and endothelial cells is observed. Scattered inflammatory cells within the interstitium are stained as well **B**, Staining of endothelial cells and fibroblasts/myofibroblasts in a remodeled microvessel. Note positive epithelial cells (type 1-pneumocytes) within the alveoli (top and right) and scattered inflammatory cells and fibroblasts in the surrounding tissue. Scale bar = 100 μm.

Figure 7A-B: Overexpression of MMP10 protein by immunohistochemistry in lungs of Fra-2 transgenic mice. Representative photomicrographs of distal pulmonary arteries in lung sections from fra-2 transgenic mice. An overexpression of MMP10 was detected in the lung of Fra-2 transgenic mice (B) compared to wild type mice (A). MMP10 expression localized to the vessels, mainly in endothelial cells and the smooth muscle cell layer (B). Scale bar = 100 μm.

Figure 8A-B: Evaluation of the efficacy of MMP10 inhibition through a molecular targeted strategy using antibodies against the catalytic domain of MMP10 on the

progression pulmonary arterial hypertension (PAH) in fra-2 transgenic mice. **(A)** Right ventricular systolic pressure (RVSP) and **(B)** right ventricular hypertrophy (RVH, Fulton index). A total of 30 mice were used (n= 7 wild type, WT, receiving control IgG, n=8 WT receiving anti-MMP10 antibodies, n=7 fra-2 receiving control IgG and n=8 Fra2 receiving ani-MMP10 antibodies). Statistical analyses: post hoc Tukey test; * $P<0.05$, *** $P<0.001$, **** $P<0.0001$

Figure 9A-D: reduction of vascular remodeling and proliferation among MMP10 inhibition in fra-2 transgenic mice. **(A)** Representative images of hematoxylin-eosin staining, **(B)** Significant reduction of wall thickness in Fra-2 transgenic mice treated with anti-MMP10 antibodies compared with fra-2 transgenic mice receiving control IgG. **(C)** Representative images of a-smooth muscle actin (α-SMA) staining, **(D)** Significant reduction of numbers of muscularized pulmonary arteries in Fra-2 transgenic mice treated with anti-MMP10 antibodies compared to Fra-2 transgenic mice receiving control IgG. A total of 30 mice were used (n= 7 wild type, WT, receiving control IgG, n=8 WT receiving anti-MMP10 antibodies, n=7 fra-2 receiving control IgG and n=8 Fra2 receiving ani-MMP10 antibodies). Scale bar = 100 μm. Statistical analyses: Mann-Whitney U-test; * $P<0.05$.

DETAILED DESCRIPTION OF THE INVENTION

Definition of most frequently used terms

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

As used in the present description, the term **"matrix metalloprotease"** or **"matrix metalloproteinase"** or **"MMP"** relates to an endopeptidase subfamily that hydrolyze extracellular proteins, especially collagens and elastin. By regulating the integrity and composition of the extracellular matrix, these enzymes play a pivotal role in the control of signals elicited by matrix molecules that regulate cell proliferation, differentiation, and death. The following subtypes of matrix metalloproteases are known:

- collagenases, which digest triple-helical fibrillar collagens, major components of bone and cartilage, and which members are: MMP-1, MMP-8, MMP-13, MMP-18, MMP-14, MMP-2 ;
- gelatinases, which digest type IV collagen and gelatin and which members are: MMP-2, MMP-9;
- stromelysins, which digest extracellular matrix proteins but not triple-helical collagens and which members are: MMP-3, MMP-10, MMP-11, and

- Membrane-type MMPs, which have a furin cleavage site (which is also seen in MMP-11, a stromelysin) and which members are: MMP-14 to MMP-17, MMP-24, MMP-25.

As used in the present description the term **"precursor of MMP"** or **"pro-MMP"** relates to the inactive form of a metalloprotease which is generated by translation of said MMP mRNA and has not yet been activated by cleavage by an extracellular proteinase and thus does not have enzymatic activity. The active MMP form is generated by cleavage by an extracellular proteinase.

As used in the present description, the term **"MMP10"** or **"matrix metalloprotease 10"** refers to a stromelysin type of matrix metalloprotease, which is an enzyme encoded by the *MMP10* human gene. This gene is part of a cluster of MMP genes which localize to chromosome 11q22.3.

The nucleotide sequence of cDNA corresponding to mRNA (RNA messenger) encoding pro-MMP10 is available on NCBI site under the reference NM_002425 corresponding to SEQ ID NO:1. mRNA encodes pro-MMP10 protein which amino acid sequences is available on NCBI site under reference NP_002416.1 (entire sequence: amino acids 1-476) corresponding to SEQ ID NO: 2. Inactive pro-MMP10 amino acid sequence is then cleaved in vivo by an extracellular proteinase to obtain active MMP10 enzyme which amino acid sequence is available on NCBI site under reference NP_002416.1 (partial sequence: amino acids 99-476) corresponding to SEQ ID NO:3.

As used in the description, the term **"pro-MMP10"** refers to a precursor of MMP10 which has not been cleaved by an extracellular proteinase and which does not have enzymatic activity.

Sequences of pro-MMP10 mRNA and protein and of MMP10 protein are summarized in **Table 1** below.

Table 1. Sequences of pro-MMP10 mRNA and protein and of MMP10 protein

Sequences of human MMP10	NCBI Reference	SEQ ID NO:	Full sequence
mRNA	NM_002425.2	SEQ ID NO:1	aaagaaggta agggcagtga gaatgatgca tcttgcattc cttgtgctgt tgtgtctgcc agtctgctct gcctatcctc tgagtggggc agcaaaagag gaggactcca acaaggatct tgcccagcaa tacctagaaa agtactacaa cctcgaaaag gatgtgaaac agtttagaag aaaggacagt aatctcattg ttaaaaaaat ccaaggaatg cagaagttcc ttgggttgga ggtgacaggg aagctagaca ctgacactct ggaggtgatg cgcaagccca ggtgtggagt tcctgacgtt ggtcacttca gctccttcc

			tggcatgccg aagtggagga aaacccacct tacatacagg attgtgaatt atacaccaga tttgccaaga gatgctgttg attctgccat tgagaaagct ctgaaagtct gggaagaggt gactccactc acattctcca ggctgtatga aggagaggct gatataatga tctctttcgc agttaaagaa catggagact tttactcttt tgatggccca ggacacagtt tggctcatgc ctacccacct ggacctgggc tttatggaga tattcacttt gatgatgatg aaaaatggac agaagatgca tcaggcacca atttattcct cgttgctgct catgaacttg gccactccct ggggctcttt cactcagcca acactgaagc tttgatgtac ccactctaca actcattcac agagctcgcc cagttccgcc tttcgcaaga tgatgtgaat ggcattcagt ctctctacgg acctcccccc gcctctactg aggaacccct ggtgcccaca aaatctgttc cttcgggatc tgagatgcca gccaagtgtg atcctgcttt gtccttcgat gccatcagca ctctgagggg agaatatctg ttctttaaag acagatattt ttggcgaaga tcccactgga accctgaacc tgaatttcat ttgatttctg cattttggcc ctctcttcca tcatatttgg atgctgcata tgaagttaac agcagggaca ccgtttttat ttttaaagga aatgagttct gggccatcag aggaaatgag gtacaagcag gttatccaag aggcatccat accctgggtt ttcctccaac cataaggaaa attgatgcag ctgtttctga caaggaaaag aagaaaacat acttctttgc agcggacaaa tactggagat ttgatgaaaa tagccagtcc atggagcaag gcttccctag actaatagct gatgactttc caggagttga gcctaaggtt gatgctgtat tacaggcatt tggatttttc tacttcttca gtggatcatc acagtttgag tttgacccca atgccaggat ggtgacacac atattaaaga gtaacagctg gttacattgc taggcgagat agggggaaga cagatatggg tgtttttaat aaatctaata attattcatc taatgtatta tgagccaaaa tggttaattt ttcctgcatg ttctgtgact gaagaagatg agccttgcag atatctgcat gtgtcatgaa gaatgtttct ggaattcttc acttgctttt gaattgcact gaacagaatt aagaaatact catgtgcaat aggtgagaga atgtattttc atagatgtgt tattacttcc tcaataaaaa gttttatttt gggcctgttc ctt

pro-MMP10	NP_002416.1, entire sequence (amino acids 1-476)	SEQ ID NO: 2	MMHLAFLVLLCLPVCSAYPLSGAAKEEDSNKDLAQQYLE KYYNLEKDVKQFRRKDSNLIVKKIQGMQKFLGLEVTGKL DTDTLEVMRKPRCGVPDVGHFSSFPGMPKWRKTHLTYRI VNYTPDLPRDAVDSAIEKALKVWEEVTPLTFSRLYEGEA DIMISFAVKEHGDFYSFDGPGHSLAHAYPPGPGLYGDIH FDDDEKWTEDASGTNLFLVAAHELGHSLGLFHSANTEAL MYPLYNSFTELAQFRLSQDDVNGIQSLYGPPPASTEEPL VPTKSVPSGSEMPAKCDPALSFDAISTLRGEYLFFKDRY FWRRSHWNPEPEFHLISAFWPSLPSYLDAAYEVNSRDTV FIFKGNEFWAIRGNEVQAGYPRGIHTLGFPPTIRKIDAA VSDKEKKKTYFFAADKYWRFDENSQSMEQGFPRLIADDF PGVEPKVDAVLQAFGFFYFFSGSSQFEFDPNARMVTHIL KSNSWLHC
MMP10	NP_002416.1, amino acids 99-476		FSSFPGMPKWRKTHLTYRIVNYTPDLPRDAVDSAIEKAL KVWEEVTPLTFSRLYEGEADIMISFAVKEHGDFYSFDGP GHSLAHAYPPGPGLYGDIHFDDDEKWTEDASGTNLFLVA AHELGHSLGLFHSANTEALMYPLYNSFTELAQFRLSQDD VNGIQSLYGPPPASTEEPLVPTKSVPSGSEMPAKCDPAL SFDAISTLRGEYLFFKDRYFWRRSHWNPEPEFHLISAFW PSLPSYLDAAYEVNSRDTVFIFKGNEFWAIRGNEVQAGY PRGIHTLGFPPTIRKIDAAVSDKEKKKTYFFAADKYWRF DENSQSMEQGFPRLIADDFPGVEPKVDAVLQAFGFFYFF SGSSQFEFDPNARMVTHILKSNSWLHC

As used in the present description, the term **"systemic sclerosis"** or **"SSc"** refers to a connective tissue disease that is essentially characterized by vasomotor disturbances, fibrosis, subsequent atrophy of the skin, subcutaneous tissue, muscles, and internal organs (eg, alimentary tract, lungs, heart, kidney, CNS), and immunologic disturbances.

As used in the present description, the term **"pulmonary arterial hypertension" or "PAH" or "pulmonary hypertension"** refers to an increase of blood pressure in the pulmonary artery, pulmonary vein, or pulmonary capillaries, together known as the lung vasculature, leading to shortness of breath, dizziness, fainting, leg swelling and other symptoms. Pulmonary hypertension can be a severe disease with a markedly decreased exercise tolerance such as heart failure. PAH may be idiopathic or associated to various other diseases.

Pulmonary arterial hypertension can be classified by the Pulmonary Hypertension World Health Organisation (WHO) clinical classification system (Dana Point 2008) (Simonneau et *al*. 2013) comprising:

Group 1. Pulmonary arterial hypertension (also referred to in the present description as PAH1);

Group 1'. Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary haemangiomatosis (PCH);

Group 2. Pulmonary hypertension due to left heart diseases (also referred to in the present description as PAH2);

Group 3. Pulmonary hypertension due to lung diseases and/or hypoxemia (also referred to in the present description as PAH3 or "PAH ILD" for Interstitial lung disease);

Group 4. Chronic thromboembolic pulmonary hypertension (CTEPH, also referred to as PAH4); and

Group 5. PH with unclear multifactorial mechanisms (also referred to as PAH5).

By **"advanced PAH"**, it is meant a PAH which is characterised by a class 3 or a class 4 dyspnea as defined by the New York Heart Association. According to the New York Heart Association classification, the functional Assessment of Pulmonary Arterial Hypertension is divided into the following classes:

Class 1: No symptoms with ordinary physical activity.

Class 2: Symptoms with ordinary activity. Slight limitation of activity.

Class 3: Symptoms with less than ordinary activity. Marked limitation of activity.

Class 4: Symptoms with any activity or even at rest.

As used in the present description, the term **"idiopathic pulmonary arterial hypertension" or "iPAH"** refers to PAH arising spontaneously or from an obscure or unknown cause. Idiopathic pulmonary arterial hypertension is uncommon, representing only a tiny fraction of all cases of pulmonary arterial hypertension, which has a very long list of secondary causes.

As used in the present invention the expression **"pulmonary arterial hypertension resulting from systemic sclerosis"** or **"SSc-PAH"** refers to a PAH in a SSc patient.

As used herein the term **"patient"** relates to a human being, whatever its age or sex.

In the context of the present invention, **"SSc patient"** refers to a human being afflicted with systemic sclerosis.

In the context of the present invention, **"SSc-PAH patient"** refers to a human being suffering from systematic sclerosis (SSc) and who has developed pulmonary arterial hypertension (PAH).

As used herein, **"SSc-patient without PAH"** refers to a human being suffering from systematic sclerosis (SSc) but who has not, or has not yet, developed PAH.

Other definitions will be given below in the description of the embodiments of the present invention.

Inhibitors of pro-MMP10 expression and/or MMP10 activity for treatment or prevention of PAH in SSc patients

In the context of the present invention, the inventors found that MMP10 contributes to the lung vessel remodelling process leading to lung arteries progressive obstruction. The inventors have thus demonstrated that MMP10 is implicated on the development of pulmonary arterial hypertension in patients suffering from systemic sclerosis.

Following this finding, the inventors then demonstrated that inhibition of MMP10 significantly decreases right ventricular systolic pressure (RVSP) and Right Ventricular Hypertrophy (RVH) and markedly reduces vascular remodelling and myointimal proliferation, and could thus be used in the treatment and the prevention of PAH.

A first aspect of the invention thus relates to an inhibitor of pro-MMP10 expression and/or MMP10 activity, for use in the treatment or prevention of PAH in a SSc patient.

According to a preferred embodiment, the SSc patient is suffering from an advanced PAH.

According to another preferred embodiment, the SSc patient is suffering from a PAH due to lung diseases and/or hypoxemia.

According to another preferred embodiment, the inhibitor of pro-MMP10 expression and/or MMP10 activity is administered to an SSc patient that has previously been diagnosed to suffer from PAH using the method according to invention.

In an embodiment, the invention thus relates to an inhibitor of pro-MMP10 expression and/or MMP10 activity, for use in the treatment or prevention of PAH in a SSc patient, wherein said use further comprises a preliminary step of diagnosing PAH in the SSc patient according to the above described method according to the invention, and the inhibitor of pro-MMP10 expression and/or MMP10 activity is administered only if the SSc patient has been diagnosed to suffer from PAH. When said patient has been diagnosed to suffer from PAH using the method according to invention, the inhibitor of pro-MMP10 expression and/or MMP10 activity is used for treating SSc-PAH. When the SSc-PAH patient has been identified to be at risk of developing advanced PAH, the inhibitor of pro-MMP10 expression and/or MMP10 activity is used for preventing advanced PAH in the SSc-PAH patient.

In the present description, the term "**treating**" or "**treatment**" means an improvement of the patient's disease, which may be observed at the clinical, histological, biochemical level. In particular, any alleviation of a clinical, histological or biochemical symptom of the disease is included in the terms "treating" and "treatment". In the context of SSc-PAH, any decrease of blood pressure in the pulmonary artery, pulmonary vein, or pulmonary capillaries, together known as the lung vasculature; any decrease of

shortness of breath, dizziness, fainting, or leg swelling; or any increase in exercise tolerance is to be considered as a treatment. Treatment may require administration of an agent and/ or treatment more than once.

In the present description, the term **"preventing"** or **"prevention"** means the fact to preclude or delay the onset or reduce the intensity of clinical, histological or biochemical events associated with the disease. In the context of SSc-PAH, **"preventing"** or **"prevention"** thus relates to the fact to preclude or delay the onset or reduce the intensity of increase of blood pressure in the pulmonary artery, pulmonary vein, or pulmonary capillaries, together known as the lung vasculature; of shortness of breath, dizziness, fainting, leg swelling; or of decreased exercise tolerance such as heart failure. Prevention may require administration of an agent and/ or treatment more than once.

According to the present invention, any compound able to inhibit pro-MMP10 expression or MMP10 activity may be used to prevent or treat PAH in a SSc patient, provided that this inhibitor is appropriate to pharmaceutical use in a human being. It is a common practice for those skilled in the art to select appropriate inhibitors from all inhibitors known to inhibit pro-MMP10 expression or MMP10 activity.

Inhibitors of pro-MMP10 expression or MMP10 activity according to the invention include:

a) broad spectrum MMPs inhibitors, which will be able to inhibit pro-MMP10 expression or MMP10 activity as well as expression or activity of other MMPs (e.g. tetracycline);
b) medium spectrum MMP inhibitors inhibiting expression or activity of several MMPs but with a stronger inhibition on pro-MMP10 expression or MMP10 activity; and
c) specific inhibitors of pro-MMP10 expression or MMP10 activity (e.g. anti-MMP10 antibodies).

Preferably, inhibitors of pro-MMP10 expression or MMP10 activity according to the invention are selected from inhibitors of categories b) and c) above (i.e. inhibitors with at least some specificity for MMP10), more preferably inhibitors of pro-MMP10 expression or MMP10 activity according to the invention are selected in category c) above (i.e. specific inhibitors of pro-MMP10 expression or MMP10 activity).

In a preferred embodiment of the present invention, the inhibitor of pro-MMP10 overexpression and/or MMP10 activity is selected from:

- small chemical compounds, such as tetracycline compounds, batimastat, marimastat, ilomastat, prinomastat, neovastat, all of which are broad or medium spectrum MMPs inhibitors (category a) or b) above),
- TIMP-1 (TIMP metallopeptidase inhibitor 1), a natural MMP10 inhibitor,
- anti-MMP10 antibodies or antigen fragments thereof (i.e. specific inhibitors of MMP10 activity, category c) above), and

nucleic acids inhibiting the expression of pro-MMP10, such as antisense oligonucleotides, interfering RNAs, ribozymes and aptamers (i.e. specific inhibitors of pro-MMP10 expression, category c) above)

In a more preferred embodiment, the invention relates to an inhibitor of pro-MMP10 expression and/or MMP10 activity, which is selected from:

- small chemical compounds selected from the group comprising tetracycline compounds, batimastat, marimastat, ilomastat, prinomastat and neovastat,
- TIMP-1 (TIMP metallopeptidase inhibitor 1),
- anti-MMP10 antibodies or antigen fragments thereof, and
- nucleic acids inhibiting the expression of pro-MMP10, such as antisense oligonucleotides, interfering RNAs, ribozymes and aptamers,

for use in the treatment or prevention of pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient.

In an even more preferred embodiment, the invention relates to an inhibitor of pro-MMP10 expression and/or MMP10 activity, which is selected from anti-MMP10 antibodies or antigen fragments thereof, for use in the treatment or prevention of pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient.

According to the present invention, the small chemical compounds used to inhibit pro-MMP10 overexpression and MMP10 activity can be selected from tetracycline antibiotics, batimastat, marimastat, ilomastat, prinomastat, neovastat. Preferably, this small chemical compound is a doxycycline.

In the context of the present invention the term **"tetracycline"** relates to a broad class of antibiotic compounds produced by *Streptomyces* genus of Actinobacteria.

As used herein the term **"doxycycline"** relates to a broad-spectrum antibiotic of the tetracycline class, which kills bacteria and protozoa by inhibiting protein production. Doxycycline is well known in the art for its ability to inhibit metalloproteases activity. Its MMP inhibition properties were recently demonstrated by Neto-Neves and al. (2013).

In another preferred embodiment of the invention, TIMP-1 (TIMP metallopeptidase inhibitor 1), a natural MMP10 inhibitor, may be also used.

In another preferred embodiment of the invention, nucleic acids inhibiting the expression of pro-MMP10, such as antisense oligonucleotides, interfering RNAs, ribozymes and aptamers may be also used.

As herein used the term **"antisense oligonucleotides"** relates to unmodified or chemically modified single-stranded DNA molecules which are relatively short in general and which is able to hybridize to a unique sequence in the total pool of targets present in cells, the sequence of said DNA molecule being complementary by virtue of Watson-Crick bp hybridization, to a specific mRNA and is able to inhibit said mRNA expression and then induce a blockade in the transfer of genetic information from DNA to protein.

In the context of the invention, **"RNA interference"** (hereinafter referred to as RNAi) is interpreted as a process by which a double stranded RNA (dsRNA) with a given sense nucleic sequence leads to the breakdown of all messenger RNA (mRNA) comprising said nucleic sequence, in a manner specific to said nucleic sequence. Although the RNAi process was originally demonstrated in *Caenorhabditis elegans*, it is now clear that the RNAi process is a very general phenomenon, and inhibition of human genes by RNAi has been achieved.

The process of RNAi can be achieved using small interfering RNA (or siRNA). These siRNAs are dsRNA of fewer than 30 nucleotides comprising in their sense sequence a sequence that is highly homologous, preferably identical, to a fragment of the targeted mRNA. When a siRNA crosses the plasma membrane, the reaction of the cell is to destroy the siRNA and all the sequences comprising an identical or highly homologous sequence. Thus a mRNA with a fragment that is identical or highly homologous to the siRNA sequence will be destroyed, the expression of this gene being thus inhibited.

shRNA may be also used as inhibitor according to the present invention. As used herein, an "shRNA molecule" includes a conventional stem-loop shRNA, which forms a precursor miRNA (pre-miRNA). "shRNA" also includes micro-RNA embedded shRNAs (miRNA-based shRNAs), wherein the guide strand and the passenger strand of the miRNA duplex are incorporated into an existing (or natural) miRNA or into a modified or synthetic (designed) miRNA. When transcribed, a conventional shRNA forms a primary miRNA (pri-miRNA) or a structure very similar to a natural pri-miRNA. The pri-miRNA is subsequently processed by Drosha and its cofactors into pre-miRNA. Therefore, the term "shRNA" includes pri-miRNA (shRNA-mir) molecules and pre-miRNA molecules.

To inhibit pro-MMP10 expression and/or MMP10 activity, ribozymes may be also used. In this context, **"ribozymes"** also called **"catalytic RNAs"** or **"RNAzymes"**, are RNA molecules that are capable of catalyzing specific biochemical reactions, similar to the action of protein enzymes. Ribozymes allow the cleavage or ligation of RNA and DNA and

peptide bond formation. Examples of ribozymes include the hammerhead ribozyme, the VS ribozyme, Leadzyme and the hairpin ribozyme.

Aptamers may be also used in the present invention for inhibiting pro-MMP10 expression and MMP10 activity. By **"aptamer"** is meant a ligand-specific DNA or RNA molecule with high affinity for a protein. Consequently, this meaning comprises "natural" aptamers and chemically-modified analogs.

Aptamers are selected by the alternation of selection and amplification, which makes it possible to direct the evolution of the population in a Darwinian manner: in the population, the most "apt" molecules are selected, hence the origin of the name "aptamers" given to oligonucleotides having the desired feature, stemming from the selection. Standard genetic engineering techniques (cloning, sequencing, expression) make it easy to identify these aptamers individually, then to characterize them and produce them in large amounts.

Aptamers can be selected by means of an optimized *in vitro* selection protocol known as systemic evolution of ligands by exponential enrichment (SELEX) (WO 91/19813).

The SELEX method makes it possible to generate in large amounts ligands of very high affinity and specificity. This approach rests on exposing the target molecule to a library of potential ligands. A system of desorption/selection cycles makes it possible to enrich the population of ligands that most specifically interact with the target molecule. The final population obtained is then isolated and characterized, allowing its large-scale resynthesis.

Highly varied targets were aimed at in order to generate aptamers: amino acids, peptides, proteins and enzymes, and also complex structures such as intact viruses and living cells. For protein targets K_d values below 10^{-9} M are not rare.

The most remarkable property of aptamers is the specificity of the interactions engaged with their ligand, making them ideal agents for target recognition.

Aptamers can be oligodeoxynucleotides (DNA) or oligoribonucleotides (RNA). RNA and DNA aptamers having comparable characteristics were selected against the same target. Additionally, both compounds are competitive inhibitors of one another, suggesting overlapping interaction sites.

Preferably, the aptamer according to the invention can bind with high affinity to pro-MMP10 or MMP10, and most preferably to the enzymatic site of MMP10.

By "high affinity" is meant, in the context of the present invention, a specific interaction of the aptamer with the target and a dissociation constant that is sufficiently low to allow significant inhibition of the catalytic activity of the enzyme.

Antisense oligonucleotides, interfering RNAs, ribozymes and aptamers may be made of natural or modified DNA or RNA.

In particular, analogs give the oligomer resistance to nucleases, a property useful in a biological environment (cell culture medium or *in vivo*) may be used. For instance, modifications of the phosphodiester bond, sugar or sugar-phosphate backbone, such as 2'-O-methyl, 2'-amino- or 2'-fluoro-pyrimidine, locked nucleic acid or boranophosphate derivatives, lead to nuclease-resistant oligomers. L-analogs are resistant to nucleases and may also be used

In another preferred embodiment of the invention, anti-MMP10 antibodies or antigen-binding fragments thereof (which belong to the specific inhibitors of pro-MMP10 expression, category c) above) may be used for inhibiting MMP10 activity.

Particularly, said anti-MMP10 antibody or antigen-binding fragment thereof may be selected from polyclonal antibodies, monoclonal antibodies, bispecific antibodies, and from fragments selected from Fv, scFv, Fab, F(ab')2, Fab', scFv-Fc, diabodies, and any antigen-binding fragment whose half-life has been increased by chemical modification.

The term "**antibody**" is used herein in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies) of any isotype such as IgG, IgM, IgA, IgD, and IgE, polyclonal antibodies, multispecific antibodies, chimeric antibodies, and antigen-binding fragments. An antibody reactive with a specific antigen can be generated by recombinant methods such as selection of libraries of recombinant antibodies in phage or similar vectors, or by immunizing an animal with the antigen or an antigen-encoding nucleic acid.

In one embodiment, the present application relates to polyclonal antibodies as inhibitors of MMP10 activity.

A "**polyclonal antibody**" is an antibody which was produced among or in the presence of one or more other, non-identical antibodies. In general, polyclonal antibodies are produced from a B-lymphocyte in the presence of several other B-lymphocytes producing non-identical antibodies. Usually, polyclonal antibodies are obtained directly from an immunized animal.

According to preferred embodiment, the anti-MMP10 antibody, or antigen-binding fragments thereof, used in the invention is a monoclonal antibody.

As used herein, the term "**monoclonal antibody**" refers to an antibody arising from a nearly homogeneous antibody population. More particularly, the individual antibodies of a population are identical except for a few possible naturally-occurring mutations which can be found in minimal proportions. In other words, a monoclonal antibody consists of a homogeneous antibody population arising from the growth of a single cell clone (for example a hybridoma, a eukaryotic host cell transfected with a DNA molecule coding for the homogeneous antibody, a prokaryotic host cell transfected with a DNA molecule coding

for the homogeneous antibody, etc.) and is generally characterized by heavy chains of one and only one class and subclass, and light chains of only one type. Monoclonal antibodies are highly specific and are directed against a single antigen.

Moreover, the antibodies used in the present invention may be chimeric or humanized antibodies, or antigen-binding fragments, which can be obtained by genetic engineering or by chemical synthesis.

The term **"chimeric antibody"** as used herein refers to an antibody containing a natural variable region (light chain and heavy chain) derived from an antibody of a given species in combination with constant regions of the light chain and the heavy chain of an antibody of a species heterologous to said given species. Thus, a "chimeric antibody", as used herein, is an antibody in which the constant region, or a portion thereof, is altered, replaced, or exchanged, so that the variable region is linked to a constant region of a different species, or belonging to another antibody class or subclass. "Chimeric antibody" also refers to an antibody in which the variable region, or a portion thereof, is altered, replaced, or exchanged, so that the constant region is linked to a variable region of a different species, or belonging to another antibody class or subclass.

As used herein, the term "**humanized antibody**" refers to a chimeric antibody which contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a CDR of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, rabbit or non-human primate having the desired specificity, affinity, and/or capacity. In some instances, framework ("FR") residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance, such as binding affinity. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin sequence, and all or substantially all of the FR regions are those of a human immunoglobulin sequence, although the FR regions may include one or more individual FR residue substitutions that improve antibody performance, such as binding affinity, isomerization, immunogenicity, etc. The number of these amino acid substitutions in the FR is typically no more than 6 in the H chain, and in the L chain, no more than 3. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

According to a preferred embodiment of the invention, the anti-MMP10 antibody is a bispecific monoclonal antibody.

For inhibiting pro-MMP10 expression and/or MMP10 activity, an antigen-binding antibody fragment may also be used. An **"antigen-binding antibody fragment"** comprises a portion of an intact antibody that maintains binding to the antigen, preferably the antigen binding and/or the variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments; diabodies; linear antibodies (see U.S. Pat. No. 5,641,870, Example 2; Zapata et al., *Protein Eng.* 8(10): 1057-1062 [1995]); single-chain antibody molecules and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produced two identical antigen-binding fragments, called **"Fab"** fragments, and a residual **"Fc"** fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire L chain along with the variable region domain of the H chain (V_H), and the first constant domain of one heavy chain (C_H1). Each Fab fragment is monovalent with respect to antigen binding, i.e., it has a single antigen-binding site. Pepsin treatment of an antibody yields a single large **$F(ab')_2$** fragment which roughly corresponds to two disulfide linked Fab fragments having different antigen-binding activity and is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having a few additional residues at the carboxy terminus of the C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known

In a preferred embodiment of the invention, said anti-MMP10 antibody or antigen-binding fragment thereof blocks the catalytic domain of MMP10. Examples of such anti-MMP10 antibodies that block the catalytic domain of MMP10 include a rabbit polyclonal antibodies against the catalytic domain of MMP10 and a monoclonal anti-MMP10 antibody denoted "[5E4]" commercialized by Abcam, Paris, France (catalog references: ab59437 for the rabbit polyclonal antibody and ab49473 for the [5E4] monoclonal antibody). Monoclonal antibodies or antigen-binding fragments thereof that block the catalytic domain of MMP10 may also be generated by those skilled in the art based on technologies well known in the art. In particular, a response against human MMP10 may be generated in an animal model, hybridomas cloned and antibodies produced by the cloned hybridomas tested for their ability to block the catalytic domain of MMP10.

Further inhibitors of pro-MMP10 expression and/or MMP10 activity, in addition to those described above, may be obtained by implementation of classical screening methods well known to skilled artisans.

The inhibitors of pro-MMP10 expression or MMP10 activity as described above may be also included in pharmaceutical composition for treating SSC or for preventing or treating PAH of SSc patient.

In another aspect, the invention thus relates to a pharmaceutical composition comprising at least one inhibitor of pro-MMP10 expression or MMP10 activity as described above and at least one pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant, for use in the prevention or in the treatment of a pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient.

Preferably, the pharmaceutical composition of the invention may contain, in addition to the inhibitor of pro-MMP10 expression or MMP10 activity as described above, various diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art.

In still another aspect, the present invention relates to a method for treating or preventing PAH in a SSc patient, comprising administering an inhibitor of pro-MMP10 expression or MMP10 activity or a pharmaceutical composition according to the invention as described above.

An *in vitro* method for diagnosing a pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient

As indicated above, the inventors have also found that endothelial cells derived from circulating progenitors obtained from SSc-PAH patients had increased pro-MMP10 mRNA levels compared to SSc patient without PAH and even lower in healthy patients. The inventors also found that serum pro-MMP10 levels have high positive and negative predictive value for the diagnosis of SSc-PAH.

In a second aspect, the present invention thus relates to an *in vitro* method for diagnosing a pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient from a biological sample of said patient, comprising the following steps:

a) measuring *in vitro* the expression level of the precursor of matrix metalloproteinase-10 (pro-MMP10) in said sample;

b) comparing the expression level of pro-MMP10 measured in step a) to the expression level of pro-MMP10 in at least one reference sample, and

c) diagnosing PAH from said comparison.

According to the invention, **"diagnosing PAH"** or **"diagnosis of PAH"** means detecting the presence or absence of PAH in a SSc patient. It also includes determining a risk stratification among patients, patients being classified in several distinct classes depending on the risk level that they are suffering from PAH. SSc patients who have been diagnosed as suffering from PAH or as having a high risk to suffer from PAH according to the method of the invention should undergo a right heart catheterism for confirmation of the diagnosis.

It may also allow monitoring a SSc-PAH patient treated for PAH until PAH is over. It may further allow detecting the risk that a SSc-PAH patient will develop advanced PAH. If a high risk of developing advanced PAH is detected, said patient may be treated in order to in order to prevent or to delay advanced PAH development

According to the present invention, the term **"biological sample"** relates to any sample derived from a SSc patient, which contains nucleic acids or proteins. Examples of such samples include body fluids (including blood, plasma, and serum), or solid samples such as tissues, cell samples, organs, biopsies, etc. Preferably, the biological sample is selected from a blood sample, a serum sample, a plasma sample, a blood-derived circulating endothelial progenitor cells (EPCs) sample and a lung tissue sample. The sample may be collected according to conventional techniques and used directly for diagnosis or stored. In a more preferred embodiment of the invention, the biological sample is a blood sample, a serum sample or a plasma sample.

Step a): measuring in vitro the expression level of the precursor of matrix metalloproteinase-10 (pro-MMP10) in a biological sample

The expression level of pro-MMP10 may be determined *in vitro* either at the protein or at the nucleic level, using any technology known in the art.

For instance, at the protein level, the *in vitro* measure of the expression level of pro-MMP10 may be performed by any dosage method known by a person skilled in the art, including but not limited to ELISA or mass spectrometry analysis. These technologies are easily adapted to any fluid or solid sample. Indeed, proteins of the fluid or solid sample may be extracted using various technologies well known to those skilled in the art for measure by ELISA or mass spectrometry in solution. Alternatively, the expression level of a protein in a biological sample may be analyzed by using mass spectrometry directly on the tissue slice. For determination of pro-MMP10 expression at the protein level, ELISA is a preferred technology.

According to a preferred embodiment of the method of the invention, the expression level of pro-MMP10 is measured at the protein level, preferably in a biological sample selected from a blood sample, a serum sample, and a plasma sample.

In a particularly preferred embodiment of the method of the invention, the expression level of pro-MMP10 is measured at the protein level, in a biological sample selected from a blood sample, a serum sample, and a plasma sample, and using an ELISA assay.

At the nucleic level, the *in vitro* measure of the expression level of a gene may be carried out either directly on messenger RNA (mRNA), or on retrotranscribed complementary DNA (cDNA). Any method to measure the expression level may be used, including but not limited to microarray analysis, quantitative PCR, southern analysis.

In particular, the expression level may be determined *in vitro* using a nucleic acid microarray, in particular an oligonucleotide microarray. In another embodiment of the invention, the expression level may be determined *in vitro* using quantitative PCR. In any case, the expression level of any gene is preferably normalized. There are many methods for normalizing obtained expression data, depending on the technology used for measuring expression. Such methods are well known to those skilled in the art. In some embodiments, normalization may be performed in comparison to the expression level of an internal control gene, generally a household gene, including but not limited to ribosomal RNA (such as for instance 18S ribosomal RNA) or genes such as HPRT1 (hypoxanthine phosphoribosyltransferase 1), UBC (ubiquitin C), YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide), B2M (beta-2-microglobulin), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), FPGS (folylpolyglutamate synthase), DECR1 (2,4-dienoyl CoA reductase 1, mitochondrial), PPIB (peptidylprolyl isomerase B (cyclophilin B)), ACTB (actin ß), PSMB2 (proteasome (prosome, macropain) subunit, beta type, 2), GPS1 (G protein pathway suppressor 1), CANX (calnexin), NACA (nascent polypeptide-associated complex alpha subunit), TAX1BP1 (Tax1 (human T-cell leukemia virus type I) binding protein 1), and PSMD2 (proteasome (prosome, macropain) 26S subunit, non-ATPase, 2).

In the context of the present invention, **"expression levels"** (also referred to as **"expression values"**) of pro-MMP10 include both:

- non-normalized raw expression values, and
- derivatives of raw expression values, which may further have been normalized no matter with method is used for normalization.

In particular, when quantitative PCR is used for measuring *in vitro* expression values of genes used for prognosis, derivatives of raw expression values selected from ΔCt, -ΔCt, ΔΔCt, or -ΔΔCt values may be used.

When a microarray is used for measuring *in vitro* expression values of pro-MMP10, log derivatives (in particular log2 derivatives) of raw expression values (which may further have been normalized or not) are usually used.

These technologies are also easily adapted to any fluid or solid sample used in the invention. Indeed, several well-known technologies are available to those skilled in the art for extracting mRNA from a biological sample and retrotranscribing mRNA into cDNA.

When the expression level of pro-MMP10 is determined at the nucleic acid level, quantitative PCR is a preferred technology.

In an embodiment of the method of the invention, the expression level of pro-MMP10 is measured at the nucleic acid level, preferably in a blood-derived EPCs cells sample.

More preferably, the expression level of pro-MMP10 mRNA or cDNA is measured using quantitative PCR.

In an embodiment of the method of the invention, the expression level of pro-MMP10 is measured at the nucleic acid level, in a blood-derived EPCs cells sample, and using quantitative PCR.

As used herein the term **"blood-derived EPCs cells"** relates to endothelial cells derived from circulating endothelial progenitor cells. These cells represent an original and valuable cellular model to investigate endothelial biology since they have the phenotype of genuine endothelial cells, exhibit *in vitro* angiogenic properties and have the capacity to constitute and orchestrate vascular remodeling *in vivo*. Methods for obtaining and isolating EPCs are described by Avouac et al. (Arthritis Rheum. 2008, 58:3550-61 and 67: 1455-60) and by Ingram et al. (2004). The skilled artisan could easily adapt these methods for the purpose of the present invention.

In another embodiment, the expression level of pro-MMP10 may be measured in a lung sample (for example a biopsy), at the nucleic or protein level, as defined above.

Steps b) and c): comparing the expression level of pro-MMP10 measured in step a) to the expression level of pro-MMP10 in at least one reference sample in step b) and diagnosing PAH in step c) from the comparison performed in step b)

According to the method of the invention, in step b), the expression level of pro-MMP10 measured in step a) is compared to the expression level of pro-MMP10 in at least one reference sample.

According to the present invention, a **"reference sample"** is selected from a biological sample (fluid or solid sample as defined above) of a healthy subject (subject not suffering from SSc), a biological sample of a SSc patient without PAH and a biological sample of a SSc patient with PAH. Preferably, a pool of reference samples is used, which comprises at least one (preferably several, more preferably at least 5, more preferably at least 6, at least 7, at least 8, at least 9, at least 10) sample from healthy subject(s), at least one (preferably several, more preferably at least 6, at least 7, at least 8, at least 9, at least 10) sample from SSc patient(s) without PAH and at least one (preferably several, more preferably at least 6, at least 7, at least 8, at least 9, at least 10) sample from SSc patient(s) with PAH. The highest the number of samples from healthy subjects, from SSc patients without PAH and from SSc patients with PAH reference samples, the better for the reliability of the method of prediction according to the invention.

According to the present invention, the inventors found that the higher the expression level of pro-MMP10 is, the higher is the risk that the SSc patient suffers from PAH.

Based on this observation, the comparison of the expression level of pro-MMP10 measured in step a) to the expression level of pro-MMP10 in at least one reference sample (step b) and the diagnosis of PAH from the comparison (step c) may be performed in various manners.

In a particularly simple way, a simple comparison of the expression level of pro-MMP10 measured in step a) with one or more threshold values calibrated based on said reference samples may be performed. In this case, based on reference samples ("training data"), one or more threshold values may be determined, over which the tested subject will be diagnosed as suffering from SSc-PAH. In an embodiment, the expression level of pro-MMP10 measured in step a) may be compared to predetermined expression level threshold value(s). Alternatively, the ratio of the expression level of pro-MMP10 measured in step a) to the expression level of pro-MMP10 in at least one reference sample may be compared to predetermined expression level threshold value(s).

Particularly, if the expression level of pro-MMP10 in the tested biological sample measured in step a) is much higher than the expression level in at least one sample obtained from healthy subject(s) or from reference SSc patients without PAH, it is concluded that the tested SSc patient is suffering or suspected to suffer from PAH.

Similarly, if the expression level of pro-MMP10 in the tested sample obtained from SSc patient measured in step a) is higher than the expression level of pro-MMP10 in at least one reference sample obtained from SSc patient(s) with PAH, it is concluded that the SSc patient is suffering or suspected to suffer from SSc-PAH.

Such SSc-PAH diagnosing can be combined with already known diagnostic methods for SSc-PAH such as cardiac catheterization. Diagnostic method according to the invention is advantageous compared to more invasive known methods, since it may be performed using a simple serum sample and may limit the use of more invasive methods only to SSc patients considered as suffering or as suspected to suffer from PAH with the method according to the invention.

In contrast, if the expression level of pro-MMP10 in the tested biological sample measured in step a) is lower than the expression level of pro-MMP10 in at least one reference sample obtained from SSc patient(s) without PAH, it is concluded that the SSc patient is not suffering from SSc-PAH.

However, more sophisticated manners to perform the comparison and reach a diagnosis may be used.

In particular, many algorithms may be used for comparing expression levels in step b) and diagnosing PAH in step c). In particular, the algorithm may be selected from PLS (Partial Least Square) regression, Support Vector Machines (SVM), linear regression or derivatives thereof (such as the generalized linear model abbreviated as GLM, including logistic regression), Linear Discriminant Analysis (LDA, including Diagonal Linear Discriminant Analysis (DLDA)), Diagonal quadratic discriminant analysis (DQDA), Random Forests, k-NN (Nearest Neighbour) or PAM (Predictive Analysis of Microarrays) algorithms. Cox models may also be used. Centroid models using various types of distances may also be used.

In this case, a group of reference samples (those with which comparison is performed in step b)), which is generally referred to as “training data”, is used to select an optimal statistical algorithm that best separates 1) SSc-PAH patients from SSc-without PAH (diagnosis) or 2) good from bad prognosis (like a decision rule). The best separation is usually the one that misclassifies as few samples as possible and that has the best chance to perform comparably well on a different dataset.

For a binary outcome such as 1) presence/absence of SSc-PAH or 2) good/bad prognosis, linear regression or a generalized linear model (abbreviated as GLM), including logistic regression, may be used.

Linear regression is based on the determination of a linear regression function, which general formula may be represented as:

$$f(x_1, \ldots, x_N) = \beta_0 + \beta_1 x_1 + \ldots + \beta_N x_N .$$

Other representations of linear regression functions may be used (see below).

Logistic regression is based on the determination of a logistic regression function:

$$f(z)=\frac{e^z}{e^z+1}=\frac{1}{1+e^{-z}},$$

in which z is usually defined as

$$z=\beta_0+\beta_1 x_1+\ldots+\beta_N x_N.$$

In the above linear or logistic regression functions, x_1 to x_N are the expression values (or derivatives thereof such as ΔCt, -ΔCt, ΔΔCt, or -ΔΔCt for quantitative PCR or logged values for microarray) of the N genes for which expression is measured, β_0 is the intercept, and β_1 to β_N are the regression coefficients. In the present case, only one gene is analysed, but the method may also be used if additional parameters are added to the pro-MMP10 expression level for diagnosis.

The values of the intercept and of the regression coefficients are determined based on a group of reference samples ("training data"). The value of the linear or logistic regression function then defines the probability that a test subject suffers (or is suspected to suffer) or not from SSc-PAH (when defining the linear or logistic regression function based on training data, the user decides if the probability is a probability of presence (or suspicion of presence) or absence of SSc-PAH). A test subject is then classified as suffering (or as suspected to suffer) or not from SSc-PAH depending if the probability that the subject suffers (or is suspected to suffer)or not from SSc-PAH is inferior or superior to a particular threshold value, which is also determined based on training data. Sometimes, two threshold values are used, defining an undetermined area. Other types of generalized linear models than logistic regression may also be used.

Alternative methods such as nearest neighbour (abbreviated as k-NN) are also commonly used for a new sample, based on whether the sample is closer 1) to the group of SSc-PAH patients or to the group of SSc patients without PAH. The notion of "closer" is based on a choice of a distance (metric, such as but not limited to Euclidian distance) in the n-dimension space defined by a signature consisting of N genes useful for diagnosis (thus excluding potential housekeeping genes used for normalization purpose). In the present case, only one gene is analysed, but the method may also be used if additional parameters are added to the pro-MMP10 expression level for diagnosis. The distances between a test sample and all reference samples are calculated and the sample is classified by analysis of the k closest reference samples (k being an positive integer of at least 1 and most commonly 3 or 5), a rule of classification being pre-established depending on the number of SSc-PAH or SSC-without PAH reference samples among the k closest reference expression profiles. For instance, when k is 1, a test sample is classified as SSc-without PAH if the closest reference sample is a SSc-without PAH sample, and as SSc-PAH if the closest reference expression profile is a SSc-PAH sample. When k is 2, a test sample is

classified as SSc-without PAH if the two closest reference samples are SSc-without PAH samples, and as SSc-PAH if the two closest reference samples are SSc-PAH samples, and undetermined if the two closest reference samples include a SSc-PAH sample and a SSc-without PAH sample. When k is 3, a test sample is classified as SSc-without PAH if at least two of the three closest reference samples are SSc-without PAH, and as SSc-PAH if at least two of the three closest reference samples are SSc-PAH samples. More generally, when k is p, a test sample is classified as SSc-without PAH if more than half of the p closest reference samples are SSc-without PAH samples, and as SSc-PAH if more than half of the p closest reference samples are SSc-PAH samples. If the numbers of SSc-without PAH and SSc-PAH reference samples are equal, then the test sample is classified as undetermined.

Other methodologies from the field of statistics, mathematics or engineering exist, for example but not limited to decision trees, Support Vector Machines (SVM), Neural Networks and Linear Discriminant Analyses (LDA). Cox models may also be used. Centroid models using various types of distances may also be used. These approaches are well known to people skilled in the art.

In summary, an algorithm (which may be selected from linear regression or derivatives thereof such as generalized linear models (GLM, including logistic regression), nearest neighbour (k-NN), decision trees, support vector machines (SVM), neural networks, linear discriminant analyses (LDA), Random forests, or Predictive Analysis of Microarrays (PAM)) is calibrated based on a group of reference samples (preferably including several SSc-without PAH reference samples and several SSc-PAH or reference samples) and then applied to the test sample. In simple terms, a patient will be classified as SSc-without PAH (or SSc-PAH) based on how the expression level measured in step a) compares to a reference expression level that was developed from a group of SSc-without PAH and preferably also of SSc-PAH reference samples (training data). Methods using such algorithms are particularly suited for cases in which more than one parameters are compiled, and would be particularly suitable if at least one other parameter than pro-MMP10 expression level is used for diagnosis/prognosis.

According to an embodiment, the method of diagnosis of the invention is used for determining the presence or the absence of PAH, including all of groups 1 to 4 (as defined above using WHO clinical classification system)in a systemic sclerosis in a (SSc) patient.

According to a particular embodiment, the method of diagnosis of the invention is used for determining the presence or the absence of one or more particular groups of PAH (as defined above using WHO clinical classification system).

According to a preferred embodiment, the method of the invention may be used for detecting the presence or absence of PAH due to lung diseases and/or hypoxemia (also called PAH3 or PAH ILD in the present description).

In a preferred embodiment, the diagnosis method according to the invention is intended for diagnosing an absence of PAH or an absence of any of the groups of PAH according to WHO classification. Indeed, when the read-out is the presence of PAH (or one of the groups of the WHO classification), the methods according to the invention have been shown to present high negative predictive value (NPV) and high specificity (see Example 1).

For such diagnosis method (read-out = presence of PAH or presence of any one of the groups of PAH according to WHO classification), PAH (or any one of the groups of PAH) condition or PAH (or any one of the groups of PAH) test outcome is considered a positive result, while absence of PAH (or any one of the groups of PAH) condition or absence of PAH (or any one of the groups of PAH) test outcome is considered a negative result. True and false positive results, negative predictive value (NPV), positive predictive value (PPV), specificity, sensitivity and error rate are then defined and calculated as follows:

		Condition (presence of PAH or **any one of the groups of PAH)**	
		Positive	***Negative***
Test outcome (**presence of PAH or** any one of the groups of **PAH)**	***Positive***	True Positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)

PPV = TP / (TP+FP), representing the proportion of patients really suffering from PAH (or any one of the groups of PAH) among patients with positive test outcome, i.e. with test indicating that they suffer from PAH (or any one of the groups of PAH).

NPV = TN / (TN+FN), representing the proportion of patients really not suffering from PAH (or any one of the groups of PAH) among patients with negative test outcome, i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH).

Specificity = TN / (TN+FP), representing the proportion of patients with negative test outcome (i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH)) among patient that really do not suffer from PAH (or any one of the groups of PAH).

Sensitivity = TP / (TP+FN), representing the proportion of patients with positive test outcome (i.e. with test indicating that they suffer from PAH (or any one of the groups of PAH)) among patient that really suffer from PAH (or any one of the groups of PAH).

Error rate = (FP+FN)/ Total number of patients, representing the proportion of patients whose test outcome does not correspond to their actual condition.

The methods according to the invention with a read-out corresponding to presence of PAH (or any one of the groups of PAH) (i.e. methods for detecting the presence of PAH (or any one of the groups of PAH)) have been shown to present very high negative predictive value (NPV) and high specificity (see Example 1), meaning that:

- patients with negative test outcome (i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH)) have very high probability to really not suffer from PAH (or any one of the groups of PAH)) (high NPV value).
- patients that really do not suffer from PAH (or any one of the groups of PAH)) have a high probability to obtain a negative test outcome (i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH)) (high specificity).

Conversely, for a corresponding method directed to detecting the absence of PAH (or any one of the groups of PAH according to WHO classification) (read-out = absence of PAH (or any one of the groups of PAH)), absence of PAH (or any one of the groups of PAH) condition or absence of PAH (or any one of the groups of PAH) test outcome is considered a positive result, while presence of PAH (or any one of the groups of) condition or presence of PAH (or any one of the groups of PAH) test outcome is considered a negative result, and PPV; NPV, sensitivity and specificity are defined as follows:

		Condition (absence of PAH or any one of the groups of PAH)	
		Positive	***Negative***
Test outcome (absence of PAH or any one of the groups of PAH)	***Positive***	True Positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)

PPV = TP / (TP+FP), representing the proportion of patients really not suffering from PAH (or any one of the groups of PAH) among patients with positive test outcome, i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH).

NPV = TN / (TN+FN), representing the proportion of patients really suffering from PAH (or any one of the groups of PAH) among patients with negative test outcome, i.e. with test indicating that they suffer from PAH (or any one of the groups of PAH).

Specificity = TN / (TN+FP), representing the proportion of patients with negative test outcome (i.e. with test indicating that they suffer from PAH (or any one of the groups of PAH)) among patient that really suffer from PAH (or any one of the groups of PAH).

Sensitivity = TP / (TP+FN), representing the proportion of patients with positive test outcome (i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH)) among patient that really do not suffer from PAH (or any one of the groups of PAH).

Error rate = (FP+FN)/ Total number of patients, representing the proportion of patients whose test outcome does not correspond to their actual condition.

Such a method directed to detecting the absence of PAH (or any one of the groups of PAH) (read-out = absence of PAH (or any one of the groups of PAH)) will have very high PPV and high sensitivity, meaning that:

- patients with positive test outcome (i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH)) have very high probability to really not suffer from PAH (or any one of the groups of PAH)) (high PPV value).
- patients that really do not suffer from PAH (or any one of the groups of PAH)) have a high probability to obtain a positive test outcome (i.e. with test indicating that they do not suffer from PAH (or any one of the groups of PAH)) (high sensitivity).

In Example 1 below, the inventors have demonstrated that NPV of a method for determining the presence of PAH (including all groups according to WHO classification) is high (about 80%), for a threshold (or "cut-off") value of pro-MMP10 expression of 920 pg/ml. In a method of diagnosis of presence of PAH in a patient, diagnosis may thus be performed by simple comparison of the expression level of pro-MMP10 measured in step a) with a threshold (or "cut-off") value of about 900 to 950 pg/ml, in particular about 920 pg/ml, over which the tested subject will be diagnosed as suffering from SSc-PAH.

According to one preferred embodiment, the method of the invention is particularly efficient for diagnosing any of the groups of PAH according to WHO classification. More particularly, it is particularly efficient for determining an absence of any of the groups of PAH according to WHO classification.

According to another preferred embodiment, the method of the invention is particularly efficient for diagnosing PAH3. More particularly, it is particularly efficient for determining an absence of PAH3 according to WHO classification.

Particularly, all definitions and embodiments disclosed above relating to a method of diagnosis of PAH (including all groups of PAH according to WHO classification) in SSc patients may be applied when the method of diagnosis is used to specifically detect the presence or the absence of any one of the groups of PAH according to WHO classification, and notably PAH3.

In Example 1 below, the inventors have demonstrated that NPV of a method for determining the presence of PAH3 is very high (about 97%), for a threshold (or "cut-off") value of pro-MMP10 expression of 1177 pg/ml. In a method of diagnosis of presence of PAH in a patient, diagnosis may thus be performed by simple comparison of the expression level of pro-MMP10 measured in step a) with a threshold (or "cut-off") value of about 1150 to 1200 pg/ml, in particular about 1177 pg/ml, over which the tested subject will be diagnosed as suffering from SSc-PAH3.

The high NPV and specificity values found by the inventors mean that when using the method of diagnosis of the invention, if an SSc patient is determined as negative for PAH (or any one of the groups of PAH according to WHO classification), this result should be considered reliable. In such case, it may be decided not to perform additional tests in order to conclude definitely that this SSc patient does not suffer from PAH (or any one of the groups of PAH according to WHO classification). This is advantageous because presence of PAH (or any one of the groups of PAH according to WHO classification) may potentially be excluded based on an easy, not very expensive and not invasive test based on a simple biological sample, and in particular a blood sample, a serum sample, a plasma sample, a blood-derived circulating endothelial progenitor (EPCs) cells sample. When a lung biopsy is already available, the test may also be performed based on lung tissue.

Another advantage of the method of diagnosis according to the invention is that it may also allow patients stratification based on their expression level of pro-MMP10 and thus, the possibility to propose an adapted treatment for each patient. Such stratification is also of particular interest in the design of clinical trials, thereby allowing assigning subjects of the trial to different test groups as a function of their expression level of pro-MMP10.

While a test indicating that the patient suffers from PAH (or any one of the groups of PAH according to WHO classification) may be used directly, according to another embodiment, it may be contemplated to perform additional tests in order to conclude if the SSc patient really suffers from PAH (or any one of the groups of PAH according to WHO classification) or not. Such additional method may notably be selected from cardiac catheterization, echocardiogram; a functional expiratory exploration (FEE) along with measuring of diffusing capacity (DLCO) and forced vital capacity (FVC); measuring NT-

proBNP (N-terminal pro-brain natriuretic peptide) and ultra-sensitive troponin concentrations. When the method of the invention is used to determine a presence or an absence of PAH3, and the test result suggests that the patient suffers from PAH3, a CT scan (chest scan, preferably high resolution CT chest scan) in order to evaluate the lung fibrosis may be performed as additional test.

The methods according to the invention thus limit the potential use of more invasive and more expensive tests to only a fraction of SSc patients, found to be at risk of PAH using the method according to the invention.

As indicated above, the method of the invention allows easily and early diagnosing PAH (or any one of the groups of PAH according to WHO classification) in a SSc patient and according to the obtained diagnosis, the patient may or not be treated with adapted treatment. The diagnosis by the method of the invention is easy to implement since the tested sample may be obtained from the blood and the techniques used for measuring and comparing the expression of pro-MMP10 are standard techniques well known to those skilled in the art. When the SSc patient is determined as having a high risk of developing advanced PAH, an appropriate preventive treatment may be administered.

Following the diagnosis of the presence of SSc-PAH, the treatment administered to the SSc patient may notably and preferably be the treatment described above, using inhibitors of pro-MMP10 expression and/or MMP10 activity.

In a further embodiment, the present invention thus relates to a method for treating PAH in a SSc subject, comprising the steps of:

a) diagnosing a pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient from a biological sample of said subject by the method of the invention and

b) if said patient is diagnosed as suffering from SSc-PAH, administering an inhibitor of pro-MMP10 expression or MMP10 activity or a pharmaceutical composition according to the invention.

EXAMPLES

Example 1.

Materials and methods

Late outgrowth EPC isolation

Late outgrowth EPC-derived cells were obtained from the peripheral blood of 36 patients (28 females) who met the American College of Rheumatology criteria for SSc [14].

Among these patients, 6 (3 females) had PAH confirmed by right heart catheterization (RHC), with a mean age of 67±10 years and mean disease duration of 13±8 years. Thirty SSc patients without PAH (25 females with systolic pulmonary artery pressure <40 mmHg on echocardiography and a diffusing capacity for carbon monoxide >75% predicted) were included, with a mean age of 58±11 years and mean disease duration of 9±8 years. Controls EPC-derived cells were obtained from 10 healthy controls (7 females), with a mean age of 57±11 years old. Local ethic review committees approved all experiments and written informed consent was obtained for all patients and controls.

Blood sample collection was performed on a 50 ml heparinised venous blood sample, collected at the time of patient hospitalization, in the morning, at rest, at forearm, together with routine analysis. Patient and control samples were immediately transported to the laboratory for testing. The blood mononuclear cell fraction was collected by Ficoll (Pancoll, Dutcher, France) density gradient centrifugation and was resuspended in complete EGM-2 medium (Lonza, Basel, Switzerland). Cells were then seeded onto separate wells of a 12-well tissue culture plate precoated with type I rat tail collagen (BD Biosciences) and stored at 37°C, under 5% CO2, in a humidified incubator. After 24 hours of culture, non-adherent cells and debris were aspirated, adherent cells were washed once with PBS1x, and complete EGM-2 medium was added to each well. Medium was changed daily for 7 days and then every other day until the first passage. Colonies of endothelial cells appeared between 8 and 26 days of culture and were identified as well-circumscribed monolayers of cells with a cobblestone appearance. After the third passage, endothelial phenotyping of EPC-derived cells was performed by flow cytometry, as previously described by Avouac et al (2008). After confirmation of endothelial phenotype, cells were suspended in fetal bovine serum supplemented with 20% DMSO, frozen in liquid nitrogen, and stored until used.

Hypoxia treatment

Gene expression profiles of EPC-derived endothelial cells were determined in basal conditions and also after hypoxic exposure. Identification of new targets in endothelial cells stimulated with hypoxic exposition may particularly relevant for SSc-PAH, since severe tissue hypoxia is a hallmark of this condition and contributes directly to its progression.

For hypoxia experiments, 1 x 10^6 cells were seeded into 100-mm dishes. After 3 days, when cells were subconfluent, the medium was changed and cells were exposed to hypoxia for 6 h at 1% O2, 5% CO2, 37°C, in a humidified incubator.

Microarray analysis

Microarray analysis was performed on 35 samples: 6 SSc patients with precapillary PAH confirmed by RHC, 20 SSc patients free of PAH and 9 healthy controls. All the 36 samples were defrosted, cultured, extracted and hybridized at the same time. Affymetrix Microarray technology was used to analyze gene expression levels. Labelling and Microarray processing were performed according to the manufacturer's protocol.

Data were RMA normalized with Bioconductor. First the data in an unsupervised way by PCA were controlled and analysed and then ANOVA was used to extract DEGs with PartekGS©. Enrichment analysis were carried out through the use of IPA© and Pathway Studio©.

RNA preparation

Total RNA was extracted from cultured cells in RLT® RNA extraction buffer (Qiagen Rneasy kit, Qiagen, Courtaboeuf, France) and treated by DNase I to eliminate genomic DNA contamination. The integrity and purity of the total RNA, and of the cRNA, were analyzed twice by Bioanalyser 2100, and RNA kit 6000 LabChip (Agilent Technologies). Only total RNAs with a 28S/18S ratio ≥1.9 were used. cRNA concentrations were measured by using NanoDrop (NanoDrop Technologies, Wilmington, DE).

cRNA synthesis and probe array hybridization

cRNA synthesis was performed with 3µg of total RNA using the GeneChip Expression 3' Amplification One-Cycle Target Labelling and Control Reagents, hybridized onto the Human Genome GeneChip® Human Exon 1.0 ST (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's protocol (GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual version 4). Briefly, the majority of the ribosomal RNA (rRNA) was removed from the total RNA samples for increased sensitivity, using the RiboMinus™ Human/Mouse Transcriptome Isolation Kit (Invitogen). Then, RNA depleted from rRNA was reverse transcribed using a T7-Oligo (dT) promoter primer in the first strand cDNA synthesis reaction. Following RNase H-mediated second strand cDNA synthesis, the double strand cDNA was purified and served as a template in the subsequent in vitro transcription (IVT). The IVT reaction was carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets were then cleaned up and fragmented. Fragmentation was evaluated on Bioanalyser. Selected fragmented cRNA were hybridized to GeneChip expression arrays. After washing and staining using the Affymetrix

fluidics station 450 (Affimetrix, Inc.), the probe arrays were scanned into the Affymetrix GCS 3000 scanner.

Bioinformatics

Fluorescence data were imported into two analysis softwares: Affymetrix® Expression Console™ and R Bioconductor. Gene expression levels were calculated using the RMA algorithm at gene level in Expression Console and flags were computed using a custom algorithm within R. Then all quality controls and statistics were performed using Partek GS ® (version 6.6 Copyright © 2012 Partek Inc., St. Louis, MO, USA).

Firstly a hierarchical clustering (Pearson's dissimilarity and average linkage) and Principal Component Analysis as unsupervised exploratory data analysis were made. This multivariate technical aimed to control data from experimental bias or outlier samples. To find differentially expressed genes, a classical analysis of variance (ANOVA) was applied for each gene and made pair wise Tukey's post hoc tests between groups. Then, *P*-values were used and fold changes to filter and select differentially expressed genes. Herein, genes with average fold-change ≥1.5 and a $P<0.05$ were selected. Interactions, pathways and functional enrichment analysis were carried out through the use of Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, USA www.ingenuity.com) and Pathway Studio© (Ariadne Genomics).

Pro-MMP10 serum levels

Serum concentrations of pro-MMP10 were measured in a cohort of 52 SSc patients with confirmed PAH, 93 SSc patients without PAH and 50 unrelated control subjects. Quantitative sandwich ELISAs (R&D Systems, Minneapolis, MN, USA) was used to measure pro-MMP10 serum levels according to manufacturer recommendations. The analytical range extended from undetectable to 5000 pg/ml. The intra-assay coefficient of variation was 4.4% for a concentration of 515 pg/ml and 4.0% for a concentration of 3442 pg/ml. The inter-assay coefficient of variation was 5.9% and 4.3% respectively.

Real-time quantitative polymerase chain reaction (PCR)

Real-time quantitative polymerase chain reaction (PCR) was performed on total RNA isolated from the 6 patients with SSc-PAH, 30 patients with SSc without PAH (20 samples used in the microarray analysis and 10 additional samples) and the 10 healthy controls. Total RNA was isolated with the RNeasy mini kit according to the instructions of the manufacturer (Qiagen, Courtaboeuf, France). First-strand complementary DNA was synthetized (from 2 μg of total RNA) using random primers and Superscript II reverse

transcriptase (200 units:μl; Invitrogen, Carlsbad, CA). TaqMan quantitative reverse transcriptase-PCR (RT-PCR) was performed, as described by Avouac et al. (2008). It was used primer sequences (inventoried by life technologies, Saint Aubin, France) for human MMP10 (Hs00233987_m1) and TIMP1 (Hs00171558_m1). Hypoxanthine guanine phosphoribosyl-transferase was used as a housekeeping control (Hs99999909_m1). All PCRs were performed in triplicate. Relative mRNA levels for each sample were quantified using the C_t approach (fluorescence threshold), normalized to HPRT RNA as the standard.

Immunofluorescence for MMP10 in EPC-derived endothelial cells

The expression of MMP10 in EPC-derived endothelial cells was detected by staining with monoclonal mouse anti-human antibodies against MMP10 (R&D systems). Goat anti-mouse Alexa Fluor 594-conjugated antibodies were used as secondary antibodies. Counterstaining was performed with DAPI (Santa Cruz Biotechnology) for ten minutes at room temperature. Slides were then viewed by microscopy using appropriate fluorescence filters.

The intensity of MMP10 fluorescence was quantified with the imageJ software, as described in the following webpage (http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html).

Immunohistochemical Detection of MMP10 in Paraffin-Embedded Lung Tissues

After classical dewaxing and heat antigen retrieval at pH 6, immunohistochemistry was performed with novocastra™ lyophilized monoclonal mouse anti-human antibodies against MMP10 (Leica Biosystems, Newcastle, UK). Polyclonal rabbit anti-mouse antibodies (Dako, Glostrup, Denmark) labeled with horseradish peroxidase (HRP) were used as secondary antibodies for one hour at room temperature. The expression of MMP10 was also detected in lesioned lung sections of Fra-2 transgenic mice by staining overnight with polyclonal rabbit anti-mouse antibodies against MMP10 (Acris Antibodies, San Diego, CA). Polyclonal goat anti-rabbit antibodies (Dako) labeled HRP were used as secondary antibodies.

Animals

Thirteen-week-old Fra-2 transgenic mice (SA5446 D-H3/FRA-2 (Tg4)) were obtained from a collaboration established with SANOFI-GENZYME and 13-week-old C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All were bred and maintained at the animal care facilities of Paris Descartes University (Faculty of dental surgery, Montrouge, Paris). All experimental procedures were conducted in compliance

with Animal Health regulations and the local ethical committee approved all animal experiments.

Fra-2 mouse model

Transgenic mice expressing the fra-2 gene under control of the ubiquitous major histocompatibility complex class I antigen H2Kb promoter display systemic fibrosis, microangiopathy, and PAH. These manifestations follow a similar temporal sequence as seen in human SSc. The presence of both typical capillary changes and pulmonary arterial hypertension is unique among murine models for SSc. Regarding the occurrence of PAH, Fra-2 transgenic mice develop severe vascular remodeling of pulmonary arteries resembling human SSc-PAH. Histological features typical for SSc-PAH, such as intimal thickening with concentric laminar lesions, medial hypertrophy, perivascular inflammatory infiltrates, adventitial fibrosis are frequently detected.

Effects of MMP10 inhibition on experimental pulmonary arterial hypertension

To assess the effects of MMP10 inhibition on the development of experimental PAH, a group of 8 13-week-old fra-2 transgenic mice (4 males, 4 females) was subjected to intraperitoneal (IP) injections of rabbit polyclonal antibodies against the catalytic domain of MMP10 (Abcam, Paris, France) at a dose of 30 µg/kg (diluted in PBS, 100 µl injected/mouse) every other day for 5 weeks. This group was compared to a group of 7 13-week-old fra-2 transgenic mice (3 males, 4 females) treated with IP injections of control Rabbit polyclonal IgG (Abcam) at a dose of 30 µg/kg (diluted in PBS, 100 µ injected/mouse) every other day for 5 weeks.

Two other negative control groups were used, consisting of 15 13-week-old C57BL/6 mice (7 males, 8 females) receiving either IP injections of control IgG (n=7, 3 females, 4 males) or anti-MMP10 antibody (n=8, 4 males, 4 females).

Hemodynamic Measurements and Assessment of Pulmonary Vascular Changes

Right ventricular systolic pressure (RVSP) and heart rate were determined in unventilated mice under isoflurane anesthesia (1.5-2.5%, 2L O2/min.) using a closed chest technique, by introducing a catheter (1.4 F catheter, Millar Instruments Inc, Houston, TX) into the jugular vein and directing it to the right ventricle. After all the hemodynamic assessments were completed, blood was collected by direct cardiac puncture and sacrificed by exsanguinations. The heart and lungs were then removed *en bloc* and right ventricular hypertrophy (RVH) was determined by the Fulton index measurement (right ventricle/left ventricle plus septum RV/LV+S). The pulmonary circulation was flushed with

5mL of buffered saline at 37°C, and then the left lung was prepared for morphometric analyses and the right lung was quickly harvested, immediately snap-frozen in liquid nitrogen and kept at -80°C for Western immunoblot analysis and total RNA extraction.

Morphometric analyses were performed on paraffin-embedded lung sections stained using hematoxylin and eosin (H&E) and alpha smooth muscle actin (αSMA). Muscularization was assessed in 15-higher magnification fields/per mouse (×40 magnification) by calculating the proportion of fully and partially muscularized peripheral (alveolar duct and wall) pulmonary arteries to total peripheral pulmonary arteries. All morphometric analyses were performed by one observer (CG), blinded as to genotype and condition.

Statistical analysis

All data are expressed as mean values ± SEM. Statistical analysis was performed using GraphPad Prism 6.04 software (San Diego, CA). For a two-group comparison, a Student's t-test was used provided the pretest for normality (D'Agostino-Pearson normality test) was not rejected at the 0.05 significance level; otherwise, a nonparametric Mann-Whitney U-test was used. Kruskal-Wallis test was used for comparing data among three or more independent groups. Correlations were assessed using Spearman's rank correlation test. Multiple group comparisons (analysis of RVSP and RVH) were analyzed by using a post hoc Tukey test. $P<0.05$ was considered significant.

Results

Gene expression changes in EPC-derived endothelial cells from SSc-PAH patients compared to SSc patients without PAH

28 differentially expressed genes were detected in unstimulated EPC-derived cells issued from patients with SSc-PAH compared to SSc patients without PAH. IPA revealed significant enrichment in functional groups related to cellular assembly and organization (5 genes, *P*-value: 3.16E-04-4.66E-02) and cell-to-cell signalling and interaction (4 genes, *P*-value: 3.16E-04-4.43E-02) (**Table2**). Among these 28 genes, matrix metalloproteinase-10 (MMP10, stromelysin-2), involved in the breakdown of extracellular matrix, was the top upregulated gene in SSc-PAH, with a fold change of 2.197 (**Table 2**).

After hypoxic exposure, 31 genes were found differentially expressed in EPC-derived cells issued from patients with SSc-PAH compared to SSc patients without PAH. MMP10 was confirmed to be one of the top upregulated genes, with a fold change of 2.104 (**Table 2**).

Table 2: Top biological functions and top genes identified by microarray analysis

Comparison	Top biological functions			Top genes (fold change)	
	Molecular and Cellular functions	Number of genes	p-value	*Overexpressed*	*Underexpressed*
Basal conditions **SSc-PAH *vs.* SSc**	Cell-To-Cell signaling and interaction Cellular assembly and organization Cellular function and maintenance Cell cycle Cell morphology	4 5 7 1 6	3.16E-04 - 4.43E-02 3.16E-04 - 4.66E-02 3.16E-04 - 4.66E-02 8.07E-04 - 4.43E-02 8.07E-04 - 4.66E-02	**MMP10 (2.197)** mir-126 (1.746) PCDHB5 (1.730) TERC (1.507)	CDH2 (-3.299) ANTXR1 (-1.675) SPHAR (-1.611) ST3GAL6 (-1.604) DLG1 (-1.546) LOC645166 (-1.535)
Hypoxia **SSc-PAH *vs.* SSc**	Protein synthesis Carbohydrate metabolism Cell morphology Cellular compromise Energy production	3 2 4 2 1	7.07E-04 - 3.40E-03 8.75E-04 - 1.65E-02 8.75E-04 - 4.87E-02 8.75E-04 - 4.37E-03 8.75E-04 - 8.75E-04	CDR1 (2.479) **MMP10 (2.104)** mir-7 (1.720) LGALS9C (1.676) Mir-32 (1.650) OCLN (1.539) RASSF4 (1.526)	CDH2 (-2.977- GPR31 (-1.886) P2RX5 (-1.614) ST3GAL6 (-1.574) PABPN1 (-1.572) CRYAB (-1.514)
Basal conditions **SSc-PAH *vs.* Healthy controls**	Cell cycle Cellular assembly and organization DNA replication, recombination and repair Cellular movement Cellular function and maintenance	33 26 22 16 8	5.49E-13 - 4.93E-02 7.15E-12 - 4.93E-02 7.15E-12 - 4.93E-02 8.04E-09 - 4.87E-02 1.29E-05 - 4.22E-02	**MMP10 (2.441)** PLA2G4C (2.267) CYTL1 (2.092) SERPINE2 (2.047) CLIC2 (1.914) RND1 (1.628) SULF2 (1.626) ARL4C (1.515) COL4A2 (1.508) CEACAM1 (1.505)	SULT1B1 (-2.740) SLIT2 (-2.486) DPP4 (-2.247) HIST1H3A (-2.121) TOP2A (-2.106) DEPDC1 (-2.009) LDB2 (-2.001) ANLN (-1.929) KIF11 (-1.924) HIST1H1D (-1.913)
Hypoxia **SSc-PAH *vs.* Healthy controls**	Cell cycle Cellular assembly and organization DNA replication, recombination and repair Cell morphology Lipid metabolism	34 28 17 13 9	1.55E-10 - 3.42E-02 1.55E-10 - 3.42E-02 1.55E-10 - 3.42E-02 8.53E-05 - 3.42E-02 1.56E-04 - 3.42E-02	**MMP10 (2.545)** mir-32 (2.257) CLIC2 (2.090) TIMP3 (2.056) mir-103 (1.923) SERPINE2 (1.817) mir-7 (1.806) CD69 (1.785) RNU5B-1 (1.784) HIST1H2BJ (1.768)	SLIT2 (-2.414) SULT1B1 (-2.370) TOP2A (-2.051) LDB2 (-2.015) ASPM (-1.955) DPP4 (-1.922) GPR31 (-1.911) STEAP1 (-1.898) ANLN (-1.895) PRC1 (-1.866)

Gene expression changes in EPC-derived endothelial cells from SSc-PAH patients compared to healthy controls

Supervised analyses performed on unstimulated EPC-derived cells detected 112 differentially expressed genes between patients with SSc-PAH and healthy controls. IPA analysis showed significant enrichment in functional groups related to cell cycle (33 genes, *P*-value: 5.49E-13-4.93E-02) and cellular assembly and organisation (26 genes, *P*-value: 7.15E-12-4.93E-02) (**Table 2** above). Consistent with the results of the previous comparison between SSc patients with or without PAH, MMP10 was also found to be the top

upregulated gene in SSc-PAH patients compared to healthy controls (FC: 2.441). Hypoxic exposure did not significantly change the results obtained in basal conditions, since MMP10 remained the top upregulated gene in SSc-PAH patients (FC: 2.545) (**Table 2** above).

Increased MMP10 expression in EPC-derived EC of patients with SSc-PAH

To follow-up the hypothesis that MMP10 may be implicated in SSc-PAH, the inventors measured the expression of MMP10 in EPC-derived endothelial cells issued from SSc-PAH, SSc without PAH and healthy controls. Firstly, qPCR was performed, which showed a 2.7 and 4.1-fold increase of MMP10 mRNA levels in unstimulated SSc-PAH EPC-derived endothelial cells compared to the cells issued from SSc patients without PAH and healthy controls, respectively (**Figure 1A**). Results were similar after hypoxic exposure (**Figure 1B**). Correlations for MMP10 between microarray and qPCR both in basal (R^2=0.94, P<0.001) and hypoxic (R^2=0.85 P<0.001) conditions illustrate the close agreement using microarray and qPCR to measure gene expression, and further validate the upregulation of MMP10 in SSc-PAH EPC-derived endothelial cells (**Figures 1C and 1D**).

Immunoblot analysis confirmed that MMP10 protein levels were significantly increased in cell lysates obtained from SSc-PAH compared with SSc without PAH and healthy controls (**Figures 2A and 2B**). Immunofluorescence staining showed a cytoplasmic localization of MMP10 protein and higher activation state in SSc-PAH EPC-derived endothelial cells (**Figure 2C**).

Since MMP10 has been found to be inhibited by TIMP metallopeptidase inhibitor 1 (TIMP1), TIMP1 mRNA expression was also assessed in EPC-derived endothelial cells, and the MMP10/TIMP1 mRNA ratio between SSc PAH patients, SSc patients without PAH and healthy controls was compared. Firstly, it was observed that MMP10 and TIMP1 mRNA levels correlated in basal conditions (R^2=0.34, P=0.031) (**Figure 3A**), and after hypoxic exposure (R^2=0.35, P=0.018) (**Figure 3B**). Interestingly, the MMP10/TIMP1 mRNA ratio was found significantly increased in SSc-PAH patient compared to SSc patients without PAH and healthy controls, both in unstimulated and hypoxia-stimulated endothelial cells (**Figures 3C and 3D**). This result further underlines the MMP10 overexpression in SSC-PAH EPC-derived cells, which exceeds the negative regulation capacity of TIMP1.

Increased pro-MMP10 serum levels in SSc patients with PAH

After that, pro-MMP10 serum levels was investigated in a discovery cohort of 60 SSc-PAH patients, compared to 78 SSc patients without PAH and 50 healthy controls and in a replication cohort of 42 SSc-PAH patients, compared to 200 SSc patients without PAH.

Patients with SSc-PAH from the discovery cohort and the replication cohort were more likely to have significantly higher pro-MMP10 serum concentrations than SSc patients without SSc-PAH (Figures 4A-C).

In the combined cohort, pro-MMP10 serum was measured. Using a cut-off value of 920 pg/ml (90th percentile of population of patients without PAH), the sensitivity, the specificity, the positive and the negative predictive values of pro-MMP10 serum levels for the diagnosis of PAH were 27.5%, 78.4%, 26% and 80%, respectively in the combined cohort.

Interestingly, pro-MMP10 serum levels were markedly increased in the subset patients with PAH associated with interstitial lung disease (PAH3) (WHO group 3, Dana Point), both in the discovery and replication cohorts (Figures 4A and 4B).

In the combined cohort, pro-MMP10 serum concentration was 1073 +/- 110 pg/ml in patients with PAH group 3 compared to 696 +/- 23 pg/ml in patients without PAH (P<0.001) (Figure 4C). The diagnostic value of pro-MMP10 for PAH3 analyzed in the combined cohort was reflected by an area under the curve of 0.71. Using a cut-off value of 1177 pg/ml (90th percentile of population of patients without PAH), the sensitivity, specificity, positive and negative predictive values of pro-MMP10 serum levels for the diagnosis of PAH group 3 were 54%, 89%, 25% and 97%, respectively, in the combined cohort.

Interestingly, pro-MMP-10 serum concentrations correlated with MMP10 mRNA levels detected in EPC-derived cells (R^2=0.67 P<0.001) (**Figure 5**).

Expression of MMP10 in the lungs of patients with SSc-PAH

The first results have confirmed an overexpression of MMP10 in EPC-derived endothelial cells and in the serum of SSc patients with PAH. We next assessed by immunohistochemistry the expression of MMP10 in lungs of three SSc-PAH patients. An expression of MMP10 was detected in the lungs of these 3 patients with SSc-PAH. It was found that MMP10 expression was abundant in the walls of SSc-PAH pulmonary arteries.

Endothelial cells, fibroblasts/myofibroblasts and smooth muscle cells were the major sites of MMP10 expression (**Figure 6A and B**).

MMP10 inhibition alleviates PAH in the Fra-2 mouse model

The previous set of results suggests that MMP10 may be implicated in SSc-PAH. To demonstrate the implication of MMP10 in SSc-PAH *in vivo*, we used Fra-2 transgenic mice, which is a reliable animal model of SSc-PAH. Firstly the MMP10 expression was assessed in the lung tissue of Fra-2 transgenic mice and wild type littermates. By immunohistochemistry, it was observed an overexpression of MMP10 in the lung of Fra-2 transgenic mice compared to wild type littermates (**Figures 7A and 7B**). In particular,

MMP10 expression localized to the vessels, mainly in endothelial cells and the smooth muscle cell layer (**Figure 7B**).

Then, the effects of MMP10 inhibition on the development of PAH in fra-2 transgenic mice were investigated. To this end, Fra-2 transgenic mice were treated with IP injections of antibodies directed against the catalytic domain of MMP10. These mice were compared to Fra-2 transgenic mice that received IP injections of control IgG.

In Fra-2 transgenic mice treated with control antibody, a marked increase of RVSP (Right Ventricular Systolic Pressure, **Tables 3 and 4**) and RVH (Right Ventricular Hypertrophy, **Tables 5 and 6**) was observed compared with vehicle-treated wild type mice (**Figures 8A and 8B**).

Table 3: Comparing of MMP10 overexpression in the lung of wild type mouse and Fra-2 mouse receiving IgG or anti-MMP10 antibody in RVSP.

	Wild type IgG	Wild type anti-MMP10 Ab	Fra-2 IgG	Fra-2 anti-MMP10 Ab
Mean	0.2450	0.2580	0.3067	0.2850
SEM	0.005	0.002	0.006	0.005

Moreover, **Table 4** below shows the results obtained by Tukey's rang test.

Table 4: Tukey's multiple comparison test between wild type mouse and Fra-2 mouse receiving an MMP10 inhibitor (antibody anti-MMP10) or IgG in RVSP.

Tukey's multiple comparison test	Mean Diff.	95% CI	Summary
Fra2 IgG vs. WT IgG	-8.890	-13.17 to -4.614	***
Fra-2 IgG vs. WT anti-MMP10	-8.258	-12.53 to -3.962	***
Fra-2 IgG vs. Fra-2 anti-MMP10	4.338	0.606 to 8.070	*
Fra-2 anti-MMP10 vs. WT IgG	-4.553	-8.548 to -0.557	*

Table 5: Comparing of MMP10 overexpression in the lung of wild type mouse and Fra-2 mouse receiving IgG or anti-MMP10 antibody in RVH.

	Wild type IgG	Wild type anti-MMP10 Ab	Fra-2 IgG	Fra-2 anti-MMP10 Ab
Mean	0.2450	0.2580	0.3067	0.2850
SEM	0.005	0.002	0.006	0.005

Moreover, the **Table 6** below shows the results obtained by Tukey's rang test.

Table 6: Tukey's multiple comparison test between wild type mouse and Fra-2 mouse receiving an MMP10 inhibitor (antibody anti-MMP10) or IgG in RVH.

Tukey's multiple comparison test	Mean Diff.	95% CI	Summary
Fra2 IgG vs. WT IgG vs.	-0.062	-0.082 to -0.041	****
Fra-2 IgG vs. WT anti-MMP10	-0.049	-0.068 to -0.029	****
Fra-2 IgG vs. Fra-2 anti-MMP10	0.022	0.004 to 0.039	*
Fra-2 anti-MMP10 vs. WT IgG	-0.040	-0.059 to -0.020	****
Fra-2 anti-MMP10 vs. WT anti-MMP10	-0.027	-0.045 to -0.006	**

Increased percentage medial wall thickness, and numbers of muscularized distal pulmonary arteries were found in IgG-treated-Fra-2 mice compared with vehicle- or IgG-treated wild type mice (Figures 9A-D). The degree of pulmonary vascular remodeling was substantially attenuated in Fra-2 transgenic mice receiving anti-MMP10 antibodies compared to IgG-treated Fra-2 mice. Indeed, the percentage medial wall thickness, and numbers of muscularized distal pulmonary arteries were significantly reduced in anti-MMP10-treated Fra-2 mice when compared to IgG-treated Fra-2 mice (Figure 9A-D).

There were no significant differences between Fra-2 or C57BL/6 mice given vehicle or anti-MMP10 antibodies regarding body weight, systemic blood pressure, or heart rate.

Conclusion

The above results demonstrate the involvement of MMP10 in systemic sclerosis (SSc) and in pulmonary arterial hypertension development in SSc patients (SSc-PAH).

In particular, the above results show overexpression of MMP10 mRNA and protein in EPC-derived cells derived from SSc patients compared to healthy subject, and more particularly in SSc-PAH patients compared to SSc-without PAH patients. The involvement of MMP10 in SSc PAH has been also supported by the demonstration of increased circulating pro-MMP10 levels in the serum of SSc-PAH patients and by the detection of MMP10 in the walls of pulmonary arteries of these patients.

The above results also demonstrate the involvement of MMP10 in SSc-PAH *in vivo* in Fra-2 transgenic mice, which develop several features of the human SSc disease, in particular vasculopathy and progressive skin fibrosis. Particularly, it was demonstrated an increase in MMP10 expression within walls of remodeled pulmonary arteries of Fra-2 transgenic mice.

The above results also show that MMP10 inhibition attenuate pathologic vascular remodeling in Fra-2 transgenic mice. Particularly, treatment with anti-MMP10 antibodies significantly decreased RVSP and RVH and markedly reduced vascular remodeling and myointimal proliferation.

Therefore, blocking MMP10 (it expression or it activity) during SSc or once PAH is established holds promise as a therapeutic approach for this disease. Indeed, MMP10 inhibition on SSc-PAH patients should provide benefits far beyond a mere symptomatic relief.

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CLAIMS

1. An inhibitor of pro-MMP10 expression and/or MMP10 activity, which is selected from:

- small chemical compounds selected from the group comprising tetracycline compounds, batimastat, marimastat, ilomastat, prinomastat and neovastat,
- TIMP-1 (TIMP metallopeptidase inhibitor 1),
- anti-MMP10 antibodies or antigen fragments thereof, and
- nucleic acids inhibiting the expression of pro-MMP10, such as antisense oligonucleotides, interfering RNAs, ribozymes and aptamers,

for use in the treatment or prevention of pulmonary arterial hypertension (PAH) in a systemic sclerosis (SSc) patient.

2. The inhibitor for use according to claim 1, wherein said inhibitor is selected from anti-MMP10 antibodies or antigen fragments thereof.

3. The inhibitor for use according to claim 2, wherein said anti-MMP10 antibody or antigen fragment thereof is selected from polyclonal antibodies, monoclonal antibodies, bispecific antibodies, fragments selected from Fv, scFv, Fab, F(ab')2, Fab', scFv-Fc, diabodies, or any fragment whose half-life has been increased by chemical modification.

4. The inhibitor for use according to claim 2 or 3, wherein said anti-MMP10 antibody or antigen fragment thereof blocks the catalytic domain of MMP10.

5. The inhibitor for use according to of any one of claims 1 to 4, wherein said PAH is an advanced PAH.

6. An *in vitro* method for diagnosing PAH in SSc patient from a biological sample of said patient selected from a blood sample, a serum sample, a plasma sample, a blood-derived circulating endothelial progenitor (EPCs) cells sample and a lung tissue sample, comprising the following steps:

a) measuring *in vitro* the expression level of the precursor of matrix metalloproteinase-10 (pro-MMP10) in said sample;

b) comparing the expression level of pro-MMP10 measured in step a) to the expression level of pro-MMP10 in at least one reference sample, and

c) determining from said comparison the presence or the absence of PAH in said patient, wherein the higher the expression level of pro-MMP10 is, the higher is the risk that the SSc patient suffers from PAH.

7. The *in vitro* method according to claim 6, wherein in step a) the expression level of pro-MMP10 is measured at the protein level.

8. The *in vitro* method of claim 7, wherein the expression level of pro-MMP10 protein is measured using an ELISA assay.

9. The *in vitro* method of claims 6 to 8, wherein the expression level of pro-MMP10 protein is measured in a biological sample selected from a blood sample, a serum sample, and a plasma sample.

10. The *in vitro method* according to claim 6, wherein in step a) the expression level of pro-MMP10 is measured at the nucleic acid level.

11. The *in vitro method* according to claim 10, wherein in step a) the expression level of pro-MMP10 is measured in a blood-derived EPCs cells sample.

12. The *in vitro* method of any one of claims 6 to 11, wherein at least one reference sample used in step b) is a sample obtained from a subject selected from a healthy subject, a systemic sclerosis (SSc) patient without PAH, and a systemic sclerosis (SSc) patient with PAH.

13. The in vitro method of any one of claims 6 to 12, wherein the expression level of pro-MMP10 measured in step a) is compared to a threshold value calibrated based on said reference samples, over which the tested subject will be diagnosed as suffering from SSc-PAH.

14. The in vitro method of any one of claims 6 to 12, wherein:

- if the expression level of pro-MMP10 in the tested biological sample measured in step a) is much higher than the expression level in at least one sample obtained from healthy subject(s) or from reference SSc patients without PAH, it is concluded that the tested SSc patient is suffering or suspected to suffer from PAH; or
- if the expression level of pro-MMP10 in the tested biological sample measured in step a) is lower than the expression level of pro-MMP10 in at least one reference sample obtained from SSc patient without PAH, it is concluded that the SSc patient is not suffering from SSc-PAH.

15. The *in vitro* method of any one of claims 6 to 14, wherein the PAH is an advanced PAH.

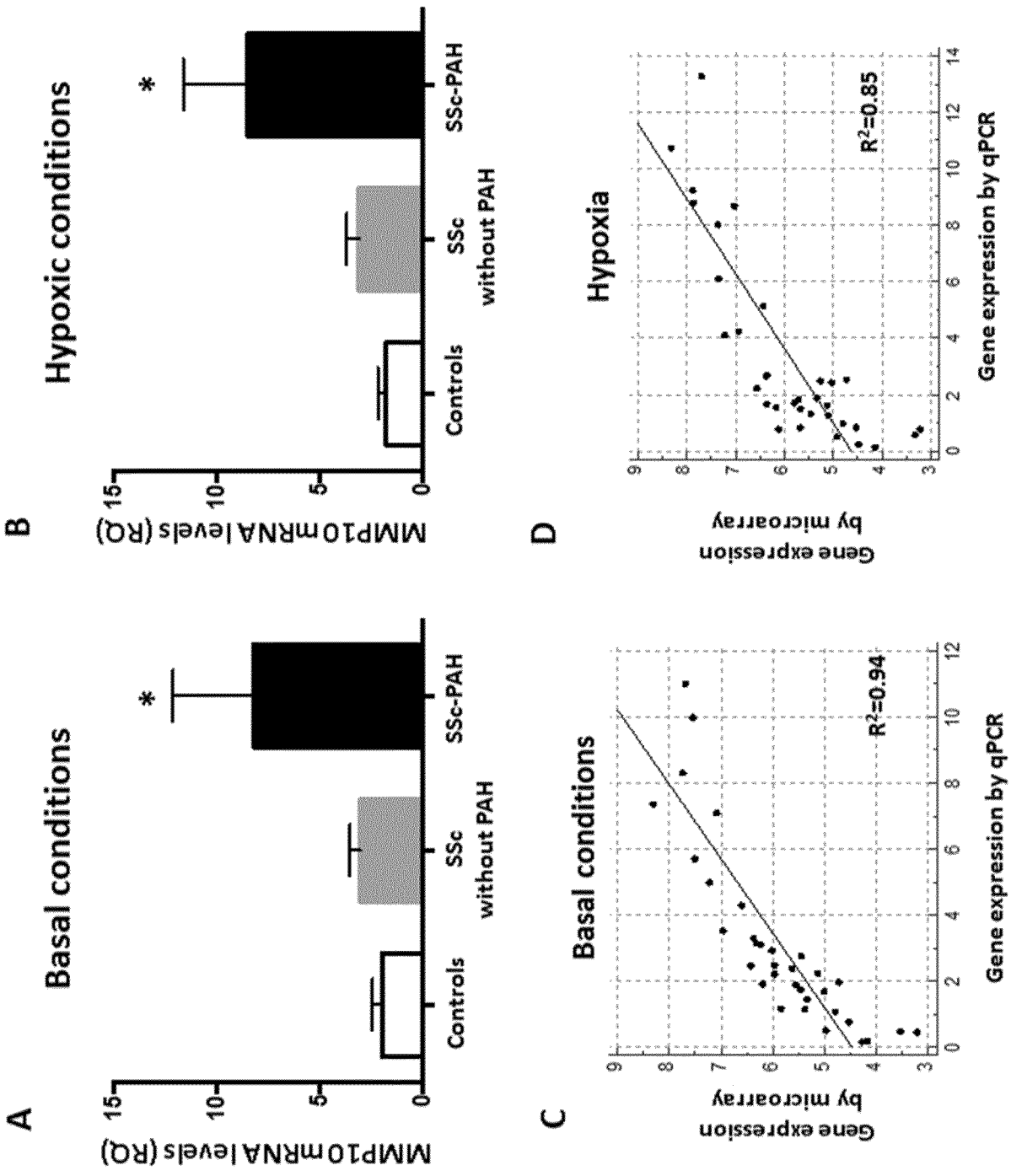

A
Basal conditions
MMP10 mRNA levels (RQ)
15
10
5
0
*
Controls
SSc without PAH
SSc-PAH
B
Hypoxic conditions
MMP10 mRNA levels (RQ)
15
10
5
0
*
Controls
SSc without PAH
SSc-PAH
C
Basal conditions
Gene expression by microarray
Gene expression by qPCR
R²=0.94
9
8
7
6
5
4
3
0
2
4
6
8
10
12
D
Hypoxia
Gene expression by microarray
Gene expression by qPCR
R²=0.85
9
8
7
6
5
4
3
0
2
4
6
8
10
12
14

Figure 1

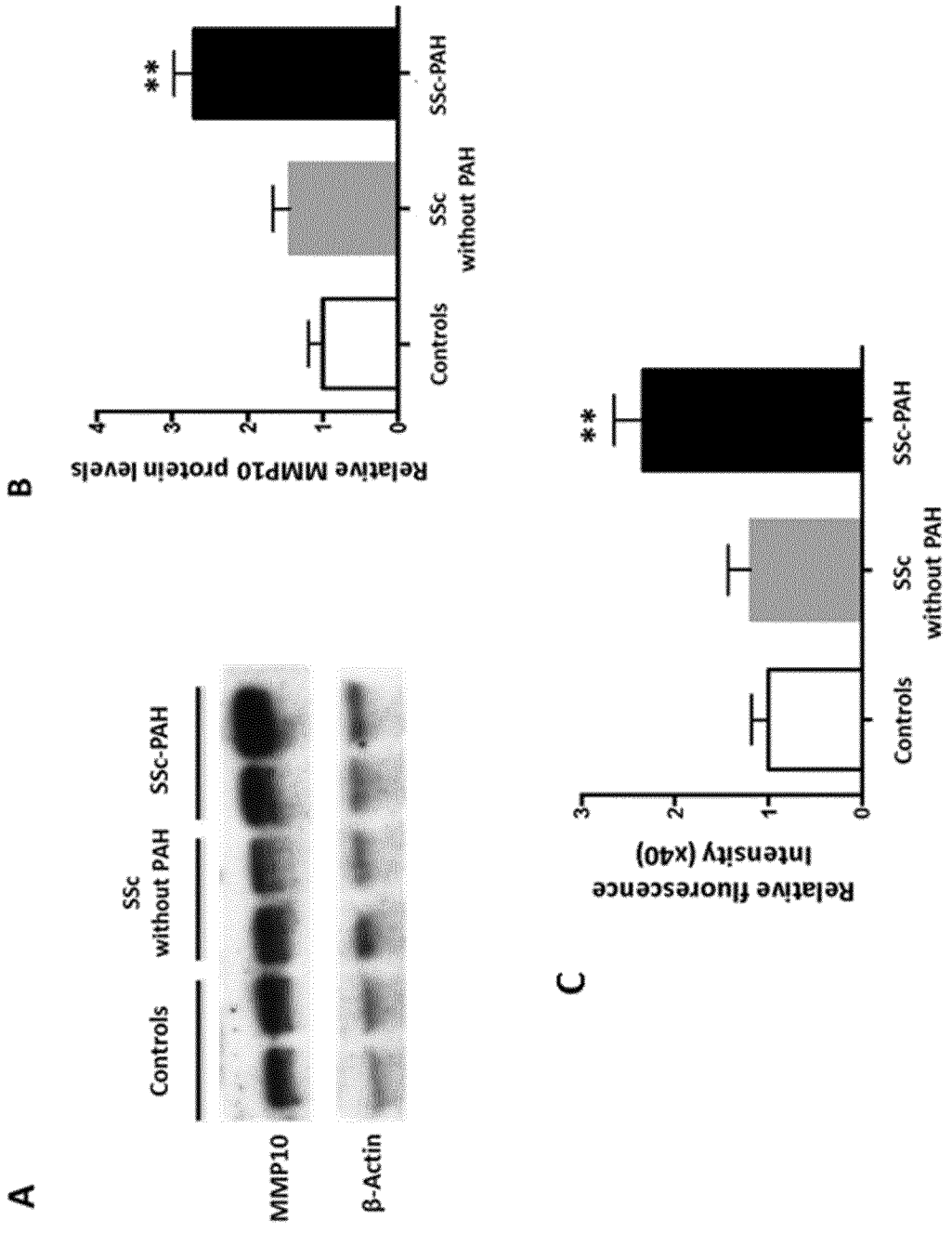

A
Controls
SSc without PAH
SSc-PAH
MMP10
β-Actin
B
Relative MMP10 protein levels
0
1
2
3
4
**
Controls
SSc without PAH
SSc-PAH
C
Relative fluorescence intensity (x40)
0
1
2
3
**
Controls
SSc without PAH
SSc-PAH

Figure 2

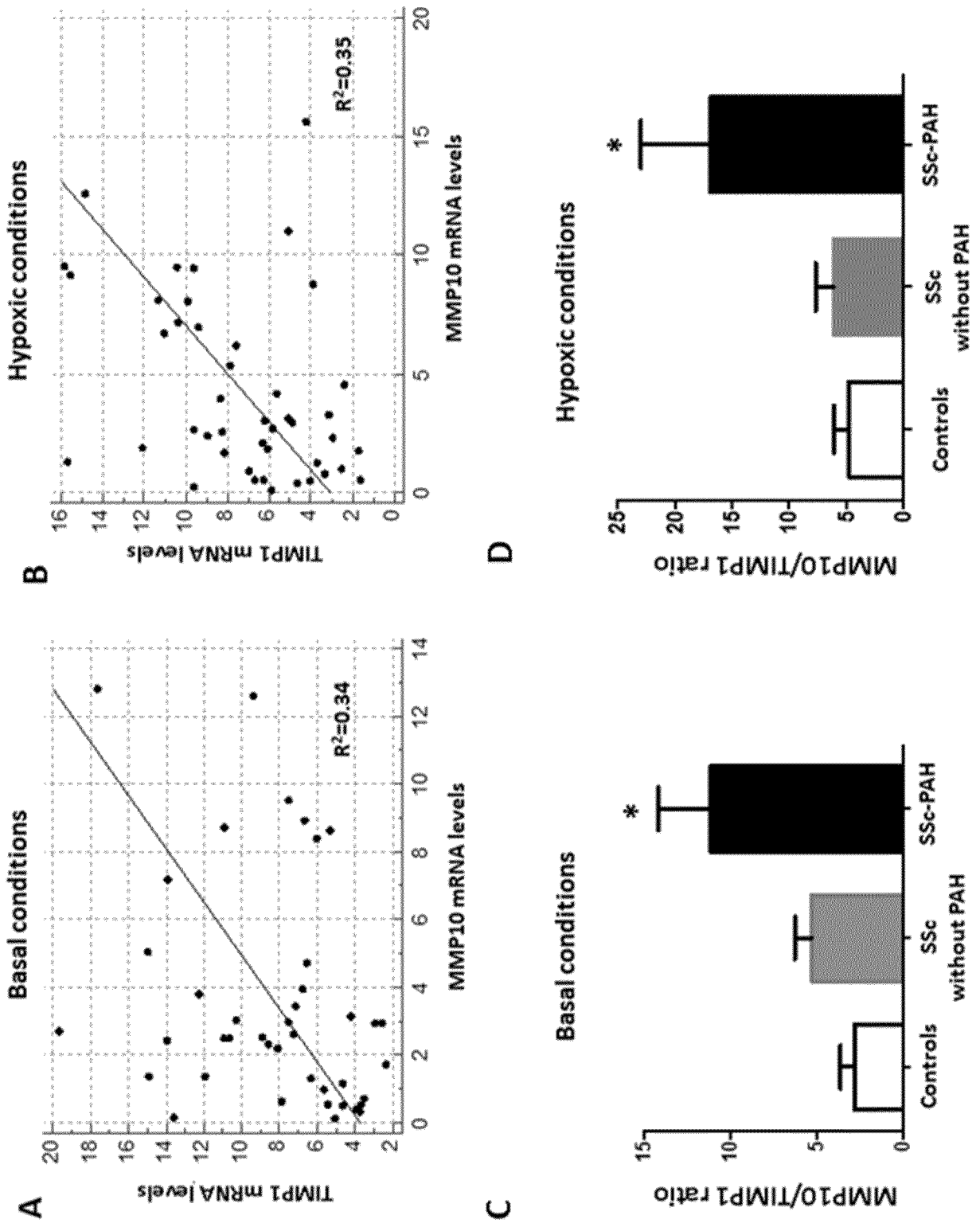

A
Basal conditions
TIMP1 mRNA levels
MMP10 mRNA levels
R²=0.34
B
Hypoxic conditions
TIMP1 mRNA levels
MMP10 mRNA levels
R²=0.35
C
Basal conditions
MMP10/TIMP1 ratio
Controls
SSc without PAH
SSc-PAH
*
D
Hypoxic conditions
MMP10/TIMP1 ratio
Controls
SSc without PAH
SSc-PAH
*

Figure 3

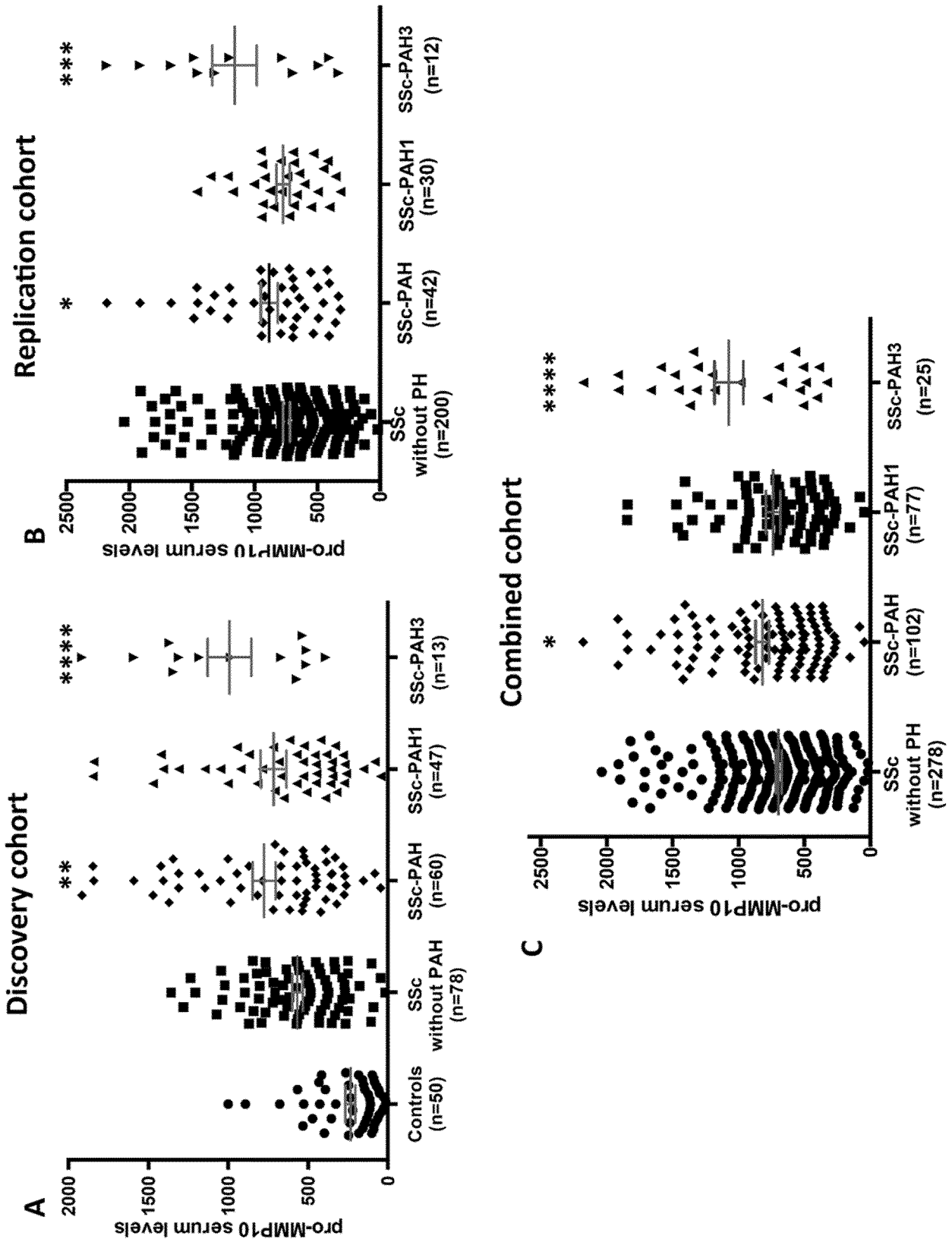

A
Discovery cohort
pro-MMP10 serum levels
2000
1500
1000
500
0
**
****
Controls (n=50)
SSc without PAH (n=78)
SSc-PAH (n=60)
SSc-PAH1 (n=47)
SSc-PAH3 (n=13)
B
Replication cohort
pro-MMP10 serum levels
2500
2000
1500
1000
500
0
*
***
SSc without PH (n=200)
SSc-PAH (n=42)
SSc-PAH1 (n=30)
SSc-PAH3 (n=12)
C
Combined cohort
pro-MMP10 serum levels
2500
2000
1500
1000
500
0
*
****
SSc without PH (n=278)
SSc-PAH (n=102)
SSc-PAH1 (n=77)
SSc-PAH3 (n=25)

Figure 4

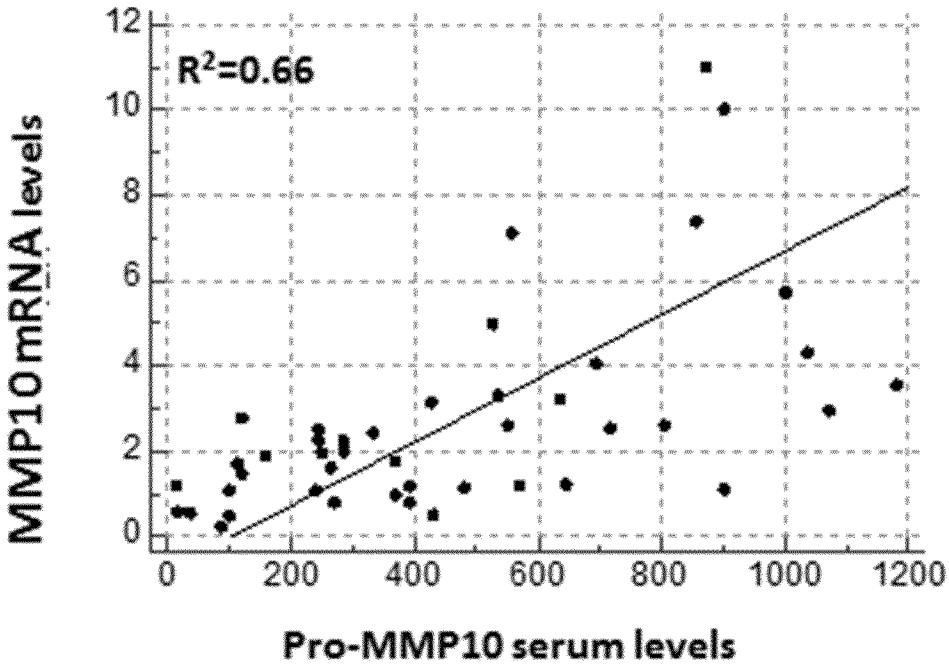

MMP10 mRNA levels
Pro-MMP10 serum levels
R²=0.66
0
2
4
6
8
10
12
0
200
400
600
800
1000
1200

Figure 5

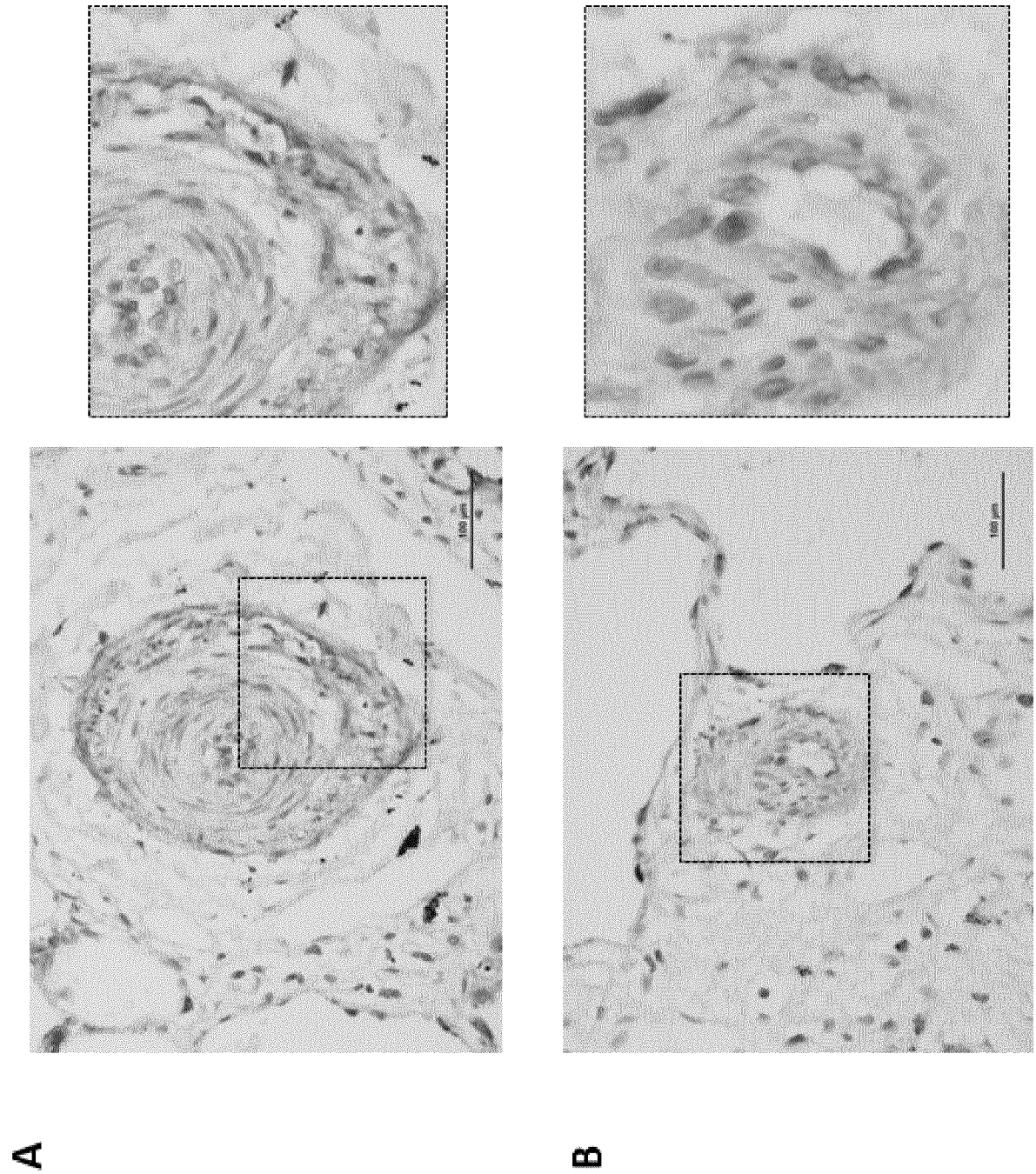


Figure 6

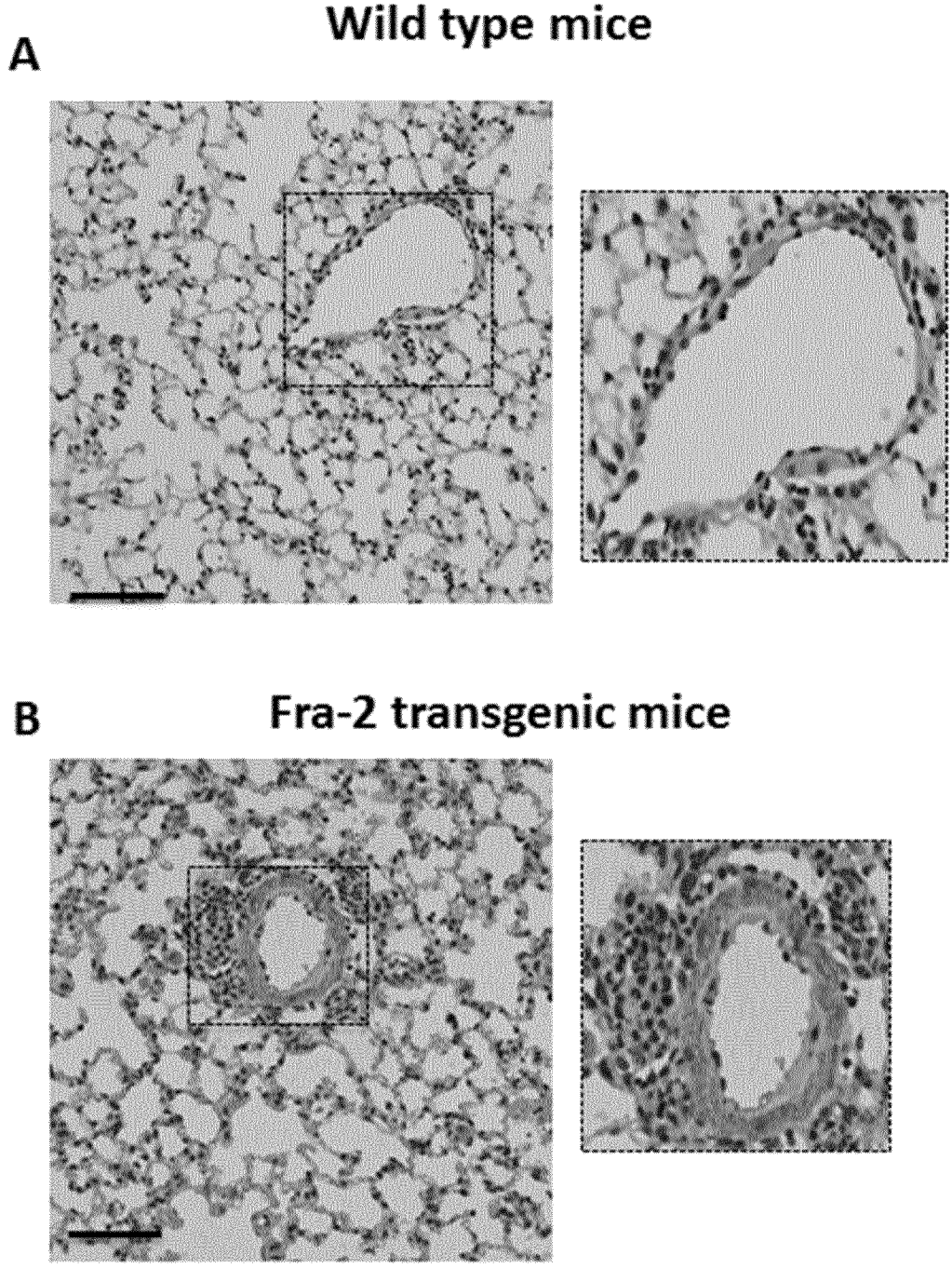


Figure 7

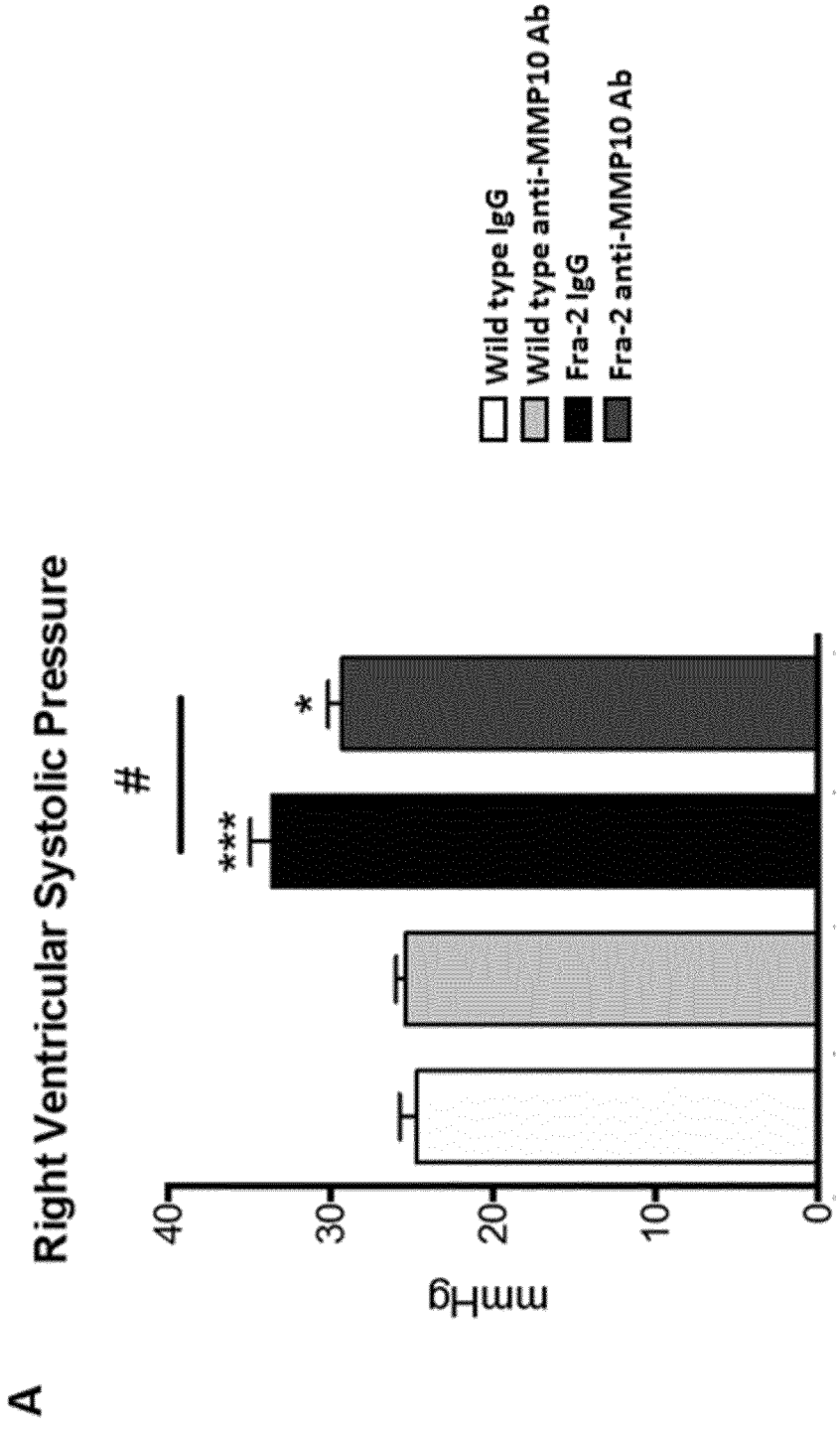

A
Right Ventricular Systolic Pressure
mmHg
0
10
20
30
40
#
***
*
Wild type IgG
Wild type anti-MMP10 Ab
Fra-2 IgG
Fra-2 anti-MMP10 Ab

Figure 8A

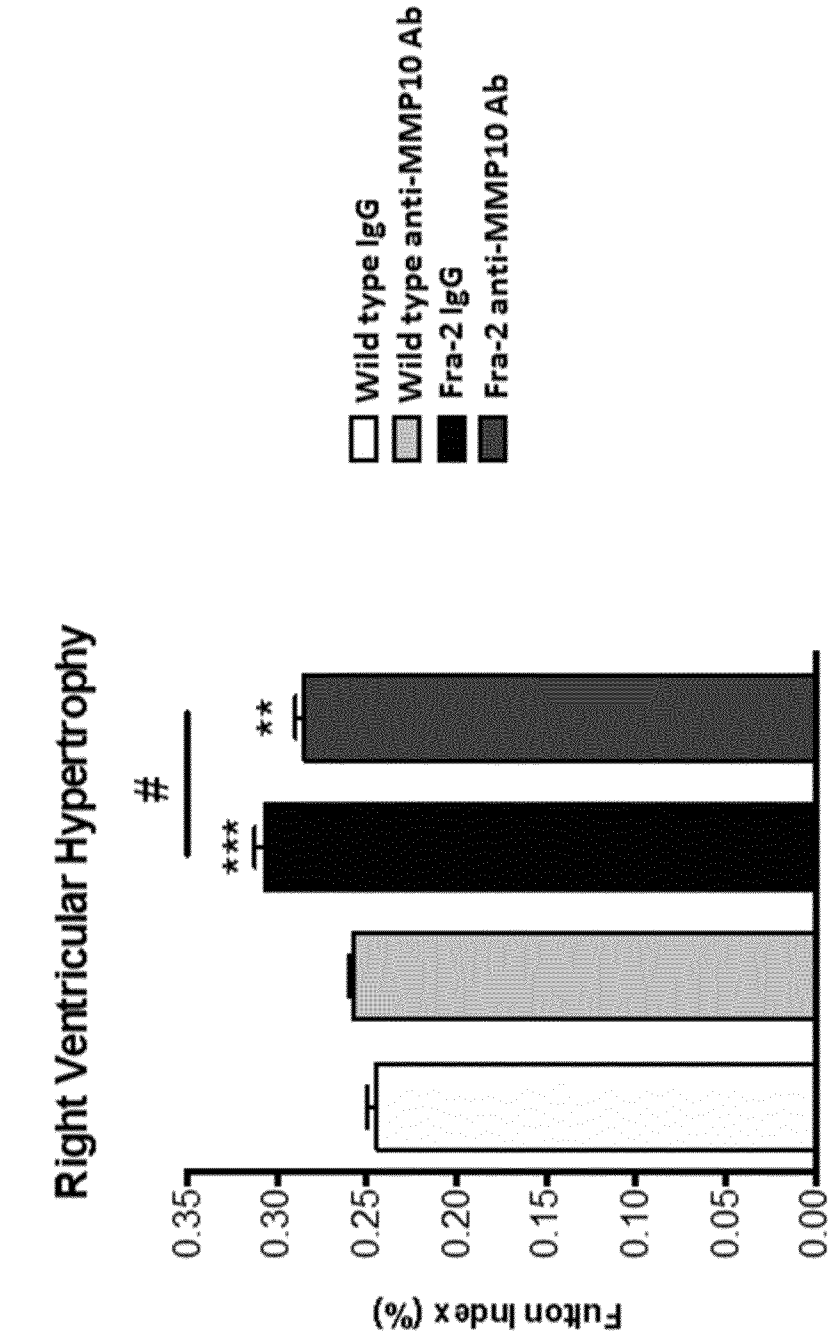
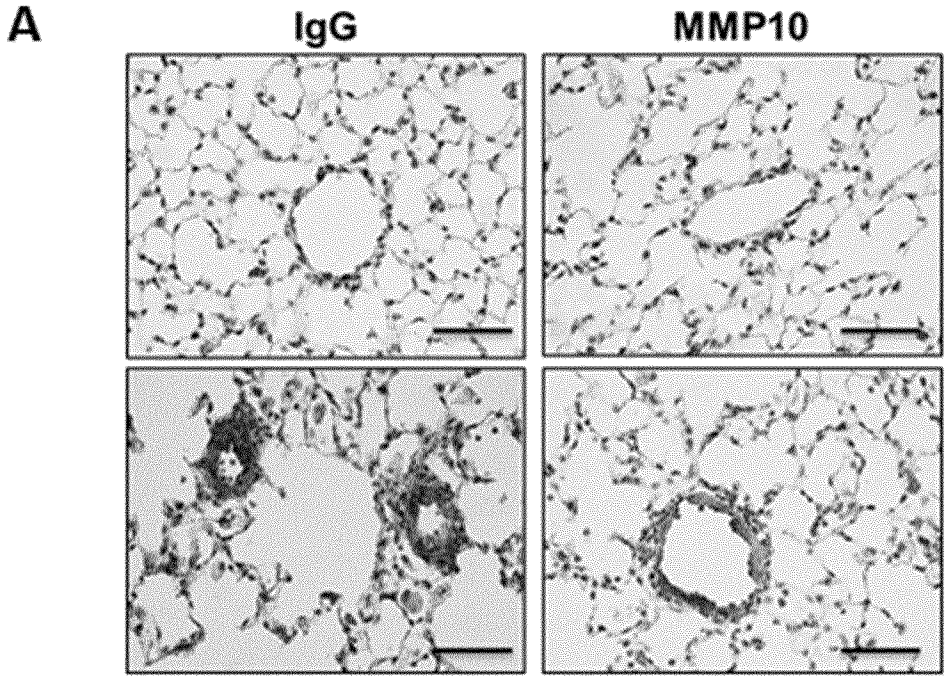
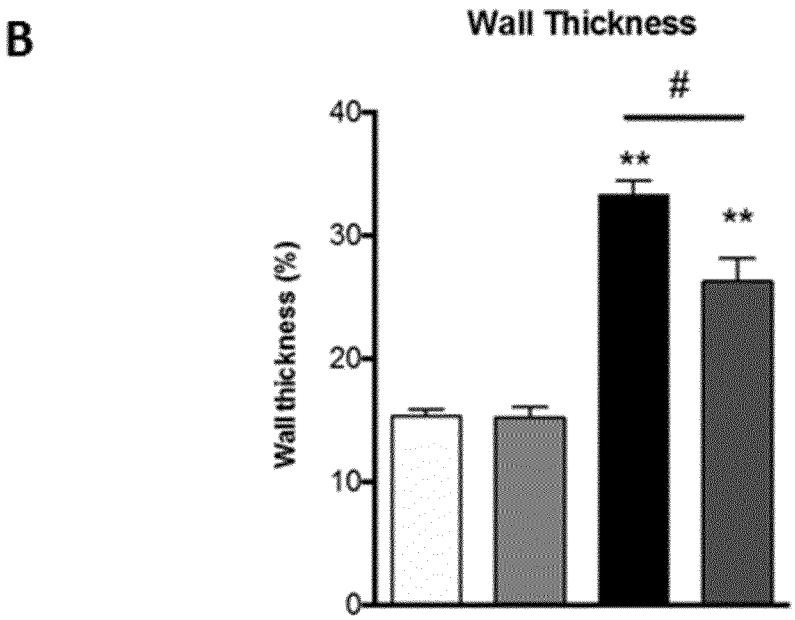

B
Right Ventricular Hypertrophy
Fulton Index (%)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
#
***
**
Wild type IgG
Wild type anti-MMP10 Ab
Fra-2 IgG
Fra-2 anti-MMP10 Ab

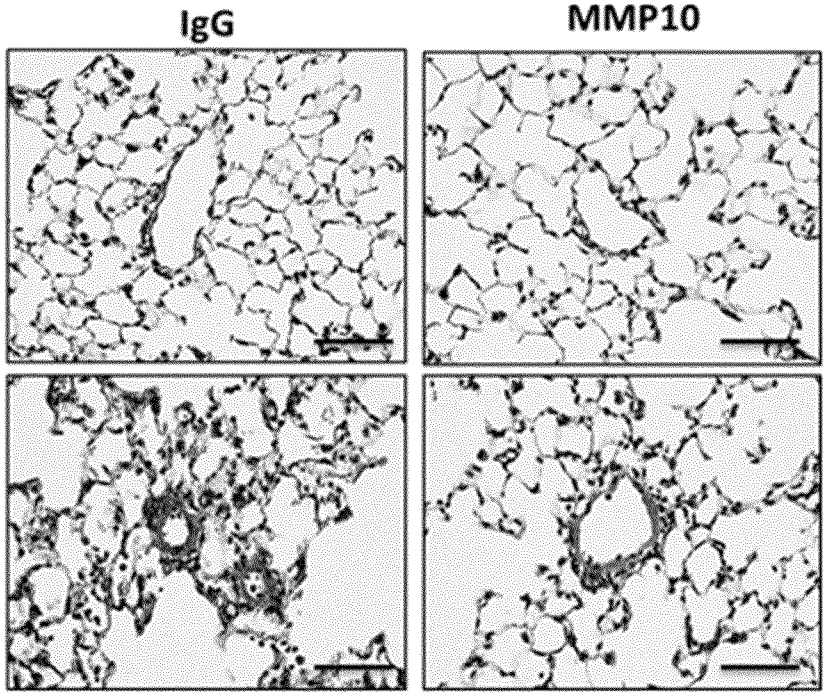
Figure 8B



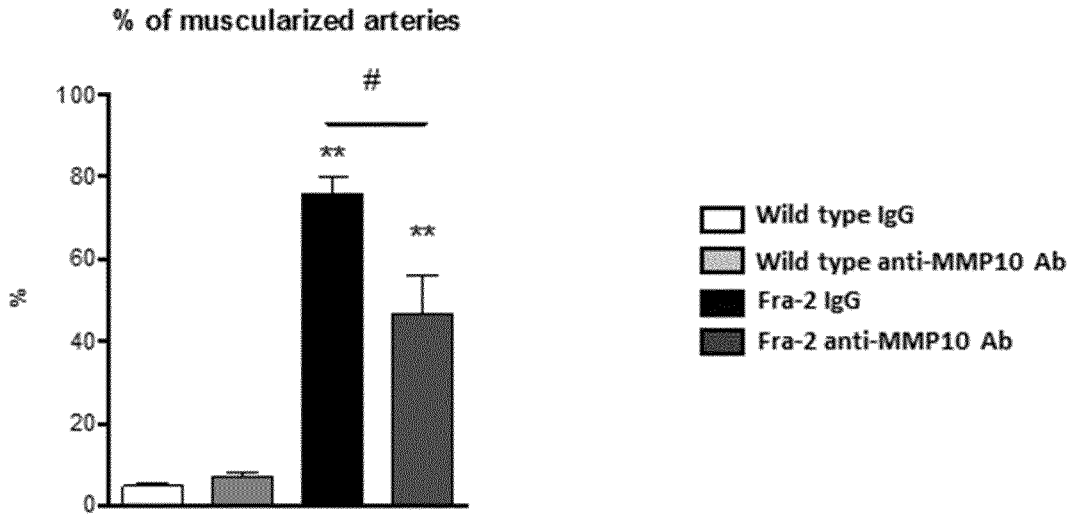


Figures 9A and 9B

C



D



Figures 9C and 9D

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/078908

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/40 A61P37/00 A61P9/12
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)
C07K A61K

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, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOFFMANN JULIA ET AL: "Compartment-specific expression of collagens and their processing enzymes in intrapulmonary arteries of IPAH patients", AMERICAN JOURNAL OF PHYSIOLOGY - LUNG CELLULAR AND MOLECULAR PHYSIOLOGY, vol. 308, no. 10, May 2015 (2015-05), pages L1002-L1013, XP009189824, abstract figure 4 ----- -/--	6-13,15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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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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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 February 2017	27/02/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bernhardt, Wiebke

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/078908

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DZIANKOWSKA-BARTKOWIAK BOZENA; ZEBROWSKA AGNIESZKA; JOSS-WICHMAN EWA; KOBOS JOZEF; WASZCZYKOWSKA ELZBIETA: "Expression of metalloproteinases and their inhibitors in skin lesions of systemic sclerosis (SSc) patients", CENTRAL-EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 31, no. 3-4, 1 January 2006 (2006-01-01), pages 94-101, XP055269035, PL ISSN: 1426-3912 abstract table 2 page 97	6-13,15
A	FRIESE RYAN S ET AL: "Matrix metalloproteinases: discrete elevations in essential hypertension and hypertensive end-stage renal disease.", CLINICAL AND EXPERIMENTAL HYPERTENSION (NEW YORK, N.Y. : 1993) OCT 2009, vol. 31, no. 7, October 2009 (2009-10), pages 521-533, XP009189825, ISSN: 1525-6006 abstract	1-15
A	ESTHER MARTINEZ-AGUILAR ET AL: "Matrix metalloproteinase 10 is associated with disease severity and mortality in patients with peripheral arterial disease", JOURNAL OF VASCULAR SURGERY., vol. 61, no. 2, 1 February 2015 (2015-02-01), pages 428-435, XP055268907, US ISSN: 0741-5214, DOI: 10.1016/j.jvs.2014.09.002 abstract	1-15
A	WEN-JIA PENG ET AL: "Matrix Metalloproteinases: A Review of Their Structure and Role in Systemic Sclerosis", JOURNAL OF CLINICAL IMMUNOLOGY, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NE, vol. 32, no. 6, 6 July 2012 (2012-07-06), pages 1409-1414, XP035153748, ISSN: 1573-2592, DOI: 10.1007/S10875-012-9735-7 whole document, in particular table 1 page 1413	1-15

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