UNIVERSITÀ DEGLI STUDI DI SASSARI

UNIVERSITY OF SASSARI

Dipartimento di Scienze Biomediche

Department of Biomedical Sciences

Scuola di Dottorato di Ricerca in Scienze Biomolecolari e Biotecnologiche

International PhD School in Biomolecular and Biotechnological Sciences

Curriculum: Biochemistry, Physiology and Molecular Biology

Cycle XXVII

Director: Prof. Leonardo A. Sechi

Cellular and Molecular Study of Vascular Damage during Systemic Sclerosis

Tutor/Supervisor: Prof.Gianfranco Pintus

PhD Student: Dr. Tulasigeri M.Totiger

ACADEMIC YEAR 2013-2014

TABLE OF CONTENTS

ABSTRACT

Systemic Sclerosis is a devastating vascular, connective and a multisystem autoimmune disease characterized by fibrosis of skin and internal organs, exhibiting various forms of disease conditions. Pulmonary arterial hypertension (PAH) is one of the clinical manifestations that arise as a result of cellular and molecular vascular damage in the vascular wall, and is one of the leading causes of death in systemic sclerosis (SSc). SSc-PAH is a progressive depleting condition that can lead to right-sided heart failure and death. Oxidative stress is largely evidenced in the development of arterial hypertension in SSc. This may give rise to unbalanced redox homeostasis that could further bring about vascular remodeling characterized by Vascular Smooth Muscle Cells (VSMC) hypertrophy and hyperplasia and ECM deposition leading to subsequent obliterative vasculopathy and vessel occlusion. So it is likely that circulating pro-oxidant factors may be involved in the pathogenesis of SSc-PAH by inducing VSMCs activation and phenotypic switch. So we speculate, that in SSc patients associated with Pulmonary Arterial Hypertension (PAH), circulating factors may drive an aberrant vascular remodeling by exerting pro-oxidant and pro-fibrotic effects on Human Pulmonary Arterial Smooth Muscle Cells (HPASMCs) and activate collagen I synthesis. To test this hypothesis, we exposed primary Human Pulmonary Artery Smooth Muscle Cells (HPASMCs) to serum obtained from SSc patients with or without PAH and healthy donors (HD) and looked for the production of reactive oxygen species (ROS) and collagen I synthesis. From our study, we found that SSc-PAH patients' circulating factors exhibited pro-oxidant effect by inducing ROS generation through NOX2 in HPASMCs and we also found that, circulating factors exhibited pro-fibrotic effect by inducing activation of collagen I synthesis in HPASMCs via ERK signaling, thus asserting the vascular damage in SSc patients.

ABBREVIATIONS

Systemic Sclerosis(SSc)

Limited cutaneous SSc (lcSSc)

Diffuse cutaneous SSc (dcSSc)

Vascular smooth-muscle cells (VSMCs)

Pulmonary Arterial Hypertension (PAH)

Vascular remodeling (VR)

Oxidative Stress (OS)

Reactive Oxygen Species (ROS)

Nicotinamide Adenine Dinucleotide Phosphate-Oxidase (NADPH Oxidase or NOX)

Human Pulmonary Artery Smooth Muscle Cells (HPASMCs)

Pulmonary Artery (PA)

Endothelin (ET)

Extracellular signal-regulated kinase (ERK)

Right heart catheterization (RHC)

Healthy donors (HD)

Right ventricular systolic pressure (RVSP)

Dichlorodihydrofluorescein-diacetate (H2-DCFDA)

Collagen type-I(COL1A1)

Lentiviral vector (LV), Forced vital capacity (FVC)

CHAPTER 1. INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1 Historical Background and Definition of Systemic Sclerosis

Systemic Sclerosis (SSc) is also called as Scleroderma or Systemic Scleroderma or Familial Progressive Scleroderma or Progressive Scleroderma. The first recitation of skin diseases consubstantial to SSc were reported as early as Hippocrates (460 - 370 B.C.), that are cited by the detailed historical account of SSc by Rodnan and Benedek in 1962 [1, 2]. However, Italian physician and dermatologist Carlo Curzio in 1753 [3] was the first to describe this disease condition. He reported about a 17-year old woman who had excessive tension and hardness of skin and she could hardly move her limbs. Goetz gave the name progressive systemic sclerosis or systemic sclerosis in 1945 [4], and this has become widely accepted term for this disease. Scleroderma was recognized as a clinical entity in mid-19th century with its present name [5].

More than 20 descriptive names had been proposed in the 19th century before this disease condition was named as *sclerodermie or scleroderma*. Systemic Sclerosis is derived from the Greek words '*scleros*' and '*derma*', means thickened, hardened skin. It is integrated by two words Systemic and Sclerosis where in Systemic refers to affecting a particular body system, or circulating through the entire body and Sclerosis refers to abnormal thickening or hardening of a body part. It is a connective tissue and a chronic multisystem autoimmune disease characterized by vasculopathy, diffuse fibrosis of skin and various internal organs. Systemic sclerosis involves hardening of internal organs and stops them working normally. It is a rare, heterogeneous and a slow-motion disease, but can be very serious. It varies greatly from person to person and hence there are many symptoms and problems that may progress or evolve with systemic sclerosis. As of now, there is no cure for scleroderma but effective treatments for some phenotypes of the disease are available.

1.2 Classification of SSc

There are mainly two classes of SSc, named as **Localized** & **Systemic Scleroderma**. Localized scleroderma does not involve internal organs. But systemic scleroderma has been given more importance due to its involvement of internal organs. It has two subsets based on the extent of skin involvement (as shown in Fig. C) , namely **Limited cutaneous SSc (lcSSc)** with skin involvement distal to the elbows and knees, with or without involvement of face and **Diffuse cutaneous SSc (dcSSc)** with skin involvement of the proximal limbs and/or trunk [6, 7]. The distinguishing features between the both the subsets are explained in the table below [8].

Figure C: Skin involvement in LSSc & DSSc

Baltimore 2002; Ferri C. et al (Medicine) 81(2):139-153.

Table 1 Diffuse vs. limited scleroderma—distinguishing features

Diffuse	*Limited*
ILD (severe in 15%)	ILD (severe in 15%)
Heart (severe in 10%)	Minimal heart
Pulmonary hypertension (5–10%)	Pulmonary arterial hypertension (10–15%)
Kidney (severe in 10–15%)	Minimal kidney
Large joint contractures	Concurrent Primary biliary cirrhosis (6–8%)
Worse survival overall	

Table 1.Distinguishing Fetures:LSSc & DSSc
Dinesh Khanna. Indian Journal of Rheumatology June 2010

1.3 Pulmonary Hypertension

There are several heterogeneous clinical manifestations of SSc involving cutaneous, vascular, pulmonary, gastrointestinal, endocrine, neurologic, cardiac, renal and muscoskeletal. Pulmonary hypertension is one of the serious manifestations that develop in SSc patients. Actually, pulmonary hypertension is an abnormal increase of the pressure in the blood vessels of

the lungs. This is usually called as the "high blood pressure" of the lungs. In normal functioning of lungs, the pressure in the blood vessels is about one-quarter of the pressure in the arteries of the body and this can temporarily adapt to increased pressures that occur during exercise [9]. But during pulmonary hypertension, in the lungs, small arteries are very much narrow, that's why the pressure rises in these vessels. Because of this, the right side of the heart, which pumps blood into the lungs, will have to pump against a higher resistance of blood flow. This would cause more difficult to pump the blood through the lungs, exactly when increased flow is needed, in a patient when exercises [9].Pulmonary arterial hypertension (PAH) is described as a progressive condition characterized by elevated pulmonary arterial pressures causing right ventricular (RV) failure [10]. In medical terms it is defined as an increase in mean pulmonary arterial pressure (mPAP) ≥ 25 mm Hg at rest as assessed by right heart catheterization [11,12]. Different hemodynamic definitions of PH are described based on pulmonary capillary wedge pressure, pulmonary vascular resistance (PVR) and cardiac output (CO).Clinical groups 1, 3, and 4 are of pre-capillary PH, group 2 compose post-capillary PH [13-19] and group 5 linked to PH with unclear or multifactorial etiologies.

1.4 Classification of Pulmonary Arterial Hypertension (PAH)

The revised classification comprises of 5 different types of PAH, with the inclusion of subclasses & their subtypes [13, 16-19]. They are as follows.

1. Pulmonary arterial hypertension

2. Pulmonary hypertension due to left heart disease

3. Pulmonary hypertension due to lung diseases and/or hypoxia

4. Chronic thromboembolic pulmonary hypertension (CTEPH)

5. Pulmonary hypertension with unclear multifactorial mechanisms

PAH or Group 1 PH include PAH having different etiologies. The pathological characteristics of this group involve pulmonary arterial endothelial cell (EC) dysfunction, pulmonary artery EC and smooth muscle cell (SMC) proliferation, vasoconstriction and in situ thrombosis [20]. The sub-groups of PAH include common clinical characteristics and share similarities in terms of management [17]. PAH remains an incurable disease process, even though there are many new available therapies from the last two decades. If PAH not interrupted, would leads to right heart failure and death [22, 23, 24].

1.5 Systemic Sclerosis-associated Pulmonary Arterial Hypertension (SSc -PAH)

The existence of a small vessel vasculopathy is one of the distinguishing features of SSc & hence SSc patients are at greater risk of developing & arriving at disease condition called PAH. This disorder is now admitted as the major cause of morbidity and mortality. So the newest (2013) American College of Rheumatology / European League Against Rheumatism (ACR/EULAR) classification criteria has given importance & recognition to it and provided equal weight and consideration to SSc-PAH as similar to other manifestations of SSc. PAH is a life-threatening ailment, which could rapidly progress to severe right heart failure [13, 14]. The prevalence of PH in connective tissue diseases is usually estimated on the basis of echocardiographic examinations, however most of the recent studies show that for systemic sclerosis (SSc), PH assessment depends on the basis of strict hemodynamic criteria [25,26,27]. Pulmonary arterial hypertension (PAH) is described as a progressive pulmonary vascular disease that involves

narrowing or tightening or constriction of the pulmonary arteries that connect the right side of the heart to the lungs. PAH is characterized by an increase in mean pulmonary arterial pressure (PAP) to ≥25 mmHg at rest, and a mean primary capillary wedge pressure of ≤15 mmHg [15]. As PAH progresses, the blood flow through the pulmonary arteries is restricted and because of this, there occurs an enhanced strain of pumping blood through the lungs, resulting in the enlargement of the right side of the heart. As a result of this strain on the heart and also due to the reduction in blood to the left heart and also to the systemic circulation through the lungs lead to usual symptoms of PAH, such as *Dyspnea (breathlessness), fatigue, weakness, Exertional syncope, angina, and abdominal distension* [12].The parameter that indicates a high risk of future development of PAH in patients with SSc is the existence of 'borderline' pressures aligning between 21 and 24mmHg [28, 29].

1.6 Epidemiology of SSc-PAH

PAH is caused by the pulmonary vascular remodeling and can either occur alone (SSc-PAH), or may advance secondary to pulmonary parenchymal involvement resulting as interstitial lung diseases (ILD-PH). ILD-PAH is the most frequent and a serious complication of SSc. It occurs in 75% of cases and is more frequent in diffuse cutaneous SSc (DcSSc) [30-33]. Based on right heart catheterization (RHC), the prevalence of PAH other than ILD-PH is between 7.85 and 12% [30, 31] and more common in later stage of limited cutaneous SSc (LcSSc). Isolated PAH (SSc-PAH) is more common in LcSSc, when compared to DcSSc, however few recent studies [30-38] suggest that the prevalence of PAH is similar in both limited and diffuse cutaneous SSc patients. SSc-PAH has worse prognosis than that of IPAH [39] and patients are at higher risk of death than IPAH patients. Even though both types share

similar histopathological & haemodynamic characteristics but estimated one-year survival rates are 55% and 84%, respectively [40]. PAH among patients with SSc is estimated to be 0.61 cases per 100 patient-years [41]. Median survival time of SSc patients with PAH is between 1 to 3 years [40, 42] and SSc patients with ILD alone have a median survival of 5–8 years [43].

1.7 Subtypes of PAH in SSc

Pulmonary hypertension, defined by mean pulmonary arterial pressure greater than 25 mm Hg, can be isolated in SSc, occurring as PAH (SSc-associated PAH or SSc-PAH), and this one is due to pulmonary vascular remodeling. The other subtype is in combination with ILD [pulmonary hypertension (PH)-ILD] or (ILD-PH) [44,45], which may develop secondary due to parenchymal involvement.

A. Isolated Pulmonary Arterial Hypertension {IPAH} (in the absence of significant pulmonary fibrosis or Interstitial Lung Disease)

PAH in SSc patients without pulmonary fibrosis or ILD is a severe complication due to narrowing or occlusion of small pulmonary arteries caused by smooth muscle hypertrophy, intimal hyperplasia, inflammation, thrombosis in situ. The breathlessness (dyspnea) progression rate from normal exercise tolerance to oxygen dependency is about 6–12 months & the mean duration of survival is 2 years. Unlikely, SSc patients with PAH with respect to interstitial lung disease have a same degree of disability, but the difference is that they progress more slowly for a period more than 2, up to 10 years [46-50]. The prevalence of Isolated PAH in SSc (without significant

pulmonary fibrosis) is between 7 and 12% of patients [11]. However, the most frequent type of pulmonary hypertension in SSc patients is Group I PAH [51].

B. PAH associated with pulmonary fibrosis

With either of both the (diffuse or limited) forms of the disease, more than one third of SSc patients have pulmonary fibrosis. The clinical tests have revealed alveolar, interstitial, peribronchial and pleural fibrosis. This kind of PAH has relatively slow progression & has gradual elevation in the resistance of pulmonary vasculature resulting in the widespread pulmonary fibrosis [47]. ILD is prevalent in up to 75% of SSc cases and is more frequent in diffuse cutaneous SSc (DcSSc), being often complicated with pulmonary hypertension, plays significant role in PAH development [45].

1.8 Introduction and Biochemistry of Oxidative Stress (OS).

From long time it has been known that Oxidative stress plays a major role in the pathogenesis and vascular damage of SSc-PAH, which is elucidated by the abnormal redox state in SSc patients [52]. It is well documented that ROS mediates vascular damage in the vascular wall [53]. “Any species which is capable of independent existence and contains one or more unpaired electron in atomic or molecular orbitals” are termed as free radicals [54] and also called by the name Reactive Oxygen Species (ROS). Since free radicals possess atomic structure that has an unpaired electron in the outer orbital, gives them a special configuration of great instability. This provides it very un-stable state, making it short-lived and extremely reactive, with a tremendous capacity to interact nonspecifically with the diverse set of molecules of the cell structure such as carbohydrates, lipids, proteins and nucleic acids [55].Pro-oxidant denotes to any endobiotic or xenobiotic that induces oxidative stress

either by generation of ROS or by inhibiting antioxidant systems. It can include all reactive, free radical containing molecules in cells or tissues [56]. Pro-oxidants may be broadly classified into two categories as ***Exogenous*** and ***Endogenous***. Exogenous pro-oxidants include pro-oxidants derived from dietary ingredients, toxicants, drugs, pathogens, environmental pollutions and climate. Endogenous pro-oxidants include pro-oxidants derived from endogenous metabolites, drug metabolites, cellular metabolism, ion flux, anxiety, pathophysiology and ischemia [56].These pro-oxidants may be present in the circulatory fluid (CF) which can induce OS in the vascular wall leading to vascular morbidities such as SSc-PAH and (CF) can be used to study the OS or vascular damage related pathophysiology of the vascular diseases.

1.9 Role of Oxidative stress in Cellular and Molecular Vascular Damage of SSc-PAH

Oxidative stress (OS) has been recognized as an important triggering agent in inflammatory processes within the vascular wall, especially in the setting of systemic arterial disease [57]. OS may mediate through activation of mononuclear cells [58, 59] with infiltration of inflammatory cells in pulmonary perivascular spaces within and around plexiform lesions [60–62]. In the vasculature of HT patients, ROS are formed in high concentrations that cannot be balanced by the normal protective antioxidant mechanisms employed by the cells, resulting in oxidative stress [64]. As a result, $\bullet O_2^-$ reacts with nitric oxide (NO) to produce a dramatic concentration of the toxic peroxynitrite ($ONOO^-$) which stimulates a variety of negative effects on cellular function involving alteration in the functioning of kinases, protein synthesis, and redox-sensitive genes [65-67]. There exists a strong relationship between BP and some parameters related to OS [68]. It has been

shown that ROS production is elevated with the redox-dependent signaling amplified and antioxidant activity reduced in cultured vascular smooth muscle cells of arteries from hypertensive rats and humans [69, 70]. Reactive oxygen species (as depicted in Figure D) are known to play a significant role in intracellular signal transduction and trigger many growth-associated signaling pathways in vascular smooth muscle cells, comprising phosphorylation-mitogen activated protein arterial pressure (MAP) kinases (p38 and ERK5) and others [71-84]. ROS stimulate STATs, activate Akt by Ang II and activate tyrosine kinases and tyrosine phosphatases, and activate ras as well [71, 83]. These signaling events would lead to redox-sensitive growth in vascular smooth muscle cells, proliferation, hypertrophy, which eventually would confer to vascular wall thickening and remodeling (as depicted in Figure D) in hypertension [71-99]. The organization of membrane myofilaments poses a great impact on the integrity of vascular smooth muscle cell morphology and ROS can cause severe morphologic and structural alterations resulting in the vast damage of the cellular cytoskeleton. Thus ROS are involved in endothelial dysfunction, increased reactivity, and vascular remodeling which are the characteristic features of vascular damage in hypertension [104] related to SSc as well as in IPAH.

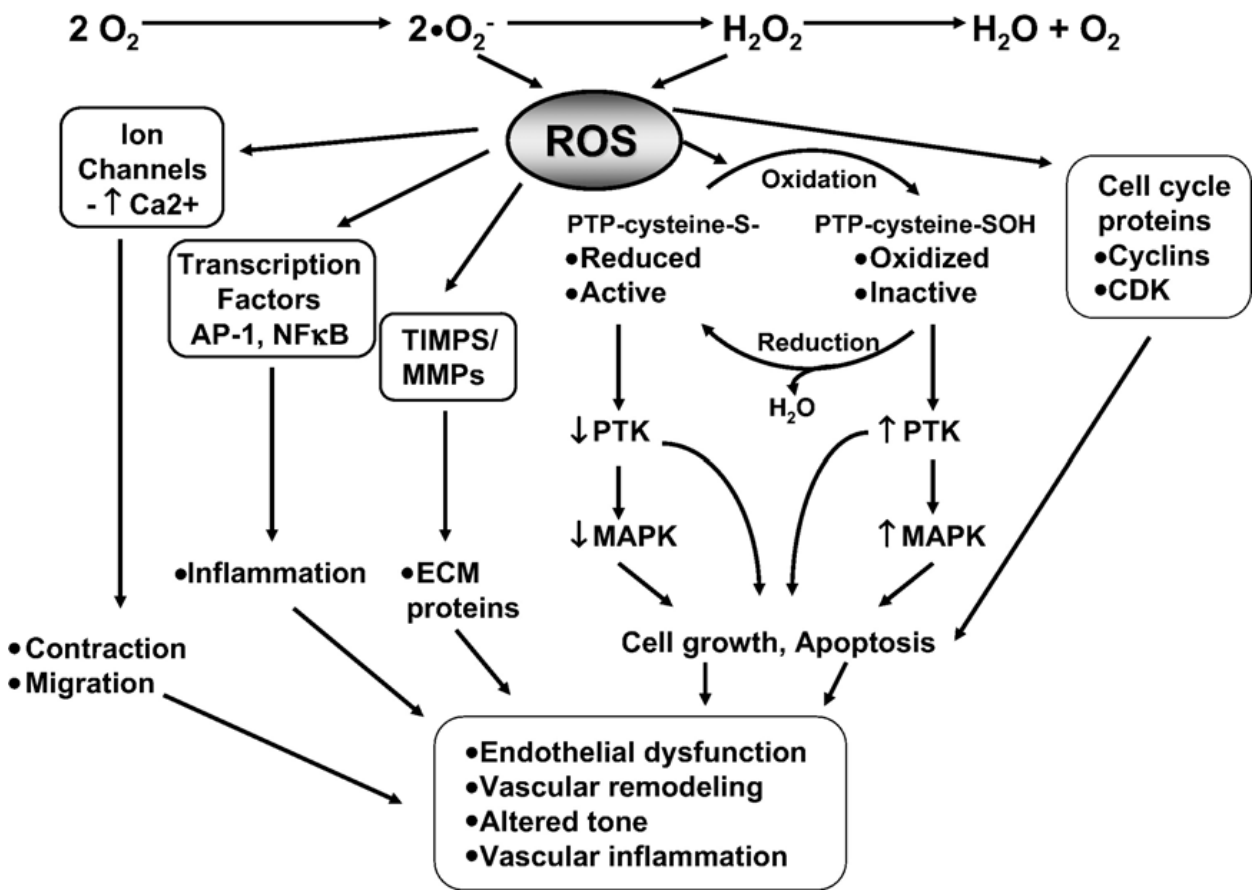

Figure D. Redox-dependent signaling pathways in vascular smooth muscle cells. Intracellular reactive oxygen species (ROS) modify the activity of protein tyrosine kinases (PTK), such as Src, Ras, JAK2, Pyk2, PI3K, and EGFR, as well as mitogen-activated protein kinases (MAPK), particularly p38MAPK, JNK and ERK5. These processes probably occur through oxidation/reduction of protein tyrosine phosphatases (PTP), which are susceptible to oxidation and inactivation by ROS. ROS also influence gene and protein expression by activating transcription factors, such as NF-nB, activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1). ROS stimulate ion channels, such as plasma membrane Ca2+ and K+ channels, leading to changes in cation concentration. Activation of these redox-sensitive pathways results in numerous cellular responses which, if uncontrolled, could contribute to hypertensive vascular damage.ECM, extracellular matrix; MMPs, matrix metalloproteinases; TIMP, tissue inhibitor of matrix metalloproteinase. Tamara M and R.M.Touyz .Redox signaling in hypertension. Cardiovascular Research 71 (2006) 247 – 258.

1.10 Role of Oxidative Stress in Vascular Remodeling in SSc-PAH

Vascular remodeling is an active process of structural change that relies on active interactions between local growth factors, vasoactive substances, and hemodynamic stimuli and is an active process that arises in retort to longstanding changes in hemodynamic conditions and leads to the pathophysiology of vascular diseases. It is well documented that OS plays significant role in Vascular Remodeling in SSc-PAH. During this process, the arterial system endures structural remodeling that involves hypertrophy of the arterial wall and increased wall-to lumen ratio and accompanying decreased arterial distensibility. The stage when the remodeling process develops maladaptive, it carries further vascular damage followed by impaired endothelial function, enhanced reactivity, and vascular inflammation. In the condition of hypertension, OS promotes VSMC proliferation and hypertrophy, collagen deposition, and alterations in activity of MMPs, which results in thickening of the vascular media and arterial remodeling [103]. It has reported that Superoxide anion and H_2O_2 excite several growth factor-like cellular responses, such as intracellular alkalinization, MAP kinase phosphorylation, and tyrosine kinase activation [71]. In hypertension, OS not only influences arterial structure, media thickening but also eventually influences & affects complete vessel redox state. So vascular wall thickening increases the distance required for diffusion of oxygen from the lumen. With this, there would be a reduced pO_2, which results in incomplete oxidation and increased concentrations of free radicals and abnormalities of the oxidant state. With this overall excess of OS state in hypertension, would further lead to vascular smooth muscle cell growth, endothelial dysfunction, and over-all vascular damage [104]. This would lead to progressive increase in pulmonary vascular resistance, pulmonary arterial pressure, and right ventricular (RV) pressure overload. Initially,

compensatory mechanisms in the right ventricle preserve the stroke volume and cardiac index, but as the limits are crossed, cardiac failure and death follow.

1.11 Involvement of NOX in SSc-PAH

The activation of vascular NAD (P) H Oxidase plays a pivotal role in vascular functional and structural changes for the development of hypertension [67]. The NOX1 (NADPH oxidase 1) and NOX2 oxidases are the major sources of

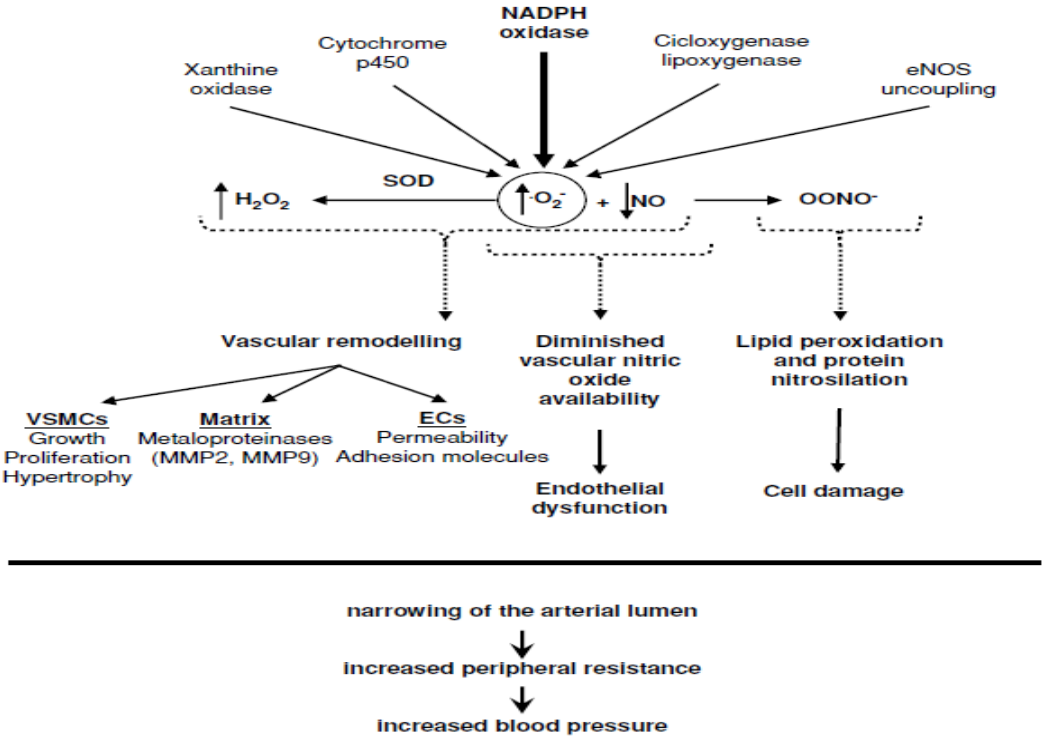

Figure E. NADPH oxidase(NOX) a major component involved in generation of ROS, leading to OS, Vascular Remodeling & Vascular Damage in SSc-PAH {Ana Fortuno et al. Oxidative stress and vascular remodeling. Exp Physiol 90.4 pp 457–462(2005)}.

ROS in the artery wall in hypertension as well as in SSc-PAH, and thus are formed to be important contributors [63] to the oxidative stress that would lead to vascular damage[105-108]even in the premature stages[109, 110] of vascular disease. The abnormal vasoconstriction in the vascular wall by hormones, such as endothelin-1, angiotensin II, or urotensin II is mediated by ROS [111] produced, majorly by NOX2.ROS derived from NOX isoforms, in particular NOX2 and NOX4, are evident to be involved in long-term responses of the pulmonary vasculature to hypoxia [112-115]. NOX2 is involved in hypoxia induced endothelial dysfunction in the intrapulmonary arteries [115].Increased level of NOX was documented in the pulmonary artery smooth muscle cells (PASMCs) in hypoxia dependent development of PAH in mice [113]. As illustrated in the above **Figure E**, NOX more specifically NOX2, by generating ROS, contribute to the narrowing of the arterial lumen and consequently to increased peripheral resistance and blood pressure, thus leading to Hypertension in SSc patients.

1.12 Involvement of ERK in Collagen Synthesis in SSc-PAH

There are many signaling pathways involved in the regulation & synthesis of Collagen. The MAPK/ERK pathway is one of the predominant one among them [116,117].In both the vasoconstriction and vascular smooth muscle cell growth, extracellular signal-regulated kinase (ERK) is involved, which is a member of the mitogen-activated protein kinase family [118]. From the reports of hypertension in animal models, it has been showed that ERK activity is raised in vascular smooth muscle cells, and inhibition of ERK activation reduces both vascular smooth muscle cell growth and vasoconstriction [118,119]. ERK1 and ERK2 are the extensively explored, even though other isozymes of ERK are present. Activation of ERK can happen through stimulation of either a G protein-coupled receptor or a growth factor

receptor, and this can be followed by activation of the Ras, Raf, mitogen-activated protein kinase pathway [118, 119]. There are evidences which reveals that, activation of ERK through G protein-coupled receptors can happen (as shown in Fig: F) through direct activation of the Ras, Raf, or mitogen-activated protein kinase pathway, or through transactivation of a growth factor receptor, such as EGFR. This might happen through activation of a matrix metalloprotease and succeeding cleavage of a membrane-bound ligand such as heparin-binding epidermal growth factor (EGF), leading to release of the ligand and activation of the receptor. In other way, activation of the G protein-coupled receptor could lead to tyrosine phosphorylation of the epidermal growth factor receptor [118]. However, there are few evidences suggesting that ERK activation occurs via ROS, but the mechanism needed to be elucidated [120].

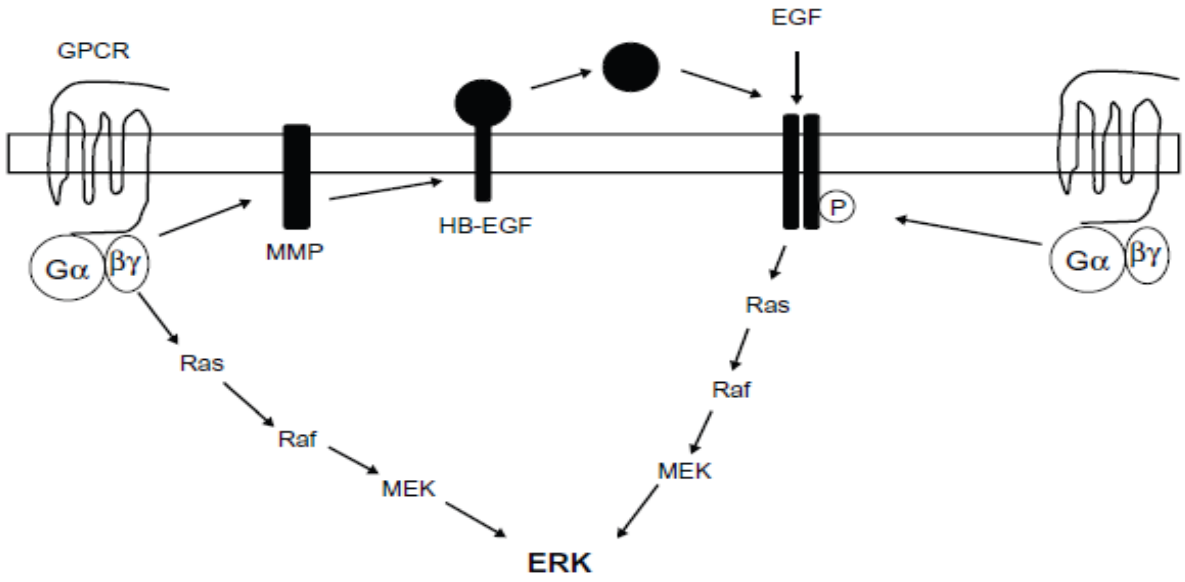

Figure F. Schematic diagram summarizing the potential mechanisms of extracellular signal-regulated kinase activation. *Richard E Roberts. The extracellular signal-regulated kinase (ERK) pathway: a potential therapeutic target in hypertension. Journal of Experimental Pharmacology Aug.2012.*

It has been shown that activation of ERK is linked with changes in gene transcription and cell proliferation [118]. Activated (as shown in Fig: F) ERK is known to be involved in Collagen synthesis and has been reported in various types of cells including VSMCs.

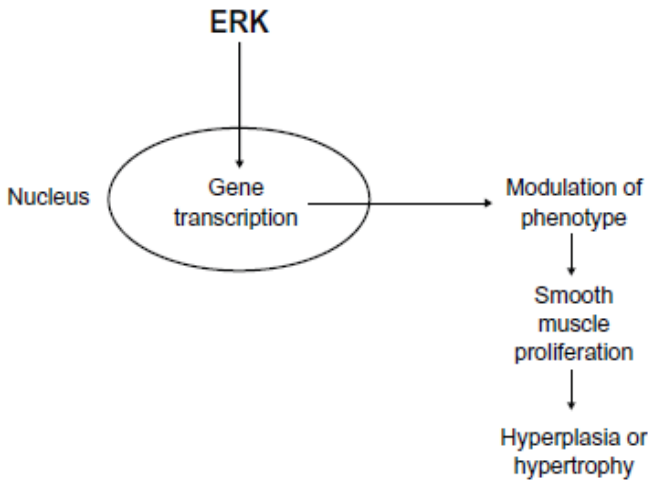

Figure G: Schematic diagram summarizing the effect of extracellular signal-regulated kinase on vascular smooth muscle cell growth and proliferation through effects on gene transcription. Richard E Roberts 2012.

Undeniably, changes of primary vascular smooth muscle cells from a contractile to a proliferating phenotype are associated with prolonged ERK activation [118] and the accompanying synthesis of collagen. This could progress the disease conditions such as IPAH, SSc-PAH and stenosis in which there is elevation in vascular smooth muscle cell growth, collagen synthesis and subsequent aberrant vascular remodeling (hypertrophy or hyperplasia).

CHAPTER 2. AIM OF THE WORK

CHAPTER 2. AIM OF THE WORK

An important note to assign SSc straight to vascular morbidity is because the vascular bed is a primary site of injury [32, 38, 44, 48, 121], and diffuse devascularization of multiple tissues is the major consequence. Hence, SSc is termed as Vascular Disease. Even, the course of the pathogenesis clearly shows the mode of vascular complications that line from the onset of the disease till late clinical manifestations. Raynaud phenomenon (RP) is observed in all most all SSc patients & is one of the earliest symptoms seen in SSc patient. When looked at cellular level, it is the clinical manifestation that involves abnormal functioning of cutaneous vessels that happens due to the thermal regulation of blood flow [122-124]. RP occurs before the onset of clinical signs of tissue fibrosis. The vascular abnormalities that begin & participate in this disease condition have prompted to better understand the disease course from the vascular point of view, which could help in finding out cellular & molecular hints that set the vascular damage during systemic sclerosis. Pulmonary involvement occurs in at least two thirds of systemic sclerosis patients and about 10-15% of them will develop severe lung disease during the course of their illness. Pulmonary disease has surpassed renal disease and is now the leading cause of death amongst patients with scleroderma [34, 44, 45]. It is estimated that 80% of patients with SSc have some evidence of pulmonary disease. The estimated mortality of pulmonary disease from all causes is said to be 33% [34, 35, 41]. Moreover, pulmonary involvement has a poorer prognosis [39]. Pulmonary arterial hypertension (PAH) is a grimly progressive life-threatening & a serious condition which prematurely takes a toll on many lives. Systemic sclerosis-related pulmonary arterial hypertension (SSc-PAH) is a major complication of both limited and diffuse systemic sclerosis that leads to substantial morbidity & mortality. SSc-PAH has emerged as a leading cause of death [38, 44]. These are some of the reasons that have prompted us take up this study. In vascular smooth muscle cells, it has been shown that there was an elevation in protein tyrosine kinase

activity and tyrosine phosphorylation in response to cooling and are linked to excessive alpha2-adrenergic response and shown that increased level ROS activate of Rho/Rho kinase pathway and up-regulates alpha2c-adrenergic receptors on the surface of vascular smooth muscle cells, thus determining an excessive vasoconstrictive response to cooling [122-124] and clearly showing that ROS[52] are involved in the early pathogenesis. Structural alterations of small and medium-sized arteries contribute to RP involving Smooth muscle cells & endothelial cells, thus leading to Vascular remodeling as result of deposition of extracellular matrix (ECM) and fibrosis. These above mentioned participations of SMCs prompted us to use this type of cells as an ideal cell model to study SSc-PAH. In hypertension, OS besides influencing arterial structure, media thickening, also eventually influences & affects complete vessel redox state [125,126]. Hence, vascular wall thickening would lead to increase in the distance required for diffusion of oxygen from the lumen thus resulting in reduced pO2, which leads to incomplete oxidation and increased concentrations of free radicals and abnormalities of the oxidant state [52]. This overall excess of OS state in hypertension, would further lead to vascular smooth muscle cell growth, endothelial dysfunction, and over-all vascular damage [125,126].Many circulating factors have been shown to be associated in the pathogenesis of Raynaud's phenomenon related to SSc, involving in platelet activation, impaired fibrinolysis, white blood cell activation, reduced red blood cell deformability and oxidative stress [122,127]. Due to OS, there occurs an unbalanced redox homeostasis, and circulatory factors may contain the endogenous derived factors which can exhibit the pathophysiology state that is caused by the OS. Thus the circulatory factors play very significant roles in the assessment of SSc-PAH, wherein they could be used to study the pathophysiology that has been exerted by OS. In such condition the blood as the circulatory fluid may have ROS or free radicals or pro-oxidant factors, activated growth factors, cytokines or molecules in it, which may direct in

activating the pulmonary arterial smooth muscle cells, endothelial cells or the complete vascular bed with the wrong signals formed by the abnormality caused by OS or other factors. From previous reports, it is well evident that NOX are the major contributors of ROS in the vascular wall. It has been shown that NOX2 subunit involved in generation of large amounts of ROS, and so inhibiting or deleting NOX2 or p47phox subunits significantly reduced vascular oxidative stress in several disease models [106, 129-136]. So there is a strong rationale for therapeutically targeting NOX2 oxidase in the arterial wall for the treatment of vascular disease such as SSc-PAH, so as to combat the oxidative stress and prevent the progression of vascular disease such as SSc-PAH.It is has been suggested that the high mortality in SSc-PAH may be due to excessive collagen content in the heart and the pulmonary arteries [137]. Vascular collagen content has impact on hemodynamics of SSc-PAH, which is due to the overproduction of collagen throughout the body. However, it has been reported that excessive PA collagen accumulation is linked to increased PA stiffening and has been shown that excessive PA(Pulmonary Artery)collagen accumulation elevates (pulmonary vascular impedance) PVZ or right ventricular afterload [138-142]. So, whether sera of SSc-PAH stimulate collagen synthesis has become one of the objectives of this study. From the reports it is known that MAPK/ERK is the predominant pathway involved in the regulation & synthesis of Collagen and there are evidences reporting that ERK activation occurs via ROS [120] but the mechanism is not clearly understood. ERK signaling contributing to the overexpression of pro-fibrotic proteins in scleroderma fibroblasts as well as VSMCs is unclear. Hence, targeting ROS and as well as collagen synthesis by inhibiting ERK or ROS-mediated ERK could potentially be an important pharmacological treatment of hypertension in SSc. By gathering the above background, it can be explained collectively, that OS is involved in the Vascular Damage of SSc-PAH. This leads to the state of unbalanced redox homeostasis and generate large amount of ROS by stimulating growth factors & cytokines. These factors

may be present in the circulatory fluid and can be detected and their effect can be analyzed in in-vitro. This ligand stimulated change in the cellular redox state could lead to vascular remodeling characterized by VSMC hypertrophy & hyperplasia and ECM deposition leading to subsequent Obliterative Vasculopathy. Hence, it can be hypothesized that in SSc patients associated with Pulmonary Arterial Hypertension (PAH), circulating factors may drive an aberrant vascular remodeling by exerting pro-oxidant & Pro-fibrotic effects on Pulmonary Arterial Smooth Muscle Cells (PASMCs) and activate collagen I synthesis.

CHAPTER 3. MATERIALS AND METHODS

CHAPTER 3. MATERIALS AND METHODS

3.1. Materials

Smooth Cell medium was purchased from ScienCell Research Labs, Fetal Bovine Serum (FBS) purchased from Invitrogen, rat tail Collagen I coating agent, ERK Inhibitor PD98059, DMEM were purchased from Sigma-Aldrich, GFP-based Lentiviral Particle (harboring the COL1A1 promoter having COL1A1-LV-tGFP and EF1α-LV-FP602) was initially purchased from Innoprot-Innovative Technologies In Biological Systems, S.L. But the production of the same Lentiviral particles was done by purchasing second generation packaging systems from Addgene,Inc. 2‟,7‟-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Molecular Probe) was purchased from Eugene, OR.NOX NOX2ds-tat was purchased from Anaspec (Fremont, CA, USA) .

3.2 Cell culture and treatments.

Human Pulmonary Artery Smooth Muscle Cells (HPASMCs) purchased from Innoprot-Innovative Technologies In Biological Systems, S.L. The cell line was supplemented with specific medium(Smooth Cell medium, ScienCell). The cell culture was done by using rat tail Collagen I as a matrix or coating agent to promote adherence and attachment to provide proper cell growth. When cultured HPASMCs reach confluent stage, they were split in to 1:2 ratio and the cells were used for experiments with passage numbers below six. In routine maintenance of HPASMCs, during splitting of cells, the neutralization is done to stop trypsinization by using Neutralization Solution and Fetal Bovine Serum (FBS) in the ratio of 1:1.Experiments were done by plating required number of HPASMCs in 96-well black plates and white plates (BD Falcon, Franklin Lakes, NJ.

For measuring intra cellular ROS 10 μM of H_2-DCFDA (Figures 1A & 1B) was used prior to stimulation by sera and the intra cellular ROS was measured kinetically for 4hrs.

In the experiments for measuring the collagen promoter activity(Figures 2A,2B,4,6), the cells were transduced with lentiviral particles obtained from the COL1A1-LV-tGFP and EF1α-LV-FP602 lentivectors, and then cultured in basal medium for stimulating the cells with sera and COL1A1 promoter activity were kinetically measured for 10 hours.

In the experiments (Figures 3, 4) for studying NOX involvement, HPASMCs were incubated for 1 hour with 5 μM NADPH oxidase specific inhibitor NOX2ds-tat (NOX) before treatment with SSc sera.

In the experiments (Figures 5, 6) for studying ERK involvement, HPASMCs were incubated for 1 hour with 15 μM of PD98059, a specific inhibitor of ERK before treatment with SSc sera.

3.3 Study Subjects.

Study subjects were enrolled by signing the informed consent and the collected data of clinical, serologic, and diagnostic criteria were according to the protocol approved by the Johns Hopkins Scleroderma Center Baltimore, Johns Hopkins University's Institutional Review Board (IRB). Through posted flyers, Healthy donors (HD) were recruited and enrolled after going through such a screening questionnaire that aimed at excluding the presence of any underlying vascular or autoimmune disease. Each SSc patient met the American College of Rheumatology criteria or had 3 of 5 features of the CREST (Calcinosis, Raynaud's syndrome; Esophageal dysmotility; Sclerodactyly; Telangiectasia) syndrome [143]. Each Patient's demographic profile, SSc

subtype, autoantibody status, Medsger severity scores, pulmonary function tests, echocardiography, and right heart catheterization parameters, modified Rodnan skin scores(mRSS) were assessed. Demographic data comprising sex, race, smoking status (never, past current) were also evaluated. Calculation of SSc disease duration was done at the time of serum sampling, by counting the years from the onset of RP or from the first non-RP symptom. RP, heart and lung severity scores were evaluated as previously defined by Medsger et al [144].

3.4 Measurements of Intracellular ROS

Intracellular ROS levels were measured by using the ROS molecular probe 2‟,7‟-dichlorodihydrofluorescein diacetate (H_2-DCFDA). Esterases cleave the acetate groups on H_2-DCFDA within the cell, consequently trapping the reduced form of the probe (H_2DCF). Intracellular ROS oxidize H_2DCF, yielding the fluorescent product, DCF. When HPASMCs reach sub-confluent were loaded with 10 μM of H_2-DCFDA. Then washed with PBS1X buffer followed by treatment with 5% (V/V) of sera from scleroderma (SSc) patients with pulmonary arterial hypertension (PAH), without PAH(No PAH) and healthy donors (HD)respectively in basal medium. Intracellular ROS levels were kinetically measured using GENios plus microplate reader (Tecan, Männedorf, CH) with excitation at 485 & emission at 535 respectively in a 4 hour time-course experiment (Figure 1A) and values at 2 hours (steady state) used for comparison (Figure 1B). Fluorescence measurements were corrected for background fluorescence and protein concentration.

3.5 GFP-based Lentiviral Particle (harboring the COL1A1 promoter) Production.

Lentiviral vectors (LV) are very efficient (can exceed 90%) at transferring viral DNA into host cells and integrate stably into the host-cell genome of dividing and non-dividing cells and can provide long-term expression of the vectored transgene in target cells and thus provide great potential for gene therapeutic applications [145-148]. Because of these advantages we employed lentiviral vectors as shown below (Fig .H & I) in our study. The 2nd generation system plasmids are less bulky to use and highly efficient and have ease of use over third-generation system led us to use these in our study.

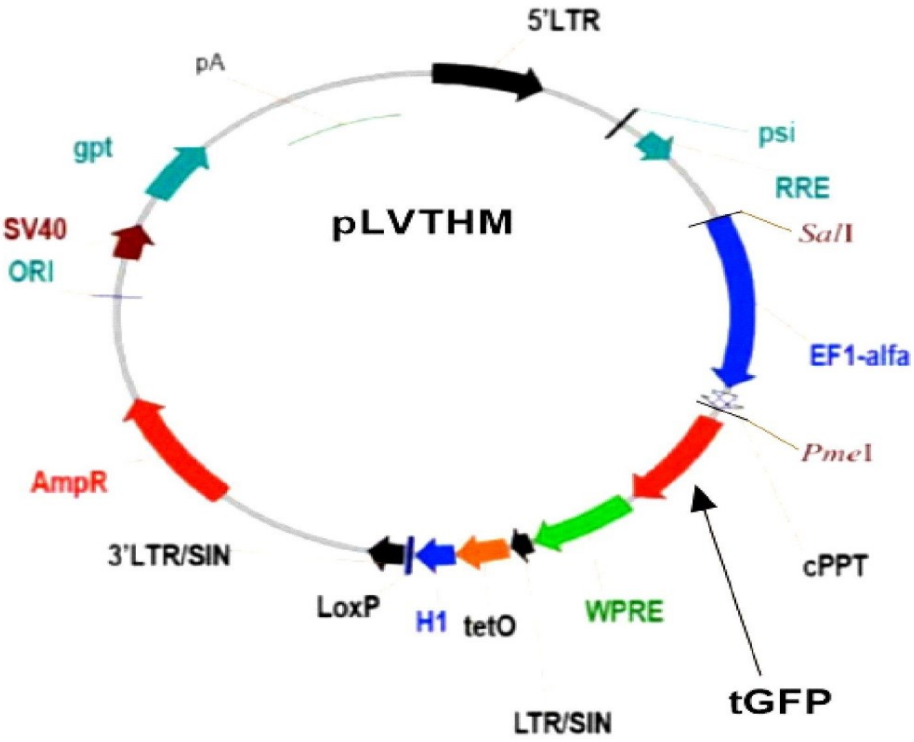

Figure H : The vector map of LVTHM transfer vector.

Description of Each Component of the Vector Map:

5'LTR 5' Long terminal repeat, contains cis regulatory regions which recruit proteins that promote transcription of full length lentivirus.Sequences here facilitate proviral integration into the host genome; an intact 5'LTR indicates that this is a 2nd generation transfer plasmid
Psi Sequence that facilitates packaging of viral RNA
RRE Rev response element: sequence to which the Rev protein binds to facilitate nuclear export of viral RNA
EF1α Promoter for elongation factor 1α, used to the expression of a marker gene such as GFP
cPPT Central poly purine tract allows nuclear import of lentivirus in host cells
GFP Green fluorescent protein used to mark successfully infected cells
WPRE The woodchuck hepatitis virus Posttranscriptonal response element augments shRNA expression
TetO Binding site for the tet repressor protein or a variant thereof which can be used to turn on or off shRNA expression
H1 Histone H1 promoter used to drive shRNA expression
LoxP Site which is recombined by the Cre recombinase in order to conditionally remove the lentiviral provirus from the host genome after successful infection and incorporation
3'LTR/SIN 3'LTR with a large deletion in the U3 region, makes the virus replication incompetent
Amp R Ampicillin resistance gene for clonal selection of transfer vector in bacteria

C) Map of Human α(I) Procollagen Gene Promoter-Lentiviral vector-tGFP

LentiFP602: These lentiviral particles express FP602 red fluorescent protein under the EF1a promoter. This promoter is not tissue specific and highly expressed in all cell types.

LentipCOL1A1tGFP*:* These lentiviral particles express tGFP green fluorescent protein under the collagen type1 promoter (-804+42). This promoter is tissue specific and expressed in smooth muscle cell types.

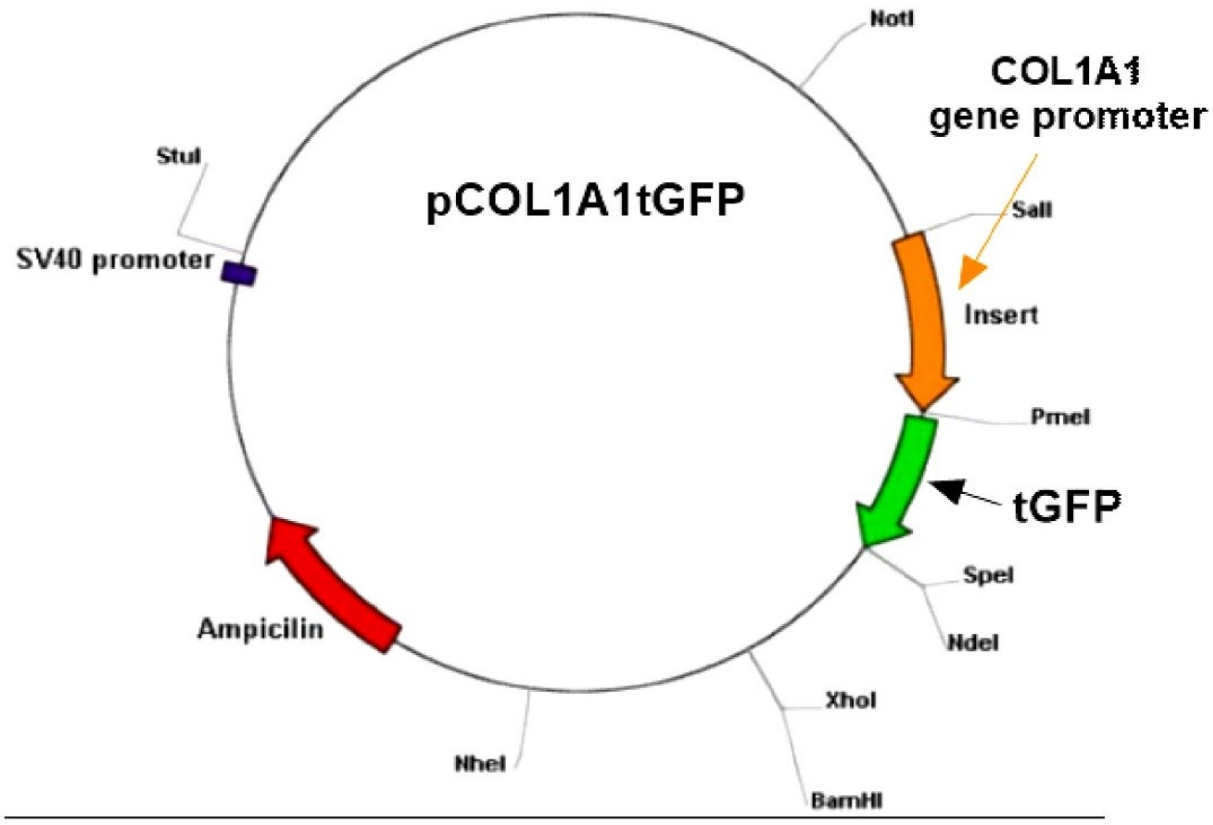

Figure I : Map of Human α(I) Procollagen Gene Promoter-Lentiviral vector-tGFP.

By using the above shown 2nd generation plasmids containing COL1A1-LV-tGFP and EF1α-LV-FP602 lentivectors, the co-transfection into human embryonic kidney 293T cells was done , and then the medium was collected 3 times at 24 hour intervals beginning 24 hours after changing the medium post-transfection. Thus lentiviral particle production was done by using 293T cells [149, 150] as described. Later purification process was performed. In this step, every sample was filtered immediately through a 0.22 mm cellulose acetate filter and stored at 40 C. The virus pellet was collected by carrying out 2hrs of ultracentrifugation at 19.4K rpm and at room temperature. Since lentiviral particles contain GFP as a reporter system, transfection was confirmed by using Fluorescent microscopy and then titration of lentiviral vectors was performed.

3.6 GFP-based Lentivirus (harboring the COL1A1 promoter) Transduction in HPASMCs.

The lentivirus transduction was done in HPASMCs by following the company protocol (Innoprot- Innovative Technologies In Biological Systems, S.L.). The determined number of HPASMCs were seeded in Smooth Muscle Cell complete medium and incubated overnight. When 50%-75% confluent,the lentiviral stock was thawed at room temperature. The culture medium was then removed and appropriate amount of this virus stock was loaded to cells and the required volume of the medium was bought by using OptiMEM. The cells were then placed in the CO_2 incubator maintained at 5% CO_2 and 37^{o}C and the plate was carefully rotated for every 15 min for 1 hour. Later Smooth Muscle Cell complete medium was added to bring to the required volume. At around 48-72h after transduction, the Green Florescent protein production or expression was checked under fluorescence microscope.

3.7 Measurement of COL1A1 Promoter Activity

HPASMCs transduced with lentiviral vector were treated with of 5% (V/V) of sera from PAH, no PAH and HD subjects in basal medium. The kinetic measurement COL1A1 promoter activation was performed for 10 hours (Figure2A) and any deviations occurred during this course of time was studied. The steady state in the promoter activation was attained at 8hrs and the values at this time point were used for comparison (Figure 2A, 2B, 4, 6) and data were normalized for transduction efficiency by reporting the ratio of COL1A1-LV-tGFP to EF1α-LV-FP602 Relative Fluorescence Units (RFU).

3.8 Study of NOX Involvement.

HPASMCs were pretreated with 5 μM NADPH oxidase specific inhibitor (NOX2ds-tat, formerly gp91ds-tat) [151] for 1hr before stimulating with sera from PAH, no PAH and HD subjects and Intracellular ROS was then measured(as shown in Fig.3) as previously described to observe the NOX inhibitor effect.HPASMCs transduced with lentiviral vector (obtained from the COL1A1-LV-tGFP and EF1α-LV-FP602) were incubated for 1 hour with 5μM of NOX before treatment with SSc sera from the three subjects (PAH, no PAH and HD). Then collagen type-I (COL1A1) promoter activity was measured (as shown in Fig.4) as previously described to determine whether NOX inhibitor has effect on collagen promoter activity.

3.9 Study of ERK Involvement

For studying the effect of ERK inhibitor PD98059 on Intracellular ROS, HPASMCs were pretreated with 15μM of ERK inhibitor PD98059 for 1hr before stimulating with sera from PAH, no PAH and HD subjects and

Intracellular ROS was then measured as described earlier.
In another set of experiments for studying the effect of ERK inhibitor PD98059 on COL1A1 promoter activity, HPASMCs were pre-incubated with 15 μM of ERK inhibitor PD98059 for 1hr before stimulating with sera from PAH, no PAH and HD subjects and collagen I synthesis was assessed as described earlier to observe the effect of this inhibitor.

3.10 Statistical Analysis

Healthy donors were matched for gender, race and smoking status. Horizontal lines indicate the median with interquartile range (Fig1A,2A). Kruskall–Wallis one-way analyses of variance followed by post-hoc Dunn's test for multiple comparisons were used to detect differences among studied groups in (Fig 1B, 2B). For studying subjects' characteristics, P values were determined by Fisher's exact test or the Wilcoxon rank-sum test, as appropriate. Wilcoxon matched-pairs signed rank test was used to determine meaningful differences between pre- and post-NOX & ERK inhibitor treatment pairs in Figures 3, 4, 5 & 6. All statistical analysis were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA) and p-values <0.05 were considered to be statistically significant.

CHAPTER 4.RESULTS

CHAPTER 4.RESULTS

4.1 Demographic Data and Clinical Characteristics of Subjects:

Mainly the middle age, white women were the SSc patients enrolled in this study. SSc subjects were concluded as "No PAH" (n = 17) if their right ventricular systolic pressure (RVSP) estimated by echocardiogram was ≤35 mm Hg, and they were defined as "PAH" (n = 19) if the RVSP was >35 mm Hg. They underwent right heart catheterization (RHC) showing a mean pulmonary artery pressure (mPAP) ≥ 25 mm Hg and pulmonary capillary wedge pressure (PCWP) ≤ 15 mm Hg. For this study, Healthy Donors [HD] (n = 14) were selected through such a screening questionnaire, so as to rule out any underlying autoimmune or vascular disease in the donors. In this study, patients with PAH were slightly older than No-PAH (64.0 ± 9.4 vs 53.3 ± 11.6; p = 0.009) and had longer disease duration (18.5 ± 9.5 vs 10.5 ± 7.3 years; p = 0.01). These observations matched with most of the reports saying that PAH occurs as late age manifestation in SSc [152-156]. It was also registered that in this study, there was slight elevation in modified Rodnan skin score(mRSS) in SSc-PAH patients as compared to No-PAH (7.3 ± 10.3 vs 5.5 ± 6.1;p=0.567) pin pointing there was no significant difference in the values of mRSS between the comparable subjects. RP severity scores (1.6 ± 0.8 2.0 ± 1.0; p= 0.194) and Heart severity scores (0.2 ± 0.7 1.2 ± 1.7; p=0.062) were little bit higher in SSc-PAH vs No-PAH, but not reaching to the significant difference between the subjects. But as anticipated, SSc-PAH subjects revealed relatively higher lung severity scores (3.1 ± 1.3 vs 1.1 ± 1.3; p < 0.001) against No-PAH subjects. Cardiac hemodynamic measurements by Right heart Catheterization showed that mean pulmonary arterial pressure (mPAP) values (35.2 ± 8.1 mm Hg) and pulmonary capillary wedge pressure (PCWP) values (11.5 ± 4.0 mm Hg) were remained in the range as expected to be in SSc-PAH subjects as defined by mPAP should be ≥25 mm Hg, as well as PCWP should be ≤ 15 mm

Hg to diagnostically characterize the individuals having PAH according to the reports [17,157]. These diagnostic measurements clearly provided the proof that severe abnormalities were present in the structural & functional mode of pulmonary vascular compartment. It was observed that there was significantly lower diffusion capacity of lung for carbon monoxide (DLCO) (48.7 ± 16.8 vs 78.2 ± 23.0; $p < 0.001$) with comparable forced vital capacity (FVC) with decreased values against No-PAH subjects, which provided the confirmed clues that there exists an underlying pulmonary vascular disease. Pulmonary function tests (PFT) revealed that values determining restrictive lung disease (RLD) remain equal in both the subjects. In this study, it was observed that SSc-PAH patients exhibited significantly higher estimated right ventricular systolic pressure (ERVSP) values (65.2 ± 19.9 vs 24.0 ± 6.3; p= <0.001) against those of Non-PAH subjects. This elevated value of ERVSP further clarified the devastating damage of pulmonary vascular function of this ailment and also the severity of the underlying pulmonary vascular disease. ERVSP values >30–45mmHg are often considered abnormal even though there are no consensus about the elevated levels of healthy individuals but ERVSP values generally does not increase in normal individuals. Studies have been reported that SSc patients having ERVSP >35mmHg showed elevated pulmonary pressures [158,159], thus proving the underlying impairment in pulmonary vascular compartment. Another study also confirmed that combination of eRVSP on TTE and PFT parameters provided in identifying of up to 97–100% of SSc patients with RHC confirmed PAH [160].The antibody status in this study revealed that SSc-PAH subjects showed not though significant but slight higher level of Anti centromere antibodies {(ACA) 10 (53) vs 4 (24); P= 0.07} which have been put very relevant to the underlying cause of PAH in SSc by the earlier reports. Demographic Studies have shown ACA is prevalent in older, female Caucasians having SSc, so the same case in this study as well. It has been reported that the presence of ACAs linked with higher risk of development of

PAH [161,162] in SSc patients particularly the lSSc patients [161]. Anti-Scl-70 (anti-topoisomerase I) antibodies in this study were found lower in SSc-PAH against the No-PAH subjects as this study includes SSc-PAH subjects mostly from lcSSc subjects and Anti-Scl-70 are mostly found in patients with dcSSc having pulmonary involvement, more specifically linked to pulmonary fibrosis & interstitial lung disease [161,163,164]. The medications used by this study's subjects were vasodilators (i.e. endothelin receptor antagonists and phosphodiesterase 5 inhibitors) which were significantly higher in SSc-PAH subjects.

Variables	**No PAH (N = 17)**	**PAH (N = 19)**	**HD (N = 14)**	**p value•**
Age at serum sampling (years)*	53.3 ± 11.6	64.0 ± 9.4	54.1 ± 10.4	0.009
Female	15 (88)	16 (84)	15 (85)	0.727
Race				
White	14 (82)	16 (84)	12 (80)	0.881
Black	3 (18)	3 (16)	3 (20)	
Smoking status				
Never	9 (53)	10 (53)	8 (53)	0.280
Past	6 (35)	9 (47)	5 (33)	
Current	2 (12)	0	2 (13)	
SSc types				
Limited	11 (65)	16 (84)		0.177
Diffuse	6 (35)	3 (16)		
mRSS* (range 0–51)	5.5 ± 6.1	7.3 ± 10.3		0.567
SSc duration (RP onset)*, years	14.0 ± 12.6	21.7 ± 9.4		0.008

SSc duration (1st non-RP symptom)*, years	10.5 ± 7.3	18.5 ± 9.5	0.010
RP severity score* (range 0–4)	1.6 ± 0.8	2.0 ± 1.0	0.194
Heart severity score* (range 0–4)	0.2 ± 0.7	1.2 ± 1.7	0.062
Lung severity score* (range 0–4)	1.1 ± 1.3	3.1 ± 1.3	<0.001
Hemodynamics (RHC)			
mPAP* (mm Hg)	NA	35.2 ± 8.1	NA
PCWP* (mm Hg)	NA	11.5 ± 4.0	NA
FVC* (% predicted)	81.9 ± 22.9	73.1 ± 9.9	0.149
DLCO* (% predicted)	78.2 ± 23.0	48.7 ± 16.8	<0.001
RLD†	6 (35)	6 (32)	0.813
eRVSP*	24.0 ± 6.3	65.2 ± 19.9	<0.001
Autoantibody status			
ACA	4 (24)	10 (53)	0.07
Anti-Scl-70	7 (41)	1 (5)	0.01
Anti-RNA-polymerase 3	2 (12)	0	0.124
Medication use (current)			
Immunosuppressants‡	5 (29)	5 (26)	0.836
Calcium channel blocker	10 (59)	7 (37)	0.187
Endothelin receptor antagonist	1 (6)	6 (32)	0.052
Phosphodiesterase 5 inhibitor	4 (24)	11 (58)	0.037
Prostanoid	0	0	NA
Statin	6 (35)	5 (26)	0.559
Aspirin	5 (29)	5 (26)	0.836

Table A. Statistical Data and Clinical Characteristics of Subjects having SSc-PAH, SSc-No PAH & Healthy Donors. All values are given as number (%) unless otherwise specified. *Mean ± SD. †The presence of RLD was defined by a FVC < 70% of predicted. ‡Use of immunosuppressants include cyclophosphamide, mycophenolate, methotrexate, hydroxycholorquine or prednisone. §P values were determined by Fisher's exact test or the Wilcoxon rank-sum test, as appropriate.

4.2 Exposure of subjects 'sera to Human Pulmonary Arterial Smooth Muscle Cells (HPASMCs) and Kinetic Measurement of Intracellular ROS

The figure below (Fig1A) depicts the Kinetic Measurement of Intracellular ROS in HPASMCs. All the three subjects' sera were used in this experiment to observe whether they could induce generation of ROS. For this, the HPASMCs were exposed to sera {5% (V/V)} and kinetic measurement of intracellular ROS was followed for the duration of 4hrs.Intracellular ROS generation was studied in HPASMCs in response to the exposure of sera using 2‟,7‟-dichlorodihydrofluorescein diacetate (H_2-DCFDA) and this probe enters the cells and get oxidized in the presence of ROS, generating the fluorescent compound, DCF.This result showed that SSc-PAH subjects' sera stimulated generation of intracellular ROS with higher level as compared to No-PAH subjects and no response was exhibited by Healthy Donors (HD) sera. As anticipated by a ligand-stimulated ROS activation, a rapid and sustained raise in the intracellular ROS levels were observed starting from the cells exposure to the sera (Time =0). The sera-induced raise of ROS was spiked at around 30 minutes, thereafter a gradual and slow decrement was visible, which is consistent with a redox-regulated signaling event. These dramatic differences in the intracellular ROS levels were noted down and they acquired the steady state from the time point of 2hrs and followed the steady state till the end time point of 4hrs of this experiment as shown in Fig 1A.Previous reports demonstrated that serum factors in-vitro induced ROS production in vascular cells [166]. Following the same response, this experiment revealed that sera of SSc of both the subjects (SSc-PAH and No-PAH) have factors that could

stimulate and induce intracellular ROS generation in vascular cells such as HPASMCs against the lack of response to (HD) Healthy Donors' sera. This result provided the clue that some factors might be present in the sera of SSc subjects which might be pro-oxidant factors but earlier reports showed that platelet activation, impaired fibrinolysis, white blood cell activation, reduced red blood cell deformability and oxidative stress [122,127] are involved and all of these circulatory factors have been implicated in the pathogenesis of Raynaud's phenomenon and so in the development of SSc disease phenomena. Since there was intracellular ROS generation in our context of this experiment, when HPASMCs were exposed to SSc sera, driven the clue that pro-oxidant factors (OS) might be involved in the induction and generation of intracellular ROS in HPASMCs. In order to draw the comparison between the subjects at this steady state (2hr Time Point), statistical analysis at this time point was done as shown in the Figure.1B to confirm that indeed pro-oxidant factors (OS) are involved in SSc-PAH and No-PAH subjects. Sera of SSc-PAH individuals showed that the generation of intracellular ROS in HPASMCs was significantly higher against the No-PAH and Healthy Donors (HD) with median (interquartile range) of 213 (158) compared to subjects without PAH [141 (48); $p = 0.027$] and HD[130 (52); $p = 0.002$]. But when it was compared between No-PAH and Healthy Donors, it was revealed that the induction of intracellular ROS by sera of No-PAH is increased against the sera of healthy donors but was not to significant enough to draw the comparison. This result showed that SSc-PAH sera had pro-oxidant effect on HPASMCs by inducing the generation of intracellular ROS.

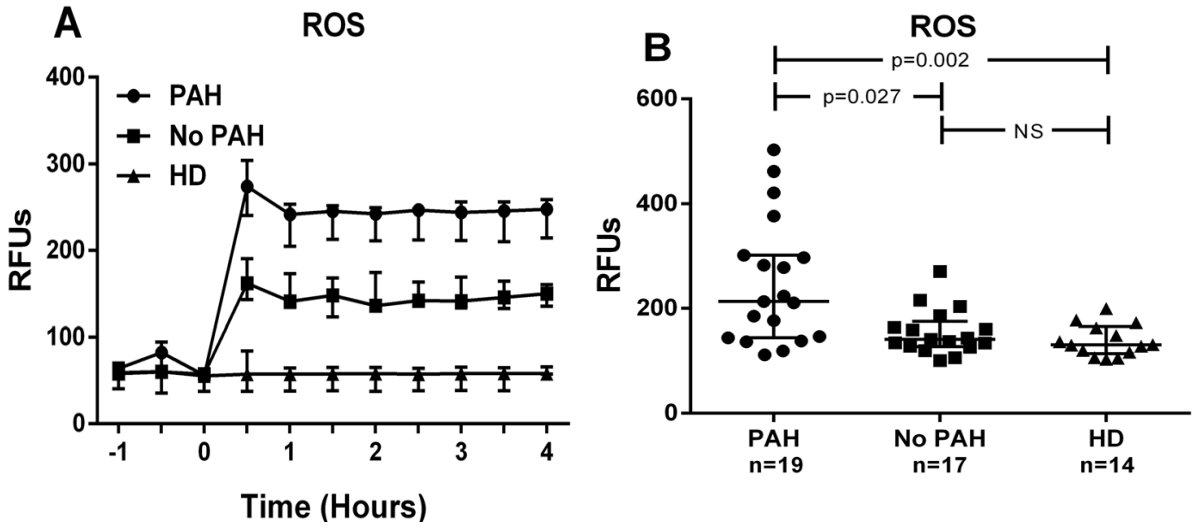

Figure 1A: Kinetic Measurement of Intracellular ROS in HPASMCs exposed to Sera of SSc-PAH,SSc-No PAH and Healthy Donors to examine the effects of SSc sera on human pulmonary artery smooth muscle cells (HPASMCs) intracellular ROS production. Here,sub-confluent HPASMCs were loaded with 10 μM of H_2-DCFDA before stimulation, and then cultured in basal medium containing 5% (V/V) of sera of the above mentioned subjects. Variations in intracellular ROS levels were kinetically determined in a 4 hour time-course experiment.

Figure 1B: Measurement of Intracellular ROS in HPASMCs exposed to Sera of SSc-PAH, SSc-No PAH and Healthy Donors taken at 2hr time point of steady state to look for the comparison between the subjects. Healthy donors were matched for gender, race and smoking status. Horizontal lines indicate the median with interquartile range (Fig 1A). Fluorescence data were normalized for protein content and expressed as Relative Fluorescence Units (RFU). Kruskall–Wallis one-way analysis of variance followed by post-hoc Dunn's test for multiple comparisons was used to detect differences among studied groups (Figure 1B). P values =0.027 compared to No PAH and P values =0.002 when compared to Healthy Donors thus illustrating the significant effect of sera in inducing intracellular ROS in HPASMCs.

4.3 Exposure of subjects 'sera to Human Pulmonary Arterial Smooth Muscle Cells (HPASMCs) and Kinetic Measurement of COL1A1 Promoter Activity

Type I collagen overproduction as ECM component is the hallmark of fibrosis in SSc. It is the most abundant collagen present in SSc patients compared to other types, with the presence of certain nucleotide repeats linked with higher expression of COL1A1 and COLA1A2 [165]. There are reports demonstrating that expression of the COL1A1 gene is primarily regulated at the transcriptional level and that its highest promoter activity in both normal and SSc fibroblasts resides in the proximal promoter region [168, 169]. And there is a report that showed that SSc fibroblasts produce ROS constitutively and this elevated ROS levels could be involved in the increased collagen expression in these cells [107]. VSMC is the main cell type most responsible for the vascular deposition of ECM proteins in hypertension [170-172] and this lead us the idea that whether ROS or pro-oxidant effect of sera induce collagen synthesis in HPASMCs. With this back ground, we speculated to see whether serum factors are involved in the COL1A1 promoter activity. For this, the GFP based lentivirus transduction in HPASMCs was done and confirmed. Then sera {5% (V/V)} of all three subjects were exposed to HPASMCs and the kinetic measurement for 10 hrs was endured to closely observe as when the sera trigger the promoter activity as shown in Fig.2A. The outcome of this experiment showed that sera of SSc triggered a progressive time-related increase of the COL1A1 promoter activity with values at 8 hours remained steady state. By looking at the kinetic measurement lines of the three different subjects it was noticed that sera of SSc-PAH had more increased influence in activating promoter activity as compared to No-PAH. According to the kinetic measurement of COL1A1 promoter activity induced by sera of subjects

under study in HPASMCs, at around 8hrs, the COL1A1 promoter activity remained stable. So at this time point the comparative statistical analysis was done to observe the significant level of COL1A1 promoter activity by all the three subject groups. Here, sera from SSc-PAH showed the significant level of increase in inducing the promoter activity in HPASMCs against the No-PAH subjects' sera with values of PAH [2.375 (1.597)] compared to no-PAH [1.825 (0.612); p = 0.028] and HD [1.844 (0.265); p = 0.007] sera. In case of No-PAH comparison against Healthy donors, it came to known that sera of No-PAH sera had increased level of COL1A1 promoter activity versus Healthy donors but not driving to the statistically significant values as shown in Fig.2B.The overall conclusion drawn from this experiment, revealed that sera from SSc-PAH subject group significantly increased the COL1A1 promoter activity and hence the Collagen synthesis in HPASMCs. This experiment proved that sera of SSc-PAH had profibrotic effect on HPASMCs.

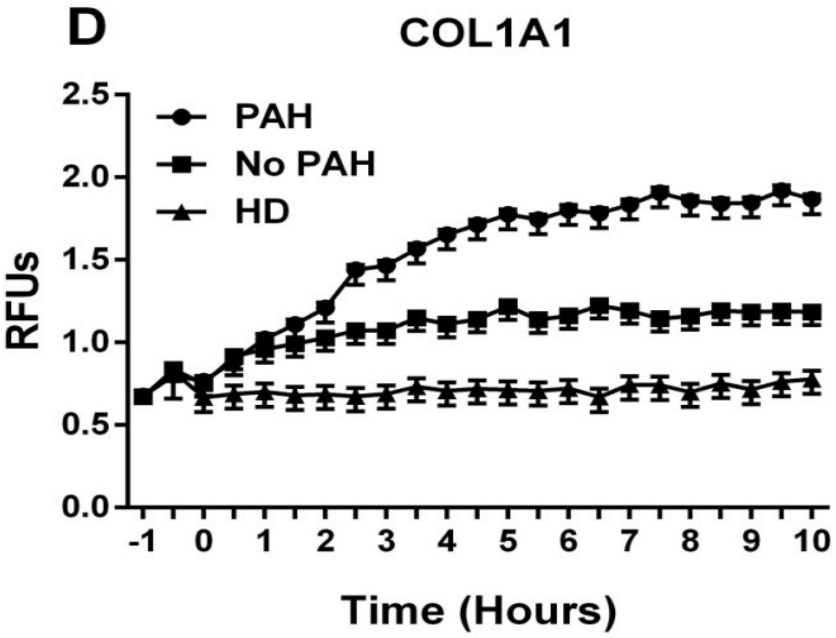

Figure 2A: Kinetic Measurement COL1A1 Promoter Activity in PASMCs transduced with COL1A1 promoter containing lentivirus and examination of the effects of SSc sera on HPASMCs collagen (COL1A1) promoter activation. Here, the sub-confluent HPASMCs were transduced with lentiviral particles obtained from the COL1A1-LV-tGFP and EF1α-LV-FP602 lentivectors, and then cultured in basal medium containing 5% (V/V) of sera from PAH, no PAH and HD subjects. Variations of COL1A1 promoter activation were kinetically followed for 10 hours. Horizontal lines indicate the median with interquartile range

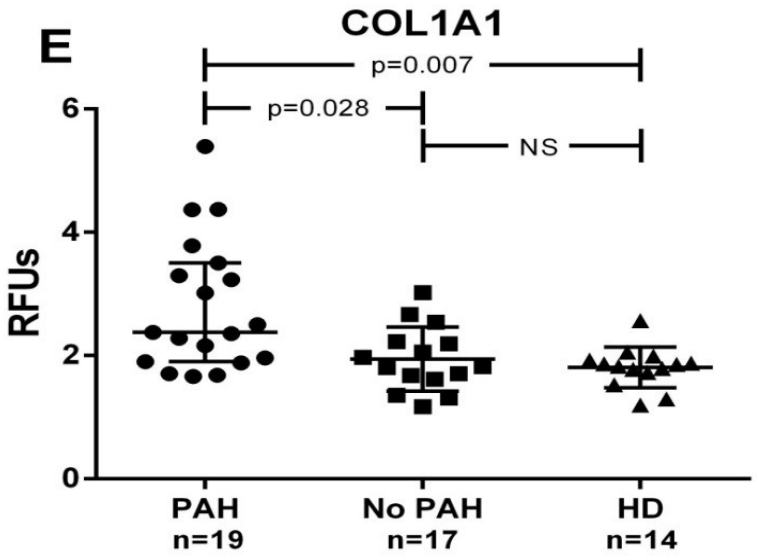

Figure 2B: Values taken at 8hr Time Point of Steady State to draw the comparison between the subjects. Data (Fig 1A & 1B) were normalized for transduction efficiency by reporting the ratio of COL1A1-LV-tGFP to EF1α-LV-FP602 Relative Fluorescence Units (RFU). Healthy donors were matched for gender, race and smoking status. Kruskall–Wallis one-way analysis of variance followed by post-hoc Dunn's test for multiple comparisons was used to detect differences among studied groups. P values =0.028 compared to No PAH and P values =0.007 when compared to Healthy Donors, thus illustrating the significant effect of sera in activating COL1A1 Promoter in HPASMCs.

4.4 Effect of N0X2 inhibitor gp91on Intracellular ROS

From many reports it has been concluded that Oxidative stress plays a pivotal role in the pathophysiology of vascular diseases such as SSc [38, 52, 58, 64, 66-68, 70,173]. And Reactive oxygen species are known to cause oxidative damage to various cells and induce organ dysfunction after ischemia-reperfusion injury. Nicotinamide Adenine Dinucleotide Phosphate-Oxidase (**NADPH Oxidase or NOX**) form the predominant biochemical source of ROS in the vasculature [174-178]. The NOX1 (NADPH oxidase 1) and NOX2 oxidases are the major sources of ROS in the artery wall in hypertension as well as in SSc-PAH, and are formed to be important contributors to the oxidative stress[106-108] in SSc-PAH. Hypertension was worsened by NOX2 overexpression in experimental models [179].So this background suggested us to employ NOX2 inhibitor gp91 and to target NOX2 to validate whether NOX2 is involved in the Oxidative damage of SSc-PAH.To test this idea, the HPASMCs were pre-incubated with 5 μM NADPH oxidase specific inhibitor NOX2ds-tat (NOX) (formerly called as gp91ds-tat) for 1 hour. Later, HPASMCs were exposed to 5% (V/V) of sera from SSc-PAH, and no PAH subjects. NOX2ds-tat effectively reduced induction of ROS by PAH-SSc sera ($p = 0.009$), confirming that NADPH oxidase is indeed involved in the generation of ROS in SSc-PAH subjects as depicted in Fig.3. When the analysis of NOX inhibitor NOX2ds-tat effect was tested for SSc-No-PAH sera on HPASMCs disclosed that there was no significant reduction in ROS levels. So from this result it was proved that inhibiting NOX2 could reduce the pro-oxidant effect induced by SSc-PAH sera.

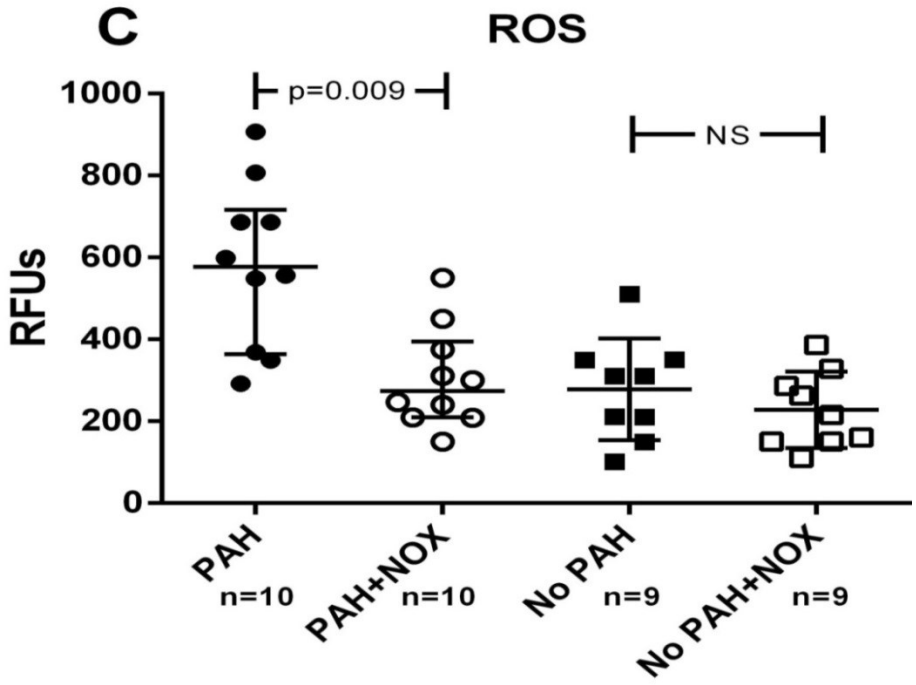

Figure 3: Effect of N0X2 inhibitor gp91on Intracellular ROS in sera stimulated HPASMCs. Here, sub-confluent HPASMCs were incubated for 1 hour with 5 μM NADPH oxidase specific inhibitor NOX2ds-tat (NOX) before treatment with SSc sera {5% (V/V)} and measurement of Intracellular ROS in PASMCs exposed to Sera of SSc-PAH and SSc-No PAH was carried out. Fluorescence data were normalized for protein content and expressed as Relative Fluorescence Units (RFU).Wilcoxon matched-pairs signed rank test was used to draw the differences between pre- and post-NOX treatment pairs. P values =0.009 compared to PAH+NOX, thus illustrating the significant effect of N0X2 inhibitor gp91 in counteracting the intracellular ROS stimulated by SSc-PAH sera but the comparison between No PAH and No PAH+ NOX remained non-significant.

4.5 Effect of N0X2 inhibitor gp91 on Collagen type I COL1A1 promoter Activity

In this experiment, GFP based lentivirus transduction in HPASMCs was done. Then HPASMCs were pre-treated with 5 μM NADPH oxidase specific inhibitor NOX2ds-tat for 1hr. And then, sera {5% (V/V)} from SSc-PAH and No-PAH subjects were exposed to transduced and NOX inhibitor treated HPASMCs and measured for COL1A1 promoter activity. This experiment revealed that NOX2ds-tat acted in very effective manner by significantly ($p = 0.005$) reducing the COL1A1 promoter activity in the SSc-PAH exposed HPASMCs, however there was no significant effect produced in the HPASMCs that were exposed to No-PAH as depicted in Fig.4.This experiment provided the result that NADPH Oxidase inhibitor NOX2ds-tat is also involved in the activation of COL1A1 promoter activity and hence in the synthesis of Collagen I in HPASMCs revealing that phenotypic switch and collagen synthesis activation in these cells may be driven by SSc-related PAH sera through NADPH-oxidase dependent ROS generation.

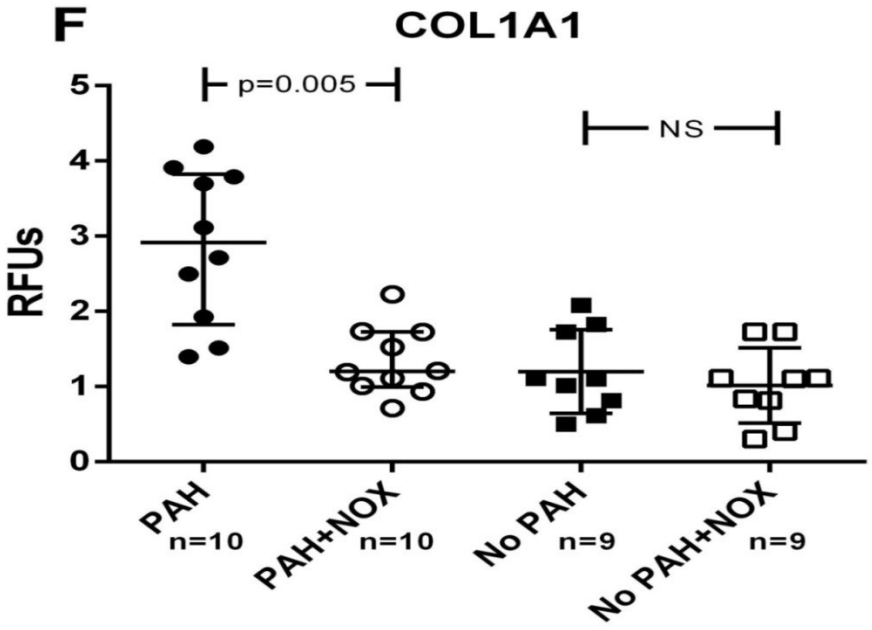

Figure 4: Effect of N0X2 inhibitor gp91on COL1A1 Promoter Activity in sera stimulated HPASMCs. Here, sub-confluent HPASMCs were transduced with lentiviral particles obtained from the COL1A1-LV-tGFP and EF1α-LV-FP602 lentivectors, and then incubated for 1 hour with 5 μM NADPH oxidase specific inhibitor NOX2ds-tat (NOX) before treatment with 5% (V/V) of sera from PAH, no PAH and COL1A1 Promoter Activity was measured. Data are normalized for transduction efficiency by reporting the ratio of COL1A1-LV-tGFP to EF1α-LV-FP602 Relative Fluorescence Units (RFU). Wilcoxon matched-pairs signed rank test was used to draw the differences between pre- and post-NOX treatment pairs. P values =0.005 compared to PAH+NOX, thus illustrating the significant effect of N0X2 inhibitor gp91 in counteracting the COL1A1 Promoter Activity stimulated by SSc-PAH sera but the comparison between No PAH and No PAH+ NOX remained non-significant.

4.6 Effect of ERK inhibitor PD98059 on Intracellular ROS

The MAPK/ERK pathway is one of the predominant signaling pathways involved in the regulation and synthesis of Collagen. Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase family is shown to be involved in SMC proliferation and also in both vasoconstriction and vascular smooth muscle cell growth [118]. There are evidences suggesting that ERK activation occurs via ROS [180,181].With these evidences, it was thought that targeting ERK would provide some clues about the pro-fibrotic effects of SSc-PAH sera. In this mode of experiment, 15uM ERK Inhibitor PD98059 of was pre-loaded to HPASMCs for 1hr before stimulation with sera. In the next step, SSc-PAH sera and No-PAH sera in {5% (V/V)} were exposed to these cells and intracellular ROS was measured as described in the method section. The outcome of this experiment showed ERK Inhibitor PD98059 did not effectively inhibit the intracellular ROS in SSc-PAH exposed HPASMCs (Fig.5). The similar effect was also observed in the No-PAH exposed HPASMCs.

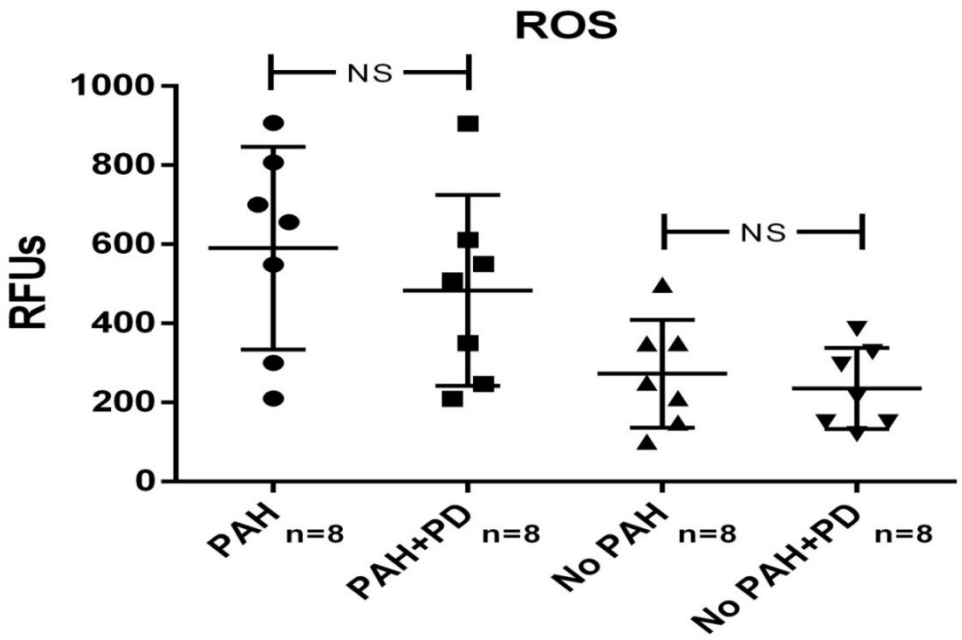

Figure 5: Effect of ERK inhibitor PD98059 on Intracellular ROS in sera stimulated HPASMCs.Here, sub-confluent HPASMCs were incubated for 1 hour with 15 μM ERK inhibitor PD98059 before treatment with SSc sera and measurement of Intracellular ROS in PASMCs exposed to Sera of SSc-PAH and SSc-No PAH was carried out. Fluorescence data were normalized for protein content and expressed as Relative Fluorescence Units (RFU).Wilcoxon matched-pairs signed rank test was used to draw differences between pre- and post ERK inhibitor PD98059 treatment pairs. P values › 0.05 when compared to PAH+PD as well as No PAH+ PD thus illustrating the non-significant effect of ERK inhibitor PD98059 in counteracting the intracellular ROS stimulated by SSc-PAH and SSc-No PAH sera .

4.7 Effect of ERK inhibitor PD98059 on Collagen type I (COL1A1) promoter Activity

In this experiment, lentivirus transduction in HPASMCs was done. This is followed by the confirmation of the transduction of lentiviral particles in the cells by using fluorescence microscope by observing the Green fluorescence provided by the GFP reporter system. The next step was done by pre-incubating the transduced cells by 15uM ERK Inhibitor PD98059 for 1hr.Then HPASMCs were exposed with 5% (V/V) sera of SSc-PAH and No-PAH. This step was followed by the measurement of COL1A1 promoter activity. The outcome of this experiment was that ERK Inhibitor PD98059 efficiently reduced (Fig.6) the COL1A1 promoter activity in SSc-PAH sera exposed HPASMCs, but the similar effect was not observed in No-PAH sera exposed HPASMCs, as the inhibitor did not significantly reduced COL1A1 promoter activity. So the result from this experiment proved that ERK signaling is involved in the activation of COL1A1 promoter activity in the HPASMCs exposed only with sera of SSc-PAH, but not the similar effect was observed in case of No-PAH, confirming that sera of SSc-PAH had pro-fibrotic effect on HPASMcs thereby suggesting that phenotypic switch and collagen synthesis activation in HPASMCs may be driven by SSc-related PAH sera through ERK signaling activation of COL1A1 promoter activity and collagen synthesis in HPASMCs.

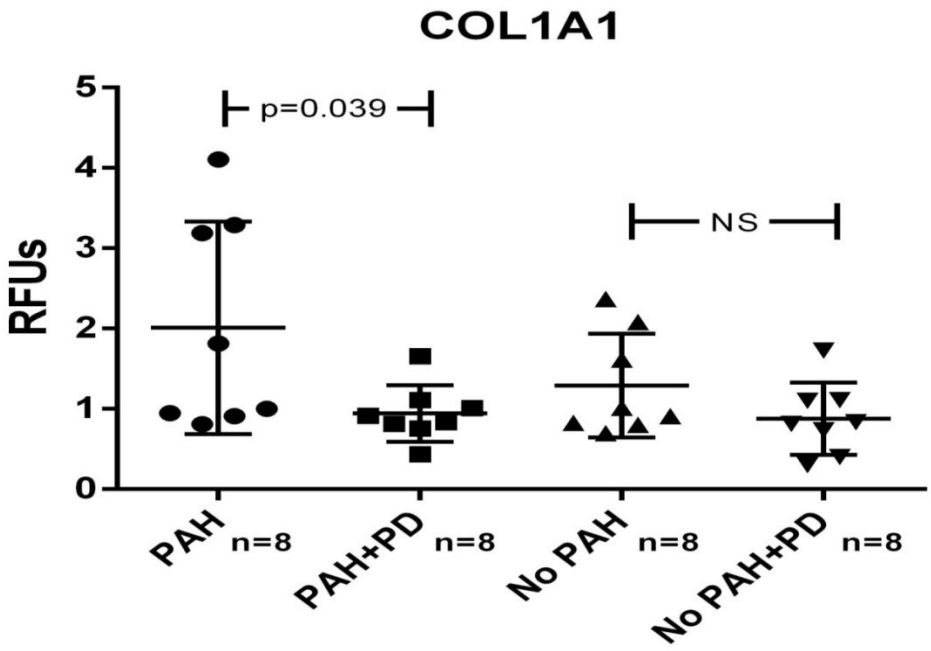

Figure 6: Effect of ERK inhibitor PD98059 on COL1A1 Promoter Activity in sera stimulated HPASMCs. Here, sub-confluent HPASMCs were transduced with lentiviral particles obtained from the COL1A1-LV-tGFP and EF1α-LV-FP602 lentivectors, and then incubated for 1 hour with15 μM ERK inhibitor PD98059 before treatment with 5% (V/V) of sera from PAH, no PAH and COL1A1 Promoter Activity was measured . Data are normalized for transduction efficiency by reporting the ratio of COL1A1-LV-tGFP to EF1α-LV-FP602 Relative Fluorescence Units (RFU). Wilcoxon matched-pairs signed rank test was used to draw differences between pre- and post ERK inhibitor PD98059 treatment pairs. P values =0.039 when compared to PAH+PD thus illustrating the significant effect of ERK inhibitor PD98059 in counteracting the COL1A1 Promoter Activity stimulated by SSc-PAH sera. But the comparison between No PAH and No PAH+ PD remained non-significant.

CHAPTER 5. DISCUSSION AND CONCLUSION

CHAPTER 5. DISCUSSION AND CONCLUSION

Most patients in SSc die due to pulmonary involvement of the disease with SSc PAH in second place to ILD PH. But SSc-PAH has worse prognosis than that of IPAH [39] and patients are at higher risk of death than IPAH patients or ILD PH. PAH is a life-threatening ailment, which could rapidly progress to severe right heart failure [13,14] and hence death of the patient occurs in very short time. So, it has thus become extreme need to study SSc-PAH, in particular the cellular and molecular events of the disease that set the vascular damage by OS during its disease pathogenesis, as OS is largely evidenced in this disease. In our Study, the first need was the selection of the subjects and so the study subjects were selected based on the diagnostic characters that divide SSc-PAH from No-PAH in overall SSc patients that came under this study and they were mostly middle age, white women. A total number of 19 SSc patients were diagnosed as SSc-PAH and 17 patients were selected as SSc No-PAH based on the diagnostic criteria. SSc-PAH is more common in LcSSc, when compared to DcSSc, however few recent studies [30-38] suggest that the prevalence of PAH is similar in both limited and diffuse cutaneous SSc patients. But in our study cases, PAH has shown higher in LcSSc with 16 patients showed PAH in total of 84 LcSSc patients against only 3patients showed PAH in total of 16 DcSSc patients as depicted in the Table A. It has been proved by many reports that PAH occurs as a late age onset[152-156] in SSc and so in our study case also the trend remains exactly the same with PAH prevailing at age of 64.0 ± 9.4 vs 53.3 ± 11.6 along with healthy donors age of 54.1 ± 10.4; p= 0.009. And in our study cohort, it is also noticed that the prevalence of PAH being present in the SSc patients having the disease for longer durations with a duration(RP onset) of 21.7 ± 9.4, against the non-PAH SSc patients duration of 14.0 ± 12.6 ; p= 0.008 matching with many earlier reports[152-156]. Increased collagen leads to skin thickness, which is due to intercellular matrix formation in the dermis and by oedema, possibly affected by both microvascular injury and

inflammation. Rodnan skin score (mRSS) is the 'gold standard' method for measuring the skin thickness in scleroderma [182].Few groups have tried to link this score to the severity of organ involvements in SSc, but, unfortunately this skin score measurement remained only to assess the thickening of skin in SSc pateints and could not be evolved as a biomarker to relate to the organ based severities such as pulmonary and cardiac involvements [183]. The Rodnan skin score (mRSS) in our study remain slight higher in SSc-PAH but not to the significant level when compared to No-PAH. However, their study [183] showed that mRSS greater than score 3 linked to pulmonary fibrosis but similar distribution was present when compared with other patients (PAH and cardiac involvement) with a higher mRSS clearly uttering the confirmation that Rodnan skin score (mRSS) did not serve as a biomarker in distinguishing the organ involvement defects in SSc.RP severity scores and Heart severity scores were little bit higher in SSc-PAH vs No-PAH, but not to the significant level of difference between the subjects. These scores indicate the level of vascular damages that are being fabricated by the disease condition and the higher scores in our studies exhibited the severity in pulmonary vascular condition that is also reported [184] by other studies. The Lung severity scores remained higher in SSc-PAH against non-PAH which are in compliance with the data of the other published reports [184-187].These features strongly show that there exists pulmonary vascular damage due to the prevalence of pulmonary vascular disease. Cardiac hemodynamic measurements by Right heart Catheterization showed that mean pulmonary arterial pressure (mPAP) and pulmonary capillary wedge pressure (PCWP) were remained in the range as expected to be in SSc-PAH subjects. There was comparatively lower diffusion capacity of lung for carbon monoxide (DLCO) (48.7 ± 16.8 vs 78.2 ± 23.0; $p < 0.001$) with comparable forced vital capacity (FVC) with decreased values against No-PAH subjects, which provided the confirmed clues that there exists an underlying pulmonary vascular disease. SSc-PAH patients showed significantly higher estimated right ventricular

systolic pressure (ERVSP) values (65.2 ± 19.9 vs 24.0 ± 6.3, p= <0.001) when compared to those of Non-PAH subjects this observation provided the severity of the underlying pulmonary vascular disease as RVSP >45 mm Hg cutoff has demonstrated to have high correlation with PAH detected by RHC [184, 189] that provides the proof of severe disturbances in the pulmonary vascular bed. Demographic Studies have shown Anti centromere antibodies (ACA) is prevalent in older, female Caucasians having SSc [161], so the same case in our study as well. The antibody status in our study revealed that SSc-PAH subjects showed not though significant but slight higher level of Anti centromere antibodies against No-PAH, because prevalence of ACAs linked with higher risk of development of PAH [161, 162] in SSc patients particularly the lSSc patients [161]. Anti-Scl-70 (anti-topoisomerase I) antibodies in this study were found lower in SSc-PAH against the No-PAH subjects very relevant to earlier studies [161,163,164] as SSc-PAH subjects mostly from lcSSc subjects but not dcSSc having interstitial lung disease showed the similar condition. There have been reports saying that there is complex interaction between endothelial cells (ECs), smooth muscle cells (SMCs), pericytes, extracellular matrix (ECM), and intravascular circulating factors [167]. Serum from long been has provided the useful tool in the diagnosis of many diseases. So also in our study has provided a very significant knowledge in understanding the SSc-PAH, by showing the proof that OS is linked to SSc-PAH. Serum factors in-vitro induced ROS production in Vascular cells [166]. The generation of ROS by vascular cells can activate several processes that elicit the development of SSc [190]. These could exhibit the underlying disease. So, this background provides us a hint that abnormalities in circulatory factors such as sera could be tested in-vitro to study the molecular and cellular events of the vascular damage during SSc, particularly in SSc-PAH. From our study the concept has been evolved which proves that ROS status can be identified as an indicator of the disease which could become a tool in the diagnosis and thereby becoming an aid in targeting the disease

mechanism and pursuing the remedy in SSc disease including SSc-PAH.In our study, when the sera from SSc were tested over the Human pulmonary artery Smooth Muscle Cells (HPASMCs), it was noticed that SSc sera induced the ROS production. When the Kinetic measurement of intracellular ROS was done in PASMCs by exposing to the SSc sera, the result showed that SSc-PAH sera induce more level of ROS significantly compared to the SSc with "No PAH" as well against the sera of Healthy Donors(HD).This proves that circulatory factors execute the pro-oxidant effect on HPASMCs. Endogenous pro-oxidants may be formed as the derivatives of the abnormal pathophysiology, cellular metabolism and ion flux [56], such kind of unusual or abnormalities are evolved due to the underlying disease, and this concept has been greatly evolved in our study which showed that pro-oxidants may be present in the sera of SSc-PAH patients which stimulated intracellular ROS and COL1A1 promoter activity in HPASMCs by inducing HPASMCs activation and phenotypic switch and if this process is not regulated would lead to the progressive narrowing and obliteration of pulmonary arterioles in SSc-PAH.This implies that circulating pro-oxidant factors may be involved in the pathogenesis of SSc-PAH through their ability to induce VSMCs activation and phenotypic switch.NADPH oxidases are widely distributed throughout different tissues and organs with only sole function to produce ROS. From our study it was revealed that NOX2 inhibitor gp91 very significantly reduced the intracellular ROS produced by the exposure of SSc-PAH sera, strongly implicating that cell membrane intracellular ROS are involved in the induction of Oxidative stress in SSc-PAH and more specifically indicating that NOX2 is involved in the process. But the same scenario was not observed in case of No-PAH subjects, evidencing, that oxidative stress is very prominently implicated in the pathogenesis and development of hypertension arterial disease of SSc condition.This observation also provided the proof that redox status is not balanced in SSc-PAH subjects which has led to the development OS in SSc-PAH. There are reports evidencing the same picture as in our study stating

that ROS are formed in high concentrations, in pathological conditions, such as hypertension, that cannot be balanced by the normal protective antioxidant mechanisms employed by the cells, resulting in oxidative stress [64, 109-111,113, 115,191-193]. The result that has been procured from our studies also showed more intracellular ROS as compared to the No-PAH SSc patients clearly indicating the higher level of intracellular ROS in this disease phenomena. Besides, the result from the kinetic measurement of intracellular ROS showed that, from the start of the measurement till the end the level, ROS was relatively higher than that of the No-PAH SSc subjects which strongly supports the earlier reports that SSc-PAH disease condition physiology is involved with the production of intracellular ROS. This condition would lead to many abnormalities in the vascular wall as follows. This could cause the subsequent pathogenic process in the vascular wall posing inflammation and deteriorations in the functions of endothelium in vasodilation, with increased pro-inflammatory states and increased prothrombotic activity. This would further cause vascular inflammation and OS may mediate through activation of mononuclear cells [58, 59] with infiltration of inflammatory cells in pulmonary perivascular spaces within and around plexiform lesions [60–62]. This further would stimulate a variety of negative effects on cellular function involving alteration of transcription factors, kinases, protein synthesis and also would cause increase in vascular contractility, also would promote vascular smooth muscle cell growth and apoptosis, monocyte migration, lipid peroxidation, inflammation, and increased deposition of ECM proteins. These all events play a pivotal role in the pathogenesis and progression of vascular damage, vascular remodeling in vascular diseases [65-67] including SSc-PAH. The major pathophysiologic event that occurs in the development of pulmonary hypertension is the proliferation of vascular smooth muscle cells and it has been shown that extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase family that has been reported to be involved in both vasoconstriction and vascular smooth muscle

cell growth [118] which prompted us to relate, whether the ROS generation and collagen promoter activity is dependent on ERK activation. That's why we employed ERK Inhibitor PD98059 in our study to look for ERK involvement. HPASMCs were pre-incubated with ERK Inhibitor PD98059 and then were exposed to sera (SSc-PAH & SSc-No-PAH). In this experiment, the ERK Inhibitor PD98059 could not form significant effect in reducing the intracellular ROS (Fig 5) in HPASMCs exposed to both the subjects of sera, confirming that, ERK was not involved in the generation of intracellular ROS. In another experiment, we planned to look for involvement of ERK in collagen promoter activity. The outcome of this experiment showed that ERK Inhibitor PD98059, very effectively ($p=0.039$)counteracted the COL1A1 promoter activity(Fig 6) in HPASMCs that were exposed to SSc-PAH sera, but the same effect was not reproduced in case of No-PAH SSc sera exposed HPASMCs. This result indeed confirmed that ERK was involved in the activation of COL1A1 promoter activity and hence in the synthesis of collagen in the HPASMCs that were exposed to SSc-PAH sera. This in-vitro study implies that in the patients of SSc-PAH, the ERK pathway is involved in the activation and synthesis of collagen I and hence in the accumulation of ECM in the vascular wall causing vascular remodeling and progressive vessel occlusion and thus involving or leading to pulmonary arterial hypertension. By looking into the complete account of the outcomes of our study, it can be summarized that SSc-PAH sera induced pro-oxidant effect on HPASMCs and hence there occurred the generation of intracellular ROS, which suggests that pro-oxidant effect existence in the sera may be due to the endogenous pathophysiology of the disease condition. This further suggests that the pathophysiology of the disease involves Oxidative Stress (OS), which may be due to the vascular damage derived from the abnormal changes in cellular and molecular status of the disease condition thereby, exhibiting the notion that OS is linked to SSc-PAH [66-70,173] and as well as giving a hint that OS can be considered as one of the etiological factor in SSc-PAH. Further, when examined for the source of

intracellular ROS by using specific inhibitor NOX2ds-tat (NOX), it was found that NADPH Oxidases (NOX) are the one participated in the generation of intracellular ROS in the vascular bed in compliance with the earlier reports [106-108,112,193-208] that have been reported in vascular diseases. The evidence from our study implicates for therapeutically targeting NOX2 oxidase in the arterial wall for the treatment of SSc-PAH, so as to combat the oxidative stress and prevent the progression of pulmonary vascular disease such as SSc-PAH in agreement with the earlier reports [106, 129, 136,194-205]. In other experiments, the outcomes were that, ERK Inhibitor PD98059 did not effectively inhibit the intracellular ROS in SSc-PAH exposed HPASMCs but efficiently reduced the COL1A1 promoter activity in SSc-PAH sera exposed HPASMCs. Prior, to this it was gathered from our result, that ROS generation exists via NADPH Oxidases (NOX2).These findings can be collectively explained that sera of SSc-PAH stimulated the activation of NOX2, which resulted in the generation of intracellular ROS. The intracellular ROS generated by NOX through its activation by the pro-oxidant effect of sera, further activated ERK resulting in the activation of COL1A1 promoter activity and hence the synthesis of Collagen as illustrated by the figure below. Hence, the sera of SSc-PAH, in our study, exhibited the pro-oxidant effect by activating NOX enzymes. It is well evidenced in various reports [210-227] that NADPH Oxidase is activated by various cellular stresses, chemical factors, and physical challenges, cellular environments, and inflammatory stimuli, stress-related humoral and neural factors. And, in our studies, it is emerging that the pro-oxidant effect executed by the SSc-PAH sera may be due to the one of the above said factors and it is speculated that circulating pro-oxidant factors may be involved in the pathogenesis of SSc-PAH through their ability to induce VSMCs activation and phenotypic switch. With these findings our study provides new evidence supporting the possibility that, vascular disease and in particular PAH may be driven or maintained in SSc patients by pro-oxidant

circulating factors acting, at least in part, through the activation of collagen synthesis in VSMCs.

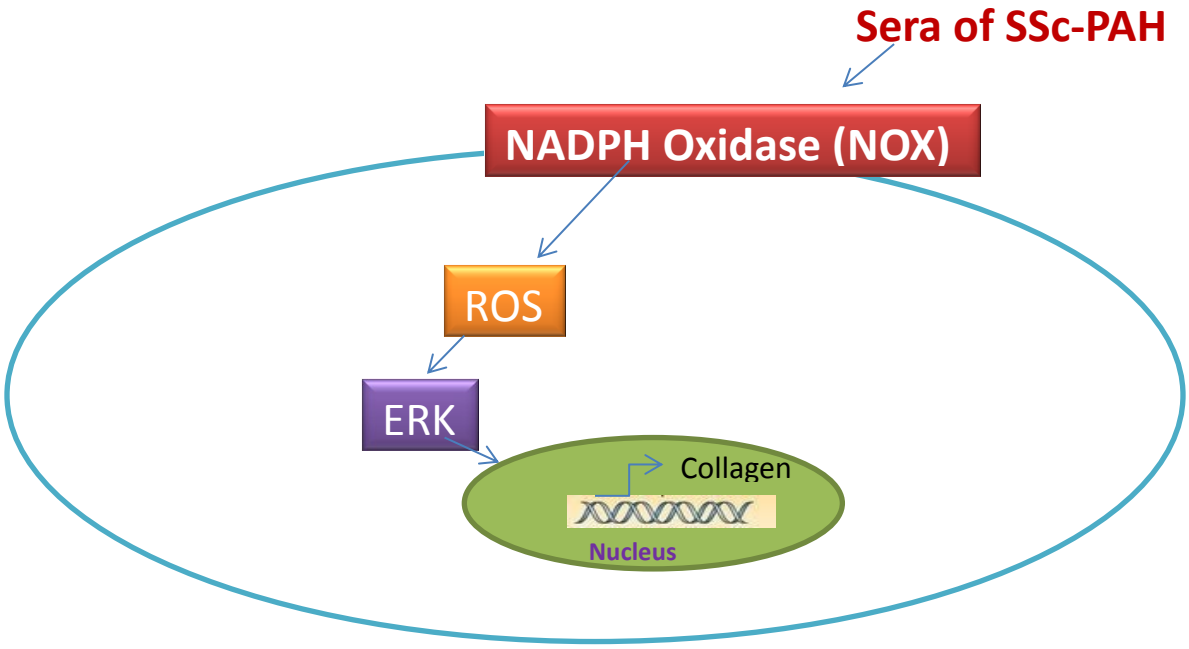

Figure 7: Representative Diagram to show that Sera from SSc-PAH subjects stimulate NOX in HPASMCs for ROS generation thus exhibiting the Pro-oxidant effect, which subsequently exhibit the Pro-fibrotic effect by synthesizing Collagen I via the stimulation of ERK.

To conclude, our study for the first time, shows that an increase of NOX2-derived ROS production induced by sera from SSc patients with PAH drives pro-oxidant and as well as pro-fibrotic responses in HPASMCs by activating ERK-mediated collagen synthesis. Hence, our study further suggests that antioxidant therapies should be explored in the treatment or prevention of Systemic Sclerosis related pulmonary vascular disease.

CHAPTER 6.BIBLIOGRAPHY

CHAPTER 6.BIBLIOGRAPHY

1. Rodnan,G.P. and T.G.Benedek. *An historical account of the study of progressive systemic sclerosis (diffuse scleroderma)*. Ann Intern Med 57:305-319 (1962).
2. Carol M. Artlett. *Immunology of Systemic Sclerosis*. Frontiers in Bioscience 10, 1707-1719, May 1, 2005.
3. Curzio, C. *Discussioni anatomico-pratiche di un raro, e stravagante morbo cutaneo in una giovane Donna felicemente curato in questo grande Ospedale degl' Incurabili, Giovanni di Simone, Naploi*. Anatomico-Pratiche (1753).
4. Goetz, R. H. *Pathology of progressive systemic sclerosis (generalized scleroderma) with special reference to changes in the viscera.* Clin Proc (S Afr) 4:337-339 (1945).
5. Barnet AJ: *History of Scleroderma*.In Clements PJ,Furst DE, editors: Systemic Sclerosis,Baltimore,1996,Williams & Wikins 1996,pp 3-22.
6. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, et al. *Scleroderma (systemic sclerosis): classification, subsets and pathogenesis*.J Rheumatol 1988;15:202-5.
7. Ferri C, Valentini G, Cozzi F, Sebastiani M, Michelassi C, La Montagna G, Bullo A, Cazzato M, Tirri E, Storino F, Giuggioli D, Cuomo G, Rosada M, Bombardieri S, Todesco S, Tirri G; Systemic Sclerosis Study Group of the Italian Society of Rheumatology(SIR-GSSSc).*Systemics clerosis: demographic, clinical, and serologic features and survival in 1,012 Italian patients.* Medicine (Baltimore).2002Mar;81(2):139-53.
8. Dinesh Khanna.*Diagnosis and management of systemic sclerosis*. Indian Journal of Rheumatology 2010 June; Volume 5, Number 2; pp. 69–75.

9. Jacques I. Benisty. *Pulmonary Hypertension*. Circulation. 2002;106:e192-e194.
10. Kelly M. Chin, Lewis J. Rubin. *Pulmonary Arterial Hypertension*. Journal of the American College of Cardiology, Vol. 51, No. 16, 2008.
11. Galie, N., Hoeper, M. M., Humbert, M., Torbicki, A., Vachiery, J. L., Barbera, J. A., et al. (2009a). *Guidelines for the diagnosis and treatment of pulmonary hypertension*. Eur Respir J 34(6), 1219–1263.
12. Galie, N., Hoeper, M. M., Humbert, M., Torbicki, A., Vachiery, J. L., Barbera, J. A., et al. (2009b). *Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT).*Eur Heart J 30(20), 2493–2537.
13. Galiè N, Torbicki A, Barst R, Dartevelle P, Haworth S, Higenbottam T, Olschewski H, Peacock A, Pietra G, Rubin LJ, Simonneau G, Priori SG, Garcia MA, Blanc JJ, Budaj A, Cowie M, Dean V, Deckers J, Burgos EF, Lekakis J, Lindahl B, Mazzotta G, McGregor K, Morais J, Oto A, Smiseth OA, Barbera JA, Gibbs S, Hoeper M, Humbert M, Naeije R, Pepke-Zaba J; Task Force. *Guidelines on diagnosis and treatment of pulmonary arterial hypertension. The Task Force onDiagnosis and Treatment of Pulmonary Arterial Hypertension of the European Society of Cardiology.* Eur Heart J 2004;25:2243–78.
14. Hachulla E, Coghlan JG. *A new era in the management of pulmonary arterial hypertension related to scleroderma: endothelin receptor antagonism.* Ann Rheum Dis 2004; 63: 1009–1014.
15. Sweiss NJ, Hushaw L, Thenappan T, Sawaqed R, Machado RF, Patel AR, Gomberg-Maitland M, Husain AN, Archer SL. *Diagnosis and Management*

of Pulmonary Hypertension in Systemic Sclerosis. Curr Rheumatol Rep. 2010; 12(1): 8–18.

16. Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, Gomez Sanchez MA, Krishna Kumar R, Landzberg M, Machado RF, Olschewski H, Robbins IM, Souza R.*Updated Clinical Classification of Pulmonary Hypertension.* Journal of the American College of Cardiology.Vol. 62, No. 25, 2013.
17. Simonneau G, Robbins IM, Beghetti M, Channick RN, Delcroix M, Denton CP, Elliott CG, Gaine SP, Gladwin MT, Jing ZC, Krowka MJ, Langleben D, Nakanishi N, Souza R. *Updated clinical classification of pulmonary hypertension.* JAmColl Cardiol 2009;54:S43–54.
18. The Task Force for Diagnosis and Treatment of Pulmonary Hypertension of European Society of Cardiology (ESC) and the European Respiratory Society (ERS) endorsed by the International Society of Heart and Lung Transplantation (ISHLT). Galiè N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, Beghetti M, Corris P, Gaine S, Gibbs JS, Gomez-Sanchez MA, Jondeau G, Klepetko W, Opitz C, Peacock A, Rubin L, Zellweger M, Simonneau G. *Guidelines for the diagnosis and treatment of pulmonary hypertension.* Eur Respir J 2009; 34: 1219–63.
19. Oudiz, R. J. *Pulmonary hypertension associated with left-sided heart disease.* Clin Chest Med 2007;28(1), 233–241.
20. David Montani, Marie-Camille Chaumais, Christophe Guignabert, Sven Günther,Barbara Girerd, Xavier Jaïs, Vincent Algalarrondo, Laura C. Price , Laurent Savale, Olivier Sitbon, Gérald Simonneau, Marc Humbert. *Targeted therapies in pulmonary arterial hypertension*. Pharmacology & Therapeutics 141 (2014) 172–191.
21. Eric Hachulla & David Launay. *Diagnosis and Classification of Systemic Sclerosis.* Clinic Rev Allerg Immunol (2011) 40:78–83.

22. Chin, K. M., & Rubin, L. J. (2008). *Pulmonary arterial hypertension.* J Am Coll Cardiol 51(16), 1527–1538.
23. Humbert, M., Sitbon, O., Chaouat, A., Bertocchi, M., Habib, G., Gressin, V., et al. (2010). *Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era*. Circulation 122(2), 156–163.
24. Humbert, M., Sitbon, O., Yaici, A., Montani, D., O'Callaghan, D. S., Jais, X., et al. (2010).*Survival in incident and prevalent cohorts of patients with pulmonary arterial hypertension*. Eur Respir J 36(3), 549–555.
25. Dweik RA, Rounds S, Erzurum SC, Archer S, Fagan K, Hassoun PM, Hill NS, Humbert M, Kawut SM, Krowka M, Michelakis E, Morrell NW, Stenmark K, Tuder RM, Newman J; ATS Committee on Pulmonary Hypertension Phenotypes.*An Official American Thoracic Society Statement: Pulmonary Hypertension Phenotypes*. Am J Respir Crit Care Med. 2014 Feb 1;189(3):345-55. doi: 10.1164/rccm.201311-1954ST.
26. Sullivan WD, Hurst DJ, Harmon CE, Esther JH, Agia GA, Maltby JD, Lillard SB, Held CN, Wolfe JF, Sunderrajan EV, et al. *A prospective evaluation emphasizing pulmonary involvement in patients withmixed connective tissue disease.* Medicine (Baltimore) 1984;63:92–107.
27. Jimenez SA, Derk CT. *Following the molecular pathways toward an understanding of the pathogenesis of systemic sclerosis.* Ann InternMed 2004;140:37–50**.**
28. Bae S, Saggar R, Bolster MB, et al. *Baseline characteristics and follow-up in patients with normal haemodynamics versus borderline mean pulmonary arterial pressure in systemic sclerosis: results from the PHAROS registry.*Ann Rheum Dis 2012; 71:1335–1342.
29. Marius M. Hoeper, Harm Jan Bogaard, Robin Condliffe, Robert Frantz,Dinesh Khanna,Marcin Kurzyna,David Langleben,Alessandra

Manes,Toru Satoh,Fernando Torres, Martin R. Wilkins, David B. Badesch. *Definitions and Diagnosis of Pulmonary Hypertension*. J Am Coll Cardiol. Vol. 62, No. 25, Suppl D, 2013.

30. Hachulla E, Gressin V, Guillevin L, Carpentier P, Diot E, Sibilia J, Kahan A, Cabane J, Frances C, Launay D, et al. *Early detection of pulmonary arterial hypertension in systemic sclerosis: a French nationwide prospective multicenter study.* Arthritis Rheum 2005;52: 3792–3800.
31. Mukerjee D, St George D, Coleiro B, Knight C, Denton CP, Davar J, Black CM, Coghlan JG. *Prevalence and outcome in systemic sclerosis associated pulmonary arterial hypertension: application of a registry approach.* Ann Rheum Dis 2003; 62:1088–1093.
32. Steen VD, Medsger TA. *Changes in causes of death in systemic sclerosis*, *1972-2002*. Ann Rheum Dis 2007; 66(7):940–4.
33. Steen V, Medsger Jr TA. *Predictors of isolated pulmonary hypertension in patients with systemic sclerosis and limited cutaneous involvement.* Arthritis Rheum 2003; 48(2):516–22.
34. Avouac J, Airo` P, Meune C, et al. *Prevalence of pulmonary hypertension in systemic sclerosis in European Caucasians and meta-analysis of 5 studies.* JRheumatol 2010; 37:2290–2298**.**
35. Hunzelmann N, Genth E, Krieg T, et al. *The registry of the German network for systemic scleroderma: frequency of disease subsets and patterns of organ involvement.* Rheumatology 2008; 47:1185–1192.
36. Domsic RT, Chung L, Gomberg-Maitland M, et al. *Pulmonary hypertension assessment and recognition of outcomes in scleroderma (PHAROS): comparison of outcomes in subtypes of pulmonary hypertension*. Arthritis Rheum 2010; 62 (10S):S245.
37. Yaqub A, Chung L.*Epidemiology and risk factors for pulmonary hypertension in systemic sclerosis.* Curr Rheumatol Rep 2013; 14:302.

38. Kristin B. Highland. *Recent advances in scleroderma-associated pulmonary hypertension.* Curr Opin Rheumatol 2014, 26:637–645.
39. Bull TM. *Screening and therapy of pulmonary hypertension in systemic sclerosis.* Current opinion in rheumatology 2007;19(6):598–603**.**
40. Kawut SM, Taichman DB, Archer-Chicko CL, Palevsky HI, Kimmel SE. *Hemodynamics and survival in patients with pulmonary arterial hypertension related to systemic sclerosis*. Chest 2003;123(2):344–50.
41. Hachulla E, de Groote P, Gressin V, Sibilia J, Diot E, Carpentier P, Mouthon L, Hatron PY, Jego P, Allanore Y, Tiev KP, Agard C, Cosnes A, Cirstea D, Constans J, Farge D, Viallard JF, Harle JR, Patat F, Imbert B, Kahan A, Cabane J, Clerson P, Guillevin L, Humbert M; Itinér AIR-Sclérodermie Study Group.*The three-year incidence of pulmonary arterial hypertension associated with systemic sclerosis in a multicenter nationwide longitudinal study in France.* Arthritis Rheum 2009; 60:1831–1839.
42. Fisher MR, Mathai SC, Champion HC, Girgis RE, Housten- Harris T, Hummers L, et al. *Clinical differences between idiopathic and scleroderma-related pulmonary hypertension.* Arthritis Rheum 2006;54:3043–50.
43. Altman RD, Medsger TA Jr, Bloch DA, Michel BA. *Predictors of survival in systemic sclerosis (scleroderma)*. Arthritis Rheum 1991;34:403–13.
44. Paul M. Hassoun.*Lung involvement in systemic sclerosis*. Presse Med. 2011 Jan; 40(1 Pt 2):e3-e17.
45. Demir N, Şahin A, Küçükşahin O, Kayacan O, Dinçer İ, Sayın T, Karnak D, Turgay M.*Pulmonary Arterial Hypertension and Systemic Sclerosis Relation: A Single Centre Experience.* Heart, Lung and Circulation (2014) 23, 667–673.
46. McLaughlin VV, Archer SL, Badesch DB, Barst RJ, Farber HW, Lindner JR, Mathier MA, McGoon MD, Park MH, Rosenson RS, Rubin LJ, Tapson VF, Varga J; American College of Cardiology Foundation Task Force on Expert Consensus Documents; American Heart Association; American College of

Chest Physicians; American Thoracic Society, Inc; Pulmonary Hypertension Association. *ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association*. JAmCollCardiol 2009;53:1573–619.

47. Silver RM, Medsger Jr TA, Bolster MB. *Systemic sclerosis and scleroderma variants: clinical aspects.* In: Koopman WJ, editor. Arthritis and allied conditions. 15th ed. Lippincot Williams & Wilkins; 2005. p. 1633–80.
48. Jeffery TK, Morrell NW. *Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension*. Prog Cardiovasc Dis 2002;45:173–202.
49. Humbert M, Morrell NW, Archer AL, Stenmark KR, MacLean MR, Lang IM, et al. *Cellular and molecular pathobiology of pulmonary arterial hypertension.* J AmColl Cardiol 2004;43:13S–24S.
50. Sevdalina Lambova, Ulf Müller-Ladner. *Pulmonary arterial hypertension in systemic sclerosis*. Autoimmunity Reviews 9 (2010) 761–770.
51. Price LC, Wort SJ, Perros F, Dorfmüller P, Huertas A, Montani D, Cohen-Kaminsky S, Humbert M.*Inflammation in pulmonary arterial hypertension*.Chest 2012; 141:210–211.
52. Murrel DF. *A radical proposal for the pathogenesis of scleroderma.* J Am Acad Dermatol 1993, 28: 78-85**.**
53. Pasciu V, Posadino AM, Cossu A, Sanna B, Tadolini B, Gaspa L, Marchisio A, Dessole S, Capobianco G, Pintus G. Akt downregulation by flavin oxidase induced ROS generation mediates dose-dependent endothelial cell damage elicited by natural antioxidants. Toxicol Sci 2010, 114:101–112.
54. Halliwell B. *Reactive oxygen species in living systems: source, biochemistry and role in human disease*. Am J Med 1991; 91: 145- 225.

55. Venereo JR. Daño oxidativo, radicales libres y anti-oxidantes. Rev Cubana Med Milit. 2002; 31(2):126-33.
56. Anu Rahal, Amit Kumar,Vivek Singh, Brijesh Yadav, Ruchi Tiwari, Sandip Chakraborty, and Kuldeep Dhama. *Oxidative Stress, Prooxidants, and Antioxidants: The Interplay.* BioMed Research International Volume 2014, Article ID 761264**.**
57. Satoh K, Nigro P, Matoba T, O'Dell M, Cui Z, Shi X, Mohan A, Yan C, Abe J, Illig K, Berk B. *Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. Nat Med 2009, 15:649-65.*
58. Nicolas Dumoitier,Sébastien Lofek,Luc Mouthon. *Pathophysiology of systemicsclerosis: State of the art in2014*. Presse Med.2014.
59. Badimón L, Martínez-González J. *Endothelial Dysfunction*. Rev Esp Cardiol. 2006;6(Supl A):21-30.
60. Voelkel NF, Cool C, Lee SD, Wright L, Geraci MW, Tuder RM. *Primary pulmonary hypertension between inflammation and cancer*. Chest 1998; 114:225S-30S.
61. Nicolls MR, Taraseviciene-Stewart L, Rai PR, Badesch DB, Voelkel NF. *Autoimmunity and pulmonary hypertension: a perspective*. Eur Respir J 2005; 26:1110-8**.**
62. Voelkel NF, Gomez-Arroyo J, Abbate A, Bogaard HJ, Nicolls MR. *Pathobiology of pulmonary arterial hypertension and right ventricular failure*. Eur Respir J 2012; 40: 1555-65.
63. Lassègue B, Clempus RE. *Vascular NAD(P)H oxi-dases: specific features, expression, and regulation.* Am J Physiol Regul Integr Comp Physiol. 2003; 285(2):277-97.
64. U. Landmesser and D. G. Harrison. "*Oxidative stress and vascular damage in hypertension,"* Coronary Artery Disease, vol. 12, no. 6, pp. 455–461, 2001. http://dx.doi.org/10.1097/00019501-200109000-00004.

65. Mulvany MJ, Baumbach GL, Aalkjaer C, Heagerty AM, Korsgaard N, Schiffrin EL, Heistad DD. *"Vascular remodeling,"* Hypertension, vol. 28, pp. 505–506, 1996.
66. R. M. Touyz and E. L. Schiffrin. *"Reactive oxygen species in vascular biology: implications in hypertension,"* Histochem Cell Biol. 2004 Oct;122(4):339-52. Epub 2004 Aug 26.
67. Agostino Virdis, Emiliano Duranti, and Stefano Taddei. *Oxidative Stress and Vascular Damage in Hypertension:Role of Angiotensin II.* International Journal of Hypertension Volume 2011.
68. Rodrigo R, Prat H, Passalacqua W, Araya J, Guichard C, Bächler JP. *Relationship between oxidative stress and essential hypertension.* Hypertens Res. 2007; 30(12):1159-67.
69. Touyz RM, Schiffrin EL. *Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD(P)H oxi-dase-sensitive pathways.* J Hypertens. 2001;19(7): 1245-54.
70. Touyz RM. *Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance?* Hypertension. 2004; 44:248–252.
71. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. *Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction.* Science 1995, 270:296–299.
72. Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK.*p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells.* J Biol Chem. 1996;271:23317–23321.
73. Jones SA, O'Donnell VB, Wood JD. *Expression of phagocyte NADPH oxidase components in human endothelial cells.* Am J Physiol 1996, 271:H1626–H1634.

74. Rajagopalan S, Kurz S, Munzel T. *Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vasomotor tone.* J Clin Invest 1996, 97:1916–1923.
75. De Leo FR, Ulman KV, Davis AR, Jutila KL, Quinn MT. *Assembly of the human neutrophil NADPH oxidase involves binding of p67phox and flavocytochrome b to a common functional domain in p47phox.* J Biol Chem 1996, 271:17013–17020.
76. Griendling KK, Ushio-Fukai M.*NADH/NADPH oxidase and vascular function.* Trends Cardiovasc Med 1997, 301–307.
77. Abe J-I, Berk BC. *Reactive oxygen species of signal transduction in cardiovascular disease.* Trends Cardiovasc Med 1998, 8:59–64.
78. De Keulener GW, Alexander RW, Ushio-Fukai M. *Tumour necrosis factor alpha activates a p22phox-based NADH oxidase in vascular smooth muscle.* Biochem J 1998, 329:653–657.
79. Finkel T. *Oxygen radicals and signaling*. Curr Opin Cell Biol 1998, 10:248–253.
80. Puig JG, Ruilope LM. *Uric acid as a cardiovascular risk factor in arterial hypertension.* J Hypertens 1999, 17:869–872.
81. Azumi H1, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M. *Expression of NADH/ NADPH oxidase p22phox in human coronary arteries.* Circulation 1999, 100:1494–1498.
82. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD. *Cell transformation by the superoxide-generating oxidase Mox1*. Nature 1999, 410:79–82.
83. McIntyre M, Bohr DF, Dominiczak AF. *Endothelial function in hypertension. The role of superoxide anion.* Hypertension 1999, 34:539–545.

84. Marumo T, Schini-Kerth VB, Brandes RP, Busse R. *Glucocorticoids inhibit superoxide anion production and p22phox mRNA expression in human aortic smooth muscle cells.* Hypertension 1999, 32:1083–1088.
85. Wei EP, Kontos HA, Christman CW. *Superoxide generation and reversal of acetylcholine-induced cerebral arteriolar dilation after acute hypertension.* Circ Res 1985, 57:781–787.
86. Chin JH, Azhar S, Hoffman BB. *Inactivation of endotheliumderived relaxing factor by oxidized lipoproteins.* J Clin Invest 1992, 89:10–18.
87. Rao GN, Berk BC. *Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression.* Circ Res 1992, 70:593–599.
88. Darley-Usmar V, Wiseman H, Halliwell B. *Nitric oxide and oxygen radicals; a question of balance.* FEBS Lett 1995, 369:131–135.
89. Sharma RC, Hodis HN, Mack WJ, Sevanian A, Kramsch DM. *Probucol suppresses oxidant stress in hypertensive arteries. Immuno histochemical evidence*. Am J Hypertens 1996, 9:577–590.
90. Harrison DG. *Cellular and molecular mechanisms of endothelial cell dysfunction*. J Clin Invest 1997, 2153–2157.
91. Fridovich I. *Superoxide anion radical, superoxide dismutases, and related matters.* J Biol Chem 1997, 272:18515-18517.
92. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. *Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension.* Circulation 1997, 95:588-593.
93. Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, Taylor WR, Griendling KK.*Role of NADH/ NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy*. Hypertension 1998, 32:488–495.
94. Touyz RM, Schiffrin EL.*Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells.* Hypertension 1999, 34(Part 2):976–982.

95. Chappell LC, Seed PT, Briley AL, Kelly FJ, Lee R, Hunt BJ, Parmar K, Bewley SJ, Shennan AH, Steer PJ, Poston L. *Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomized trial.* Lancet 1999, 354:810–816.
96. Schnackenberg CG, Welch W, Wilcox CS. *Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic. Role of nitric oxide.* Hypertension 1999, 32:59–64.
97. Brown MR1, Miller FJ Jr, Li WG, Ellingson AN, Mozena JD, Chatterjee P, Engelhardt JF, Zwacka RM, Oberley LW, Fang X, Spector AA, Weintraub NL. *Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular smooth muscle cells.* Circ Res 1999, 85:524–533.
98. Di Wang H, Hope S, Du Y, Quinn MT, Cayatte A, Pagano PJ, Cohen RA. *Paracrine role of adventitial superoxide anion in mediating spontaneous tone of the isolated rat aorta in angiotensin II-induced hypertension.* Hypertension 1999, 33:1225–1232.
99. Kerr S, Brosnan J, McIntyre M. *Superoxide anion production is increased in a model of genetic hypertension. Role of endothelium*. Hypertension 1999, 33:1353-1358**.**
100. Tsai MH, Yu CL, Stacey DW. *A cytoplasmic protein inhibits the GTPase activity of H-Ras in a phospholipid-dependent manner*. Science 1990, 250:982-985.
101. Griendling KK, Harrison DG. *Dual role of reactive oxygen species in vascular growth.* Circ Res 1999, 85:562–563**.**
102. Cosentino F, Sill JC, Katusic ZS. *Role of superoxide anions in the mediation of endothelium-dependent contractions*. Hypertension 1994, 23:229–235.
103. Rajagopalan S, Meng XP, Ramasamy S, et al.*Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of*

vascular matrix metalloproteinases in vitro. J Clin Invest 1996, 98:2572–2579.

104. Rhian M. Touyz. *Oxidative Stress and Vascular Damage in Hypertension.* Current Hypertension Reports 2000, 2:98–105.
105. Ana Fortuno˜ , Gorka San Jose´, Mar´ıa U. Moreno1, Javier D´ıez and Guillermo Zalba. Oxidative stress and vascular remodeling. Exp Physiol (2005) 90.4 pp 457–462.
106. Grant R. Drummond, Stavros Selemidis, Kathy K. Griendling and Christopher G. Sobey. *Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets*. Nat Rev Drug Discov. 2011 Jun; 10(6):453-71
107. Sambo P, Baroni SS, Luchetti M,Paroncini P, Dusi S, Orlandini G, Gabrielli A. *Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway.* Arthritis Rheum. 2001 Nov;44(11):2653-64.
108. Pei-Suen Tsou, Nadine N. Talia,Adam J. Pinney,Ann Kendzicky,Sonsoles Piera-Velazquez, Sergio A. Jimenez, James R. Seibold, Kristine Phillips, and Alisa E. Koch. *Effect of Oxidative Stress on Protein Tyrosine Phosphatase 1B in Scleroderma Dermal Fibroblasts*. Arthritis & Rheumatism Vol. 64, No. 6, June 2012, pp 1978–1989.
109. Brandes, R. P. & Schroder, K. *Composition and functions of vascular nicotinamide adenine dinucleotide phosphate oxidases*. Trends Cardiovasc. Med. 18, 15–19 (2008).
110. Miller, A. A., Drummond, G. R. & Sobey, C. G. *Novel isoforms of NADPH-oxidase in cerebral vascular control*. Pharmacol. Ther. 111, 928–948 (2006).
111. Ramón Rodrigo,Matías Libuy, Felipe Feliú, and Daniel Hasson. *Oxidative Stress-Related Biomarkers in Essential Hypertension and Ischemia-*

Reperfusion Myocardial Damage. Disease Markers Volume 35 (2013), Issue 6, Pages 773–790.

112. .Liu JQ, Zelko IN, Erbynn EM, Sham JS, and Folz RJ. *Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox)*. Am J Physiol Lung Cell Mol Physiol 290: L2–L10, 2006.
113. Mittal M, Roth M, Konig P, Hofmann S, Dony E, Goyal P, Selbitz AC, Schermuly RT, Ghofrani HA, Kwapiszewska G, Kummer W, Klepetko W, Hoda MA, Fink L, Hanze J, Seeger W, Grimminger F, Schmidt HH, and Weissmann N. *Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature*. Circ Res 101: 258–267, 2007.
114. Fike CD, Slaughter JC, Kaplowitz MR, Zhang Y, and Aschner JL. *Reactive oxygen species from NADPH oxidase contribute to altered pulmonary vascular responses in piglets with chronic hypoxia-induced pulmonary hypertension*. Am J Physiol Lung Cell Mol Physiol 295: L881–L888, 2008.
115. Fresquet F, Pourageaud F, Leblais V, Brandes RP, Savineau JP, Marthan R, and Muller B. *Role of reactive oxygen species and gp91phox in endothelial dysfunction of pulmonary arteries induced by chronic hypoxia.* Br J Pharmacol 148: 714–723, 2006.
116. Gary L.Johnson and Razvan Lapadat.*Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases.* Science Vol 298 6 December 2002.
117. Cobb MH. *MAP kinase pathways*. Prog Biophys Mol Biol. 1999;71(3–4): 479–500.
118. Richard E Roberts. *The extracellular signal-regulated kinase (ERK) pathway: a potential therapeutic target in hypertension.* Journal of Experimental Pharmacology Aug.2012.

119. Rhian M. Touz and Ernesto L. Schiffrin. *Signal Transduction Mechanisms Mediating the Physiological and Pathophysiological Actions of Angiotensin II in Vascular Smooth Muscle Cells.* Pharmacol Rev 52:639–672, 2000.
120. Victoria Vellarde, Paula M De La Cerda, Claudia Duarte, Francisca Arancibia, Eduardo Abbott, Alejandro Gonzalez,Francesca Moreno and Ayad A Jaffa. *Role of reactive oxygen species in bradykinin-induced proliferation of vascular smooth muscle cells*. Biol Res 37: 419-430, 2004.
121. Vishalakshi Viswanath, Meghana M Phiske, and Vinay V Gopalani. Systemic Sclerosis: Current Concepts in Pathogenesis and Therapeutic Aspects of Dermatological Manifestations. Indian J Dermatol. 2013 Jul-Aug; 58(4): 255–268. 46. Vishalakshi.
122. A. L. Herrick.Pathogenesis of Raynaud's phenomenon. Rheumatology 2005;44:587–596.
123. John P Cookea and Janice M Marshallb. Mechanisms of Raynaud's disease. Vascular Medicine 2005; 10: 293–307.
124. Prete M, Fatone MC, Favoino E, Perosa F. Raynaud's phenomenon: from molecular pathogenesis to therapy. Autoimmun Rev. 2014 Jun;13(6):655-67.
125. Nicolás F. Renna, Natalia de las Heras, and Roberto M. Miatello. Pathophysiology of Vascular Remodeling in Hypertension. International Journal of Hypertension.Volume 2013, Article ID 808353, 7 pages
126. Armando Gabrielli, Silvia Svegliati, Gianluca Moroncini and Donatella Amico. New Insights into the Role of Oxidative Stress in Scleroderma Fibrosis. The Open Rheumatology Journal, 2012, 6, (Suppl 1: M4) 87-95.
127. Ariane L. Herrick and Maurizio Cutolo. Clinical Implications From Capillaroscopic Analysis in Patients With Raynaud's Phenomenon and Systemic Sclerosis. Arthritis & Rheumatism Vol. 62, No. 9, September 2010, pp 2595–2604 DOI 10.1002/art.27543.

128. Mohamed A. Gashouta,Marc Humbert,Paul M. Hassoun. *Update in systemic sclerosis-associated pulmonary arterial hypertension.* Presse Med. 2014; 43: e293–e304.
129. Selemidis, S., Sobey, C. G., Wingler, K., Schmidt, H. H. & Drummond, G. R. *NADPH oxidases in the vasculature: molecular features, roles in disease and pharmacological inhibition*. Pharmacol. Ther. 120, 254–291 (2008).
130. Jackman, K. A. et al. *Reduction of cerebral infarct volume by apocynin requires pretreatment and is absent in Nox2‑deficient mice.* Br. J. Pharmacol. 156, 680–688 (2009).
131. Chen, H., Song, Y. S. & Chan, P. H. *Inhibition of NADPH oxidase is neuroprotective after ischemia–reperfusion*. J. Cereb. Blood FlowMetab. 29, 1262–1272 (2009).
132. Kahles, T. et al. *NADPH oxidase plays a central role in blood–brain barrier damage in experimental stroke*. Stroke 38, 3000–3006 (2007).
133. Jung, O. et al. *gp91phox‑containing NADPH oxidase mediates endothelial dysfunction in renovascularhypertension.* Circulation 109, 1795–1801 (2004).
134. Rey, F. E., Cifuentes, M. E., Kiarash, A., Quinn, M. T. & Pagano, P. J. *Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O2 – and systolic blood pressure in mice*. Circ. Res. 89, 408– 414 (2001).
135. Brennan, A. M. et al. *NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation*. Nature Neurosci. 12, 857–863 (2009).
136. Park, L. et al. *Nox2‑derived radicals contribute to neurovascular and behavioral dysfunction in mice overexpressing the amyloid precursor protein.* Proc. Natl Acad. Sci. USA 105, 1347–1352 (2008).

137. Diana M. Tabima, Alejandro Roldan-Alzate, Zhijie Wang, Timothy A. Hacker, Robert C. Molthen, and Naomi C. Chesler. *Persistent vascular collagen accumulation alters hemodynamic recovery from chronic hypoxia.* J Biomech. 2012 March 15; 45(5): 799–804.
138. Mahapatra S, Nishimura RA, et al. *The prognostic value of pulmonary vascular capacitance determined by Doppler echocardiography in patients with pulmonary arterial hypertension*. Journal of the American Society of Echocardiography : official publication of the American Society of Echocardiography. 2006; 19(8):1045–1050.
139. Hemnes AR, Champion HC. *Right heart function and haemodynamics in pulmonary hypertension*. International journal of clinical practice. 2008; (160):11–19.
140. Gan CT, Lankhaar JW, et al. *Noninvasively assessed pulmonary artery stiffness predicts mortality in pulmonary arterial hypertension*. Chest. 2007; 132(6):1906–1912.
141. Hunter KS, Lee PF, et al. *Pulmonary vascular input impedance is a combined measure of pulmonary vascular resistance and stiffness and predicts clinical outcomes better than pulmonary vascular resistance alone in pediatric patients with pulmonary hypertension.* American Heart Journal. 2008; 155(1):166–174.
142. Ooi CY, Wang Z, et al. *The role of collagen in extralobar pulmonary artery stiffening in response to hypoxia-induced pulmonary hypertension.* Am J Physiol Heart Circ Physiol. 2010; 299(6):H1823–1831.
143. Subcommittee For Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee: *Preliminary criteria for the classification of systemic sclerosis (scleroderma).* Arthritis Rheum 1980, 23:581–590.

144. Medsger TA Jr, Silman AJ, Steen VD, Black CM, Akesson A, Bacon PA, Harris CA, Jablonska S, Jayson MI, Jimenez SA, Krieg T, Leroy EC, Maddison PJ, Russell ML, Schachter RK, Wollheim FA, Zacharaie H: *A disease severity scale for systemic sclerosis*: *development and testing*. J Rheumatol 1999, 26:2159–2167.
145. Bukrinsky MI, Haggerty S, Dempsey MP, Sharova N, Adzhubel A, Spitz L, Lewis P, Goldfarb D, Emerman M, Stevenson M: A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature 1993, 365:666-669.
146. Naldini L: Lentiviruses as gene transfer agents for delivery to non-dividing cells. Curr Opin Biotechnol 1998, 9:457-463.
147. Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE: Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science 1999, 283:682-686.
148. Baekelandt V, Claeys A, Eggermont K, Lauwers E, De Strooper B, Nuttin B, Debyser Z: Characterization of lentiviral vectormediated gene transfer in adult mouse brain. Human Gene Therapy 2002, 13:841-853.
149. Francesco Galimi, Meenakshi Noll, Yoshiyuki Kanazawa, Timothy Lax, Cindy Chen, Markus Grompe andInder M. Verma. *Gene therapy of Fanconi anemia: preclinical efficacy using lentiviral vectors.* Blood, 15 October 2002 Volume 100, Nunber 8.
150. CRE recombinase-inducible RNA interference mediated by lentiviral vectors. Gustavo Tiscornia, Vinay Tergaonkar, Francesco Galimi, and Inder M. Verma. PNAS May 11, 2004 vol. 101 no. 19 7347–7351.
151. Altenhofer S, Radermacher KA, Kleikers P, Wingler K, Schmidt HH.*Evolution of NADPH oxidase inhibitors: selectivity and mechanisms for target engagement*. Antioxid Redox Signal 2014, doi:10.1089/ars.2013.5814.

152. Schachna L, Wigley FM, Chang B, White B, Wise RA, Gelber AC*. Age and risk of pulmonary arterial hypertension in scleroderma.* Chest. 2003 Dec;124(6):2098–104.
153. Hachulla E, Launay D, Mouthon L, Sitbon O, Berezne A, Guillevin L, et al. *Is pulmonary arterial hypertension really a late complication of systemic sclerosis?* Chest. 2009 Nov;136(5):1211–9.
154. Chang B, Schachna L, White B, Wigley FM, Wise RA. *Natural history of mild-moderate pulmonary hypertension and the risk factors for severe pulmonary hypertension in scleroderma.* J Rheumatol. 2006 Feb;33(2):269–74.
155. Hachulla E, de Groote P, Gressin V, Sibilia J, Diot E, Carpentier P, et al. *The three-year incidence of pulmonary arterial hypertension associated with systemic sclerosis in a multicenter nationwide longitudinal study in France.* Arthritis Rheum. 2009 Jun;60(6):1831–9.
156. Rebecca L. Manno, Fredrick M. Wigley, Allan C. Gelber, and Laura K. Hummers.*Late-Age Onset Scleroderma*. J Rheumatol. Jul 2011; 38(7): 1317–1325.
157. Galie N, Palazzini M, Manes A. *Pulmonary hypertension and pulmonary arterial hypertension: a clarification is needed*. Eur Respir J 2010;36:986–90.
158. Alkotob ML, Soltani P, Sheatt MA, Katsetos MC, Rothfield N, Hager WD, Foley RJ, Silverman DI. *Reduced exercise capacity and stress-induced pulmonary hypertension in patients with scleroderma*. Chest, 2006;130(1):176–81.
159. Collins N, Bastian B, Quiqueree L, Jones C, Morgan R, Reeves G. *Abnormal pulmonary vascular responses in patients registered with a systemic autoimmunity database: Pulmonary Hypertension Assessment and*

Screening Evaluation using stress echocardiography (PHASE-I). Eur J Echocardiogr, 2006;7(6):439–46.

160. Heather Gladue, Virginia Steen, Yannick Allanore, Rajeev Saggar, Rajan Saggar, Paul Maranian, Veronica J. Berrocal, Jerome Avouac, Christophe Meune, Mona Trivedi, and Dinesh Khanna.*Combination of Echocardiographic and Pulmonary Function Test ParametersImproves Sensitivity for the Diagnosis of Systemic Sclerosis-Associated Pulmonary Arterial Hypertension- Analysis of Two Cohorts.* J Rheumatol. 2013 October; 40(10).
161. Khanh T Ho and John D Reveille. *The clinical relevance of autoantibodies in scleroderma*. Arthritis Res Ther 2003, 5:80-93.
162. Virginia D. Steen. *Autoantibodies in Systemic Sclerosis*. Semin Arthritis Rheum 2005,35:35-42.
163. Basu D, Reveille JD. *Anti-scl-70.* Autoimmunity 38, 65-72 (2005).
164. Czömpöly T, Simon D, Czirják L, Németh P. *Anti-topoisomerase I autoantibodies in systemic sclerosis.* Autoimmun Rev 8, 692-6 (2009**)**.
165. Laurence Goffin, Queralt Seguin-Estévez, Montserrat Alvarez, Walter Reith and Carlo Chizzolini.*Transcriptional regulation of matrix metalloproteinase-1 and collagen 1A2 explains the anti-fibrotic effect exerted by proteasome inhibition in human dermal fibroblasts*. Goffin et al. Arthritis Research & Therapy 2010, 12:R73.
166. A Servettaz, P Guilpain, C Goulvestre, C Che´reau, C Hercend, C Nicco, L Guillevin, B Weill, L Mouthon, F Batteux. *Radical oxygen species production induced by advanced oxidation protein products predicts clinical evolution and response to treatment in systemic sclerosis*. Ann Rheum Dis 2007;66:1202–1209.

167. Gabrielli A, Avvedimento EV, Krieg T (2009) Scleroderma. N Engl J Med 360: 1989–2003. doi: 10.1056/nejmra0806188.
168. Hitraya, E. G., and Jimenez, S. A. *Transcriptional activation of the alpha 1(I) procollagen gene in systemic sclerosis dermal fibroblasts. Role of intronic sequences*. (1996) Arthritis Rheum. 39, 1347–1354.
169. Hitraya, E. G., Varga, J., Artlett, C. M., and Jimenez, S. A. *Identification of elements in the promoter region of the alpha1(I) procollagen gene involved in its up-regulated expression in systemic sclerosis.* Arthritis Rheum. 1998 Nov;41(11):2048-58.
170. Mayne R. *Vascular connective tissue: normal biology and derangement in human diseases. In: Uitto J, Perejda Z, eds. Diseases of Connective Tissue: The Molecular Pathology of the Extracellular Matrix*. New York:Marcel Dekker Inc; 1984:271–308.
171. Burke JM, Ross R. *Synthesis of connective tissue macromolecules by smooth muscle.* Int Rev Connect Tissue Res. 1979;8:119–157.
172. Christopher J. O'Callaghan, Bryan Williams. *Mechanical Strain–Induced Extracellular Matrix Production by Human Vascular Smooth Muscle Cells Role of TGF-b1.* Hypertension. 2000;36:319-324.
173. Armando Gabrielli, Silvia Svegliati, Gianluca Moroncini and Donatella Amico.New Insights into the Role of Oxidative Stress in Scleroderma Fibrosis.The Open Rheumatology Journal, 2012, 6, (sup 1: M4) 87-95.
174. Karen Bedard and Karl-Heinz Krause. *The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology*. Physiol Rev 87: 245–313, 2007.
175. M Beg, A Gupta, VN Khanna.*Oxidative Stress in Essential Hypertension and Role of Antioxidants.* JIACM 2010; 11(4): 287-93.

176. Brian Griffith,Srikanth Pendyala,Louise Hecker,Patty J. Lee, Viswanathan Natarajan and Victor J. Thannickal. *NOX Enzymes and Pulmonary Disease.* Antioxidants & Redox Signaling Volume 11 Number 10, 2009.
177. Yosit Ponce Gutiérreza, Arik Ponce Gutiérreza, Arnaldo Rodríguez Leónb and Katherin Cabrera Garcíaa. *Role of oxidative stress in the pathogenesis of hypertension.* CorSalud 2014 Apr-Jun;6(2):181-192.
178. Weijing Cai,Massimo Torreggiani,Li Zhu,1 Xue Chen,John Cijiang He,Gary E. Striker and Helen Vlassara. *AGER1 regulates endothelial cell NADPH oxidase-dependent oxidant stress via PKC-ϒ: implications for vascular disease.* Am J Physiol Cell Physiol 298: C624–C634, 2010.
179. Bendall JK, Rinze R, Adlam D, Tatham AL, de Bono J, Wilson N, Volpi E, Channon KM. *Endothelial Nox2 overexpression potentiates vascular oxidative stress and hemodynamic response to angiotensin II: studies in endothelial-targeted Nox2 transgenic mice.* Circ Res.N 2007;100:1016–1025.
180. Xiaohui Wang , Zhuyao Wang , Yuzhen Yao, Jingjin Li , Xiaojin Zhang , Chuanfu Li , Yunlin Cheng , Guoxian Ding , Li Liu , Zhengnian Ding.*Essential role of ERK activation in neurite outgrowth induced by α-lipoic acid.* Biochimica et Biophysica Acta 1813 (2011) 827–838.
181. Lee SL, Wang WW, Finlay GA, Fanburg BL. *Serotonin stimulates mitogen-activated protein kinase activity through the formation of superoxide anion*. Am J Physiol. 1999;277(2 Pt 1):L282–L291.
182. L. Czirja´k , I. Foeldvari and U. Mu¨ller-Ladner. *Skin involvement in systemic sclerosis.* Rheumatology 2008;47:v44–v45
183. L. G. Hanitsch, G.-R. Burmester, C. Witt, N. Hunzelmann, E. Genth, T. Krieg, W. Lehmacher,I. Melchers, M. Meurer, U.Mu¨ ller-Ladner, E. Schulze-Lohoff, M. Becker, C. Sunderkoetter,the DNSS centers and G. Riemekasten. *Skin sclerosis is only of limited value to identify SSc patients*

with severe manifestations—an analysis of a distinct patient subgroup of the German Systemic Sclerosis Network (DNSS) Register. Rheumatology 2009;48:70–73.

184. Francesco Boin, Stefano Franchini, Elizabeth Colantuoni, Antony Rosen,Fredrick M. Wigley, and Livia Casciola-Rosen. *Independent Association of Anti–2-Glycoprotein I Antibodies With Macrovascular Disease and Mortality in Scleroderma Patient.* Arthritis & Rheumatism Vol. 60, No. 8, August 2009, pp 2480–2489.
185. American Thoracic Society. Lung function testing: selection of reference values and interpretative strategies. Am Rev Respir Dis. 1991; 144:1202–1218.
186. Denton CP, Cailes JB, Phillips GD, Wells AU, Black CM, Bois RM. Comparison of Doppler echocardiography and right heart catheterization to assess pulmonary hypertension in systemic sclerosis. Br J Rheumatol. 1997; 36:239–243.
187. Medsger TA Jr, Silman AJ, Steen VD, Black CM, Akesson A, Bacon PA, et al. A disease severity scale for systemic sclerosis: development and testing. J Rheumatol. 1999; 26:2159–2167.
188. Jörg HW Distler, Marius M Hoeper and Oliver Distler. Diagnosis of pulmonary arterial hypertension in a patient with systemic sclerosis. Nature Clinical Practice Rheumatology (2008) 4, 160-164doi:10.1038/ncprheum0728.
189. D.Mukerji, St George D, Knight C, Davar J, Wells AU, Du Bois RM, Black CM, Coghlan JG.*Echocardiography and pulmonary function as screening tests for pulmonary arterial hypertension in systemic sclerosis*. Rheumatology (Oxford). 2004 Apr;43(4):461-6.
190. Wioleta K. Marut, Niloufar Kavian, Ame´lie Servettaz, Carole Nicco, Lalla A. Ba, Mandy Doering, Christiane Che´reau ,Claus Jacob, Bernard Weill and

Fre´de´ric Batteux. *The Organotelluride Catalyst (PHTE) 2NQ Prevents HOCl-Induced Systemic Sclerosis in Mouse.* Journal of Investigative Dermatology (2012) 132, 1125–1132.

191. Ismail S, Sturrock A, Wu P, Cahill B, Norman K, Huecksteadt TP, Sanders KA, Kennedy TP, and Hoidal JR. *NOX4 mediates hypoxia-induced proliferation of human pulmonary artery smooth muscle cells: the role of autocrine production of TGF-b1 and IGFBP-3.* Am J Physiol Lung Cell Mol Physiol 296: L489–L499, 2009.
192. Jiang Y, Dai A, Li Q, and Hu R. *Hypoxia induces transforming growth factor-beta1 gene expression in the pulmonary artery of rats via hypoxia-inducible factor-1alpha.* Acta Biochim Biophys Sin (Shanghai) 39: 73–80, 2007.
193. Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K,Sanders K, Karwande SV, Stringham JC, Bull DA, Gleich M, Kennedy TP, and Hoidal JR. *Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol 290: L661–L673, 2006.
194. Bedard K and Jaquet V. *Cell-free screening for NOX inhibitors*. Chem Biol 19: 664–665, 2012.
195. Altenho¨fer S, Kleikers PWM, Radermacher KA, Scheurer P, Rob Hermans JJ, Schiffers P, Ho H, et al. *The NOX toolbox: validating the role of NADPH oxidases in physiology and disease*. Cell Mol Life Sci 69: 2327–2343, 2012.
196. Liu J, Yang F, Yang X-P, Jankowski M, and Pagano PJ*. NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy*. Arterioscler Thromb Vasc Biol 23: 776–782, 2003.
197. Liu J, Ormsby A, Oja-Tebbe N, and Pagano PJ. *Gene transfer of NAD(P)H oxidase inhibitor to the vascular adventitia attenuates medial smooth muscle hypertrophy.* Circ Res 95: 587–594, 2004.

198. Lasse`gue B, San Martin A, and Griendling KK. *Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system*. Circ Res 110: 1364–1390, 2012.
199. Lee MY, San Martin A, Mehta PK, Dikalova AE, Garrido AM, Datla SR, Lyons E, et al. *Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation*. Arterioscler Thromb Vasc Biol 29: 480–487, 2009.
200. Jaquet V, Scapozza L, Clark RA, Krause K-H, and Lambeth JD. Small-molecule NOX inhibitors. *ROSgenerating NADPH oxidases as therapeutic targets.* Antioxid Redox Signal 11: 2535–2552, 2009.
201. Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CXC, and Laurindo FRM. *Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells.* J Biol Chem 280: 40813–40819, 2005.
202. Jacobson GM, Dourron HM, Liu J, Carretero OA, Reddy DJ, Andrzejewski T, and Pagano PJ. *Novel NAD(P)H oxidase inhibitor suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery.* Circ Res 92: 637–643, 2003.
203. Ibi M, Matsuno K, Shiba D, Katsuyama M, Iwata K, Kakehi T, Nakagawa T, et al. *Reactive oxygen species derived from NOX1/NADPH oxidase enhance inflammatory pain*. J Neurosci 28: 9486–9494, 2008.
204. Hecker L, Cheng J, and Thannickal VJ. *Targeting NOX enzymes in pulmonary fibrosis.* Cell Mol Life Sci 69: 2365–2371, 2012.
205. Griendling KK and FitzGerald GA. *Oxidative stress and cardiovascular injury: part II: animal and human studies*. Circulation 108: 2034–2040, 2003

206. Chen Z, Keaney JF, Schulz E, Levison B, Shan L, Sakuma M, Zhang X, et al. *Decreased neointimal formation in Nox2-deficient mice reveals a direct role for NADPH oxidase in the response to arterial injury*. Proc Natl Acad Sci U S A 101: 13014–13019, 2004.
207. Braunersreuther V, Montecucco F, Ashri M, Pelli G, Galan K, Frias M, Burger F, et al. *Role of NADPH oxidase isoforms NOX1, NOX2 and NOX4 in myocardial ischemia/ reperfusion injury.* J Mol Cell Cardiol 64: 99–107, 2013.
208. Block K and Gorin Y. *Aiding and abetting roles of NOX oxidases in cellular transformation.* Nat Rev Cancer 12: 627–637, 2012.
209. D Mukerjee, D St George, B Coleiro, C Knight, C P Denton, J Davar, C M Black, J G Coghlan. Prevalence and outcome in systemic sclerosis associated pulmonary arterial hypertension: application of a registry approach. Ann Rheum Dis 2003;62:1088–1093.
210. Matsunaga Y, Kawai Y, Kohda Y, and Gemba M.*Involvement of activation of NADPH oxidase and extracellular signal-regulated kinase (ERK) in renal cellinjury induced by zinc.* J Toxicol Sci (2005)30:135–144.
211. Amara N, Bachoual R, Desmard M, Golda S, Guichard C, Lanone S, Aubier M, Ogier-Denis E, and Boczkowski J. *Diesel exhaust particles induce matrix metallopro- tease-1 in human lung epithelial cells via a NADP(H) oxidase/NOX4 redox-dependentmechanism.* Am J Physiol Lung Cell Mol Physiol (2007)293:L170–L181.
212. Hasegawa T, Kikuyama M, Sakurai K, Kambayashi Y, Adachi M, Saniabadi AR,Kuwano H, and Nakano M. *Mechanism of superoxide anion production byhepatic sinusoidal endothelial cells and Kupffer cells during short-term ethanolperfusion in the rat*. Liver (2002) 22:321–329.

213. Hayashi T, Yamashita C, Matsumoto C, Kwak CJ, Fujii K, Hirata T, Miyamura M,Mori T, Ukimura A, Okada Y, et al. *Role of gp91phox-containing NADPHoxidase in left ventricular remodeling induced by intermittent hypoxic stress*.Am J Physiol Heart Circ Physiol (2008) 294:H2197–H2203.
214. Heinloth A, Heermeier K, Raff U, Wanner C, and Galle J. *Stimulation of NADPH oxidase by oxidized low-density lipoprotein induces proliferation of humanvascular endothelial cells.* J Am Soc Nephrol (2000) 11:1819–1825.
215. Hingtgen SD, Tian X, Yang J, Dunlay SM, Peek AS, Wu Y, Sharma RV, EngelhardtJF, and Davisson RL. *Nox2-containing NADPH oxidase and Akt activationplay a key role in angiotensin II-induced cardiomyocyte hypertrophy.* PhysiolGenomics (2006)26:180–191.
216. Hishikawa K, Oemar BS, Yang Z, and Lu¨ scher TF. *Pulsatile stretch stimulatessuperoxide production and activates nuclear factor-kappa B in human coronarysmooth muscle*. Circ Res (1997) 81:797–803.
217. Jiang F, Drummond GR, and Dusting GJ. *Suppression of oxidative stress in the endothelium and vascular wall.* Endothelium (2004) 11:79–88
218. Katsuyama M, Fan C, and Yabe-Nishimura C. *NADPH oxidase is involved in prostaglandin F2alpha-induced hypertrophy of vascular smooth muscle cells: inductionof NOX1 by PGF2alpha*. J Biol Chem(2002) 277:13438–13442.
219. Liu H, Zhang H, Iles KE, Rinna A, Merrill G, Yodoi J, Torres M, and Forman HJ. *The ADP-stimulated NADPH oxidase activates the ASK-1/MKK4/JNK pathway in alveolar macrophages*. Free Radic Res(2006) 40:865–874.
220. Lopes NH, Vasudevan SS, Gregg D, Selvakumar B, Pagano PJ, Kovacic H,and Goldschmidt-Clermont PJ. *Rac-dependent monocyte chemoattractant protein-1 production is induced by nutrient deprivation*. Circ Res(2002) 91:798–805

221. Luchtefeld M, Drexler H, and Schieffer B. *5-Lipoxygenase is involved in theangiotensin II-induced NAD(P)H-oxidase activation*. Biochem Biophys Res Commun 308 (2003):668–672.
222. Muzaffar S, Shukla N, Lobo C, Angelini GD, and Jeremy JY. *Iloprost inhibits superoxide formation and gp91phox expression induced by the thromboxane A2 analogue U46619, 8-isoprostane F2alpha, prostaglandin F2alpha, cytokines and endotoxin in the pig pulmonary artery.* Br J Pharmacol (2004b) 141:488–496.
223. Narayanan PK, Goodwin EH, and Lehnert BE. *Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells.* Cancer Res (1997) 57:3963–3971
224. Parinandi NL, Kleinberg MA, Usatyuk PV, Cummings RJ, Pennathur A, Cardounel AJ, Zweier JL, Garcia JG, and Natarajan V. *Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells*. Am J Physiol Lung Cell Mol Physiol (2003) 284:L26–L38
225. Yamakawa T, Tanaka S, Yamakawa Y, Kamei J, Numaguchi K, Motley ED, Inagami T, and Eguchi S. *Lysophosphatidylcholine activates extracellular signalregulated kinases 1/2 through reactive oxygen species in rat vascular smooth muscle cells.* Arterioscler Thromb Vasc Biol (2002) 22:752–758.
226. Chapman KE, Sinclair SE, Zhuang D, Hassid A, Desai LP, and Waters CM. *Cyclic mechanical strain increases reactive oxygen species production in pulmonary epithelial cells.* Am J Physiol Lung Cell Mol Physiol (2005) 289:L834–L841.
227. Chowdhury AK, Watkins T, Parinandi NL, Saatian B, Kleinberg ME, Usatyuk PV, and Natarajan V. *Src-mediated tyrosine phosphorylation of p47phox in hyperoxiainduced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells.* J Biol

ACKNOWLEDGEMENT

I am very much thankful and grateful to my supervisor Prof.Gianfranco Pintus for all the support and guidance throughout my PhD Studies.

My deepest thanks to Dr.Roberta Giordo, Dr.Annalisa Cossu and Dr.Anna Maria Posadino, who have greatly contributed to this work.

I would like to extend many thanks to our collaborator Dr. Francesco Boin, Johns Hopkins University School of Medicine, Baltimore for his contribution to the clinical part of this study.

I would like to thank other lab members and our other collaborators Prof.Giuseppe Passiu & Dr.GianLuca Erre from Unit of Rheumatology, University of Sassari, who also have contributed to this work.

I am very much grateful to our International PhD School, faculty and to our University of Sassari for all the support provided to me during my PhD studies. I am also thankful to my all friends and those who have directly or indirectly given their support during my studies.

At last, I would like to immensely thank my parents and all my family members and my wife Smitha for providing all the support during my entire PhD Studies.