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POTENTIAL MOLECULAR TARGETS FOR MODULATING COLLAGEN TYPE I EXPRESSION IN SYSTEMIC SCLERODERMA

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BACHELOR'S DEGREE FINAL PROJECT

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THE PROJECT

The bachelor thesis allows undergraduate students to put into practice the knowledge acquired throughout the degree. As a biotechnology student, I consider that it is important to be able to translate the theory learnt in class to the benchwork. In line with this, this bachelor thesis was supposed to be an experimental project about the research performed during my internship in collaboration with Dr Vivek Malhotra's research group. One of their research lines is focused on revealing how bulky cargoes, such as collagens, are secreted. They found that TANGO1, a transmembrane protein located at the endoplasmic reticulum exit sites, is responsible for the export of collagens. Understanding the functional mechanism by which this protein operates is an arduous task that they are accomplishing day-by-day. Currently, the group is working on reducing extracellular collagen deposition by inhibiting TANGO1. To achieve this, Vivek Malhotra's research group has designed several peptides that are thought to inhibit the interactions between TANGO1 and the proteins involved in the export of collagens. According to this, my bachelor thesis was previously based on testing the effect of these inhibitors to reduce extracellular collagen accumulation *in vitro.* Unfortunately, due to the COVID-19 situation, my internship was suspended and thus the experiments planned could not be completed. Despite not being able to continue with this investigation, the idea of reducing extracellular collagen accumulation gave me the solution to proceed with my bachelor thesis outside the lab. Considering the results obtained after completing one of the experiments, I thought about its promising application in treating dermal fibrosis. Dermal fibrosis is a disease commonly caused by the excessive accumulation of collagen type I in the extracellular matrix. Therefore, this bachelor presents TANGO1 as a molecular target that modulates collagen I secretion by experimentally demonstrating the effect of the TANGO1 inhibitor P1 *in vitro*. Besides, to complement the experimental results obtained, this project also presents a literature review of molecular targets that modulate collagen I secretion and thus, may also ameliorate cutaneous fibrosis. For this, the articles selected must have a common background of study so results can be compared. Systemic scleroderma is an autoimmune disorder characterised by the development of cutaneous fibrosis, hence, the bibliographic search is performed using this disease as a common field of study.

In conclusion, this bachelor thesis is a combination of a bibliographic and an experimental project that aims to present potential molecular targets that modulate collagen I expression in systemic scleroderma to mitigate cutaneous fibrosis.

ABSTRACT

Scleroderma comprehends a group of autoimmune rheumatic diseases in which dermal fibrosis is the hallmark of the disease. Depending on which tissues are affected by the fibrotic process, scleroderma can be divided into two types. While localised scleroderma is the mildest form of the condition by only affecting the skin, systemic scleroderma is mainly characterised by the excessive production and accumulation of collagen in the dermis and internal organs. Although the mortality associated with this disease is caused by other clinical manifestations such as scleroderma renal crisis, dermal fibrosis affects considerably the patients' quality of life. In line with this, this bachelor thesis presents molecular targets that modulate collagen I expression to mitigate cutaneous fibrosis in systemic scleroderma. For this, a literary review is carried out by consulting three different databases and ten scientific articles are selected for further discussion. Besides, the bibliographic search is complemented with an experiment which assesses TANGO1 as a plausible molecular target by investigating the effect of the inhibitor P1 upon collagen I secretion *in vitro.* Finally, all targets presented in this project are discussed to lead us to a better understanding of the therapeutic application of these for treating cutaneous fibrosis in systemic scleroderma.

Keywords*: systemic scleroderma; molecular target; collagen type I.*

L'esclerodèrmia comprèn un grup de malalties reumàtiques autoimmunes on la fibrosi dèrmica és la característica principal de la malaltia. Depenent de quins teixits es veuen afectats pel procés fibròtic, la esclerodèrmia es pot dividir en dos tipus. Mentre que la esclerodèrmia localitzada és la forma més lleu de fibrosi, afectant només a la pell, la esclerodèrmia sistèmica es caracteritza principalment per l'excessiva producció i acumulació de col·lagen en la dermis i òrgans interns. Tot i que la mortalitat associada a aquesta malaltia és causada per altres manifestacions clíniques com la fibrosi pulmonar o la crisi renal esclerodèrmica, la fibrosi dèrmica afecta considerablement la qualitat de vida dels pacients. Seguint en aquesta línia, en aquest treball de fi de grau es presenten diferents dianes moleculars que modulen l'expressió de col·lagen I per tal de mitigar la fibrosi cutània en l'esclerodèrmia sistèmica. Per tal de realitzar aquest treball, es duu a terme una revisió bibliogràfica consultant tres bases de dades i deu articles científics son seleccionats per a una posterior discussió. A més, la cerca bibliogràfica es complementa amb un experiment *in vitro* on s'estudia TANGO1 com una potencial diana molecular investigant l'efecte de l'inhibidor P1 en la secreció de col·lagen I. Finalment, totes les dianes moleculars presentades en aquest treball son discutides per conduir-nos a una millor comprensió de les seves futures aplicacions terapèutiques pel tractament de la fibrosis dèrmica en l'esclerodèrmia sistèmica.

Paraules clau*: esclerosi sistèmica; diana molecular; col·lagen tipus I*

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1. INTRODUCTION: SCLERODERMA

Scleroderma is a chronic autoimmune disease characterized by the thickening and hardening of the skin due to a fibrotic process, which can lead to problems in internal organs such as the kidney, lungs or heart. Scleroderma can be divided into two types: systemic scleroderma and localised scleroderma (Rongioletti et al., 2018).

Adults are more commonly affected than children, being 45 years the average onset of the disease (Gelber et al., 2013). Although scleroderma has a worldwide incidence, several epidemiological studies have shown that ethnicity may play an important role in the severity of the disease. It has been suggested that African-Americans tend to have severer symptoms than Caucasians (Leitenberger et al., 2009). Statistically, localized scleroderma has an incidence of 3 cases per 100.000 individuals, while the incidence of systemic scleroderma varies globally from 8 to 56 cases per million. It is estimated that women are 4 times more affected than men for reasons that are still unknown (Ingegnoli et al., 2018), (Pope, 2017).

1.1. LOCALIZED SCLERODERMA

Localised scleroderma is commonly limited to the skin and underlying tissue. Although it doesn't have serious systemic consequences, it can cause morbidity as the skin and underlying tissues are affected by fibrotic processes. Localised scleroderma can be divided into several categories depending on their clinical diagnoses, nonetheless, symptoms and signs often overlap with each other. Therefore, localized scleroderma is generally grouped into two categories: plaque morphea and linear morphea (Bielsa, 2013).

- Plaque morphea: Characterised by the presence of round areas of indurated skin in no more than 2 different anatomical regions. When plaques of morphea appear in more than 2 distinct anatomical regions it is called generalized morphea.
- Linear morphea: Presented as a single lesion that has a linear distribution that commonly affects face and extremities.

1.2. SYSTEMIC SCLERODERMA

Systemic scleroderma, also known as systemic sclerosis (SSc), is the most acute form of scleroderma by not only affecting the skin but also internal organs. Gradually, patients go through a significant reduction of life quality as clinical manifestations progress with an uncertain outcome which might become lethal (Denton & Khanna, 2017).

The American Rheumatism Association (ARA) in conjunction with the European League Against Rheumatism (EULR), updated in 2013 the criteria to improve the classification of different subtypes of systemic scleroderma.

Thus, systemic scleroderma is classified into 3 different subtypes depending on the extension of the affected skin (Hoogen et al., 2013).

- Limited cutaneous SSc: this subtype of scleroderma only affects extremities such as the face, hands and arms. It was known as “CREST syndrome”, as there were common features with this condition such as Raynaud phenomenon, oesophageal motility dysfunction, sclerodactyly, calcinosis and telangiectasia.
- Diffuse cutaneous SSc: is characterised by the involvement of internal organs as well as the thickening of the skin in large areas, such as the trunk or extremities.
- Sine SSc: in this case, internal organs are affected by a fibrotic process but there is no apparent cutaneous involvement.

1.2.1. Clinical Manifestations

Raynaud phenomenon is the most common manifestation of systemic sclerosis, present in more than 95% of patients, whose symptomatology includes cyanosis and signs of ischemic damage to the fingers (Ranque & Mouthon, 2010).

Skin fibrosis is the hallmark of the disease, leading to ulcerations and puffy hands, moreover, internal organs such as lungs and heart can also be affected by a fibrotic process. Indeed, pulmonary fibrosis is present in 80% of the patients and represents the main cause of death along with pulmonary hypertension. Besides this, systemic sclerosis can also be associated with arrhythmias and myocardial disease (Rubio-Rivas et al., 2014).

1.2.2. Pathogenesis

Even though the driving events of the pathogenic process of systemic scleroderma remain elusive, it is known that a combination of environmental and genetic factors may trigger the pathogenesis of the disease (Sierra-Sepúlveda et al., 2019). Silica dust, infectious agents and drugs are thought to be the most related substances at the beginning of the pathogenesis in systemic sclerosis (Asano, 2018). The disease seems to start with the development of vasculopathy and autoimmunity, followed by tissue fibrosis in multiple organs (Yanaba, 2016). Therefore, to define SSc just as a fibrotic disease would be an oversimplification.

The pathogenesis of systemic scleroderma (Figure 1) seems to start with the damage of endothelial cells followed by a disrupted repair process, thus activating a chronic inflammatory response (Asano, 2018). Vascular damage appears as the earliest event in systemic sclerosis, resulting in tissue hypoxia and oxidative stress. Vascular damage is induced by microvascular injury and endothelial cell activation. Besides, endothelial cell activation increases the expression of cell adhesion molecules such as intracellular adhesion molecule (ICAM), resulting in the infiltration of inflammatory cells into the perivascular areas (Allanore et al., 2015). Such inflammatory cells are macrophages, B cells, dendritic cells and lymphocytes T-CD4$^+$. Among T-CD4$^+$ lymphocytes, two subgroups are highly present on systemic

scleroderma, T helper 2 (Th2) and T helper 17 (Th17). It has been demonstrated that Th2 secrete cytokines IL-4, IL-5, IL-10 and IL-13, which stimulate the synthesis of collagen in dermal fibroblasts. Interestingly, IL-13 can also stimulate tumour growth factor-β (TGF-β) which regulates cell adhesion and chemokine gradient to recruit leukocytes and promote fibroblasts activation. Besides, TGF-β is also a potent inducer of collagen synthesis (Asano, 2018).

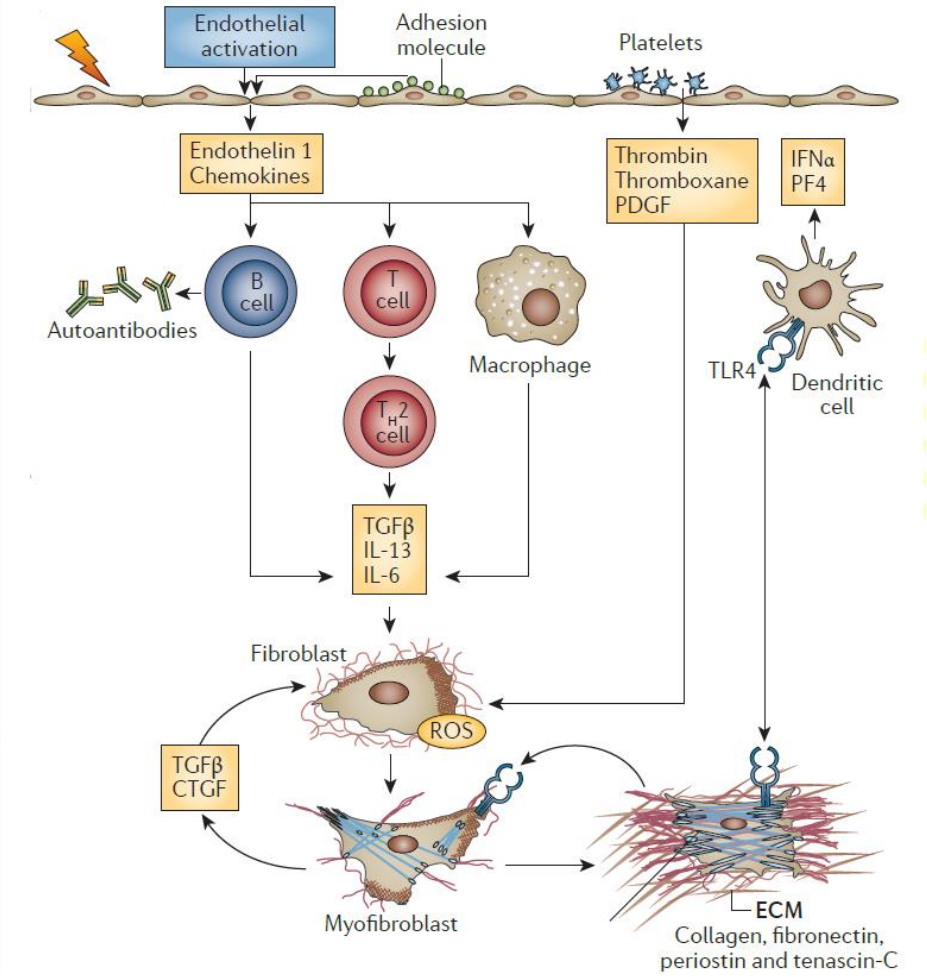

Figure 1. Schematic representation of the pathophysiology in systemic scleroderma. Tissue injury triggers endothelial cell activation, which stimulates the production of endothelin-1, fibrotic chemokines, adhesion molecules and platelet activation. Inflammatory cells are recruited by adhesion molecules and chemokines. Among these inflammatory cells, we can find Th2 cells which secrete TGF-β and IL-13 and B cells which secrete autoantibodies and IL-6. The secretion of pro-fibrotic and pro-inflammatory cytokines activates fibroblasts, resulting in the secretion of ROS species and differentiation into myofibroblasts. Finally, myofibroblasts produce excessive ECM causing fibrosis of the tissue. The synthesis of ECM is aggravated by the activation of Toll-like receptors 4 (TLR4) on dendritic cells and myofibroblasts through positive feedback (Allanore et al., 2015).

Moreover, Th2 cytokines enhance the production of immunoglobulins and IL-6 of B cells. The dysregulation of the immune response in systemic scleroderma leads to chronic production of autoantibodies which are also used as clinical markers: anti-topoisomerase 1, anticentromere and anti-RNA polymerase III (Yanaba, 2016).

On the other hand, the release of pro-fibrotic and pro-inflammatory cytokines induces the activation of fibroblasts. The persistent activation of fibroblast increases the generation of reactive oxygen species (ROS) species and the extracellular matrix (ECM) proteins (Allanore et al., 2015).

SKIN FIBROSIS IN SYSTEMIC SCLERODERMA

Dermal fibrosis or cutaneous fibrosis is not considered a life-threatening symptom of systemic scleroderma. However, it has a significant impact on patients' life quality as well as on their understanding of the disease. It may also induce severe complications in the musculoskeletal system, generating musculoskeletal pain, subcutaneous calcinosis, joint contractures and tendon friction rubs (Denton & Khanna, 2017).

The ECM comprehends non-cellular components of tissues that bind cells together, playing a pivotal role in different cellular activities such as differentiation, migration and proliferation (Kular et al., 2014). In this line, cutaneous fibrosis is defined as the accumulation of ECM components in the dermis due to a dysregulation of the synthesis and degradation of the ECM (Figure 2). Activated fibroblasts and myofibroblasts are the main cells synthesizing ECM and its origin can be diverse. In some cases, fibroblasts can be activated driving fibroblasts proliferation, but transdifferentiation into myofibroblasts may also occur (Asano, 2018).

Collagen type I is the main component of the mammalian ECM in the skin. It consists of two chains, α1 and α2, which form a triple helix, creating a protein of approximately 275nm long. Each of these chains is encoded by the genes *COL1A1* and *COL1A2* (Henriksen & Karsdal, 2019). Even though it has been demonstrated that TGF-β is the principal inducer of collagen synthesis, there are several signalling pathways induced by TGF-β that promote collagen type I synthesis. The TGF-β/Smad signalling triggers the phosphorylation of Smad2 and Smad3 proteins. After these proteins are activated, they associate with Smad4 creating a complex which is translocated to the nucleus. The complex regulates the transcription of the targeted collagen genes (Jinnin, 2010). Nonetheless, collagen synthesis may also

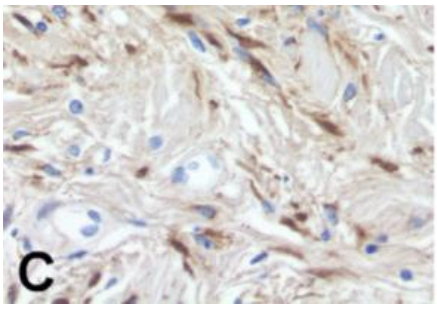

Figure 2. Histopathology sample of the skin in systemic sclerosis. The dermis is characterized by broad sclerotic collagen bundles that replace the subcutaneous tissue. Procollagen I stained with dark brown fibrocytes and light brown immature background collagen matrix (Cowper et al., 2008).

be induced by other TGF-β Smad independent pathways, such as p38 MAPK, ERK/MAPK and PI13K/AKT (Van Caam et al., 2018).

Interestingly, pro-inflammatory cytokines as IL-6 and IL-4 are also implicated in the induction of collagen type I synthesis (Varga, 2017). On the other hand, these inflammatory cytokines can promote the degradation of matrix metalloproteinases (MMPs), which are responsible for ECM degradation. Supporting this finding, recent studies affirm that IL-4 and IL-10 are highly expressed in systemic scleroderma patients and inhibit MMP expression. Therefore, the dysregulation of tissue homeostasis drives to ECM accumulation that impairs tissue architecture and causes loss of organ function (Do & Eming, 2016).

1.2.3. Treatments

Unfortunately, no treatment can reverse systemic scleroderma, nonetheless, current therapies have the aim of improving quality of life by minimising specific organ involvement. Among the clinically available treatments available, methotrexate has been recommended for treating skin manifestations (Chung & Chung, 2020). On the other hand, mycophenolate mofetil has also shown promising results in ameliorating skin thickness, however, slight gastrointestinal-associated adverse effects are sometimes a limitation of this treatment (Omair et al., 2015). Some clinicians may suggest a therapy based immunosuppressive agents such as nintedanib, which mitigates dermal thickness. Combination of conventional and novel treatments as well as occupational therapies are also suggested by experts to improve musculoskeletal strength and help to reduce associated pain. Sometimes, surgery may be also a necessary option for severe complications (Kowal-Bielecka et al., 2017).

2. HYPOTHESIS

Under the hypothesis that fibrosis in the skin and internal organs is due to excessive collagen accumulation, the primary approach in our research is based on reducing collagen deposition. Collagen type I is the most abundant collagen expressed on vertebrates and it is mainly found in skin, bones and connective tissue (Henriksen & Karsdal, 2019). Therefore, for the scientific community it seems to be essential to reveal whether modifying collagen type I gene and protein expression may ameliorate cutaneous fibrosis. In this project, we hypothesize that molecular targets that reduce collagen I mRNA and protein expression in systemic scleroderma may also ameliorate cutaneous fibrosis.

3. AIM OF THE PROJECT

As mentioned before, cutaneous fibrosis in systemic scleroderma is characterised by the excessive deposition of collagen type I, thus it is interesting to investigate how collagen type I expression can be modulated to reduce its accumulation.

This project presents a literature review about molecular targets that modulate collagen I expression in systemic scleroderma. For this, 3 databases will be consulted and the articles will be selected according to the inclusion and exclusion criteria. Besides, this bachelor thesis also assesses TANGO1 as a plausible molecular target by experimentally inhibiting collagen I secretion *in vitro.* Therefore, this bachelor thesis has two aims:

1. To present molecular targets that modify collagen type I expression *in vitro* and *in vivo* in systemic scleroderma by carrying out literary research.
2. To validate TANGO1 as a possible molecular target for treating cutaneous fibrosis by testing the efficiency of the inhibitor P1 *in vitro* upon collagen I secretion.

4. MATERIALS AND METHODS

Considering the aims proposed for this bachelor thesis, two procedures are performed in this project. On one hand, the bibliographic research is carried out in three databases to present potential molecular targets that modulate collagen type I gene and protein expression *in vitro* as well as *in vivo*. On the other hand, the effect of the TANGO1 inhibitor P1 is tested *in vitro* to modulate collagen I secretion.

4.1. Bibliographic research

The literary review has been accomplished by consulting three databases: PubMed, Web of Science and Scopus. The words used for carrying out this research have been obtained by a previous search on MeSH thesaurus (Table 1). Finally, taken into account the inclusion and exclusion criteria established, 10 articles have been selected for this project. A schematic representation of the searching process is shown in figure 3.

Table 1. MeSH terms obtained on NCBI.

Keywords	MeSH terms
Collagen I	Collagen type I Procollagen type I
Molecular target	Molecular target Targeted therapy
Scleroderma	Systemic scleroderma Systemic sclerosis

4.1.1. INCLUSION AND EXCLUSION CRITERIA

The inclusion and exclusion criteria have been decided considering the aim of this project. The searching parameters used for this literary research are publication date of the articles, types of experiments performed, relevant data upon collagen type I expression and type of cells.

INCLUSION CRITERIA:

- Publication date: articles published between 2015 and 2020 have been selected.
- Experiments: selected articles include *in vivo* and *in vitro* experiments.
- Relevant data: articles considered for this project present in their results the modulation of collagen type I expression.
- Cell type: human dermal fibroblasts are the type of cells used for performing the experiments in the articles selected.

EXCLUSION CRITERIA:

- Type of publication: publications such as reviews, abstracts and clinical trials have been excluded, therefore, only articles have been selected.
- Databases results: duplicated results among the different databases have been discarded.

4.1.2. LITERATURE SEARCH STRATEGY

Once the inclusion and exclusion criteria have been established, a search strategy has been performed on the 12th of July. For this project 3 databases have been consulted: PubMed, Scopus and Web of Science. The search strategy used in each of these databases is presented in table 2.

PUBMED

The literary research strategy followed has reduced the number of articles obtained from 25.699 to 39 by using the advanced search tool. After reading the titles and abstracts, 21 articles were selected for further use. Finally, 3 articles were selected for this project after discarding 9 repeated articles and applying the inclusion and exclusion criteria.

WEB OF SCIENCE

Searching systemic scleroderma as a topic retrieved 69.975 results within all databases included in Web of Science. 68 articles were obtained after applying the strategy search using the basic search tool. After reading the titles and abstracts and subsequently applying the inclusion and exclusion criteria, 1 article was selected among the 11 candidates.

SCOPUS

The search started with 38.503 results, which were reduced to 46 after applying the search strategy presented in table 2. Subsequently, 22 articles were obtained by reading the titles and abstracts. This literary search was performed using the basic search tool and 9 repeated articles were found. Finally, 6 articles were chosen for this project considering the inclusion and exclusion criteria previously mentioned.

Table 2. The search strategy used in each database.

DATABASE	SEARCH STRATEGY
PUBMED	((((systemic scleroderma OR systemic sclerosis) AND (skin fibrosis OR dermal fibrosis)) AND (collagen type 1 OR procollagen type 1)) AND (("2015"[Date - Publication] : "3000"[Date - Publication]))) NOT (review[Publication Type] OR clinical trial[Publication Type] OR multicenter study[Publication Type])
SCOPUS	(TITLE-ABS-KEY ((systemic AND sclerosis) OR (systemic AND scleroderma)) AND TITLE-ABS-KEY ((“skin fibrosis”) OR (“dermal fibrosis”)) AND TITLE-ABS-KEY ((collagen AND type 1) OR (procollagen AND type I)) AND ALL (molecular AND target) OR (novel AND target) OR (targeted AND therapy)) AND DOCTYPE (ar) AND PUBYEAR > 2014
WEB OF SCIENCE	TOPIC: (systemic scleroderma OR systemic sclerosis) AND TOPIC: (collagen type I OR procollagen type I) AND TOPIC: (skin fibrosis OR dermal fibrosis) AND TOPIC: (molecular target) TYPE OF DOCUMENT: (ARTICLE) TIME PERIOD: 2015-2020 DATABASES: WOS, CCC, DIIDW, KJD, MEDLINE, RSCI, SCIELO

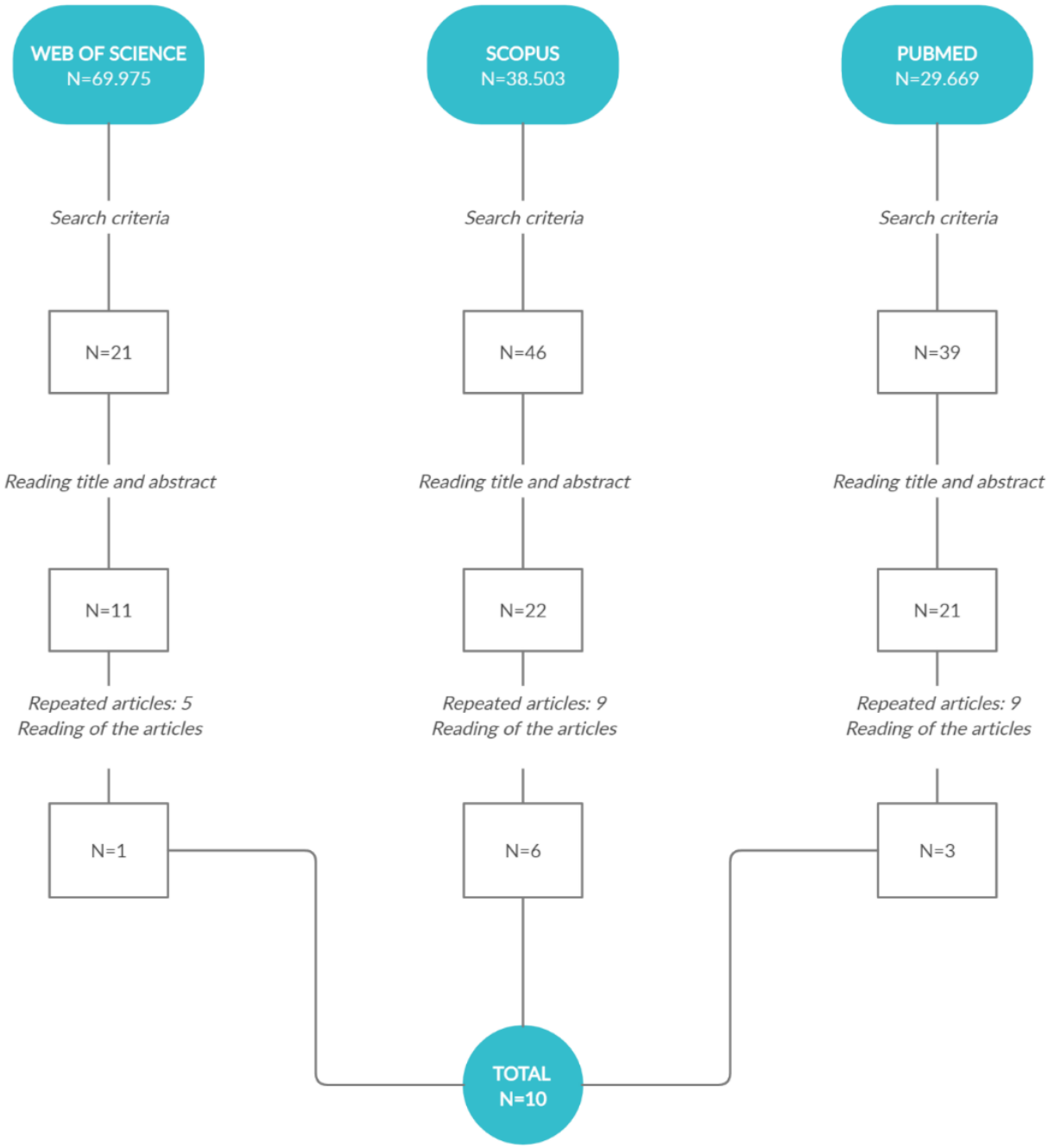

Figure 3. Representation of the literary research performed. Firstly, the initial numbers of the articles obtained by searching “systemic scleroderma” or “systemic sclerosis” are represented on top. Secondly, the search criteria used for the bibliographic search, search strategy as well as the inclusion and exclusion criteria, reduce the number of articles obtained in the first place. Subsequently, an easy read of the titles and abstracts allows discarding repeated and unrelated articles to this project. Finally, the most relevant articles for this project are selected after fully reading the plausible candidates and applying the inclusion and exclusion criteria again. N: number of articles

4.2. Experiment: TANGO1 protein

Considering that the excessive deposition of collagen type I is the main cause of dermal fibrosis, it appears interesting to understand how this collagen is synthesised and secreted to mitigate its excessive extracellular accumulation. It has been demonstrated that TANGO1, a transmembrane protein located at the endoplasmic reticulum exit sites (ERES), is responsible for the export of these bulky cargoes (Annexed figure 1S), (Raote et al., 2018). Vivek Malhotra's research group has focused on investigating how collagen type I secretion can be modulated by targeting TANGO1 protein. For this, they designed several peptides, among them, the inhibitor P1, which is thought to inhibit an interaction between TANGO1 and TALI (The functional mechanism and TANGO1 family proteins are annexed on extended data 2). Thus, the following experiment, carried out in collaboration with Dr Vivek Malhotra's research group at the Centre for Genomic Regulation, aims to modulate collagen I secretion by using TANGO1 inhibitor P1 *in vitro.*

4.2.1. Cell Culture and Counting

The cell line used for this experiment was U-2OS (obtained from the eukaryotic cell line collection maintained by the Centre for Genomic Regulation, Spain). These cells are osteoblasts derived from human bone osteosarcoma which overexpress collagen type I.

Cells were grown at 37ºC with 5% CO_2 in a complete Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher) with 10% FBS (Fetal Bovine Serum) in a T-75 flask. For the experiment, cells were counted using a Neubauer chamber, thus 750.000 U-2OS cells/well were seeded in a 6-well plate. Finally, cells were grown on this plate at 37ºC with 5% CO_2 in a complete Dulbecco's modified Eagle's medium with 10% FBS.

4.2.2. Collagen Type I Secretion Assay

To compare the effect of the inhibitor P1 upon collagen secretion, it is necessary to obtain the collagen I content on cell lysates and media. For this, a secretion assay allows us to separate the intracellular content from the media secreted (Complete protocol on extended data 1). Firstly, the medium was replaced with fresh Opti-MEM reduced serum media (Gibco; Thermo Fisher) and a solution of ascorbic acid (250mM ascorbic acid and 1M phosphor ascorbate) was added subsequently for inducing collagen synthesis. The secretion assay was performed in duplicates using MiliQ water as control and the inhibitor P1. Cells were stored at 37ºC with 5% CO_2 for 4 hours before collecting the medium. Afterwards, cells were placed on ice and 750µL of media were collected to be centrifugated at 2.000rpm at 4ºC for 6 minutes. The remaining media was removed, and cells were rapidly incubated with lysis buffer for 20 minutes on ice. Cell lysates were then obtained by collecting 500µL which were further centrifugated at 16.000rpm for 20 minutes at 4ºC. After completing these centrifugations, two samples of 200µL were collected from lysates and media supernatants to be stored at -80ºC.

4.2.3. Western Blot

For visualizing the effect of the inhibitor P1 upon collagen I secretion, it is necessary to perform SDS-PAGE electrophoresis followed by a western blot. The percentage of acrylamide for the gel is determined by the size of the targeted protein. In this case, given that collagen I weights 225kDa approximately, 8% acrylamide gels with a width of 1.5mm were prepared. An equal amount of cell lysate and medium samples (50 µL) were subjected to electrophoresis in Trys-glycine 1X buffer at 100V for 1.5h. Two protein ladders were used: High-Range Spectra (Thermo Fishier) and NYZColour Protein Marker (NYZTech). Afterwards, the western blot allowed us to visualize the protein of interest by antibody binding. For this, proteins separated in polyacrylamide gels were transferred to nitrocellulose membranes at 80V for 3h in protein transfer buffer 10X. Subsequently, membranes were blocked for 1h using 5% skimmed milk in PBS. To detect the desired proteins, anti-collagen type I (1:1000; Abcam) and anti-β-tubulin (1:10000; Abcam) primary antibodies were used. The ladder as loading and lysing control. Membranes were incubated with their primary antibodies overnight at 4ºC and continuous shaking. Primary antibodies were recovered afterwards, and membranes were washed 5 times with PBS-T under constant agitation for 5 minutes. Membranes were incubated with their respectively secondary fluorescent antibodies (Table 3) for 1h in the dark. Finally, membranes were washed again 5 times with PBS-T in constant shaking for 5 minutes and protein bands were visualized using an imager scanner (LiCor Odyssey 9120).

Table 3. List of primary and secondary antibodies used for western blotting.

Protein of interest and size	Primary antibody; source; reference	Dilution	Secondary antibody; dilution; reference
Collagen Type I (220kDa)	Anti-collagen type I; Rabbit; ab260043 (Abcam)	1:1000 in 2.5% BSA	Anti-Rabbit IgG; 1:20000 in PBS-T; Alexa Fluor ab175773 (Abcam)
β-Tubulin (55kDa)	Anti-β-Tubulin; Mouse; ab231082 (Abcam)	1:10000 in 2.5% BSA	Anti-Mouse IgG; 1:20000 in PBS-T; Alexa Fluor 680 ab175775 (Abcam)

4.2.4. Statistical analysis

Protein bands width was measured using ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). This software allowed us to obtain the respective areas of each protein band. These areas were further used for calculating the ratio of intracellular and extracellular collagen I. Lastly, Xlstat software (Addisfot, Paris, France) was used to analyse data and unpaired *t*-test was used for statistical analysis. Data were normalized to protein control β-tubulin. Results are expressed as the mean ± SD (Standard deviation). $P<0.05$ was considered statistically significant.

5. RESULTS

5.1. Molecular targets of SSc

Following the research strategy mentioned above, 10 articles were selected for this literature review. The literature research process is represented in figure 3. All articles have been chosen accordingly to the inclusion and exclusion criteria. A summary of each article with its respectively molecular target is mentioned in tables 4 and 5.

5.1.1. Micro RNA

Several micro RNA (miRNA) have an altered expression in systemic scleroderma (SSc). Luo et al., 2015 propose miRNA130b as a molecular target which is upregulated in SSc dermal fibroblasts. Interestingly, a conserved seed sequence of a nuclear hormone receptor was found to be complementary to miRNA130b. This nuclear hormone receptor was the peroxisome proliferator-activated receptor gamma (PPAR-γ), which was found to be downregulated by miRNA130b. The upregulation of miRNA130b was related to an increased expression of collagen type I and a decreased expression of PPAR-γ. *In vitro* experiments demonstrated that using miRNA130b inhibitors increased the expression of PPAR-γ whereas the expression of type I collagen was decreased among other fibrotic proteins.

5.1.2. Receptors

Extracellular and intracellular receptors have been identified as targets for treating skin fibrosis in SSc. Purinergic P2 receptors are a class of plasma membrane receptors that bind specifically to ATP. Perera et al., 2019 show that ATP is enhanced in SSc fibroblasts and it can act as a damage-associated molecular pattern molecule by binding to the purinergic receptor $P2Y_2$. *In vitro* experiments demonstrated that blocking the $P2Y_2$ receptor with an antagonist decreased the production of type I collagen. Moreover, it is known that IL-6 increases the production of collagen type I. In their article it is suggested that binding of ATP may induce the phosphorylation of p38 protein kinases via $P2Y_2$. The activation of these protein kinases may, in turn, enhance the production of IL-6 and consequently, the production of collagen type I.

5.1.3. Cytokines

The persistent mechanism used by cytokines to activate fibroblasts in SSc may be considered an important approach for treating this disease. Kudo et al., 2015 present EBI3 as a molecular target for mitigating skin fibrosis. They demonstrate that EBI3, a subunit of IL-35, can downregulate protein and mRNA expression of collagen type I *in vitro* and *in vivo* on SSc dermal fibroblasts. The authors suggested that the decrease in collagen expression may be related to a decreased stability of collagen type I mRNA. The results obtained in their

experiments led to hypothesize that the stability of the mRNA might be perturbed by miRNA-4500.

On the other hand, CX_3CL_1, also known as fractalkine, is a soluble cytokine that exerts a chemoattractant activity by binding to its receptor CX_3CR_1. The receptor CX_3CR_1 is expressed on the surface of a diverse type of cells such as macrophages and T cells. Elevated levels of CX_3CL_1 have been reported in SSc patients. Vu H. Luong et al., 2019 propose the axis CX_3CL_1/CX_3CR_1 as a potential target for treating skin fibrosis on SSc. In their study, the use of an anti-CX_3CL_1 monoclonal antibody (mAb) reduced significantly the number of infiltrating cells as well as collagen I accumulation. Besides, constitutive phosphorylation of Smad2 and Smad3 proteins have been found in SSc patients. The activation of these Smad proteins is related to the overexpression of fibrotic genes such as collagen I. Their experiments reveal that anti-CX_3CL_1 mAb decreased collagen type I accumulation by inhibiting the phosphorylation of Smad3.

5.1.4. Transcription factors

Excessive levels of type I interferons (INF1) are found on the skin of SSc patients. It is known that interferon regulatory factor 7 (IRF7) regulates INF type I signalling in a positive feedback loop. *In vivo* and *in vitro* experiments performed by Wu et al., 2019 show a decrease in INF-α and collagen I accumulation by using siRNA IRF7. Of notice, their study demonstrates that IRF7 interacts with Smad3, a key component of the TGF-β/Smad-dependent pathway, which induces the expression of fibrotic genes such as *COL1α2.* Interestingly, Vu Huy Luong et al., 2018 also consider the TGF-β/Smad-dependent pathway as an attractive target for treating SSc. In their study, the transcription factor Smad3 is presented as the targeted protein. The inhibition of Smad3 phosphorylation using the histidine-pyridine-histidine ligand 15 (HPH-15) reduced the expression of *COL1α2* gene as well as its protein expression. Similar results were obtained *in vivo* by treating a bleomycin-induced mouse with HPH-15.

5.1.5. Enzymes

Sirtuin type1 (SIRT1), a class of histone deacetylases, has been reported to be downregulated in human SSc dermal fibroblasts. Wei et al., 2015 propose SIRT1 as a molecular target whose activation reduced collagen I deposition. In their study, the authors demonstrate that SIRT1 negatively regulates the acetyltransferase p300. *In* vitro and *in* vivo experiments performed using an activator of SIRT1 showed an increased expression of SIRT1 while cellular levels of p300 were decreased. These results translated into a reduction of collagen I accumulation and amelioration of cutaneous fibrosis.

On the other hand, the soluble guanylate cyclase (sGC) is an intracellular enzyme which is proposed as a target for treating skin fibrosis in SSc by Beyer et al., 2015**.** The authors showed in their study that stimulating sGC interfered with the ERK/MAPK signalling pathway. Their

experiments demonstrated a significant decrease in collagen type I release by stimulating sGC *in vitro* and *in vivo.*

Other authors such as Dosoki et al., 2017 suggest targeting the enzyme NOX4 as oxidative stress plays an important role in the pathogenesis of SSc. Dosoki et al., 2017 and Morry et al., 2015 demonstrated that inhibiting NOX4 decreased collagen type I expression in human SSc dermal fibroblasts and a bleomycin-induced mouse. Furthermore, in Morry et al., 2015 research, heat-shock protein 47 (HSP47) is also proposed as another target for treating skin fibrosis. HSP47 is a collagen-specific molecular chaperone that ensures the proper assembly of procollagen during its secretion. Elevated levels of HSP47 have been found in the serum of SSc patients. Therefore, it might be an interesting target for reducing collagen expression. In this study, the authors demonstrate that silencing HSP47 *in vitro* and *in vivo* downregulate protein expression of collagen type I.

5.2. Inhibition of TANGO1 *in vitro*

To assess the ability of TANGO1 inhibitor P1 on reducing extracellular collagen I deposition *in vitro*, it is necessary to compare the intracellular and extracellular deposition of collagen on cells. For this, a secretion assay was performed and collagen I accumulation was detected by western blotting. As it was expected, the results show that extracellular deposition of collagen I was significantly reduced by the addition of the inhibitor P1 when compared to control in U-2OS cells. On the other hand, the inhibitor had no significant effect on intracellular collagen deposition (Figure 4). Cell lysis was confirmed by the absence of β-tubulin band on the media (Figure 5).

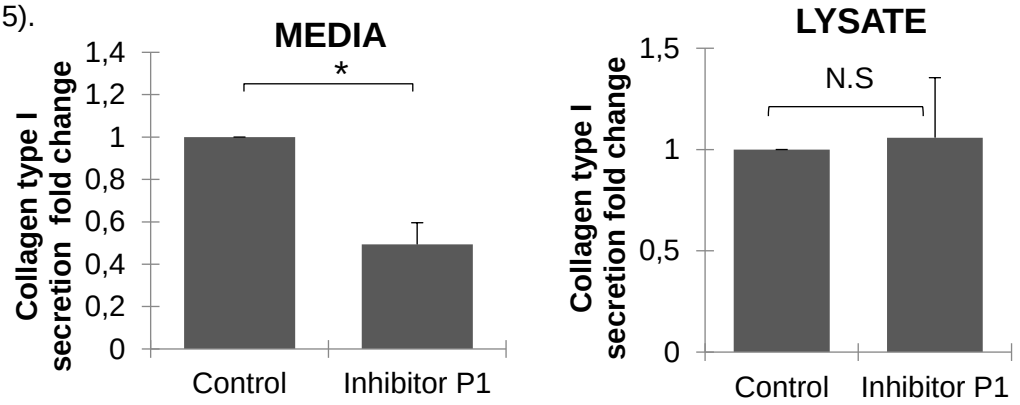

Figure 5. Western blot results showed a statistically significant reduction (*P<0.05) in extracellular collagen I accumulation by TANGO1 inhibitor P1 compared to control (Media). No significant difference (N.S) was found in intracellular collagen I accumulation (Lysate). Data are presented as the mean ± SD (Standard deviation) represented in error bars (n=2).

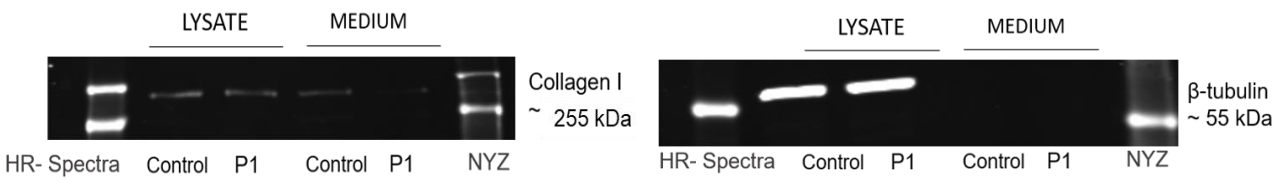

Figure 4. Bands obtained by western blot show a decrease of collagen I accumulation in the media but not in the lysate (Left). β-tubulin is used as a loading and lysing control (Right).

Table 4. Summary of the *in vitro* experiments performed using human dermal fibroblasts. The table lists the articles with its molecular targets as well as the modulation of collagen type I mRNA and protein expression. The signalling pathway abrogated by the activation or inhibition of each target is shown. N.D: No data.

IN VITRO						
Reference	**Human dermal fibroblasts Source**	**Type of target**	**Molecular target**	**Collagen type I mRNA expression**	**Collagen type I protein expression**	**Targeted TGF-β signalling pathway**
Kudo et al., 2015	Patients	Cytokine	EBI3	Reduced	Reduced	Smad-dependent
Vu H. Luong et al., 2019	Neonatal human dermal fibroblasts (Kurabo Industries, Osaka, Japan)	Cytokine	CX_3CL_1	N.D	Reduced	Smad-dependent
Wu et al., 2019	Patients	Cytokine	IRF7	Reduced	Reduced	Smad-dependent
Beyer et al., 2015	Patients	Enzyme	sGC	Reduced	Reduced	Smad-independent
Dosoki et al., 2017	Patients	Enzyme	NOX4	Reduced	N.D	Smad-dependent
Wei et al., 2015	Patients	Enzyme	SIRT1	Reduced	Reduced	Smad-dependent
Luo et al., 2015	Patients	miRNA	miRNA130b	Reduced	Reduced	Smad-dependent
Morry et al., 2015	Adult human dermal fibroblast cell line (HDFa, Life Technologies, Carlsbad, CA)	miRNA and Enzyme	NOX4 and HSP47	Reduced	Reduced	Smad-dependent
Perera et al., 2019	Patients	Receptor	$P2Y_2$	Reduced	Reduced	Smad-independent
Vu Huy Luong et al., 2018	Neonatal human dermal fibroblasts (Kurabo Industries, Osaka, Japan)	Transcription factor	Smad3	Reduced	Reduced	Smad-dependent

Table 5. Summary of the *in vivo* experiments performed using mouse models. The table lists the articles with its molecular targets as well as the modulation of collagen type I mRNA and protein expression. The inhibitors and activators that modulate collagen I expression in each article are also shown.
ND: No Data

IN VIVO						
Reference	**Mouse model**	**Molecular target**	**Target**	**Collagen type I mRNA expression**	**Collagen type I protein expression**	**Inhibitor/Activator of the target**
Kudo et al., 2015	Bleomycin-induced	Cytokine	EBI3	Reduced	Reduced	Activation by supplementation of EBI3
Vu H. Luong et al., 2019	Bleomycin-induced	Cytokine	CX_3CL_1	N.D	Reduced	Inhibition by anti- CX_3CL_1 mAb
Beyer et al., 2015	TGF-β driven	Enzyme	sGC	N.D	Reduced	Activation by BAY 41-2272
Dosoki et al., 2017	Bleomycin-induced	Enzyme	NOX4	Reduced	Reduced	Inhibition by DPI and siRNA NOX4
Morry et al., 2015	Bleomycin-induced	Enzyme and chaperone	NOX4 and HSP47	Reduced	Reduced	Inhibition by siHSP47-MSN-PEI-PEG
Wei et al., 2015	Bleomycin-induced	Histone deacetylase	SIRT1	N.D	Reduced	Activation by resveratrol
Luo et al., 2015	Bleomycin-induced	miRNA	miRNA130b	N.D	N.D	Inhibition by miRNA130b inhibitor
Perera et al., 2019	Bleomycin-induced	Receptor	$P2Y_2$	N.D	N.D	$P2Y_2$ receptor antagonist
Vu Huy Luong et al., 2018	Bleomycin-induced	Transcription factor	Smad3	N.D	Reduced	Inhibition by HPH-15
Wu et al., 2019	Bleomycin-induced and Tsk/IRF7 KO double congenic	Transcription factor	IRF7	Reduced	Reduced	Inhibition by IRF7 siRNA

6. DISCUSSION

Systemic scleroderma (SSc) is described as an autoimmune disease characterised by the development of fibrosis in the skin and internal organs. Indeed, skin fibrosis is the hallmark of systemic scleroderma mainly caused by the overexpression of collagen type I, thus it is interesting to investigate how its expression can be modulated to ameliorate dermal fibrosis. In this bachelor project, a systematic review has been performed to obtain and dissect different articles which present molecular targets that modulate collagen type I expression in systemic scleroderma, and consequently, may mitigate cutaneous fibrosis. A total of 10 articles have been selected accordingly to the inclusion and exclusion criteria established.

A chronic inflammatory response is thought to precede the development of fibrosis in systemic scleroderma. Hence, it is not surprising that several molecular targets presented in this project have key roles in modulating the inflammatory cascade. As an example, EBI3 and CX_3CL_1 are pro-inflammatory cytokines which are presented as molecular targets because their downregulation reduces collagen I expression *in vitro* as well as *in vivo*. On the other hand, the persistent activation of fibroblasts is also a crucial step in the development of fibrosis as the continuous generation of reactive oxygen species (ROS) allows the differentiation of fibroblasts into myofibroblasts. In line with this, NOX4, an enzyme that reduces oxygen to hydrogen peroxide, is presented in two of the articles selected as another potential target.

Interestingly, most of the molecular targets already proposed in other investigations for treating fibrosis are predominately extracellular molecules, as they are more accessible for drug therapy (Li et al., 2017). In contrast with these findings, the targets presented in this project are mostly intracellular. Indeed, $P2Y_2$ is a plasma membrane receptor and is the only extracellular target proposed in this bachelor thesis. Moreover, G-couple receptors, which account for approximately 34% FDA-approved drug targets, have neither been obtained in this bibliographic search (Hauser et al., 2018).

Of note, all targets obtained from the bibliographic search have significantly reduced collagen type I mRNA and protein expression *in vitro*. Indeed, the molecular targets presented have the aim of downregulating collagen I synthesis except for the collagen-specific chaperon HSP47. This protein is located at the endoplasmic reticulum (ER) and Morry et al., 2015 propose HSP47 as a molecular target which modulates collagen I secretion. It has been found that HSP47 interacts with TANGO1 allowing the export of collagens from the ER to Golgi (Ito & Nagata, 2019). In this bachelor thesis, we have hypothesized that inhibiting TANGO1 might reduce extracellular collagen I deposition. The data obtained from our experiment reveal that TANGO1 may be a promising molecular target in fibrosis as results show that the inhibitor P1 reduces extracellular collagen I deposition *in vitro* (Figure 4). However, we cannot state that

TANGO1 is a promising target for specifically treating dermal fibrosis in systemic scleroderma as the experiments have been performed using osteoblasts instead of dermal fibroblasts. Besides, the specificity of the inhibitor cannot be assured by the experiment performed and it cannot be confirmed that the inhibitor P1 is exerting its activity on TANGO1. It is worth to mention that despite these limitations, the experiment has fulfilled its purpose as extracellular collagen I deposition has been reduced by inhibiting TANGO1. Furthermore, the fact that HSP47 and TANGO1 are involved in collagen secretion as well as in their assembly leads us to suggest that they might be promising targets for treating not only fibrosis but also misfolding collagen diseases.

On the other hand, all *in vitro* experiments presented in the articles selected have been performed using human dermal fibroblasts, most of them were obtained from skin biopsies samples of patients presenting SSc. Nonetheless, some authors induced TGF-β in human dermal fibroblasts derived from cell lines to achieve a systemic scleroderma phenotype. It is well known that TGF-β is a potent inducer of collagen synthesis and it exerts its biological effects primarily via Smad-dependent signalling (Sisto et al., 2018). Since SSc patients show constitutive phosphorylation of Smad2/Smad3, targeting TGF-β/Smad signalling would be an interesting approach. Hence, as shown in table 4, most of the authors present a molecular target that interferes with TGF-β/Smad-dependent signalling. Indeed, Vu Huy Luong et al., 2018 propose targeting the transcription factor Smad3 by directly blocking its phosphorylation using HPH15. In this line, another transcription factor proposed as a target is IRF7 by Wu et al., 2019 due to the decreasing effect that IRF7 exerts on collagen type I mRNA and protein expression by forming a complex with Smad3. However, other authors demonstrate that interfering with TGF-β/Smad-independent signalling is also a promising approach. Beyer et al., 2015 showed that stimulating sGC interfered with TGF-β/ERK signalling, which in turn lead to a decrease of collagen I accumulation *in vivo* as well as *in vitro.*

It is important to mention that *in vitro* results related to collagen type I modulation are similar to those *in vivo* results: collagen I expression is significantly reduced in both. When it comes to performing *in vivo* experiments in systemic scleroderma, it is important to choose a proper animal model for studying particular aspects of the disease pathophysiology. Most of the *in vivo* experiments have been performed using a bleomycin-induced mouse. Bleomycin is a chemotherapeutic agent that induces skin fibrosis by thickening the dermis and allowing the infiltration of mononuclear cells and anti-nuclear antibodies into the lesioned skin (Marangoni et al., 2016). Supporting this finding, Wu et al., 2019 complemented their *in vivo* experiments using a bleomycin-induced mouse with a Tsk/IRF7 KO mouse model by crossbreeding a Tsk/+ and IRF KO mice. The Tsk/+ mouse model develops fibrosis independent of inflammation. On the other hand, TGF-β injections are also used to induce dermal fibrosis as Beyer et al., 2015

did. Therefore, the fact that most of the *in vivo* experiments have been performed with a bleomycin-induce mouse model, suggests that this is the most accurate mouse model nowadays to study skin fibrosis in systemic scleroderma. Nonetheless, some authors defend the convenience of validating a molecular target in two complementary models of systemic scleroderma before going into clinical trials (Perera et al., 2019). In that case, the use of a single animal model is a limitation in most of the studies presented and hence, further *in vivo* investigations using a complementary animal model must be carried out.

Regardless of the limitations that the articles might have in their research, this literature search has its drawbacks. On one hand, although all of the articles presented have been selected according to the inclusion and exclusion criteria, two of them do not fulfil the aim of the project. As it can be seen in table 5, Perera et al., 2019 demonstrate that antagonizing $P2Y_2$ receptor in a bleomycin-induced mouse reduced skin fibrosis, however, no data related to collagen type I expression *in vivo* is shown. Unfortunately, Luo et al., 2015 neither showed in their article how collagen type I expression was modulated *in vivo.* Therefore, despite these two articles present potential molecular targets, they do not fulfil the aim of this project. Another limitation of this bachelor thesis is that other potential targets have been excluded because they did not match the inclusion and exclusion criteria. For example, miRNA-21 is a target proposed by Jafarinejad-Farsangi et al., 2019, which is upregulated in the skin of SSc patients but has been excluded from this bachelor project due to the absence of *in vivo* experiments. Besides, any of the drugs presented can be considered as an ideal drug candidate by exclusively considering the information obtained from the articles selected. There are many different properties, such as an organ-specific expression or prediction of side-effects that must be previously assessed (Gashaw et al., 2011).

Finally, despite the limitations mentioned above, this bachelor thesis has allowed us to obtain information about molecular targets that may have an important therapeutic impact on treating cutaneous fibrosis in systemic scleroderma in the future. Among them, the blockade of the axis CX_3CL_1/CX_3CR_1 proposed by Vu H. Luong et al., 2019 using an anti-CX_3CL_1 monoclonal antibody (mAb) is considered a potential future therapy for treating skin fibrosis. The authors showed a significant reduction in cutaneous fibrosis *in vivo* and recent studies have confirmed the efficiency of anti-CX_3CL_1 mAb in two complementary models of systemic scleroderma as dermal fibrosis was ameliorated with no apparent side-effects (Hasegawa et al., 2019). This can lead us to suggest that the CX_3CL_1 might be considered the most promising candidate among the molecular targets presented in this bachelor thesis. Moreover, the hypothesis of this project has been confirmed as the decrease in collagen type I accumulation was linked to reduced levels of dermal fibrosis *in vitro* as well as *in vivo*. This suggests that modulating collagen type I expression would be an interesting approach for treating skin fibrosis.

7. FUTURE PERSPECTIVES

In this bachelor thesis, we have presented several molecular targets that mitigate collagen I expression *in vitro* as well as *in vivo* by dissecting the articles obtained from literature research. Interestingly, some of the investigations performed in these articles show the efficiency of the drugs used for targeting the molecular candidates. These drugs are summarised in table 5 and might become potential therapies in the future. For instance, the lead compound of Riociguat, BAY41-2272, has been proposed for ameliorating skin fibrosis (Beyer et al., 2015). Riociguat, a stimulator of sGC, is approved for pulmonary arterial hypertension and it has been demonstrated to have anti-inflammatory and antifibrotic effects in animal models of tissue fibrosis. Several clinical trials have been conducted during these years to demonstrate Riociguat's efficiency in ameliorating skin fibrosis in systemic scleroderma. Unfortunately, a phase II clinical trial had an unsuccessful outcome when patients with early diffuse systemic sclerosis treated with Riociguat showed no different results in skin thickness when they were compared with those patients who were administrated with a placebo (Khanna et al., 2020). However, no clinical trials have been found regarding its lead compound BAY41-2272. On the other hand, even though the anti-CX_3CL_1 mAb is considered the most promising therapy in this bachelor thesis, there are no ongoing clinical trials regarding the investigation of anti-CX_3CL_1 mAb as a potential therapy for treating skin fibrosis in systemic scleroderma by now.

It is interesting that most of the targets proposed in this bachelor thesis, as well as those that have been excluded, are focused on regulating the synthesis of collagen. Nonetheless, it has been proposed that regulating collagen secretion might also be a promising therapeutic approach. In fact, this bachelor thesis has presented the chaperone HSP47 and the protein TANGO1 as potential targets that modulate collagen type I secretion. In line with this, we have demonstrated that targeting TANGO1 using the inhibitor P1 significantly reduces extracellular collagen I deposition. However, we cannot state that TANGO1 is a potential molecular target for treating cutaneous fibrosis as the experiments have been performed using osteoblasts. Thus, it would be necessary to investigate TANGO1 in dermal fibroblasts which show a dermal fibrosis phenotype. On the other hand, the inhibitor P1 might become a potential therapeutic drug. For this, a better comprehension of the inhibitor's activity is required. Dr Vivek Malhotra's research group is currently working on characterising the effects of three different TANGO1 inhibitors. These investigations will focus on revealing how is collagen secretion modulated, where is the collagen being accumulated within the cell and determining the specificity of the inhibitors. Besides, revealing how bulky cargoes are secreted is an intriguing issue for cell biologists. Dr Vivek Malhotra's research group is also focused on explaining the molecular events that take place during the export of such cargoes, especially regarding TANGO1. Their recent investigations suggest that TANGO1 creates a tunnel between the endoplasmic

reticulum and ERGIC-53 membranes, thus their future investigations will be visualising the export of these bulky cargoes through the tunnel.

In conclusion, there are just a few therapeutic drugs available nowadays that delay the development of cutaneous fibrosis, unfortunately, none has been shown to reverse disease progression. Therefore, future investigations should focus on characterising the effects of these drug candidates on reversing the effects of dermal fibrosis in systemic scleroderma.

8. CONCLUSIONS

This bachelor thesis had the aim of presenting potential molecular targets that modulate collagen I expression and hence ameliorate cutaneous fibrosis in systemic scleroderma. For this, a literature search was carried out and ten articles were selected according to inclusion and exclusion criteria. Each of these articles presented a molecular target that reduced collagen I expression *in vitro.* Their investigations were complemented with *in vivo* experiments that demonstrated how cutaneous fibrosis is ameliorated by inhibiting or activating the target. Besides, this project also presents TANGO1 as a promising target for modulating collagen I deposition. The use of the inhibitor P1 *in vitro* reduced extracellular collagen I accumulation, hence, suggesting that TANGO1 is an interesting molecular target for treating cutaneous fibrosis and the inhibitor P1 might become a potential therapeutic drug for treating cutaneous fibrosis.

In this context, we can conclude:

1. The hypothesis of this bachelor thesis has been confirmed and thus, reducing collagen type I gene and protein expression ameliorates cutaneous fibrosis.
2. Bleomycin-induced mouse model is the most accurate animal model nowadays to study the development of cutaneous fibrosis and test anti-fibrotic compounds.
3. CX_3CL_1 is considered the most promising molecular candidate among those obtained from the literature search.
4. TANGO1 protein is a potential target for ameliorating collagen I deposition. Future research should focus on investigating the effects of the inhibitor P1 on dermal fibroblasts.
5. The inhibitor P1 might become an important drug for reducing collagen I secretion. Further investigations are needed to characterise its activity.

9. ASSESSMENT OF THE PROJECT

During my bachelor's degree, I have acquired a wide knowledge of different topics ranging from bioinformatics to molecular pathology. Most of the subjects focus on giving students the resources for solving problems that appear when we are performing our experiments at the bench. Nonetheless, this bachelor thesis has made me realize that carrying out accurate literary research is as important as performing your experiment. A bibliographic search can help scientists to develop a theoretical framework and methodology for their experimental research. In this case, basing my project on a literature review has broadened my knowledge about systemic scleroderma and cutaneous fibrosis. I have discovered that there is a research pattern followed in all the articles selected, and thus, this would be useful in case I needed to plan an investigation. Moreover, I have been able to dissect the articles and understand their experiments and results by applying the critical thinking and theoretical knowledge acquired during this degree.

However, it is noteworthy to mention that this bachelor thesis was supposed to be an experimental project but unfortunately, due to the COVID-19 situation, the experiments planned for this project could not be completed. Nonetheless, one of the experiments has been included and discussed in this thesis. The experiment has been conducted during my internship at CRG in collaboration with Vivek Malhotra's research group. I am glad to have had the opportunity to learn from such professional scientists. Although my internship period has been relatively short, I must say that it has been an enriching experience that has helped me to grow as a scientist. I had the opportunity to collaborate in their investigation, attend lab meetings and talks given by excellent scientists. On the other hand, I have discovered the cell biology research field, which made me realize how little we know about the basic processes of cells, such as protein secretion. It was not until I met Vivek Malhotra that I questioned how bulky cargoes as collagens, which may have a size of approximately 300nm, are transported to Golgi by COPII vesicles if these structures have a diameter up to 70nm. One of the most remarkable things learnt in his lab is that a scientist must question everything, even those things we think we already know.

In conclusion, this combination of experimental and bibliographic bachelor thesis has allowed me to develop many skills such as critical thinking, time-management and communication. I personally think that this project has been a great opportunity to grow as a scientist by applying theory to practice and vice-versa.

10. BIBLIOGRAPHY

Allanore, Y., Simms, R., Distler, O., Trojanowska, M., Pope, J., Denton, C. P., & Varga, J. (2015). Systemic sclerosis. *Nature Reviews Disease Primers*, *1*(1), 1–21. https://doi.org/10.1038/nrdp.2015.2

Asano, Y. (2018). Systemic sclerosis. *The Journal of Dermatology*, *45*(2), 128–138. https://doi.org/10.1111/1346-8138.14153

Beyer, C., Zenzmaier, C., Palumbo-Zerr, K., Mancuso, R., Distler, A., Dees, C., Zerr, P., Huang, J., Maier, C., Pachowsky, M. L., Friebe, A., Sandner, P., Distler, O., Schett, G., Berger, P., & Distler, J. H. W. (2015). Stimulation of the soluble guanylate cyclase (sGC) inhibits fibrosis by blocking non-canonical TGFβ signalling. *Annals of the Rheumatic Diseases*, *74*(7), 1408–1416. https://doi.org/10.1136/annrheumdis-2013-204508

Bielsa, I. (2013). Update on the classification and treatment of localized scleroderma. *Actas Dermo-Sifiliograficas*, *104*(8), 654–666. https://doi.org/10.1016/j.adengl.2012.10.012

Chung, M. P., & Chung, L. (2020). Drugs in phase I and phase II clinical trials for systemic sclerosis. *Expert Opinion on Investigational Drugs*, *29*(4), 349–362. https://doi.org/10.1080/13543784.2020.1743973

Cowper, S. E., Rabach, M., & Girardi, M. (2008). Clinical and histological findings in nephrogenic systemic fibrosis. *European Journal of Radiology*, *66*(2), 191–199. https://doi.org/10.1016/j.ejrad.2008.01.016

Denton, C. P., & Khanna, D. (2017). Systemic sclerosis. *The Lancet*, *390*(10103), 1685–1699. https://doi.org/10.1016/S0140-6736(17)30933-9

Do, N. N., & Eming, S. A. (2016). Skin fibrosis: Models and mechanisms. *Current Research in Translational Medicine*, *64*(4), 185–193. https://doi.org/10.1016/j.retram.2016.06.003

Dosoki, H., Stegemann, A., Taha, M., Schnittler, H., Luger, T. A., Schröder, K., Distler, J. H. W., Kerkhoff, C., & Böhm, M. (2017). Targeting of NADPH oxidase in vitro and in vivo suppresses fibroblast activation and experimental skin fibrosis. *Experimental Dermatology*, *26*(1), 73–81. https://doi.org/10.1111/exd.13180

Gashaw, I., Ellinghaus, P., Sommer, A., & Asadullah, K. (2011). What makes a good drug target? *Drug Discovery Today*, *16*(23–24), 1037–1043. https://doi.org/10.1016/j.drudis.2011.09.007

Gelber, A. C., Manno, R. L., Shah, A. A., Woods, A., Le, E. N., Boin, F., Hummers, L. K., & Wigley, F. M. (2013). Race and association with disease manifestations and mortality in scleroderma. *Medicine (United States)*, *92*(4), 191–205. https://doi.org/10.1097/MD.0b013e31829be125

Hasegawa, M., Luong, V. H., Utsunomiya, A., Chino, T., Oyama, N., Matsushita, T., Obara, T., Kuboi, Y., Ishii, N., Machinaga, A., Ogasawara, H., Ikeda, W., & Imai, T. (2019). LB1141 Anti-mouse CX3CL1 monoclonal antibody therapy in mouse models of systemic sclerosis. *Journal of Investigative Dermatology*, *139*(9), B25. https://doi.org/10.1016/j.jid.2019.06.114

Hauser, A. S., Chavali, S., Masuho, I., Jahn, L. J., Martemyanov, K. A., Gloriam, D. E., & Babu, M. M. (2018). Pharmacogenomics of GPCR Drug Targets. *Cell*, *172*(1–2), 41–54. https://doi.org/10.1016/j.cell.2017.11.033

Henriksen, K., & Karsdal, M. A. (2019). Type I collagen. In K. A. Morten (Ed.), *Biochemistry of Collagens, Laminins and Elastin* (Second, pp. 1–12). Elsevier. https://doi.org/10.1016/B978-0-12-817068-7.00001-X

Hoogen, F. Van den, Baron, M., & Matucci-cerinic, M. (2013). Classification Criteria for Systemic Sclerosis: An ACR-EULAR Collaborative Initiative. *Arthritis and Rheumatology*, *65*(11), 2737–2747. https://doi.org/10.1002/art.38098.Classification

Ingegnoli, F., Ughi, N., & Mihai, C. (2018). Update on the epidemiology, risk factors, and disease outcomes of systemic sclerosis. *Best Practice & Research Clinical Rheumatology*, *32*(2), 223–240. https://doi.org/10.1016/j.berh.2018.08.005

Ito, S., & Nagata, K. (2019). Roles of the endoplasmic reticulum–resident, collagen-specific molecular chaperone Hsp47 in vertebrate cells and human disease. *Journal of Biological Chemistry*, *294*(6), 2133–2141. https://doi.org/10.1074/jbc.TM118.002812

Jinnin, M. (2010). Mechanisms of skin fibrosis in systemic sclerosis. *The Journal of Dermatology*, *37*(1), 11–25. https://doi.org/10.1111/j.1346-8138.2009.00738.x

Khanna, D., Allanore, Y., Denton, C. P., Kuwana, M., Matucci-Cerinic, M., Pope, J. E., Atsumi, T., Bečvář, R., Czirják, L., Hachulla, E., Ishii, T., Ishikawa, O., Johnson, S. R., De Langhe, E., Stagnaro, C., Riccieri, V., Schiopu, E., Silver, R. M., Smith, V., … Distler, O. (2020). Riociguat in patients with early diffuse cutaneous systemic sclerosis (RISE-SSc): randomised, double-blind, placebo-controlled multicentre trial. *Annals of the Rheumatic Diseases*, *79*(5), 618–625. https://doi.org/10.1136/annrheumdis-2019-216823

Kowal-Bielecka, O., Fransen, J., Avouac, J., Becker, M., Kulak, A., Allanore, Y., Distler, O., Clements, P., Cutolo, M., Czirjak, L., Damjanov, N., del Galdo, F., Denton, C. P., Distler, J. H. W., Foeldvari, I., Figelstone, K., Frerix, M., Furst, D. E., Guiducci, S., … Müller-Ladner, U. (2017). Update of EULAR recommendations for the treatment of systemic sclerosis. *Annals of the Rheumatic Diseases*, *76*(8), 1327–1339. https://doi.org/10.1136/annrheumdis-2016-209909

Kudo, H., Wang, Z., Jinnin, M., Nakayama, W., Inoue, K., Honda, N., Nakashima, T., Kajihara, I., Makino, K., Makino, T., Fukushima, S., & Ihn, H. (2015). EBI3 Downregulation Contributes to Type I Collagen Overexpression in Scleroderma Skin. *The Journal of Immunology*, *195*(8), 3565–3573. https://doi.org/10.4049/jimmunol.1402362

Kular, J. K., Basu, S., & Sharma, R. I. (2014). The extracellular matrix: Structure, composition, age-related differences, tools for analysis and applications for tissue engineering. *Journal of Tissue Engineering*, *5*, 1–17. https://doi.org/10.1177/2041731414557112

Leitenberger, J. J., Cayce, R. L., Haley, R. W., Adams-Huet, B., Bergstresser, P. R., & Jacobe, H. T. (2009). Distinct Autoimmune Syndromes in Morphea. *Archives of Dermatology*, *145*(5), 545–550. https://doi.org/10.1001/archdermatol.2009.79

Li, X., Zhu, L., Wang, B., Yuan, M., & Zhu, R. (2017). Drugs and Targets in Fibrosis. *Frontiers in Pharmacology*, *8*(855), 1–21. https://doi.org/10.3389/fphar.2017.00855

Luo, H., Zhu, H., Zhou, B., Xiao, X., & Zuo, X. (2015). MicroRNA-130b regulates scleroderma fibrosis by targeting peroxisome proliferator-activated receptor γ. *Modern Rheumatology*, *25*(4), 595–602. https://doi.org/10.3109/14397595.2014.1001311

Luong, Vu H., Utsunomiya, A., Chino, T., Doanh, L. H., Matsushita, T., Obara, T., Kuboi, Y., Ishii, N., Machinaga, A., Ogasawara, H., Ikeda, W., Kawano, T., Imai, T., Oyama, N., & Hasegawa, M. (2019). Inhibition of the Progression of Skin Inflammation, Fibrosis, and Vascular Injury by Blockade of the CX3CL1/ CX3CR1 Pathway in Experimental Mouse Models of Systemic Sclerosis. *Arthritis & Rheumatology*, *71*(11), 1923–1934. https://doi.org/10.1002/art.41009

Luong, Vu Huy, Chino, T., Oyama, N., Matsushita, T., Sasaki, Y., Ogura, D., Niwa, S., Biswas, T., Hamasaki, A., Fujita, M., Okamoto, Y., Otsuka, M., Ihn, H., & Hasegawa, M. (2018). Blockade of TGF-β/Smad signaling by the small compound HPH-15 ameliorates experimental skin fibrosis. *Arthritis Research & Therapy*, *20*(46), 1–13. https://doi.org/10.1186/s13075-018-1534-y

Marangoni, R. G., Varga, J., & Tourtellotte, W. G. (2016). Animal models of scleroderma. *Current Opinion in Rheumatology*, *28*(6), 561–570. https://doi.org/10.1097/BOR.0000000000000331f

Morry, J., Ngamcherdtrakul, W., Gu, S., Goodyear, S. M., Castro, D. J., Reda, M. M., Sangvanich, T., & Yantasee, W. (2015). Dermal delivery of HSP47 siRNA with NOX4-modulating mesoporous silica-based nanoparticles for treating fibrosis. *Biomaterials*, *66*, 41–52. https://doi.org/10.1016/j.biomaterials.2015.07.005

Omair, M. A., Alahmadi, A., & Johnson, S. R. (2015). Safety and Effectiveness of Mycophenolate in Systemic Sclerosis. A Systematic Review. *PLOS ONE*, *10*(5), 1–13. https://doi.org/10.1371/journal.pone.0124205

Perera, L. M. B., Sekiguchi, A., Uchiyama, A., Uehara, A., Fujiwara, C., Yamazaki, S., Yokoyama, Y., Ogino, S., Torii, R., Hosoi, M., Ishikawa, O., & Motegi, S. (2019). The Regulation of Skin Fibrosis in Systemic Sclerosis by Extracellular ATP via P2Y2 Purinergic Receptor. *Journal of Investigative Dermatology*, *139*(4), 890–899. https://doi.org/10.1016/j.jid.2018.10.027

Pope, J. E. (2017). Reflections on the EULAR recommendations for the treatment of systemic sclerosis. *Nature Reviews Rheumatology*, *13*(3), 134–136. https://doi.org/10.1038/nrrheum.2016.212

Ranque, B., & Mouthon, L. (2010). Geoepidemiology of systemic sclerosis. *Autoimmunity Reviews*, *9*(5), 311–318. https://doi.org/10.1016/j.autrev.2009.11.003

Raote, I., Ortega-Bellido, M., Santos, A. J. M., Foresti, O., Zhang, C., Garcia-Parajo, M. F., Campelo, F., & Malhotra, V. (2018). TANGO1 builds a machine for collagen export by recruiting and spatially organizing COPII, tethers and membranes. *ELife*, *7*, 1–21. https://doi.org/10.7554/eLife.32723

Rongioletti, F., Ferreli, C., Atzori, L., Bottoni, U., & Soda, G. (2018). Scleroderma with an update about clinico-pathological correlation. *Giornale Italiano Di Dermatologia e Venereologia : Organo Ufficiale, Societa Italiana Di Dermatologia e Sifilografia*, *153*(2), 208–215. https://doi.org/10.23736/S0392-0488.18.05922-9

Rubio-Rivas, M., Royo, C., Simeón, C. P., Corbella, X., & Fonollosa, V. (2014). Mortality and survival in systemic sclerosis: Systematic review and meta-analysis. *Seminars in Arthritis and Rheumatism*, *44*(2), 208–219. https://doi.org/10.1016/j.semarthrit.2014.05.010

Sierra-Sepúlveda, A., Esquinca-González, A., Benavides-Suárez, S. A., Sordo-Lima, D. E., Caballero-Islas, A. E., Cabral-Castañeda, A. R., & Rodríguez-Reyna, T. S. (2019). Systemic Sclerosis Pathogenesis and Emerging Therapies, beyond the Fibroblast. *BioMed Research International*, *2019*, 4569826. https://doi.org/10.1155/2019/4569826

Sisto, M., Lorusso, L., Ingravallo, G., Tamma, R., Ribatti, D., & Lisi, S. (2018). The TGF-β1 Signaling Pathway as an Attractive Target in the Fibrosis Pathogenesis of Sjögren's Syndrome. *Mediators of Inflammation*, *2018*, 1–14. https://doi.org/10.1155/2018/1965935

Van Caam, A., Vonk, M., Van den Hoogen, F., Van Lent, P., & Van der Kraan, P. (2018). Unraveling SSc Pathophysiology; The Myofibroblast. *Frontiers in Immunology*, *9*(2452), 1–22. https://doi.org/10.3389/fimmu.2018.02452

Varga, J. (2017). Etiology and Pathogenesis of Scleroderma. In G. S. Firestein, R. C. Budd, S. E. Gabriel, I. B. McInnes, & J. R. O'Dell (Eds.), *Kelley and Firestein's Textbook of Rheumatology* (10th ed., pp. 1400–1423). Elsevier. https://doi.org/10.1016/B978-0-323-31696-5.00083-8

Wei, J., Ghosh, A. K., Chu, H., Fang, F., Hinchcliff, M. E., Wang, J., Marangoni, R. G., & Varga, J. (2015). The Histone Deacetylase Sirtuin 1 Is Reduced in Systemic Sclerosis and Abrogates Fibrotic Responses by Targeting Transforming Growth Factor β Signaling. *Arthritis & Rheumatology*, *67*(5), 1323–1334. https://doi.org/10.1002/art.39061

Wu, M., Skaug, B., Bi, X., Mills, T., Salazar, G., Zhou, X., Reveille, J., Agarwal, S. K., Blackburn, M. R., Mayes, M. D., & Assassi, S. (2019). Interferon regulatory factor 7 (IRF7) represents a link between inflammation and fibrosis in the pathogenesis of systemic sclerosis. *Annals of the Rheumatic Diseases*, *78*(11), 1583–1591. https://doi.org/10.1136/annrheumdis-2019-215208

Yanaba, K. (2016). Strategy for treatment of fibrosis in systemic sclerosis: Present and future. *The Journal of Dermatology*, *43*(1), 46–55. https://doi.org/10.1111/1346-8138.13026

EXTENDED DATA 1: SECRETION ASSAY PROTOCOL

Procedure:

1. Prepare falcons for each condition (Control and Inhibitor P1).
2. Vacuum DMEM media and wash cells with 2mL of PBS 1X. Repeat the wash 5 times.
3. Prepare the following mixed solution:
    - Opti-MEM media (previously warmed-up).
    - A solution of ascorbic acid. Add 1µL/mL of mixed solution.
    - MiliQ water (control). 10µL/mL of mixed prepared.
    - Inhibitor P1. Add 10µL/mL of mixed prepared.
4. Pipette up and down the mixed solution and add 750µL in each well.
5. Incubate cells at 37°C with 5% CO_2.
6. Collect media after 4h.
7. Place cells on ice and collect 500µL of media. Centrifugate the samples at 2.000rpm for 6 minutes at 4°C. After centrifugation, pipette 200µL of the supernatant obtained (twice) and store samples at -80°C.
8. Remove the remaining media of cells and wash with PBS 1X twice.
9. Add 500µL of lysis buffer in each well. Incubate cells with the lysis buffer for 20 minutes (rotate the plate every few minutes) on ice.
10. Scrape cells gently and collect 500µL of cell lysates. Spin these samples at 16.000rpm for 20 minutes at 4°C. After centrifugation, pipette 200µL of the supernatant obtained (twice) and store samples at -80°C.

Prepare:

- A solution of ascorbic acid (dilute in Opti-MEM media):
    - 1M phosphor-ascorbate
    - 250mM of ascorbic acid
- Lysis buffer:
    - MiliQ water
    - NaCl 150mM
    - NP-40 1%
    - Tris-Cl 50M pH=8
    - Aprotinin 1X
    - Leupeptin 1X
    - Pepstatin 1X

EXTENDED DATA 2: TANGO1 PROTEIN

TANGO1 is described as a transmembrane protein located at the endoplasmic reticulum exit sites (ERES), which assembles into rings around COPII vesicles and it is responsible for exporting collagens. Nonetheless, recent investigations reveal how the export of collagen is orchestrated by different TANGO1 family proteins, which include:

- TANGO1 has a length of 1907 amino acids and it is inserted into the ER membrane by a transmembrane domain.
- TANGO1-short is a spliced isoform of TANGO1 and lacks the ability of binding to procollagens.
- TANGO1-like protein (TALI) is expressed as two isoforms. While the long isoform is expressed in selected tissues, the short isoform called cTAGE5 is ubiquitously expressed.

Although the functional mechanism by which TANGO1 exports collagen is still unclear, it has been proposed that TANGO1 family proteins interact with each other, thus creating a structure which reassembles into a ring with a diameter up to 300nm at ERES (Figure 1S). It is suggested that TANGO1 interacts with NRZ protein tether, which in turn recruits ERGIC-53 membranes. Subsequently, the heat-shock protein HSP47 interacts with the luminal part of TANGO1 and finally, a tunnel is created between the ER and ERGIC-53

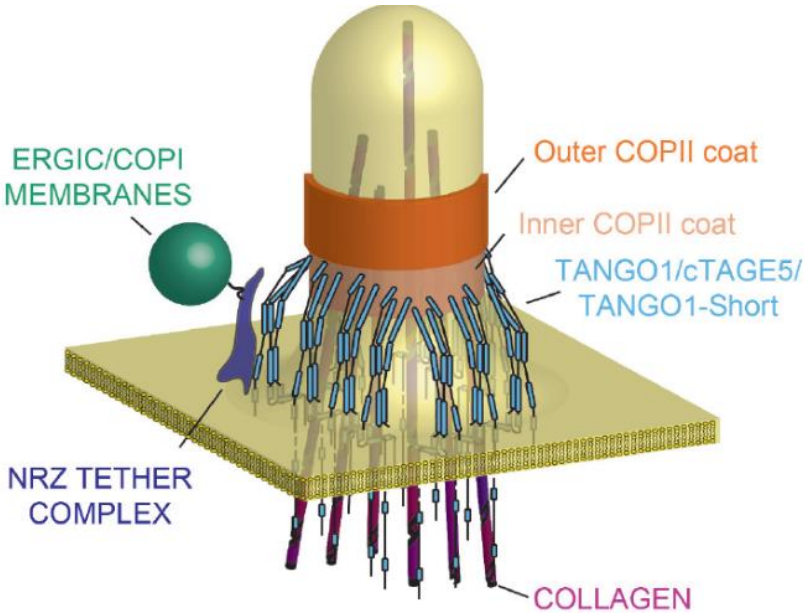

Figure 1S. Model of TANGO1 assembly at ERES. TANGO1 interacts with COPII components and corrals these vesicles by forming a ring of 300nm of diameter. (Raote et al., 2018)

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