Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in

Scienze Biomediche Integrate e Bioetica

XXXIII ciclo a.a. 2017-2018

Titolo tesi

STRIAE DISTENSAE: ASSESSMENT OF A COMBINED TREATMENT ON FIBROBLASTS AND AN IN VIVO STUDY

Nome e cognome candidato

SIMONE LA PADULA

Coordinatore

Prof PAOLO POZZILLI

Tutore

Prof. PAOLO PERSICHETTI

16 Giugno 2021

Summary

Thanks.....p4

Abstract……p5

1. Skin and fibroblasts.....p7

2. Striae Distensae.....p10

EXPERIMENTAL PART - IN VITRO STUDY.

1.Introduction..... p11

2. Material and methods …….p17

3. Results…….p30

IN VIVO STUDY……p46

4. Discussion……p54

5. Future prospects …….p56

REFERENCES……p57

ABREVIATIONS

alpha SMA: α-smooth muscle actin

mRNA: Messenger RNA

GF: Grow Factors

ECM: extracellular matrix

NS: Normal skin (without SD)

PrP: Platelet Rich Plasma

RT-PCR: Reverse Transcription - Polymerase Chain Reaction

SM: stretch marks

SD: Striae Distensae

SA: Striae Albae

SR: Striae Rubrae

SMF: stretch marks derived fibroblasts

SAF: SA derived fibroblasts

SRF: SR derived fibroblasts

NSF: Normal skin fibroblasts

I wanted to thank the people who followed me and helped me with this project and in particular:

Professor Persichetti for believing in me since the first time we met in Paris, for his support and his help in choosing the thesis topic.

Professor Pozzilli for believing in this project and his constant support.

Christophe Chesné, Ismail, Agnes Jamin and the entire Biopredic International Team.

Professor Jean Paul Meningaud and Dr Barbara Hersant for their enormous support.

Professor D'Andrea for his support and for believing in me; thanks to him I started my university career in Italy in Naples, my hometown, in July 2020.

Professor Fabrizio Schonauer, thanks to him in 2006 I discovered my passion for plastic surgery.

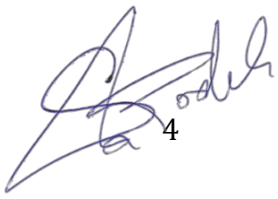
My colleagues and friends who were by my side and who helped and supported me during the realization of this project: Chiara (for coming often with me to Rennes where I carried out some of the in vitro experiments, and for her collaboration in the completion of two articles on the subject of this thesis) Titti, Giovanni and Edoardo who gave a sample of his fat to do a test before carrying out the study in vivo.

The entire team of the Plastic Surgery Departments of Campus Biomedico (Rome) and Henri Mondor University Hospital (France) where I spent my last 7 years.

My Rose, the woman who changed my life, thank you for being by my side!

My mother my father and my brother who have always inspired and encouraged me.

Alone we go fast, together we go far



ABSTRACT

Introduction

Stretch marks or Striae Distensae (SD) appear clinically as parallel streaks, perpendicular to the lines of tension in the skin. SD evolves into two clinical phases, an initial inflammatory phase or striae rubrae (SR) and a chronic phase or striae albae (SA). Fibroblasts appear to play a key role in the pathogenesis of stretch marks. The results of studies explaining the etiology of SD are discordant, which is why we have tried to analyze the phenotype and function of fibroblasts in initial and old lesions and compare them with those in healthy skin (NS) of the same patient. Currently, there is no cure for SA. The non-ablative fractional laser can achieve satisfactory results, but only on the initial lesions (SRs). The goals of this study were: to describe and analyze fibroblasts from stretch marks and the differences between SRF and SAF compared to fibroblasts from healthy tissue from the same donor (NSF); to test innovative treatments (sodium ascorbate and PrP) on cell cultures of fibroblasts from SA (SAF) in vitro; performing an in vivo study using PRP + nanofat combined treatment.

Material and methods

In order to characterize the SMF, the expression of alpha smooth muscle actin (alpha SMA) was investigated. Type I collagen expression was measured in SAF, before and after adding different PrP concentrations and sodium ascorbate in the culture medium. The products tested were: Standard PrP 1% and 5%, PrP concentrated 1% and 5%, sodium ascorbate (100µg / M), PrP standard 1% and 5% + sodium ascorbate (100µg / M), PrP concentrated 1% and 5% + sodium ascorbate (100µg / M), no treatment (control). For the in vivo study, the products tested were: PRP, PRP 20% + nanofat 80%, nanofat. Results were processed through statistical analysis models using the Student's t-test.

Results

A significant increase in alpha SMA (P <0.001) was observed in SRF. SAF treated with PrP and sodium ascorbate showed a resumption of their metabolic activity by an increase in collagen type I production and cell proliferation. After 24 hours of incubation with PrP 1% and PrP 5% + Sodium ascorbate, cell viability was increased by 140% and 151% and by 156 and 178% after 48 hours respectively compared to the control. Regarding the in vivo study: the PrP and the nanofat contributed to increasing the biosynthesis of collagen in the treated areas significantly compared to the control. A greater effect was observed for AS treated with the combined PrP-Nanofat treatment (p <0 .0001).

Conclusion

Our study shows that a biologically mediated improvement of SMF metabolic activity is possible. Our promising results require further trials to be able to confirm the reproducibility of these combined treatments, particularly in vivo.

Keywords: Stretch marks, platelet rich plasma, ascorbic acid, nanofat, fibroblast.

THE SKIN

The embryological origin of the epidermis is the ectoderm, the superficial layer that covers the embryo, which also gives rise to the central and peripheral nervous system and to the hair bulbs. This embryological connection between the skin and the nervous system certainly has important physiopathological consequences on homeostasis and skin repair processes. The dermis, on the other hand, comes from the somites which differentiate from the mesoderm. The interactions between the epidermis and the dermis, between, mainly, the keratinocytes and the fibroblasts, are essential; as the epidermis is not vascularized, its growth and differentiation as well as its regeneration depend on the dermis, which must be able, thanks to a properly irrigated vascular tree, to provide it with all the necessary nutrients. In the dermis, fibroblasts play major roles, in particular through the secretion of an extracellular matrix which, in addition to its supporting roles, is also involved in cell migration and manages the reservoirs of growth factors it contains. In addition, fibroblasts are key cells in maintaining the balances that contribute to good skin health. Finally, when they transform into myofibroblasts, they then become essential players in skin tissue repair (1).

THE FUNCTION OF FIBROBLASTS AND MYOFIBROBLASTS

Fibroblasts and myofibroblasts are major players in skin repair processes and the dermal compartment is an essential part of this organ which is the skin. The good health of the epidermis depends in part on the quality of the nutrients and the information that reaches it from the dermis. Indeed, on both sides of the dermo-epidermal junction (DEJ), an intense dialogue between these two compartments exists. Different fibroblast subpopulations have been observed in the dermis. It is possible to distinguish them, of course according to their location (papillary or reticular fibroblasts), but also according to their ability to play different roles, particularly during skin repair. Recently, it has been shown that dermal subpopulations

exhibit particular differentiation capacities (2). It is commonly believed that myofibroblasts are derived from dermal fibroblasts present in uninjured areas deep down or on the periphery of the injured area. However, other sources may be involved, especially when the lesion is very extensive, and this is of course the case with burns (in this case myofibroblasts can derive from cells in the bone marrow) (3). Furthermore, and this is extremely important, fibroblasts and myofibroblasts are linked to the extracellular matrix through complex mechano-transducers. Thus, the quality of the extracellular matrix (especially the proteins and their amino acid composition) surrounding these cells, but also its rigidity, will drastically influence the cell phenotype. A rigid matrix will promote the expression of smooth alpha-muscle actin in myofibroblasts and enhance their contractile properties (4). Moreover, the rigidity of the matrix will also influence the bioavailability of TGF-β1(fig.1). Thus, myofibroblasts are the main players in the retraction processes that occur in hypertrophic scars. Aarabi et al. have shown, using a relevant model, that mechanical stress is able to induce a hypertrophic-type lesion in rodents (it should be noted that animals never develop a hypertrophic scar) (5). Therefore, the role of tensile forces in the treatment of hypertrophic scars must be particularly taken into account. Recently, medical devices have emerged that "manage" the mechanical environment of the wound to prevent the development of a hypertrophic scar highlighting the importance of mechanical stresses that must be taken into account in scar management (6). Finally, research on stem cells, and particularly stem cells from the dermis and adipose tissue, could allow the implementation of innovative therapies for healing, in particular severe burns. Fibroblasts stimulated by appropriate concentrations of Platelet Rich Plasma (PrP) using platelet-derived growth factors also appear to be a promising treatment for stimulating the formation of ECM and autologous dermal tissue by promoting healing of injured areas. Stimulation and proliferation of fibroblasts may be useful as has been recently demonstrated (in vitro study on the co-culture of keratinocytes and fibroblasts) to

promote the proliferation of keratinocytes and the restoration of skin tissue in severe burns (7).

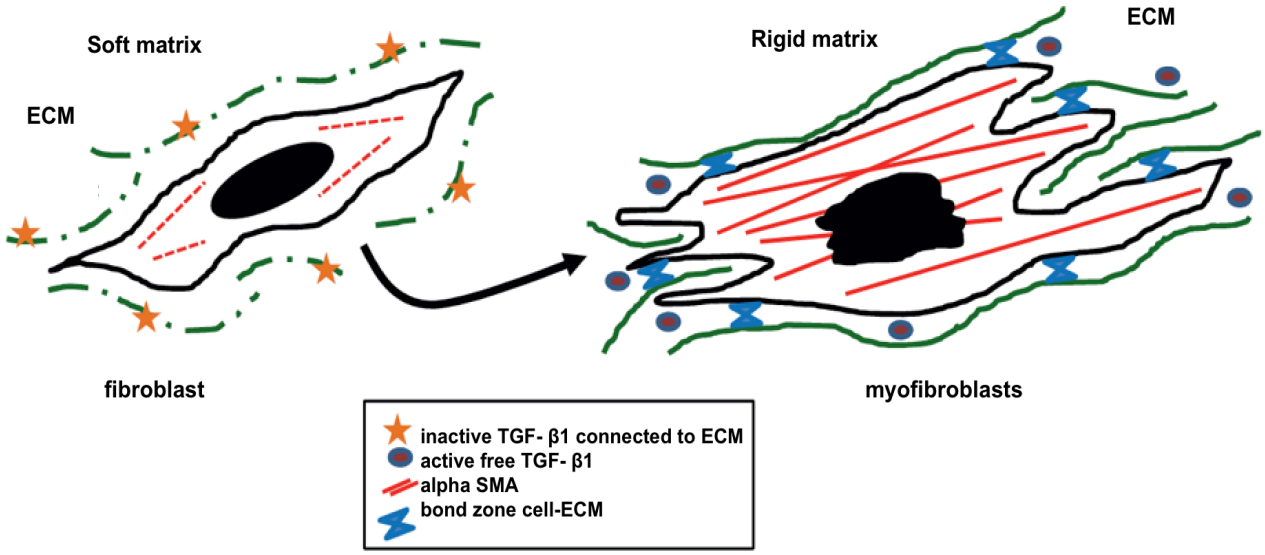


Figure 1.

THE STRIAE DISTENSAE

Stretch marks or Striae distensae (SD) appear clinically as parallel streaks, perpendicular to the lines of tension of the skin. SD evolves through two clinical phases: an initial inflammatory phase or a striae rubrae (SR) phase and a chronic phase or striae albae (SA) (8). Stretch marks appear especially during adrenal cortical hyperfunction or when corticosteroids are administered systemically or locally. They also appear when the volume of an organ or subcutaneous adipose tissue (pregnancy, weight gain) increases rapidly, exposing the skin to severe stretching. These two etiological factors are often associated (9). Stretch marks are permanent scars and their pathogenesis remains poorly understood (10).

Recently Youssef et al. (11) demonstrated a significant increase in the expression of the androgen receptor and glucocorticoids in early lesions (SR), while a declined expression of the estrogen receptor was observed. The results obtained by these authors underline the importance of these hormone receptors in the development of striae. This information is important for studying new targets for the prevention and treatment of SD.

EXPERIMENTAL PART - IN VITRO STUDY

STRIAE DISTENSAE: IN VITRO STUDY AND ASSESSMENT OF A COMBINED TREATMENT WITH SODIUM ASCORBATE AND PLATELET RICH PLASMA ON FIBROBLASTS

INTRODUCTION

SD (Striae Distensae) have two clinical phases: an initial inflammatory clinical phase (striae rubrae SR) and a chronical phase (Striae Albae, SA). SD are the consequence of lesions of the dermal connective tissue. These lesions are initially associated to inflammatory phenomena that evolve eventually into scarring (12). What actually underlies the SD creation is a chronic quantitative and qualitative alteration of dermal collagen associated to certain metabolic anomalies of fibroblasts.

The trigger event seems to be an excessive cutaneous distension which causes mechanical damage of the dermal components (13). The initial changes found on electronic microscopy are the mastocytes degranulation and the activation of macrophages which is associated to a moderate elastolysis of the dermis. These findings suggest that the production and release of elastases by the mastocytes represent some of the most important trigger phenomena in SD pathogenesis. (14). When the SD start becoming clinically visible, collagen fibers shows structural alterations and fibroblast anomalies are present. At this stage the mastocytes disappear and there is an increase in dermal oedema with infiltrating lymphocytes (12-13). The initial phase of SR is characterised by inflammatory lesions. The SA phase is characterised by epidermal atrophy and collagen anomalies, which mimic microscopically the dermal scar tissue. The results of the studies aiming at explaining the aetiology of SD vary significantly and to date there are no unanimous definitive treatments available for SD (14).

A very encouraging and innovative approach is based on the use of platelet rich plasma (PrP)

and micro-needling (15).

Another method that appears to improve the appearance of the skin with SD is the non-ablative fractional laser. However, this treatment allows satisfactory results to be obtained only on the initial lesions – SR (16).

Dermal fibroblasts and their stimulation

It has been speculated that SA fibroblasts are in a quiescent state and produce lower amounts of collagen compared to fibroblasts in healthy skin (11-15). Platelet rich plasma (PrP) and ascorbic acid have been shown to increase collagen production in normal fibroblasts (17). No study has ever tested the effects of combined stimulation by PrP and ascorbic acid on SMF in vitro. In particular the PrP preparation methods and platelet concentration (which represent fundamental elements for the validity and reproducibility of the results) have not been described in the current studies (17-19).

Platelet Rich Plasma (PRP) is defined as a sample of blood with a platelet concentration (PLT) above baseline values. The PRP sample is obtained by centrifugation of the blood and separation of the appropriate blood fraction. Depending on the method used, it may contain 1.3 to 8 times the concentration of PLT in plasma than in whole blood (18).

PRP is also a rich source of many cytokines and growth factors (GFs). Platelets (PLT), and in particular the platelet-derived growth factors (PDGF) contained in their alpha granules, are responsible for the biological effects of PRP (19). The interaction between these GFs and surface receptors on target cells activates intracellular signaling pathways, which induces the expression of genes necessary for regenerative processes such as cell proliferation and extracellular matrix formation (20).

The most abundant component of connective tissue responsible for maintaining its architecture and integrity is collagen.

Collagen also plays an important role in the interaction with integrin receptors on the cell surface, through which it can participate in the regulation of gene expression, differentiation, growth and other cellular functions. These interactions are involved in cytoskeletal reorganization, intracellular ion transport, lipid metabolism, kinase activation, gene expression, cell cycle regulation, and cancer metastasis (21).

In addition to the role of a structural protein, collagen is a ligand of integrin receptors that transmit signals for cell growth, upregulation of prolidase activity and stimulation of collagen biosynthesis. All of these processes are necessary for tissue regeneration during wound healing. PRP effectively aids in tissue repair by stimulating collagen metabolism (22). Little is known about the mechanism of PRP on collagen biosynthesis. Interest in PRP is focused on its application to the healing of damaged tissue.

Certain aspects may be common to injured tissues and stretch marks: an inflammatory phase which characterizes SR and skin atrophy (pathological scar) responsible for SA.

It seems clear to us that the effectiveness of this process depends on how the PrP is prepared. However, despite the many benefits associated with PRP and the promising results reported for its therapeutic potential, clinical results are heterogeneous and sometimes contradictory. These controversial results are mainly due to the lack of standardization of PRP preparation procedures. This has led to the availability of a large number of different products in terms of cell types and quantity, and therefore GF and cytokine content and release times. Platelet activation is a crucial step that can influence the availability of bioactive molecules and therefore the therapeutic effects of PrP (23).

An activation step before the administration of PRP is included in many protocols used, generally by adding thrombin and / or calcium chloride (CaCl2).

According to other authors, we prefer to inject the inactivated PRP because it has been shown that platelets can be spontaneously activated after contact with native collagen present in connective tissue (24).

Platelet Rich Plasma (PrP)

According to Marx et al. (18) PRP is defined as a small volume of plasma at high platelet concentration. In healthy adults, the normal platelet count varies between 150,000 and 350,000 / ml. Studies have shown that a concentration of about 1 million platelets per ml produced clinical benefits (25). Platelets contain an average of 50 to 80 alphas granules. These granules contain different growth factors that are essential for the healing process (26):

• platelet-derived growth factor (PDGF-AA, PDGF-BB, and PDGF-AB)

• transforming growth factor-b (TGF-b1 and TGF- b2)

• vascular endothelial growth factor (VEGF)

• epithelial growth factor (EGF)

• insulin-like growth factor (IGF-I, IGF-II)

• fibroblast growth factor (FGF)

• endothelial cell growth factor (ECGF)

• platelet-derived angiogenesis factor (PDAF)

Numerous studies (27-28) have shown the role of autologous PRP preparations, obtained from the patient's own blood, in soft and hard tissue healing. It has been specifically demonstrated that the use of PRP for the treatment of wounds led to faster regeneration of damaged tissue, as well as a reduction in inflammation and pain, due to the release of platelets derived GF acting locally, and which are essential for cell differentiation and new tissue regeneration of. The active secretion of these growth factors is initiated by the contact of platelets with the extracellular matrix. Activation is a degranulation process in which alphas

granules fuse with the platelet membrane, thus releasing the GF.

Various techniques for activating PRP exist to date. Marx et al. described a activation technique which consists of mixing 6ml of PRP with 1ml of hydrochloride calcium / bovine thrombin. This activation allows a release of growth factors in 1 hour (29). Another activation process consists of mixing the PRP only with calcium hydrochloride without thrombin, allowing the formation of a fibrin matrix including the growth factors which will thus be released into the medium very gradually over a period of 7 days.

Platelets activation induces the release of many early growth factors: FGF, PDGF, IGF, and late GFs such as EGF, VEGF, TGF. The platelets also secrete other proteins, such as fibrinogen, vitronectin and fibronectin, which play a critical role in the regulation of cellular interactions and the spatial organization of cells. Platelet GFs are capable of stimulate the replication of mesenchymal cells, such as fibroblasts, and endothelial cells, and promote chemotaxis of macrophages, monocytes and polymorphonuclear cells. Once released, these growth factors induce various signaling cascades that activate angiogenesis, proliferation and cell differentiation and the synthesis of a new extracellular matrix for tissue regeneration.

Attempts to use isolated GFs have been proved unsuccessful due to their high cost of production and the inability of a single GF to regulate the entire process of regeneration.

On the contrary, autologous PRP represents an economically favorable, safe and effective method by delivering an effective and appropriate GFs cocktail directly to the site of administration, in their natural and physiological relative proportions. Autologous PrP has been used safely in many fields, including dentistry, maxillofacial surgery, plastic and reconstructive surgery, orthopedic surgery, dermatology, cardiovascular surgery and for the treatment of acute and chronic wounds. Autologous PRP can also be used as a delivery system in tissue engineering. Indeed, many active substances are contained in the granules of platelets; these active substances could stimulate the quiescent SAF to make them able again

to secrete significant quantities of ECM proteins.

L-ascorbic acid (vitamin C)

L-Ascorbic Acid (Vitamin C) is a natural, water-soluble, antioxidant acid that has been used in cosmetics as a preservative, as a pH adjuster, and / or as an active compound. This molecule has been shown to promote collagen synthesis in human skin fibroblasts in vitro. In particular, Sodium Ascorbate appears to be the most effective form in stimulating collagen production (30). Sodium ascorbate concentrations, tested in a recent study, which demonstrated the ability to improve collagen secretion from healthy human fibroblast cultures was between 50 and 200 μM (31).

Higher concentrations (400 μM) did not show better efficacy and even higher concentrations (800 μM) had an inhibitory effect on collagen synthesis (32). Simultaneous use of sodium ascorbate and PrP has not yet been studied. We want to test these two products alone and in combination to verify whether there is a potentiation of their effects on the stimulation of SMF.

The difficulty of creating an in vitro SD model, makes it difficult to experiment treatments that could prove to be very effective. Given that SDs are very frequent (70% of women after pregnancy) and poorly tolerated psychologically, there is much interest in developing an effective treatment that can act on the pathogenic mechanisms of this condition.

HYPOTHESIS:

1) SRF and SAF show different functional and phenotypic alterations which are responsible for the appearance and chronicization of SD.

2) SMFs stimulation mediated by growth factors, would increase the production of collagen in vitro and in vivo, with a consequent increase in the thickness of the dermis capable to revert the lesions of old stretch marks (SA).

AIM

The aim of this study is to investigate and analyze stretch marks derived fibroblasts (SMF) and the differences between SR and SA derived fibroblasts (SRF, SAF) compared to normal skin derived fibroblasts (NSF) and to test a new treatment (sodium ascorbate and PrP) on cell cultures of SAF in vitro.

MATERIALS AND METHODS

The first part of the study was based on the analysis of the SMF and on the observation of the differences between SRF and SAF compared with the fibroblasts derived from the healthy skin of the same patients. On the other side, we tried to determine the effects of PrP and sodium ascorbate on cell cultures of SAF in vitro.

The rationale of using these substances **exclusively on SAF** depends on the fact that SR can be greatly improved through multiple techniques such as non-ablative fractioned laser resurfacing (16) and that the majority of patients who come to see us, present lesions that are already old (AS), for which no treatment has shown real effectiveness. In addition, several conditions are present in the initial lesions (SR) (inflammation etc.) that we will not be able to reproduce in vitro for the moment.

Obtaining, isolating and growing fibroblasts

We conducted a prospective, randomized single-blind study in sixty-nine women aged between 30 and 40 years (35 ± 5.2) old with no comorbidities and a mean BMI < 30 of 29±1.8, presenting with SR in the abdominal region and an equal number of women with SA with the same characteristics. These patients had voluntarily sought a conventional abdominoplasty procedure that had been planned at least 3 months before the beginning of the study. Each operation was performed at the same plastic and reconstructive surgery center by the same surgeon.

Exclusions criteria were ongoing treatment with corticosteroids and Cushing syndrome (both conditions alter the normal dermis metabolism) (14).

From March 2016 to July 2019, two weeks before the operation, patients had been informed of the subject and characteristics of our study and of the possibility of donating the excess skin, which was otherwise going to be discarded after the surgery. The respect of their privacy was strictly guaranteed and an appropriate written consent was signed by all the patients.

For the purpose of **randomization**, hundred sealed envelopes were prepared by our medical secretary. Inside the envelopes there was a piece of paper indicating whether their skin would be used for the study (50 envelopes) or not (50 envelopes).

Patients were asked to choose a sealed envelope, by the same blind surgeon, that didn't know what was written on the paper inside the envelopes (if the patient would be included in the study or not). At the end of the randomization process **23 patients** were selected to be included in the study. For each abdominoplasty derived skin sample (Fig.2), 4 skin biopsies were performed using a 2 mm diameter punch that was centered on areas with SD. At the same time two healthy skin biopsies were performed using the same technique. This was to allow 4 primary cell cultures of SMF and two NSF cultures derived from the same patient.

This choice has helped to minimize all inter-individual variations that could interfere with the final results.

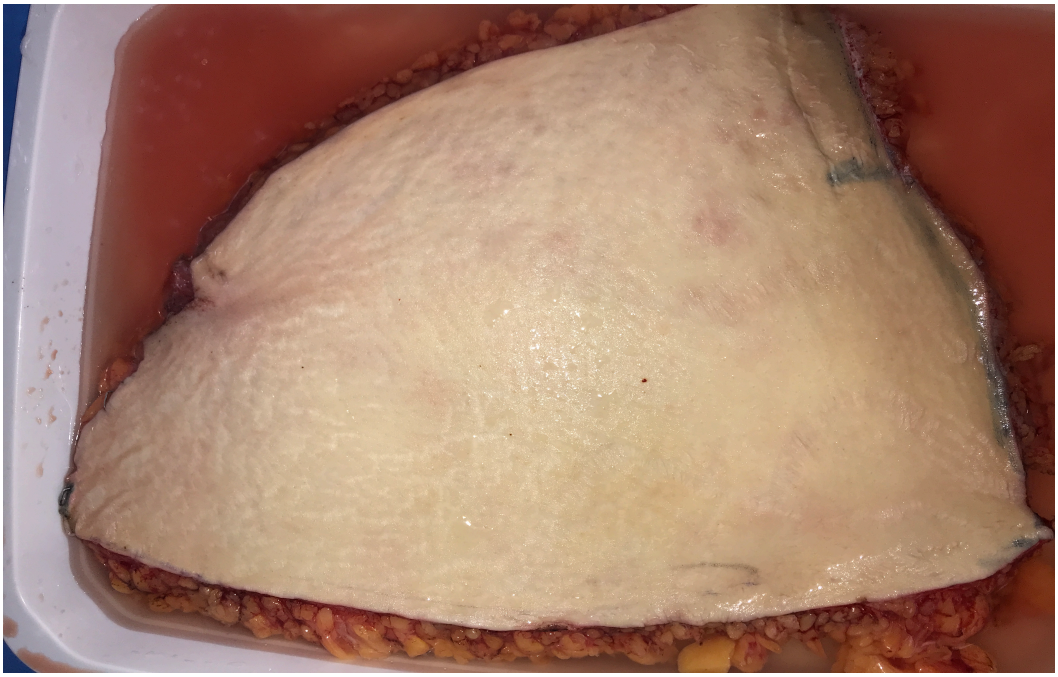


Figure 2: Example of an abdominoplasty derived skin sample with SA used for the study.

FIBROBLASTS ISOLATION AND CULTURE

The same protocol was used to produce SAF and SRF. To isolate dermal fibroblasts, the CELLNTEC protocol was used. For optimal performance, fibroblasts were cultured at 37 ° C with 5% CO2. To detach the cells for the passages, we used Accutase in place of trypsin / EDTA. Accutase (Cat # CnT-Accutase-100) is a mixture of proteolytic and collagenolytic enzymes isolated from crustaceans. It is free from mammalian components. Accutase is the detachment enzyme recommended by Cellntec because of its gentle action which leaves most of the surface proteins intact, and the fact that it does not require a separate reagent to stop the reaction (just dilute with the medium immediately after cell detachment). Skin biopsies of SD and NS were placed in CnT-PR-F medium (15 ml of medium in a 50 ml tube). CnT-PR-F

(CnT-Prime Fibroblast) medium is characterized by a 1% serum component offering better cell isolation and growth efficiency compared to traditional 10% serum media. The skin pieces were placed in a 15 mL centrifuge tube containing 10 mL of 1x diaspase solution, and 2 x antibiotics / antimycotics (Penicillin-Streptomicin 1/1000 + Fungizone). The skin was incubated overnight (~ 16h) at 4 ° C in a horizontal position. The next day, the skin with the Dispase solution was transferred to a Petri dish. Each piece of tissue was then transferred to a new Petri dish containing PBS to remove excess diaspase. While keeping the skin submerged in the PBS, the dermis was gently separated (pink, opaque, sticky) from the epidermis (whitish, semi-transparent) with two pairs of curved forceps, securing the tissue with a pair of forceps while detaching the epidermis with the second pair. The dermis was then cut into very small pieces using a new slide, the dermis pieces were transferred to a centrifuge tube containing 5 ml of collagenase A (Roche # 10103578001, concentration 1 mg / ml in CnT-PR-F), plus 0.5 x CnT-GAB10 antibiotic / antimycotic. Tubes were then incubated at 37 ° C for 8 hours with occasional mixing. We then added 15 ml of CnT-PR-F medium to dilute the collagenase, and mixed by pipetting up and down, then the resulting suspension was passed through a 70 µm cell strainer (BD # 08-771- 2) to obtain a single-cell suspension. The single cell suspension was then centrifuged for 5 min, 200 x g at room temperature. The supernatant is discarded and the pellet has been resuspended in 1 ml of CnT-PR-F. A cell count was performed and we seeded approximately 4000 cells per cm2 in the CnT-PR-F MEDIUM. The cells were grown at 37 ° C and 5% CO2, with a first change of medium 24-48 hours later. Then the medium was changed every 2-3 days. For the cell passages accutase was used. Fibroblasts were used between the 3rd and 8th subculturing, because fibroblasts grown in passages below 8 have been shown to retain their characteristics in vivo. Beyond that, they adapt to the conditions of in vitro (17).

1) CHARACTERIZATION OF SMF

In order to characterize SMFs, the expression of alpha smooth muscle actin (alpha SMA) and the contractile forces generated by NSFs SRFs and SAFs were studied.

Contractile forces generated by fibroblasts in collagen lattices were measured with the **Forcebox** device of our laboratory (INSERM-Henri Mondor Hospital, Créteil, France). To better characterize SMF fibroblasts, we have used a dermis equivalent model in vitro, the collagen lattice, consisting of a mixture of fibroblasts in a gel of collagen. This three-dimensional culture makes it possible to study the capacity of fibroblasts to retract the gel in which they are seeded and to form a compact structure.

For this first experimental phase of our study this 3D culture model can therefore better reproduce the "in vivo" characteristics of this cell population. The aim of this test was therefore to reconstitute a dermal equivalent in vitro using fibroblasts from the stretch marks in order to study their mechanical behaviour within the collagen matrix, compared to the lattices produced using the healthy fibroblasts harvested from the same patient.

THE FORCE-BOX

This device was used to study the modification of SAF, SRF and NSF (healthy skin) derived lattices. The cell chamber was composed of 8 rectangular culture wells (26x33 mm) in which lattices developed. Two opposite silicon beams (200 µm thick, 20 mm wide and 45 mm high) hanged down into each well at a distance of 27 mm apart. The lattice was attached to this sensor through a grid directly etched on the lower part of the beams. A strain gauge of 4.5 kW resistance was deposited at the beam surface by phase vapor deposition (PVD) etched by photolithography method and connected to form a Wheastone bridge.

The strain gauges signal output was amplified, then converted and collected by a computer, which included an acquisition card and a specific program for giving directly the forces in real time. The Forcebox was calibrated against weights, which ranged from 0 g to 1.9 g.

Calibration showed the high linearity of the system and sensitivity of 0.1 mN. The whole cell chamber was sterilized at 120°C for 20 min.

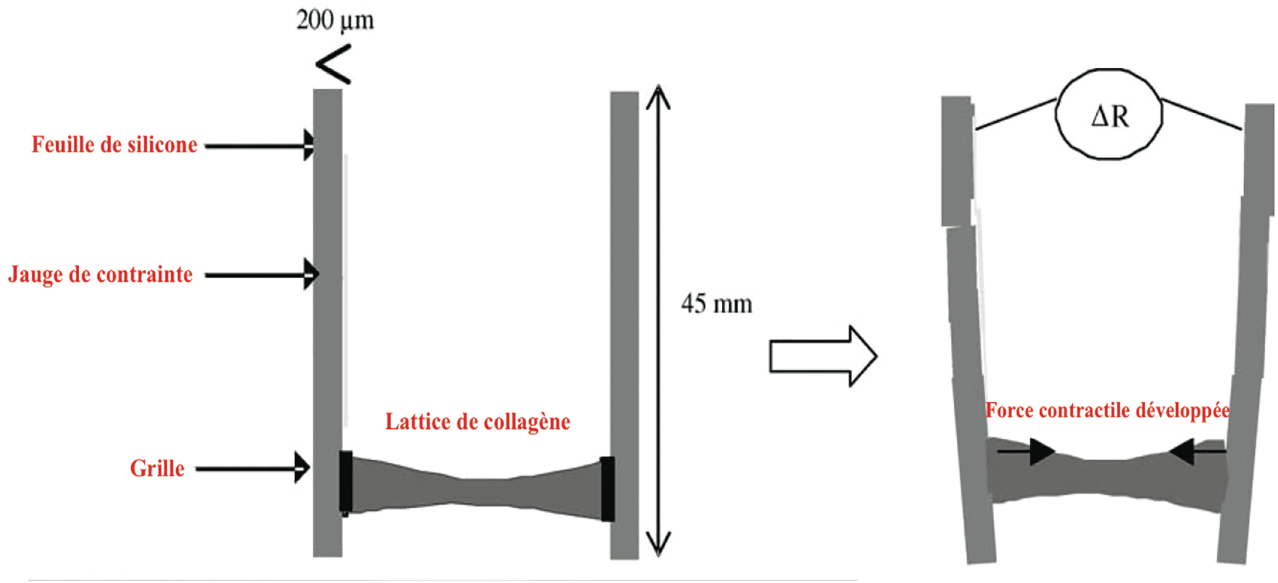


Figure 3 : Force-Box Schema

LATTICE PREPARATION AND MEASUREMENT OF CONTRACTILE FORCES

Fibroblasts were suspended just before confluence with a trypsin-EDTA (1 X) solution. A sample was taken to determine the number of cells obtained. The suspension was centrifuged, and a final count was performed before bringing the fibroblasts to the concentration of $8x10^5$ C/ml. Fibroblasts were embedded three-dimensionally in hydrated collagen gel. Two ml of 1.76 X concentrated DMEM medium (with gentamycin, 0.1 N NaOH and NaHCO3) were added to 1 ml of a solution of type I rat tail collagen (2 mg/ml) (Jacques Boy, Reims, France), and to 0.33 ml of the suspension of fibroblasts in culture medium. The lattice mixture was poured into a rectangular culture well of the Forcebox and polymerized in less than 5 min at 37°C. Two milliliters of culture medium were added. The Forcebox was then placed into a humidified incubator at 37°C, and force measurements were started immediately.

Figure 4 : Force-Box

ALPHA SMA DETECTION – (FLUORESCENCE MICROSCOPY)

The lattices used for the contraction force measurements were 2 days old. They were fixed under tension in the culture plate with a 3% solution of PFA in PBS for 30 min. After rinsing with PBS, fibroblasts in the lattice were permeabilized by treatment with 0.4% Triton X100 for 15 min followed by the three changes of PBS. Non-specific binding sites were blocked at room temperature by a 10 minute incubation in a solution containing 1% glycine in PBS followed by an hour incubation in a solution of 3% BSA in PBS containing 10% normal goat serum and 0.1% Triton X100. Then, the samples were incubated overnight at room temperature and in a humid medium with anti-alpha SMA mouse monoclonal antibody (diluted 1: 100 in PBS containing 1% BSA and 0.1% Triton) (Sigma , Saint Quentin Fallavier, France). For the control, a piece of lattice was incubated in PBS instead of the primary

antibodies. The excess antibody was removed through three 10 min changes of PBS + 1% BSA + 0.1% Triton X 100 each.

The cell cultures used to measure the **alpha SMA expression** were 2 days old.

Anti-mice IgG goat antibodies combined with rhodamine (diluted at 1:40 in PBS containing 1% BSA and 0.1% Triton) (Sigma, Saint Quentin Fallavier, France) were applied for 1 hour at room temperature in a wet environment.

After washing and three PBS changes, the lattice fragments were incubated with 2.5 µg / ml Hoechst 33258 (Sigma, Saint Quentin Fallavier, France) in PBS for 10 min in the dark at room temperature.After washing again, they were gently transported to a histological slide and laminated with Vectashield mounting medium (Biosys, Compiegne, France).

Preparations were examined by a fluorescence microscope (Olympus IX50).

FLOW CYTOMETRY

The two days aged cell cultures, were digested by Clostridium Histolyticum type I collagenase (0.150 U/mg) at 37oC for 1 hour. Cell suspensions were fixed with 3% PFA in PBS for 10 minutes, permeabilized in Triton X100 at 0.4% for 15 min and coloured with alpha SMA actin. Only secondary antibodies were used to control the incubated cells. All samples were analyzed on a FACScan Flow Cytometer (Beckman Coulter) with excitation at 488 nm and filter set at 610 nm for rhodamine. For all flow cytometry analysis, 20,000 cells were analyzed.

2) EFFECTS OF DIFFERENT TREATMENTS ON SAF

For this phase of our study, adult dermal fibroblasts isolated from SA were incubated in different environments containing 1% and 5% standard PRP and the same concentrations of concentrated PrP for 24 and 48 hours. We also wanted to test the effects of ascorbic acid at the same time (100 mg/M). The same measurements were made for control environments in which no molecules were tested. Each procedure was performed three times for each cell culture derived from the same donor. The products tested were:

Standard 1% and 5% PrP, concentrated PrP 1% and 5%, sodium ascorbate (100g /M), standard 1% and 5% PrP and sodium ascorbate (100g /M), concentrated 1% and 5% PrP and sodium ascorbate (100g /M), no added treatment (control). The control model was aimed at assessing whether fibroblasts had reacquired a "normal" phenotype thanks to the previously mentioned molecules or thanks to the cultures. The cell cultures were incubated with different treatments for 24 and 48 hours. We intended to examine and quantify type I collagen in fibroblasts in healthy skin, SA before and after the administration of different treatments.

PRP PREPARATION

Our 23 patients with SA who had accepted to donate the excess skin of their abdominoplasty procedures had not taken any medications, including aspirin and other non steroidal anti-inflammatory drugs, in the 2 weeks prior to the operation and harvesting of PrP. The PrP was produced using the RegenKit®-BCT kit. For the preparation of PrP, two 8 ml tubes of venous blood were collected for each patient. Each tube was centrifuged at 1500 rpm for 5 minutes, resulting in 4 ml of PrP. To obtain the concentrated PrP, the resulting plasma (PRP) in the second tube was centrifuged again with a higher spin (2330 rpm, for 10 min).

The 2 ml of the platelet poor plasma (PPP) were withdrawn and eliminated to obtain the concentrated PrP. A small fraction of each sample was used for haematological analysis.

Platelet concentration was measured with an automated blood cell counter (XS-1000i; Sysmex Co.). Before testing we measured the platelet concentration in the PrP.

SODIUM ASCORBATE

In all the experiments described, we used Sigma-Aldrich (St. Louis, MO) sodium L-ascorbate (CAS 134-03-2) prepared in pure powder form 99.0%, adapted to cell cultures (A4034).

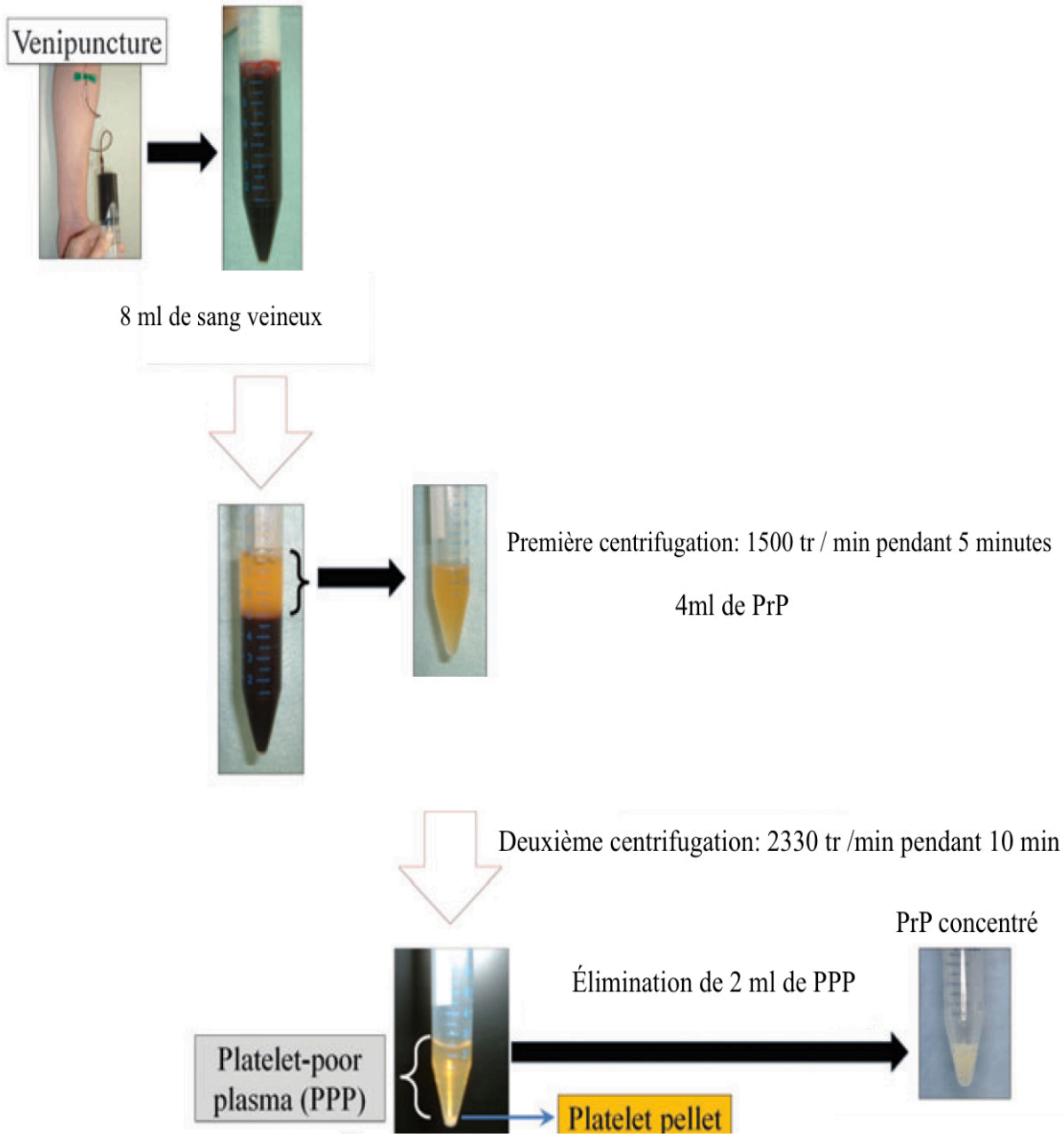


Figure 4 : PrP Preparation

QUANTIFICATION OF TYPE I COLLAGEN THROUGH ELISA ASSAY

The concentration of type I collagen in the supernatant of cultured cells was determined through the coliA1 dosage, made through an Enzyme Linked Immunosorbent Assay (ELISA) technique according to the recommendations of the supplier (Biotechne). Absorption at 450 nm was determined in a microplate reader (E max; Molecular Devices, Sunnyvale, California, USA).

REAL-TIME PCR ANALYSIS FOR TYPE I COLLAGEN

RNA was isolated from cultured fibroblasts. For experiments to measure type I collagen mRNA levels, a relative quantitative RT-PCR was performed. The RNAs were isolated using trizol (life technologies) then the reverse transcriptase (Superscript First-Strand Synthesis System by Invitrogen, Villebon on Yvette, France) was used for cDNA synthesis and amplification using the SYbR green (PCR cycle and machine model used). The quantitative RT-PCR's reactions were performed through a 7900 PCR real-time detection system (Applied Biosystems) using the SYBR Green qPCR Platinium SuperMix (Invitrogen).

PCR reactions were performed using the following primers: human COL I (forward: 5'-atgatgagaaatcaaccgga-3'; reverse: 5'-ccagtagcaccatcatttcc-3') and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward: 5'-gtcttcaccaccatggagaaggc-3'; reverse: 5'-cggaaggccatgccagtgagctt-3'). Type I collagen expression levels were normalized to the endogenous GAPDH levels (endogenous control). In all these experiments SAF cultures, to which no treatment had been added, were used as a calibrator control.

CELL PROLIFERATION

Cell proliferation was measured by CellTiter 96 Aqueous One solution (Promega, Madison, WI, USA). CellTiter 96® AQueous One Solution reagent contains tetrazolium compound [3-(4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium, internal salt); MTS] and an electronic coupling reagent (phenazine ethosulfate; PES). PES has improved chemical stability, which allows it to be combined with MTS to form a stable solution.

Testing was performed by adding a small amount of the CellTiter 96® AQueous One Solution reagent directly to the culture plates, incubating for 1-4 hours, then recording the absorbance at 490 nm with a 96-well plate reader. The amount of formazan measured by the amount of absorbance of 490 nm is directly proportional to the number of living cells in culture.

IMMUNOCYTOLOGY

Culture fibroblasts were subsequently incubated with 10g of polyclonal antibodies against type I collagen (Chemicon, Temecula, CA). The cultures were then incubated with secondary antibodies combined with fluorescein. The nuclei were detected with propidium iodine (Sigma, Sigma, St. Louis, MO). All cultures were then examined with a Nikon Eclipse E1000 Microscope.

STATISTICS

Data and statistics management:

Our results were processed using the Prism software in its Version 5 (Graph Pad, USA). The statistical analysis was conducted using JMP 10 (SAS institute Inc. software).

In all experiments, the average values for the three SD-test sets were calculated. The results were processed through statistical analysis using the Student's t-test. The results were

considered statistically significant when the likelihood was less than 0.05 (p.05).

RESULTS

1) CHARACTERIZATION OF SMF

Contractile Force generation

The Forcebox was used to measure contractile forces in tense collagen lattices. Force measurements were recorded continuously for 2 days. The profiles of contractile force generated by NSF SRF and SAF were broken down into three phases. The first phase occurred within 2 h of the start of the measurement and did not consist of generated contractile force. The second phase, seen between 2 h and approximately 22 h, represented a near linear increase in force. In comparison with NSF, the force generation with SRF fibroblasts was much enhanced. A significant increase for SRF compared with NSF was observed from the 12th hour ($P<0.05$). The peak contractile force developed by fibroblasts from SR was 28% greater than fibroblasts from NS. In contrast, the contractile force developed by SAF did not differ significantly from that produced by NSF.

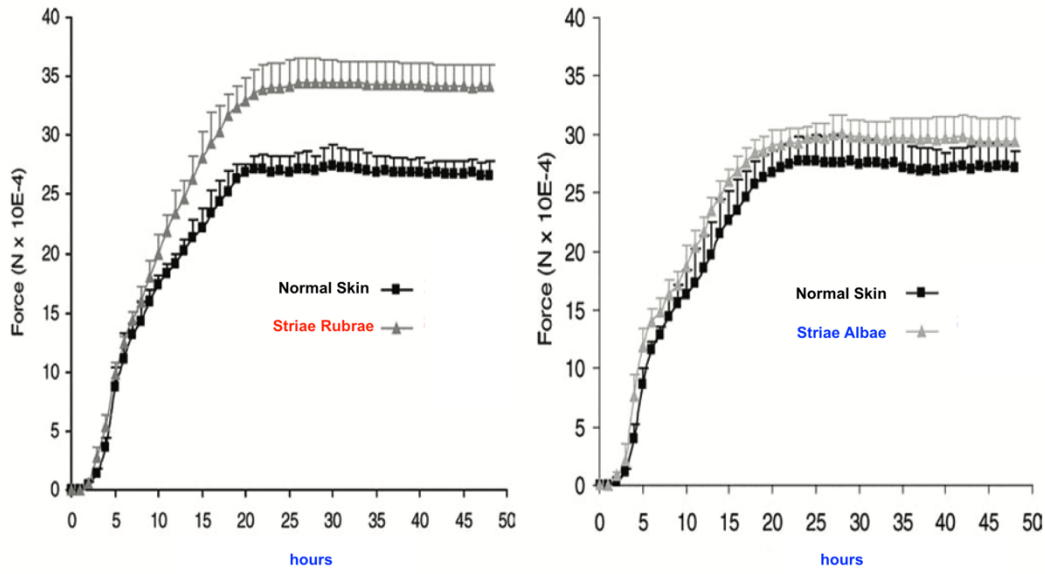


Figure 5: Forces generated by Striae Rubrae, Striae Albae and Normal Skin.

EXPRESSION OF ALPHA SMA

The fibroblasts were cultured in the three-dimensional collagen lattices for 2 days. We used immunofluorescence techniques for the demonstration of alpha-SMA and histofluorescence for nuclei.

SRF showed an important positivity for alpha SMA. We were not able to observe alpha SMA filaments in SA and NSF (fig. 6). Flow cytometry analyses of alpha SMA expression on NSF, SRF and SAF showed an average coloration intensity of 2,3 ± 0,6; 5,7 ± 0,4 and 3,7 ± 0,3 respectively (fig. 7).

Compared to the Fibroblasts of healthy skin, a significant increase in alpha SMA was observed for SR fibroblasts ($P<0.001$). There was no significant difference between SAF and NSF.

Figure 6: Alpha SMA marking (red), fibroblasts from SR. SRF showed an important positivity for alpha SMA (a). Alpha SMA marking (red), fibroblasts from SA. SAF showed no alpha SMA expression (b). NSF showed no alpha SMA expression (c).

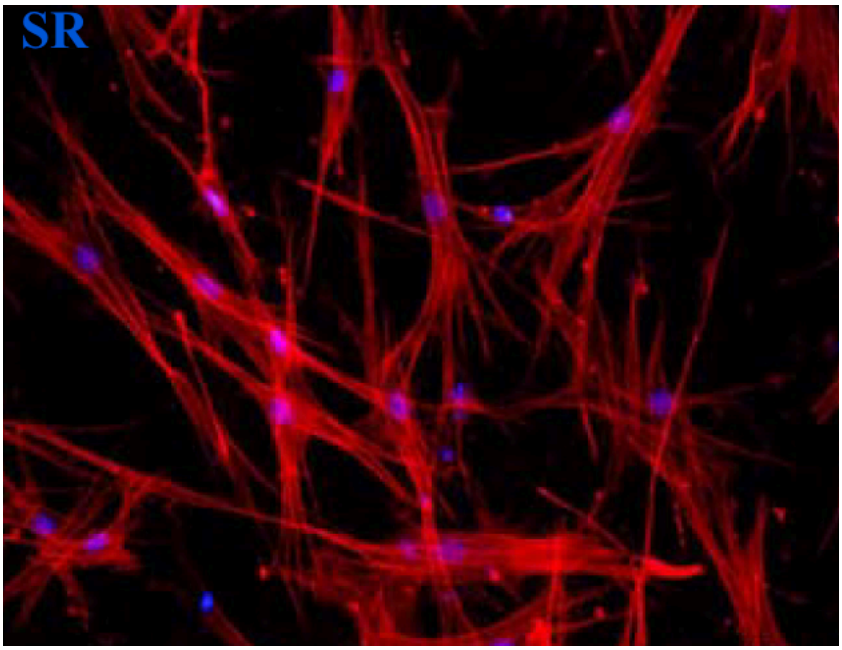


Figure 6a

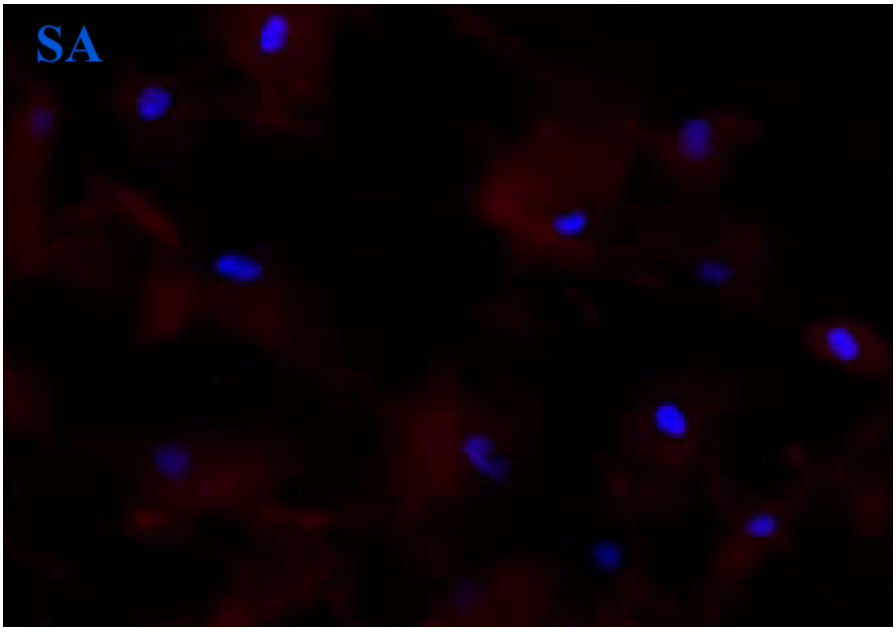


Figure 6b

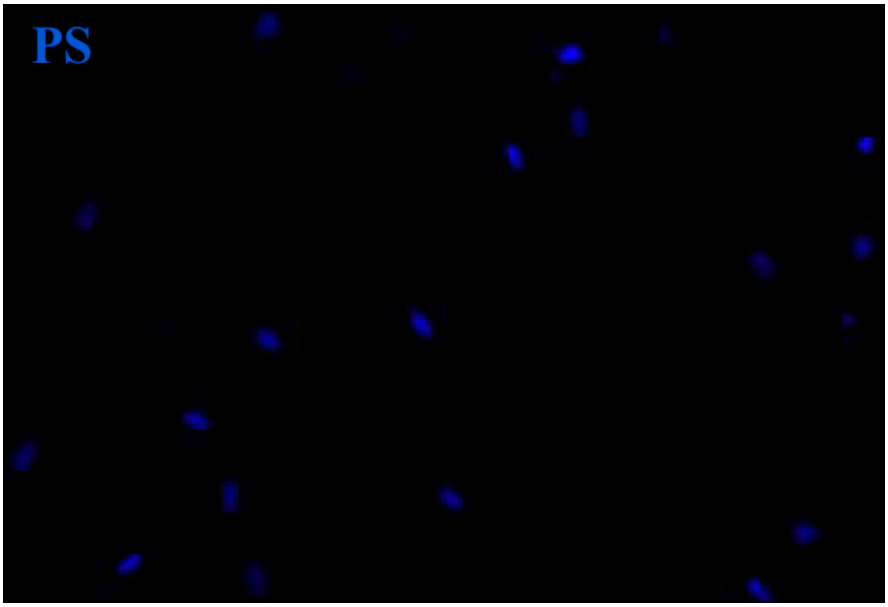
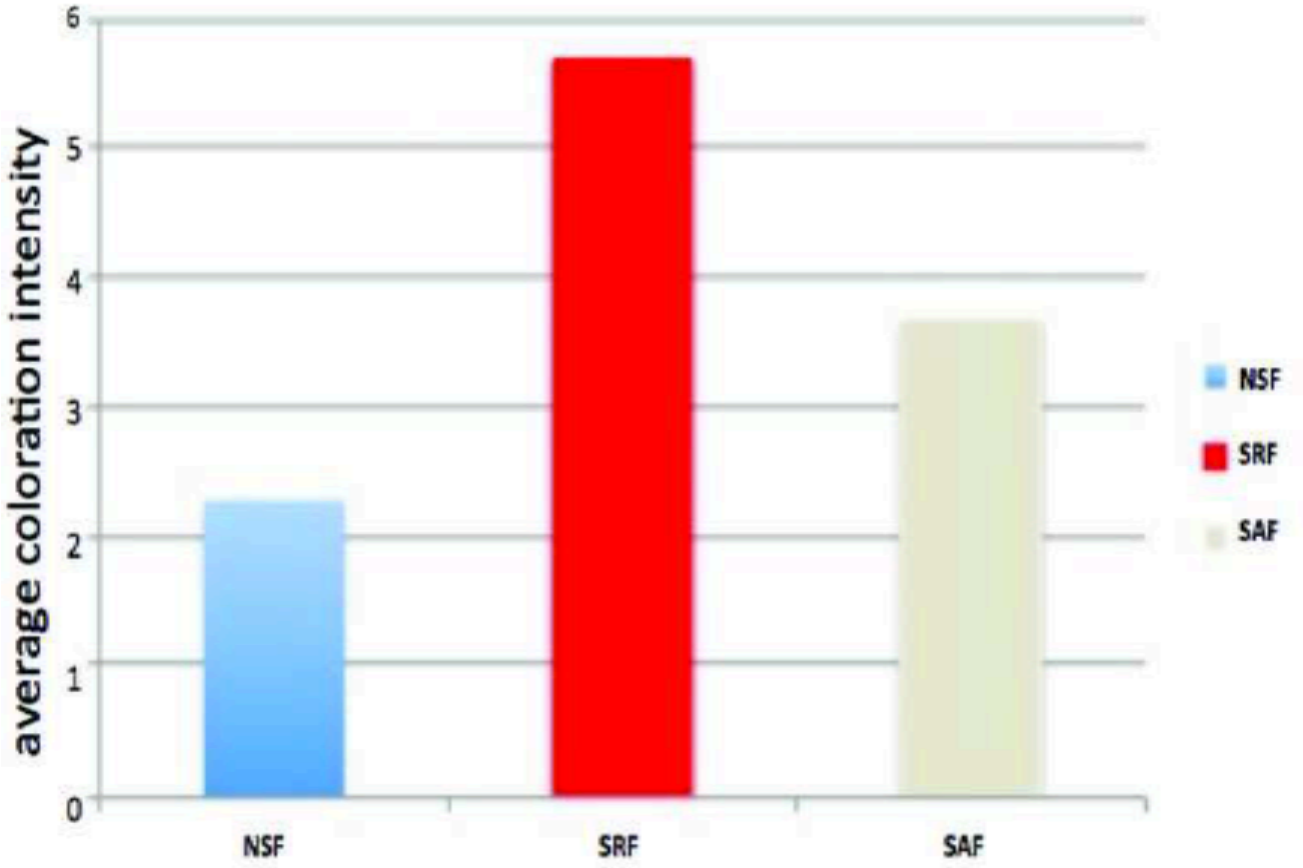


Figure 6c

Figure 7: Flowcytometry Fluorescence intensity (positivity for alpha SMA) detected by flow cytometry.



2)EFFECTS OF DIFFERENT TREATMENTS ON SAF

COLLAGEN I QUANTIFICATION BY ELISA

The average concentration of type I collagen obtained by dosing the COLIA1 from the supernatant of NSF cultures was on average 1750 ± 390 pg/mL, 550 ± 239 pg/mL in fibroblasts extracted from Striae Albae and 1313± 247 pg/mL in fibroblasts extracted from the skin with Striae Rubrae. Statistically significant differences ($p<0.05$) were observed only between the collagen production of fibroblasts from SA and healthy skin.

These results were confirmed observing SA samples at confocal microscope (fig. 8). Confocal

microscopy, which is based on tissue self-fluorescence, was used to get more reliable observations and to avoid the biases that are often associated with immunomarking.

Figure 8. Image obtained using confocal microscopy that shows SA in healthy skin. Collagen fibers appear disorganized and in reduced amounts compared to surrounding stretchmarks free skin.

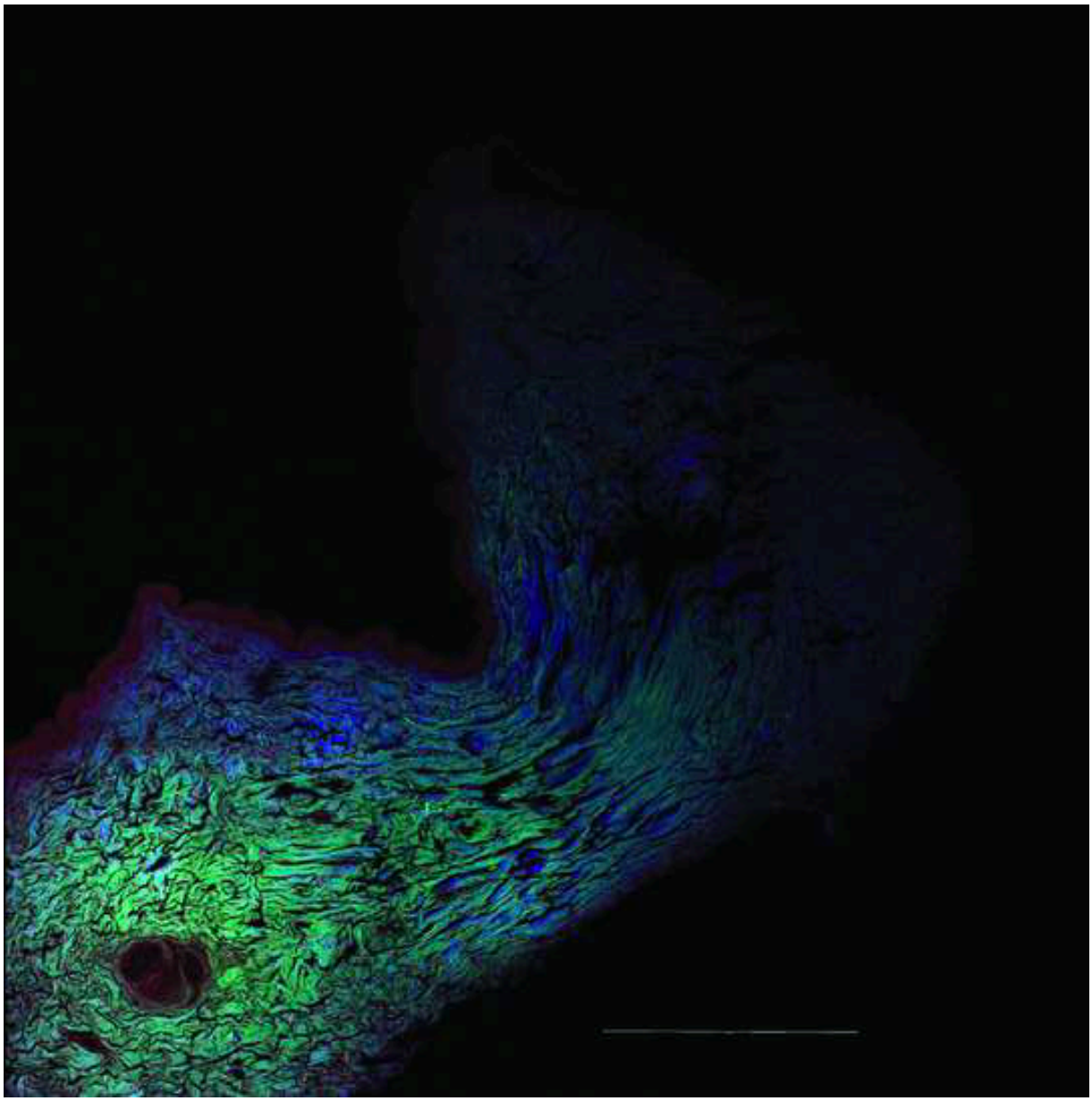
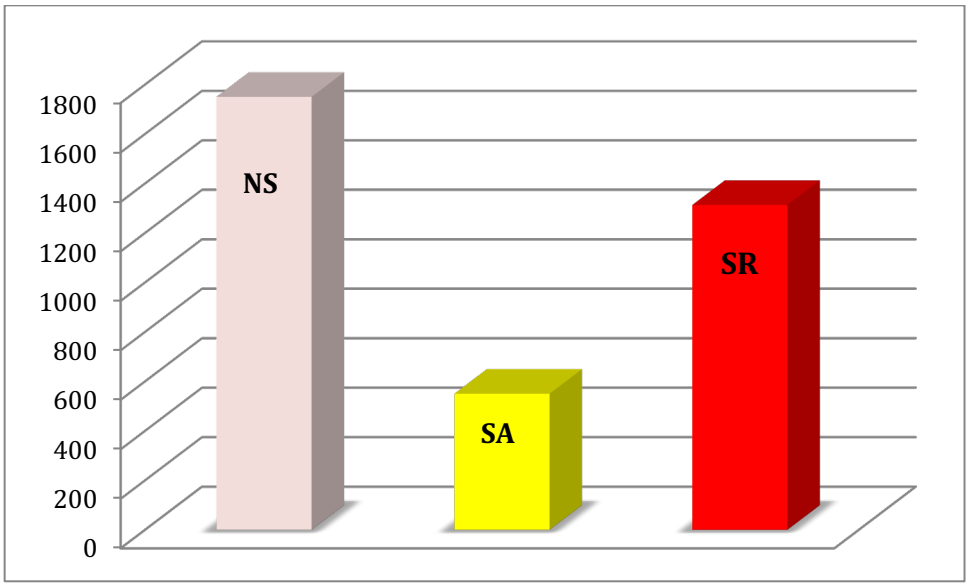


Figure 9 : Amount of pro collagen type I. X = Normal Skin (NS), Striae Albae (SA), Striae Rubrae (SR); Y = Concentration pg / ml

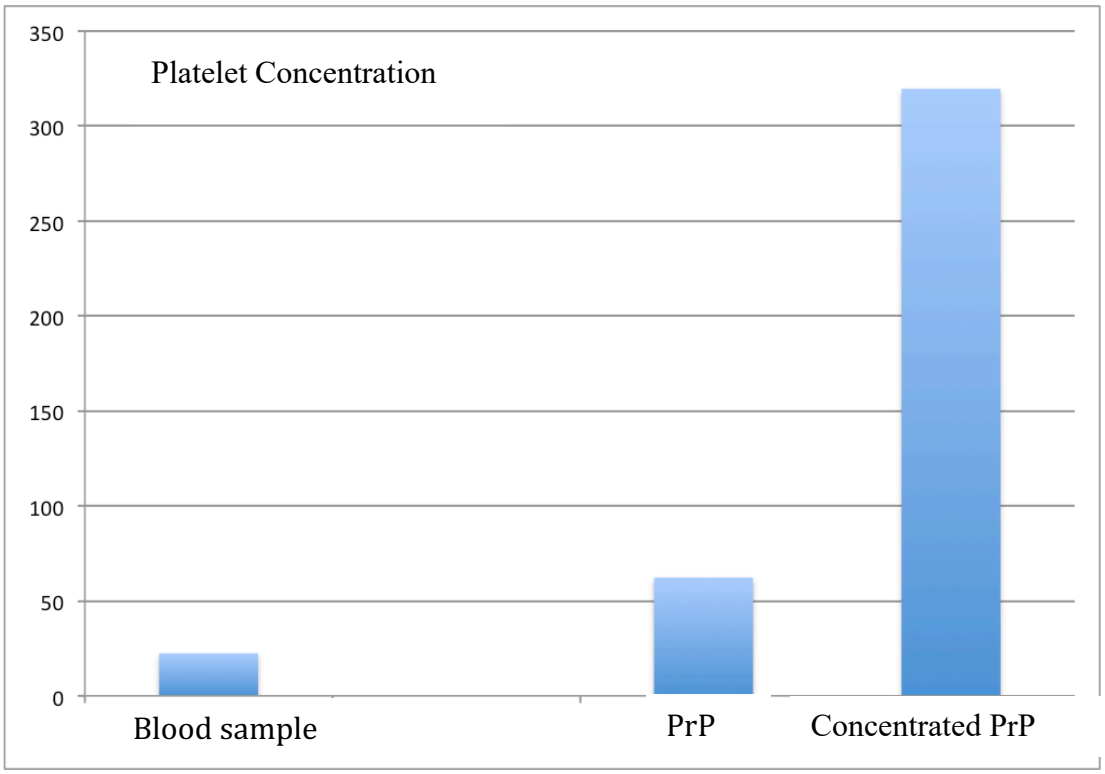


PrP Characterization

After 24 hours of serum deprivation, fibroblasts derived from SA were grown in a culture medium without serum and incubated for 24 and 48 hours with different concentrations (1%-5%) of “standard” PrP or "concentrated" PrP (cPrP) combined or not with 100 µM sodium ascorbate. The outcomes were compared to the control cultures.

The average platelet concentration (x10$_4$ µL) was 22 .4±4.59 (whole blood) before the first cycle of centrifugation was 62.3 ±13.8 for the PrP and 319.6 ± 31.8 for the concentrated PrP.

Figure 10: Average platelet concentration. X = Before centrifugation (whole blood), standard PrP, concentrated PrP; Y = Concentration (x104 µL).



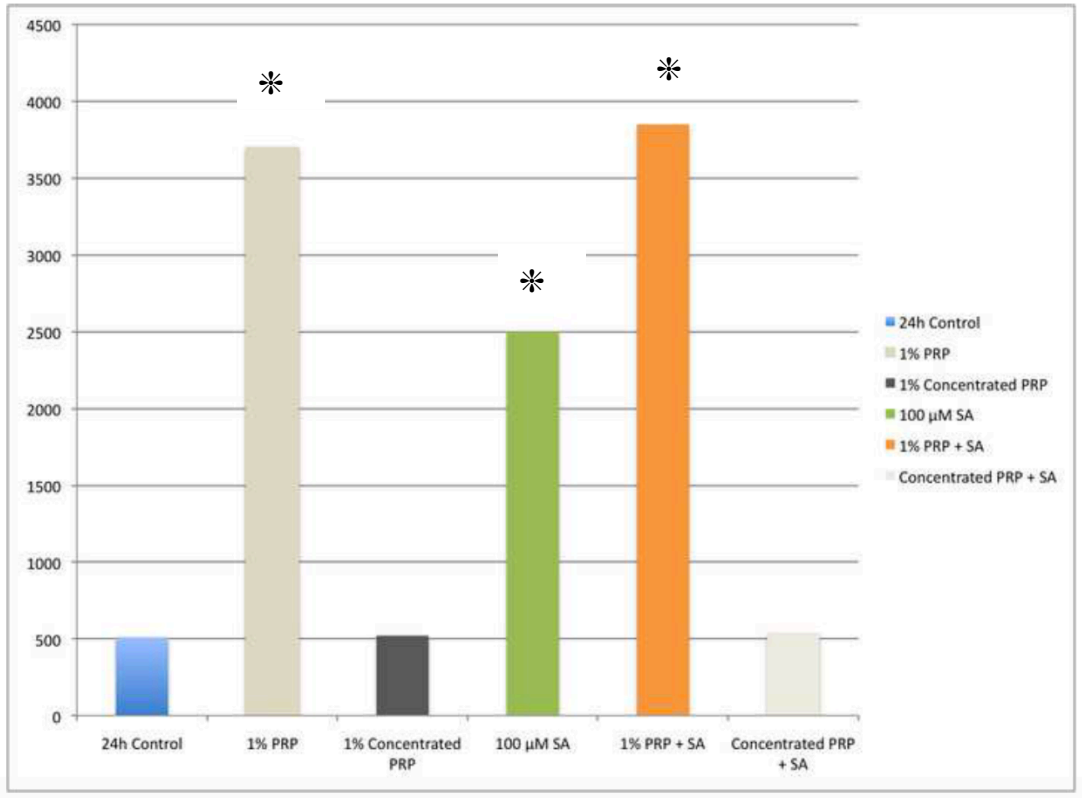
Type I Collagen quantification by ELISA after treatment administration

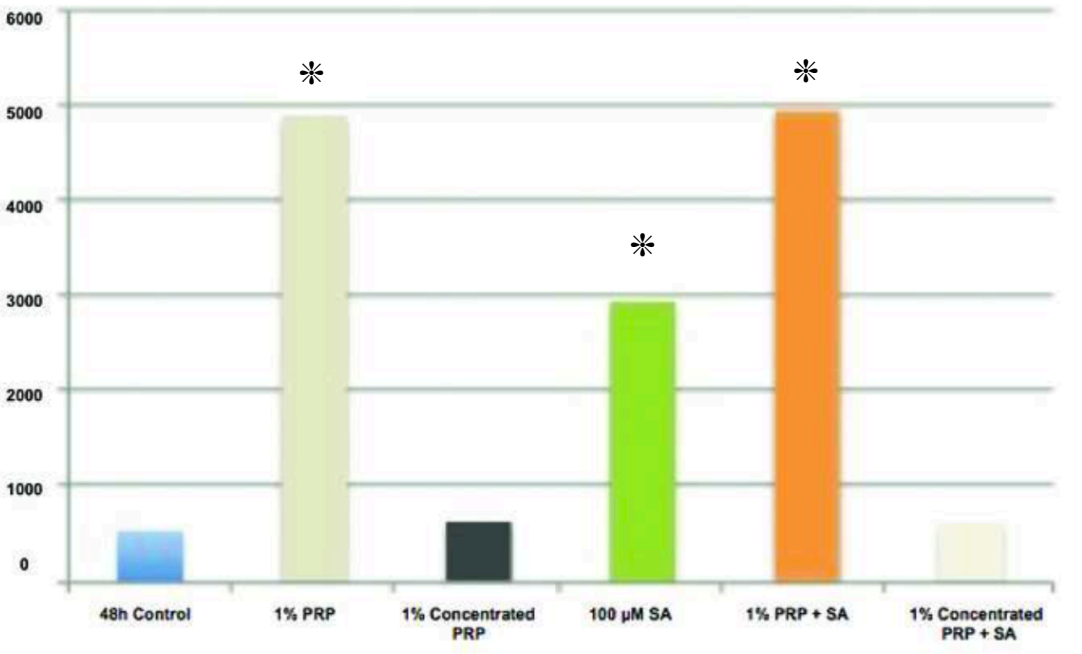
Collagen biosynthesis was measured in dermal fibroblasts from SA treated with 1% and 5% PRP, 1% and 5% concentrated PrP combined or not with sodium ascorbate (table 1). As shown, (fig. 11), PrP 1% and 5% with sodium ascorbate contributed after 24 and 48 hours of incubation to the increase in collagen biosynthesis significantly compared to the control. A greater effect was observed for cell cultures that had received a combination of Sodium PrP9 Ascorbate treatment ($p< 0 .0001$). Concentrated PrP showed a non-significant effect on collagen production ($p=0.123$).

Table 1. Representative Table of type I collagen quantification through ELISA assay. Type I pro-collagen quantity after 24 and 48 hours (pg/ml). Results are presented as mean values ± standard deviation. C-PRP= concentrated PrP; SA= 100 µM sodium ascorbate

Patients: 24h and 48h SAF cultures	Control: no treatment	1%PRP	1% C-PRP	SA	1% PRP + SA	1% C-PRP+SA
1	499,73±0,84	4354±771,7	510±0,57	2734±339,41	**4837,5**±1339	560±3,2
2	488,61±0,63	4395±760,3	521±0,23	2733±331,21	**4843,2**±1234	521±3,1
3	494,23±0,82	4377±784,2	450±0,11	2756±323,22	**4837,5**±1344	560±2,2
4	493,12±0,86	4390±821,7	500±0,22	2732±334,32	**4845,3**±1359	522±2,4
5	495,22±0,74	4399±780,5	600±0,65	2766±421,12	**4730,51**±1355	553±4,1
6	501,10±0,58	4552±774,8	522±0,21	2778±390,32	**4790,**±1249	533±3
7	490,12±0,87	4532±771,9	511±0,33	2800±321,22	**4811,5**±1431	521±2,2
8	521,73±0,84	4377±766,3	500±0,5	2766±332,45	**4690,6**±1332	585±1,9
9	478,54±0,34	4390±789,3	490±0,7	2754±190,76	**4790,1**±1321	590±3,7
10	490,34±0,23	4344±520,2	500±0,76	2733±439,11	**4921,9**±1232	600±1,8
11	552,65±0,18	4310±777,7	522±0,32	2799±336,43	**4844,6**±1039	510±2,1
12	467,45±0,67	4343±790,9	518±0,11	2890±334,76	**4830,1**±1620	470±3,2
13	497,88±0,45	4355±798,1	519±0,89	2789±332,56	**4211,32**±1300	562±4,3
14	445,98±0,76	4399±721,4	399±0,41	2711±334,43	**4867,11**±1290	577±3,9
15	511,1±0,43	4514±795,5	535±0,44	2690±339,44	**5558,9**±1258	460±3,5
16	498,43±0,23	4219±776,1	602±0,99	2766±452,67	**4233,5**±1345	569±3,1
17	499,65±0,98	4322±734,8	510±0,23	2780±344,27	**4850,8**±1365	398±3,4
18	469,34±0,45	4341±720,8	522±0,54	2934±319,54	**4890,4**±1333	597±3,8
19	514,65±0,34	4366±756,3	565±0,66	2864±509,32	**4899,7**±1292	584±3,3
20	560,89±0,56	4397±787,2	532±0,1	2751±339,51	**4696,23**±1344	490±3,8
21	499,93±0,86	4401±788,7	514±0,23	2934±379,76	**4928,11**±1506	560±3,4
22	500,56±0,83	4332±741,8	516±0,65	2532±379,83	**4887,2**±1400	571±3,1
23	496,21±0,23	4400±811,5	499±0,32	2765±329,33	**5231,3**±1106	533±3,3

Figure 11: Quantification of type I collagen through ELISA assay. X= type I pro-collagen quantity after 24 (a) and 48 hours (b); Y=Concentration pg/ml. In the images the small star (⁕) indicates that the observed differences were statistically significant **compared to the control cultures.**

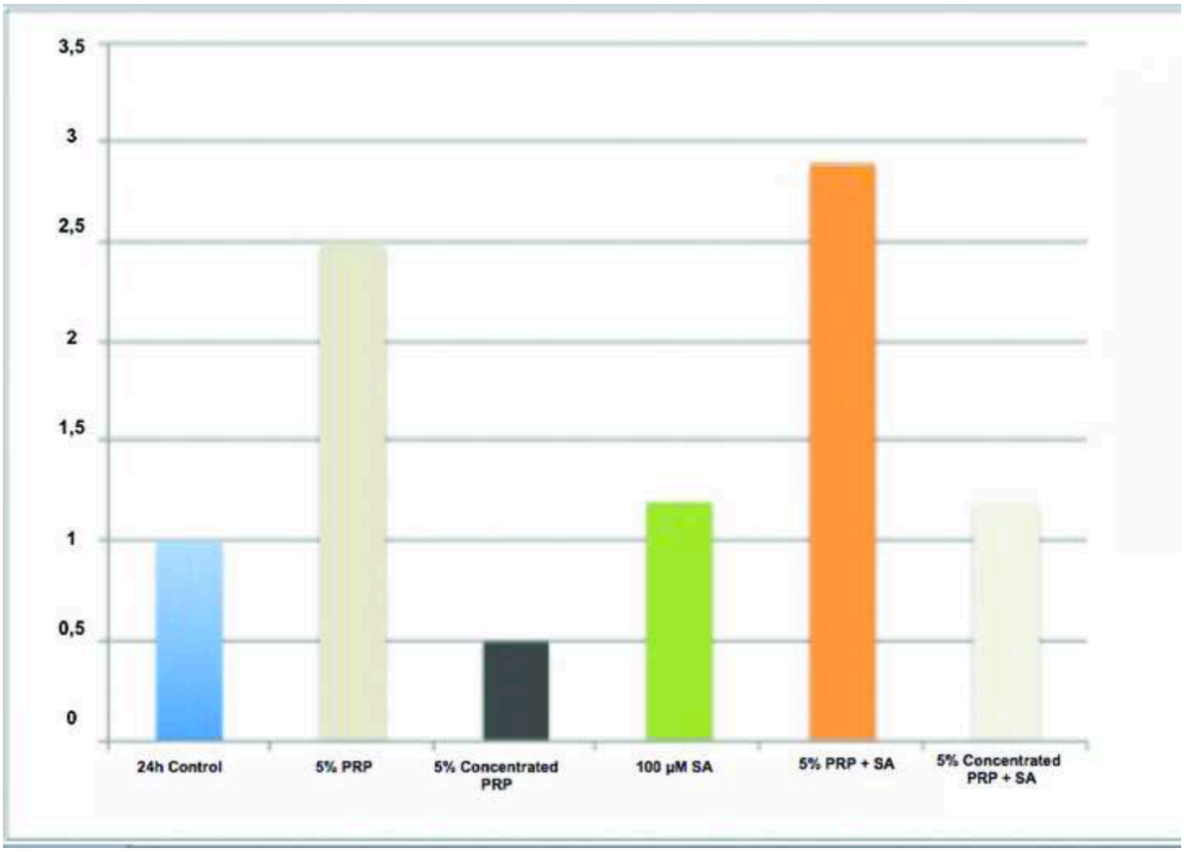


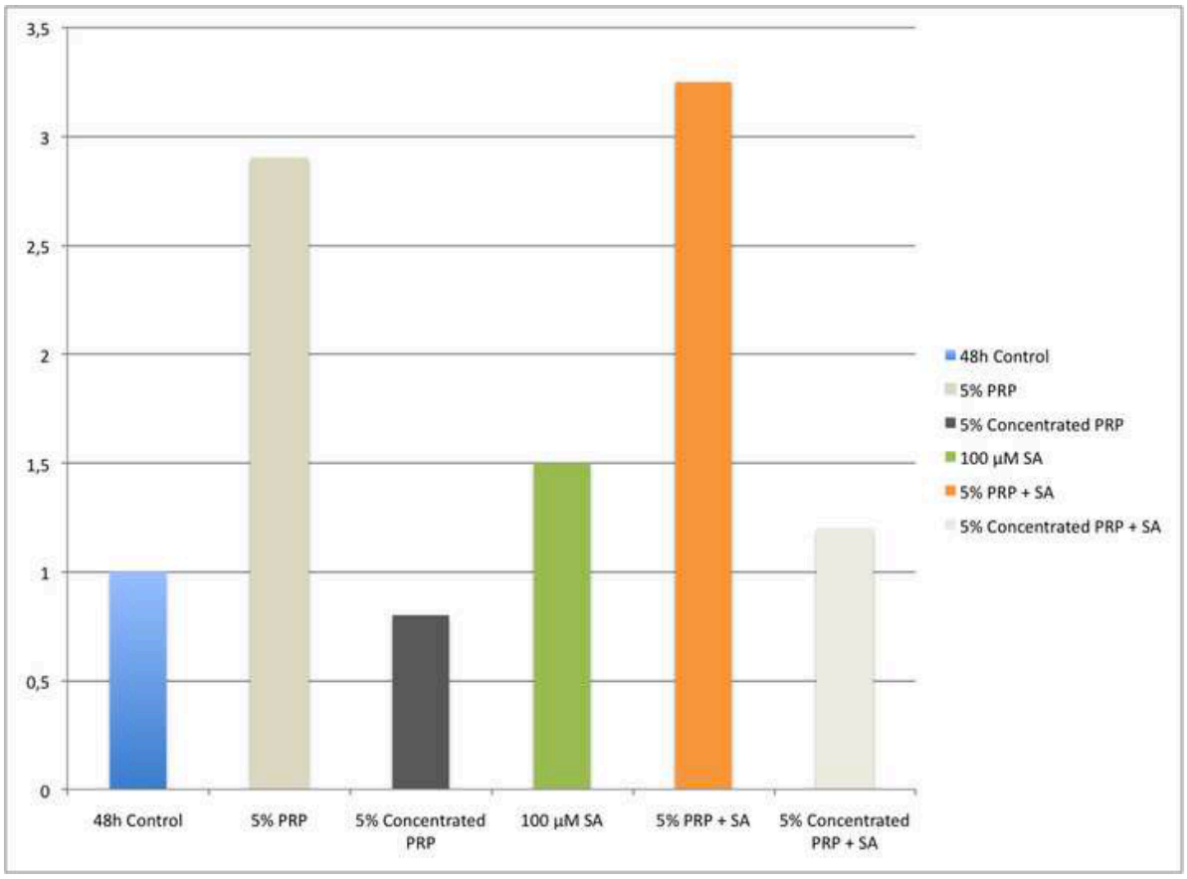


Rt-PCR for Type I Collagen quantification

Similar results were observed with RT-PCR. The expression of type I collagen mRNA was significantly increased for fibroblast cultures from SA treated with 1% and 5% PrP for 24 and 48 hours. The PrP/Ascorbate sodium combination showed an increase in the mRNA expression of type I collagen 3,2 times greater than the control after 24 and 48 hours of incubation. Concentrated PrP showed a non-significant effect on the mRNA expression of type I collagen (fig. 12).

Figure 12: Real-time PCR Analysis (type I Collagen): The expression of type I collagen mRNA after (a) 24 and (b) 48 hours (PrP 5%).





CELL PROLIFERATION

After 24 hours of incubation with PrP 1% and PrP 5% and sodium Ascorbate, cellular viability was increased by 140% and 151% and by 156 and 178% after 48 hours respectively compared to the buffer. These results were statistically significant ($p<0.05$).

Surprisingly, concentrated PrP had a small or even negative effect on cellular viability in all experiments.

Figure 13 : 24-hour SAFs proliferation (5% PrP). In the images the small star (*) indicates that the observed differences were statistically significant compared to control.

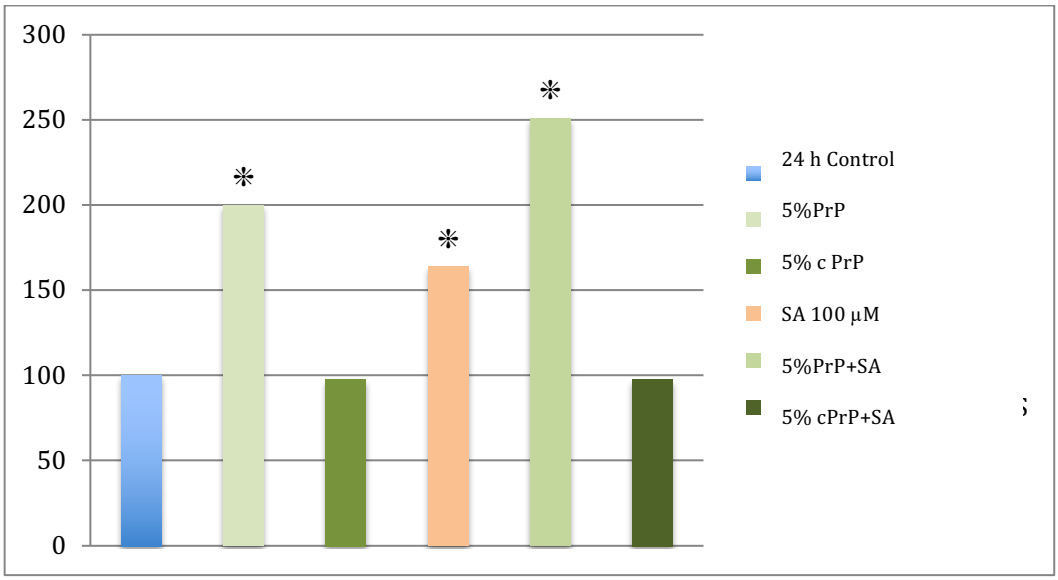
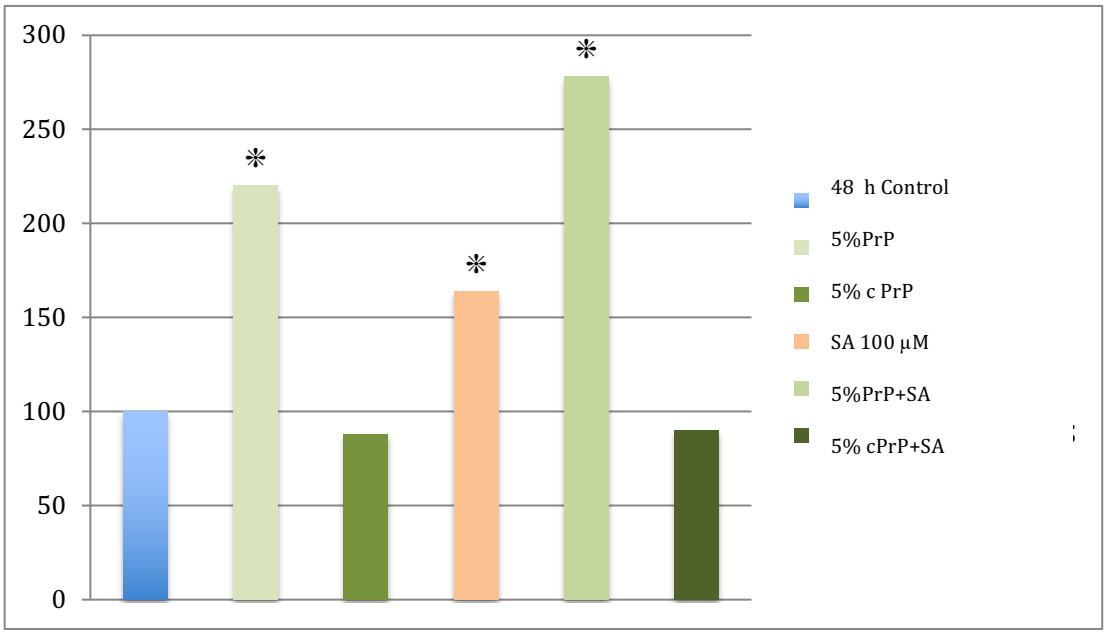


Figure 14: 48h SAFs proliferation (5% PrP). In the images the small star (*) indicates that the observed differences were statistically significant compared to control.



IMMUNOCYTOLOGY

The cytological immunoanalysis confirm our previous observations. SAF produce less type I collagen compared to healthy skin fibroblasts in the same patient. The combination of PrP and Sodium Ascorbate has been proven as the most effective treatment for the induction of collagen production in vitro (fig. 15-8).

Figure 15. Immunocytology. Fibroblasts from SA; immunomarking for collagen type I (green) and fibroblasts (red); Fibroblasts from SA 24h (a-f) and 48h (g-n) after addition of treatments to the cultures. The combination of PrP and Sodium Ascorbate has been proven as the most effective treatment for the induction of collagen production. Immunomarking collagen type I (green) and fibroblasts (red).

Fig.15a

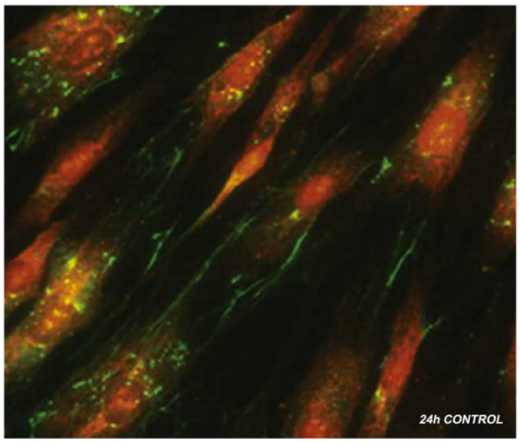


Fig.15b

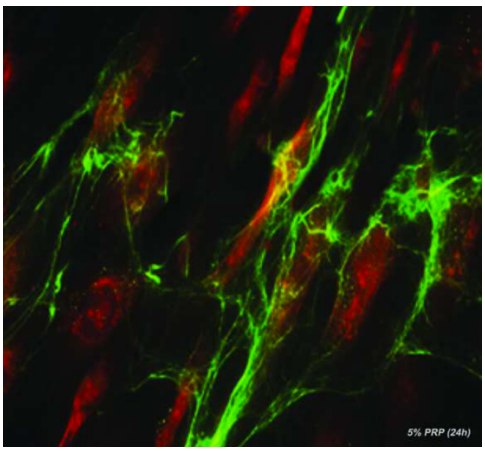


Fig. 15c

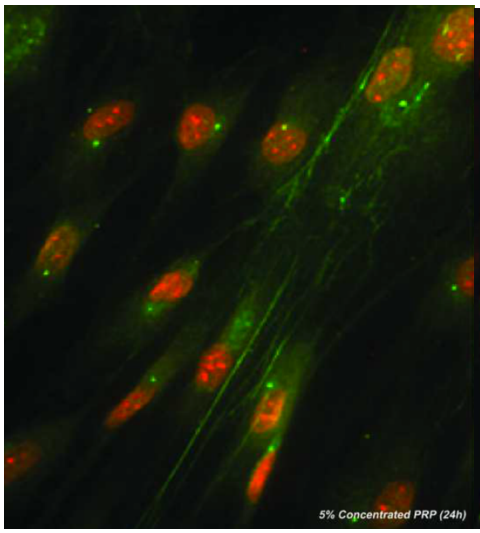


Fig.15d

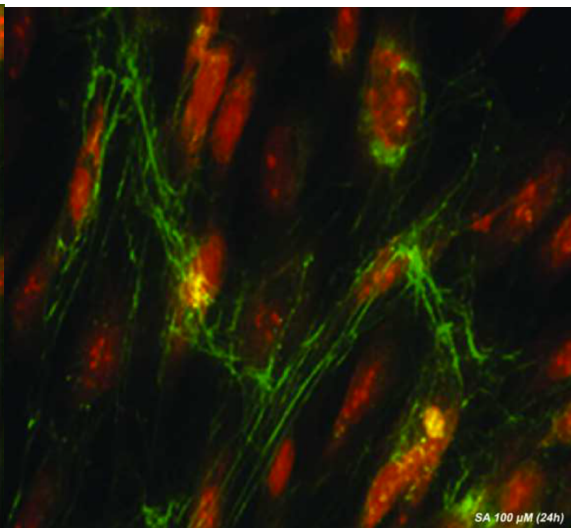


Fig.15 e

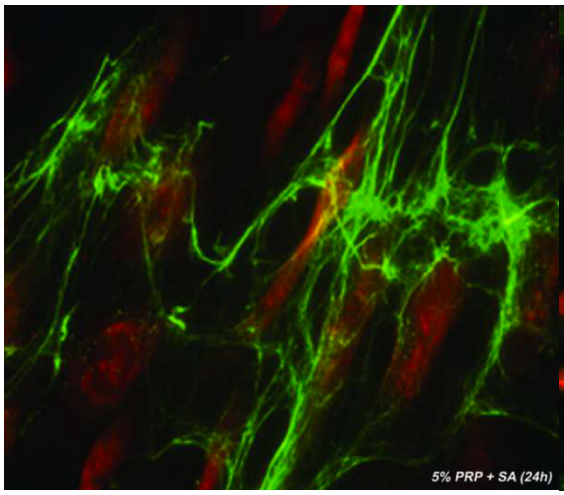


Fig.15f

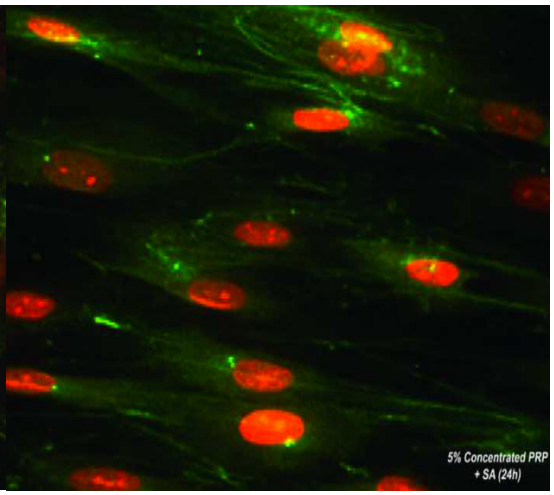


Fig. 15g

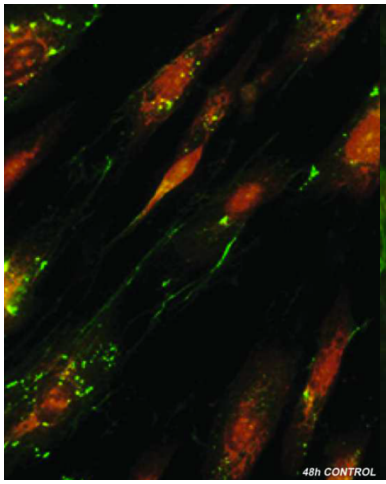


Fig.15h

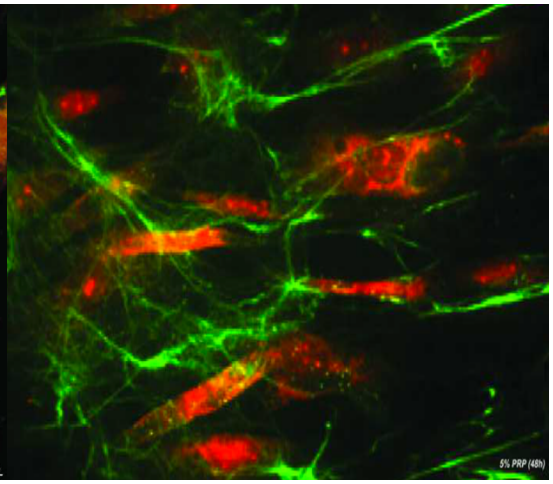


Fig. 15i

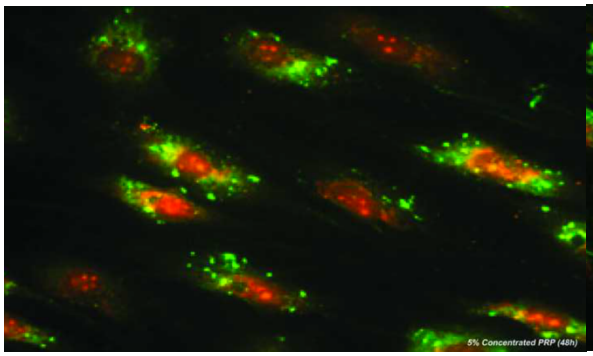


Fig.15L

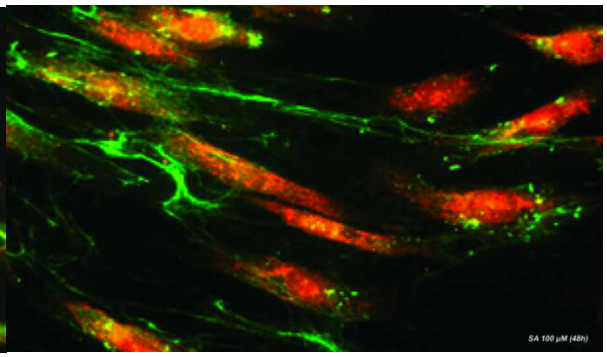


Fig 15 M

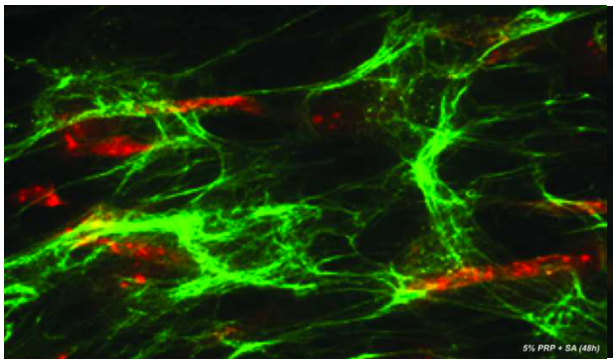


Fig 15 N

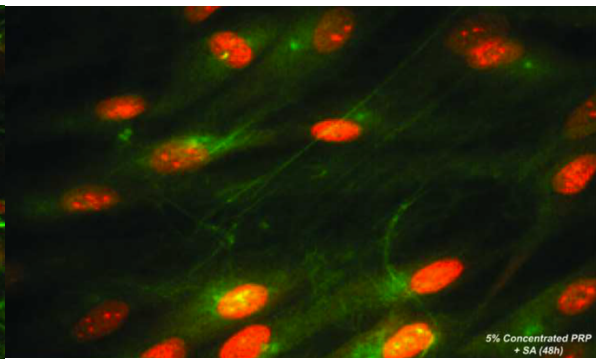
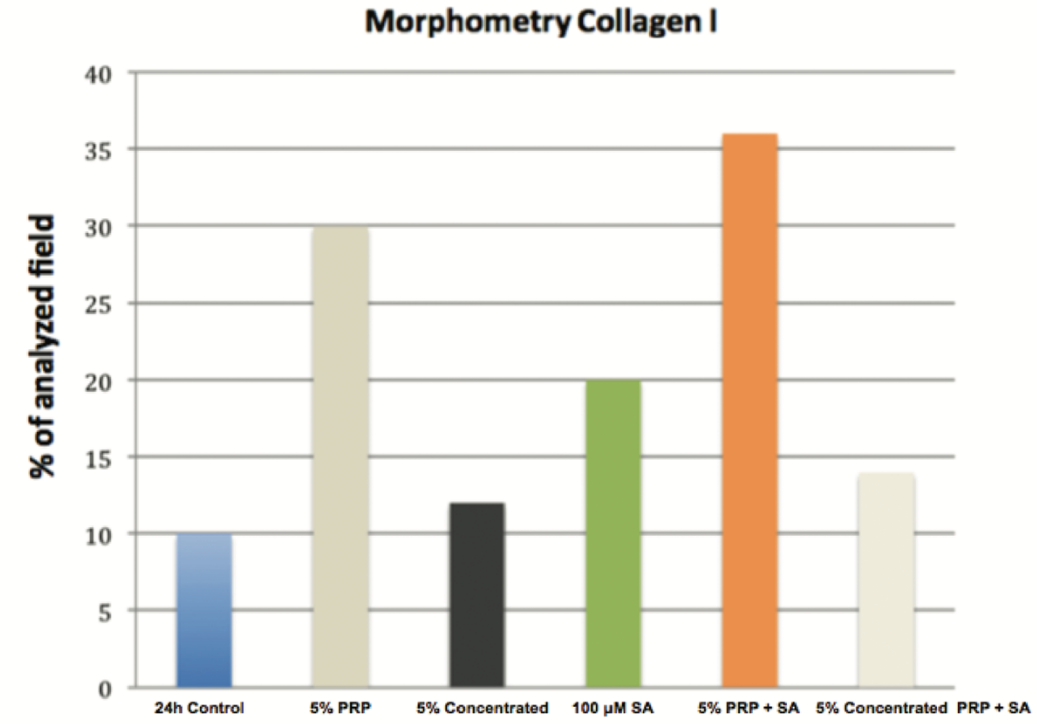
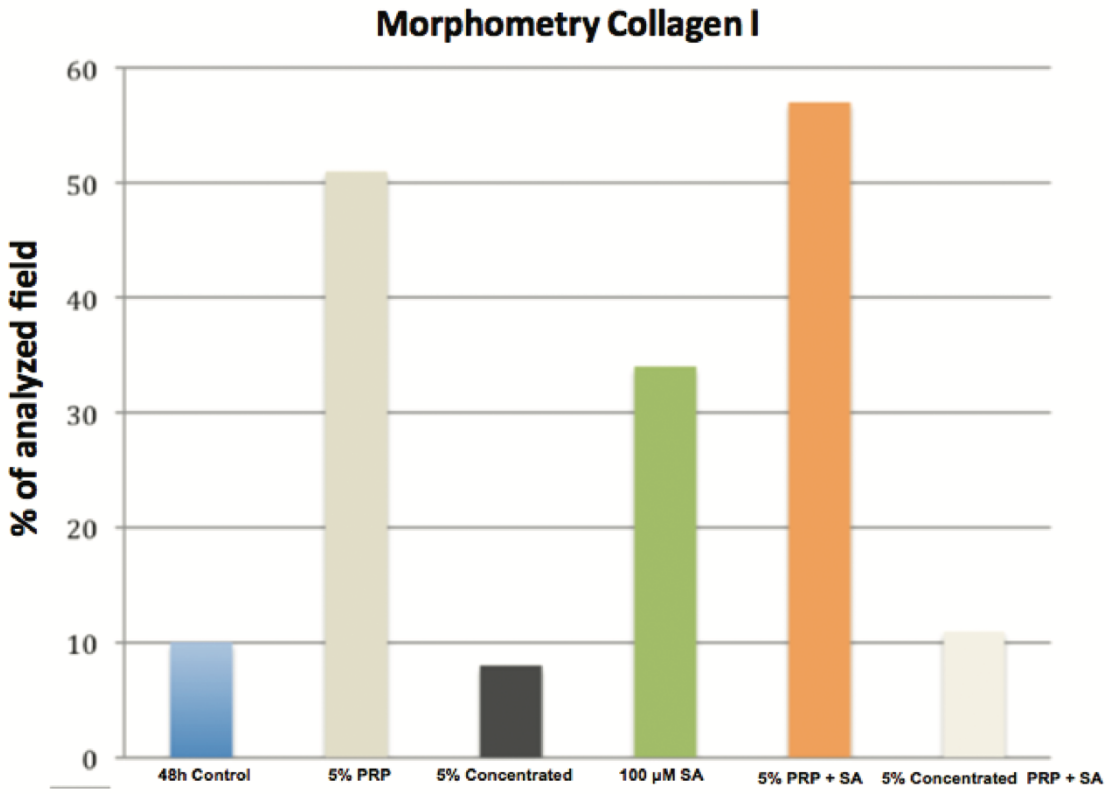


Figure 16. Results of the morphometric evaluation and quantification of type I collagen at 24h(a) and 48h (b) after addition of treatments to the cultures.





IN VIVO STUDY (OCTOBER 2019-OCTOBER 2020):

A prospective, randomized single-blind study has been conducted in fifty women aged between 37 and 50 years (43 ± 6.8) old with no comorbidities and a mean BMI < 30 of 29±1.3, presenting with SA in the abdominal region. These patients had voluntarily sought a treatment for their SMs and a conventional abdominoplasty procedure at the same time, that had been planned at least 3 months before the beginning of the study.

Exclusions criteria were ongoing treatment with corticosteroids and Cushing syndrome (both conditions alter the normal dermis metabolism).

From October 2017 to October 2019, patients had been informed of the subject and characteristics of our study. The project has been approved by the French ethics committee.

For the purpose of randomization, hundred sealed envelopes were prepared by our medical secretary. Inside the envelopes there was a piece of paper indicating whether their skin would be used for the study (50 envelopes) or not (50 envelopes). Patients were asked to choose a sealed envelope, by the same blind surgeon, that didn't know what was written on the paper inside the envelopes (if the patient would be included in the study or not).

At the end of the randomization process 25 patients were selected to be included in the study. They all accepted to test a new procedure on the SAs located on the area to be excided during the abdominoplasty procedure, and to donate their abdominoplasty remaining excess skin (which included the treated areas), which was otherwise going to be discarded after the surgery. The respect of their privacy was strictly guaranteed, and an appropriate written consent was signed by all the patients.

Each operation was performed at the Henri Mondor University Hospital by the same surgeon.

After discussion with a multidisciplinary team made up of 7 plastic surgeons and 5 dermatologists, we have decided to use **nanofat** (autologous product) and not the sodium ascorbate for the in vivo study to avoid any kind of allergic reaction which could be caused by some excipients including **sodium metabisulfite** (33).

Since the in vitro study showed a statistically significant increase in collagen production when SMFs were treated using PrP, for the in vivo study it was decided to treat the SA using:

PrP 10 ml; PrP 2ml (20%) + nanofat 8ml (80%); Nanofat 10ml.

To avoid any inter-individual variations that may interfere with the results, 4 grade 4 SA (according to our scale: The Objective Stretch Marks photo-numerical Assessment Scale-OSMAS) were selected on each patient, three were treated and the other was used as a control.

Plastic and Reconstructive Surgery
The Objective Stretch Marks photo-numerical Assessment Scale (OSMAS): a new and complete method to assess Striae Distensae.

Table1.Development of the OSMAS

SEVERITY of SD for area	No SD	Slight intensity	Mild intensity	Moderate intensity	Severe intensity
Abdomen	0	1	2	3	4
Breasts	0	1	2	3	4
Hips	0	1	2	3	4
Gluteal area	0	1	2	3	4
Back area	0	1	2	3	4
Thighs	0	1	2	3	4
Calves	0	1	2	3	4
Arms	0	1	2	3	4

Nanofat is emulsified (liquified) and filtered fat with a high concentration of adipose stem cells. Recent (2013) clinical research by Dr Tonnard (34) has shown remarkable improvements in skin quality six months after the injection of nanofat, superficially, in the dermis and subdermis. In recent few years, it has gained more popularity amongst aesthetic practitioners, though only relatively few clinics offer this advanced treatment option.

In 2013, Tonnard et al. reported on the efficiency and safety of treating skin aging with nanofat, which is different from traditional fat transplant, using intradermal injection of an emulsified fat suspension to promote skin rejuvenation.

Numerous mesenchymal stem cells (MSCs) are present in an isolated culture of nanofat and Have been designated nanofat-derived stem cells (NFSCs) (35).

NFSCs belong to a subcategory of adipose-derived stem cells (ASCs), which are known to participate in repair after injury, and their functions in skin rejuvenation have been described (36). Regeneration of collagen, elastin, extra cellular matrix, and microcirculation is crucial for reversing the signs of skin aging. An observational study of histologic and ultrastructural changes in skin after injection of fat grafts and ASCs revealed that this treatment was effective in modifying the structure of the dermis with an increase of type I collagen fibers deposition by dermis fibroblasts (37). Multiple differentiation and paracrine functions of ASCs contribute to their tissue-repairing properties, which are improved by stimulation with platelet-rich plasma (37-39).

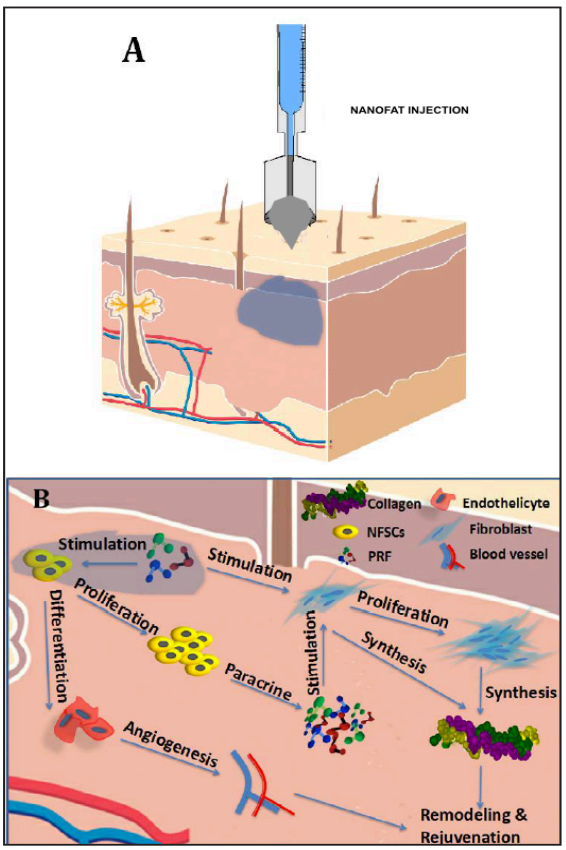


Figure 17: NANOFAT Mechanism of action

This founding let us decide to test this new treatment (nanofat + PRP) on SA.

Three months before surgery (abdominoplasty), the patients were treated in our hospital offices. The procedure was carried out under local anaesthesia. After an infiltration of adrenaline-saline solution XYLOCAÏNE 10 mg/ml with ADRENALINE 0,005 mg/ml, a 10 cc lipoaspirate simple was obtained from the abdominal region of each patient via a 10cc louer lock syringe and a "tulip" cannula with 1mm orifices .

Nanofat was obtained from the autologous fat collection; fat was then filtered via a Tulip kit by passing the adipose graft through 3 reducers (2.4mm, 1.4mm, 1.2mm) and a nano filter to obtain an emulsion. For the SAs to be treated with Nanofat + PrP, Nanofat 80% was mixed to PrP 20% (40). The SDs were located in the area of skin to be removed by abdominal dermolipectomy, that was performed three months after the treatment. PrP was obtained with the same method described for the in vitro study.

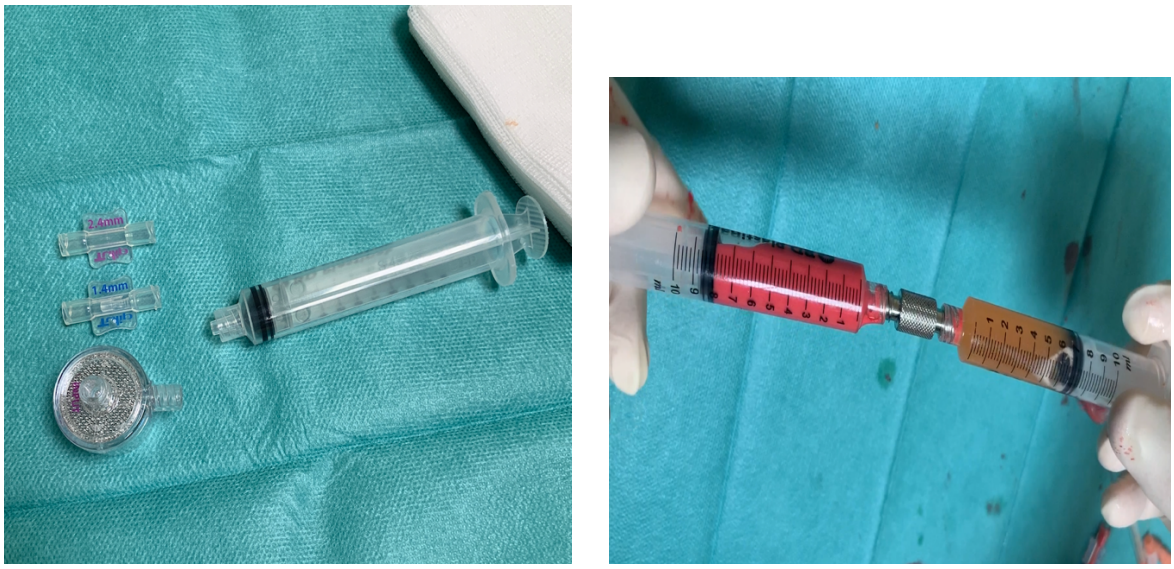


Figure 18 : Nanofat 80% + PRP 20%

The products were injected intradermally using a 10 cc syringe with a 30 gauge needle.

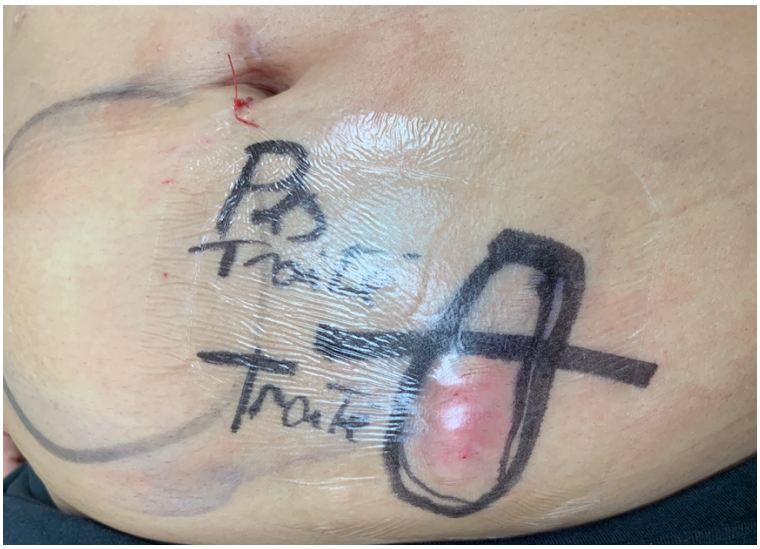


Figure 19 : Example of two SAs, one of which was treated with 80% nanofat + 20% PRP and the other received no treatment.

Following the abdominal skin and adipose *panniculus* excision, 2 biopsies (with a 2mm punch) from the treated areas (nanofat, PrP, PrP and nanofat) those not treated and from the healthy skin were performed. The biopsies were placed in formalin, and analysed using the Biotechne Elisa Human Pro-Collagen I α1 / COLIA1 kit for the quantification of type I collagen.

PROTEIN EXTRACTION AND PROCOLLAGEN I ENZYME-LINKED IMMUNOSORBENT ASSAY - Type I Collagen quantification by ELISA

Human Pro-Collagen I α1/COLIA1 Biotechne (France)

Serial frozen sections of 7, 200, and 7 μm were prepared from (optimal cutting temperature compound) OCT-embedded skin biopsy specimens. Dermal areas of 7-μm sections were measured with Image ProPlus software (Media Cybernetics, Bethesda, Maryland) and used to calculate the volume of the 200-μm sample. Soluble proteins were extracted from the 200-μm sample in ice-cold extraction buffer (50mM Tris hydrochloride, pH 7.4; 0.15M sodium chloride; 1% Triton X-100; protease inhibitors [Complete Mini; Roche Diagnostics, Indianapolis,

Indiana]). After 5-minute centrifugation at 10 000g at 4°C, supernatants were assayed for procollagen I by means of a commercial enzyme-linked immunosorbent assay kit (Human Pro-Collagen I α1/COLIA1 Biotechne - France). Procollagen I concentrations were normalized to the volume of tissue used for each sample.

The statistical analysis of the data were carried out with the PRISM Software (Graph Pad, USA). The difference in the obtained results have been analyzed using the T-test. (Statistical significance $p < 0.05$).

QUANTIFICATION OF COLLAGEN I BY ELISA

The mean concentration of type I collagen obtained by the COLIA1 assay of the healthy skin biopsies (normal skin-NS) was on average 15850 ± 390 pg / mL, and 3950 ± 239 pg / mL for the biopsies of skin with Striae Albae (untreated SA: SA-NT).

As can be seen (figure 20), the PrP and the nanofat contributed to increase the biosynthesis of collagen significantly compared to the control (SA-NT). A greater effect was observed for SA treated with the combined PrP-Nanofat treatment (p <0 .0001).

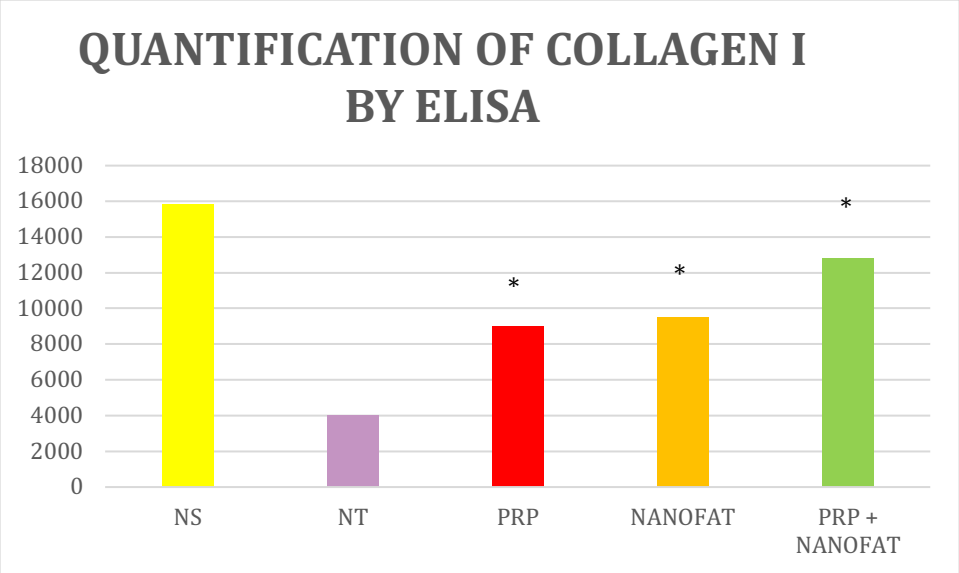


Figure 20. NS : normal skin; NT : control not treated (SA-NT)

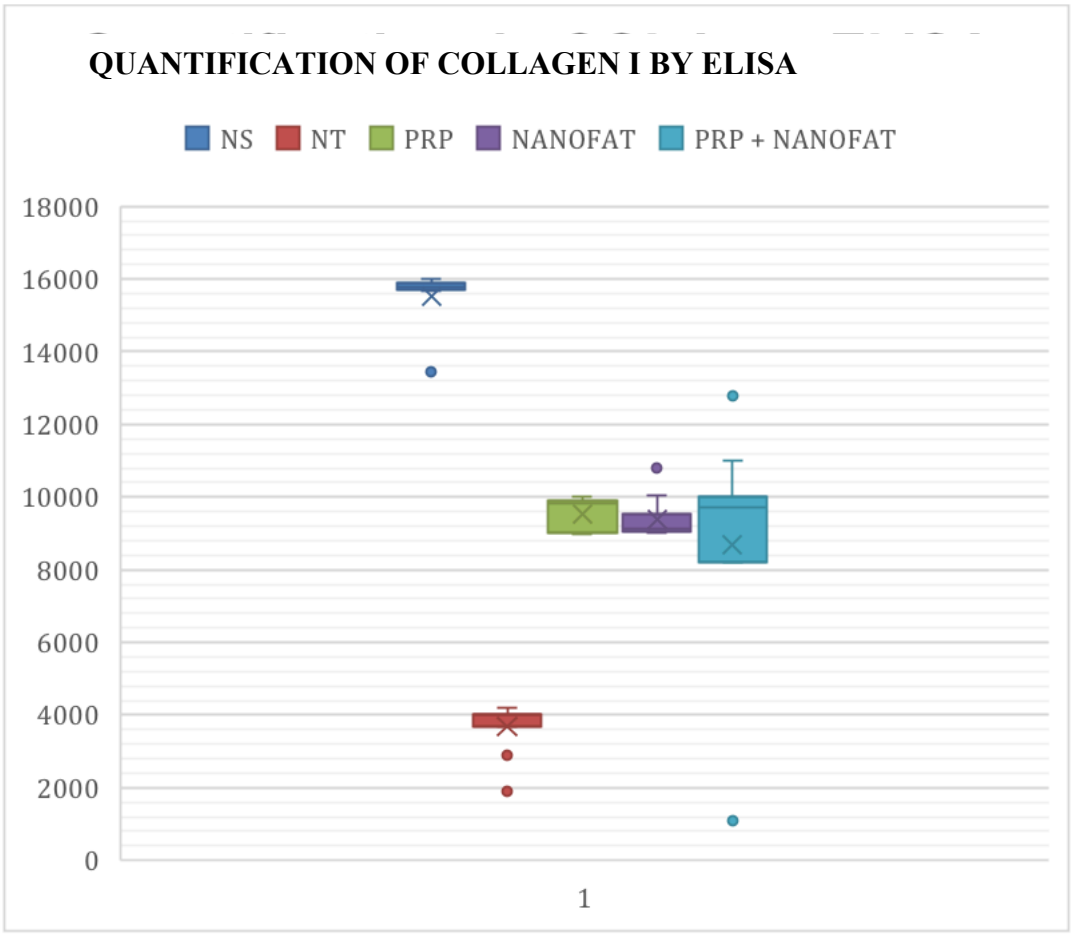


Figure 21 : Amount of pro collagen type I; Y = Concentration pg / ml

In the images the small star (⁕) indicates that the observed differences were statistically significant compared to the control.

DISCUSSION

Several mechanisms appear to be at the basis of the onset and chronicization of Striae Distensae (15). An alteration in the fibroblast phenotype that presents itself differently in initial and old lesions appears to be the necessary and fundamental event. These cells seem to react to the mechanical stress that occurs in the skin, first turning into myofibroblasts (alpha SMA +) then into quiescent fibroblasts (alpha SMA-). The mechanical forces generated by the cells have a very important biological role at the cellular level (as for mechanical transduction, vasoregulation etc.) and tissue level (maintains tone). These forces can be out of order and be the basis of certain anomalies, sometimes with a loss of function (41-42). Anomalies and especially differences in forces generated by the fibroblasts resulting from the SD were observed through our experiments. Our results highlight the different mechanical behavior of fibroblasts in vitro depending on the origin of the cells. Based on their mechanical properties, SMF appear as a different population from healthy fibroblasts. This observation is consistent with the idea of fibroblast heterogeneity (43).

This observation also explains a key moment in the appearance of SDs, the mechanical stress that skin undergoes during pregnancy or every weight change in an individual. The alpha SMA+ phenotype that characterizes the SRF is significantly reduced in SAF.
In a stressed matrix, in the case of strained collagen lattices, the strength of recent stretch mark fibroblasts is significantly greater than that developed by healthy fibroblasts and old stretch mark fibroblasts. This observation would therefore explain one of the key moments in the appearance of SD, that is to say the mechanical stress to which the skin is subjected during pregnancy or with each weight change in the individual. The contraction of fibroblasts in strained lattices results from the contractile activity of the actin-filament cytoskeleton, which develops into stress fibers characteristic of myofibroblasts. The alpha SMA positive phenotype which characterizes the fibroblasts of SRs is strongly reduced in the fibroblasts

from old stretch marks. Our results confirm and provide additional information to that published by Viennet et al. (44).

SAFs combined stimulation with standard PrP and sodium ascorbate showed a resumption of the metabolic activity of these cells by an increase in the production of type I collagen and cell proliferation. Guszczyn et al. (45) recently published his results on the stimulation of normal fibroblasts by PrP (46). His study also demonstrated an increase in the synthesis of type I collagen by fibroblasts in cultures that had been incubated with different concentrations of PrP. It seems to us that so far no study has tested PrP on cultures of SAFs.
In our study the stimulation with the combined PrP / Sodium Ascorbate therapy was found to be superior compared to other treatments, these treatments had never been tested in combination. Also the results of the *in vivo* study demonstrated that the PrP and the nanofat contribute to increasing collagen biosynthesis significantly compared to the control (untreated SA). A greater effect was observed for SA treated with the combined PrP-Nanofat treatment. This is the first time that this type of treatment has been used for SD.

The importance of finding an effective strategy for the treatment of SD and especially SA, is due to the fact that the occurrence of stretch marks is a significant event, even traumatic especially for women and that currently, there is no has no resolving treatment for SD. Our results, although encouraging, require further testing to confirm their reproducibility.
Our study shows encouraging results and also opens the doors to other fields of application of this therapy such as, for example, stimulation of skin regeneration in burn patients, chronic wounds or all types of conditions where stimulation of the fibroblasts would be necessary.

FUTURE PROSPECTS:

The interest of a combined treatment capable of stimulating pathological fibroblasts to produce an effective amount of collagen could be useful not only in the context of SDs, but also in other conditions. The stimulation and proliferation of fibroblasts and keratinocytes induced by these methods, could be used to restore the skin in severe burns.

Other applications can be envisaged, for example in the treatment of chronic wounds. It has recently been demonstrated that dermal fibroblasts of patients with Ehlers-Danlos Syndrome can return to a normal phenotype if exposed to physiological concentrations of collagen (47). Other trials on the use of our treatments could be performed on the dermal fibroblasts of these patients, etc.

Recently Youssef et al. (11) demonstrated a significant increase in the expression of the androgen receptor and glucocorticoids in early lesions (SR), while a declined expression of the estrogen receptor was observed. The results obtained by these authors underline the importance of these hormone receptors in the development of striae. This information is important for studying new targets for the prevention and treatment of SD.

REFERENCES

1. Paus R. Exploring the‘‘brain-skin connection’’: Leads and lessons from the haïr follicle. Curr Res Transl Med 2016;64:207-14.
2. Rinkevich Y, Walmsley GG, Hu MS,Maan ZN, Newman AM, Drukker M, et al. Skin fi brosis. Identifi cation and isolation of a dermal lineage with intrinsic fi brogenic potential. Science 2015;348:aaa2151.
3. Kwan P, Desmoulière A, Tredget EE. Molecular and cellular basis of hypertrophic scarring. In: Herndon DN (ed). Total Burn Care. (4th edition). Paris;Elsevier, 2012:497-505.
4. Hinz B. The myofi broblast: paradigm for a mechanically active cell. J Biomech 2010;43:146-55.
5. Aarabi S, Bhatt KA, Shi Y, Paterno J, Chang EI, Loh SA, et al. Mechanical load initiates hypertrophic scar formation through decreased cellular apoptosis. FASEB J 2007;21:3250-61.
6. Yagmur C, Akaishi S, Ogawa R, Guneren E. Mechanical receptorrelated mechanisms in scar management: a review and hypothesis. Plast Reconstr Surg. 2010;126:426-34.
7. Britt ter Horst, Gurpreet Chouhan, Naiem S. Moiemen, Liam M. Grover. Advances in keratinocyte delivery in burn wound care.. Adv Drug Deliv Rev. 2018; 123: 18–32.
8. Gilmore SJ1, Vaughan BL Jr, Madzvamuse A, Maini PK. A mechanochemical model of striae distensae. Math Biosci. 2012 Dec;240(2):141-7.
9. A.J. Arem, C.W. Kischer, Analysis of striae, Plast. Reconstr. Surg. 65 (1) (1980) 22-9.
10. Cohen-Letessier A., Cutaneous striae : a disorder of fibroblasts? Aetiologic theories. Nouv Dermatol, 1998, 17. 431-435.

11. Youssef SES1, El-Khateeb EA, Aly DG, Moussa MH. Striae distensae: Immunohistochemical assessment of hormone receptors in multigravida and nulligravida. J Cosmet Dermatol. 2017 Jun;16(2):279-286. doi: 10.1111/jocd.12337. Epub 2017 Apr 4.
12. H.M. Sheu, H.S. Yu, C.H. Chang, Mast cell degranulation and elastolysis in the early stage of striae distensae, J. Cutan. Pathol. 18 (6) (1991) 410.
13. G. Singh, L.P. Kumar, Striae distensae, Indian J. Dermatol. Ve. 71 (5) (2005) 370.
14. K.S. Lee, Y.J. Rho, S.I. Jang, M.H. Suh, J.Y. Song, Decreased expression of collagen and fibronectin genes in striae distensae tissue, Clin. Exp. Dermatol. 19 (4) (1994) 285.
15. Hersant B, Niddam J, Meningaud JP. Comparison between the efficacy and safety of platelet-rich plasma vs microdermabrasion in the treatment of striae distensae: clinical and histopathological study. Cosmet Dermatol. 2016; 15:565.
16. Guida S, Galimberti MG, Bencini M, Pellacani G, Bencini PL. Treatment of striae distensae with non-ablative fractional laser: clinical and in vivo microscopic documentation of treatment efficacy. Lasers Med Sci. 2018 Jan;33(1):75-78.
17. Antonopoulou I, Varriale S, Topakas E, Rova U, Christakopoulos P, Faraco V. Enzymatic synthesis of bioactive compounds with high potential for cosmeceutical application. Appl Microbiol Biotechnol. 2016 Aug;100(15):6519-6543.
18. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant Dent. 2001;10(4):225–228.
19. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 2009;23(4):177–189.

20. Amable PR, Carias RB, Teixeira MV, et al. Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors. Stem Cell Res Ther. 2013;4(3):67
21. Donjacour AA, Cunha GR. Stromal regulation of epithelial function. Cancer Treat Res. 1991;53:335–364.
22. Veronesi F, Pagani S, Torricelli P, Filardo G, Cavallo C, Grigolo B, Fini M. PRP and MSCs on tenocytes artificial wound healing: an in vitro study comparing fresh and frozen PRP. Histol Histopathol. 2018 (3):18018. doi: 10.14670/HH-18-018.
23. Jun Araki, Masahiro Jona, Hitomi Eto, Noriyuki Aoi, Harunosuke Kato, Hirotaka Suga, Kentaro Doi, Yutaka Yatomi, Kotaro Yoshimura. Optimized Preparation Method of Platelet-Concentrated Plasma and Noncoagulating Platelet-Derived Factor Concentrates: Maximization of Platelet Concentration and Removal of Fibrinogen. Tissue Eng Part C Methods. 2012 Mar; 18(3): 176–185.
24. Carola Cavallo, Alice Roffi, Brunella Grigolo, Erminia Mariani, Loredana Pratelli, Giulia Merli, Elizaveta Kon, Maurilio Marcacci, Giuseppe Filardo. Platelet-Rich Plasma: The Choice of Activation Method Affects the Release of Bioactive Molecules. Biomed Res Int. 2016; 2016: 6591717.
25. Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg Off J Am Assoc Oral Maxillofac Surg. 2004 Apr;62(4):489–96.
26. Cervelli V, Gentile P, Scioli MG. Application of platelet-rich plasma in plastic surgery: clinical and in vitro evaluation. Tissue Eng Part C Methods 2009; 15(4): 625-634.
27. Cervelli V, De Angelis B, Lucarini L, Sapallone D, Balzani A, Palla L, Gentile P, Cerulli P. Tissue regeneration in loss of substance on the lower limbs through us e of

platelet-rich plasma, stem cells form adipose tissue, and hyaluronic acid. Adv Skin Wound Care 2010; 23(6):262-272.

28. Roubelakis MG, Trohatou O, Roubelakis A, Mili E, Kalaitzopoulos I, Papazoglou G, Pappa KI, Anagnou NP. Platelet-rich plasma (PRP) promotes feta mesenchymal stem/stromal cell migration and wound healing process. Stem Cell Rev 2014Jun;10(3):417-28.
29. Eppley BL, Pietrzak WS, Blanton M. Platelet-rich plasma: a review of biology and applications in plastic surgery. Plast Reconstr Surg. 2006;118(6):147–159.
30. Tajima S, Wachi H, Hayashi A. Accumulation of tropoelastin by a short-term ascorbic acid treatment in the culture medium of aortic smooth muscle cells in vitro. Keio J Med 1995;44:140–5.
31. Corti A, Casini AF, Pompella A. Cellular pathways for transport and efflux of ascorbate and dehydroascorbate. Arch Biochem Biophys 2010;500:107–15.
32. Godoy A, Ormazabal V, Moraga-Cid G, Zuniga FA, Sotomayor P, Barra V, et al. Mechanistic insights and functional determinants of the transport cycle of the ascorbic acid transporter SVCT2. Activation by sodium and absolute dependence on bivalent cations. J Biol Chem 2007;282:615–24.
33. Cussans, Amelia; McFadden, John; Ostlere, Lucy. Systemic sodium metabisulfite allergy. Contact dermatitis. 2015, Vol.73(5), p.316-317
34. Tonnard P, Verpaele A, Peeters G, Hamdi M, Cornelissen M, Declercq H. Nanofat grafting: basic research and clinical applications. Plast Reconstr Surg. 2013;132(4):1017-26.
35. Liang ZJ et al. Precise Intradermal Injection of Nanofat-Derived Stromal Cells Combined with Platelet-Rich Fibrin Improves the Efficacy of Facial Skin Rejuvenation. Cell Physiol Biochem 2018;47:316–329

36. Charles-de-Sá L, Gontijo-de-Amorim NF, Maeda-Takiya C, Borojevic R, Benati D, Bernardi P, Sbarbati A, Rigotti G: Antiaging treatment of the facial skin by fat graft and adipose-derived stem cells. Plast Reconstr Surg 2015;135:999–1009.

37. Xu FT, Liang ZJ, Li HM, Peng QL, Huang MH, Li de Q, Liang YD, Chi GY, Li DH, Yu BC, Huang JR: Ginsenoside Rg1 and platelet-rich fibrin enhance human breast adipose-derived stem cell function for soft tissue regeneration. Oncotarget 2016;7:35390–35403.

38. Keyhan SO, Hemmat S, Badri AA, Abdeshahzadeh A, Khiabani K: Use of platelet-rich fibrin and platelet-rich plasma in combination with fat graft: which is more effective during facial lipostructure? J Oral Maxillofac Surg 2013;71:610–621.

39. Cameli N, Mariano M, Cordone I, Abril E, Masi S, Foddai ML: Autologous pure platelet-rich plasma dermal injections for facial skin rejuvenation: clinical, instrumental, and flow cytometry assessment. Dermatol Surg. 2017;43:826–835.

40. Picard F , Hersant B , La Padula S, Meningaud JP. Platelet-rich plasma-enriched autologous fat graft in regenerative and aesthetic facial surgery: Technical note. J Stomatol Oral Maxillofac Surg. 2017 Sep;118(4):228-231.

41. Pushkarsky I, et al. (2018). Elastomeric sensor surfaces for high-throughput single-cell force cytometry. Nature Biomedical Engineering 2: 124-137.

42. Wang N., Butler J.-P., Ingber D.-E., Mechanotransduction across the cell surface and through the cytosqueleton. Science, 1993, 260, 1124-1127.

43. Sappino A.-P., Schürch W., Gabbiani G., Biology of disease : différentiation repertoire of fibroblastic cells : expression of cytoskeletal proteins as marker of phenotypic modulations. Lab. Invest., 1990, 63, 144-161.

44. Viennet C, Bride J, Armbruster V, Aubin F, Gabiot AC, Gharbi T, Humbert P. Contractile forces generated by striae distensae fibroblasts embedded in collagen lattices. Arch Dermatol Res. 2005 Jul;297(1):10-7.

45. Guszczyn T, Surażyński A, Zaręba I, Rysiak E, Popko J, Pałka J. Differential effect of platelet-rich plasma fractions on β1-integrin signaling, collagen biosynthesis, and prolidase activity in human skin fibroblasts. Drug Des Devel Ther. 2017 Jun 22;11:1849-1857.

46. Peng GL. Platelet-Rich Plasma for Skin Rejuvenation: Facts, Fiction, and Pearls for Practice. Facial Plast Surg Clin North Am. 2019. 27(3):405-411.

47. Viglio S, Zoppi N, Sangalli A, Gallanti A, Barlati S, Mottes M, Colombi M, Valli M. Rescue of migratory defects of Ehlers-Danlos fibroblasts in vitro by type V collagen but not insulin-like binding protein-1. 2008, J Invest Dermatol 128:1915-19.