UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head Office: Università degli Studi di Padova

Department of Women's and Children's Health

Ph.D. Course in Developmental Medicine and Healthcare Planning Sciences

Curriculum: Oncohematology, Medical Genetics, Rare Diseases and Predictive Medicine

Series XXXV

MICROVASCULOPATHY IN SYSTEMIC SCLEROSIS: THE ROLE OF LONG NON-CODING RNA *H19X*

Thesis written with the financial contribution of PENTA Foundation

Coordinator: Ch.mo Prof. Gianni Bisogno

Supervisor: Ch.mo Prof. Francesco Zulian

Ph.D. Student: Dott.sa Francesca Tirelli

A chi ha sempre creduto in me

ABSTRACT

Background: Systemic sclerosis (SSc) is an autoimmune disease characterized by inflammation, vasculopathy, and fibrosis of the skin and internal organs. Long non-coding RNAs (lncRNAs) are non-coding transcripts involved in the regulation of gene expression. This study aimed to characterize the functional relevance of *H19X* in SSc microvasculopahty

Methods: *H19X* expression in SSc skin biopsies was computed on single–cell RNA–sequencing data (scRNA-seq). Analysis of differential expression of endothelial cell (EC) injury markers and pathway enrichment analysis (KEGG) between *H19X* expressing cells and H19X negative cells was carried out. The function of *H19X* was investigated in human dermal microvascular EC (HDMEC) by silencing, using locked nucleic acid antisense oligonucleotides (LNA GapmeRs). H19X expression was analyzed by qPCR after HDMEC stimulation with proinflammatory cytokines. Gene expression was measured by qPCR. Protein levels were analyzed by Western Blot. Endothelial adhesion was evaluated by co-culture of HDMEC and fluorescently labelled peripheral blood mononuclear cells (PBMCs). Phalloidin staining was used to study cell contractility.

Results: scRNA-seq data showed a significant upregulation of H19X in SSc compared to healthy EC. In HDMEC, *H19X* was consistently induced by IFNα, IFNβ and IFNγ. Adhesion molecule VCAM1 was identified as its target, as *H19X* knockdown lead to a consistent and significant decrease both at mRNA and protein level. Moreover, activity of Rac1 and PAK, two downstream VCAM1 effectors, and VCAM1-induced cell contractility were also reduced following H19X knockdown. Correlation between adhesion molecules and H19X was confirmed by co – expression analysis and KEGG pathway analysis performed on ScRNA-seq data. The functional relevance of H19X on endothelial adhesion was confirmed by PBMCs with *H19X* silenced HDMEC, where it was demonstrated a significant decrease in leucocyte-to-endothelial cell adhesion.

Conclusion: This study provides evidence for a functional role of lncRNA *H19X* in microvascular EC. By acting as a regulator of adhesion molecules, *H19X* contributes significantly to SSc – associated microvasculopathy.

TABLE OF CONTENTS

INTRODUCTION

1. SYSTEMIC SCLEROSIS

Systemic Sclerosis (SSc), or scleroderma, is a multisystem connective tissue disease characterized by hardening of the skin and fibrosis of the internal organs, resulting from a pathogenic triad of vascular damage, immune dysregulation and fibrosis (1). Although rare, with a reported annual incidence that varies between 8 to 56 new cases per million persons (2), the disease is burdened by very high morbidity and mortality, greater than any other rheumatic condition, and is considered an orphan disease (3). The pharmacological treatment of SSc, remains a major challenge, as current treatment strategies are mainly based on the management of single organ involvement (4), while, to date, no single agents have been demonstrated to significantly modify overall disease course. In this heterogeneous condition with such complex etiology, advanced research is needed to guide novel approaches in the management of the disease and to address the several unmet clinical needs faced by SSc patients.

1.1 Clinical manifestations

Clinical manifestations of SSc can be very heterogeneous, potentially involving all major organs and systems as the disease progresses over time. From Greek, the term "scleroderma" means "hard skin" and, indeed, induration of the skin is a clinical hallmark of this disease. Skin changes are characterized initially by edema and then by fibrosis of the skin, that typically involves the face and extremities, however can be diffuse. Cutaneous induration of the face causes a typical expressionless face while involvement of the fingers, known as sclerodactyly, can be associated with significant disability as severe induration causes a limited range of motion (3, 5) (Figure 1A-B). Raynaud's phenomenon (RP) is an

almost universal finding in SSc that may precede the development of other organs involvement by years. (1, 6). It is characterized by typical color changes (white/purple/red – Figure 1C) of the fingers and sometimes toes, and it is caused by arterioles and capillary spasm caused by several triggers, especially cold exposure

Disease progression is associated with development of vascular manifestations along with fibrotic changes affecting internal organs. Other than RP, nailfold capillaroscopy changes are frequent and show typical patterns related with different disease stages (7); other manifestations include digital pitting ulcers that can complicate with digital infarction (Figures 1D-E).

Gastrointestinal involvement in typical and may be found in nearly 90% of patients, although most are asymptomatic (8, 9). Common manifestations include symptoms of gastro – esophageal reflux as wells as altered esophageal dysmotility. Muscle – skeletal symptoms, including arthralgias, arthritis and poor muscle strength, are often reported and might correlate with disease progression (10). Renal impairment is common, while scleroderma renal crisis, a life – threatening complication, presents in less than 10 of cases (11). Finally, pulmonary manifestations, including Interstitial Lung Disease (ILD) and Pulmonary Arterial Hypertension (PAH) can complicate up to 80% of SSc cases and present since the early phases of the disease, while cardiovascular involvement presents overtly in 10 – 30%, particularly as heart failure and arrythmias (12, 13). Together, cardiopulmonary manifestations represent that main cause of death in SSc (14, 15).

As for laboratory findings, autoantibodies are typically detected is typical and are useful in the classification of the disease. Antinuclear antibodies (ANA) are positive in more than 90% of patients, while scleroderma – specific ones, including anti – topoisomerase (anti-Scl-70), anti – centromere (ACA) and anti – polimerase III (anti – RNAP) associate with different organ involvement.

1.2 Juvenile Systemic Sclerosis

Juvenile Systemic Sclerosis (JSSc) is even more rare than its adult counterpart. Annual incidence is reported as 0.27 per million children under the age of 16 years (16) , and onset of systemic sclerosis in pediatric age is uncommon, accounting for only 5% of cases (17). Mean age of presentation is 8 years and the disease affects equally boys and girls under the age of 8, while in older children female to male ration is 3:1 (6). Severe morbidity and high mortality are common also in the pediatric age, especially in those children with rapidly progressive course leading to organ failure, severe disability and eventually death (18). Clinically, the onset of the disease in children is often insidious. Raynaud's phenomenon (RP) is the most common presenting sign (6) while skin changes resemble those of adult SSc. Vascular manifestations are common, involving more than three quarter of cases, as reported in a large international cohort involving 153 children with JSSc (6). Muscle – skeletal and gastrointestinal are also often reported, while cardiopulmonary involvement is rarer than in adults (6), however remaining the leading cause of mortality also in JSSc. Finally, renal involvement is rarely reported in children, varying from isolated proteinuria to potentially fatal renal crisis. As for laboratory findings, high titer ANA are reported in 78–97% of patients in the jSSc cohorts (19) and scleroderma – specific antibodies are also present as reported in adults, however in divergent frequencies, mirroring the slightly different clinical phenotype in children with SSc (6, 19). Among scleroderma specific autoantibodies, anti - RNAP are very rare in children (5). Other autoantibodies, such as Anti-PM-Scl and anti-U1RNP, are associated with scleroderma overlap syndromes with other connective tissue disorders, such as dermatomyositis or mixed connective tissue disease (5).

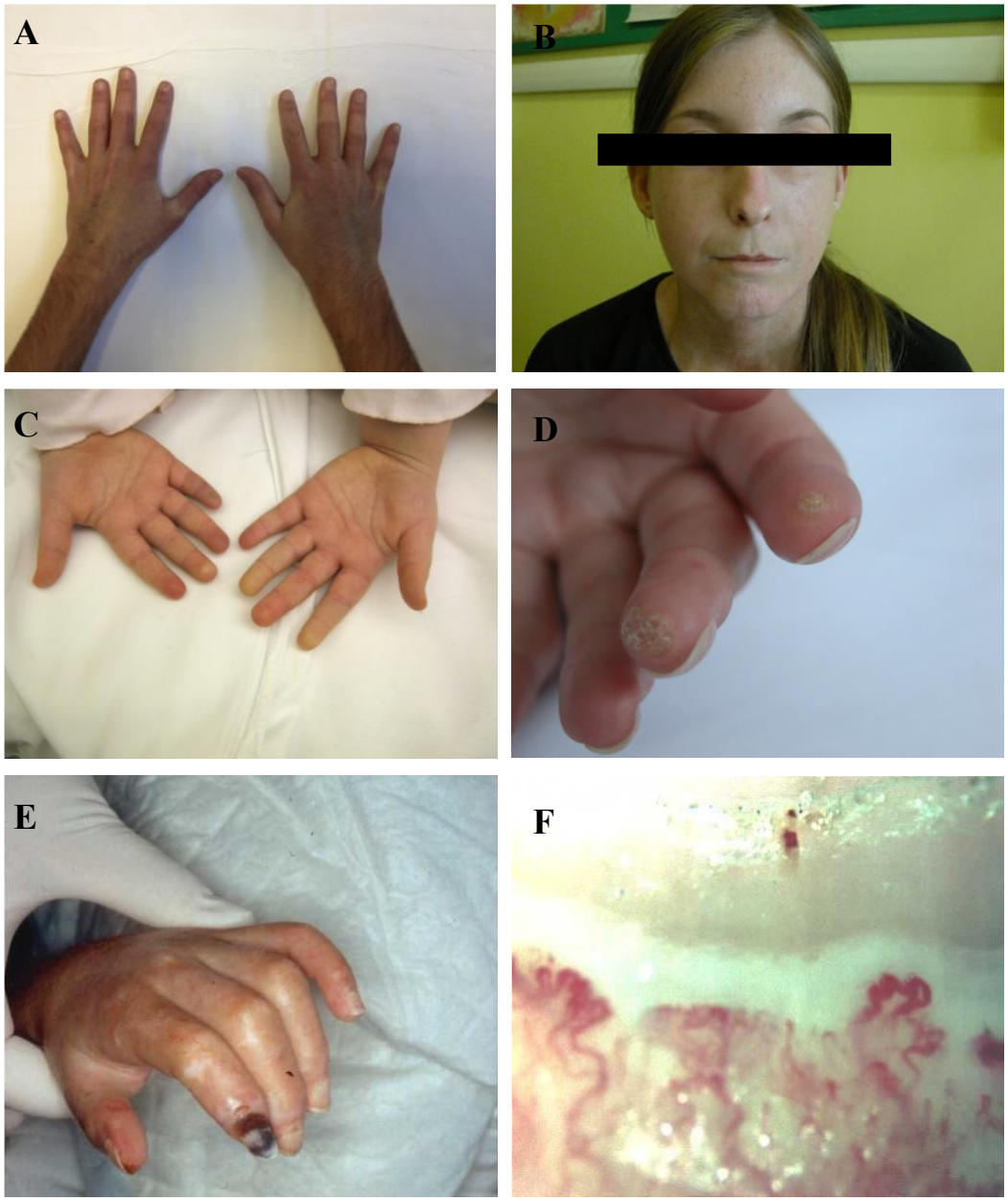


Figure 1. Clinical features of Systemic Sclerosis (A) Sclerodactily **(B)** Typical expressionless face **(C)** Raynaud's phenomenon **(D)** Digital pitting ulcers **(E)** Fingertip necrosis **(F)** Capillaroscopy scleroderma pattern.

Courtesy of Prof. Francesco Zulian

1.3 Classification

Clinical classification of SSc is primarily based on the extent of cutaneous fibrosis, identifying those patients with only distal involvement as having limited form of the disease (lcSSc), while those with proximal and truncal involvement as having the diffuse form (dcSSc) (20, 21). Along with the typical cutaneous pattern, the prevalence of internal organ involvement and other clinical manifestations also differs across the subtypes, as well as autoantibody positivity. Moreover, a minority of cases have peculiar characteristics that define two more disease variety, including SSc sine scleroderma, characterized by the absence of typical skin induration, which has been recently described in a few cases also in children (22), and overlap syndrome, which shares features with other autoimmune connective tissue disease (3, 5) .

Table 1 summarizes the main clinical features of the four SSc subtypes.

SSc subtype	Main Clinical Features	Autoantibodies
Diffuse cutaneous SSc (dcSSc)	• Proximal and distal skin fibrosis of the skin, involvement of the face and trunk • More common and severe involvement of internal organs	Anti – topoisomerase (Anti – Scl70)
Limited cutaneous SSc (lcSSc)	• Skin fibrosis distal to elbows and knees • Esophageal involvement • Cutaneous calcinosis	Anti – centromere (ACA)
SSc Sine Scleroderma (SSSS)	• Absence of skin induration • Typical vascular and internal organ manifestation • In children, higher cardiac mortality	
Overlap Syndrome	• Combination of symptoms typical of Systemic Lupus Erythematosus, Dermatomyositis or Arthritis	Associated with overlapping condition

Table 1. Clinical and laboratory features of SSc subtypes

1.4 Pathogenesis

Etiology of scleroderma remains unclear, but it is believed that a combination of genetic and environmental factors can start a complex pathogenic cascade that leads to the development of inflammation, vasculopathy and fibrosis.

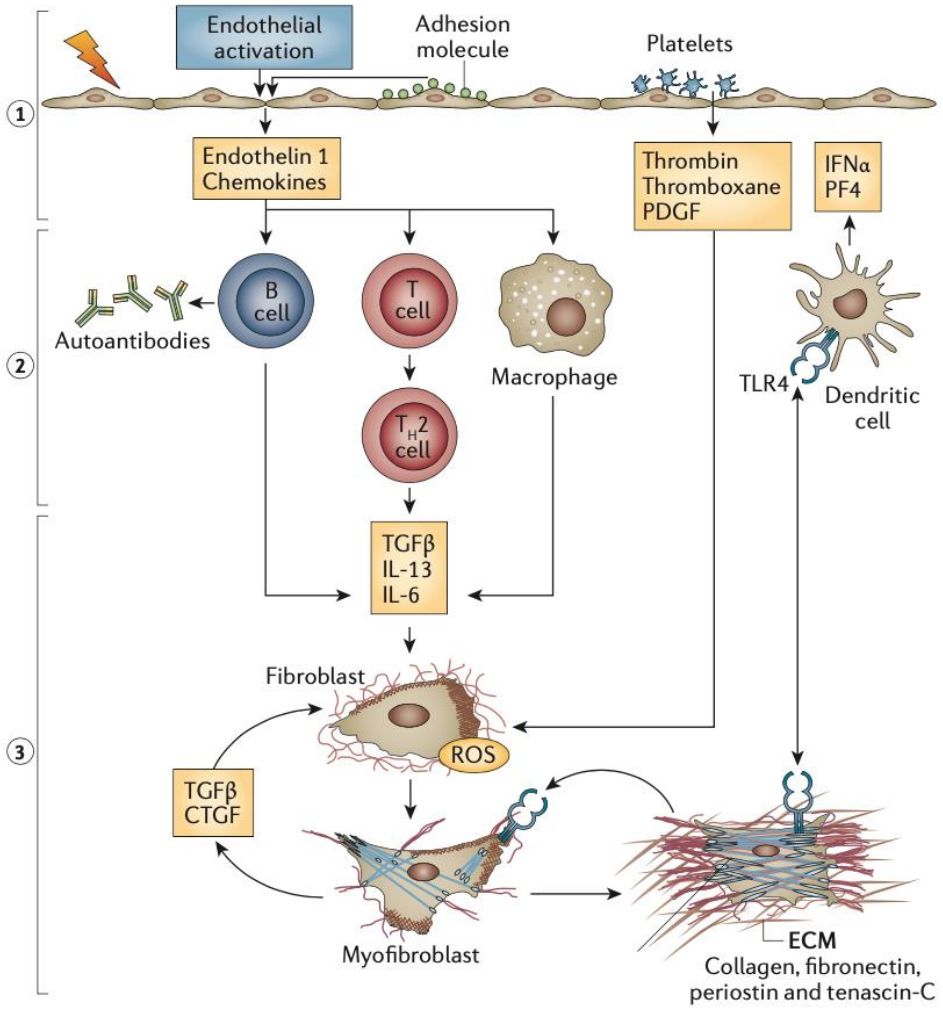


Figure 2. The three pathogenic hallmarks of Systemic Sclerosis (1) Endothelial activation and systemic widespread vasculopathy occur since the very early phases of the disease (2) Dysregulation of both innate and adaptive immune systems contributes to development of inflammation (3) Fibrosis, characterized by myofibroblast activation and excessive extracellular matrix deposition, develops as final, not reversible alteration. *TGF Tumor Growth Factor; CTGF Connective Tissue Growth Factor; PDGF Platelet-Derived Growth Factor; TRL Toll-like Receptor; IFN Interferon; PF Platelet Factor; IL Interleukin; ECM Extracellular Matrix; ROS Reactive Oxygen Species*

From Allanore Y, et al. Nat Rev Dis Primer 2015 (1)

1.4.1 Inflammation and immune system dysregulation

Dysregulation of both innate and adaptive immunity occurs in SSc. As seen, autoantibodies are commonly observed in these patients, strongly indicating activation of the immune system against self-antigens. Circulating and tissue-infiltrating monocytes and macrophages, plasmacytoid dendritic cells and stromal cells show a type I Interferon signature, that reflects activation of Toll-like receptor (TLR)-mediated immune signaling (23-26). TLR activation in these cells is thought to be triggered by endogenous ligands, such as nucleic acid-containing immune complexes, as well as by damage-associated molecular patterns (DAMPs), such as variants of extracellular matrix components generated during tissue injury. Fibrotic tissue also displays prominent infiltration of bone marrow-derived immune cells that include CD4+ T cells, macrophages, activated B cells. Among CD4+ T cells, type 2 T Helper Cells (TH2) – characterized by secretion of IL-4 and IL-13 — predominate over TH1 cells (27). Finally, Monocyte/macrophages seem to be involved in the development of fibrosis in SSc by affecting immune responses and influencing fibroblast activation and differentiation. In the skin of adult patients with SSc, tissue macrophages are shown to produce several pro-inflammatory mediators, such as chemokines that promote T cell recruitment, and pro-fibrotic mediators (28). Likewise, circulating monocytes are able to produce several key mediators that influence fibrosis directly or through the production of pro-fibrotic mediators. Peripheral blood mononuclear cells (PBMC) from SSc patients show, for example, enhanced levels of Tumor Growth Factor β1 (TGFβ1), the key cytokine involved in fibrogenesis (29) as well as several cytokines, and chemokines (28). Interestingly, some studies have also suggested that the activation of TLRs as well as the inflammasome might contribute to the pathogenesis of SSc (30, 31).

1.4.2 Fibrosis

Fibrosis, the hallmark of scleroderma syndromes, is the result of excessive extracellular matrix (ECM) deposition by activated fibroblasts. Under physiologic conditions, tissue-resident fibroblasts, upon induction of tissue injury stimuli, can transiently differentiate into myofibroblasts, alpha-Smooth Muscle Actin (α-SMA) expressing cells with contractile properties. Myofibroblasts, under control of TGFβ1 and other profibrotic mediators, participate in tissue healing processes with deposition of ECM components and subsequently undergo deactivation and apoptosis (32). In scleroderma, a very complex combination of intracellular signaling cascade, involving the key players TGFβ1 and Platelet – Derived Growth Factor (PDGF), result in pathological activation, differentiation and apoptosis resistance of fibroblasts, leading to progressive aberrant ECM deposition and remodeling (1).

1.4.3 Vasculopathy

Widespread systemic vasculopathy occurs in SSc. Macroscopically, vascular manifestations, including Raynaud's phenomenon, digital ulcers and pulmonary hypertension are prominent features that present since the earliest stages of the disease, indicating from a clinical standpoint that the vascular component is very relevant in SSc (33) (Figure 3). Most importantly, the main alterations involve the microcirculation and small vessels. Microvascular changes, such as loss of capillaries, vessel wall thickening of small arteries and perivascular mononuclear infiltration are found even in sites not affected by fibrosis and might be detected before the onset of overt symptoms (33). Impairment of endothelial function, therefore, occurs very early in disease course, suggesting that the microvascular endothelial cells (EC) are primary targets in SSc pathogenesis.

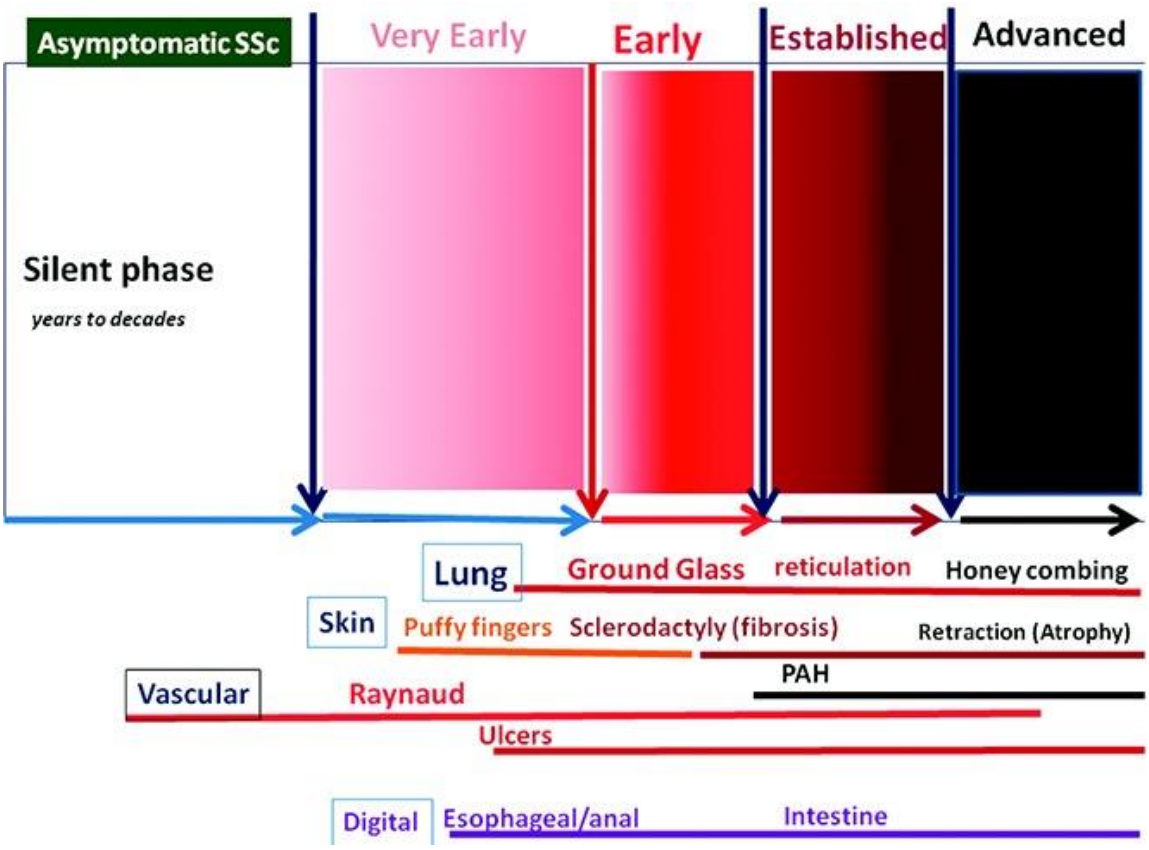


Figure 3 Macrovascular and other organ manifestations development over time in SSc. Alteration of the microvasculature are detected even before symptoms development. *PAH Pulmonary Arterial Hypertension From Matucci-Cerinic M et al, Arthritis Rheum. 2013(33)*

Endothelial activation and microvascular injury are well described in SSc and are believed to play a crucial role in disease onset and progression (34, 35).

Microvascular injury in SSc is characterized by development of vascular structural changes and impairment of numerous endothelial functions, which are supposed to be triggered by external factors that have not been fully identified. It is hypothesized that, on a background of genetic predisposition, some triggers such as anti – endothelial antibodies, specific lymphocytes and/or environmental factors can induce damage to the endothelium and initiate the microvasculopathy cascade (36)(Figure 4).

Endothelial cell damage injury increases cellular death and apoptosis, which lead to aberrant compensatory neoangiogenesis and vasculogenesis, as demonstrated by a significant imbalance circulating levels of pro- and anti – angiogenetic factors described in SSc (35, 36). Consequently, capillaries reduce in number and vascular remodeling occurs, causing progressive obliterative vasculopathy that favors tissue hypoxia and oxidative stress (1, 36) (Figure 4). At the same time, activated EC are crucial in the development of inflammation and fibrosis, as they produce higher levels of pro – inflammatory cytokines, pro – fibrotic factors and vasoactive mediators such as endothelin – 1 (1, 36) (Figure 4).

A large systematic review on almost 200 papers (35) has summarized the most recent evidence on biomarkers associated with SSc vasculopathy, emphasizing how endothelial injury involves several different aspects of EC functions, such as angiogenesis, apoptosis, coagulation and others. Interestingly, among the dysregulated functions, altered endothelial adhesion resulted one of the most relevant and best described in the literature. Biomarkers of endothelial adhesion such as Vascular cell adhesion protein 1 (VCAM – 1) and E – Selectin are elevated in the serum of SSc patients, as their expression is augmented upon endothelial activation (35, 37, 38). This mechanism is pivotal for inflammatory conditions, as it initiates leucocyte rolling and adhesion, promoting immune cells recruitment into the tissues, and initiating, in fact, the inflammatory infiltration of the tissues.

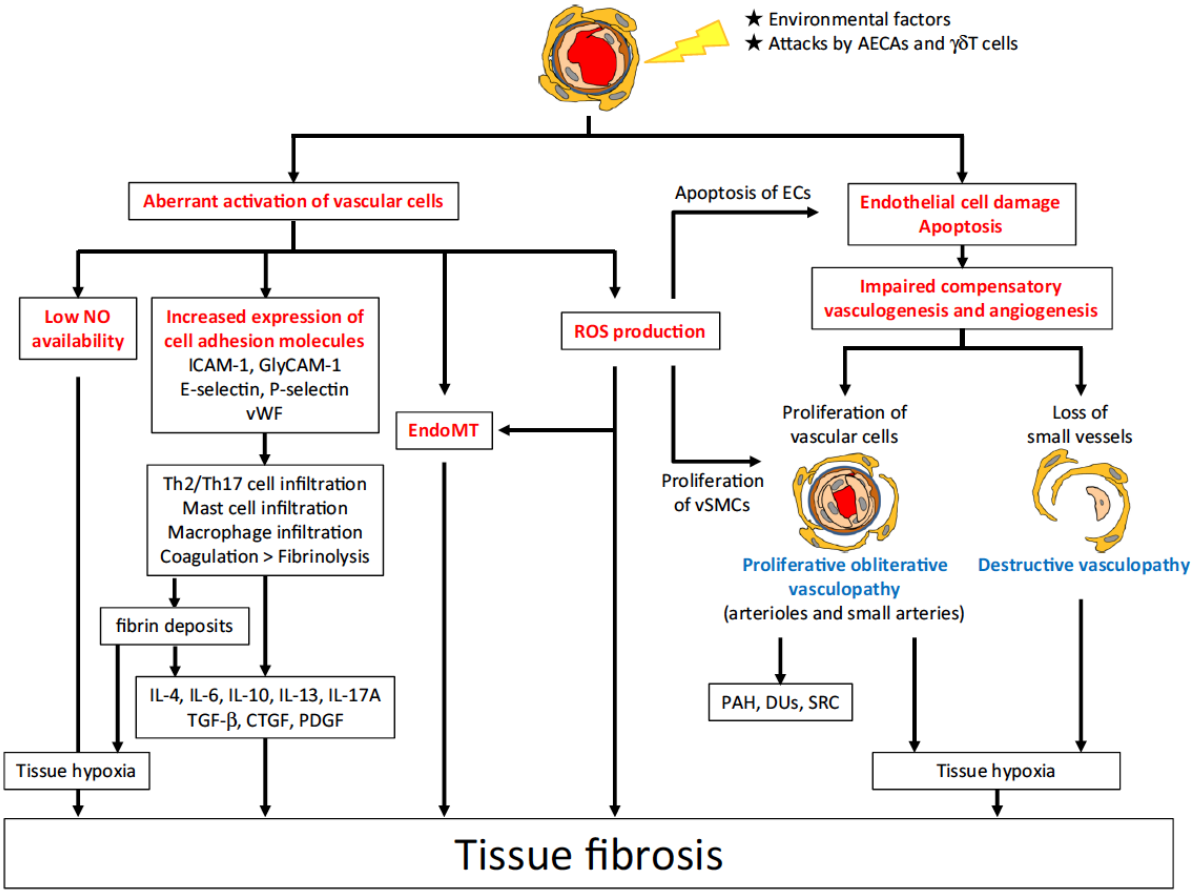


Figure 4 Schematic representation of the pathogenic mechanism involved in endothelial activation in Systemic Sclerosis. Following damage unidentified external triggers, injured endothelial cells display both functional and structural alterations that contribute to development of vasculopathy and favors tissue fibrosis. *EC Endothelial Cells; NO Nitric Oxide; ICAM-1 Intercellular Adhesion Molecule 1; GlyCAM-1 Glycosylation-dependent cell adhesion molecule-1; vWF von Willebrandt Factor; IL interleukin; TGF- β Tumor Growth Factor-β; CTGF connective tissue growth factor; PDGF Platelet – Derived Growth Factor; EndoM Endothelial – to. – mesenchymal Transition; ROS Reactive Oxygen Species; vSMC vascular Smooth Muscle Cells; PAH Pulmonary Arterial Hypertension; DU Digital Ulcers; SRC Scleroderma Renal Crisis Form Asano Y, Sato SA. Semin Immunopathol (2015) 37 (36)*

2. LONG NON – CODING RNAs AND THEIR ROLE IN HUMAN DISEASES

2.1 Classification and roles of non – coding RNAss

Protein – coding genes, in spite of being the most known and best characterized, account for less than 2% of the whole human genome (39). In the last decade, non – coding regions of the genome have been annotated thanks to the implementation of high – throughput techniques, and it has become increasingly clear not only that most of the non – protein – coding regions are transcribed, but also non – coding transcripts play a crucial role as functional regulators in cellular biology. Likewise, and not surprisingly, it is now emerging how alterations in the expression and/or function of non-coding RNAs (ncRNA) are relevant in the development of many diseases (40).

NcRNA can be distinguished into two categories: housekeeping ncRNA, such as transfer RNA (tRNA), ribosomal RNA (rRNA) or small nucleolar RNA (snoRNA) are expressed ubiquitously and have a known role in the regulation of cell functions. On the other hand, other ncRNA display a tissue – specific expression and exert their function in gene expression regulation. Based on the size, we recognize long non – coding RNAs (lncRNA) which are longer than 200 nucleotides and are transcribed without prior processing, while small ncRNA, including microRNA (miRNA), small interfering RNA (siRNA), and PIWI-interacting RNAs (piRNA) are smaller molecules that are generally processed from longer precursors (41, 42).

2.2 Small non – coding RNAs

miRNA are small ncRNA of 22 nucleotides in length, and are so far the most widely studied. They are known to act as post – transcriptional regulators of gene expression, either by silencing single target genes by binding messenger RNA (mRNA), or by controlling hundreds of genes at a time and being involved in signaling pathways, functioning as master

regulators of processes like cell proliferation or apoptosis (40). As such, several different miRNAs are being identified as novel potential diagnostic and prognostic biomarkers in numerous human diseases, such as cancer, infections, cardiovascular disease and nephropathy (43). The other types of small ncRNA have been described more recently and their function is less characterized: piRNA are 24 – 30 nucleotides long and they interact with PIWI proteins, which are mainly expressed in the germline, and the piRNA/PIWI complex is crucial in transposon silencing (40, 44, 45); snoRNA are 60–300 bp in length, are located in the nucleoli and are involved in post – transcriptional modifications of ribosomal RNA (40). Some studies have investigated the role of piRNA and snoRNA in human cancer, but their role in human diseases is still under study.

2.3 Long non – coding RNAs

lncRNA are conventionally defined as transcripts longer than 200 nucleotides, which are not translated into proteins, that function as gene expression regulators (40).

LncRNA are transcribed by RNA Polymerase II; as such, they likely undergo transcriptional and post transcriptional processes similar to mRNA, and many have 5′-end m7G caps and 3′-end poly(A) tails; however, different transcriptional processes for these transcripts are emerging (46, 47). In contrast with mRNA, lncRNA are mainly located in the nucleus, where they occupy the chromatin fraction; those that are exported to the cytosol associate with organelles, especially with the ribosome, however it is still unknown whether they are engaged in translational processes (46, 47). Interestingly, it is also emerging how lncRNA tend to have tissue and cell – type specific expression patterns, likely related with their processing and functions (46, 47).

As for their function, lncRNA can be differentiated in those that exert their role in *cis*, thus regulating the expression of neighboring genes, and those that act in *trans*, influencing

cellular functions in sites other from the site of synthesis (48). Consequently, lncRNA exert a regulatory effect on gene expression at multiple levels (Figure 5). Transcriptional regulation is mediated by recruitment and modulation of transcription factors in specific DNA sequences in their locus, while post – transcriptional regulation includes regulation of processes of splicing, capping and other that influence mRNA stability. Moreover, numerous lncRNA, such as X-inactive specific transcript (XIST) and HOX transcript antisense RNA (HOTAIR), act as strong epigenetic modifiers by regulating chromatin accessibility throughout different mechanisms, including recruitment of chromatin modifiers. Finally, some lncRNA can also regulate signaling pathways by post – translational modifications, for instance by directly binding to the phosphorylation sites of some transcription factors to influence their activation status (48, 49).

2.4 Long non – coding RNAs in human diseases

Given the growing evidence that supports their relevance in many biologic processes, lncRNA are being extensively investigated as novel pathogenetic targets across many different conditions, although the exact mechanisms underlying their functions in disease development remain largely to be understood.

Many of the current evidence comes from the field of oncology: dysregulation of lncRNA has been described in different types of solid cancers and in leukemia, both with pro – oncogenic and anti – tumorigenic effects; thanks to their tissue specificity, these transcripts are being investigated as potential diagnostic biomarkers or even as therapeutic targets (35). Moreover, as it is emerging that lncRNA exert key functional roles in cardiac and neural development, they are also being investigated in the pathogenesis of cardiovascular and neurodegenerative disorders (36-39).

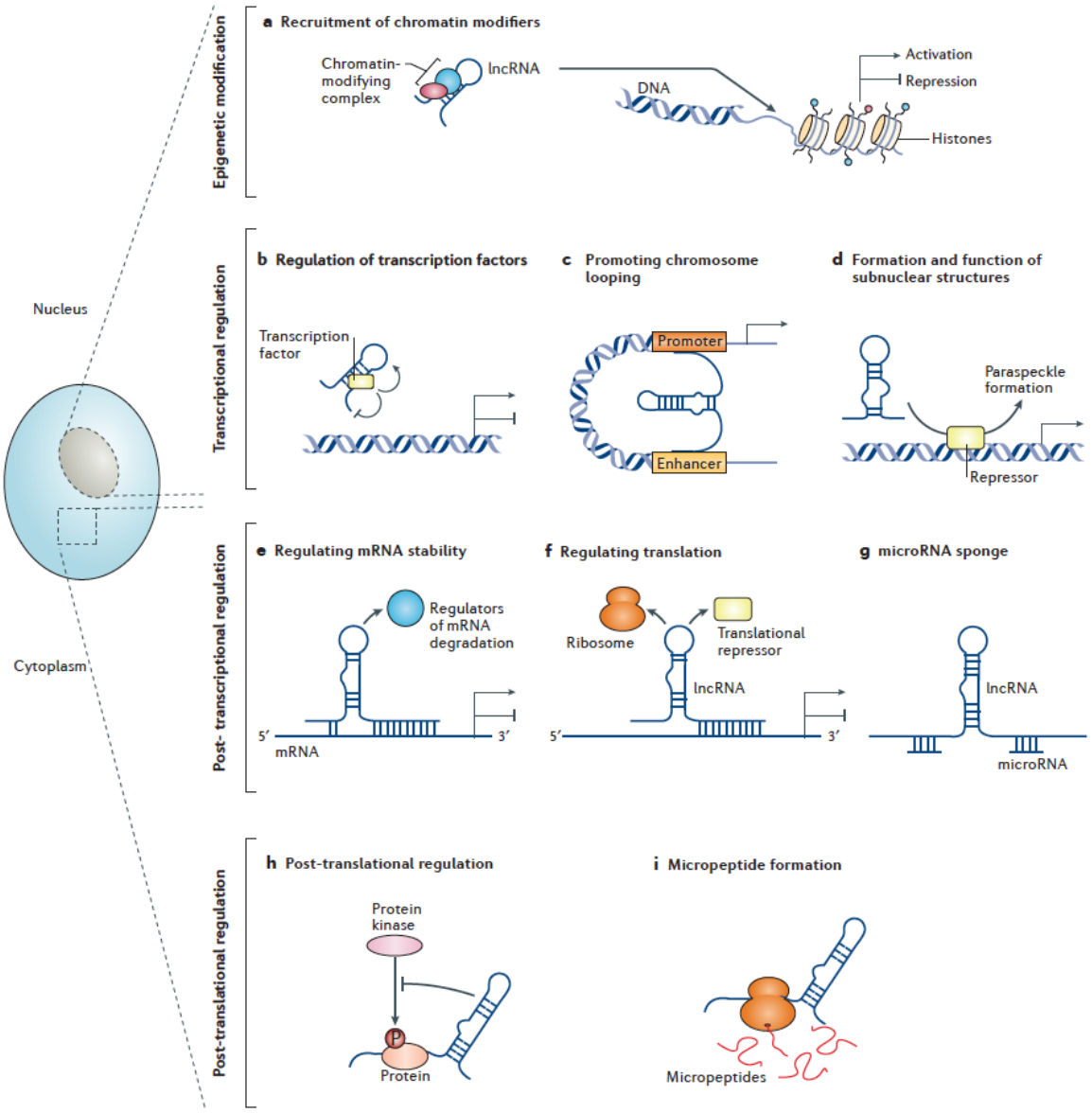


Figure 5. LncRNAs exert their regulatory function in gene expression regulation at multiple levels. LncRNA exert their gene expression regulation functions both on neighboring genes (in *cis*), especially by chromatin remodeling and recruitment of transcription factors, as well as in other cellular compartments (in *trans*) at transcriptional, post – transcriptional and post – translational level.

From Tang Y et al. Nat Rev Rheumatol 2017 (49)

The relevance of non – coding RNAs is recently emerging for rheumatic and autoimmune diseases, chronic conditions with unknown cause. Dissecting the role of lncRNA in these conditions might shed new light on their complex pathophysiology, helping to find new diagnostic and therapeutic strategies. For instance, in Systemic Lupus Erythematosus (SLE), lncRNA Gas5 has been associated with disease susceptibility, while lncRNA linc0949 results upregulated in circulating PBMC and correlates well with disease activity indexes and response to immune suppressants, thus representing a potential disease activity marker (49, 50). In Rheumatoid Arthritis (RA), both lncRNA HOTAIR and H19 have been found upregulated in the synovium and might contribute to promote inflammation, one by recruiting inflammatory cells and the other by increasing the expression of pro – inflammatory cytokines (49).

Few evidence is available in SSc: lncRNA OTUD6B-AS1 has been shown downregulated in skin of SSc patients and seems to correlate with fibroblast and vascular smooth muscle cells apoptosis, while lncRNA H19X, which is found upregulated in SSc skin, appears to be a pivotal regulator of TGFβ driven fibrosis (51, 52).

2.5 Long non – coding RNA H19X

The human H19X locus, located on Xq26.3, encodes several ncRNA, which include six miRNA (miR-424, 503, 542, 450-1, 450-2, 450b) and lncRNA H19X, also known as MIR503 hosting gene (MIR503HG), which is the hosting gene for the miRNA cluster (Figure 6).

H19X locus – encoded ncRNA appear to be involved in crucial cellular functions, including differentiation of striate muscle fibers and monocytes, proliferation and apoptosis (53). Interestingly, lncRNA H19X in endothelial cells seems to act as a sensor for hypoxia, and might be involved in angiogenesis regulation (54).

The role of lncRNA H19X so far has been described in a few diseases. In some cancers, H19X acts as a tumor suppressor, reducing cell proliferation, limiting cell invasiveness and inducing apoptosis (55-59). Recently, H19X has been investigated in endothelial cells (EC) from pulmonary artery hypertension (PAH) vasculature and PAH models, and its depletion has been associated with endothelial – to – mesenchymal transition, indicating that it might be involved in the processes of vascular remodeling (60).

In another study, it was showed that H19X is upregulated in the skin from biopsies of SSc as well as across several fibrotic disorders (52). The study demonstrated that the presence of H19X is a pivotal mediator required for the tissue remodeling effects of TGFβ that lead to fibrosis, and is also needed for myofibroblast differentiation and survival. In fibroblasts, DNA damage-inducible transcript 4-like protein (DDIT4L) was identified as an effector of H19X and it was showed that H19X regulates DDIT4L by regulating chromatin accessibility. Therefore, H19X might be a novel relevant marker associated with the processes of fibrosis in SSc.

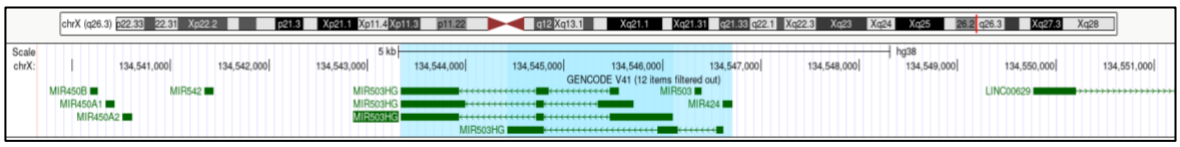


Figure 6. H19X locus on Xq26.3, encodes for lncRNA H19X and several miRNA

Created with UCSC Genome Browser

AIM OF THE THESIS

Following a previous study showing the relevance of lncRNA H19X in SSc – associated fibrosis and based on the evidence that H19X is upregulated in SSc skin biopsies (52) , we hypothesized that this lncRNA might be a mediator of more cellular mechanism involving other cellular types relevant to the pathogenesis of SSc.

In the present study, therefore, we investigated whether lncRNA H19X is involved in endothelial dysfunction and microvascular injury, in order to highlight its potential role in the development of SSc vasculopathy.

The study described in this thesis was performed in collaboration with the Center of Experimental Rheumatology of the University Hospital of Zurich, Switzerland. It represents the first part of a project, which will be carried out at the Department of Women's and Children's Health of the University Hospital of Padua also after the end of this PhD, which plans to apply sequencing techniques and to study the role of non-coding RNAs also in the pathogenesis of juvenile scleroderma syndromes.

METHODS

Subjects

Full-thickness 3 mm skin biopsies were obtained from 27 SSc adult patients and from 10 healthy controls (61). Patients were recruited at the Scleroderma Clinic of the University of Pittsburgh Medical Center (UPMC) and at the Scleroderma Program of the University of Michigan Medical School. All had confirmed diagnosis of active diffuse cutaneous SSc (dcSSc) according to the current criteria (20, 21). Active disease was defined as moderate to high skin scores, and/or early in their disease process. Twenty – one patients (74%) had either a high skin score >25 and/or were within 2 years of first non-Raynaud's disease manifestation. Only one patient had a disease duration greater than five years. Healthy control (HC) skin samples were obtained from age and sex-matched donors (61). All participants gave written informed consent in accordance with the Declaration of Helsinki. The conduct of the study was approved by the University of Pittsburgh Medical Center Institutional Review Board (Pittsburgh, PA, USA).

Single cell RNA sequencing

Single cell RNA sequencing (scRNA-seq) experiments, including data processing and analysis, were performed at Pittsburgh University by the research group of Dr. Robert Lafyatis. In order to obtain cell suspensions, skin biopsies were minced and digested enzymatically (Whole Dissociation Skin Kit, Miltenyi Biotec) for 2 hours at 37°C and dispersed using the Miltenyi gentle MACS Octo Dissociator. Preparation of the scRNA-seq library was performed using the 10X Genomics Chromium Single Cell 3' Library V2, according to the manufacturer's protocol. Briefly, cell suspensions were partitioned vesicles called Gel Beads in emulsion, or GEMs, which contain reverse transcription

reactants, oil and barcodes for each cell and each transcript (unique molecular identifier, UMI). Following GEM formation, the emulsions were transferred to a PCR cycler for cDNA synthesis and amplified by PCR (C1000, Bio-Rad). The library was quantified using the KAPA Library Quantification Kit (Illumina), and further characterized for cDNA length by bioanalyzer using a High Sensitivity DNA kit. Libraries were sequenced (~200 million reads/sample), using the Illumina NextSeq-500 platform through the University of Pittsburgh Genomics Core Sequencing Facility. The sequencing reads were examined by quality metrics, transcripts mapped to reference human genome (GRCh38) using RNA-seq aligner STAR and assigned to individual cells according to cell barcodes, using Cell Ranger pipeline (10X Genomics).

scRNA-Seq data processing and analysis

Seurat package (version 3.0) for R was used for data analysis, normalization of gene expression, and identification and visualization of cell populations. Endothelial cells were identified based on previously described gene markers. Differentially expressed genes (DEGs) analysis was conducted between ECs expressing and not expressing *H19X* (*H19X*^{high} and *H19X*^{low}). Specifically, after normalization EC expressing *H19X* levels above 0 were defined as *H19X*^{high} EC, and the rest as *H19X*^{low}. In the analysis, genes expressed by >25% cells within each population were included.

The identified DEGs were mapped to the KEGG (Kyoto Encyclopedia of Genes and Genomes Pathway Database) database, and searched for significantly enriched KEGG pathways (62). Significance threshold was set for $p<0.05$.

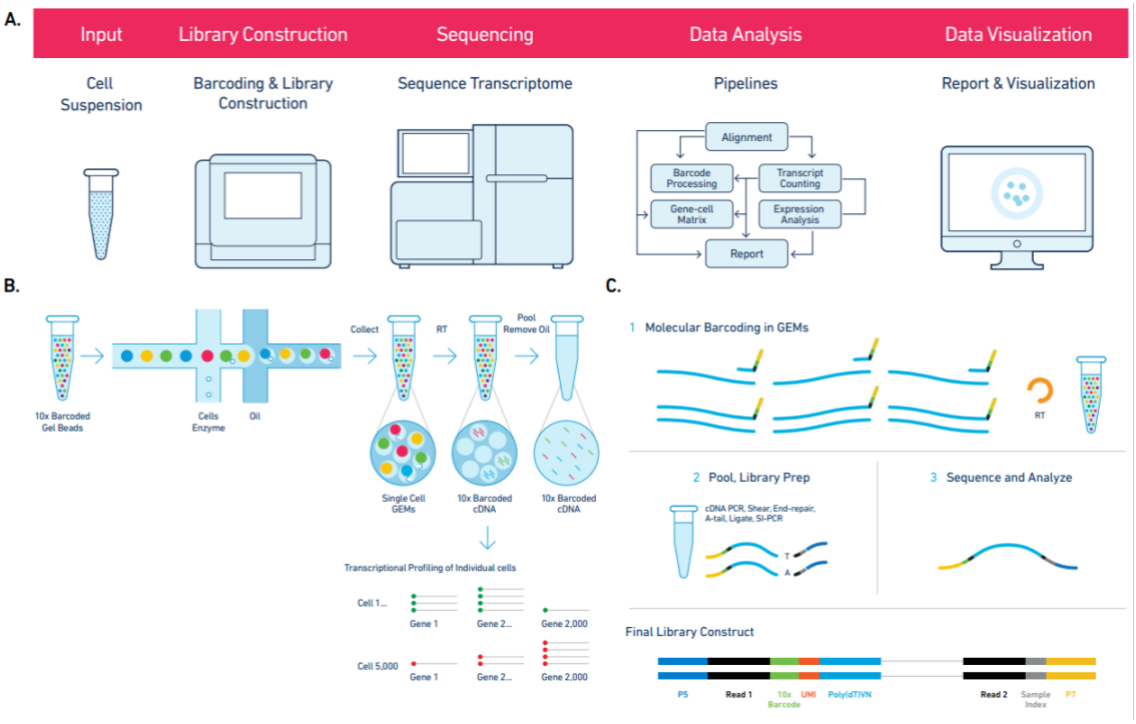


Figure 7. Chromium single – cell RNA sequencing (10X Genomics) (A) Schematic Workflow overview **(B)** Formation of GEMs **(C)** Barcoding and library preparation

Cell Culture

Human dermal microvascular endothelial cells (HDMECs) were purchased from Lonza Pharma & Biotech (Basel, Switzerland) and grown in Lonza Endothelial Cell Growth Basal Medium-2 (EBM - 2) supplemented with the Microvascular Endothelial Cell Growth Medium-2 SingleQuotsTM Kit (EGM-2-MV) at 37°C in 5% CO2.

Blood samples were collected in EDTA tubes (BD Vacutainer) from healthy donors and processed within 24h. PBMC were isolated by gradient centrifugation on cell separation medium (Lymphoprep, Stemcell Technologies) and co – cultured with HDMEC for functional assays.

Cytokines

For HDMEC stimulation experiments, endothelial cell complete growth medium was supplemented with 10 ng/ml TNFα (R&D Systems), 10 ng/ml IFNα (ImmunoTools), 10 ng/ml IFNβ (ImmunoTools), 10 ng/ml IFNγ (ImmunoTools), 10 ng/ml IL-1β (ImmunoTools), 10 ng/ml IL-4 (ImmunoTools), 50 ng/ml IL-6 (R&D Systems), and 10 ng/ml TGFβ (PeproTech) for 3, 6, 24, 48 and 72 hours. In functional assays, 10 ng/ml TNFAα was added to endothelial growth medium for 3 hours to enhance adhesion and for 24 hours to enhance apoptosis. For proliferation assay, 10 ng/ml VEGF (R&D Systems) was used.

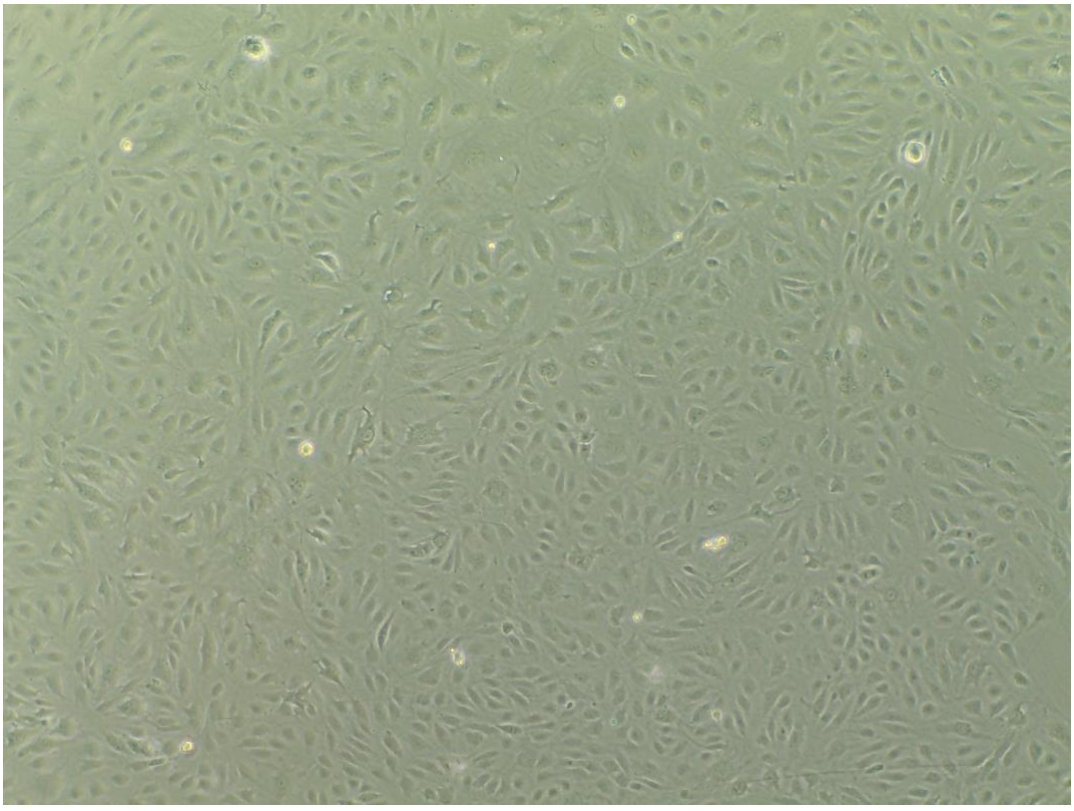


Figure 8. HDMEC in culture. 20X magnification

Transient Transfection of HDMEC

In order to study its functions in endothelial cells, *H19X* knockdown experiments were performed. HDMECs were transfected with 5 nM ASO (Antisense LNA GapmeRs, QIAGEN) targeting *H19X* (knockdown 5'-CGCGGGCTTGGTCTTT-3'; Scrambled 5'-AACACGTCTATACGC-3') using Lipofectamine 2000 (Thermo Fisher scientific) at a final concentration of 0.83 µl/ml. Cells were maintained in the transfection mix for 6 hours and washed with phosphate buffered saline (PBS) afterwards. Eventually, fresh complete medium was added to the culture. Forty – eight hours after transfection, cells were harvested for gene and protein expression analysis or prepared for functional assays.

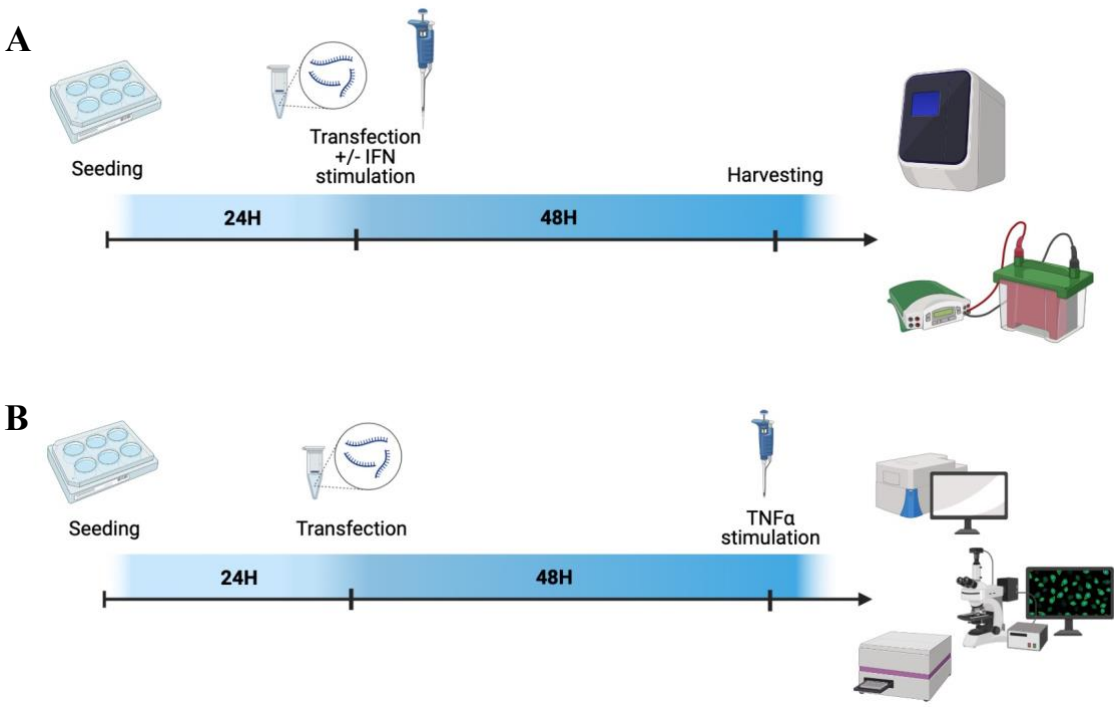


Figure 9 Experimental workflow for HDMEC transfection. Cells were transfected and stimulated with IFNs for qPCR and WB analysis (A) and with TNFα for functional assay (B**)**

Picture created with BioRender.com

Real Time quantitative PCR (RT-qPCR) Analysis

Total RNA from cells was extracted with the Zymo Quick-RNA MicroPrep RNA isolation kit. RNA concentration of isolated RNA was assessed by spectrophotometry (NanoDrop). Two hundred ng of total RNA was reverse-transcribed with random hexamers and reverse transcriptase using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher scientific). Subsequent RT-qPCRs were performed with 2x SYBR Green Master Mix (Promega) on an Agilent Technologies Stratagene Mx3005P RT-qPCR system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein lateral stalk subunit P0 (RPLP0) were used as reference housekeeping genes for expression level normalization. Specific amplification was verified measuring dissociation curves. Relative fold change in gene expression was calculated using the comparative threshold cycle method (2^{-DDCt} method). Primer sequences are listed in Table 1.

Western blot

Cells were lysed with RIPA buffer (MilliporeSigma) supplemented with phosphatase inhibitors (PhosphoStop, Roche) and a protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche). Protein concentration was measured by the colorimetric BCA method according to the manufacturer's protocol (Thermo Fisher Scientific). To maximize nucleic acid disruption, three cycles of 20 second sonication in ice-cold water followed by 20 seconds of cooling in ice were performed. Insoluble material was removed with 16000 g centrifugation for 20 minutes at 4°C. Proteins were separated on 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane overnight at 4°C. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline and Tween-20 (Thermo Fisher Scientific) (TBST) containing 5% milk and probed overnight with the following antibodies: VCAM1 (1:1000, E1E8X, Cell Signaling Technologies), P-

Selectin (1:1000, ab182135, Abcam), E-Selectin (1:500, BBA16, R&D systems), p21 (RAC1) activated kinase 1/2/3 (PAK 1/2/3, 1:1000, 2604, Cell Signaling), Phospho-PAK1 (Thr423)/ PAK2 (Thr402) (1:1000, 2601, Cell Signaling), Phospho-PAK1 (Ser144)/ PAK2 (Ser141) (1:1000, 2606, Cell Signaling) and α-tubulin as loading control (1:5000, ab7291, Abcam). Horseradish peroxidase (HRP) conjugated secondary antibodies were used for detection with enhanced chemiluminescence (ECL) substrate (SuperSignal West Pico Plus, Thermo Fisher Scientific). Semi-quantitative determination of protein expression was performed with ImageJ software.

RAC1 Pulldown assay

The RAC family small GTPase 1 (Rac1) Pulldown assay was performed by using the Active RAC1 Pull-Down and Detection Kit (16118, Thermo Fisher Scientific). Briefly, HDMEC were lysed with the Lysis/Binding/Washing Buffer, the lysate was transferred to a microcentrifuge tube, briefly vortexed and incubated on ice for 5 minutes. After centrifuging at 16000x g at 4°C for 15 minutes, the total lysate was transferred to a new tube. One-hundred µL of the 50% resin slurry provided with the kit was added to the spin cup and centrifuged at 600x g for 30 seconds. After washing, 20 µg of GST-fusion protein containing the PAK-binding domain (GST-PAK-PBD) were added to the spin cup. Proteins were then immediately transferred. The mixture was incubated at 4°C for 1 hour with gentle rocking before centrifuging at 6000x g for 30 seconds. The spin columns were washed three times with Lysis/Binding/Washing Buffer. Samples were eluted with a 50 µL mixture of 1:20 β-mercaptoethanol in SDS sample buffer. The protein samples were run on a 12% polyacrylamide gel. The blocking was performed with 3% bovine serum albumin (BSA) TBST for 1.5 hours at room temperature. Anti-RAC1 mouse monoclonal antibody (1:1000 in 3% BSA + 0.1% NaN_3 TBST) was incubated overnight at 4°C. Anti-mouse IgG HRP

conjugate were used as secondary antibodies, and proteins were visualized with ECL substrate (SuperSignal West Pico Plus, Thermo Fisher Scientific).

Phalloidin staining

Cells were fixed with 4% paraformaldehyde (PFA, MilliporeSigma) for 10 minutes at room temperature, washed two times with PBS, permeabilized with 0.1% Triton X-100 (MilliporeSigma), washed again two times with PBS and blocked with 10% fetal bovine serum (FBS) for 30 minutes at room temperature. Cells were then stained with 4U/ml of fluorescent-labeled phalloidin (Biolegend) for 40 minutes at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) solution (1 µg/ml, Roche). Images were acquired with an Olympus BX53 microscope equipped with a DP80 camera. Uncovered area was calculated by ImaJ software and normalized on nuclei count.

Adhesion Assay

HDMEC were seeded in 96-well black plates at a density of $4x10^4$ cells per well to create an endothelial monolayer, and transfected with the ASO negative control or ASO targeting *H19X*, as described above. In order to maximize the knockdown efficiency, 10nM concentration for GapmeR was used for this assay. Twenty-four hours after transfection, HDMEC were treated for 3 hours with 10 ng/ml TNFAα to enhance adhesion molecules expression. PBMC were incubated for 15 minutes at 37°C, protected from light, in a PBS solution containing 2 µM carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific). RPMI medium (Thermo Fisher Scientific) with 10% FBS was added and cells were incubated for another 15 minutes at 37°C. Cells were resuspended in RPMI with 10% FBS and $1x10^5$ PBMC per well were added on top of the endothelial monolayer. The plates were centrifuged for 30 seconds at 400 g to facilitate PBMC interaction with HDMEC.

Following overnight co-culture, cells were rinsed twice with PBS in order to remove PBMCs in suspension. A solution of 5 μM Hoechst 33342 (Thermo Fisher Scientific) in 4% PFA was used to fix the cells and counterstain endothelial nuclei.

High content imaging

For visualization and quantification of leucocytes and HDMEC in co – culture, the 96-well black plates were loaded into the Cellinsight CX7 high-content imaging platform (Thermo Fisher Scientific) for automated widefield image capture in two channels. Each plate was read twice. In the first read, channel 1 was used to measure the morphological and intensity properties of the ECs, which were stained with Hoechst 33342 dye for the nuclei, and channel 2 was used to measure the morphological and intensity properties of CFSE – labelled PBMCs. Images were produced using a dichroic filter and corresponding emitter filters that pass the fluorescent light onto a high-resolution charge-coupled device camera for image acquisition. Objects were identified and validated as cells when had a valid nuclei and cell body measurements. Identification and validation of the cells both based on physical and fluorescence intensity measurements was used to discriminate from artifacts. The acquired images were analyzed simultaneously by the high-content screening (HCS) Studio software, using the compartmental analysis bioapplication to quantify the number of endothelial and PBMCs for each study condition.

EC and PBMCs were discriminated using mean area and mean average intensity for both channels. Images were acquired at 10X objective for the whole well (25 fields per well). The number of fields per well were adjusted to nine fields per well when 20X objective was used.

Figure 10. CellInsight™ CX7 High Content Analysis Platform (Thermofisher Scientific). The platform is based on an inverted epifluorescence microscope featuring a nipkow-spinning disc confocal that automatically focuses and scans fields in individual wells using a motorized stage

BrdU Cell Proliferation ELISA

Cell proliferation was quantified using an ELISA colorimetric immunoassay with 5-bromo-2′-deoxyuridine (BrdU)(Roche/Sigma-Aldrich). BrdU is a synthetic thymidine analog which is incorporated in newly synthetized DNA molecules, allowing to detect actively proliferating cells.

HDMEC were seeded in 96-well plates at a density of 1 x 10^4 cells per well and transfected with the ASO negative control or ASO targeting H19X as described above. Forty – eight hours after transfection, medium was removed and new endothelial growth medium with either 1% or 5% FBS concentration and supplemented with 10 ng/ml VEGF (R&D system) was added. As VEGF is included in the EGM-2-MV supplement kit, it was not used during medium preparation, in order to guarantee a fixed VEGF concentration. BrdU at a concentration of 10 mg was then added to each well and incubated overnight at 37°C. After removing the labelling medium, cells were fixed and denatured and then incubated with anti – BrdU – POD for 90 minutes. After accurate rinsing with PBS, substrate solution was

added. Reaction product was quantified by measuring the absorbance at the respective wavelength with a multi – plate reader (Synergy HT, BioTek).

Caspase 3/7 Assay

To quantify apoptosis following H19X knockdown, caspase-3 and caspase-7 activities were measured using the Caspase-Glo® 3/7 Assay System (Promega).

HDMEC were seeded in 96-well plates at a density of a density of 1 x 10^4 cells per well and transfected with either ASO negative control or ASO targeting H19X. Forty-eight hours after transfection, equal volumes of Caspase-Glo® 3/7 Reagent (Promega) were added into the cell culture medium and incubated for two hours at room temperature, as per manufacturer protocol. Luminescence was recorded with a plate-reading luminometer (Synergy HT, BioTek).

Statistical analysis

All data are presented as mean ± standard deviation (SD). Normality was assumed using Shapiro – Wilk's W test. Normally distributed data were analyzed by two-tailed paired t-test. Mann – Whitney U test was used to compare H19X average expression level between SSc and HC skin biopsies. Comparison of multiple groups was performed by one-way ANOVA with Dunnett's multiple comparisons test. $P<0.05$ were considered statistically significant. All statistic tests were performed with GraphPad Prism version 9.0.

Table 2. Primer sequences

Target	Forward sequence 5' → 3'	Reverse sequence 5' → 3'
H19X	GGTGCCAGCCAGCCTTC	CGTCCACTGGAGGAAGCC
VCAM1	GCAAGTCTACATATCACCCAAG	ATCTCTGGAGCTGGTAGACC
ICAM1	CCTCAGCCTCGCTATGGCT	CCGAGCAGGACCAGGAGT
SELE	CTCTCACTTTGGTGCTTCTCAT	GTGTACCTTTGCTGACAATAAGC
SELP	ATGCCAAGCCATTCCCTGC	CCTCGTCACAGATGAATTGAC
PECAM1	ACTCTTTCACAATCAACAGTGTTG	ACGTGAGAGGTGGTGCTGA
EDN1	AAAGAACTCAGGGCTGAAGACA	CTTCCTCTCACTAACTGCTGAT
KDR	GGCATGGTCTTCTGTGAAGC	CGGACTCAGAACCACATCATAAA
TEK	GCCGCTACCTACTAATGAAGAA	TGGATGGTGAATATGGCTACTGA
VEGFA	CCATGCAGATTATGCGGATC	GTTGTGCTGTAGGAAGCTTA
THBS1	CAGCTCTACCAGTGTCCTCCTC	GCAAGTCCTTTGTCTTGTGGC
AIF-1	AATTGGAGAGGTGTCCAGTGG	CCTCATACATCAGGATCATTTTTAG
CX3CL1	GCAATCATCTTGGAGACGAGA	GCCATTTCGAGTTAGGGCAG
CXCR1	TCTTGGCACGTCATCGTGTT	GCCAGATCACCTTCCACACA
JAM2	GAAGTATTAGTGGCTCCAGCA	ATTCCCTTCTTTGTCTTGACATC
JAM3	GCACTTTGGTGTTCACTGCTG	CCAATGTTCAGGTCATAGACTTC
GAPDH	GGGAAGCTTGTCATCAATGGA	TCTCGCTCCTGGAAGATGGT
RPLP0	ACACTGGTCTCGGACCTGAGAA	AGCTGCACATCACTCAGAATTTCA

RESULTS

LncRNA *H19X* is upregulated in the endothelium from skin biopsies of SSc patients

Previous studies demonstrated that lncRNA *H19X* is upregulated in SSc skin. To investigate whether this upregulation involves cutaneous EC, scRNA – seq data from skin biopsies were analyzed for *H19X* expression, in collaboration with R. Lafyatis (Pittsburgh, USA). Analysis of scRNA-seq data from 27 dcSSc and 10 HC skin biopsies demonstrated that 1.5% of identified SSc EC and 1.3% HC ECs expressed detectable levels of *H19X*.

Comparison of average expression levels of *H19X* showed that *H19X* expression was significantly upregulated in SSc skin EC compared to HC ($p<0.01$) (Figure 11).

These data therefore suggest that *H19X* upregulation found in the skin is attributable to different cell subtypes which include not only fibroblast, as already described, but also EC. Given its significant upregulation in SSc EC, *H19X* might be involved in the molecular mechanism leading to microvascular EC injury characteristic for SSc.

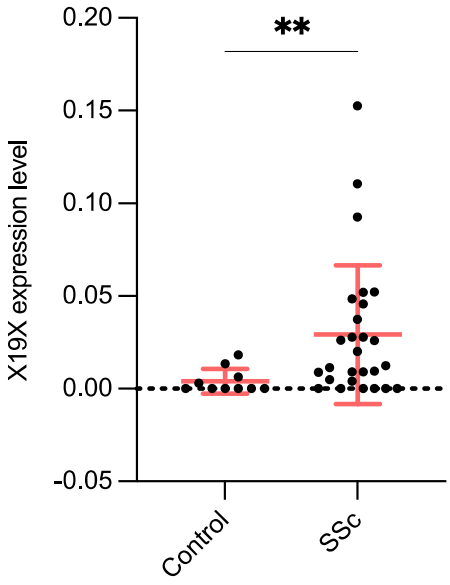


Figure 11. Analysis of single cell RNA sequencing data for H19X expression in skin endothelial cells
Comparison of average expression levels of H19X in HC (n=10) vs SSc (n=27) endothelial cells (Mann – Whitney test) **P<0.01

Type I and II interferons regulate H19X expression in endothelial cells

In order to investigate the potential role of *H19X* in EC and identify whether it could be involved in mechanism leading to EC injury, *in vitro* experiments were carried out.

In SSc, microvascular endothelial injury manifests as an early physio-pathological event triggered by inflammation. We hypothesized that *H19X* expression in ECs might be regulated by pro-inflammatory cytokines. Based on literature search, we identified cytokines relevant in endothelial injury in SSc (63) and analyzed *H19X* expression in HDMEC after stimulation with TNFα, IFNα, IFNβ, IFNγ, IL-1β, IL-4, IL-6 and TGFβ, at several time points. Initial screening experiments did not show consistent effects or showed downregulation of *H19X* after stimulation with TNFAα, IL-1β, IL-4 and IL-6 (Figure 12A). Interestingly, opposite to what was previously observed in fibroblasts (52), *H19X* expression in HDMEC was downregulated between 3 and 24 hours after TGFβ stimulation (Figure 12A). However, *H19X* expression was significantly upregulated after prolonged stimulation (48-72 hours) with IFNα. Although not significant, a very similar trend with increased *H19X* expression following IFNβ and IFNγ was also observed, starting 24 hours after stimulation (Figure 12B). These findings show that *H19X* expression in the endothelium might be regulated by both type I and type II IFNs.

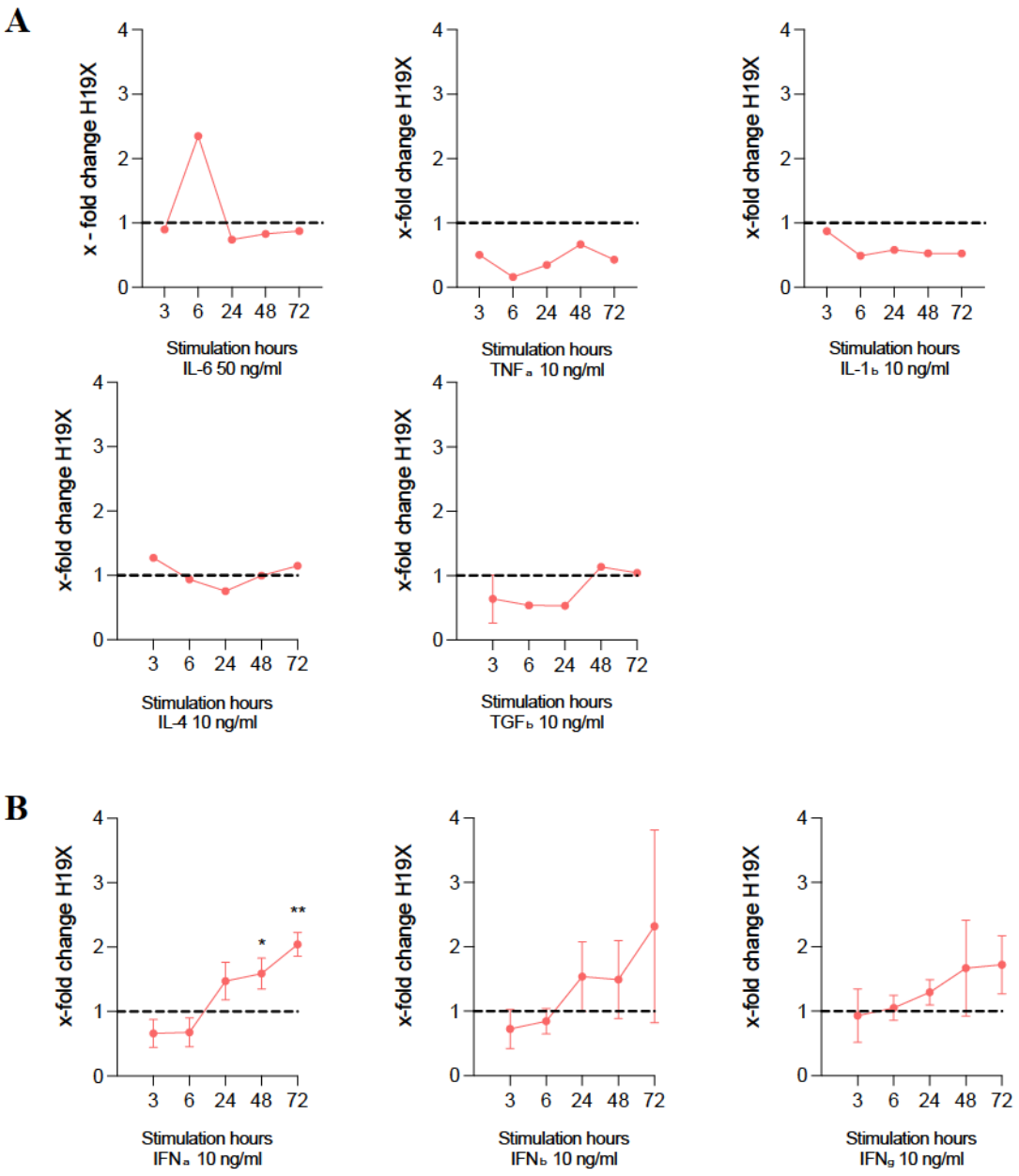


Figure 12. Time course analysis of the expression of H19X in HDMEC after pro – inflammatory cytokines stimulation. HDMEC were stimulated for 3-72h with several pro – inflammatory cytokines relevant to endothelial injury in SSc at biologically relevant concentration. Expression levels were measured by qPCR, normalized by GAPDH and RPLP0 and compared with non-stimulated dermal HDMEC. **(A)** Screening tests representative of *n*=1-2 biological replicates (**B)** Pictures are representative of *n*=1-5 biological replicates. *P<0.05, **P<0.01

H19X knockdown decreases the expression of adhesion molecules in HDMEC

Endothelial activation in SSc is a complex process that encompasses numerous alterations of endothelial functions, including adhesion, angiogenesis, inflammation and production of vasoactive molecules. Based on literature search, we identified several markers of endothelial activation which are altered in SSc (35) and analyzed their expression in HDMEC following *H19X* knockdown (Figure 13A) with or without IFN stimulation. Among several adhesion molecules tested, the expression of *VCAM1*, E-Selectin (*SELE*) and P-Selectin (*SELP*) was significantly downregulated at the mRNA level after transfection (Figure 13B). However, we did not observe a significant downregulation of *VCAM1* and *SELE* after *H19X* knockdown followed by IFNα, IFNβ and IFNγ stimulation (Figure 13B). VCAM1, but not E-selectin nor P-Selectin, was also consistently downregulated after *H19X* knockdown at the protein level (Figure 14A-C). Additionally, IFN effect on expression of adhesion molecules was tested. *VCAM1* and *SELE* expression was rapidly induced by IFNs peaking between 3 and 6 hours after stimulation (Figure 15). Less consistent results were noted for other endothelial activation markers. Specifically, among angiogenesis markers, VEGFA (*KDR*), VEGF receptor (*VEGFR*), and TEK receptor tyrosine kinase (Tie2) (*TEK*) were slightly upregulated, but with statistical significance only for VEGFA. No changes were noted for other adhesion molecules (*ICAM1*, *PECAM1*, junctional adhesion molecules 2/3 [*JAM2* and *JAM3*]), fractalkine (*CX3CL1*), C-X-C motif chemokine receptor 1 (*CXCR1*), as well as for other vascular markers including endothelin-1 (*EDN1*), thrombospondin-1 (THBS1) and allograft inflammatory factor 1 (*AIF1*) (Figure 16).

These data suggest that *H19X* in EC might be involved in the regulation of endothelial adhesion molecules expression, especially VCAM1, which is described as one of the most important EC activation markers and is upregulated in SSc skin.

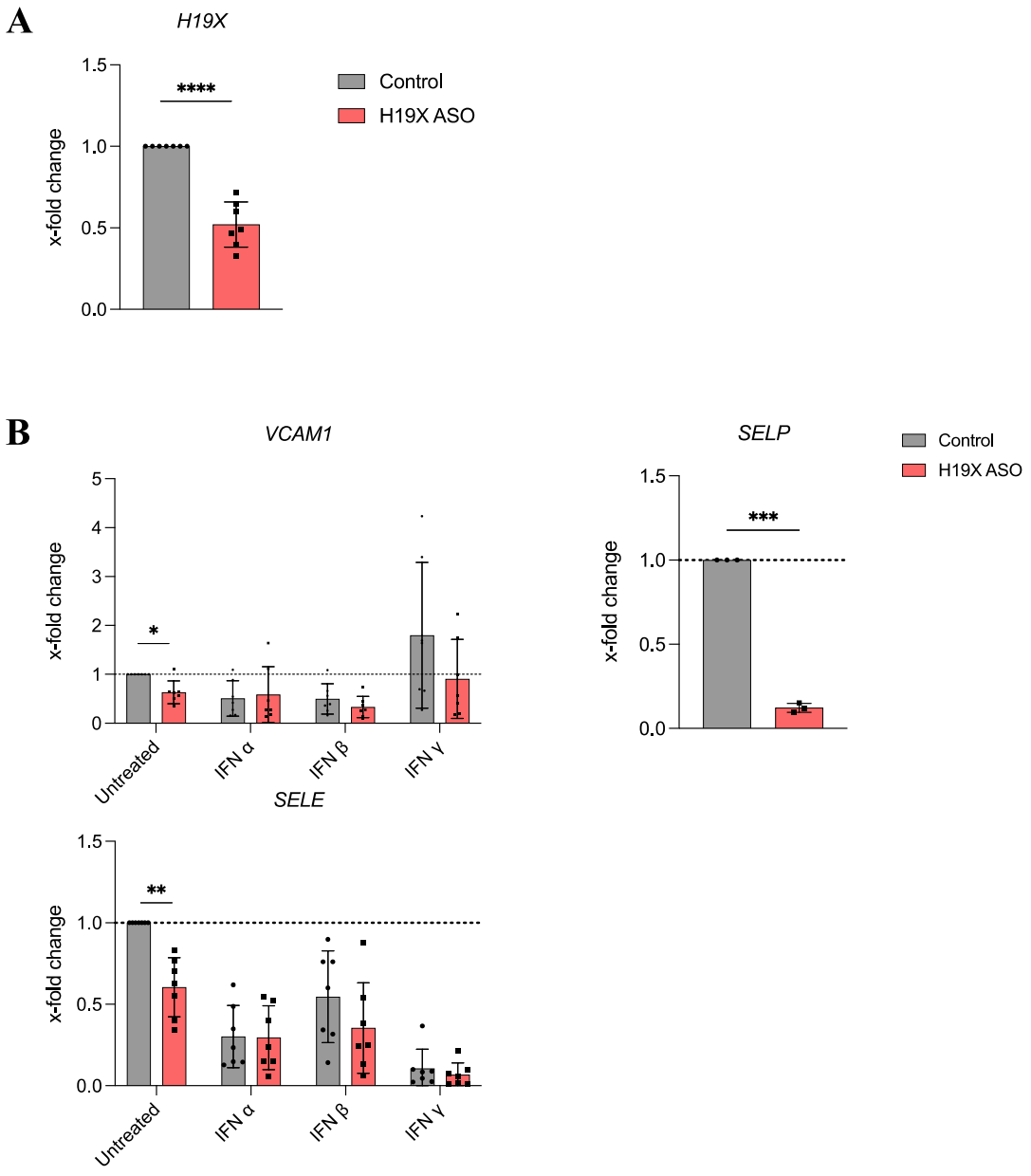


Figure 13. Adhesion molecules expression after H19X knockdown: mRNA level HDMEC were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting *H19X* (**A)** *H19X* expression following transfection was measured by qPCR **(B)** mRNA expression of VCAM1 (*VCAM1*) and E-Selectin (*SELE*) was measured by qPCR 48 h after transfection and stimulation in untreated condition an after stimulation with 10 ng/ml of IFNα, IFNβ and IFNγ (n = 7); mRNA expression of P-Selectin (*SELP*) was measured in untreated condition (n = 3). mRNA expression levels were normalized by GAPDH and RPLP0. *P<0.05**P<0.01, *** P<0.001, ****P<0.0001

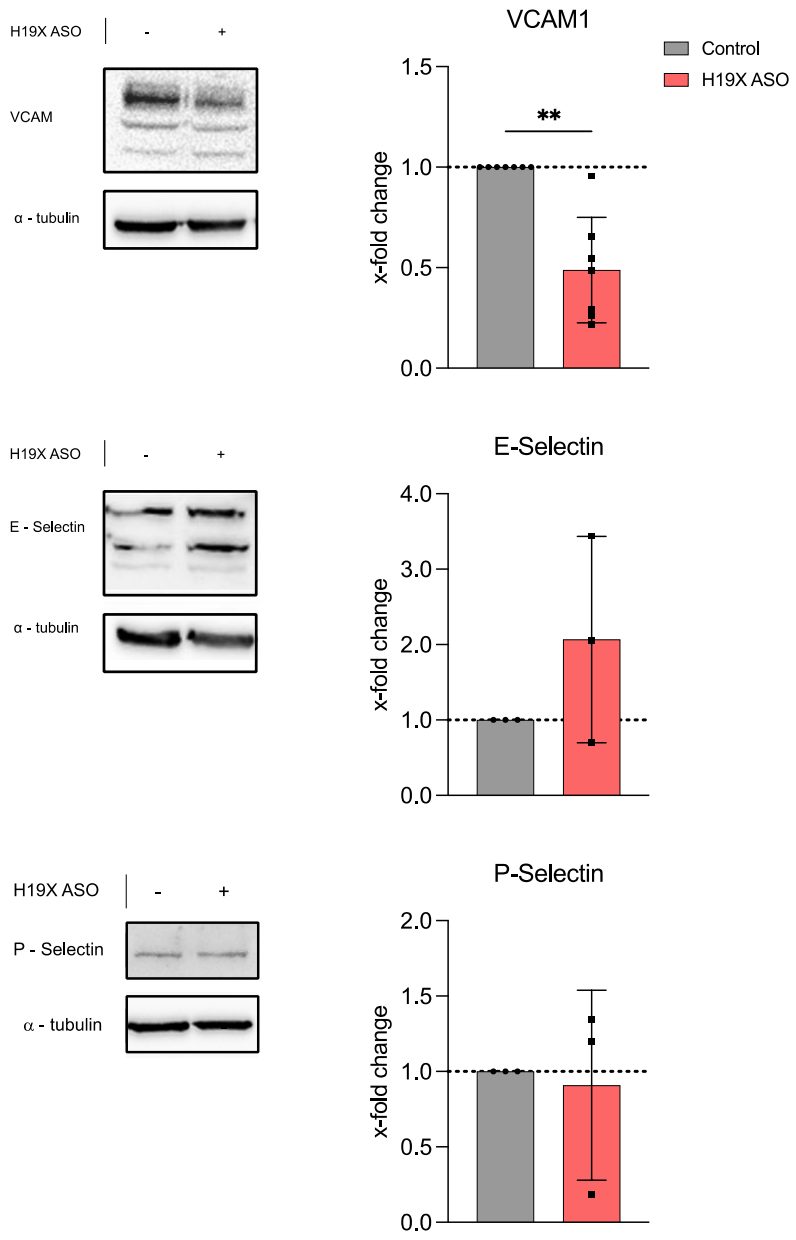


Figure 14. Adhesion molecules expression after H19X knockdown: protein level HDMEC were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting *H19X* VCAM1, E-Selectin and P-Selectin protein level was measured by western blot 48h after transfection by WB analysis in untreated condition (n = 3-7). Protein expression levels were normalized by a-tubulin. **P<0.01, *** P<0.001

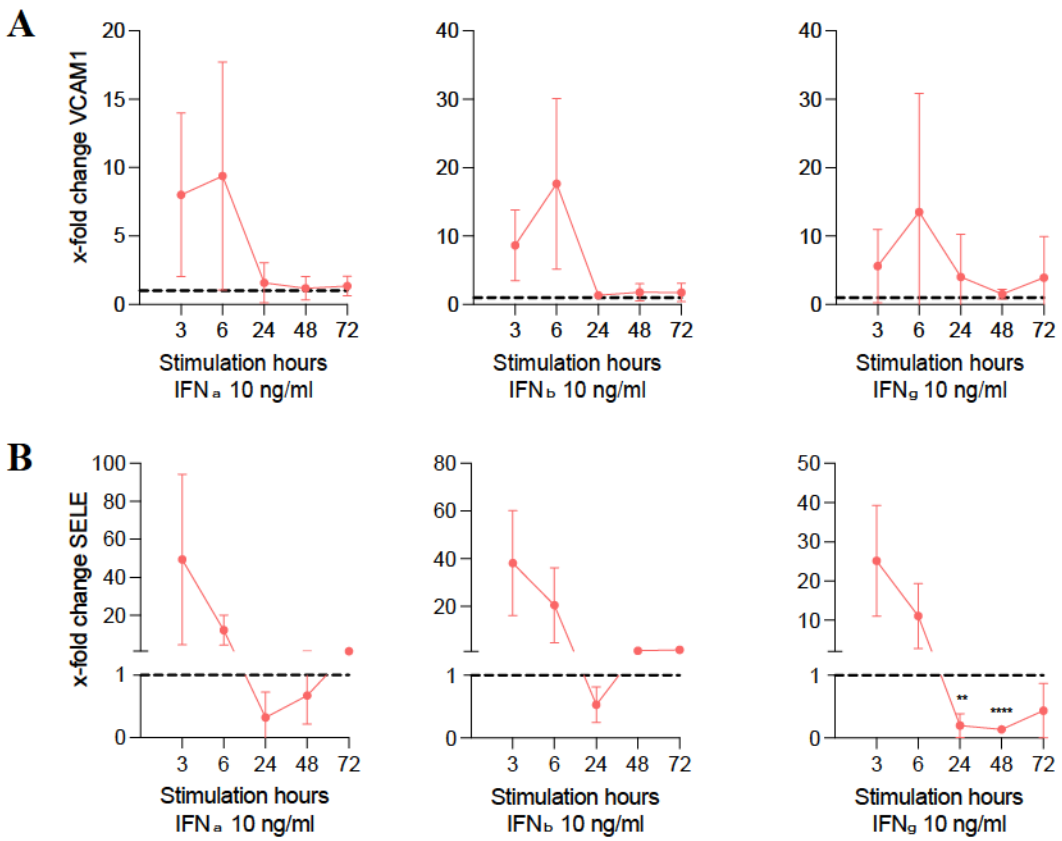


Figure 15. Time course analysis of the expression of Adhesion molecules in HDMEC after Interferon (IFN) stimulation. Time-dependent induction of VCAM1 **(A)** and E-Selectin **(B)** by of type I and II IFN. HDMEC were stimulated for 3-72h with 10 ng/ml IFNα, IFNβ, IFNγ. Expression levels were measured by qPCR, normalized by GAPDH and RPLP0 and compared with non-stimulated HDMEC. Pictures are representative of *n*=5 biological replicates. **P<0.01, ****P<0.0001

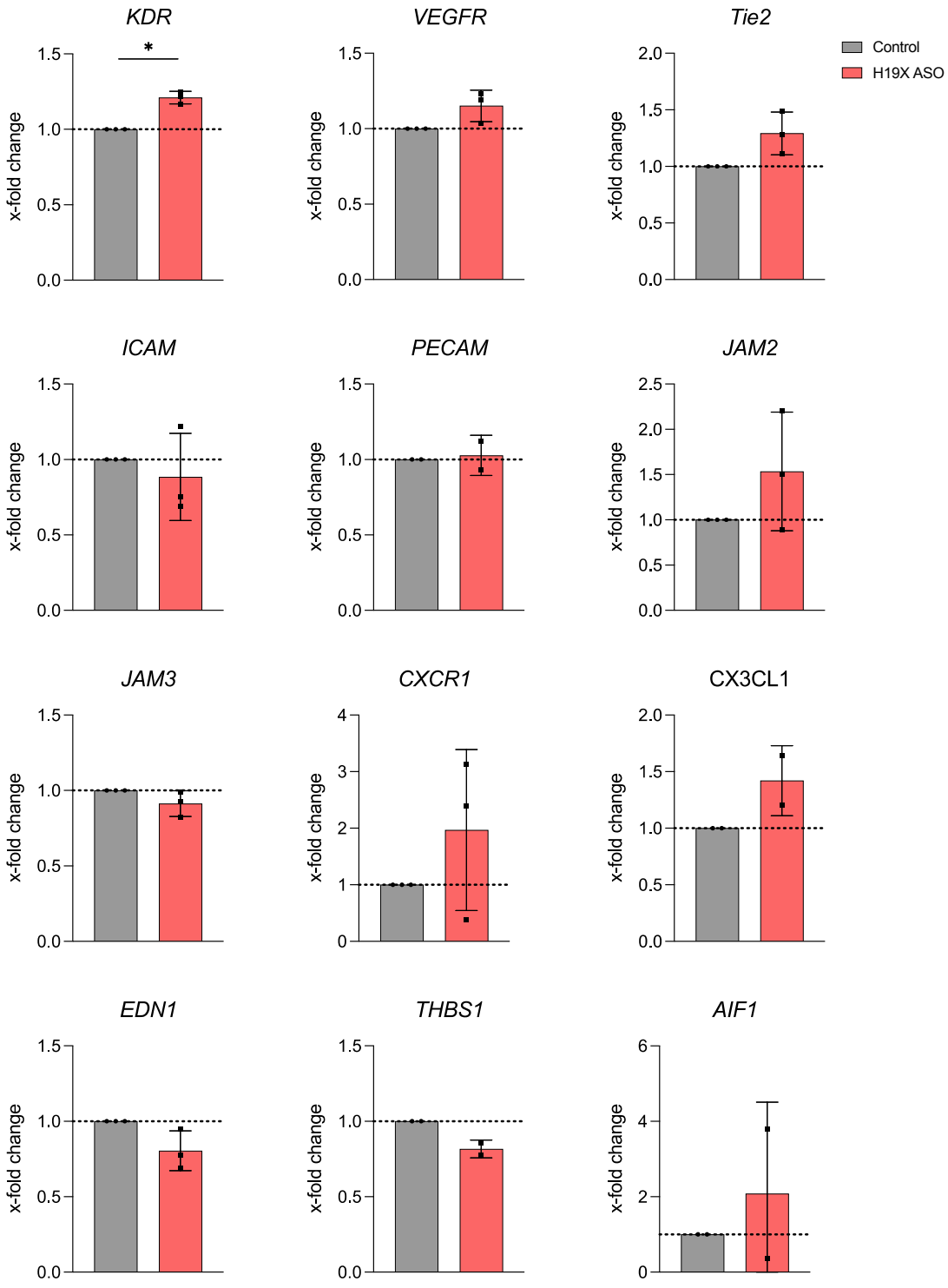


Figure 16. Endothelial activation markers expression after H19X knockdown. HDMEC were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting H19X for a total of 48H. mRNA levels of endothelial activation markers were measured by qPCR. mRNA expression was normalized by GAPDH and RPLP0. Figures are representative for *n*=2–3 biological replicates

H19X expressing ECs present with higher levels of adhesion molecules

The role of *H19X* on cell adhesion was further confirmed by KEGG (Kyoto Encyclopedia of Genes and Genomes Pathway Database) pathway analysis on differentially expressed genes between $H19X^{high}$ and $H19X^{low}$ ECs (62). This analysis of the scRNA-seq dataset revealed that genes expressed by $H19X^{high}$ cells were strongly associated with the 'Cell adhesion molecule' pathway (p=2.209e-7, Figure 17A), with integrin subunit beta 1 (*ITGB1*), *VCAM1*, intercellular adhesion molecule 2 (*ICAM2*), *SELE*, v-set immunoregulatory receptor (*VSIR*), endothelial cell adhesion molecule (*ESAM*), integrin subunit alpha 6 (*ITGA6*), Cluster of differentiation 99 (*CD99*), nectin cell adhesion molecule 2 (*NECTIN2*), and major histocompatibility complexes, class I, C (*HLA-C*), class II, DP alpha 1 (*HLA-DPA1*) , DQ alpha 1 (*HLA-DQA1*), DQ beta 1 (*HLA-DQB1*), DR alpha (*HLA-DRA*) DR beta 1 (*HLA-DRB1*), among the top deregulated genes (Figure 17B).

Next, we analyzed our scRNA-seq data in order to evaluate whether there was correlation between the expression of *H19X* and adhesion molecules. Interestingly, in skin ECs both from SSc and HC samples, VCAM1 resulted higher in EC expressing *H19X* (Figure 18). Correlation was noted also for different isoforms of ICAM, another adhesion marker.

These skin biopsy data further support our *in vitro* functional data suggesting a role of *H19X* in inducing the expression of VCAM1 in the SSc endothelium.

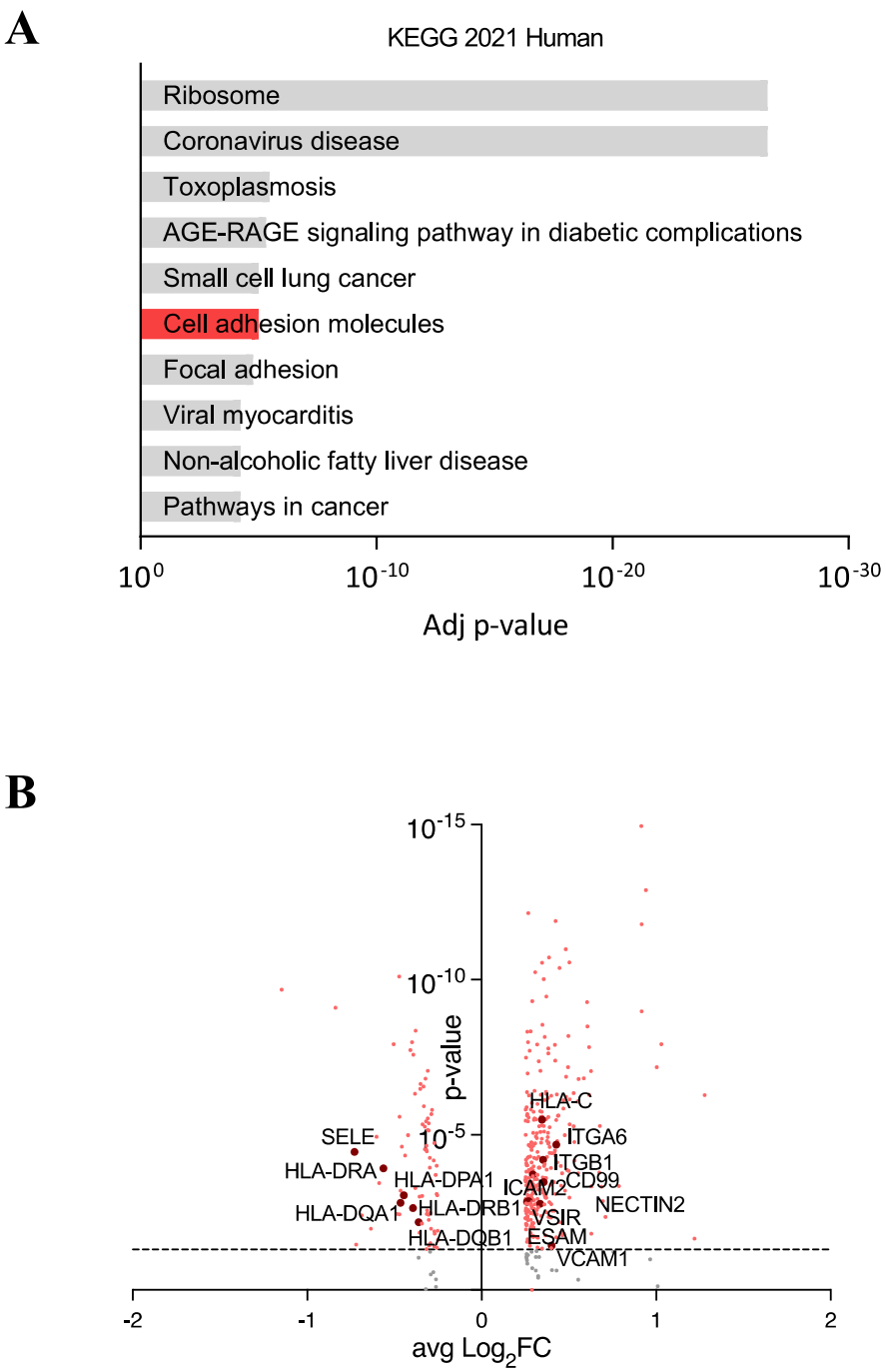


Figure 17. H19X correlates with cell adhesion molecular pathways in endothelial cells. (A) Pathway enrichment analysis (KEGG Pathway Database) performed on significantly deregulated genes (P<0.05) in scRNA-seq data between endothelial cells expressing high levels of H19X and endothelial cells expressing low levels of H19X **(B)** Differentially expressed genes between endothelial cells expressing high levels of H19X and endothelial cells expressing low levels of H19X. Significant correlations are highlighted in pink (Pearson p<0.05). Genes that belong to `Cell adhesion molecule´ pathway are marked in red

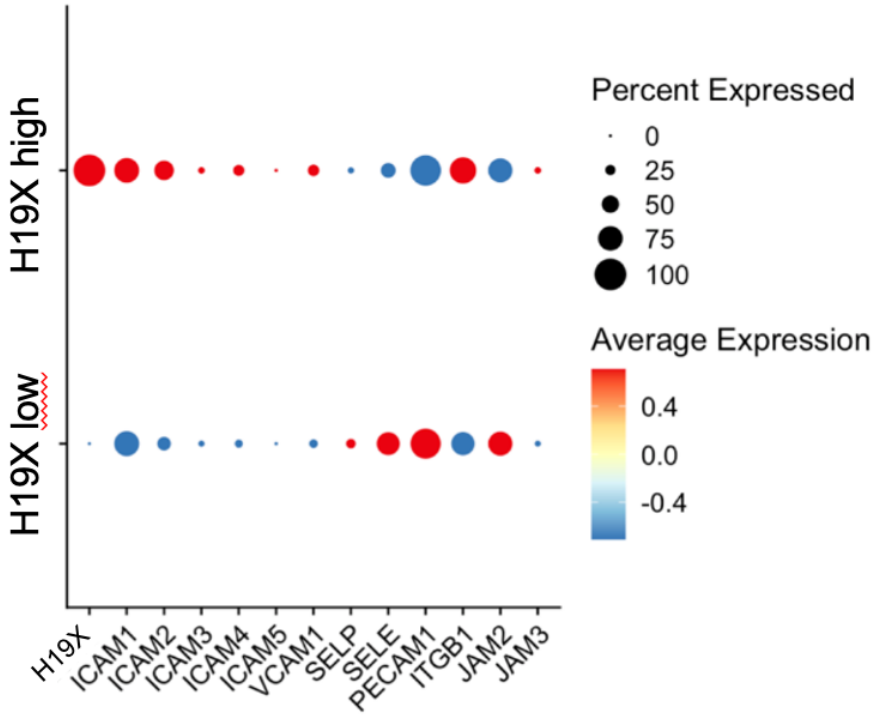


Figure 18. H19X correlates with adhesion molecules expression in skin endothelial cells Co-expression analysis of H19X and endothelial activation markers was performed on scRNA-seq data from skin biopsies using Seurat package in R. Endothelial cells expressing ($H19X^{high}$) and not expressing ($H19X^{low}$) *H19X* were compared. The size of the dots represents the percentage of endothelial cells expressing the gene of interest. Upregulated genes are marked in red and downregulated genes are marked in blue.

H19X knockdown decreases the activation of VCAM1 downstream signaling

VCAM1 is expressed on the endothelial surface following inflammatory stimuli. Upon recruitment, it activates intracellular signalling. The activation of RAC1 is a characteristic VCAM1 downstream event promoting the phosphorylation of PAK which in turn activates myosin light chains leading to cytoskeletal changes and leukocyte transmigration into the tissue (64). To analyze whether *H19X* does not only promote induction of VCAM1 expression, but also leads to downstream activation, we analyzed the activation of RAC1 after TNFα stimulation to induce VCAM1 expression (Figure 19) and *H19X* knockdown. Activation of RAC1 was reduced by 50% in *H19X* knockdown HDMECs as revealed by GTP-RAC1 pulldown followed by Western Blot (Figure 20). Total protein levels of PAK2, the main isoform expressed in endothelial cells (65), as well as the phosphorylation of the PAK2 Thr^{402} site were not significantly changed after *H19X* knockdown, neither in basal conditions nor after TNFα stimulation. However, phosphorylation levels at Ser^{141} of PAK2 were reduced following *H19X* knockdown both in basal conditions and in TNFα stimulated cells (Figure 21). These data support the hypothesis that *H19X* does not only induce VCAM1 expression but also induces downstream signalling in ECs.

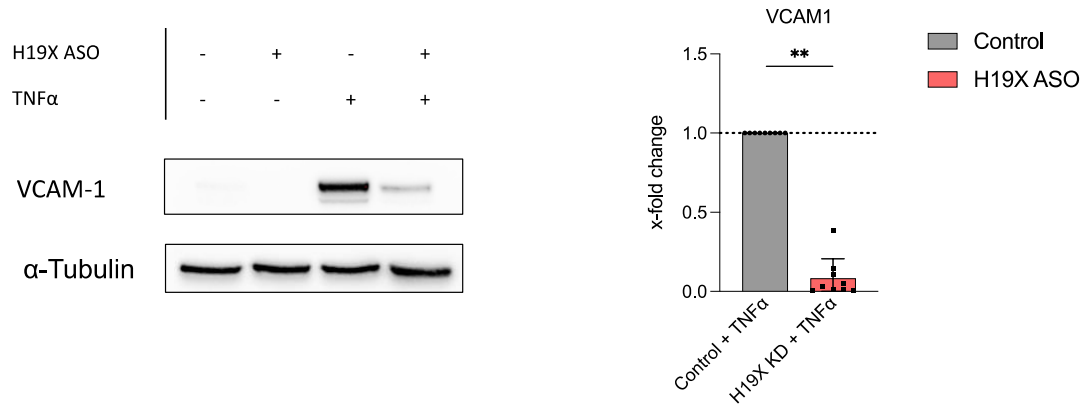


Figure 19. VCAM1 is induced by TNFα. Following transfection for 48H, HDMEC (*n*=9) were treated with 10 ng/ml to stimulate adhesion Protein levels of VCAM1 were measured by western blot. Protein expression was normalized by a-tubulin. **P<0.01

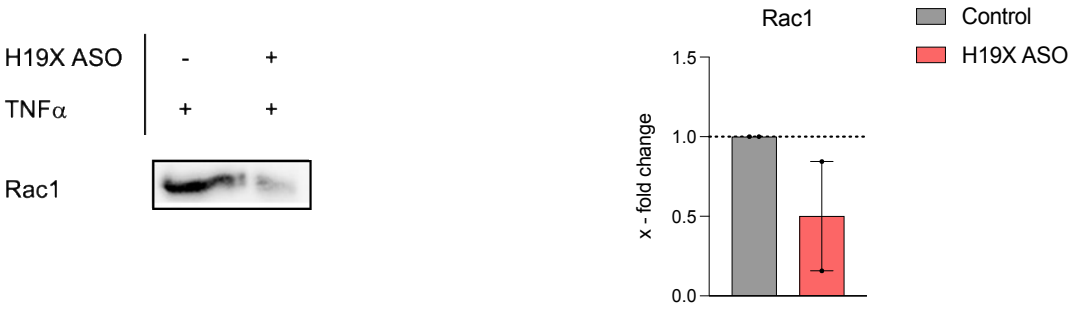


Figure 20. H19X knockdown silencing decreases the activation of VCAM1 downstream pathway. 48H after transfection HDMEC (*n*=2) were stimulated with 10 ng/ml TNFAα for 3 h to enhance pathway activation. Protein levels of Rac1 were analyzed by pull – down assay

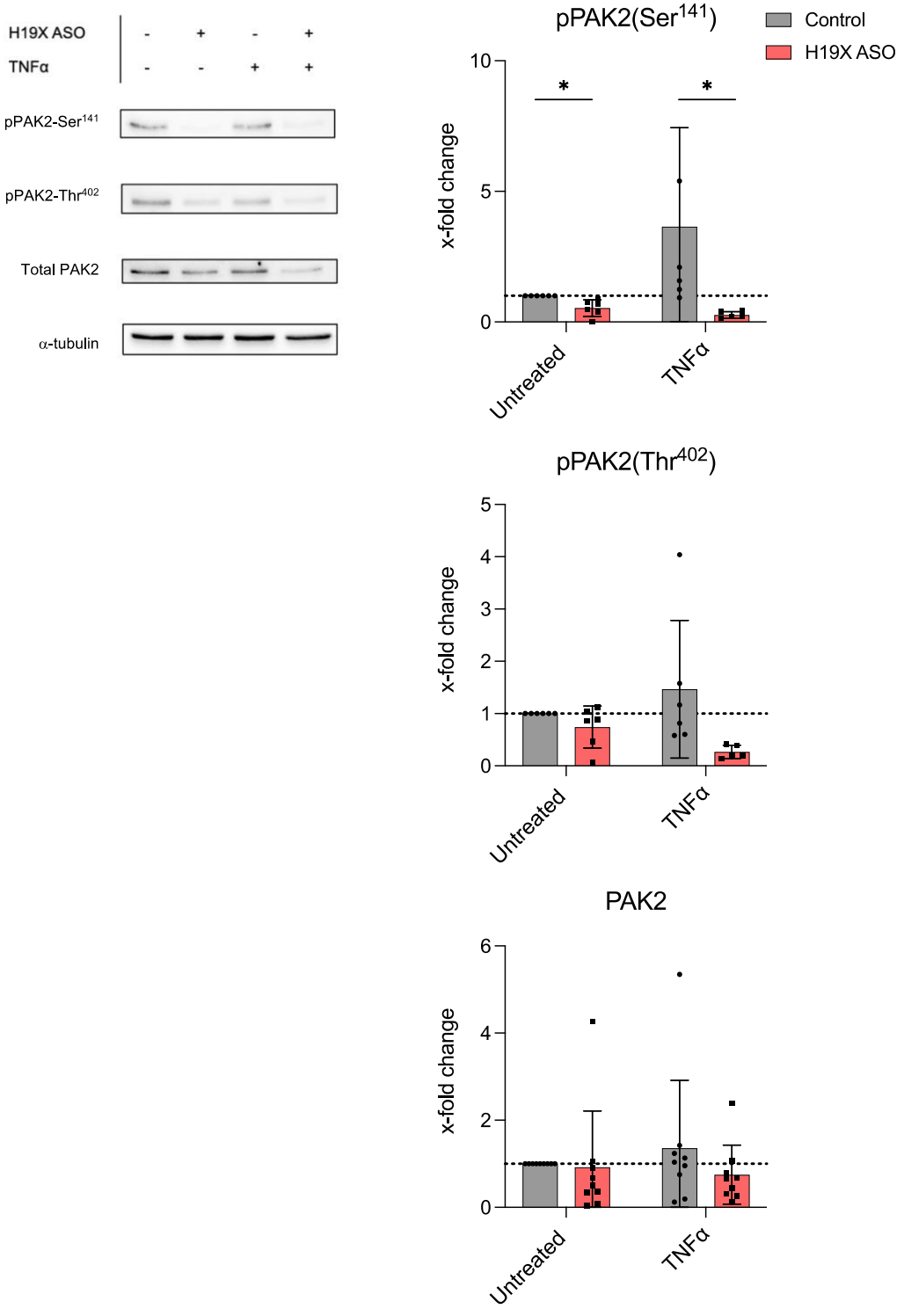


Figure 21. H19X knockdown silencing decreases the activation of VCAM1 downstream pathway. 48H after transfection HDMEC (*n*= 6-7) stimulated with 10 ng/ml TNFα for 3 h to enhance pathway activation. Protein levels of phosphorylated PAK at site Ser^{141} and Thr^{402} were analyzed by Western blot, compared to total PAK levels and normalized by α-tubulin. Pictures are representative of *n*=6-7 biological replicates. *p<0.05

H19X knockdown decreases EC contractility and PBMC adhesion to ECs

To further corroborate the role of *H19X* in VCAM1 mediated microvascular activation, we analyzed cytoskeletal rearrangements occurring after *H19X* knockdown in untreated and TNFα stimulated HDMEC. As revealed by F-actin staining with phalloidin, (Figure 7A) HDMECs had larger cytoplasmic area after *H19X* knockdown as compared to control scrambled cells, indicating reduced cell contractility after *H19X* knockdown required for leucocyte transmigration. Consistently, a significant reduction in the area not covered by cells was detected after *H19X* knockdown in basal conditions. The morphological changes seen after *H19X* knockdown was also observed following TNFα stimulation, however, the reduction in the uncovered area was not significant (Figure 22).

Following our observations on the effects of *H19X* on VCAM1 expression and its pathway activation, we evaluated leucocyte to HDMEC adhesion as an important functional effect of EC activation in SSc (Figure 8A). Under basal conditions, without specific pro-inflammatory stimuli to HDMEC, no difference was observed. However, when HDEMC where stimulated with TNFα, *H19X* silencing induced a significant reduction of 21.2% (95.00% CI=0.06243-0.3618; p=0.0156) in the number of PBMCs binding to HDMEC (Figure 23).

Taken together, these results provided additional functional evidence that *H19X* upregulation in SSc ECs leads to VCAM1 expression and VCAM1 pathway activation resulting in increased cytoskeletal rearrangements and adhesion of leucocytes to the activated endothelium.

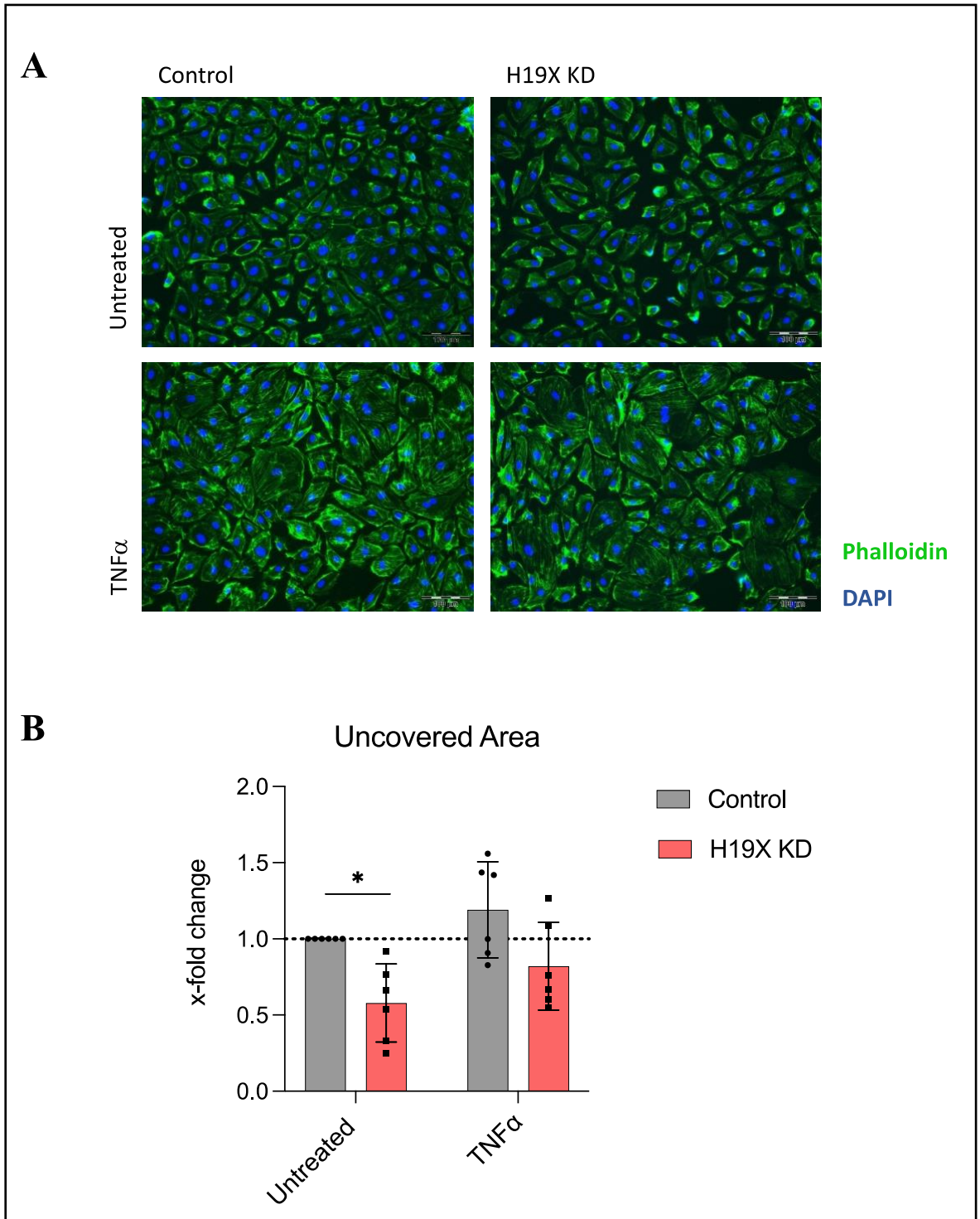
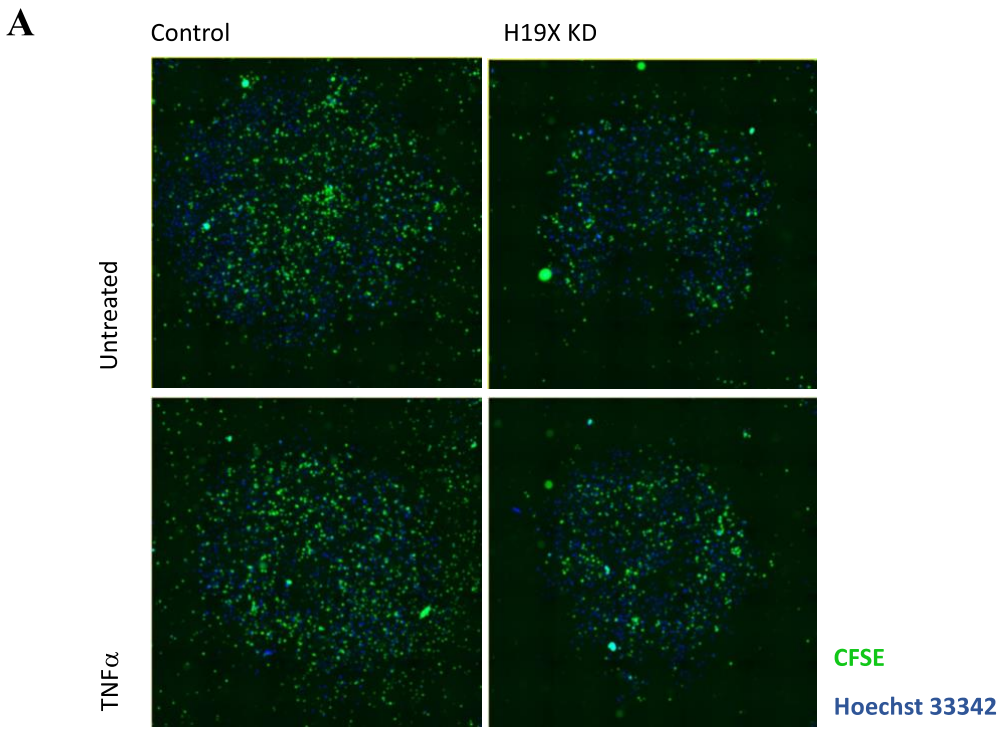


Figure 22. H19X knockdown reduces cytoskeletal rearrangements HDMEC (*n*=7) were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting H19X for a total of 48H and then stimulated with 10 ng/ml TNFα for 3 h to enhance pathway activation. **(A)** Stress fibers (F-actin) were stained using fluorescent labelled phalloidin (green) and nuclei were counterstained with DAPI (blue). Magnification 20x. (B) Uncovered area was quantified by ImageJ software and normalized to cell nuclei count.



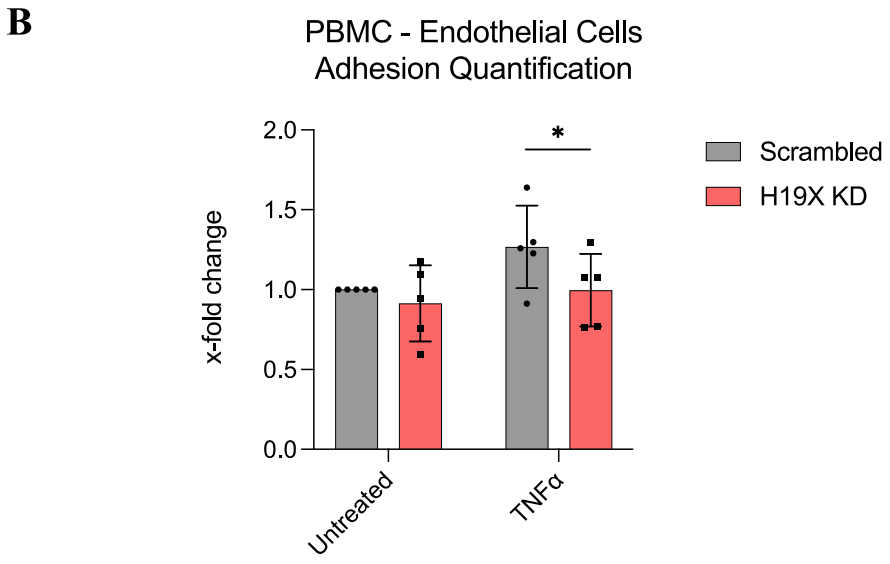


Figure 23. H19X knockdown reduces PBMC adhesion to the endothelium HDMEC (*n*=5) were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting H19X for a total of 48H and then stimulated with 10 ng/ml TNFα for 3 h to enhance adhesion activation. **(A)** PBMC were stained with CFSE (green) added to a HDMEC monolayer and co-incubated overnight. HDMEC nuclei were counterstained with Hoechst 33342 (blue). After elimination of cells in suspension, adherent PBMC and HDMEC were counted using the Cellinsight CX7 high-content imaging platform. **(B)** PBMC count was normalized on HDMEC count and compared to the negative control. Magnification 10x. *P < 0.05.

H19X does not affect apoptosis and proliferation in endothelial cells

To further understand potential roles of *H19X* in the endothelium, functional assays to measure apoptosis and proliferation were carried out.

In SSc, apoptosis represents an important mechanism to respond to microvascular injury, and markers of EC apoptosis have been described in the serum of patients (35). We assessed EC apoptosis by using Caspase 3/7 assay. In HDMEC, Caspase 3/7 activity did not differ following H19X silencing both in untreated condition and after IFN stimulation (Figure 24A) When HDMEC were stimulated with 10 ng/ml TNFα, a significant increase in apoptosis was noted compared with the untreated condition, as expected as this cytokine is a potent inducer of programmed cell death; however, even following TNFα stimulation, no changes in Caspase 3/7 activity were detected (Figure 24B).

Endothelial proliferation is another crucial cellular process involved in angiogenesis and vasculogenesis, which are impaired in SSc as an expression of endothelial injury (35). Cell proliferation following H19X knockdown was assessed using the BrdU colorimetric ELISA assay. After overnight incubation, no differences were measured under basal conditions, following IFN stimulation (Figure 25A) and stimulation with 10ng/ml VEGF (Figure 25B), the most relevant growth factor for EC.

Based on these findings, we concluded that *H19X* does not appear to be involved in the regulation of cell proliferation and apoptosis characteristic on injured endothelial cells.

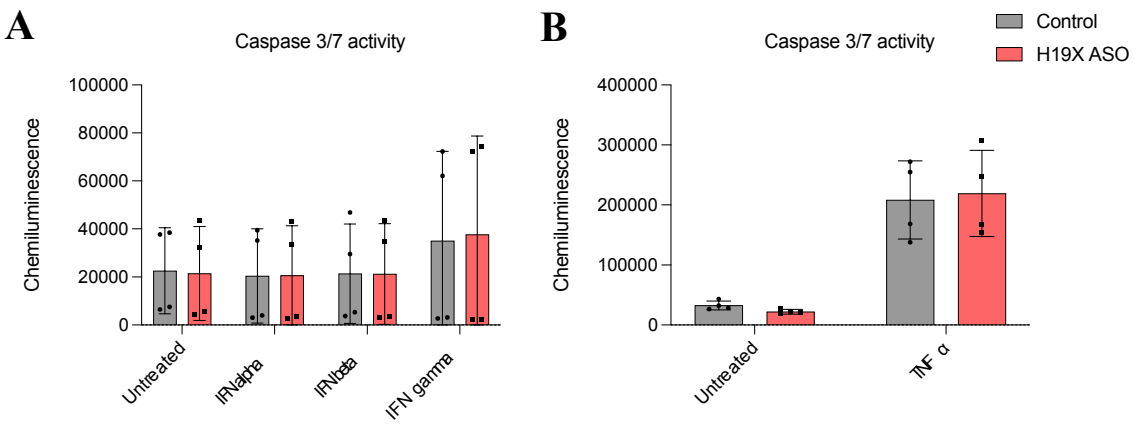


Figure 24. H19X knockdown does not affect apoptosis in endothelial cells Apoptosis was measured by Caspase 3/7 assay 48H after transfection **(A)** HDMEC (*n*=4) were stimulated with 10 ng/ml IFNs for 48H **(B)** HDMEC (*n*=4) were treated with 10 ng/ml TNFα for 24h to induce apoptosis.

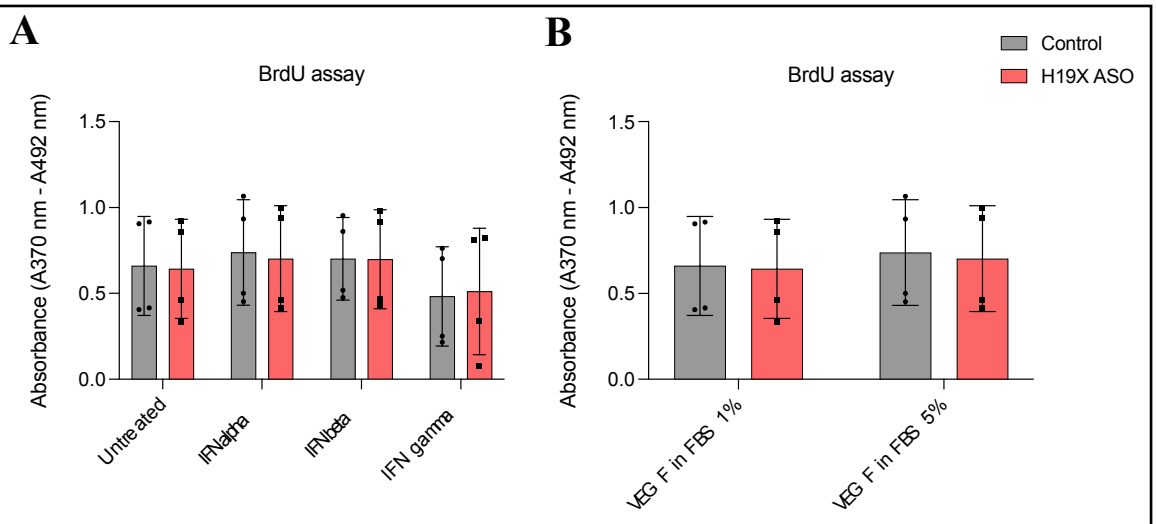


Figure 25. H19X knockdown does not affect proliferation in endothelial cells Proliferation was measured by BrdU colorimetric ELISA assay 48H after transfection **(A)** HDMEC (*n*=4) were stimulated with 10 ng/ml IFNs for 48H **(B)** HDMEC (*n*=4) were treated with 10 ng/ml VEGF in either 1% or 5%FBS medium for 24h to induce proliferation.

DISCUSSION

SSc is a multisystemic disease characterized by vasculopathy, autoimmunity and diffuse organ fibrosis. SSc is rare, especially in childhood, as pediatric onset disease accounts only for 5% of systemic sclerosis cases (17). Both adult and juvenile forms of SSc are burdened by very severe morbidity and mortality and there is an unmet need for specific treatments. Identification of novel potential pathogenic biomarkers is therefore crucial for diagnostic and therapeutic purposes. In this scenario, as their biological relevance is becoming increasingly clear, dissecting the role of non – coding RNAs in SSc might provide unprecedented insights into the molecular mechanisms involved in disease pathophysiology and open the way to innovative approaches in the management of this condition.

In this study, we characterized the functions of lncRNA *H19X* in the endothelium, which represents a crucial disease target in SSc. We could demonstrate that *H19X* is involved in the regulation of endothelial adhesion molecules, especially VCAM1, and that its expression in EC is regulated by IFNs.

The role of lncRNA *H19X*, also known as MIR503 hosting gene (*MIR503HG*), so far has been described in a few diseases. In some cancers, *H19X* acts as a tumor suppressor, reducing cell proliferation, limiting cell invasiveness and inducing apoptosis (55-57, 59). Recently, *H19X* depletion has been associated with endothelial – to – mesenchymal transition in vascular remodeling and pulmonary artery hypertension (60). In a previous study, it was shown that *H19X* is upregulated in SSc skin as well as across several fibrotic disorders (52). It was demonstrated that the presence of *H19X* is required for the remodeling effects of TGFβ, as well as myofibroblast differentiation and survival.

Given the observation that *H19X* is upregulated in SSc skin, we first wanted to investigate whether this was also due to endothelial involvement, as microvascular EC injury is a crucial pathogenic mechanism described since the first phases of the disease. Analysis of scRNA-seq data from skin biopsies of a dsSSc cohort was used to clarify which cell populations express *H19X*. In fact, scRNA-seq is a novel high throughput sequencing technique that detects gene expression at a single – cell resolution, which allows to clearly distinguish the different cellular subpopulations present in a given sample. Analysis of the scRNA – seq dataset confirmed that H19X was not only expressed by fibroblasts, as already described before (52) but also by endothelial cells. Subsequently, we demonstrated that *H19X* is significantly upregulated in the endothelium of the skin of SSc patients compared to healthy controls. This indicates that endothelial cells from SSc skin express higher levels of lncRNA *H19X*, suggesting that it might play a relevant role in endothelial injury typical for this disease.

After confirming its upregulation in SSc endothelium, we investigated which cellular mechanism could be involved in H19X function and regulation in EC by carrying out *in vitro* experiments using HDMEC, primary dermal microvascular endothelial cells which are isolated by human skin.

First, in order to identify potential regulators of *H19X* expression in the endothelium, we performed stimulation experiments on HDMECs using several pro – inflammatory and pro – fibrotic cytokines involved in EC activation in SSc and we could demonstrate that *H19X* is upregulated by type I and type II IFNs. IFNs are a class of cytokines involved in the regulation of innate immune responses that play an especially relevant role in defense against viral infections. Type I IFNs include IFNα and IFNβ, while IFNγ is the only component of Type II IFN family. An increase in circulating levels of IFNs and a dysregulation of IFN – inducible genes has been described for many chronic autoimmune

disorders (66). In SSc, the expression of type I IFN – inducible genes is increased in peripheral blood as well in affected tissue (25, 26, 67); in SSc skin this altered expression is observed both in lesional and non – lesional area (67). Of interest, Brkic and colleagues (25) showed that a type I IFN signature is present in peripheral monocytes of very early SSc, even before detection of fibrotic manifestation. With regards to type II IFNs, higher circulating levels of IFNγ are also described in SSc, and a higher expression of IFNγ has been shown in Raynaud phenomenon, an early vascular manifestation of SSc (68). Moreover, it seems to modulate vascular permeability and influence leucocyte transmigration through the endothelium (69).

Considered that the endothelium is one of the very first targets in SSc pathogenesis and given the effect of IFN in SSc and its effects on vascular and endothelial functions, the results of our stimulation experiments allow to hypothesize that *H19X*, since the earliest phases of the disease, is involved in IFN – mediated pathogenic mechanisms that lead to endothelial injury.

Among the various cytokines tested in stimulation experiment, TGFβ was also used. TGFβ is the main pro – fibrotic cytokine; in EC, it to be able to induce endothelial – to – mesenchymal transition (Endo–MT), a process where endothelial cells acquire myofibroblast – like features, contributing to fibrosis. Endo–MT induced by TGFβ involved in pathological vascular remodeling and development of skin fibrosis in SSc (70). Interestingly, in our experiments TGFβ induced downregulation of *H19X*, in contrast with what has been previously observed in fibroblasts, where TGFβ acts a strong inducer of *H19X* (52). H19X downregulation following TGFβ regulation has been recently described also in an in vitro model of Endo–MT, in line with our findings (60). Overall, in the endothelium, H19X activity does not seem to correlate with development of fibrotic

processes. Different mechanisms compared to what is described in fibrosis can be expected as lncRNAs display important tissue specificity in their expression and function.

We then wanted to characterize the possible functions of *H19X* in the endothelium. Therefore, in a hypothesis – driven manner, we selected EC injury and EC activation biomarkers relevant for SSc (35) and performed *H19X* knockdown experiments on HDMECs. *H19X* expression was closely associated with deregulated expression of adhesion molecules, including VCAM1, E-Selectin and P-Selectin, suggesting that *H19X* could act as a regulator of cell adhesion process. Remarkably, a correlation between *H19X* and adhesion function was also confirmed on scRNA-seq data. Indeed, pathway analysis performed with KEGG database on data from the skin biopsies showed a significant correlation between *H19X* – expressing EC and 'Cell Adhesion Molecules' pathway.

Exposure of surface adhesion molecules allows leukocyte recruitment and transmigration of immune cells to the tissues, which is crucial for the initiation of the inflammatory process. In vivo, leucocyte trafficking from the bloodstream is fundamental to mediate immune responses that counteract harmful stimuli in the tissues. Upon activation by pro – inflammatory cytokines, endothelial cells expose on their surface adhesion molecules such selectins, that allow leucocyte rolling on the endothelial layer through low – affinity binding. Subsequently, tight adhesion mediated by leucocyte integrins and their endothelial ligands induces morphological rearrangements in endothelial cells that allow leucocyte extravasation to the tissue (71, 72). While, in physiological conditions, upon cessation of the pathologic stimulus the effect of anti – inflammatory cytokines prevails and basal conditions are restored, in inflammatory diseases, such as many rheumatological conditions, an excessive, pathological inflammation develops, which leads to progressive tissue damage and subsequent organ disfunction. In these conditions, microvascular injury develops and adhesion molecules expression is typically increased as marker of endothelial

activation. Indeed, an increase in tissue and circulating adhesion markers levels is well characterized in SSc (35).

In our experiments, among the identified adhesion molecules, VCAM1 was the most consistently dowregulated in EC following *H19X* silencing. Moreover, a positive correlation between *H19X* and *VCAM1* expression was confirmed in SSc endothelium by co – expression analysis performed on scRNA-seq data analysis.

VCAM1 is an inducible surface glycoprotein that belongs to the immunoglobulin superfamily, which is mainly expressed by EC in response to pro-inflammatory cytokines TNFAα and IL-1β. It binds integrin-α4β1 on leukocyte surface, allowing leukocyte recruitment. Upon activation, it mediates intracellular mechanisms that determine cytoskeletal rearrangements that cause an increase in endothelial permeability, allowing leukocyte trans – endothelial migration (72, 73). Various works (38, 74, 75) have described elevated circulating VCAM1 levels in SSc patient sera, thus suggesting that VCAM1 – mediated adhesion is impaired in the disease and might contribute to its pathogenesis. Therefore, we evaluated leucocyte to HDMEC adhesion as an important functional effect of EC activation in SSc. When performing endothelial and PBMC co-culture with inflammatory stimuli, *H19X* silencing determined a significant reduction in leucocyte to HDMEC adhesion. The reduction of leucocyte adhesion, however significant, was limited to around 20%. This might be explained by the fact that VCAM1 function in the endothelium is redundant and partially overlaps with that of other adhesion mediators, which are likely not affected by *H19X*; thus, only a partial reduction of adhesion capacity was expected.

These observations lead to speculate that lncRNA *H19X* does not only influence VCAM1 expression, but also plays a functional role in VCAM1 mediated microvascular activation. To further corroborate this hypothesis, we evaluated the activity of intracellular

downstream effectors and performed functional assays to test cell contractility, demonstrating that VCAM1 signaling pathway is less active following *H19X* knockdown. Specifically, we evaluated the effects of Rac1 activation and its effectors. Rac1 – GTPase is an intracellular transducer with a crucial role in the regulation of endothelial permeability. Following activation of VCAM1, Rac1 in its GTP – bound activated form regulates two pathways: on one hand, it is involved in disruption of intracellular junctions by increasing reactive oxygen species (ROS) intracellular production and subsequent activation of protein tyrosine phosphatase 1B (PTP1B). At the same time, it regulates cell permeability through the activation of PAK - myosin light chain kinase (MLCK) pathway (73, 76, 77). PAK is an intracellular Serin/Threonine kinase that, following activation by phosphorylation of Ser^{141} and Thr^{402}, activates MLCK, a major regulator of actomyosin contraction. In endothelial cells, MLCK activation leads to reorganization of actin fibers, promoting cell retraction with disruption of endothelial integrity (78, 79). It is reported that majority of $pSer^{141}$ PAK are localized to areas of cell-cell contact and that displacement of active PAK from sites of cell-cell contact prevents increases of vascular permeability (80). In our experiments, when downstream effects of VCAM1 activation were evaluated, we observed less GTP - Rac1 and reduced levels of $pSer^{141}$ PAK following *H19X* silencing. Moreover, *H19X* knockdown reduced the growing area occupied by the cells, restoring the integrity of the monolayer of EC and indicating reduced cell contractility. Taken together, these data support our hypothesis that *H19X* – mediated expression of VCAM1 is crucial for EC cytoskeletal rearrangements and, in turn, for leukocyte transmigration into the tissues.

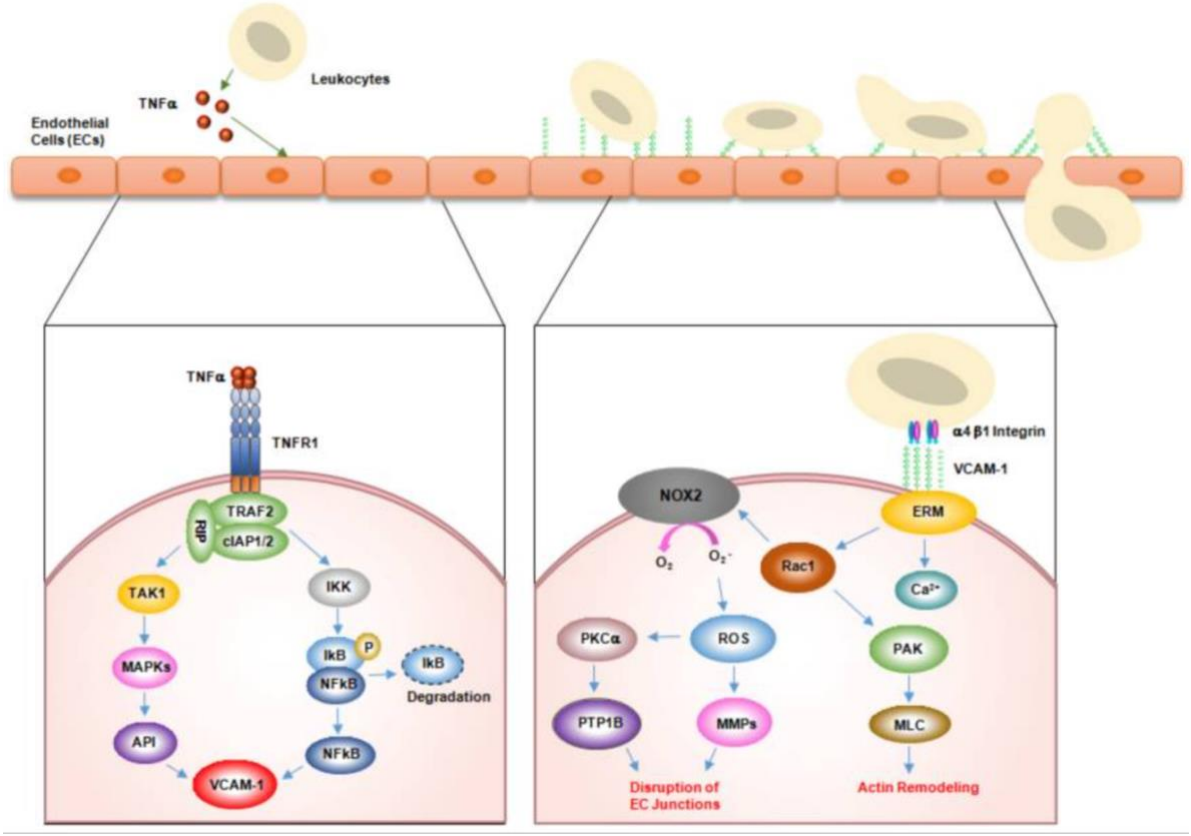


Figure 26. Schematic representation intracellular events that follow leucocyte recruitment On the left, TNFα enhances VCAM1 expression. On the right, intracellular pathways activated upon VCAM1 tight binding with an immune cell. Through mechanisms regulated by Rac1, vascular permeability increases, allowing leucocyte transmigration through the endothelial layer.
From Kong DH, Int J Mol Sci 2018 (72)

Finally, other potential functions of *H19X* in EC were explored by investigating its role in apoptosis and cellular proliferation. Apoptosis and proliferation are important mechanisms that are put in place as responses to microvascular injury. In SSc, these processes are impaired and contribute to aberrant vasculogenesis and angiogenesis and to development of vasculopathy (36); increased levels of circulating markers of EC apoptosis and proliferation have been described in SSc (35). In SSc fibroblasts, *H19X* seem to regulate both mechanisms (51, 52) and what is described in some cancers, where it seems to positively regulate proliferation (56-59). Moreover, another study that investigated the role

of lncRNA as sensors for hypoxia, described reduced cellular proliferation following H19X silencing, however without effects on angiogenesis (54). Our results did not confirm these previous data, as we did not observe changes in apoptosis and cell proliferation following *H19X* silencing. Instead, in screening knockdown experiments on EC injury markers, we reported a slight increase in angiogenesis markers, which was significant only for *VEGFA*. However, our work did not primarily focus on *H19X* effects on angiogenesis, thus more evidence should be gathered before drawing firm conclusions on this point.

LIMITATIONS

Finally, some limitations to this study need to be addressed. Importantly, we must underline that the scRNA-seq assay used for this work is designed for detecting 3'-polyadenylated (poly(A)) transcripts. Of the ten transcript variants so far reported in Ensembl database for *H19X* (81), not all present a poly(A) tail; this means that our analysis likely lacks complete data on *H19X* expression in EC. Although we recognize this as a relevant limitation, this could at least partially explain the overall low percentage of cells expressing our target. Moreover, we might also hypothesize that, in case all variants were detected, an even higher expression of *H19X* in EC from our samples could have been found.

As for the *in vitro* experiments, it should be noted that some of those discussed in this study, such as some of the stimulation experiments with cytokines different from IFNs, were performed as screening experiments on only a small number of replicates (1-2) which may limit the interpretation of the results.

Lastly, as we focused on the functional effects on regulation of adhesion molecule expression, we did not provide a mechanistic insight into how *H19X* could be involved in gene transcription regulation. Notably, one study reported that miR-503 induced adhesion molecules expression in coronary artery EC (82); this might lead to hypothesize that *H19X* might act as a reservoir for neighboring miRNAs. Another work on its role in endo – MT, has suggested that *H19X* interacts with PTBP1 protein expression mainly at protein level, suggesting that it can also act at post – translational level. However, these and other hypotheses will need to be investigated in adhesion molecules pathways in future projects.

CONCLUSIONS

In summary, this work provides evidence for a functional role of lncRNA *H19X* in microvascular EC. We showed that *H19X* had a higher expression in EC of SSc skin and that in HDMEC it was upregulated by both type I and type II IFNs. Moreover, we could demonstrate that *H19X* silencing significantly affected adhesion molecules expression, predominantly VCAM1, with consequent reduction of adhesion capacity and EC cytoskeletal rearrangements.

Based on these data, we can conclude that *H19X* contributes significantly to endothelial activation, a crucial mechanism in the pathophysiology of SSc. Elevated *H19X* levels might be induced by prolonged and sustained IFN – mediated inflammation since the very early phases of the disease. This could lead to consistent, aberrant activation of adhesion molecules, particularly VCAM1, which in turns leads to disruption of endothelial integrity, with development of microvascular injury, and promotes leucocyte infiltration, perpetuating pathologic tissue inflammation.

While at functional level relevant evidence has been provided, future studies will be required to shed light on the exact mechanism involved in *H19X* – mediated gene expression regulation of adhesion molecules in the endothelium.

Finally, although our knowledge on the effective role of non-coding RNAs in disease is still only at the beginning, it is also possible to speculate on a future clinical implication of the results of the following study. Once future analyses will confirm that *H19X* is a marker of vascular damage, an early event in SSc, it might be identified as a potential marker of early-stage disease, which would ensure to start clinical monitoring precociously, or it could be used to identify those patients at high risk of developing severe vascular disease, thus targeting tailored treatments. Again, further studies will be needed, that should also

include correlations with clinical data. This is already foreseen by our research group, which plans in particular to extend the results obtained so far also to a population of pediatric patients, in order to clarify the role of non-coding RNAs also in juvenile scleroderma.

REFERENCES

1. Allanore Y, Simms R, Distler O, Trojanowska M, Pope J, Denton CP, et al. Systemic sclerosis. Nat Rev Dis Primers. 2015;1:15002. Epub 20150423. doi: 10.1038/nrdp.2015.2. PubMed PMID: 27189141.

2. Ingegnoli F, Ughi N, Mihai C. Update on the epidemiology, risk factors, and disease outcomes of systemic sclerosis. Best Pract Res Clin Rheumatol. 2018;32(2):223-40. Epub 20180914. doi: 10.1016/j.berh.2018.08.005. PubMed PMID: 30527428.

3. Denton CP, Khanna D. Systemic sclerosis. Lancet. 2017;390(10103):1685-99. Epub 20170413. doi: 10.1016/s0140-6736(17)30933-9. PubMed PMID: 28413064.

4. Kowal-Bielecka O, Fransen J, Avouac J, Becker M, Kulak A, Allanore Y, et al. Update of EULAR recommendations for the treatment of systemic sclerosis. Ann Rheum Dis. 2017;76(8):1327-39. Epub 20161109. doi: 10.1136/annrheumdis-2016-209909. PubMed PMID: 27941129.

5. Zulian F. Scleroderma in children. Best Pract Res Clin Rheumatol. 2017;31(4):576-95. Epub 20180327. doi: 10.1016/j.berh.2018.02.004. PubMed PMID: 29773274.

6. Martini G, Foeldvari I, Russo R, Cuttica R, Eberhard A, Ravelli A, et al. Systemic sclerosis in childhood: clinical and immunologic features of 153 patients in an international database. Arthritis Rheum. 2006;54(12):3971-8. doi: 10.1002/art.22207. PubMed PMID: 17133611.

7. Smith V, Herrick AL, Ingegnoli F, Damjanov N, De Angelis R, Denton CP, et al. Standardisation of nailfold capillaroscopy for the assessment of patients with Raynaud's phenomenon and systemic sclerosis. Autoimmun Rev. 2020;19(3):102458. Epub 20200110. doi: 10.1016/j.autrev.2020.102458. PubMed PMID: 31927087.

8. Turner R, Lipshutz W, Miller W, Rittenberg G, Schumacher HR, Cohen S. Esophageal dysfunction in collagen disease. Am J Med Sci. 1973;265(3):191-9. doi: 10.1097/00000441-197303000-00002. PubMed PMID: 4701683.

9. Akesson A, Wollheim FA. Organ manifestations in 100 patients with progressive systemic sclerosis: a comparison between the CREST syndrome and diffuse scleroderma. Br J Rheumatol. 1989;28(4):281-6. doi: 10.1093/rheumatology/28.4.281. PubMed PMID: 2787182.

10. Avouac J, Walker UA, Hachulla E, Riemekasten G, Cuomo G, Carreira PE, et al. Joint and tendon involvement predict disease progression in systemic sclerosis: a EUSTAR prospective study. Ann Rheum Dis. 2016;75(1):103-9. Epub 20140827. doi: 10.1136/annrheumdis-2014-205295. PubMed PMID: 25165035.

11. Turk M, Pope JE. The Frequency of Scleroderma Renal Crisis over Time: A Metaanalysis. J Rheumatol. 2016;43(7):1350-5. Epub 20160501. doi: 10.3899/jrheum.151353. PubMed PMID: 27134252.

12. Desai CS, Lee DC, Shah SJ. Systemic sclerosis and the heart: current diagnosis and management. Curr Opin Rheumatol. 2011;23(6):545-54. doi: 10.1097/BOR.0b013e32834b8975. PubMed PMID: 21897256; PubMed Central PMCID: PMC3678364.

13. Parks JL, Taylor MH, Parks LP, Silver RM. Systemic sclerosis and the heart. Rheum Dis Clin North Am. 2014;40(1):87-102. Epub 20131107. doi: 10.1016/j.rdc.2013.10.007. PubMed PMID: 24268011.

14. Tyndall AJ, Bannert B, Vonk M, Airò P, Cozzi F, Carreira PE, et al. Causes and risk factors for death in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database. Ann Rheum Dis. 2010;69(10):1809-15. Epub 20100615. doi: 10.1136/ard.2009.114264. PubMed PMID: 20551155.

15. Elhai M, Meune C, Boubaya M, Avouac J, Hachulla E, Balbir-Gurman A, et al. Mapping and predicting mortality from systemic sclerosis. Ann Rheum Dis. 2017;76(11):1897-905. Epub 20170823. doi: 10.1136/annrheumdis-2017-211448. PubMed PMID: 28835464.

16. Herrick AL, Ennis H, Bhushan M, Silman AJ, Baildam EM. Incidence of childhood linear scleroderma and systemic sclerosis in the UK and Ireland. Arthritis Care Res (Hoboken). 2010;62(2):213-8. doi: 10.1002/acr.20070. PubMed PMID: 20191520.

17. Mayes MD, Lacey JV, Jr., Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ, et al. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. Arthritis Rheum. 2003;48(8):2246-55. doi: 10.1002/art.11073. PubMed PMID: 12905479.

18. Martini G, Vittadello F, Kasapçopur O, Magni Manzoni S, Corona F, Duarte-Salazar C, et al. Factors affecting survival in juvenile systemic sclerosis. Rheumatology (Oxford). 2009;48(2):119-22. Epub 20081014. doi: 10.1093/rheumatology/ken388. PubMed PMID: 18854345.

19. Stevens AM, Torok KS, Li SC, Taber SF, Lu TT, Zulian F. Immunopathogenesis of Juvenile Systemic Sclerosis. Front Immunol. 2019;10:1352. Epub 20190625. doi: 10.3389/fimmu.2019.01352. PubMed PMID: 31293569; PubMed Central PMCID: PMC6603145.

20. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol. 1988;15(2):202-5. PubMed PMID: 3361530.

21. LeRoy EC, Medsger TA, Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol. 2001;28(7):1573-6. PubMed PMID: 11469464.

22. Zulian F, Lanzoni G, Castaldi B, Meneghel A, Tirelli F, Zanatta E, et al. Systemic sclerosis sine scleroderma in children. Rheumatology (Oxford). 2022;61(6):2555-62. doi: 10.1093/rheumatology/keab738. PubMed PMID: 34605913; PubMed Central PMCID: PMC9157094.

23. Assassi S, Mayes MD, Arnett FC, Gourh P, Agarwal SK, McNearney TA, et al. Systemic sclerosis and lupus: points in an interferon-mediated continuum. Arthritis Rheum. 2010;62(2):589-98. doi: 10.1002/art.27224. PubMed PMID: 20112391; PubMed Central PMCID: PMC2879587.

24. York MR, Nagai T, Mangini AJ, Lemaire R, van Seventer JM, Lafyatis R. A macrophage marker, Siglec-1, is increased on circulating monocytes in patients with systemic sclerosis and induced by type I interferons and toll-like receptor agonists. Arthritis Rheum. 2007;56(3):1010-20. doi: 10.1002/art.22382. PubMed PMID: 17328080.

25. Brkic Z, van Bon L, Cossu M, van Helden-Meeuwsen CG, Vonk MC, Knaapen H, et al. The interferon type I signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high BAFF gene expression and high collagen synthesis. Ann Rheum Dis. 2016;75(8):1567-73. Epub 20150914. doi: 10.1136/annrheumdis-2015-207392. PubMed PMID: 26371289.

26. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, et al. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. Ann Rheum Dis. 2011;70(11):2029-36. Epub 20110728. doi: 10.1136/ard.2011.150326. PubMed PMID: 21803750.

27. Chizzolini C, Parel Y, Scheja A, Dayer JM. Polarized subsets of human T-helper cells induce distinct patterns of chemokine production by normal and systemic sclerosis dermal fibroblasts. Arthritis Res Ther. 2006;8(1):R10. doi: 10.1186/ar1860. PubMed PMID: 16356198; PubMed Central PMCID: PMC1526552.

28. Kania G, Rudnik M, Distler O. Involvement of the myeloid cell compartment in fibrogenesis and systemic sclerosis. Nat Rev Rheumatol. 2019;15(5):288-302. doi: 10.1038/s41584-019-0212-z. PubMed PMID: 30953037.

29. Hasegawa M, Sato S, Takehara K. Augmented production of transforming growth factor-beta by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. Arch Dermatol Res. 2004;296(2):89-93. Epub 20040507. doi: 10.1007/s00403-004-0472-5. PubMed PMID: 15133694.

30. Artlett CM, Sassi-Gaha S, Rieger JL, Boesteanu AC, Feghali-Bostwick CA, Katsikis PD. The inflammasome activating caspase 1 mediates fibrosis and myofibroblast differentiation in systemic sclerosis. Arthritis Rheum. 2011;63(11):3563-74. doi: 10.1002/art.30568. PubMed PMID: 21792841.

31. Ciechomska M, O'Reilly S, Przyborski S, Oakley F, Bogunia-Kubik K, van Laar JM. Histone Demethylation and Toll-like Receptor 8-Dependent Cross-Talk in Monocytes Promotes Transdifferentiation of Fibroblasts in Systemic Sclerosis Via Fra-2. Arthritis Rheumatol. 2016;68(6):1493-504. doi: 10.1002/art.39602. PubMed PMID: 26814616.

32. Garrett SM, Baker Frost D, Feghali-Bostwick C. The mighty fibroblast and its utility in scleroderma research. J Scleroderma Relat Disord. 2017;2(2):69-134. Epub 20170519. doi: 10.5301/jsrd.5000240. PubMed PMID: 29270465; PubMed Central PMCID: PMC5736140.

33. Matucci-Cerinic M, Kahaleh B, Wigley FM. Review: evidence that systemic sclerosis is a vascular disease. Arthritis Rheum. 2013;65(8):1953-62. doi: 10.1002/art.37988. PubMed PMID: 23666787.

34. Altorok N, Wang Y, Kahaleh B. Endothelial dysfunction in systemic sclerosis. Curr Opin Rheumatol. 2014;26(6):615-20. doi: 10.1097/bor.0000000000000112. PubMed PMID: 25191994.

35. Mostmans Y, Cutolo M, Giddelo C, Decuman S, Melsens K, Declercq H, et al. The role of endothelial cells in the vasculopathy of systemic sclerosis: A systematic review. Autoimmun Rev. 2017;16(8):774-86. Epub 20170529. doi: 10.1016/j.autrev.2017.05.024. PubMed PMID: 28572048.

36. Asano Y, Sato S. Vasculopathy in scleroderma. Semin Immunopathol. 2015;37(5):489-500. Epub 20150708. doi: 10.1007/s00281-015-0505-5. PubMed PMID: 26152638.

37. Ihn H, Sato S, Fujimoto M, Takehara K, Tamaki K. Increased serum levels of soluble vascular cell adhesion molecule-1 and E-selectin in patients with systemic sclerosis. Br J Rheumatol. 1998;37(11):1188-92. doi: 10.1093/rheumatology/37.11.1188. PubMed PMID: 9851267.

38. Andersen GN, Caidahl K, Kazzam E, Petersson AS, Waldenström A, Mincheva-Nilsson L, et al. Correlation between increased nitric oxide production and markers of endothelial activation in systemic sclerosis: findings with the soluble adhesion molecules E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1. Arthritis Rheum. 2000;43(5):1085-93. doi: 10.1002/1529-0131(200005)43:5<1085::aid-anr19>3.0.co;2-7. PubMed PMID: 10817563.

39. Alexander RP, Fang G, Rozowsky J, Snyder M, Gerstein MB. Annotating non-coding regions of the genome. Nat Rev Genet. 2010;11(8):559-71. Epub 20100713. doi: 10.1038/nrg2814. PubMed PMID: 20628352.

40. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet. 2011;12(12):861-74. Epub 20111118. doi: 10.1038/nrg3074. PubMed PMID: 22094949.

41. Brosnan CA, Voinnet O. The long and the short of noncoding RNAs. Curr Opin Cell Biol. 2009;21(3):416-25. Epub 20090515. doi: 10.1016/j.ceb.2009.04.001. PubMed PMID: 19447594.

42. Zhang P, Wu W, Chen Q, Chen M. Non-Coding RNAs and their Integrated Networks. J Integr Bioinform. 2019;16(3). Epub 20190713. doi: 10.1515/jib-2019-0027. PubMed PMID: 31301674; PubMed Central PMCID: PMC6798851.

43. Paul P, Chakraborty A, Sarkar D, Langthasa M, Rahman M, Bari M, et al. Interplay between miRNAs and human diseases. J Cell Physiol. 2018;233(3):2007-18. Epub 20170427. doi: 10.1002/jcp.25854. PubMed PMID: 28181241.

44. Thomson T, Lin H. The biogenesis and function of PIWI proteins and piRNAs: progress and prospect. Annu Rev Cell Dev Biol. 2009;25:355-76. doi: 10.1146/annurev.cellbio.24.110707.175327. PubMed PMID: 19575643; PubMed Central PMCID: PMC2780330.

45. Ozata DM, Gainetdinov I, Zoch A, O'Carroll D, Zamore PD. PIWI-interacting RNAs: small RNAs with big functions. Nat Rev Genet. 2019;20(2):89-108. doi: 10.1038/s41576-018-0073-3. PubMed PMID: 30446728.

46. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet. 2016;17(1):47-62. doi: 10.1038/nrg.2015.10. PubMed PMID: 26666209.

47. Statello L, Guo CJ, Chen LL, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. Nat Rev Mol Cell Biol. 2021;22(2):96-118. Epub 20201222. doi: 10.1038/s41580-020-00315-9. PubMed PMID: 33353982; PubMed Central PMCID: PMC7754182.

48. Kopp F, Mendell JT. Functional Classification and Experimental Dissection of Long Noncoding RNAs. Cell. 2018;172(3):393-407. doi: 10.1016/j.cell.2018.01.011. PubMed PMID: 29373828; PubMed Central PMCID: PMC5978744.

49. Tang Y, Zhou T, Yu X, Xue Z, Shen N. The role of long non-coding RNAs in rheumatic diseases. Nat Rev Rheumatol. 2017;13(11):657-69. Epub 20171005. doi: 10.1038/nrrheum.2017.162. PubMed PMID: 28978995.

50. Hur K, Kim SH, Kim JM. Potential Implications of Long Noncoding RNAs in Autoimmune Diseases. Immune Netw. 2019;19(1):e4. Epub 20190225. doi: 10.4110/in.2019.19.e4. PubMed PMID: 30838159; PubMed Central PMCID: PMC6399094.

51. Takata M, Pachera E, Frank-Bertoncelj M, Kozlova A, Jüngel A, Whitfield ML, et al. OTUD6B-AS1 Might Be a Novel Regulator of Apoptosis in Systemic Sclerosis. Front Immunol. 2019;10:1100. Epub 20190517. doi: 10.3389/fimmu.2019.01100. PubMed PMID: 31156645; PubMed Central PMCID: PMC6533854.

52. Pachera E, Assassi S, Salazar GA, Stellato M, Renoux F, Wunderlin A, et al. Long noncoding RNA H19X is a key mediator of TGF-β-driven fibrosis. J Clin Invest. 2020;130(9):4888-905. doi: 10.1172/jci135439. PubMed PMID: 32603313; PubMed Central PMCID: PMC7456219.

53. Wang F, Liang R, Tandon N, Matthews ER, Shrestha S, Yang J, et al. H19X-encoded miR-424(322)/-503 cluster: emerging roles in cell differentiation, proliferation, plasticity and metabolism. Cell Mol Life Sci. 2019;76(5):903-20. Epub 20181124. doi: 10.1007/s00018-018-2971-0. PubMed PMID: 30474694; PubMed Central PMCID: PMC6394552.

54. Fiedler J, Breckwoldt K, Remmele CW, Hartmann D, Dittrich M, Pfanne A, et al. Development of Long Noncoding RNA-Based Strategies to Modulate Tissue Vascularization. J Am Coll Cardiol. 2015;66(18):2005-15. doi: 10.1016/j.jacc.2015.07.081. PubMed PMID: 26516004; PubMed Central PMCID: PMC4631810.

55. Fu J, Dong G, Shi H, Zhang J, Ning Z, Bao X, et al. LncRNA MIR503HG inhibits cell migration and invasion via miR-103/OLFM4 axis in triple negative breast cancer. J Cell Mol Med. 2019;23(7):4738-45. Epub 20190506. doi: 10.1111/jcmm.14344. PubMed PMID: 31062436; PubMed Central PMCID: PMC6584514.

56. Qiu F, Zhang MR, Zhou Z, Pu JX, Zhao XJ. lncRNA MIR503HG functioned as a tumor suppressor and inhibited cell proliferation, metastasis and epithelial-mesenchymal transition in bladder cancer. J Cell Biochem. 2019;120(6):10821-9. Epub 20190122. doi: 10.1002/jcb.28373. PubMed PMID: 30672010.

57. Han H, Li H, Zhou J. Long non-coding RNA MIR503HG inhibits the proliferation, migration and invasion of colon cancer cells via miR-107/Par4 axis. Exp Cell Res. 2020;395(2):112205. Epub 20200730. doi: 10.1016/j.yexcr.2020.112205. PubMed PMID: 32738347.

58. Hu YL, Zhang YX, Liu N, Liu H, Yuan YC. LncRNA MIR503HG regulated cell viability, metastasis and apoptosis of cervical cancer via miR-191/CEBPB axis. Eur Rev Med Pharmacol Sci. 2021;25(8):3200-10. doi: 10.26355/eurrev_202104_25728. PubMed PMID: 33928605.

59. Xu S, Zhai S, Du T, Li Z. LncRNA MIR503HG Inhibits Non-Small Cell Lung Cancer Cell Proliferation by Inducing Cell Cycle Arrest Through the Downregulation of Cyclin D1. Cancer Manag Res. 2020;12:1641-7. Epub 20200305. doi: 10.2147/cmar.s227348. PubMed PMID: 32184667; PubMed Central PMCID: PMC7062398.

60. Monteiro JP, Rodor J, Caudrillier A, Scanlon JP, Spiroski AM, Dudnakova T, et al. MIR503HG Loss Promotes Endothelial-to-Mesenchymal Transition in Vascular Disease. Circ Res. 2021;128(8):1173-90. Epub 20210311. doi: 10.1161/circresaha.120.318124. PubMed PMID: 33703914; PubMed Central PMCID: PMC7610629.

61. Gaydosik AM, Tabib T, Domsic R, Khanna D, Lafyatis R, Fuschiotti P. Single-cell transcriptome analysis identifies skin-specific T-cell responses in systemic sclerosis. Ann Rheum Dis. 2021;80(11):1453-60. Epub 20210524. doi: 10.1136/annrheumdis-2021-220209. PubMed PMID: 34031030; PubMed Central PMCID: PMC8516708.

62. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27-30. doi: 10.1093/nar/28.1.27. PubMed PMID: 10592173; PubMed Central PMCID: PMC102409.

63. Sierra-Sepúlveda A, Esquinca-González A, Benavides-Suárez SA, Sordo-Lima DE, Caballero-Islas AE, Cabral-Castañeda AR, et al. Systemic Sclerosis Pathogenesis and Emerging Therapies, beyond the Fibroblast. Biomed Res Int. 2019;2019:4569826. Epub 20190123. doi: 10.1155/2019/4569826. PubMed PMID: 30809542; PubMed Central PMCID: PMC6364098.

64. Marchese ME, Berdnikovs S, Cook-Mills JM. Distinct sites within the vascular cell adhesion molecule-1 (VCAM-1) cytoplasmic domain regulate VCAM-1 activation of calcium fluxes versus Rac1 during leukocyte transendothelial migration. Biochemistry. 2012;51(41):8235-46. Epub 20121001. doi: 10.1021/bi300925r. PubMed PMID: 22970700; PubMed Central PMCID: PMC3558628.

65. Radu M, Lyle K, Hoeflich KP, Villamar-Cruz O, Koeppen H, Chernoff J. p21-Activated Kinase 2 Regulates Endothelial Development and Function through the Bmk1/Erk5 Pathway. Mol Cell Biol. 2015;35(23):3990-4005. Epub 20150921. doi: 10.1128/mcb.00630-15. PubMed PMID: 26391956; PubMed Central PMCID: PMC4628059.

66. Rönnblom L. The importance of the type I interferon system in autoimmunity. Clin Exp Rheumatol. 2016;34(4 Suppl 98):21-4. Epub 20160721. PubMed PMID: 27586799.

67. Farina GA, York MR, Di Marzio M, Collins CA, Meller S, Homey B, et al. Poly(I:C) drives type I IFN- and TGFβ-mediated inflammation and dermal fibrosis simulating altered gene expression in systemic sclerosis. J Invest Dermatol. 2010;130(11):2583-93. Epub 20100708. doi: 10.1038/jid.2010.200. PubMed PMID: 20613770; PubMed Central PMCID: PMC3086558.

68. Molteni M, Della Bella S, Mascagni B, Bazzi S, Zulian C, Compasso S, et al. Increased interferon-gamma (IFN-gamma) levels produced in vitro by alloactivated T lymphocytes in systemic sclerosis and Raynaud's phenomenon. Clin Exp Immunol. 1999;116(1):164-8. doi: 10.1046/j.1365-2249.1999.00842.x. PubMed PMID: 10209521; PubMed Central PMCID: PMC1905213.

69. Ciechomska M, Skalska U. Targeting interferons as a strategy for systemic sclerosis treatment. Immunol Lett. 2018;195:45-54. Epub 20171026. doi: 10.1016/j.imlet.2017.10.011. PubMed PMID: 29106987.

70. Manetti M, Romano E, Rosa I, Guiducci S, Bellando-Randone S, De Paulis A, et al. Endothelial-to-mesenchymal transition contributes to endothelial dysfunction and dermal fibrosis in systemic sclerosis. Ann Rheum Dis. 2017;76(5):924-34. Epub 20170106. doi: 10.1136/annrheumdis-2016-210229. PubMed PMID: 28062404.

71. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13(3):159-75. doi: 10.1038/nri3399. PubMed PMID: 23435331.

72. van Wetering S, van den Berk N, van Buul JD, Mul FP, Lommerse I, Mous R, et al. VCAM-1-mediated Rac signaling controls endothelial cell-cell contacts and leukocyte transmigration. Am J Physiol Cell Physiol. 2003;285(2):C343-52. Epub 20030416. doi: 10.1152/ajpcell.00048.2003. PubMed PMID: 12700137.

73. Kong DH, Kim YK, Kim MR, Jang JH, Lee S. Emerging Roles of Vascular Cell Adhesion Molecule-1 (VCAM-1) in Immunological Disorders and Cancer. Int J Mol Sci. 2018;19(4). Epub 20180402. doi: 10.3390/ijms19041057. PubMed PMID: 29614819; PubMed Central PMCID: PMC5979609.

74. Cook-Mills JM, Marchese ME, Abdala-Valencia H. Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. Antioxid Redox Signal. 2011;15(6):1607-38. Epub 20110511. doi: 10.1089/ars.2010.3522. PubMed PMID: 21050132; PubMed Central PMCID: PMC3151426.

75. Cerinic MM, Valentini G, Sorano GG, D'Angelo S, Cuomo G, Fenu L, et al. Blood coagulation, fibrinolysis, and markers of endothelial dysfunction in systemic sclerosis. Semin Arthritis Rheum. 2003;32(5):285-95. doi: 10.1053/sarh.2002.50011. PubMed PMID: 12701039.

76. Thayse K, Kindt N, Laurent S, Carlier S. VCAM-1 Target in Non-Invasive Imaging for the Detection of Atherosclerotic Plaques. Biology (Basel). 2020;9(11). Epub 20201029. doi: 10.3390/biology9110368. PubMed PMID: 33138124; PubMed Central PMCID: PMC7692297.

77. Bosco EE, Mulloy JC, Zheng Y. Rac1 GTPase: a "Rac" of all trades. Cell Mol Life Sci. 2009;66(3):370-4. doi: 10.1007/s00018-008-8552-x. PubMed PMID: 19151919; PubMed Central PMCID: PMC6669905.

78. Shen Q, Rigor RR, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase in microvascular endothelial barrier function. Cardiovasc Res. 2010;87(2):272-80. Epub 20100517. doi: 10.1093/cvr/cvq144. PubMed PMID: 20479130; PubMed Central PMCID: PMC2895546.

79. Goeckeler ZM, Masaracchia RA, Zeng Q, Chew TL, Gallagher P, Wysolmerski RB. Phosphorylation of myosin light chain kinase by p21-activated kinase PAK2. J Biol Chem. 2000;275(24):18366-74. doi: 10.1074/jbc.M001339200. PubMed PMID: 10748018.

80. Stockton RA, Schaefer E, Schwartz MA. p21-activated kinase regulates endothelial permeability through modulation of contractility. J Biol Chem. 2004;279(45):46621-30. Epub 20040827. doi: 10.1074/jbc.M408877200. PubMed PMID: 15333633.

81. Howe KL, Achuthan P, Allen J, Alvarez-Jarreta J, Amode MR, Armean IM, et al. Ensembl 2021. Nucleic Acids Res. 2021;49(D1):D884-d91. doi: 10.1093/nar/gkaa942. PubMed PMID: 33137190; PubMed Central PMCID: PMC7778975.

82. Wang Y, Xu Z, Wang X, Zheng J, Peng L, Zhou Y, et al. Extracellular-vesicle containing miRNA-503-5p released by macrophages contributes to atherosclerosis. Aging (Albany NY). 2021;13(8):12239-57. Epub 20210419. doi: 10.18632/aging.103855. PubMed PMID: 33872218; PubMed Central PMCID: PMC8109059.

Acknowledgments

Prof Oliver Distler welcomed me at the laboratories of the Center of Experimental Rheumatology and provided scientific guidance and the resources for the present project.

Dr. Elena Pachera supervised my work in the laboratory, taught me most of the research techniques that were applied in the experiments and took care of the experiments after the end of my period in Zurich, together with medical student Sabrina Gmür, allowing our project to be completed.

Dr. Eva Camarillo Retamosa supervised me in the use and analyses performed with the Cellinsight CX7 high-content imaging platform.

Dr. Robert Lafyatis and Dr. Huang Menqi of the University of Pittsburgh carried out scRNA-sequencing experiments at their Center and collaborated with our research group to perform the relevant analyses for this project regarding the expression of H19X in SSc endothelium.

Prof. Francesco Zulian, as my PhD supervisor, allowed this experience and this project to be started in the first place, and has been my clinical and research mentor in the field of Pediatric Rheumatology, in particular for Juvenile Scleroderma.

I also thank the association for children with rheumatic diseases "IL VOLO" for their contribution, which supported the extension of my visiting period in Zurich, allowing me to complete the research activities related to this project.