“VASCULAR MEMORY” AS A PREDICTIVE FACTOR FOR ENDOTHELIUM FUNCTION-ASSOCIATED CONDITIONS. COMPARATIVE STUDY OF 3 CLINICAL MODELS - RAYNAUD’S DISEASE, SYSTEMIC SCLEROSIS AND DIABETES MELLITUS

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“VASCULAR MEMORY” AS A PREDICTIVE FACTOR FOR ENDOTHELIUM FUNCTION-ASSOCIATED CONDITIONS.

COMPARATIVE STUDY OF 3 CLINICAL MODELS - RAYNAUD’S DISEASE, SYSTEMIC SCLEROSIS AND DIABETES MELLITUS

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Abstract

Introduction: Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterized by small vessel vasculopathy, autoantibody production and collagen deposition in the skin and internal organs. Although SSc is considered a fibrosing disease, vascular involvement together with immune activation, inflammatory response and oxidative stress seem to play major roles in the pathogenesis of organ dysfunction. There is some evidence that endothelial dysfunction may precede the typical perivascular abnormalities present in the disease, with the microvasculature becoming aberrant and exhibiting dilations, tortuosities and microhaemorrages, with extense avascular areas, resulting in tissular hypoxia, but the published data are still controversial.

Despite the phenotypic variety, almost all patients with systemic sclerosis have Raynaud's phenomenon (RP), an episodic vasospastic ischaemic disorder. Raynaud's phenomenon can precede other symptoms and signs of systemic sclerosis by up to 30 years, but its presence alone is not sufficient for the development of SSc. Therefore, it has been particularly difficult to understand what mechanisms come first in the pre-clinical phase of systemic sclerosis, as it is a rare disease with a small direct genetic impact. Nevertheless, studying the offspring of these patients could be the closest to a control group for genetics and early in life (at least) environmental factors.

This thesis was developed from the hypothesis that a 'vascular memory' characterized by anatomical microcirculatory changes, could be the earliest manifestation and the influencing factor for endothelial dysfunction.

Methods: The study included patients with systemic sclerosis, n=124, two different endothelium dysfunction-associated diseases (disease control groups): Raynaud's disease (RD), n=158, and type 2 diabetes mellitus (T2DM), n=98, and their respective healthy offspring (SSc offspring, n=55, RD offspring, n=11, T2DM offspring, n=50), and healthy controls (n=59). All the groups were studied concerning the demographic, clinical and general biochemical features. Circulatory anatomy was studied using Nailfold VideoCapillaroscopy (NVC) and the characterisation of the inflammation,

immune activation and oxidative stress was addressed by the quantification of VCAM-1, ICAM-1, VEGF-A, 3-NT and TAC.

Main findings: The healthy offspring of patients with SSc showed significant changes in NVC when compared to controls [number of capillaries per field inferior to eight ($p=0.003$), enlarged capillaries ($p=0.003$), major morphologic abnormalities ($p=0.027$), oedema (0.031), avascular areas ($p<0.001$), neoangiogenesis ($p=0.041$), reduced velocity of blood flow ($p<0.001$), sludge ($p<0.001$)], despite having normal levels of VCAM-1, ICAM-1, VEGF and 3-NT, with a high level of TAC. When compared to the patients with SSc, their offspring had similar changes regarding major morphologic abnormalities ($p=0.6$), "sludge" ($p=0.06$) and avascular areas ($p=0.78$). This two groups showed differences only in capillaries per field ($p<0.001$), enlarged ($p<0.001$) and giant ($p<0.001$) capillaries, minor dysmorphias ($p<0.001$), haemorrhages ($p<0.001$) and interstitial oedema ($p<0.001$). Capillary rarefaction and "sludge" were even more pronounced in the offspring of SSc when compared to patients with RD ($p<0.001$ for both parameters).

Patients with RD differed from controls only in the number of enlarged capillaries ($p=0.02$), blood flow ($p=0.002$) and neoangiogenesis ($p=0.04$); their offspring had a practically normal NVC. Regarding the biologic products, none were higher in the RD and respective offspring group compared to controls.

The offspring of patients with T2DM also showed significant differences from controls in NVC [edema ($p=0.009$), avascular areas ($p<0.001$), neoangiogenesis $p=0.001$, and reduced blood flow ($p=0.046$)]. There was only difference in haemorrhages ($p=0.002$) between patients with T2DM and their offspring. The T2DM offspring group had serum levels of 3-NT higher than controls ($p=0.027$).

Conclusions: The anatomy of microvasculature in the offspring of patients with SSc appears to change before the immune system and inflammation markers get activated. This may be due to the fact that the microcirculatory structure is genetically driven whilst the disease onset may need further "hits" to develop. RD might have a different physiopathology from RP in the context of SSc, highlighting the different nature of the two conditions regarding their early (pre-clinical)

mechanisms. The offspring of patients with T2DM showed the same phenomena that the SSc group, (despite the differences in the terminal vessels morphology), suggesting the presence of a genetic-based background which may develop into the metabolic disease after exposure to other stimuli. Therefore, we may postulate the existence of a 'vascular anatomical memory' in systemic sclerosis.

Resumo

Introdução: A esclerose sistémica (ES) é uma doença autoimune do tecido conjuntivo caracterizada por vasculopatia de pequenos vasos, produção de autoanticorpos e deposição de colagénio na pele e órgãos internos. Embora seja considerada uma doença fibrosante, o envolvimento vascular, em conjunto com a activação imunológica, a resposta inflamatória e o stress oxidativo, parecem desempenhar um papel importante na patogénese da disfunção de órgão. Há alguma evidência de que a disfunção endotelial poderá preceder as alterações perivasculares típicas presentes na doença, com a microvasculatura a tornar-se aberrante e a exibir dilatações, tortuosidades e micro-hemorragias, com extensas áreas avasculares, resultado em hipoxia tissular, mas os dados publicados são ainda controversos.

Apesar da variabilidade fenotípica, quase todos os doentes com esclerose sistémica apresentam fenómeno de Raynaud (FR), um distúrbio isquémico vaso-espástico episódico. O fenómeno de Raynaud pode preceder em 30 anos os outros sintomas e sinais da esclerose sistémica, mas a sua presença por si só não é suficiente para o desenvolvimento da ES. Tem sido particularmente difícil compreender quais os mecanismos que surgem primeiro na fase pré-clínica da esclerose sistémica, por se tratar de uma doença rara com um pequeno impacto genético direto. No entanto, estudar a descendência destes doentes pode ser o mais próximo que conseguimos como grupo controlo para fatores genéticos e ambientais em início de vida.

Esta tese foi desenvolvida a partir da hipótese de que uma "memória vascular", caracterizada por alterações anatómicas micro-circulatórias, poderia ser a manifestação mais precoce e o factor influenciador da disfunção endotelial.

Métodos: O estudo incluiu doentes com esclerose sistémica , n=124, e outras duas doenças associadas à disfunção endotelial (grupos controlo da doença): doença de Raynaud (DR, n=158) e diabetes mellitus tipo 2 (DM2, n=98); e os seus respectivos descendentes saudáveis (descendentes de ES, n=55, descendentes de DR, n=11, descendentes de DM2, n=50) e controlos (n=59). Estes grupos foram estudados quanto às características demográficas, clínicas e bioquímicas gerais. A anatomia circulatória foi estudada por vídeo-capilaroscopia peri-ungueal

(VCP) e a caracterização da inflamação, ativação imunológica e stress oxidativo, foi abordada pela quantificação de VCAM-1, ICAM-1 , VEGF-A, 3-NT e TAC.

Resultados principais: A descendência saudável dos doentes com ES apresentou alterações significativas na VCP quando comparada aos controlos [número de capilares por campo inferior a oito (p=0,003), capilares dilatados (p=0,003), dismorfias major (p=0,027), edema (p=0,031), áreas avasculares (p<0,001), neoangiogénese (p=0,041), redução da velocidade do fluxo sanguíneo (p<0,001), e estase eritrocitária (p<0,001)], apesar de apresentar níveis normais de VCAM-1, ICAM-1, VEGF e 3-NT, com níveis elevados de TAC. Quando comparados aos doentes com ES, os seus descendentes tiveram alterações semelhantes em relação a dismorfias *major* (p=0.6), "sludge" (p=0.06) e áreas avasculares (p=0.78). Estes dois grupos apresentaram diferenças apenas no número de capilares por campo (p<0,001), nos capilares dilatados (p<0,001) e megacapilares (p<0,001), nas dismorfias *minor* (p<0,001), nas hemorragias (p <0,001) e no edema intersticial (p<0,001). A rarefação capilar e a estase eritrocitária foram ainda mais pronunciadas nos descendentes de ES quando comparados com doentes com DR (p<0.001 para os dois parâmetros).

Os doentes com DR diferiram dos controlos apenas nos capilares dilatados (p=0.02), na velocidade reduzida de fluxo circulatório (p=0.002) e na neoangiogénese (p=0.04); a descendência de doentes com DR apresentou uma VCP quase normal. Em relação aos biomarcadores, nenhum foi maior nos descendentes de DR em comparação com os controlos.

A descendência dos doentes com diabetes mellitus tipo 2 também apresentou diferenças significativas na VCP em relação aos controlos [edema (p=0,009), áreas avasculares (p<0,001), neoangiogénese (p=0,001) e redução da velocidade do fluxo sanguíneo (p=0,046)]. Houve diferença apenas nas hemorragias (p=0,002) entre os doentes com DM2 e os filhos de doentes com DM2. O grupo de descendentes apresentou níveis séricos de 3-NT superiores aos controlos (p=0.027).

Conclusões: A anatomia da microvasculatura presente nos descendentes dos doentes com ES parece mudar antes do sistema imunológico e os marcadores de inflamação serem ativados. Isto

pode dever-se ao facto da estrutura microcirculatória ser determinada geneticamente, enquanto que o início da doença pode necessitar de mais "eventos" para se desenvolver. A DR pode ter uma fisiopatologia diferente do FR no contexto da ES, destacando-se a natureza diferente das duas condições quanto aos seus mecanismos precoces (pré-clínicos). A descendência de doentes com DM2 apresentou os mesmos fenómenos do grupo da ES (apesar das diferenças na morfologia da circulação terminal), sugerindo a presença de uma base genética, que pode evoluir para a doença metabólica após exposição a outros estímulos. Desta forma, podemos postular a existência de uma memória anatómica vascular na esclerose sistémica.

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Abbreviations

ANA – anti-nuclear antibodies

ACA – anti-centromere antibodies

ATA – anti-topoisomerase antibodies

ARA – anti- RNA polymerase antibodies

BSA - Bovine serum albumin

CT scan – computed tomography scan

ELISA – Enzyme-linked immunosorbent assay

EMT - Epithelial-to-mesenchymal transition

EndoMT - Endothelial-to-mesenchymal transition

hsCRP – high sensitivity C-Reactive Protein

GERD - Gastro-esophageal reflux disease

HbA1c - Haemoglobin A1c

HDL- High Density Lipoproteins

ICAM-1 – Intercellular adhesion molecule 1

ILD - Interstitial lung disease

NADP - Nicotinamide adenine dinucleotide phosphate

NO - Nitric Oxide

3-NT – 3- Nitrotyrosine

PBS - Phosphate-buffered saline

PBS-T - Phosphate-buffered saline with 0.1% Tween-20

RD – Raynaud's disease

ROS - Reactive Oxygen Species

RP – Raynaud's phenomenon

SSc – Systemic sclerosis

T2DM – Type 2 diabetes mellitus

TAC – Total antioxidant capacity

VCAM-1 – Vascular cell adhesion molecule 1

VEGF-A – Vascular endothelial growth factor A

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Scientific articles used in the preparation of this thesis

Marta C Amaral, F Seguro Paula, Joana Caetano, Paul RJ Ames, J Delgado Alves. Re-evaluation of nailfold capillaroscopy in discriminating primary from secondary Raynaud's phenomenon and in predicting systemic sclerosis: a randomised observational prospective cohort study. Expert Rev Clin Immunol. 2024 Mar 11:1-8. doi: 10.1080/1744666X.2024.2313642. Epub ahead of print. PMID: 38465507.

I. BACKGROUND

1. Systemic sclerosis

1.1 Introduction

Systemic sclerosis (SSc) is a complex connective tissue disease of unknown etiology with multiorgan involvement and heterogeneous clinical manifestations. The clinical and pathologic features of the disease are the result of three distinct processes: (1) innate and adaptive immune system abnormalities leading to the production of autoantibodies and cell-mediated autoimmunity, (2) microvascular endothelial cells (MVEC) dysfunction, fibroproliferative vasculopathy of the small vessels, and (3) fibroblast dysfunction leading to excessive collagen and other matrix components accumulation in the skin, blood vessels and internal organs (Pattanaik et al., 2015).

There are two major forms of SSc: limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc); these two forms differ mainly with regards to the extent of the skin involvement, autoantibody association and the pattern of organ involvement. A third less common form is “SSc sine scleroderma”, in which there is internal organ involvement with only occasional fibrosis of distal digits (Pattanaik et al., 2011).

The incidence of SSc is estimated between 4 and 20 new cases per 1,000,000 per year and the prevalence between 30 and 450 cases per 1,000,000 (Coral-Alvarado et al., 2009; Hochberg MC, 2010). SSc is observed predominantly in females with a peak incidence between 45 and 64 years of age. The epidemiology of SSc is not definitively established due to the relative rarity of the disease, the large variability of the clinical manifestations and severity (Coral-Alvarado et al., 2009), with the consequent difficulty in establishing a diagnosis.

Mild SSc may be more frequent than has previously been suspected; therefore, early diagnosis is important to avoid irreversible damage and the identification of more homogeneous subsets of patients is an important objective in SSc research (Johnson et al., 2020). Results from a multicentric SSc cohort of 2281 patients (247 men) recruited in the Italian Systemic Sclerosis

PRogression INvestiGation (SPRING) registry showed significant sex-related differences in several aspects of SSc, including more severe disease with more extensive skin involvement, digital vasculopathy, internal organ involvement and a higher prevalence of sicca syndrome in men, and a serum autoantibody profile characterised by ANA, anti-ENA, anti-CENP-B, and anti-La/SSB antibodies in women (De Angelis et al., 2022).

Major organ involvement leads to decreased survival in SSc. Pulmonary fibrosis [interstitial lung disease (ILD)] and pulmonary arterial hypertension (PAH) cause more than half of all the SSc-related deaths (Le Pavec et al., 2011). However, patients with SSc begin to live longer due to a better control of these manifestations, and therefore cardiovascular-associated deaths are increasing (Pattanaik et al., 2015). Systemic sclerosis is associated with an increased prevalence of atherosclerosis. Patients with SSc have a higher prevalence of carotid plaques than matched controls and patients with SSc and plaques *versus* patients without plaques have elevated serum proteins implicated in both vasculopathy and fibrosis, including IL-2, IL-6, CRP, keratinocyte growth factor, intercellular adhesion molecule 1, endoglin, plasminogen activator inhibitor 1 and insulin-like growth factor binding protein 3 (Schiopu et al., 2014).

No animal model develops systemic sclerosis in a way that faithfully replicates the human form and this has impeded our understanding of the disease. There are many unanswered questions related to the etiopathogenesis of SSc. For example, it is unclear whether the innate/adaptive immune system abnormalities, the vasculopathy and the fibroblast dysfunction are unrelated processes or are mechanistically linked, and which of the three processes is of utmost importance and how the interaction among the three leads to the development of the disease (Pattanaik et al., 2015).

Microvascular injury is one of the early events in the pathogenesis of systemic sclerosis and is characterized by endothelial-cell damage, proliferation of basal-lamina layers, occasional entrapment of peripheral-blood mononuclear cells in the vessel wall, and initial perivascular mononuclear-cell infiltrates. Endothelial cells show signs of increased programmed cell death. One or more reactive oxygen species (ROS)–generating triggering agents could be responsible for this stage. ROS may be generated inside the vascular lumen by peripheral-blood cells or within

the vessel wall by macrophages, endothelial cells, vascular smooth-muscle cells, or adventitial fibroblasts in response to one or more noxious agents. Although low levels of ROS are necessary for a normal vascular function, excessive production is responsible for functional and structural damage. Uncontrolled production of ROS activates local mesenchymal cells, inducing chemotaxis, proliferation, extracellular-matrix production and the release of cytokines and growth factors that amplify the inflammatory focus. An autocrine circuitry maintains ROS at levels that are high because of the reduced turnover of cytokine receptors. Structural and functional abnormalities of the vessel walls and intravascular changes then occur, leading to overt clinical symptoms. The next stage is dominated by fibrosis, derangement of visceral-organ architecture, rarefaction of blood vessels and consequently hypoxia, which contributes to further development of fibrosis. Triggering, amplifying and maintenance factors are not necessarily confined to a single stage. Environmental, local, and genetic factors can and do influence disease progression (Gabrielli et al., 2009). Once the single or multiple mechanisms responsible for the mesenchymal-cell activation subside or recede or the mesenchymal cells themselves undergo senescence or apoptosis, the disease burns out.

1.2 Vasculopathy in systemic sclerosis

The role of vasculogenesis in systemic sclerosis is not clear, and there are conflicting reports regarding the presence and role of circulating endothelial progenitor cells in SSc (Distler et al., 2009). Tissue ischemia usually leads to the expression of angiogenic growth factors [e.g., vascular endothelial growth factor (VEGF)], which causes vasodilatation, proliferation, and migration of endothelial cells and the stabilization of the lumina to form new vessels (Distler et al., 2002).

Angiogenesis is a physiological process in which new blood vessels are formed from pre-existing blood vessels. It is finely regulated by many pro-angiogenic factors, including vascular endothelial growth factor (VEGF), which in turn can upregulate the expression of VCAM-1 on endothelial cells (Troncoso et al., 2021). In fact, plasma levels of VEGF are elevated in SSc and this could stimulate angiogenesis (Distler et al., 2004). In addition to elevated levels of VEGF, other proangiogenic

mediators [such as endothelin-1 (ET-1), adhesion molecules, and chemokines] are also found in the circulation of SSc patients (Koch et al., 1993). Finally, it was demonstrated that homozygosity for rs2235611, a SNP of SRp55 which is the regulatory splicing factor responsible for the switch from proangiogenic VEGF-A165 to antiangiogenic VEGF-A165b, significantly influences the predisposition to develop SSc and is strictly associated with ILD and with a "late pattern" in the nailfold videocapillaroscopy (NVC) (Romano et al., 2022).

There is some evidence that endothelial dysfunction precedes the typical perivascular abnormalities of the disease. The initial lesion may involve the microvasculature, widening the space between endothelial cells with the loss of their integrity. Then, in a process which seems to involve predominantly platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR) and transforming growth factor beta (TGF-β) signaling, pericytes would be stimulated to acquire the fenotype of fibroblasts and miofibroblasts, in an epithelial-mesenchimal transition, proliferating and producing components of the extracellular matrix (collagen IV, fibronectin and fibrillarin) that are responsible for the typical fibrosis of SSc (Cipriani et al., 2011; Gabrielli et al., 2009). The Wnt and the NOTCH signaling pathways may be involved in this endothelial–mesenchymal transformation process (Jimenez, 2013).

Additionally, pericytes proliferate and contribute to the increased vascular wall thickness (Helmbold et al., 2004). Microvasculature becomes aberrant and exhibits dilations, tortuosities and microhemorrhages, with extensive avascular areas, leading to tissular hypoxia. Despite the increase in the expression of VEGF in these hypoxic tissues, the formation of new microvessels seems to be insufficient to create an effective support (Liakouli et al., 2011). The acidic microenvironment in SSc may lead to the transition of monocytes into myofibroblast-like cells, to the impairment of neoangiogenesis, and to the transition of endothelial cells into myofibroblasts (Andreucci et al., 2021).

It is reported that the intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1) are elevated in SSc (Pendergrass et al., 2010). Once induced by fibroblasts and endothelial cells, ICAM-1 and VCAM-1 subsequently recruit and activate monocytes to repair damaged endothelial cells. Monocytes can then boost the co-stimulation and transmigration of

inflammatory cells into the extracellular matrix (ECM) and contribute to the dysregulated angiogenesis. When overexpressed, these adhesion molecules can be detected in a circulating soluble form and are considered markers of underlying endothelial activity and damage. Hence, ICAM-1 and VCAM-1 expression are associated with SSc disease activity and severity (Zhang et al., 2019).

There is a reduction of the eNOS gene expression and NO release in MVECs derived from lesional and non-lesional skin biopsies of patients with SSc, both collected in the steady-state and after shear stress. This is probably associated with a deficient endothelium-dependent relaxation (Anderson et al., 2003). Impaired NO production results in the alteration of the vascular tone, enhancement of platelet aggregation and increased susceptibility of the endothelial cells to oxidative injury. NO also limits cytokine-induced endothelial cell activation, monocyte adhesion and inhibits the endothelial cell release of IL-6 and IL-8 (Berk et al., 2001). Furthermore, NO inhibits vascular smooth muscle cell proliferation through the elevation of cyclic GMP and inhibition of the mitogenic proteins TGF-β and PDGF. Therefore, impaired NO production in SSc may contribute to the pathogenesis of arteriolar intimal proliferation and may have a prominent role in the pathophysiology of the disease.

1.3 Inheritance in systemic sclerosis

While the exact cause of SSc is unknown, the disease is believed to be a result of environmental factors on top of a genetic predisposition (Patnaik et al., 2023). It is widely believed that SSc may develop in individuals with a permissive genetic background. A triggering event such as an infection or environmental toxin may then start the process that will lead eventually to the development of the disease (Pattanaik et al., 2015).

In subjects with a family history of SSc, the incidence ranges from 1.5 to 1.7% (Luo et al., 2013). In 3 large US cohorts of patients, family histories of SSc were prospectively surveyed: SSc occurs significantly more in families with scleroderma (1.6%) than in the general population (0.026%). In fat, a positive family history of SSc is the strongest risk factor yet identified for SSc; however, the

absolute risk for each family member remains quite low (<1%)(Arnett et al., 2001). Therefore, having a family history of SSc increases the risk of developing the disease 15–19-fold in siblings and 13–15-fold in first-degree relatives (Broen et al., 2012).

Microchimerism has been hypothesized to play a role in SSc (Nelson, 1996). Maloney et al. observed the persistence of maternal microchimerism (MMc) into the adult life of healthy individuals as well as in patients with systemic sclerosis (Maloney et al., 1999). Cells that have trafficked between mother and fetus can persist in their respective hosts for many years, creating a state of microchimerism. The long-term persistence of MMc suggests that these cells could, under certain conditions, participate in the development of autoimmunity later in life (Nelson, 2002). Fetal–maternal and maternal–fetal microchimerisms have in fact been proposed as mechanisms triggering autoimmunity in SSc as well as in other immune-mediated diseases (Adams Waldorf & Nelson, 2008; Giacomelli et al., 2004). This microchimerism, in susceptible individuals, could initiate a type of Graft versus Host Disease (cGVHD) that would induce SSc, with the microchimeric cells acting as effectors or as targets of an immune response. It is noteworthy that, in women with SSc who have given birth to male children, the male offspring Th2-oriented T cells that express high levels of IL-4 are found in the skin and blood of the mothers (Scaletti et al., 2002).

More recently, a multicentric study generated a GRS (genomic risk score) based on the allelic effects identified in the largest SSc genome-wide association study to date; this proved able to discriminate between SSc and healthy controls as well as between SSc and other immune-mediated inflammatory diseases, with a remarkable predictive value (Bossini-Castillo et al., 2021). However, several limitations and challenges, such as non-European ancestry or sample size, must be overcome to implement this strategy in clinical management.

The Systemic Sclerosis vasculopathy, characterized by both noninflammatory macrovascular and microvascular changes, has also been linked to genetic abnormalities in the expression of type 1 interferon (IFN) and in the regulator of the G protein signaling 5 (RGS-5), two molecules associated with vascular rarefaction (Fleming et al., 2009). Thus, a genetic predisposition to abnormal

endothelial cell senescence and apoptosis may also be important to the pathogenesis of the vasculopathy in this disease (Frech et al., 2010).

1.4 Nailfold capillaroscopy in Systemic sclerosis

Nailfold capillaroscopy (NVC) is a safe well-established method for assessing capillary microcirculation in SSc. Subjects can be diagnosed as having SSc based on specific patterns of the capillary architecture known as “scleroderma patterns”, which can be further divided into three types: early, active and late, based on the stage of disease. The early pattern is recognized as having giant capillaries but with a normal capillary distribution; the active pattern is characterised by giant capillaries, hemorrhage and a disorganized distribution with loss of capillaries; the late pattern is identified as a significant loss of capillaries and the presence of abnormal shapes in the remaining ones (Cutolo et al., 2000).

These vascular patterns are reflective of the severity and progression of the disease (Arana-Ruiz et al., 2016; Camargo et al., 2015; Ingegnoli et al., 2013), as they correlate with visceral involvement (Bredemeier et al., 2004; Caetano et al., 2019; Marino Claverie et al., 2013; Ong et al., 1998; Sato et al., 2009) and can predict death (Kayser et al., 2013). Therefore, direct observation of the terminal circulation through nailfold capillaroscopy can provide valuable information about the prognosis and even evaluate the response to therapy (Aschwanden et al., 2008; Fleming et al., 2008).

Predictive NVC models were developed, namely the PRINCE index to identify patients at higher risk of developing SSc (Ingegnoli et al., 2008), (but autoantibodies had to be added later on) (Ingegnoli et al., 2010), and the CSURI index used to predict the development of digital ulcers (Sebastiani et al., 2009; Sebastiani et al., 2012).

A recent study found that arterial stiffening and cardiovascular risk scores were positively associated with the degree of progression of the peripheral microvasculopathy assessed by NVC, suggesting an association between NVC abnormalities and a higher cardiovascular risk in these patients (Pagkopoulou et al., 2021). Interestingly, a recent narrative review highlighted that the specific SSc-related autoantibodies and the NVC patterns, so far considered independent

prognostic markers for patients, may be interconnected, with the identification of a link between faster microvascular progression and specific autoantibody profiles that would be associated with worse clinical outcomes (Hysa et al., 2023). In this context, the assessment of SSc-associated autoantibodies and NVC profiles in combination with some relevant circulating vascular biomarkers could represent an additional tool to improve the accuracy of early diagnosis and guide targeted therapy (Fioretto et al., 2023).

2. Raynaud's phenomenon and disease

2.1 Introduction

The Raynaud's phenomenon (RP) is an episodic vasospastic ischemic disorder first described by Maurice Raynaud in 1862 (Raynaud, 1862). It is characterized by an initial white discoloration (pallor) of the digits as a reaction to cold (or emotional stress), followed by cyanosis, pain and numbness and finally by a post-ischemic red flush upon re-warming (Block & Sequeira, 2001). It is more common in the extremities, especially in the hands, but it can occur in any part of the body, including the vascular territories of organs or systems, particularly the lung and the kidney. Worldwide it has a prevalence of 3 to 21%, depending on weather conditions and ethnic origins (Brand et al., 1997; Maricq et al., 1997).

The Raynaud's phenomenon can be primary (Raynaud's disease) or secondary to a wide spectrum of different diseases, ranging from peripheral vascular diseases and connective tissue diseases to paraneoplastic disorders (Allen EV, 1932). Raynaud's disease occurs frequently in otherwise healthy subjects in whom the symptoms are generally milder and more responsive to vasodilator treatment (Kingdon et al., 2006).

The aetiology and the pathophysiology of this syndrome have not yet been completely established (Block & Sequeira, 2001). The exact defect associated with RP will vary depending on the underlying cause of the process. RP is generally known to be the clinical expression of a disturbance in the regulation of the cutaneous thermoregulatory vessels (Herrick, 2005). It is a functional vascular disorder, but little is known about whether, when and why it becomes a structural disease.

The transition rates from primary (pRP) to secondary RP (sRP) vary widely according to studies from 1% to 17%, and the incidence of the transition depends on the geographical origin of the patient cohort (Suter et al., 2005; Ziegler et al., 2003). Previous studies identified various clinical and laboratory criteria that helped to explain the possible outcomes of patients with RP: criteria were set up to differentiate sRP from pRP but were non-diagnostic and simply reflected the result of a screening process (LeRoy & Medsger, 1992).

The distinction between primary RP and secondary RP is diagnostically and prognostically important, but their discrimination turned out to be difficult during the first months or even years of presentation (Amaral et al., 2024; LeRoy & Medsger, 1992; Maverakis et al., 2014). Primary RP presents at a younger age, may have a family clustering, associates with smoking (Garner et al., 2015) and is more common in women. Indeed, the incidence of RP is remarkably higher in premenopausal or post-menopausal females on oestrogen replacement therapy (Fardoun et al., 2016), suggesting that gender-associated mechanisms may influence the expression of RP in men and women (Fraenkel et al., 1999).

Although many aspects and factors contributing to this disease have been dissected, the molecular mechanisms underlying the onset and progression of RP still require further investigations. This is, in no small part, due to the multifactorial aetiology (hormonal, neuronal, and endothelial) of the disease. (Fardoun et al., 2016).

Despite the context or background behind this phenomenon, it is frequently associated to a significant morbidity that limits personal and professional life. However, this negative impact has been underestimated, due to the absence of an effective treatment.

2.2 Inheritance in Raynaud's disease

Evidence that Raynaud's disease is inherited is supported by the fact that about 25% of first degree family members will have RP (Freedman & Mayes, 1996) and an early occurrence of RP seems to correlate with a family history for the presence of this phenomenon (Brand et al., 1997).

In a study using 298 microsatellite markers, a two-stage whole genome screen of six extended families having at least three patients with RP in each family, was undertaken with linkage analysis identifying five chromosomal areas of possible linkage. Of these, three candidate genes emerged (the β-subunit of the muscle acetylcholine receptor and the 1E and 1B serotonin receptors), which could be associated with RP. However, sequencing results showed no mutations in candidate genes (Susol et al., 2000). Nonetheless, others continued to suggest that there is a genetic factor contributing to the prevalence of this disease. This assertion is supported

by familial studies and twin analysis (Pistorius et al., 2006). Interestingly, studies of RP patients that were exposed to vinyl chloride monomer suggest that the interaction between a certain genetic background and environmental conditions may play a role in the anticipation of the onset of RP (Fontana et al., 2006).

2.3 Raynaud's phenomenon in Systemic sclerosis

Despite its phenotypic complexity, almost all patients with systemic sclerosis have Raynaud's phenomenon. When the related risk of RP in relatives of patients with SSc and their controls were compared, first and second degree relatives had a significant increase in risk: first degree relatives were 6 times more likely and second degree relatives were twice as likely to have RP (Frech et al., 2010).

In 50-70% of patients, Raynaud's phenomenon can precede other symptoms and signs of systemic sclerosis by 10 to 30 years, but its presence alone is not sufficient for the development of SSc. Only a minority develops SSc features, suggesting that the pathogenic mechanisms associated with the structural vascular changes present in primary and secondary Raynaud's phenomenon, may be distinct (Kingdon et al., 2006).

In Systemic Sclerosis there is dysfunction of the endothelium that results in an imbalance of vasoactive factors with a decrease in the release of vasodilatory neuropeptides from sensory nerves, which enhances the responses to stress or cold stimuli. Evidence suggests that RP in SSc results from a vasculopathy involving all layers of the peripheral blood vessels. The endothelium is a metabolically active tissue that, under normal circumstances, regulates regional blood flow, transportation of nutrients, coagulation and fibrinolysis, and migration of blood cells, while maintaining an antithrombotic lining in the vasculature. These important biologic functions are achieved through production of a complex array of molecules including vasodilators (eg. nitric oxide and prostacyclin), vasoconstrictors (eg. endothelin-1 and platelet activating factor), and cell adhesion molecules (eg. selectins and integrins). Therefore, an abnormal function of the endothelium results in an imbalance of vasoactive factors including overproduction of the

vasoconstrictor endothelin-1 and underproduction of the vasodilators nitric oxide and prostacyclin (Wigley, 2009). In fact, previous studies had already suggested that topical application of a nitric oxide donor may improve the microcirculation of patients with Raynaud's phenomenon (Tucker et al., 1999).

2.4 Nailfold capillaroscopy in Raynaud's phenomenon

Nailfold capillaroscopy has been a widely used technique to investigate and monitor patients with Raynaud's phenomenon (Caetano et al., 2019; Cutolo et al., 2005; Ingegnoli et al., 2008; Kayser et al., 2013; Sebastiani et al., 2009).

Nevertheless, the appreciable differentiation of secondary from primary RP is sometimes difficult (Kim et al., 2008), as altered capillaries can even occur in healthy individuals (Hoerth et al., 2012) or in diseases from another spectrum (Seguro Paula et al., 2016).

However, it is important to identify secondary RP early, as it might precede a potentially severe underlying disease.

3. Type 2 diabetes mellitus

3.1 Introduction

Type 2 diabetes mellitus (T2DM) can be defined as a heterogeneous group of metabolic disturbances that has hyperglycemia as a common finding. Besides affecting the ability of the body to use glucose, it is characterised by multiple vascular complications. In fact, the precise mechanism by which type 2 diabetes mellitus leads to the development of vascular complications is complex and not yet fully elucidated but seems to be strongly related with the toxic effects derived from hyperglycemia as well as by hyperlipidemia originated from obesity - gluco and lipo toxicity, respectively. In addition, insulin resistance promotes endothelial dysfunction, which in turn precedes the early development of micro and macrovascular complications. Hyperglycemia induces oxidative stress through mitochondrial dysfunction and enhanced reactive oxygen species generation, whilst hyperlipidemia contributes to the release of pro-inflammatory cytokines by the adipocytes. The consequent oxidative stress and ongoing low-grade inflammation have been considered major contributors for the progression of T2DM and its complications (Santilli et al., 2015).

The worldwide prevalence varies from 3.8–10.2% and the rate of non-diagnosed cases is 50%, making it a major global health problem (McCulloch & Hayward, 2016). The risk of developing type 2 diabetes increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior gestational diabetes mellitus, in those with hypertension or dyslipidemia, and in certain racial/ethnic subgroups (African American, American Indian, Hispanic/Latino, and Asian American).

It is a chronic disease with a high mortality rate due to its long-term cardiovascular and renal complications (Association, 2016; Cowie et al., 2009). Chronic hyperglycemia is the most important risk factor for the development of microvascular complications in patients with type 2 diabetes ("Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group," 1998).

3.2 Inheritance

Type 2 diabetes mellitus is associated with a strong genetic predisposition or at least a strong family history in first and second degree relatives. Various genetic and environmental factors can result in the progressive loss of beta-cell mass and/or function that manifests clinically as hyperglycemia ("2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2019," 2019).

A study that investigated the impact of a familial history of T2DM on the new diagnosis of diabetes, found a predisposition to an increased microvascular risk in these patients (Hermans et al., 2019). Another recent study, (highlighting the association between diabetes and hypertension), aimed at evaluating the prevalence, determinants and clinical impact of masked hypertension in the offspring of patients with diabetes: 29% of the offspring presented hypertension and had a significantly reduced coronary flow reserve, a higher E/e' (a surrogate marker of left ventricular filling pressure) and higher values of high-sensitive C-reactive protein, when compared to controls (Mahfouz et al., 2019).

So far, at least 75 T2DM-associated common genetic variants have been identified. However, detailed mechanisms on how these variants exert their effects on the pathogenesis of T2DM have been largely lacking (Kwak & Park, 2016)

3.3 Nailfold capillaroscopy

Studies published in the last decades highlight the importance of capillaroscopy in diabetes, as it provides important data for the evaluation of vascular damage (Romano et al., 2015; Rajaei et al., 2015; Kuryliszyn-Moska et al., 2011). Nailfold video-capillaroscopy can be useful to identify the 'diabetic capillaropathy' (Maldonado et al., 2017) and may be used for diagnosing or monitoring microvascular complications (Shah et al., 2023) such as retinopathy (Abd El-Khalik et al., 2022; Raina et al., 2023) and peripheral neuropathy (Hsu et al., 2016).

More recently, NVC studies that include pre-diabetic individuals have showed the presence of subclinical microvascular and endothelial dysfunction and associated their presence with an increased cardiovascular risk (Hsu et al., 2016; Lamprou et al., 2023).

4. Rationale and hypothesis of this thesis

The aetiology of systemic sclerosis is subject to ongoing research, as the precise events that underlie the development of this disease remain unclear. The pathogenesis is known to involve the endothelium, epithelium, fibroblasts, the innate and adaptive immune systems and their immunologic mediators. Endothelial cell damage may be the initiating factor, but the precise triggering event(s) remain elusive (Abraham et al., 2009).

Regardless of the relative importance of each of the three main pillars that support the physiopathology and the consequent clinical picture of SSc - the vascular dysfunction, the immunoinflammatory activation and the pro-fibrotic phenotype – very little is known about what dysfunction(s) come first and which ones are the consequence of the initial disruption. Furthermore, there are no consistent data regarding whether there is a tangible genetic background that could explain it all or if, on a baseline genetic structure, there is a superimposure of other elements or circumstances.

All the studies addressing a hereditary factor in patients with SSc, tried to analyze genetic changes in relation to disease onset and clinical characteristics. The potential association between the gene burden and a baseline structural disruption of the microcirculation (rather than the clinical phenotype), which would represent the first step towards a potential development of the disease (pending the occurrence of a second or even third event), has not been addressed.

If this would be the case, one could postulate that the first factor towards the development of SSc would be a microvascular disruption (genetically-induced or otherwise) and only afterwards would the inflammation and immunologic response and fibrosis develop, as a consequence of an eventual 'second-hit'.

5. Objectives of this thesis

The general aim of this thesis is to investigate the microcirculation in patients with systemic sclerosis and their offspring in order to better understand the early (pre-clinic) mechanisms underlying the functional changes and to attempt to determine a relative timeline between the onset of anatomical vascular changes, immune activation, inflammation and oxidative stress.

The specific objectives are:

- To correlate physical, clinical, serological and immunological characteristics with nailfold video-capillaroscopic features and soluble vascular and oxidation biomarkers;
- To identify whether the offspring of patients with SSc without any clinical manifestations have vascular changes (assessed by NVC) and whether they have any serologic parameters associated with the immune-inflammatory response or oxidative stress known to be present early in the course of the disease.
- To correlate NVC findings with eventual serum vascular and oxidation biomarkers that may be present in the offspring of patients with SSc, before the onset of any clinical manifestations of disease.
- To use patients with Raynaud's disease and type 2 diabetes as disease-controls in order to understand whether the timeline of abnormalities (anatomical, immunologic and biochemical) is a unique characteristic of systemic sclerosis. These two conditions were chosen as controls, due to the clinical association with SSc (RD) and to the known genetic impact and NVC changes (T2DM).

II. METHODS

1. Subjects, consent, ethics, clinical data and serum collection

Subjects were included in four different groups, respectively:

Group 1- Systemic sclerosis (SSc) – study population

1a - **Patients with SSc**, classified according to the ACR/EULAR criteria 2013; these patients were recruited from the outpatient clinic of the Systemic Immune-Mediated Diseases Unit (UDIMS) at Hospital Professor Doutor Fernando Fonseca, Amadora, Portugal (HFF).

1b - **Offspring of patients with SSc**; these subjects were recruited after being contacted by their parents (group 1a).

Group 2 - Raynaud´s Disease (RD) – disease control (negative) group

2a - **Patients with primary Raynaud's phenomenon (RP);** these patients were recruited from the outpatient clinics at HFF;

2b – **Offspring of patients with RD**; these subjects were recruited after being contacted by their parents (group 2a).

Group 3 – Type 2 diabetes mellitus (T2DM) – disease control (positive) group

3a – **Patients with T2DM,** based on the criteria of the American Diabetes Association ("Diagnosis and classification of diabetes mellitus," 2008; Nathan, 2015)**,** recruited from the outpatient clinics of Medicine IV and Diabetes Unit, Department of Medicine, HFF

3b – **Offspring of patients with T2DM** , these subjects were recruited after being contacted by their parents (group 3a). In addition, subjects whose parents had T2DM, despite not being from group 3a, were also recruited.

Group 4 – Healthy control group

Subjects without RP, SSc/ SSc-related diseases (polymyositis, dermatomyositis, overlap syndrome, mixed connective tissue disease), T2DM, or positive family history for any of these 3 conditions, were recruited through local advertisement in HFF.

Subjects with any acute infection, vascular, liver or renal dysfunction or known disease, cancer, pregnancy or lactation period and medical or psychological conditions that would not allow them to sign the informed consent form or complete the study, were excluded.

After being introduced to the study purpose, procedures, potential risks and benefits all participants gave their informed consent. Both texts (informative brochure and informed consent) were approved by the appropriate Ethics Committees.

Demographics, biometrics, and clinical data, regarding smoking, exercise habits, personal history of cardiac, cerebral and peripheral vascular disease, concomitant medication, and respective disease's specificities, were registered.

Blood was collected for evaluation of organ function, safety and definition of vascular risk (triglycerides (mg/dL), total cholesterol (mg/dL), high-density lipoprotein cholesterol (mg/dL), and low-density lipoprotein cholesterol (mg/dL), HgA1c (mmol/mol, %), and acute phase reactants such as erythrocyte sedimentation velocity (mm/h) and high sensitivity C reactive protein (mg/dL).

Number of participants in each group: as there are no studies regarding the prevalence of the studied changes in these patients and respective offsprings, it was not possible to pre-calculate in an accurate fashion the size of the different groups. Therefore, we performed an interim

statistical analysis at different recruitment stages and patients were added on until there were no significant changes with the increase in the subject numbers (data not shown). The exception is the RD offspring group where the recruitment was more difficult. However, the overall comparison with the targeted study group (SSc) was not compromised.

2. Nailfold Video-capillaroscopy

Each subject was asked to refrain from caffeine-containing beverages and smoking for 2 hours prior to the Nailfold Videocapillaroscopy examination and to stay in a temperature controlled room (20-22°C) for a minimum of 20 minutes before the exam. After a drop of immersion oil on the nailfold bed, eight fingers of each patient were examined using a Digital Videocap 200 (DS-Medica, Milan, Italy) with a 200x optic probe magnification. Fingers affected by recent trauma were not analysed.

All nailfold areas where capillary visibility was good were scanned for 11 parameters:

- number of capillaries per field, defined as the mean number of capillaries in a 1-mm length of each finger (≥ 8 normal/< 8 abnormal)
- enlarged capillaries (≥ 20 µm loop width)
- giant capillaries (≥ 50 µm loop width)
- minor morphologic abnormalities (crossed patterns) > 30% per field
- major morphologic abnormalities (bushy and bizarre patterns)
- hemorrhages (non-traumatic)
- avascular isolated areas
- neoangiogenesis
- interstitial edema
- blood flow velocity (normal/reduced)
- sludge (erythrocyte stasis)

Images were captured, coded and stored using the Videocap 8.14 software.

All the exams were made by me (Marta Amaral).

For the quality control of the NVC image analysis, randomized images from different subjects were analysed by another observer who was blinded to the different groups (inter-observer variability). Images captured at a defined moment were reviewed by me randomly (intra-observer variability).

3. Quantification of VCAM-1, ICAM-1,VEGF-A, 3-NT, and TAC.

An additional blood sample was taken and collected in a dry tube, kept at room temperature for 2 hours and centrifuged for 15 minutes at 3500 rpm. The supernatant (serum) was aliquoted and stored at -80°C until quantification of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), vascular endothelial growth factor A (VEGF-A), 3- Nitrotyrosine (3-NT) and Total Antioxidant Capacity (TAC).

3.1 VCAM-1

A sandwich enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of VCAM-1 from the serum.

96-well plates (Nunc MaxiSorp, ref. 44-2404-21) were coated overnight at room temperature with 100 µL/well of a mouse (monoclonal IgG1) anti-human VCAM-1 antibody (MAB 809 Biotechne) at 4 µg/mL in BIC (benzoxazine-isocyanide chemistry). Plates were then washed 4 times with 200 µL/well with Tween 0,05% in PBS (PBS-T) and were blocked with BSA 1% in PBS for 60 minutes at room temperature. After being washed 4 times with PBS-T, serum samples were added in duplicate after 1:20 dilution in BSA 1% in PBS, for 120 minutes at room temperature, alongside with a recombinant human VCAM-1 (809-VR Biotechne) at concentrations ranging from 1,5 to 100 ng/mL in triplicate for the standard curve. After being washed 4 times with PBS-T, a sheep (polyclonal IgG) anti-human VCAM-1 (BAF 809 Biotechne) was added at 0,2 µg/mL in BSA 0,1% in PBS for 60 minutes at room temperature. Up until this step all incubations were performed with shaking at 200 rpm. After further 4 washes with PBS-T, a streptavidin-HRP (horseradish peroxidase) conjugate (21124 Thermo Scientific) was added at 0,1 µg/mL for 30 minutes at room temperature, without shaking. Plates were finally washed four times with PBS-T and a colorimetric signal was developed with 3,3',5,5'-tetramethylbenzidine (ultra-TMB, Thermofisher ref.34028) for 7-10 minutes, after which the reaction was stopped with sulphuric acid 2M. Optical densities (OD) absorbance was read at 450nm in a microplate reader Synergy HT, Biotek.

3.2 ICAM-1

A Human ICAM-1 Standard ABTS ELISA Development kit (Peprotech catalog #900-K464, lot #1110464) was used for the detection of ICAM-1 levels in serum.

96-well plates (Nunc MaxiSorp, ref. 44-2404-21) were coated overnight at room temperature with 100 µL/well of a rabbit anti-human ICAM-1 antibody at 1 µg/mL in PBS. Plates were then washed 4 times with 200 µL/well with Tween-20 0,05% in PBS (PBS-T) and were blocked with BSA 2% in PBS for 60 minutes at room temperature. After being washed 4 times with PBS-T, serum samples were added in duplicate after 1:200 dilution in BSA 0.1% in PBS-T, for 120 minutes at room temperature, alongside with recombinant human ICAM-1 at concentrations ranging from 12 to 3000 pg/mL in triplicate for the standard curve. After being washed 4 times with PBS-T, a biotinylated rabbit anti-human ICAM-1 was added at 0,1 µg/mL in BSA 0,1% in PBS-T for 60 minutes at room temperature. Up until this step all incubations were performed with shaking at 200 rpm. After further 4 washes with PBS-T, a streptavidin-HRP conjugate was added at 0,1 µg/mL for 30 minutes at room temperature, without shaking. Plates were finally washed four times with PBS-T and a colorimetric signal was developed with 3,3',5,5'-tetramethylbenzidine (ultra-TMB, Thermofisher ref.34028) for 7-10 minutes, after which the reaction was stopped with sulphuric acid 2M. Optical densities (OD) absorbance was read at 450nm in a microplate reader Synergy HT, Biotek.

3.3 VEGF-A

A Human VEGF standard TMB ELISA Development Kit (Peprotech catalog #900-T10, lot #0223T010) was used for quantitative measurement of VEGF-A.

96-well plates (Nunc Maxisorp, ref. 44-2404-21) were coated overnight at room temperature with 100 µL/well of a rabbit monoclonal anti-human VEGF antibody at 0,5 µg/mL in Dulbecco's PBS. Plates were then washed 4 times with 200 µL/well with Tween 0,05% in PBS (PBS-T) and were blocked with BSA 1% in PBS for 60 minutes at room temperature. After being washed 4 times with PBS-T, serum samples were added in duplicate after 1:4 dilution in BSA 0,1% in PBS-T for 120

minutes at room temperature, alongside with recombinant human VEGF at concentrations ranging from 8 to 1000 pg/mL in triplicate for the standard curve. After being washed 4 times with PBS-T, a biotinylated rabbit anti-human VEGF was added at 0,125 µg/mL in BSA 0,1% in PBS-T for 120 minutes at room temperature. Up until this step all incubations were performed with shaking at 200 rpm. After further 4 washes with PBS-T, a streptavidin-HRP (horseradish peroxidase) conjugate was added at 0,1 µg/mL for 30 minutes at room temperature, without shaking. Plates were finally washed four times with PBS-T and a colorimetric signal was developed with 3,3',5,5'-tetramethylbenzidine (ultra-TMB, Thermofisher ref.34028) for 10 minutes, after which the reaction was stopped with sulphuric acid 2M. Optical densities (OD) absorbance was read at 450nm in a microplate reader Synergy HT, Biotek.

3.4 3-NT

3-NT was measured with a ELISA kit from FineTest® (Lot:U2560I085). This kit was based on Competitive-ELISA detection method.

The microtiter plate provided in each kit has been pre-coated with 3-NT. After washing plates 2 times, 50 µL of standard or serum sample were added into each well, immediately followed by the addition of 50 µL of biotin-labelled antibody into each well. Right after tapping the plates for 1 minute to ensure thorough mixing, plates were statically incubated for 45 minutes at 37°C. Then plates were washed 3 times (with 1 minute of immersion each time) and 100 µL of HRP-Streptavidin Conjugate (SABC) working solution was added into each well, and again plates were incubated for 30 minutes at 37°C. Plates were washed 5 more times and 90 µL of TMB substrate solution was added; once again they were incubated at 37°C for approximately 10 minutes until reaction was stopped with 50 µL of sulphuric acid 2M. OD absorbance was read at 450 nm in a microplate reader Synergy HT, Biotek. Intra-assay CV < 8%, inter-assay CV < 10%.

3.5 TAC

TAC was measured with a CheKine® Micro Total Antioxidant Capacity Assay Kit (CAT#KTB1500).

In an acidic environment, antioxidants can reduce Fe3+-tripyridine triazine (Fe^{3+} - TPTZ) to produce Fe^{2+} - TPTZ, and TAC of samples was obtained by determining the content of Fe^{2+} - TPTZ at 593 nm.

96-well plates (Multipurpose U greiner) were filled with 180 µL per well of *working reagent* (substract + reaction buffer + substract diluent); then 10 µL of each serum sample were added after 1:10 dilution in *assay buffer*, alongside with vitamin C (positive control) at 0,1 mg/mL; plates were then incubated for 5 minutes at room temperature whilst shaking at 200 rpm. Absorbance was read at 593 nm in a microplate reader Synergy HT, Biotek.

A standard curve equation was calculated using a 5-parameter logistic regression model using MATLAB R2015a, which was used to back-calculate assay concentration from average OD. The concentration of the lower limit of detection (LoD) for the assay was back-calculated using the same equation from the lower limit of detection in OD, which in turn was calculated using the follow equation: **LoD(OD) = blanks + 1,645 x SDblank + 1,645 x SDlc,** where *blanks* is the arithmetic average of the OD of the blanks, *SDb* is the standard deviation of the OD of the blanks, and *SDlc* is the standard deviation of the OD of the lowest concentration well of the standard curve.

To assess inter-assay variability and reproducibility of results, the experiments were repeated in several samples, following the same protocol. To assess intra-assay variability and reproducibility, selected samples from the same experiment were repeated using 6 replicates.

4. Statistical analysis and data presentation

All data management statistical analysis and graph generation was performed using IBM SPSS Statistics version 29 (IBM Co., NY).

Descriptive statistics are presented as frequency (n), percentage (%), median (IQR 25-75), and mean ± standard deviation (SD). Pearson's chi-square or Fisher exact tests were used to assess relationships between categorical variables. Conformity to normality of the distributions was tested using the Kolmogorov-Smirnov test. For quantitative variables, the independent sample t-test or one-way analysis of variance (ANOVA) test was used (if the data were regularly distributed), or the Mann Whitney or Kruskal Wallis test with Bonferroni-Dunn post hoc test was used (if skewed data). A value of $p < 0.05$ was considered to be statistically significant. P-values were rounded to three decimal places.

The Spearman correlation test was applied to test relationships of ordinal or quantitative variables with nonnormal distribution and the Pearson's correlation test to evaluate continuous variables with normal distribution.

The analysis of pairs (parent-child) or families (parent-children) was performed by a multi-level logistic regression model of mixed-effect.

Graphic display of continuous variables by dichotomous or categorical variables was performed using box-and-whiskers plots, with the interquartile range represented by the box limits, and the whiskers representing extreme limits.

III. RESULTS

1. MORPHOLOGIC FEATURES IN THE DIFFERENT GROUPS

1.1 Characterization of subjects

The general characteristics of the subjects enrolled in this study are shown in table 1.

Table 1. General characteristics of the subjects from all the groups.

	SSc	**SSc Offspring**	**RD**	**RD Offspring**	**T2DM**	**T2DM Offspring**	**Controls**
N	124	55	158	11	98	50	59
Female, n (%)	109(87.9)	37 (67.3)	138 (87.3)	8 (72.7)	40(40.8)	37(74)	39(66.1)
Age (years), mean±SD	56.9 ± 16	35.2 ± 13.6	38.4 ± 13.8	30.3 ± 9.7	66 ± 11.4	35 ± 10.9	39.3 ± 17.2
Europe/African/Asian, n	109/ 13 / 2	48 / 5 /2	152 / 5 / 1	11/ 0 / 0	95 / 3 / 0	46 /4/ 0	55 /4 /0
Age at onset (years), mean±SD	52.0 ± 15.9	NA	32.6 ±14.2	NA	54.7± 12.3	NA	NA
Disease's duration (years), mean±SD	6.2 ± 5.9	NA	15.5 ± 8.7	NA	11.8 ± 7.8	NA	NA
Time to 1st non-RP symptom (years), mean±SD	4.3 ± 7.9	NA	NA	NA	NA	NA	NA
BMI (kg/m^2), mean±SD	24 ± 5.3	24.8 ± 4.3	24.1 ± 6.4	22.7 ± 2.8	29.5 ± 5.3	23.9 ± 4.8	23.3 ± 3.8
Hypertension, n (%)	24 (19.4)	0	0	0	65 (66.3)	2 (4)	12 (20)
Smoking, n (%)	20 (16.1)	0	38 (24)	0	13 (13.3)	2 (4)	6 (10.2)

Haemoglobin (g/L), mean±SD	12.6 ± 1.7	13.6 ± 1.3	13.5 ± 1.4	14.1 ± 1.3	13.3 ± 1.7	13.7 ± 1	13.6 ± 1.2
ESR (mm/h), median [IQR]	29.5 [8.5-75]	17.5 [5.5-34]	8 [4-19]	12 [6.8-25]	27 [9-39]	12 [3-21]	10.5 [3.8-22]
hsCRP (mg/dL), median [IQR]	0.37 [0.06-0.81]	0.16 [0.08-0.78]	0.06 [0-0.31]	0.18 [0.08-0.46]	0.31 [0.14-0.69]	0.07 [0.04-0.4]	0.12 [0.03-0.3]

Legend: BMI: body mass index; ESR: erythrocyte sedimentation rate; hsCRP: high sensitivity C- recative protein; IQR: interquartile range 25-75; NA: non-applicable; SD: standard deviation.

<u>Group 1- Systemic sclerosis</u>

1a - Patients (N=124)

The specific characteristics of patients with SSc are described in table 2.

Table 2. Specific characteristics of the patients with SSc.

SSc' specific characteristics	n (%)
<u>Subtype</u>	
limited cutaneous disease (lcSSc)	67 (54)
diffuse cutaneous disease (dcSSc)	34 (27.4)
sine scleroderma	23 (18.5)
modified Rodnan Skin Score (mRSS), median, IQR [25-75]	6 [0-12]
<u>Antibodies</u>	
anti-nuclear antibody (ANA)	123 (99.2)
anti-centromere antibody (ACA)	54 (43.5)
anti-topoisomerase antibody (ATA/aScl70)	22 (17.8)
anti-RNA polymerase antibody (ARA)	15 (12.1)

anti -Th/To antibody	5 (4)
anti-fibrillarin antibody (U3-RNP)	2 (1.6)
anti-NOR90 antibody	2 (1.6)
anti-PM/Scl antibody	
<u>Clinical features</u>	
Raynaud's phenomenon	119 (96)
Puffy fingers	80 (64.5)
Sclerodactyly	30 (24.2)
Digital Pitting	40 (32.2)
Digital ulcers	36 (29)
Calcinosis	15 (12.1)
Telangiectasia	67 (54)
Arthritis	76 (61.3)
Myositis	18 (14.5)
Gastroesophageal reflux disease (GERD)/GE	84 (67.7)
Gastric antral vascular ectasia (GAVE)	4 (3.2)
Intestine	
- Constipation	10 (8.1)
- Malabsorption	14 (11.3)
Renal crisis	0
Left ventricular disease (LVD)	
- systolic	10 (8.1)
- diastolic	20 (16.1)
Right ventricular disease (RVD)	10 (8.1)
Pulmonary hypertension	13 (10.4)
Interstitial lung disease (ILD)	45 (36.3)
<u>Treatment</u>	
Steroids	37 (29.8)

Hidroxychloroquine	38 (30.6)
Methotrexate	31 (25)
Mycophenolate mofetil (MMF)	30 (24.2)
Iloprost	16 (12.9)
Sildenafil	1 (0.8)
Endothelin receptor antagonists (ERA)	4 (3.2)
Riociguat	1 (0.8)
Tocilizumab	10 (8.1)
Rituximab	2 (1,6)
Nintedanib	4 (3.2)
Calcium channel blockers	41 (33)
Pentoxyfiline	14 (11.3)
Antiplatelet/anticoagulant	35 (28.2)
Other	
Non-specific interstitial pneumonia (NSIP)	38 (30.6)
Usual interstitial pneumonia (UIP)	7 (5.6)
ILD extent in CT scan	
- Limited	25 (20.2)
- Extended	20 (16.1)
Lung function tests (LFT)	
- Restrictive	17 (13.7)
- Obstructive	2 (1.6)
NT-proBNP (pg/mL), median [IQR]	92,90 [50.7-297.5]

The age at onset of SSc was negatively associated with digital ulcers (p=0.006) and positively associated with pulmonary hypertension (p=0.006). The duration of disease was associated with DU (p=0.003) and GAVE (p=0.005).

The subtype lcSSc was associated with ACA (p=0.007), telangiectasias (p=0.037) and puffy fingers (p<0.001); the dcSSc was associated with ATA (p<0.001), sclerodactyly (p<0.001), digital pitting (p=0.011), calcinosis (p<0.001), arthritis (p=0.038), mRSS (p<0.001), RVD (p=0.012), and the use of MMF (p=0.007), ERA (p=0.03) and monoclonal antibodies (p=0.03). The mRSS showed associations with ATA (p=0.006), DU (p=0.004) and GE (GERD p=0.023/GAVE p=0.042).

The NT-proBNP correlated with the extended ILD (p=0.01).

The body mass index of the patients with SSc was directly correlated with ACA (p=0.016) and arterial hypertension (p=0.035) and inversely correlated with ATA (p=0.016), pulmonary hypertension (p= 0.036), NSIP (p=0.005) and the extended ILD (p=0.009).

Smoking was associated with haemoglobin (p=0.004) and NT-proBNP (p=0.009); hsCRP was associated with pulmonary hypertension (p=0.032) and intestine involvement (p=0.041).

1b - Offspring (N=55)

We enrolled 55 subjects in this group.

The offspring of patients with SSc were 17 years younger than their parents when they were diagnosed with SSc. They had no co-morbidities and a normal ESR and hsCRP (table 1).

Group 2 - Raynaud´s Disease

2a – Patients (N=158)

We enrolled 158 patients with RD, with a mean duration of disease of 15 years, without any other associated symptom (table 1).

2b – Offspring (N=11)

We enrolled 11 offspring, whose characteristics are also shown in table 1.

Group 3 – Type 2 diabetes mellitus

3a – Patients (N=98)

A total of 98 patients with type 2 diabetes were enrolled in the study. General data is shown in table 1 and specific clinical and laboratory characteristics are shown in table 3.

Concerning the diabetic microvascular complications, the presence of retinopathy and neuropathy was associated with longer disease duration (p=0.026 and p=0,022, respectively), but not with the glycaemic status; nephropathy did not associate with the duration of the disease but showed a trend towards an uncontrolled glycemia (p= 0.051). Macrovascular complications showed no association with these two features of type 2 diabetes.

3b – Offspring (N=50)

A total of 50 subjects whose parents had type 2 diabetes were enrolled in the study (table 2 and 3).

The offspring were half the age of the patients with type 2 diabetes and were twenty years younger than the age at which diabetes was diagnosed in these patients.

Two subjects were hypertensive, 2 were active smokers. The body mass index, HbA1c, ESR, hsCRP, HDL and triglycerides were all within the normal range but total and LDL-cholesterol were above the superior limit.

Group 4 – Controls (N=59)

A total of 59 subjects, age and sex matched with the offspring groups , were enrolled in group 4.

Controls had a normal body mass index, HbA1c, ESR and hsCRP, but their total and LDL-cholesterol were higher than in the other two groups; 10% were active smokers, 20% had

hypertension and 15% were taking statins and angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (table 3).

Arterial hypertension showed a positive correlation with the BMI ($p<0.001$, $r=0.41$), HbA1c ($p<0.001$, $r=0.53$), hsCRP ($p=0.025$, $r=0.21$) and triglycerides ($p=0.047$, $r=0.19$), and a negative one with HDL ($p=0.001$, $r= -0.31$), TC ($p=0.001$, $r= -0.31$) and LDL (0.021, $r= -0.251$); there is a strong correlation between hypertension and statins intake in type 2 diabetics ($p<0.001$, $r=0.65$).

The practice of physical exercise correlated positively with HDL ($p=0.009$, $r=0.34$) and negatively with the BMI ($p<0.001$, $r= -0.56$), HbA1c ($p<0.001$, $r= -0.59$), ESR ($p=0.003$, $r= -0.42$) and triglycerides ($p=0.013$, $r= -0.33$).

Table 3. Characteristics of patients with T2DM, offspring of patients with T2DM and controls.

	T2DM	**T2DM Offspring**	**Controls**
N	98	50	59
Male, n (%)/Female, n (%)	58(59.2)/40(40.8)	13(26)/37(74)	20(33.9)/39(66.1)
Age (years), mean±SD	66 ± 11.4	35 ± 10.9	39.3 ± 17.2
Euro-caucasian/African, n (%)	95 (97.9)/3 (3.1)	46 (92)/4 (8)	55 (93.2)/4 (6.8)
Age at onset (years), mean±SD	54.7 ± 12.3	NA	NA
Disease duration (years), mean±SD	11.8 ± 7.8	NA	NA
BMI (kg/m^2), mean±SD	29.5 ± 5.3	23.9 ± 4.8	23.3 ± 3.8
Hypertension, n (%)	65 (66.3)	2 (4)	12 (20)
Smoking, n (%)	13 (13.3)	2 (4)	6 (10.2)
Physical exercise, n (%)	13 (13.3)	22 (44)	18 (30.5)
Statins, n (%)	66 (67.3)	0	9 (15.2)
Antiplatelet agent, n (%)	59 (60.2)	0	0
Anticoagulant, n (%)	9 (9.2)	0	0
ACEi/ARB, n (%)	55 (56.1)	1 (2)	9 (15.2)
Insulin, n (%)	31 (31.6)	0	0
Metformin, n (%)	59 (60.2)	0	0
GLP-1 receptor agonist, n (%)	10 (10.2)	0	0
SGLT-2i, n (%)	19 (19.4)	0	0
DPP-4i, n (%)	37 (37.8)	0	0
Sulfonylureas, n (%)	6 (6.1)	0	0
Cerebrovascular disease, n (%)	23 (23.5)	0	0
Peripheral arterial disease,n (%)	15 (15.3)	0	0
Coronary artery disease, n (%)	20 (20.4)	0	0

Retinopathy, n (%)	33 (33.7)	NA	NA
Nephropathy, n (%)	34 (34.7)	NA	NA
Neuropathy, n (%)	28 (28.6)	NA	NA
Haemoglobin (g/L), mean±SD	13.3 ± 1.7	13.7 ± 1	13.6 ± 1.2
ESR (mm/h), median [IQR]	27 [9-39]	12 [3-21]	10.5 [3.8-22]
hsCRP (mg/dL), median [IQR]	0.31 [0.14-0.69]	0.07 [0.04-0.4]	0.12 [0.03-0.3]
HbA_{1c} (mmol/mol), mean±SD	54.6 ± 14.5	29.2 ± 6.1	29 ± 5.8
HbA_{1c} (%), mean±SD	7.2 ± 1.3	4.8 ± 0.6	4.8 ± 0.5
Total cholesterol (mg/dL), mean±SD	152.9 ± 41.5	183.3 ± 30.9	188.8 ± 43.1
LDL (mg/dL), mean±SD	75.1 ± 38	103.5 ± 30.6	113 ± 41.4
HDL (mg/dL), mean±SD	46.6 ± 15.1	61.2 ± 13.5	58.9 ± 14.4
Triglycerides (mg/dL), mean±SD	151.4 ± 97.6	90.1 ± 41.2	102.4 ± 43.3
TC-HDL ratio, mean±SD	3.6 ± 1.7	3.1 ± 0.8	3.4 ± 1.1

Legend: ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; GLP-1: glugagon-like peptide-1; SGLT-2i: sodium glucose co-transporter-2 inhibitor; DPP-4i: dipeptidyl peptidase-4 inhibitor; TC: total cholesterol; NA: non-applicable; SD: standard deviation; IQR: interquartile range 25-75

1.2 Nailfold videocapillaroscopy

Group 1- Systemic sclerosis

1a - Patients

According to the NVC classification by Cutolo et al., 23 patients presented with an early 'scleroderma' pattern, 52 with an active pattern, 38 with a late pattern and 7 patients did not have a scleroderma-like pattern. There is a direct association between disease duration and active (p=0.032) and late (p=0.004) patterns (figure 1), as well as between the mRSS and the different patterns (p=0.041) (figure 2).

In patients with systemic sclerosis *sine scleroderma* and lcSSc the active pattern is predominant (43.5% and 44.6%, respectively); in patients with dcSSc the active pattern is present in 40.6% and the late pattern in 50% (p=0.039 r=0.32).

The BMI is lower in patients who have an active pattern (p=0.022) and even lower in the late pattern (p=0.009) (figure 3). Association of patterns and ESR did not reach statistical significance but there was a trend towards it (p=0.069 from early to active)

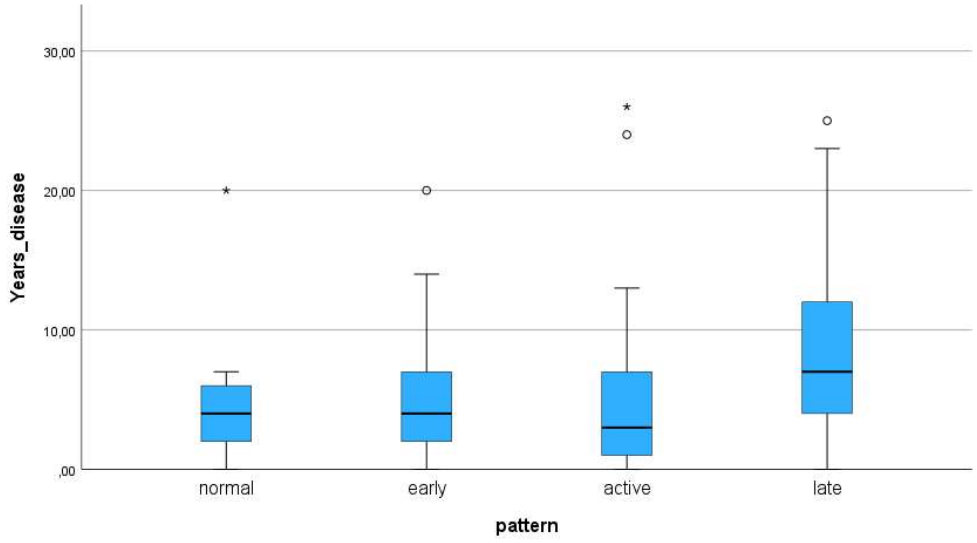

Figure 1. The duration of disease in SSc and the different 'scleroderma' patterns in NVC.

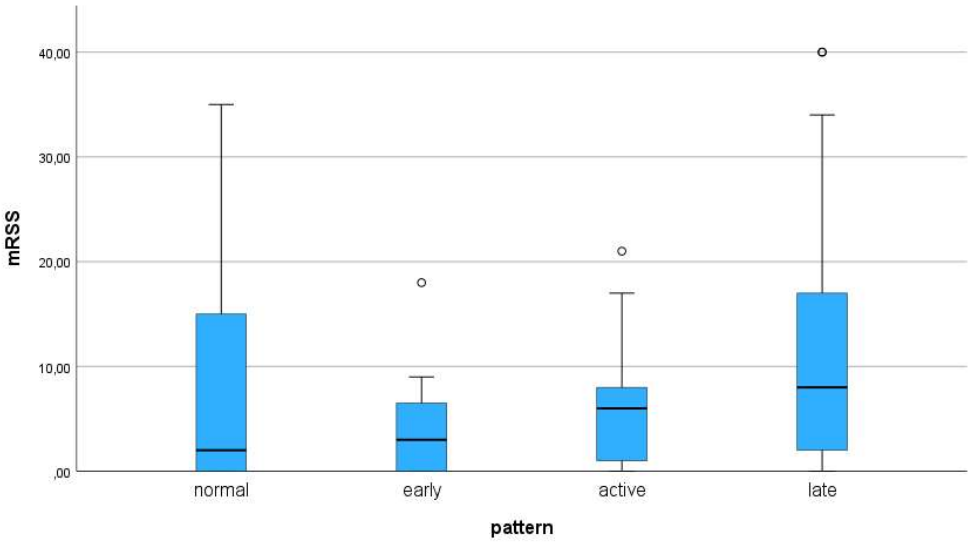

Figure 2. The modified Rodnan skin score and the different 'scleroderma' patterns in NVC.

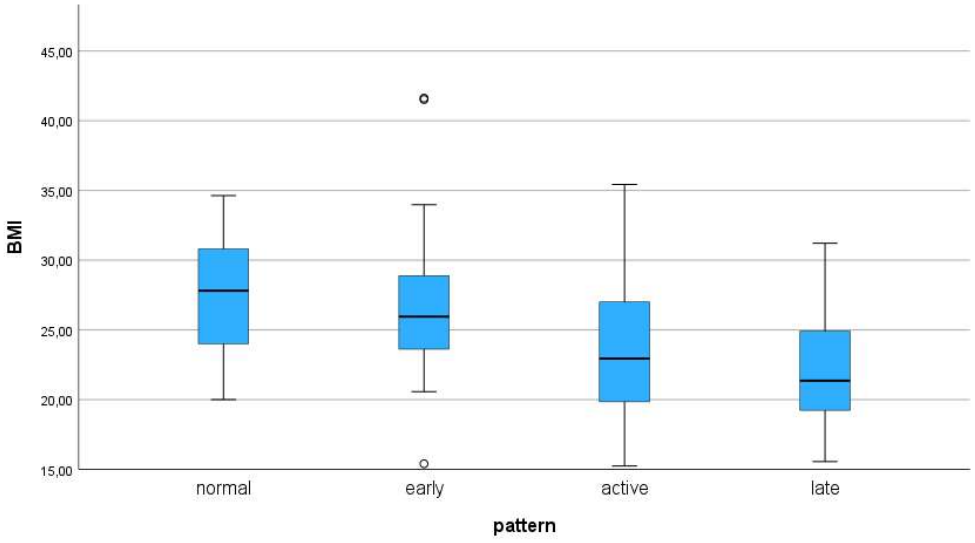

Figure 3. The body mass index and the different 'scleroderma' patterns in NVC.

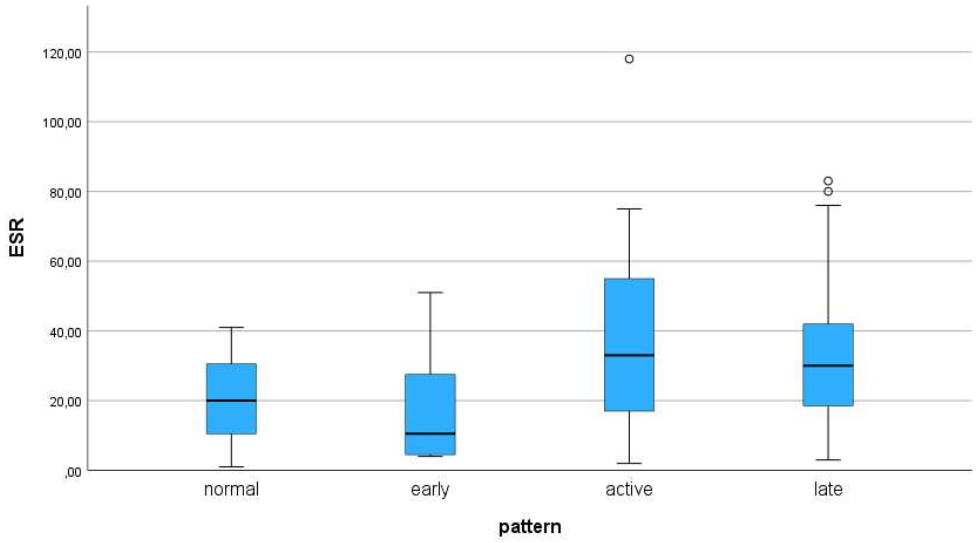

Figure 4. The erythrocyte sedimentation rate and the different 'scleroderma' patterns in NVC.

Regarding the NVC parameters and the SSc characteristics, there were positive associations between: Less than 8 capillaries per field and mRSS (p=0.019); reduced velocity and NTproBNP (p=0.041); major dysmorphias and age at onset of disease (p=0.01) and the subtype dcSSc (p=0.011); sludge and ESR (p=0.027); avascular areas and mRSS (p=0.008), dcSSc (p=0.041), NT-proBNP (p=0.003) and CRP (0.001); edema and mRSS (p=0.006); angiogenesis and age at onset (p=0.029), dcSSc (p=0.001), mRSS (p=0.004), NT-proBNP (p=0.027) and CRP (p=0.012).

All the NVC parameters from patients with systemic sclerosis differed significantly from the ones found in controls (table 4).

1b – Offspring

The offspring of patients with SSc differed from Controls in almost every parameter, except for the number of minor morphologic abnormalities (p=0.16), giant capillaries (p=0.1), and microhaemorrages (p=0.23). In fact, the NVC of patients with SSc and their Offspring were similar in most aspects including morphology (p=0.6), flux (p=0.06) and chronic ischaemia (p=0.78) markers (table 4).

Table 4. NVC parameters in patients with SSc, compared to respective offspring and controls.

	Systemic sclerosis	Controls	P	SSc Offspring	Controls	P	Systemic sclerosis	SSc Offspring	P
Age, years (mean±SD)	56.9± 16	39.3±17.2	<0.001	35.2±13.6	39.3±17.2	0.166	56.9 ±16	35.2±13.6	<0.001
< 8 cap/mm	0,64	0.04	**<0.001**	0,26	0.04	**0.003**	0,64	0,26	<0.001
Enlarged capillaries	0,87	0.19	**<0.001**	0,47	0.19	**0,003**	0,87	0,47	<0.001
Giant capillaries	0,71	0.0	**<0.001**	0,05	0.0	0,10	0,71	0,05	<0.001
Minor abnorm >30%	*0,12*	0.77	***<0.001***	0,64	0.77	0,156	*0,12*	0,64	<0.001
Major abnormalities	0,46	0.21	**0.003**	0,42	0.21	**0.027**	0,46	0,42	**0.6**
Microhaemorrhages	0,71	0.13	**<0.001**	0,22	0.13	0,23	0,71	0,22	<0.001
Interstitial edema	0,85	0.3	**<0.001**	0,51	0.3	**0.031**	0,85	0,51	<0.001
Avascular areas	0,67	0.34	**<0.001**	0,69	0.34	**<0.001**	0,67	0,69	**0.78**
Neoangiogenesis	0,43	0.08	**<0.001**	0,24	0.08	**0.041**	0,43	0,24	0.012
Low velocity	0,54	0.02	**<0.001**	0,33	0.02	**<0.001**	0,54	0,33	0.01
Sludge	0,81	0.23	**<0.001**	0,68	0.23	**<0.001**	0,81	0,68	**0.06**

Footnotes**:** values represent proportions of findings.

Analysis of pairs (parent-offspring) did not reach statistical significance regarding NVC parameters (data not shown).

Group 2 – RD

2a – patients

Patients with RD differed significantly from controls in the number of enlarged capillaries (p=0.02), blood flow (p=0.002) and neoangiogenesis (p=0.04) (table 5).

2b – offspring

There is a numeric difference between the offspring of patients with RD and controls regarding density of capillaries, number of enlarged vessels, angiogenesis-associated aspects and blood velocity. However, only the capillary density reached statistical significance (p=0.04) (table 5).

Table 5. NVC parameters in patients with RD, compared to respective offspring and controls.

	Raynaud's disease	Controls	P	RD Offspring	Controls	P	Raynaud's disease	RD Offspring	P
Age, years (mean±SD)	38.4 ± 13.8	39.3 ±17.2	0.75	30.3 ± 9.7	39.3 ±17.2	0.02	38.4 ± 13.8	30.3 ± 9.7	0.021
< 8 cap/mm	0,06	0.04	0,6	0,27	0.04	**0,04**	0,06	0,27	**0,04**
Enlarged capillaries	0,37	0.19	**0,02**	0,46	0.19	0,11	0,37	0,46	0,75
Giant capillaries	0,05	0.0	0,12	0,09	0.0	0,19	0,05	0,09	0,46
Minor abnorm >30%	*0,50*	0.77	*0,001*	0,64	0.77	0,45	*0,50*	0,64	0,54
Major abnormalities	0,27	0.21	0,41	0.18	0.21	1	0,27	0.18	0,73
Microhaemorrhages	0,17	0.13	0,42	0,18	0.13	0.64	0,17	0,18	1
Interstitial edema	0,37	0.3	0.34	0,18	0.3	0.71	0,37	0,18	0.33
Avascular areas	0,27	0.34	0,32	0,27	0.34	1	0,27	0,27	1
Neoangiogenesis	0,22	0.08	**0,04**	0,27	0.08	0.12	0,22	0,27	0,71
Low velocity	0,22	0.02	**0.002**	0,18	0.02	0,09	0,22	0,18	1
Sludge	0,35	0.23	0,14	0,36	0.23	0,45	0,35	0,36	1

Footnotes**:** values represent proportions of findings.

Patients with SSc and subjects with Raynaud's disease had significant differences in all the parameters considered (p<0.001). Morphologic abnormalities (p=0.04), capillary rarefaction [density per field (p<0.001) and isolated avascular areas (p<0.001)] and "sludge" (p<0.001) were significantly more pronounced in the offspring of patients with SSc than in patients with RD (table 6).

Table 6. NVC parameters in patients with RD compared to patients with SSc and their offspring.

	Raynaud's	Systemic	P	Raynaud's	SSc	P

	disease	sclerosis		disease	Offspring	
Age, years (mean±SD)	38.4 ± 13.8	56.9± 16	<0.001	38.4 ± 13.8	35.2 ± 13.6	0.134
< 8 cap/mm	0,06	0,64	**<0.001**	0,06	0,26	**<0.001**
Enlarged capillaries	0,37	0,87	**<0.001**	0,37	0,47	0,2
Giant capillaries	0,05	0,71	**<0.001**	0,05	0,05	0,9
Minor abnorm >30%	*0,50*	*0,12*	***<0.001***	*0,50*	0,64	0.08
Major abnormalities	0,27	0,46	**0.001**	0,27	0,42	**0.04**
Microhaemorrhages	0,17	0,71	**<0.001**	0,17	0,22	0,5
Interstitial edema	0,37	0,85	**<0.001**	0,37	0,51	0.08
Avascular areas	0,27	0,67	**<0.001**	0,27	0,69	**<0.001**
Neoangiogenesis	0,22	0,43	**<0.001**	0,22	0,24	0.74
Low velocity	0,22	0,54	**<0.001**	0,22	0,33	0.1
Sludge	0,35	0,81	**<0.001**	0,35	0,68	**<0.001**

Footnotes**:** values represent proportions of findings.

Group 3 – T2DM

3a – patients

The number of minor morphologic abnormalities showed an inverse correlation with exercise (p=0.001, r= -0.48) and SGLT-2i intake (p=0.007, r= -0.32); microhemorrhages with antiplatelet therapy (p=0.005, r= -0.33), statins (p=0.013, r= -0.3) and SGLT-2i (p=0.001, r= -0.37); oedema with antiplatelet therapy (p=0.036, r= -0.24) and neoangiogenesis with physical exercise (p=0.021, r= -0.35).

There were direct associations between neoangiogenesis and hypertension (p=0.013, r=0.19) and insulin intake (p=0.002, r=0.38).

There was no association between diabetic microvascular and macrovascular complications and the capillaroscopic abnormalities, with the exception of peripheral arterial disease which correlated positively with a reduced blood flow velocity (p=0.001; r =0,4).

Patients with type 2 diabetes had significant differences from controls in almost all parameters considered in the NVC evaluation, except for crossed patterns and the presence of enlarged and giant capillaries (table 7).

NVC in patients with Systemic sclerosis and Type 2 Diabetes showed no differences in the number of bushy and bizarre patterns, neoangiogenesis and avascular isolated areas (table 8).

Table 7. NVC parameters in patients with T2DM, compared to respective offspring and controls.

	T2DM	Controls	P	T2 DM Offspring	Controls	P	T2DM	T2DM Offspring	P
Age, years (mean±SD)	66 ±11.4	39.3 ±17.2	<0.001	35 ± 10.9	39.3±17.2	0.1	66 ±11.4	35 ± 10.9	<0.001
< 8 cap/mm	**0.27**	**0.04**	**0.001**	0.13	0.04	0.13	0.27	0.13	0.06
Enlarged capillaries	0.24	0.19	0.55	0.24	0.19	0.58	0.24	0.24	0.97
Giant capillaries	0.07	0.0	0.07	0.0	0.0	-	0.07	0.0	0.07
Minor abnorm >30%	0.84	0.77	0.27	0.87	0.77	0.2	0.84	0.87	0.68
Major abnormalities	**0.43**	**0.21**	**0.013**	0.33	0.21	0.22	0.43	0.33	0.26
Microhaemorrhages	**0.42**	**0.13**	**<0.001**	0.15	0.13	0.73	**0.42**	**0.15**	**0.002**
Interstitial edema	**0.71**	**0.3**	**<0.001**	**0.56**	**0.3**	**0.009**	0.71	0.56	0.1
Avascular areas	**0.75**	**0.34**	**<0.001**	**0.78**	**0.34**	**<0.001**	0.75	0.78	0.7
Neoangiogenesis	**0.47**	**0.08**	**<0.001**	**0.3**	**0.08**	**0.007**	0.47	0.3	0.06
Low velocity	**0.25**	**0.02**	**<0.001**	**0.13**	**0.02**	**0.046**	0.25	0.13	0.13
Sludge	**0.46**	**0.23**	**0.01**	0.39	0.23	0.10	0.46	0.39	0.44

Footnotes: values represent proportions of findings.

3b - Offspring

The offspring of the patients with type 2 diabetes differed significantly from the controls regarding oedema (p=0.009), avascular areas (p<0.001), neoangiogenesis (0.007) and the velocity of the blood flow (p=0.046); in fact, except for haemorrhages (p=0.002), we found no differences between patients with type 2 diabetes and their offspring (table 7).

When we compared the offsprings of patients with SSc and T2DM, the differences found were in enlarged capillaries (p=0.015), minor *dismorphias* (p=0.008), reduced blood velocity (p=0.021) and erythrocyte stasis (p=0.005) (table 8).

Table 8. NVC parameters in patients with T2DM compared to patients with SSc, and comparison between respective offsprings.

	T2DM	SSc	P	T2 DM Offspring	SSc Offspring	P
Age, years (mean±SD)	66 ± 11.4	56.9± 16	<0.001	35 ± 10.9	35.2 ± 13.6	0.85

< 8 cap/mm	0.27	0,64	**<0.001**	0.13	0,26	0.119
Enlarged capillaries	0.24	0,87	**<0.001**	0.24	0,47	**0.015**
Giant capillaries	0.07	0,71	**<0.001**	0.0	0,05	0.108
Minor abnorm >30%	0.84	*0,12*	**<0.001**	0.87	0,64	**0.008**
Major abnormalities	0.43	0,46	0.63	0.33	0,42	0.341
Microhaemorrhages	0.42	0,71	**<0.001**	0.15	0,22	0.398
Interstitial edema	0.71	0,85	**0.012**	0.56	0,51	0.573
Avascular areas	0.75	0,67	0.2	0.78	0,69	0.3
Neoangiogenesis	0.47	0,43	<0.001	0.3	0,24	0.442
Low velocity	0.25	0,54	**<0.001**	0.13	0,33	**0.021**
Sludge	0.46	0,81	**<0.001**	0.39	0,68	**0.005**

.....Footnotes: values represent proportions of findings.

In the same way as with SSc-offspring pairs, analysis of T2DM-offspring pairs did not achive statistical significance in any NVC parameter (data not shown).

Regarding interpretation of NVC images, global inter-observer variability was 5% and intra-observer variability was 1.5%.

1.3 Discussion and disease-based analysis

Characteristics of the groups

There is a female predominance in all the groups, except for T2DM.

The characteristics of the systemic sclerosis' group are in accordance with those described in the other cohorts, namely the average age at onset of the disease, clinical features and antibody profile (Coral-Alvarado et al., 2009; Cutolo et al., 2016; Denton & Khanna, 2017). It should be noted that the average disease duration is a little shorter than in the commonly described cohorts, as some patients were included in the early phase of the disease; also, the average *time to first non-Raynaud's manifestation* is relatively short, but with broad extreme limits as some of these patients reported only noticing RP after showing other symptoms of the disease. The percentage

of patients without cutaneous sclerosis and with limited cutaneous disease is also higher than usually described, which explains a mRSS with a median of 6. There were no unusual findings in the associations between the different clinical features of SSc.

It is worth noting a high percentage of smokers among individuals with Raynaud's disease; however, as described below, there were no significant associations of smoking with any variable in this group of patients.

In T2DM, the characteristics of the studied population were in agreement with what has been reported in universal cohorts(Nathan, 2015) ("Diagnosis and classification of diabetes mellitus," 2008), regarding clinical practice and medication profile.

NVC parameters

The offspring of patients with SSc differed from Controls in almost every parameter evaluated in the NVC; the exceptions were the giant capillaries and hemorrhages, two features much more affiliated to a diagnosis of SSc already established. In addition, the offspring of the patients with SSc share changes in morphology, flux and signs of ischaemia with their parents. Despite not having RP, the offspring of the patients with SSc have significant capillary rarefaction, major dismorphias and sludge when compared to patients with RD.

In patients with Raynaud's disease, only angiogenesis and low blood flow velocity differed from Controls, which is compatible with chronic ischemic episodes that with time lead to collateral compensatory circulation, but without an associated structural disease. They also differ in the number of enlarged capillaries, which have been considered a nonspecific feature (Ref). Patients with RD have a completely different NVC from patients with SSc. The offspring of patients with RD have a close to normal capillaroscopy. This benignity might be due to the small number of the sample (n=11) or to a different mechanistic or genetic impact in the microcirculation phenotype of RD in relation to SSc.

Similar to SSc, almost all parameters in T2DM patients differed significantly from Controls, except for the number of enlarged and giant capillaries, often considered specific to SSc. Interestingly, the offspring of the patients with type 2 diabetes also differed significantly from the Controls in features like oedema, avascular areas, and neoangiogenesis, three abnormalities typical seen in the microcirculation of patients with T2DM. In fact, patients with T2DM and their offspring have an almost identical NVC.

Although SSc and T2DM share some abnormalities, they have distinct NVC patterns overall. When we compared both offsprings, they have many NVC changes in common, suggesting that SSc and T2DM may share some physiopathologic pathways.

2. BIOCHEMISTRY FEATURES IN THE DIFFERENT GROUPS

Inter-assay and intra-assay variances were less than 10% in all experiments.

2.1 VCAM-1

VCAM-1 is a glycoprotein that is inducible and predominantly expressed in endothelial cells. Its expression is activated by pro-inflammatory cytokines and by ROS (Troncoso et al., 2021).

Table 9. Levels of VCAM-1 in the different groups.

Group	VCAM-1 (ng /mL), median [IQR]
SSc	906.8 [612.7 - 1161.5]
SSc Offspring	307.6 [169.3 - 723.3]
RD	530.4 [201.9 - 868.2]
RD Offspring	530.8 [312.3 - 773.3]
T2DM	623.2 [366.5 - 1036.4]
T2DM Offspring	481.5 [291.2 - 698.2]
Controls	505.3 [298.2 - 616.6]

In patients with SSc, VCAM-1 showed inverse correlations with ARA (p=0.017, r= -0.22) and limited extent of ILD in CT scan (p= 0.005, r= -0.28) (figure 5). Regarding VCAM-1 and features in NVC, the only correlation found was in major morphologic abnormalities (p=0.012, r=0.25).

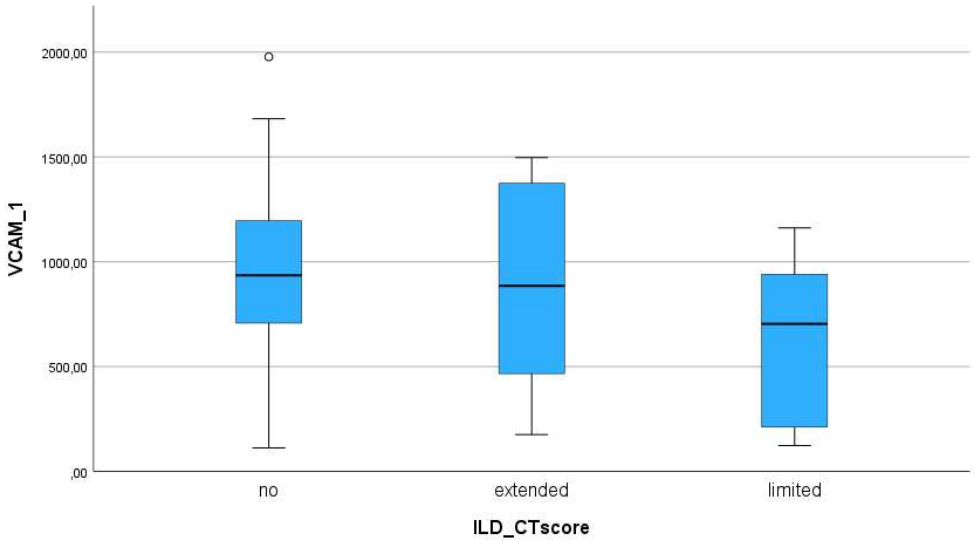

Figure 5. The levels of VCAM-1 and the extent of ILD in CT scan.

In patients with T2DM, VCAM-1 was directly associated with hypertension (p=0.011/r=0.2) and inversely with exercise (p=0.045/r= -0.24), but there was no association with micro or macrovascular disease. Considering nailfold capillaroscopic parameters in patients with T2DM and their Offspring, and independently from the group where the participants were included VCAM-1 was significantly higher in subjects with minor capillary abnormalities in more than 30% of the field (p=0.037), microhemorrhages (p=0.011), interstitial oedema (p=0.014) and neoangiogenesis (p<0.001).

Patients with SSc had significant higher levels of VCAM-1 than controls (p<0.001), patients with RD (p<0.001), offspring of RD (p<0.009), offspring of SSc (p<0.001) and offspring of T2DM (p<0.001). There were no differences between the offspring of SSc and controls (p=0.254). (figure 6).

Patients with T2DM showed significant higher levels of VCAM-1 than controls (p=0.012), patients with RD (p=0.008), and offspring of SSc. There was no difference between controls and offspring (p=0.577) (figure 6).

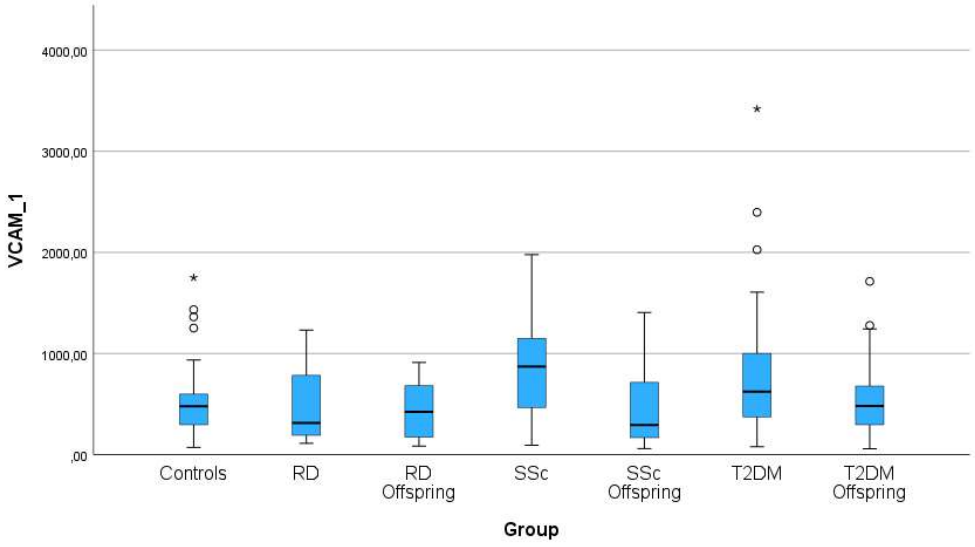

Figure 6. Comparison of the levels of VCAM-1 between the different groups.

2.2ICAM-1

ICAM-1 is an Ig superfamily protein, expressed on the surface of endothelial cells, being a critical molecule for firm adhesion and trans-endothelial migration of several leukocyte subsets. ICAM-1 itself can activate multiple cell signalling cascades (Wolf et al., 2013).

Table 10. Levels of ICAM-1 in the different groups.

Group	ICAM-1 (ng /mL), median [IQR]
SSc	130.5 [89.4 - 187.5]
SSc Offspring	121.2 [79 - 162]
RD	118.6 [60.6 - 185]
RD Offspring	58.1 [17.2 - 155.4]
T2DM	212.5 [169.9 – 318.3]
T2DM Offspring	198.1 [142.5 - 261.5]
Controls	194.4 [141.5 - 260.7]

In patients with SSc, there was an inverse association between ICAM-1 and the presence of DU (p=0.003, r= -0.28), reduced velocity of blood flow (p= 0.017, r= - 0.24) and sludge (p=0.042, r= - 0.21). In patients with T2DM, ICAM-1 was directly associated with hypertension (p=0.016/ r=0.19) and inversely with exercise (p=0.048/r= -0.24).

Although within the normal range, the control group showed higher levels than the patients with SSc (p=0.007) and respective offspring (p < 0.001). Patients with SSc had significant higher levels of ICAM-1 than their offspring (p= 0.019). There were no differences between controls and patients with T2DM (p=0.204) and respective offspring (p=0.79) (figure 7).

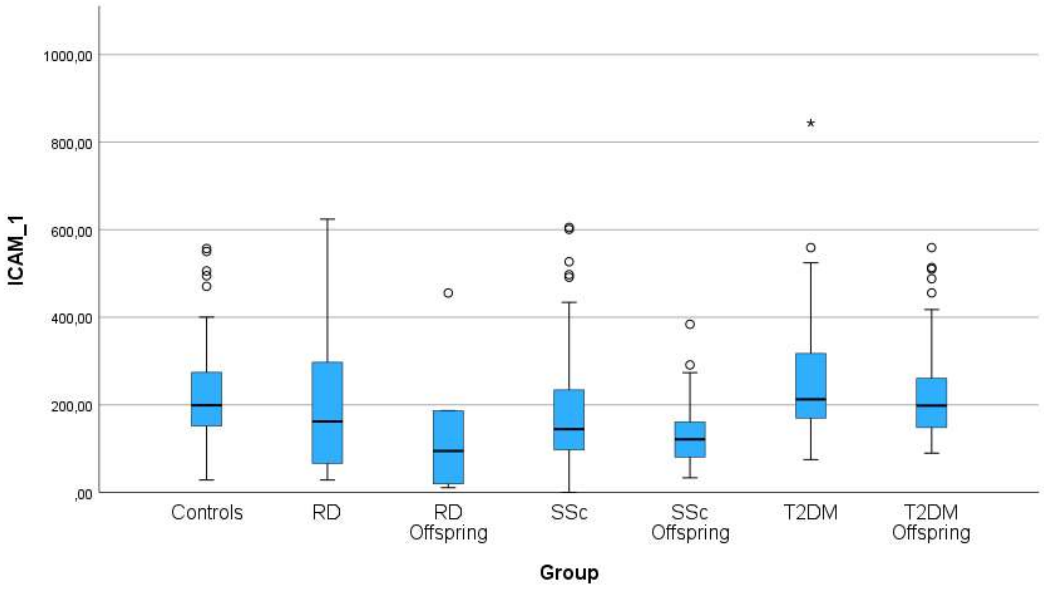

Figure 7. Comparison of the levels of ICAM-1 between the different groups.

2.3 VEGF-A

VEGF is involved in several steps of physiological and pathological angiogenesis including proliferation, survival and migration of endothelial cells (Distler et al., 2002)

Table 11. Levels of VEGF-A in the different groups.

Group	VEGF-A (pg /mL), median [IQR]
SSc	383.8 [184 - 604.7]
SSc Offspring	360 [181.8 - 649.4]
RD	404.5 [262.3 - 531.5]
RD Offspring	282.1 [129.8 - 350.4]
T2DM	410.6 [266.2 - 553.7]
T2DM Offspring	372.6 [251.6 - 535.4]
Controls	472 [339.6 - 681.9]

In patients with SSc, levels of VEGF-A showed positive correlation with both types lcSSc (p=0.013) and dsSSc (p=0.032) (figure 8), pulmonary hypertension (0.019, r=0.23), NSIP (p=0.014) (figure 9), and ILD with limited extent in CT scan (p=0.009)(figure 10).

The levels of VEGF-A correlate directly with haemorhages (p=0.017, r=0.25) and major abnormalities (p=0.001, r=0.34) in NVC.

In patients with type 2 diabetes, VEGF-A was inversely correlated with cerebrovascular disease (p=0.045, r= -0.26).

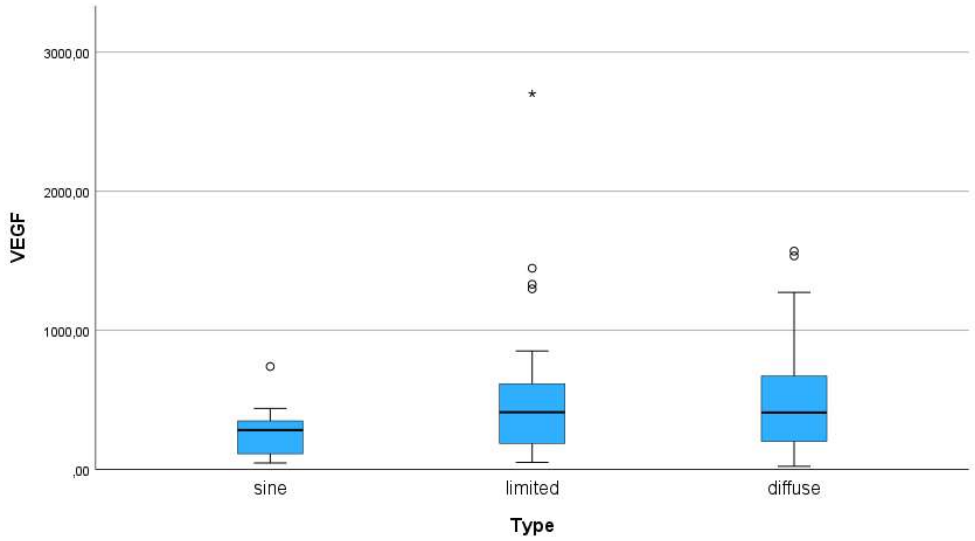

Figure 8. Levels of VEGF-A and the classification of SSc according to subtypes.

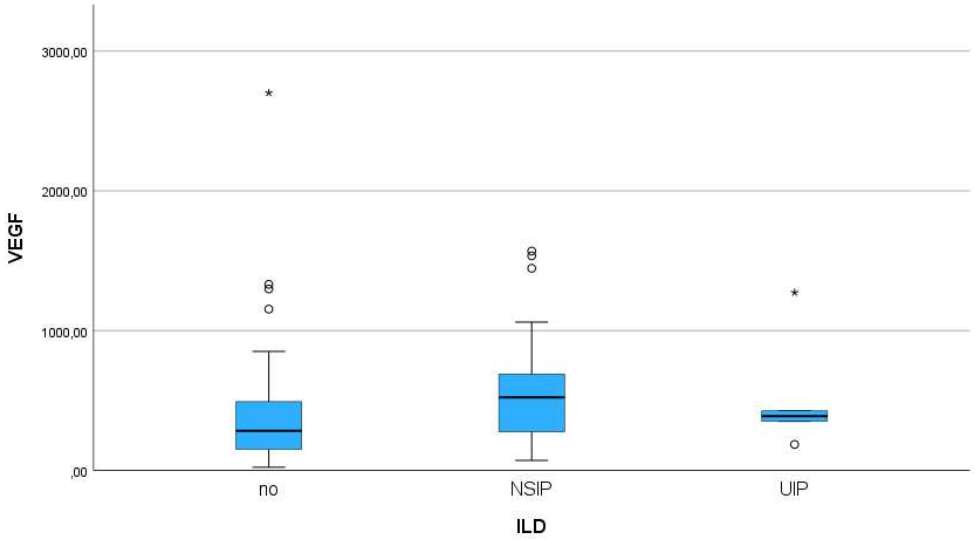

Figure 9. Levels of VEGF-A and the two more frequent subtypes of ILD in SSc.

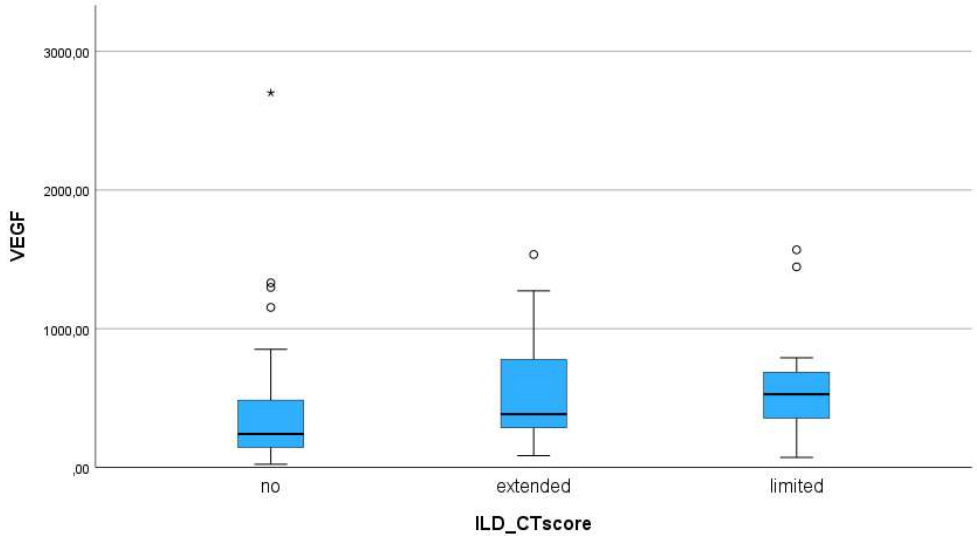

Figure 10. Levels of VEGF-A and the extent of ILD in CT scan.

Levels of VEGF-A were homogeneous between the different groups, with no relevant differences (figure 11).

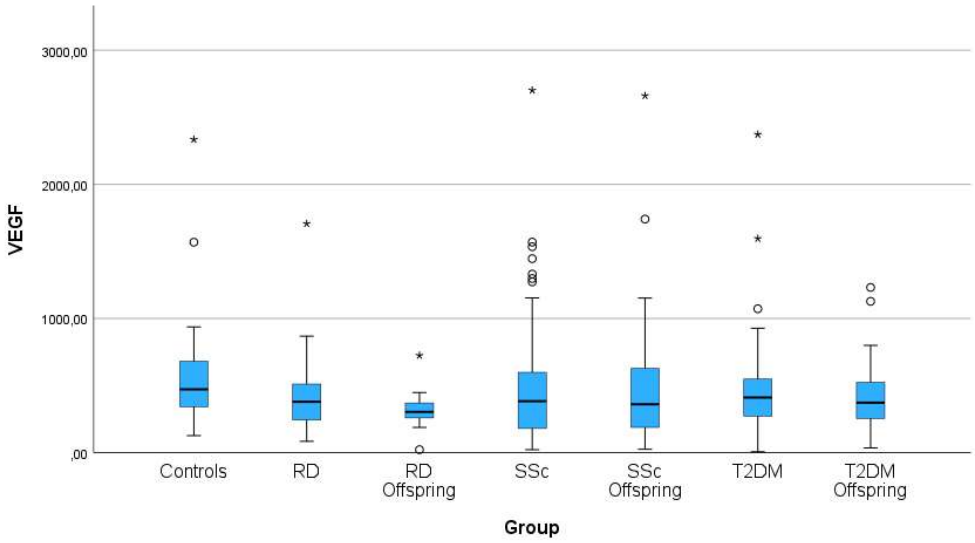

Figure 11. Comparison of the levels of VEGF-A between the different groups.

2.4 3-NT

The formation of reactive nitrogen species such as peroxynitrite can be inferred by quantification of the levels of nitrated tyrosine residues (nitrotyrosine) in proteins (Kingdon et al., 2006).

Table 12. Levels of 3-NT in the different groups.

Group	3-NT (ng /mL), median [IQR]
SSc	39.9 [33.3-52.8]
SSc Offspring	33.2 [25.5-49.9]
RD	32.8 [26.2-49.7]
RD Offspring	28.1 [25.3-37]
T2DM	32.6 [26.5 – 48.9]
T2DM Offspring	45 [35.2 - 60.4]
Controls	36.1 [25-45.1]

The offspring of SSc did not show different levels from controls (p=0.99). Additionally, there were no differences between patients with SSc (p=0.33), RD (p=0.91), T2DM (p=0.87), RD offspring (p=0.47) and controls. The offspring of T2DM showed significant higher levels of 3-NT than controls (p=0.027) (figure 12).

The levels of 3-NT did not show association with neither the general or specific characteristics of all the groups nor with the NVC parameters.

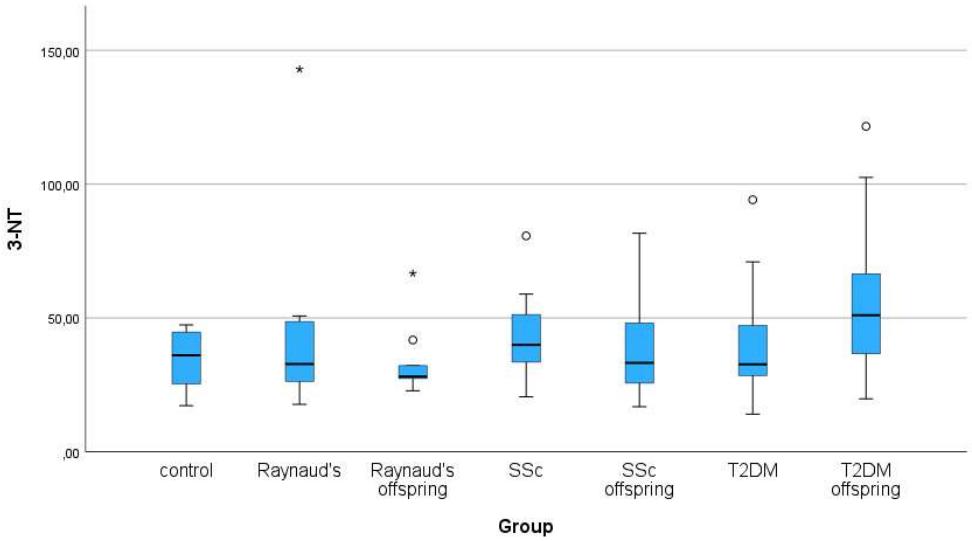

Figure 12. Comparison of levels of 3-NT between the different groups.

2.5 TAC

Oxidative stress is suggested to be involved in the pathogenesis of SSc. Since the physiological response to oxidative stress is regulated by multiple antioxidant systems, it is important to measure quantitatively the total antioxidant capacity.

Patients with SSc and respective offspring had significant higher levels of TAC than controls ($p=0.027$ and $p=0.034$, respectively). Patients with T2DM had significant higher levels of TAC than controls ($p<0.001$), respective offspring ($p=0.005$) and RD ($p=0.001$) (figure 13).

Table 13. Levels of TAC in the different groups.

Group	TAC (µmol /mL), median [IQR]
SSc	42.5 [36.6-49.75]
SSc Offspring	44.58 [37.28-51.82]
RD	38.55 [32.66-45.15]
RD Offspring	44.52 [35.73-51.6]
T2DM	47.67 [36.91-54.74]
T2DM Offspring	41.94 [34.83-46.66]
Controls	39.11 [31.18-46.37]

Levels of TAC did not show any association with either the general or specific characteristics of the different groups or with the capillaroscopic parameters.

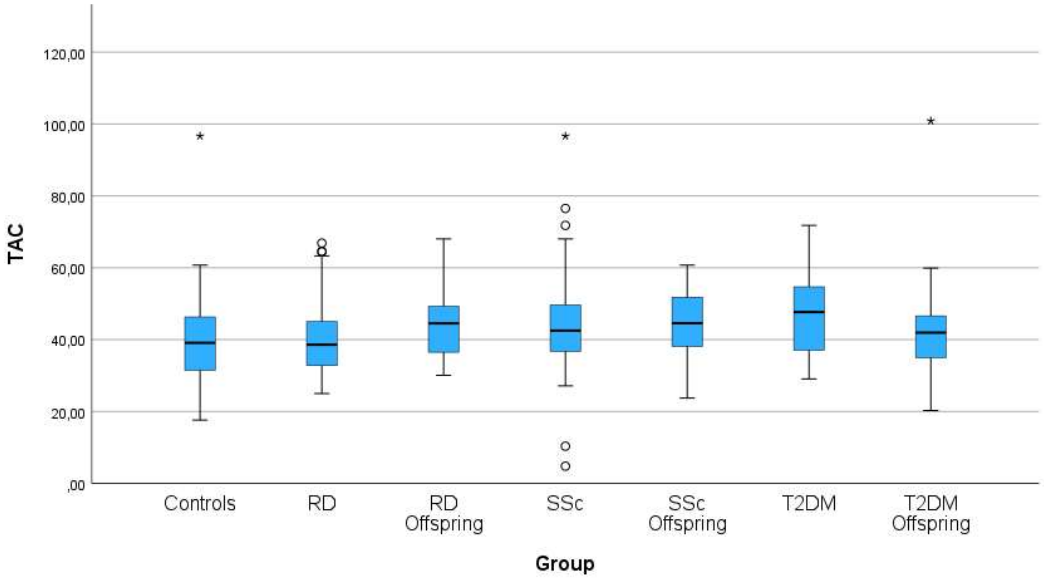

Figure 13. Comparison of the levels of TAC between the different groups.

2.6 Discussion and disease-based analysis

In order to identify evidence of immune or inflammatory activation, VCAM-1, ICAM-1 and VEGF-A were measured in all groups. There were no changes in the offspring of patients with SSc when compared to the Control group. So, endothelial cells and associated dysfunction do not appear be to the 'first hit' in SSc.

However, changes in NVC similar to the ones found in patients with SSc were already present in their offspring suggesting that the vascular changes are present before inflammation or immune response starts (at least with a significant intensity or relevance).

Naturally, a non-activation of the immune response or absence of inflammation cannot be inferred with these biomarkers alone, but these are molecules known to be elevated in the early stages of disease, hence we can suggest that the microcirculatory anatomy is one of the first factors to be altered, admittedly as a consequence of a specific genetic background.

Regarding oxidative stress and endothelium activation, the levels of 3-NT in the SSc offspring (used as a surrogate marker of those mechanisms), were not different from controls, again suggesting that the disruption of the vascular structure appears first in this disease.

Of note, the offspring of SSc had higher levels of TAC than controls. Previous studies regarding the role of TAC in SSc showed controversy: serum levels of TAC were decreased in some (Biondi et al., 2008), were no different from controls in others (Firuzi et al., 2006), or they were even increased (Brezovec et al., 2023; Ogawa et al., 2011), presenting as an indicator of the global response to oxidative stress. So, results should be interpreted with caution. The increase of these levels in the offspring of SSc may suggest that an oxidative environment does already exist, or, since the levels of 3-NT are normal, they simply represent an efficient biologic redox system.

Despite a significant variability, the age of onset of SSc takes place in the 5th and 6th decades of life for the majority of patients. In this study, the offspring of the patients with SSc were significantly younger. Together with the fact that they had no Raynaud's or any other symptom, it is reasonable to assume that the morphologic changes of the microcirculation are indeed present before the functional changes occur.

This phenomenon was not present in the RD universe as the offspring of the patients with RD did not have significant changes in the microvasculature, nor they had changes in the biomarkers when compared with the control group. These results are in line with previous studies in RD (Kingdon et al., 2006; Ringqvist et al., 1997). Thereby, one can infer that not only the hereditary weight may be less relevant in this condition, but also that the pathophysiology of this disease may be significantly different from the SSc, despite the high incidence of RP in patients with SSc.

Finally, the offspring of patients with T2DM showed changes in the NVC exam that would approach the ones reported in actual patients. Again, this changes happened before any clinical or biologic deviation from normal, reinforcing the influence of the familiar background in this condition.

IV. OVERALL DISCUSSION

(Is vasculopathy the original sin in systemic sclerosis?)

The diagnosis of systemic sclerosis and, consequently, the start of appropriate therapy is usually delayed until the appearance of skin involvement and/or clinically detectable internal organ involvement when microvascular remodeling, tissue fibrosis or atrophy are already irreversible. This limits the possibility of an early treatment and the early prevention of disease progression and tissue damage (Czirják & Matucci-Cerinic, 2011). For these reasons, amongst the rheumatic disorders, SSc is considered one of most challenging to manage.

The study of the pre-clinical phase in any medical condition may be challenging but is particularly difficult in Systemic Sclerosis as this is a rare disease where the first clinical feature – Raynaud´s phenomenon (still a non-specific manifestation), can precede the development of the disease in several decades. Furthermore, there is no clear information as to how relevant the genetic factor may be, because despite some evidence of an increased incidence in families, the percentage of offspring that develops the disease does not suggest a direct genetic link.

From the physiopathology point of view, the presence of inflammation, immune activation, endothelium dysfunction and oxidative stress since the early stages of disease, does not help determining which are the initial factors that trigger the pathologic changes, since all these mechanisms are intertwined and potentiate each other. The absence of a satisfactory and comprehensive experimental model that could allow the recreation of the first stages of the disease makes it even harder to address the initial factors that lead to this condition.

In this study, a new approach is proposed, whereby the offspring of patients with systemic sclerosis is studied in an attempt to: a) minimize the genetic impact and b) allow the observation

of a population with an increased probability of developing the disease in a time frame that precedes in several years the known period for the disease onset.

Two disease control populations were used (Raynaud´s Disease and Type 2 Diabetes Mellitus) due to the fact that they share a common feature (RD) and have a similar pattern of microcirculatory changes in NVC (although not the same), previously to the clinical onset.

This thesis showed the presence of relevant microcirculatory changes in the offspring of patients with SSc, that had no symptoms or any other signs of the disease. Furthermore, the studied subjects had normal levels of different biomarkers associated with inflammation, immune activation, oxidative stress and endothelium dysfunction. Altogether, the data presented in this thesis suggest that there might be an important role for genetics as a factor for an anatomic microcirculatory disruption (and not for clinical development) and this might be the initial factor necessary for the subsequent development of the disease. However, the still low incidence of clinical features in the offspring of patients with SSc suggests that a second 'hit' might be needed for the disease onset.

Another aspect of this work regards the different findings in the RD population and their offspring. In this context, no anatomic changes were found in the offspring group, suggesting that despite the similar phenotype between Raynaud´s disease and the Raynaud´s phenomenon present in the patients with SSc, these are two different conditions with different mechanisms and risk factors.

Finally, the analysis of the T2DM groups identified a similar model of disease to SSc, where genetic factors play an important but not definitive role in the development of the disease, with the offspring of patients with diabetes and without any clinical or biologic evidence of disease, showing anatomic changes (although different from the ones found in SSc) in their microcirculation, evaluated by NVC.

The work presented in this thesis identifies what could be the initial changes necessary for Systemic Sclerosis to develop, suggesting the need for a second 'hit' to occur in order for the clinical phenotype to emerge. This new information provides the grounds for the potential definition of protocols that may identify early changes in specific biomarkers that would allow clinicians to anticipate the disease onset, paving the way to a more precise and timely treatment.

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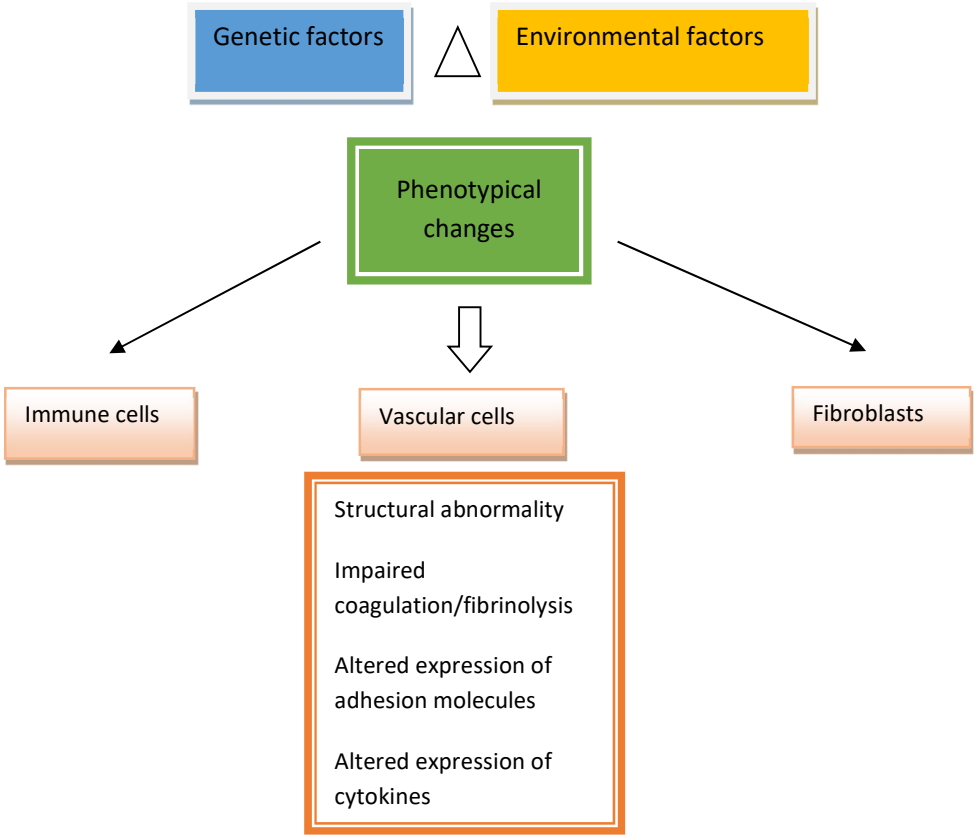

Adapted from (Asano, 2020)

Possibly we may have this

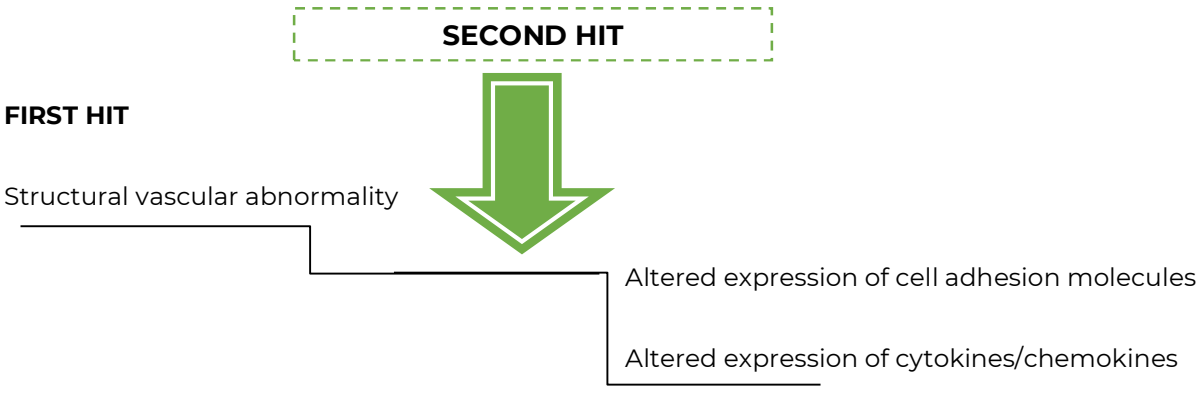

Figure 14. Hypothetical physiopathologic model for SSc.

V. FUTURE DIRECTIONS

This thesis opens up several and diverse research pathways:

1. Follow-up the Offspring with regular NVC and quantification of biomarkers (the ones already measured, and others like endothelin-1, Nox2, isoprostanes, NO, IL-6, PAI) in order to find out the 'second hit'.
2. Begin genetic studies, especially with pairs (parents - offspring) and try to associate genes to anatomic changes in microvasculature.
3. Use animal models which simulate the structural vascular abnormality and induce events to mimic 'second hits'.
4. Image processing in NVC in order to have more detail of changes in the vascular bed.

VI. SCIENTIFIC OUTPUT FROM THIS WORK

Articles

Marta C Amaral, F Seguro Paula, Joana Caetano, Paul RJ Ames, J Delgado Alves. Re-evaluation of nailfold capillaroscopy in discriminating primary from secondary Raynaud's phenomenon and in predicting systemic sclerosis: a randomised observational prospective cohort study. Expert Rev Clin Immunol. 2024 Mar 11:1-8. doi: 10.1080/1744666X.2024.2313642. Epub ahead of print. PMID: 38465507.

Marta C Amaral, Frederico Batista, Filipe Seguro Paula, João F Serôdio, Paul RJ Ames, José Delgado Alves. Type 2 diabetes mellitus' offspring have the same nailfold capillaroscopic pattern than their parents – an observational cross-sectional study (submitted to *Diabetologia*).

Abstract published

Marta C. Amaral, Filipe Seguro Paula, Frederico Batista, Joana Caetano, Susana Oliveira, Paul RJ Ames, José Delgado Alves. Systemic sclerosis' offspring have the same nailfold capillaroscopic pattern than their parents. Is there a vascular memory? Journal of Scleroderma and Related Disorders, 2024 Vol. 9 (IS)3-60; 379-80.

Presentations

Marta C. Amaral, Joana R. Batuca, João Serôdio, Frederico Batista, Filipe S. Paula, Paul R.J. Ames, J. Delgado Alves. "Vascular Memory" as a predictive factor for endothelium function in Diabetes Mellitus. 14ª Reunião Anual do NEDM, 1º Simpósio Ibérico de Medicina Interna/Diabetes, Vila Franca de Xira, Outubro 2019. Grant Helena Saldanha 2018, Boeringer Ingelheim.

Marta C. Amaral, Joana R. Batuca, João Serôdio, Frederico Batista, Filipe S. Paula, Paul R.J. Ames, J. Delgado Alves. Vascular Memory as a predictive factor for endothelium-function-associated conditions. Casual Friday with Science, Grey Building, Auditorium MMM, CEDOC, January 2020.

Poster

Marta C. Amaral, Filipe Seguro Paula, Frederico Batista, Joana Caetano, Susana Oliveira, Paul RJ Ames, José Delgado Alves. Systemic sclerosis' offspring have the same nailfold capillaroscopic pattern than their parents. Is there a vascular memory? 8th Systemic Sclerosis World Congress Prague (Czech Republic) - March 14-16, 2024.

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Grant Helena Saldanha/ Boeringer Ingelheim 2018, NEDM (Núcleo de Estudos de Diabetes Mellitus), SPMI.

Propina de Doutoramento SPMI 2023.

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