DI.VAL TOSCANA SRL
Via Madonna del Piano, 6
50019 Sesto Fiorentino (FI) CF -
VAT number: 06237650483 Tel.
+39 055 4574661
http://www.divalsrl.com Email: info@divalsrl.com

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| **Editing by** | Dr. Massimo D'Amico |
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**Protocol of**

# FINAL RELATION

**relating to the**

## "STUDY FOR THE VALIDATION, AT THE CELLULAR LEVEL, OF TREATMENTS BASED ON THE USE OF FIELDS ©LIMFA ULTRA WEAK ELECTROMAGNETIC /PHASE 1: STUDY ON COLLAGEN PRODUCTION"

**INDEX**

## GENERAL INFORMATIONS

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| Client | **Eywa srl**<br>Via E. Rodriguez 13 - 47921 Rimini (RN)<br>**Dr. Tommaso Faiella** |
| Analysis laboratory | **DI.VAL TOSCANA SRL**<br>Via Madonna del Piano, 6 – 50019 Sesto Fiorentino (FI) |
| Study director | Dr. Massimo D'Amico<br>Email: massimo.damico@divalsrl.com |

## 2. PURPOSE OF THE STUDY

The aim of the study is to evaluate the effects of a treatment with LIMFA fields ©(treatment schedule "Collagen Regeneration") on human fibroblast cell growth and collagen secretion type I in the cell supernatant.

## 3. BIBLIOGRAPHICAL REFERENCES

The study is based on indications from the literature (Rodemann, HP et al., Exp Cell Res, 182: 610-621, 1989; Rodemann HP et al., Scanning Microscop, 5: 1135-1142, 1991), according to which prolonged treatments using electromagnetic fields induce collagen secretion of human fibroblasts.

## 4. EQUIPMENT

Biohazard Laminar Flow Hood (Steril)

Incubator $CO_2$(sanyo)

Centrifuge (Beckman Coulter)

Primo Vert Inverted Light Microscope (Zeiss)

Microplate reader with ELISA filters equipped with Gen5™ sw (Bio-Tek Instruments)

LIMFA Therapy console equipped with a special transducer/applicator (supplied by the Customer)

## 5. MATERIALS

Human adult fibroblast cell line of dermal origin (HDFa Cat. No. C-013-5C, Gibco, Life technologies).

Culture medium consisting of: Medium 106 (Gibco, Life Technologies) with addition of 1X Low serum Growth Supplement (LSGS, Gibco, Life Technologies).

Recombinant Human TGFβ1 (R&D Systems).

Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich).

Trypsin-EDTA 1X in PBS (Euroclone)

Trypan blue solution 0.4% (Sigma-Aldrich).

Cell reagent proliferation Reagent WST-1 (Roche).

DuoSet Elisa-Human Pro-Collagen I α1/COL1A1 (R&D Systems).

DuoSet Ancillary Reagent Kit2 (R&D Systems).

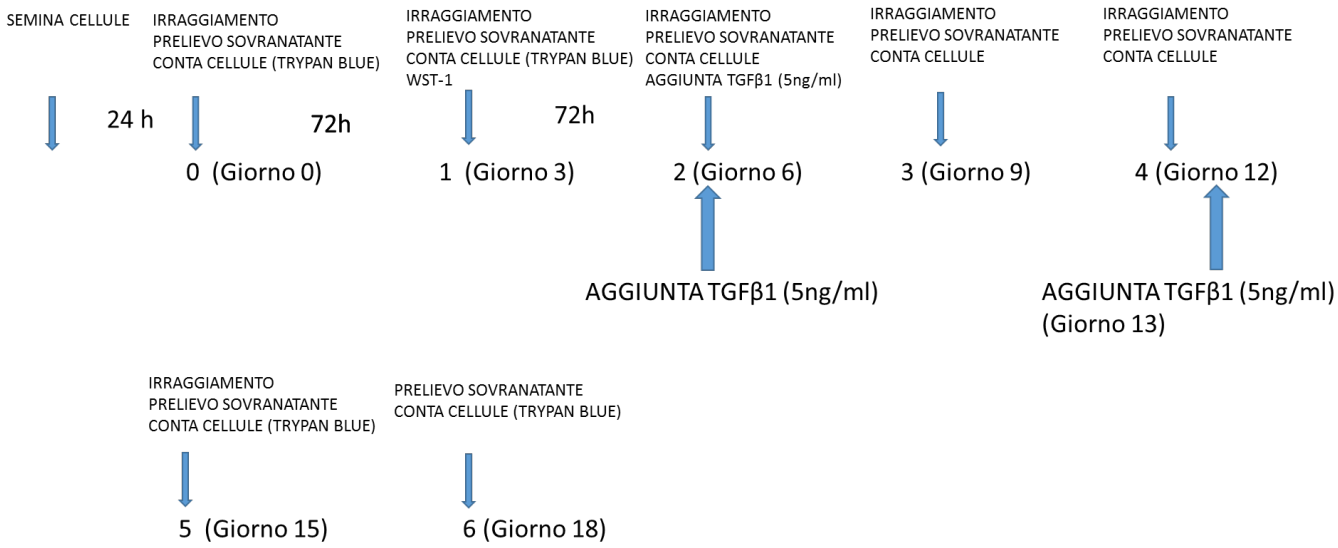
Cell culture plastic (Euroclone).

## 6. STUDY PROTOCOL AND EXPERIMENTAL DESIGN

Starting from time zero, and for the subsequent 5 treatments, at 72-hour intervals (see experimental scheme shown below), the following parameters were analysed: (1) cell viability (by both assays of exclusion of Trypan Blue and WST-1 test); (2) secretion of Pro-collagen type I α1. (For the related procedures see EXPERIMENTAL PROCEDURES). The same parameters were also evaluated a 3 days after the conclusion of the experimental treatment. It was set up, upon achievement of the cell growth plateau, also a “positive control” represented by treated HDFa cells with TGF-β 1, in which the same parameters reported above were evaluated.

In order to obtain statistical significance, 3 experiments were performed in parallel, and the respective analyzes (cell viability and collagen secretion) were performed in triplicate.

SCHEMA SPERIMENTALE




## 7. STUDY DURATION

The study had a total duration of 19 days.

## 8. EXPERIMENTAL PROCEDURE

The following operations were performed:

- Thawing of the cell line of human adult fibroblasts of dermal origin HDFa. Upon arrival, the cells were cryopreserved in liquid nitrogen. Just before thawing the line, the Low serum Growth Supplement was added to the M106 culture medium. Four 25cm flasks with 5 ml of medium per flask they were placed in an incubator at 37°C in an atmosphere of 5% $CO_2$ for 0.5h. The vial with the cells was removed from the liquid nitrogen and the lower part placed in a water bath at 37°C until its contents were completely thawed. Under the laminar hood the contents were resuspended with 1 ml of complete medium and 20 µl of the cell suspension were withdrawn in order to evaluate the number of total and viable cells. The number of cells was found to be 600,000 cells, all of which were viable. The remainder of the vial was divided equally into the 4 flasks previously incubated. After ten days the seeded cells reached confluence.

- Cell amplification (growth). The cells contained in the 4 flasks were amplified with a passage 1:2 (passage 1) in order to reach the number necessary to perform the experiment.
- Cell seeding in 24- and 96-well mutiwells . 24-well multiwells were used for each of the 3 experiments performed in parallel in order to carry out the supernatant withdrawals and cell counts, while 96-well multiwells were used for the tests involving the WST-1 dye. In the wells of the 96 multiwells, 5 X 10 were seeded in triplicate for each time and for each condition $^3$HDFa cells (step 1) for a total volume of 200 µl of complete medium per well. In those of 24, always in triplicate for each time and for each condition, 30 X 10 were seeded $^3$cells, in a volume of 1 mL of complete medium per well.

- Irradiation using the LIMFA Therapy system . At each time point (as per the experimental scheme reported above), the respective multiwells, after being parafilmed, were

subjected to irradiation using the LIMFA "Collagen Regeneration" system. This protocol lasting 19' and 10” was performed at room temperature (23-25°C). The multiwells were placed in direct contact with the surface of the transducer/applicator taking care to verify that all wells with the cells were within the surface of it. At the same time, the controls and the "positive controls", ie those treated with TGFβ1, after being parafilmed, were also kept in the same climatic conditions. At the end of the irradiation time all the multiwells were removed from the parafilm and placed in the incubator at 37°C and 5% $CO_2$.

- Treatment with TGF β1 . At the times indicated in the experimental scheme, 5 ng/ml of TGF β1 were added to each well of the respective multiwells.
- Collection and storage of cellular supernatants . After 1 hour from the irradiation and after an optical microscope check of the conditions of the cell cultures, at the relative times indicated in the Experimental Scheme, the cell supernatants were taken from all the 24 wells. These were placed in sterile 1.5 mL vials and stored at -20°C. This procedure was performed for all points except for the last point (point 6 or day 18). For this, however, the supernatant and the DPBS, with which the wells are washed before applying the trypsin, were centrifuged. This is in order to perform a correct cell count. On this day, in fact, a conspicuous presence of cells in suspension was noted.
- Cell count by Trypan Blue exclusion test. Viable and dead cells were counted in each well of the 24-well multiwell from which the supernatant was taken. After having been detached by trypsinization, the cells were counted with a Burker cell counter chamber, using the test using a Trypan blue solution. This dye is, in fact, capable of selectively coloring dead cells. The values expressed as mean ± ESM (Standard error of the mean) deriving from the triplicate count of all three experiments are reported in the Annex.

- Evaluation of cell viability using the WST-1 assay. The WST-1 or 4- [3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3 cell proliferation assay was used to verify cell viability and proliferation. -benzene disulfonate (Roche Diagnostics, Mennheim, Germany). This is a colorimetric assay for spectrophotometric quantification

of cell proliferation, viability and cytotoxicity, performed entirely in a 96-well plate. The tetrazolium salt (WST-1) is cleaved to the final, soluble and intensely colored formazan by mitochondrial dehydrogenases. This bioreduction is strictly dependent on the glycolytic production of NAD(P)H in viable cells. Thus, the amount of formazan formed correlates directly with the number of metabolically active cells in the culture. At the moment of the determination the cells were incubated with the WST-1 reagent, ready to use, for 0.25-5 hours, in a humidified atmosphere (37°C, 5% $CO_2$). During this incubation period, the formazan dye was quantified with a multi-well spectrophotometer (ELISA reader), using a wavelength at 450 nm. The measure of absorbance correlates directly with the number of viable cells. The absorbance of complete medium (blank) alone was subtracted from the absorbance measured in the samples.

- Elisa test for the quantification of pro-collagen I α1 production. At the end of the experiment all the supernatants collected during the study were thawed and a 100 µl aliquot was taken from each one (sample per well). By means of the DuoSet Elisa Kit containing the basic components for the development of an Elisa "sandwich" it was possible to quantify the concentration of pro-collagen I α1. The "capture" antibody of this kit specifically recognizes an epitope in the N-pro peptide (aa 26-161).
  The optical density of each well into which the standards and samples were loaded was determined by reading with the microplate reader at a wavelength of 450 nm. Through the use of the standards, inserting the mean of the absorbance measured against their relative concentration, the curve was calculated which allowed us to trace the concentration of the single samples applied.

## 9. STATISTICAL ANALYSIS

The values reported in Annex A are the mean ± SME (Standard Error of the Mean) of the cell count of three wells of each point (day).

Both for cell counting, for WST-1 and for the determination of pro-collagen concentrations

The α1 secreted values reported in the graphs are the weighted mean ± ESM of 3 experiments performed separately in parallel.

The statistical analyzes relating to the quantity of procollagen I secreted were carried out by applying the t Test by Student, considering significant the differences having values of $p \leq 0.05$.

## 10. RESULTS

### Radiation treatment

Below are the values of the weighted means ± ESM of the cell counts of live cells only under controlled conditions and subjected to irradiation using the LIMFA system. The averages have been graphed both as columns (Fig. 1) and as a time course of growth over the different days (Fig.2).

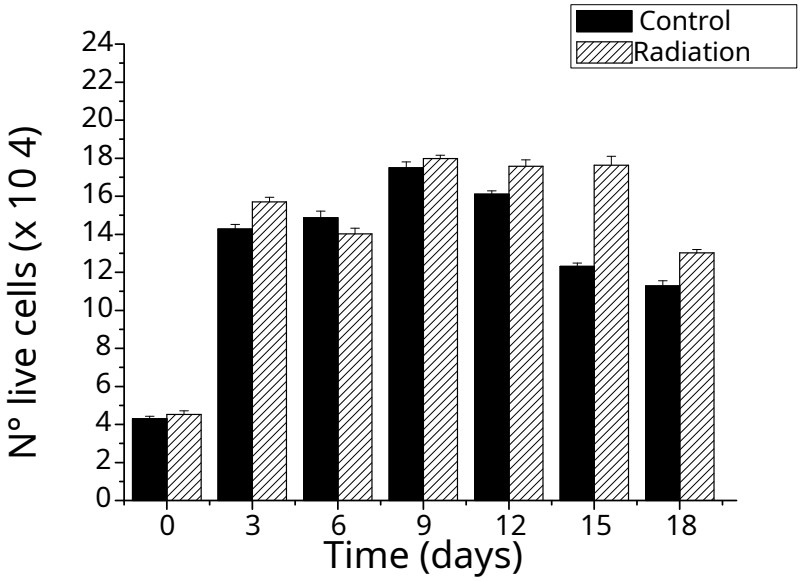



**Fig.1**. Cell viability under control conditions and after irradiation

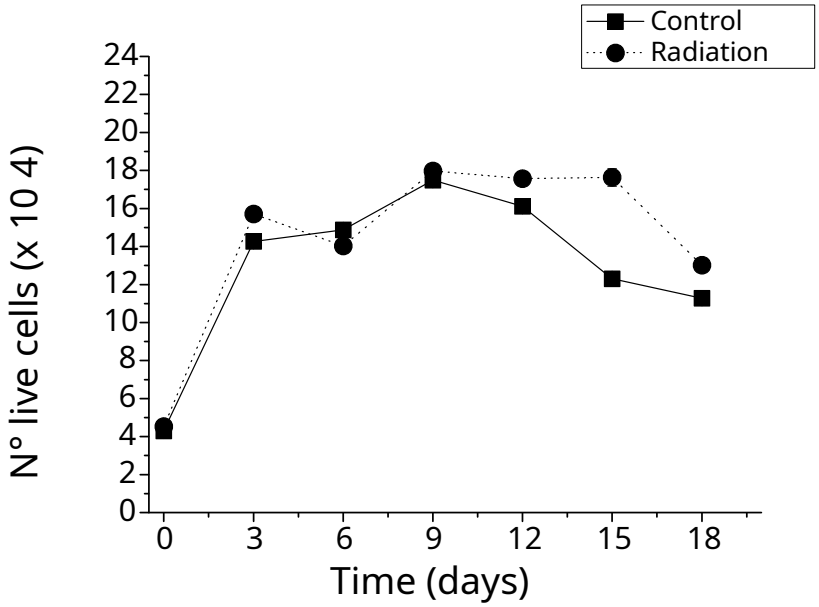



**Fig.2**Time course of cell viability of control cells and those subjected to irradiation

Furthermore, a column graph of the number of dead cells in the two different conditions was obtained experimental. A significant number of dead cells (> 0.05 X $10_4$) has only been encountered since Day 12 (Fig. 3).

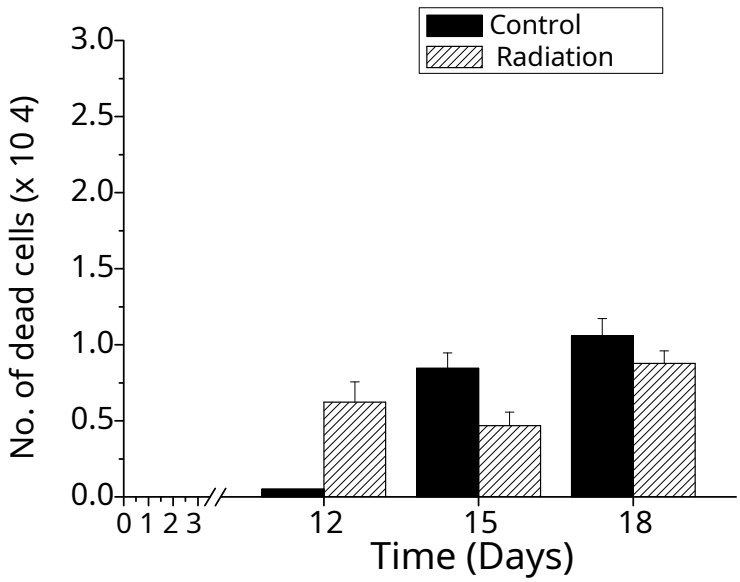



**Fig.3**Number of dead cells in control condition and after irradiation.

Below are the weighted means ± ESM of the normalized absorbances for the control obtained by WST-1 assay on days 3, 8 and 13 (Fig. 4).

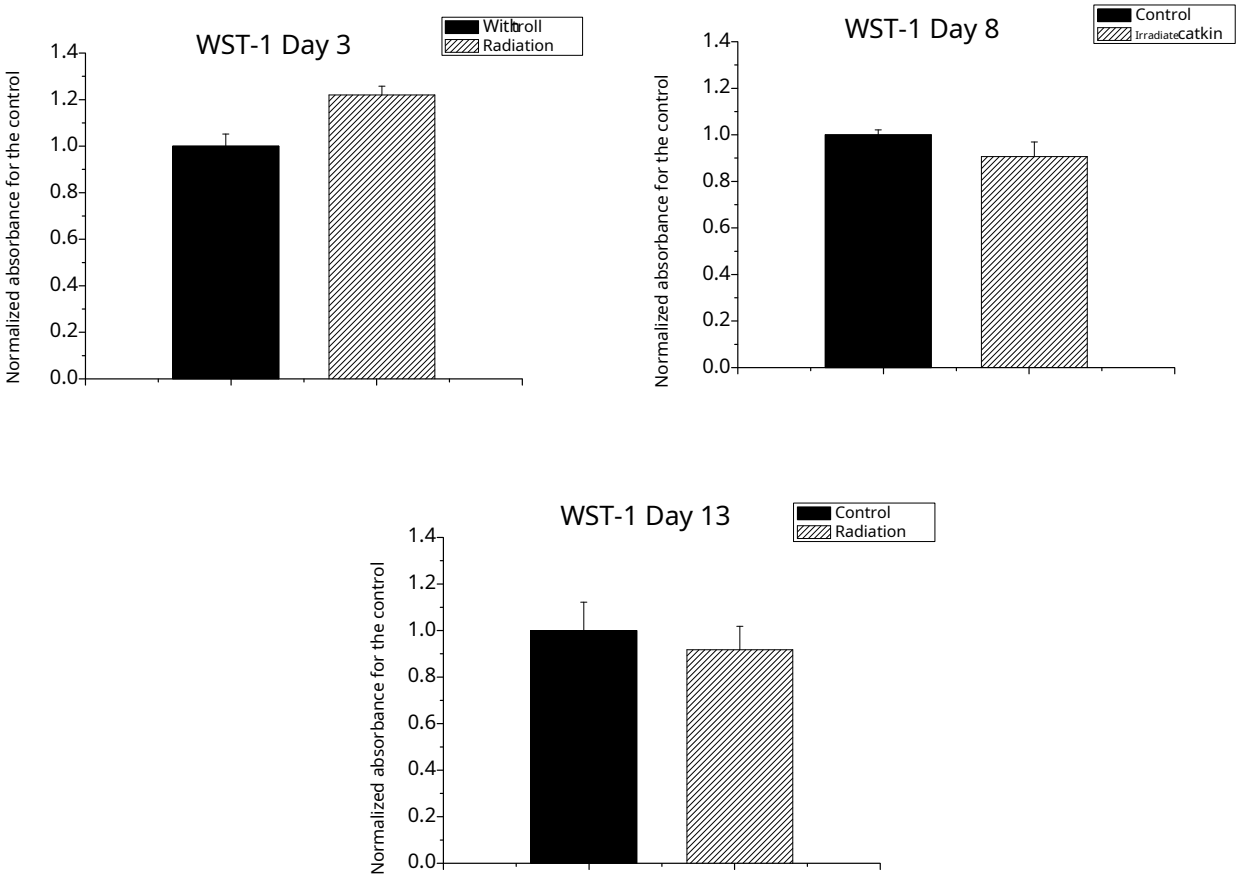



**Fig.4**Results deriving from the WST-1 assay performed on days 3, 8 and 13. The weighted means ± ESM of the normalized absorbances for the relative control of the cells subjected to irradiation are reported.

The concentration of Pro-collagen I α1 secreted by the control cells and those subjected to irradiation was determined at the end of the study by enzymatic assay. Next comes reported the concentration (pg/ml) calculated following the sampling of the cellular supernatant carried out ai several days (Fig.5 A) and the relative time course graph (Fig.5 B).

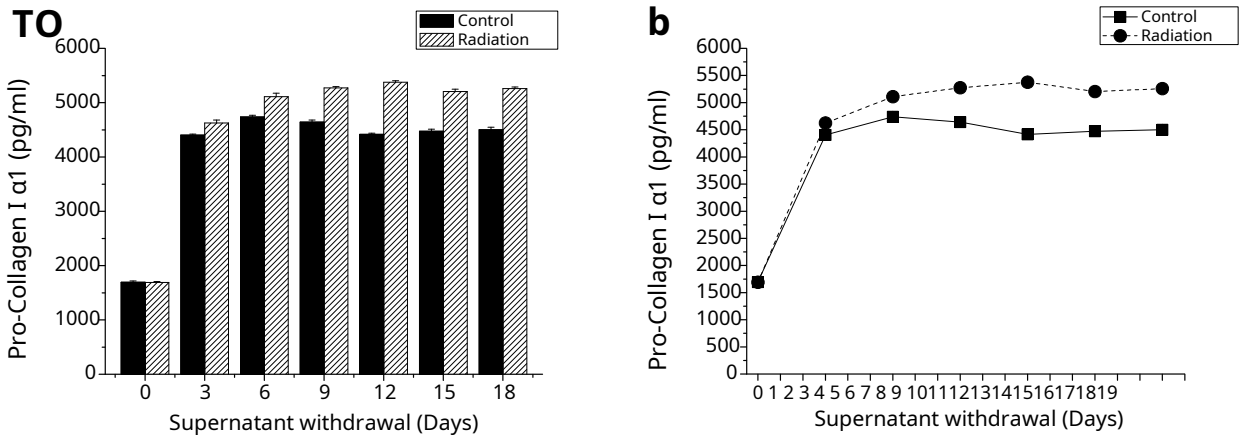



**Fig.5A-B**Concentration (pg/ml) of Pro-collagen I α1 secreted in the cell culture medium under the conditions of control and after irradiation (A) and relative time course (B).

The same concentrations were normalized for the residual volumes present in the wells on the various days (amount of pro-collagen per well) (Fig.6 AB). Due to the extended incubation time of the cell cultures, in fact, the evaporation of the culture medium distributed in the wells has caused a conspicuous decrease in starting volumes. A statistical test for was also applied in this case evaluate the significance of the differences found (see the asterisks in the two graphs and the relative value of p).

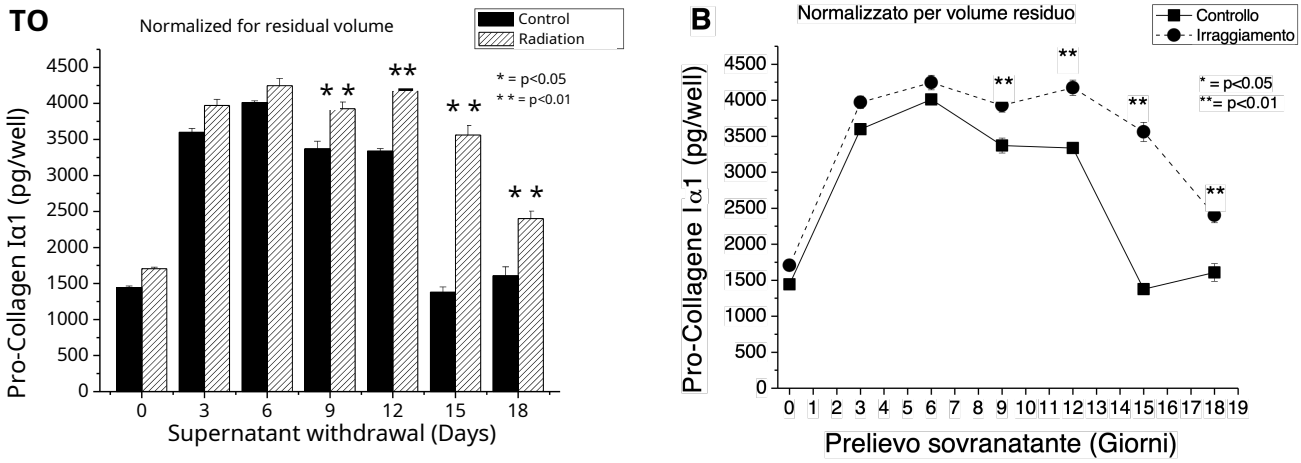



**Fig. 6A-B**Amount of Pro-collagen I α1 present at different days in the cell culture medium under the conditions control and after irradiation (A) and relative time course (B).

The averages of the amount of Pro-collagen I α1 present in the wells over the different days were in turn normalized by the average number of live cells present in the relative conditions (Fig.7A-B). Also in this case a statistical test was applied to evaluate the significance of the differences found (see the asterisks in the two graphs and the relative p-value).

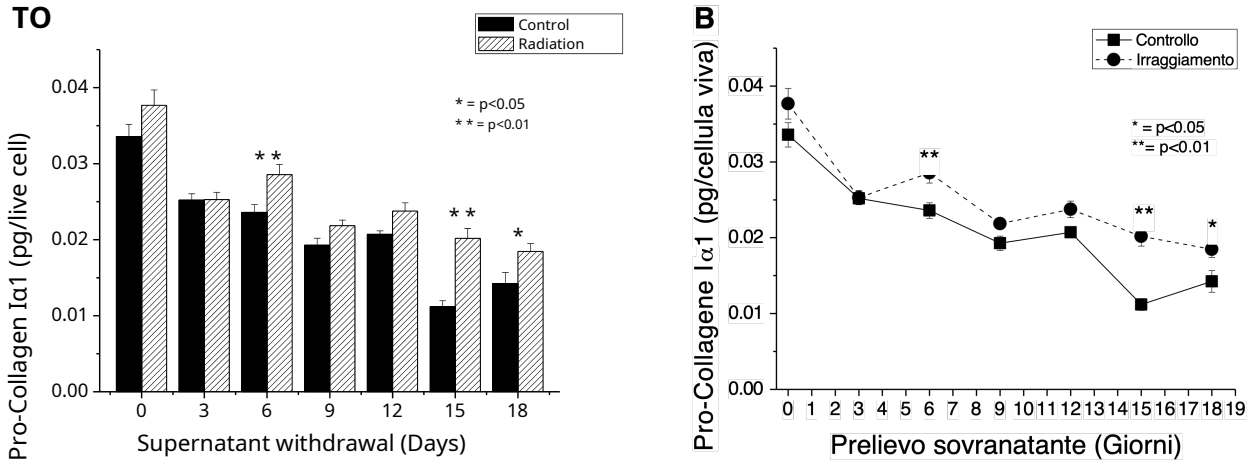



**Fig. 7A-B**Quantity of Pro-collagen I α1 per viable cell (A) and relative time course (B) over the different days in control conditions and after irradiation.

Statistical significance, indicated in the graphs with (*), was calculated using the T-Test.

**Treatment with TGFβ1**

The parameters deriving from all the tests reported above were also evaluated by treating the HDFa fibroblasts with TGF-β 1 (5ng/ml). The addition of this cytokine took place on day 6, i.e. when it was seen that the cells had reached the growth plateau. A further addition was made on day 13.

In the following column graph (Fig. 8) and in the subsequent time course (Fig. 9) the values of the weighted means ± ESM of cell counts of live cells only under control conditions and in the presence of TGF-β1. The reading of the values of the cells with TGFβ1 at day 6 was done at the end of the hour of incubation following the 19-minute period required for irradiated cells.

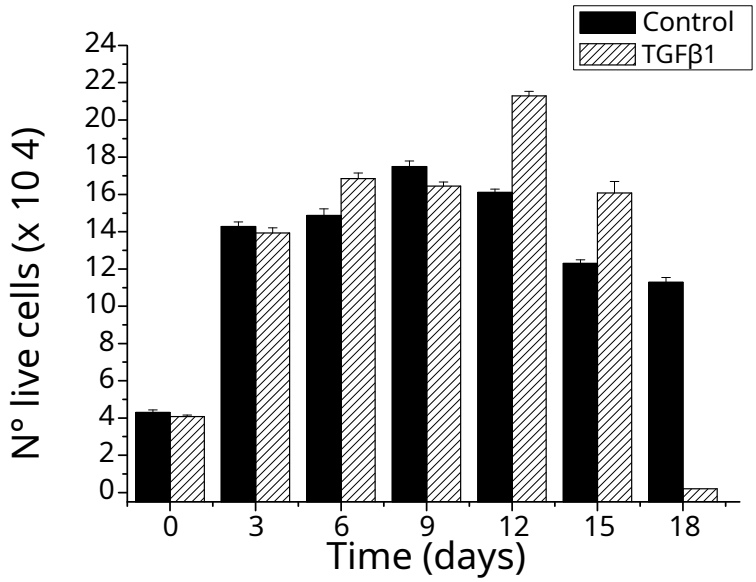



**Fig.8**Cell viability under control conditions and in the presence of TGF-β 1. This was added on Day 6 and Day 13.

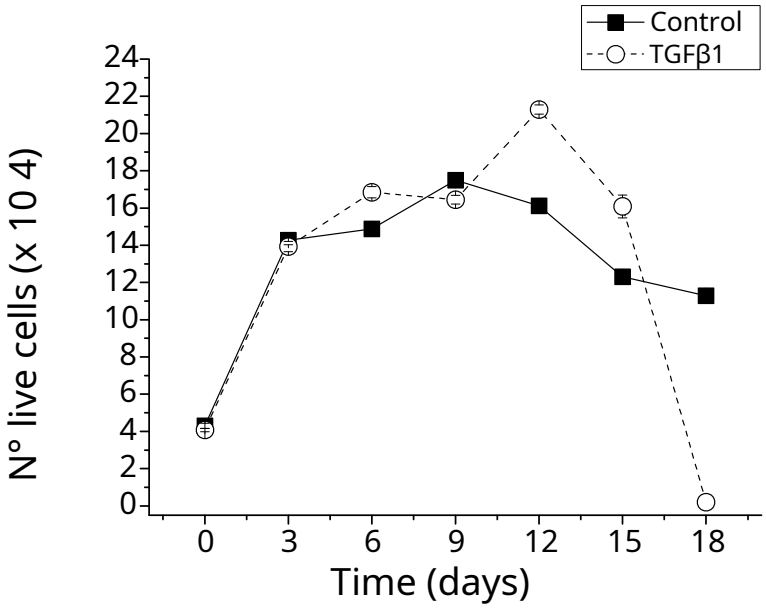



**Fig.9**Time course of cell viability of control cells and those in the presence of TGF-β 1.

Also for TGF-β 1 the dead cells were counted at different days and also in these conditions

significant number of dead cells (> 0.05 X $10_4$) was found only starting from Day 12 (Fig. 10).

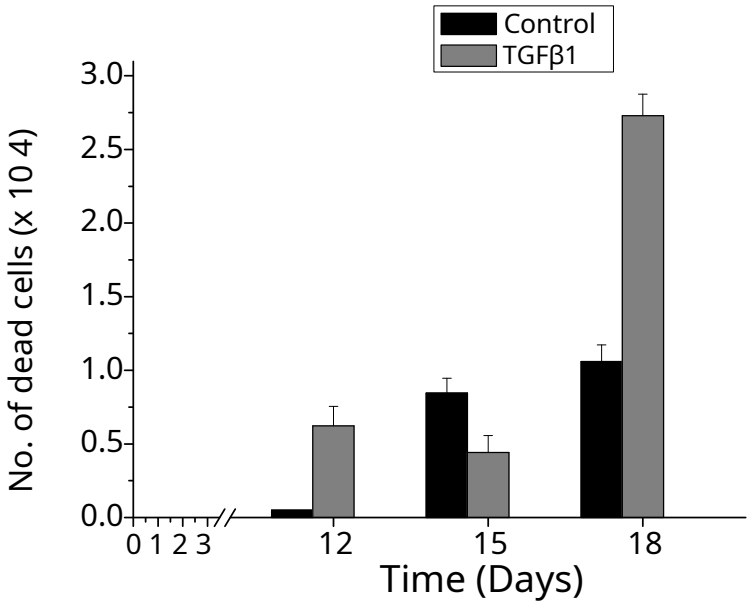



**Fig.10**Number of dead cells in control condition and after treatment with TGF-β 1.

Below are the weighted means ± ESM of the normalized absorbances for the control obtained by WST-1 assay on days 3, 8, 13 and 15 (Fig. 11).

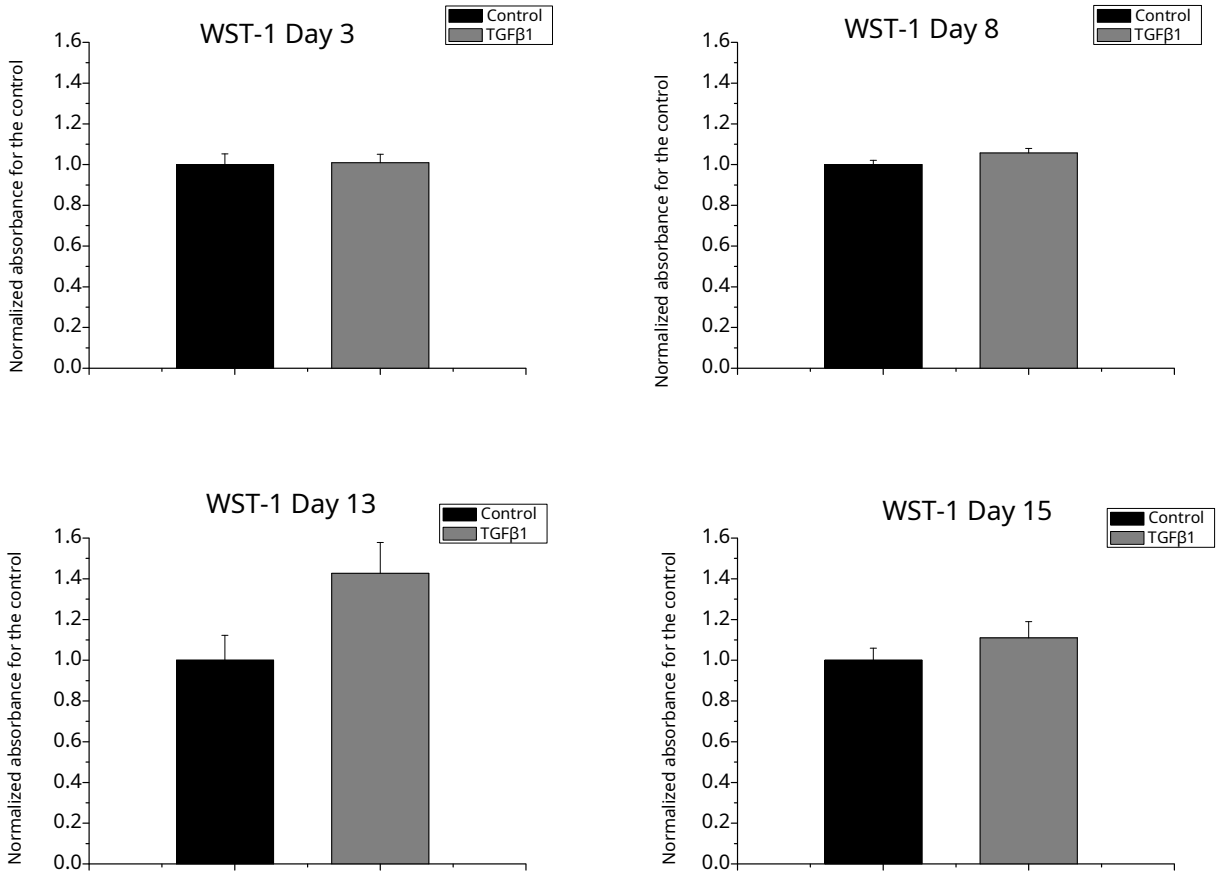



**Fig. 11M**weighted days ± ESM of normalized absorbances for control cells in the presence of TGF-β 1 ai days 3, 8, 13 and 15. Values are from WST-1 assay analysis.

Similarly to what was seen previously for the cells subjected to irradiation, they are shown below reported the calculated concentration of the secreted Pro-Collagen I α1 (Fig 12A-B), the averages of the of the same present in the wells (Fig 13A-B) and the averages of the quantities normalized by number of cells viable (Fig 14A-B) at different days of treatment with TGFβ1.

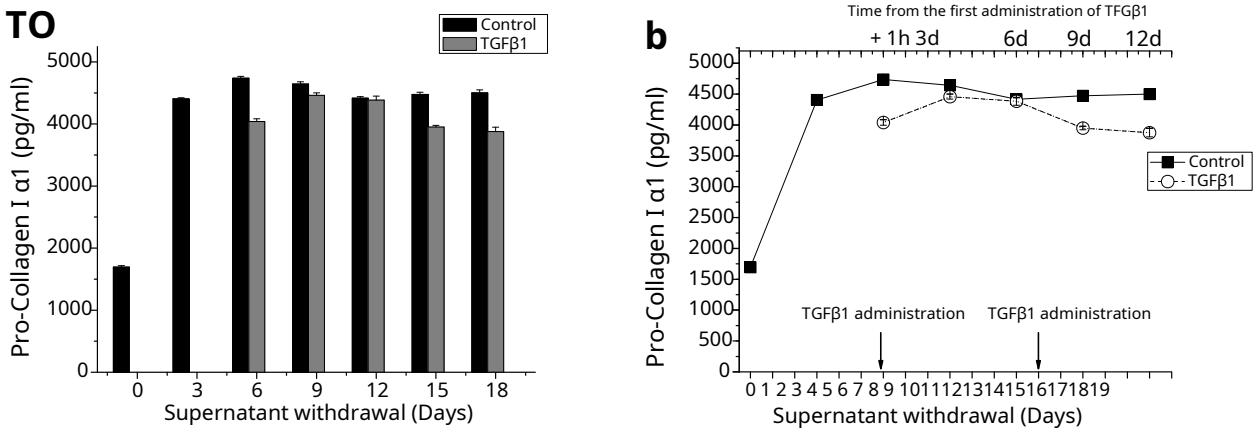



**Fig. 12A-B**Concentration of secreted Pro-Collagen I α1 (A) and relative time course (B) after treatment with TGFβ1. Graph B shows the times at which TGFβ1 was administered and the relative incubation times after the first administration.

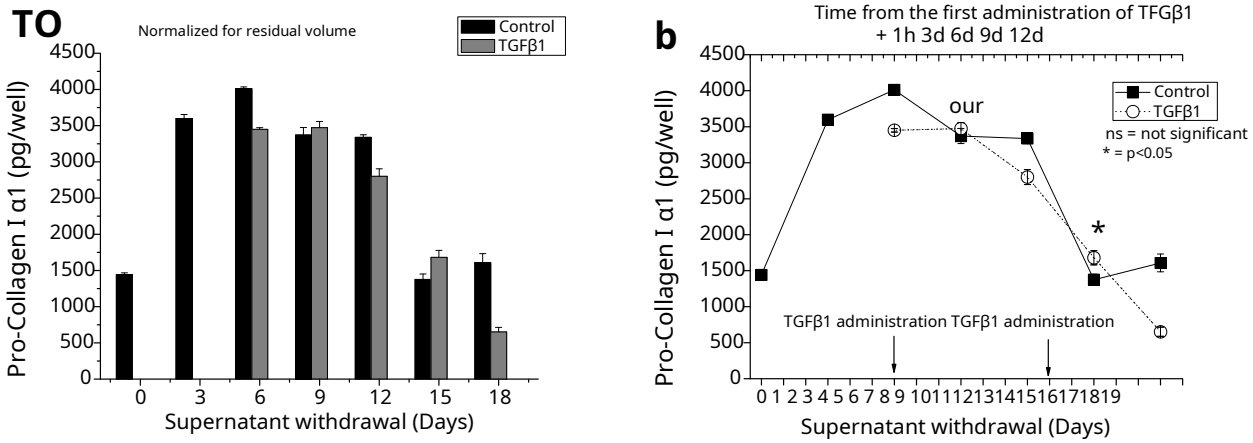



**Fig. 13A-B**Quantity of Pro-Collagen I α1 secreted per well (A) and relative time course (B) after treatment with TGFβ1. Graph B shows the times at which TGFβ1 was administered and the relative incubation times after the first administration.

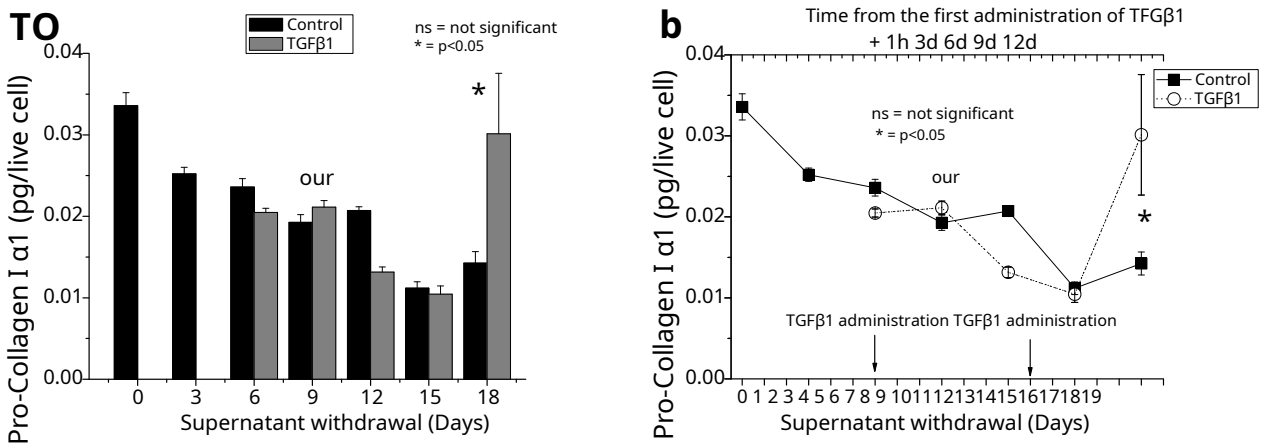



**Fig. 14A-B**Amount of Pro-Collagen I α1 secreted per number of viable cells (A) and relative time course (B) after treatment with TGFβ1. Graph B shows the times at which TGFβ1 was administered and the relative incubation times after the first administration.

## 11. FINAL CONSIDERATIONS

1)**Effects of LIMFA treatment on cell growth.**LIMFA treatment did not show induce significant effects on cell growth (viability and mortality) compared to the control until day 9. After this day, and ending on day 18 in which the experimental trial was terminated, le cultures subjected to irradiation show a higher number of live cells than the control cultures.

2)**Effects of LIMFA treatment on cell morphology.**Until day 15, the cells subjected to irradiation did not present any morphological variation, evaluable at a examination by phase contrast microscopy. At day 18, also, a certain amount of cells is detached from the substrate, index of cellular suffering. This is observed both in the crops subjected to irradiation and in the control ones, and indicates the maximum culture time that can be carried out for this type of cell. At the same time (15-18 days) a conspicuous also occurred reduction of the volume of the culture medium, attributable to an evaporation process.

3)**Effects of LIMFA treatment on procollagen I secretion.**Treatment with the system LIMFA induces an increase in the secretion of procollagen I in the culture medium, compared to

control, starting on day 6 of culture. After normalizing these values to the actual residual volume of the culture medium, this increase reaches statistical significance a starting from day 9 of culture, and is maintained until the end of the experimental procedure. The positive effect on the secretion ofprocollagen I is also observed after normalization for the number of live cells in culture, although the trend versus time appears more swinging. The increase in procollagen I secretion induced by irradiation appears, in the latter case, statistically significant starting from day 6 of culture.

4)**Comparison between LIMFA irradiation and treatment with TGFβ1.**The effect of TGFβ1 on this cell line, both in terms of cell viability and procollagen I secretion appear much less encouraging than those observable with irradiation procedures. In fact, both a negative effect of the cytokine on cell viability and a poor pro-secretory effect are observed. The quantity of Pro-Collagen I α1 secreted, in fact, turned out to be even lower than that found in the controls, and the stimulatory effect of TGF b1 on the secretion of procollagen I is observed only after normalization of the number of vve cells, and at later times (day 18), when high cytokine-induced cell mortality is also observed.

Overall the results obtained from this first preliminary test on the treatment effect LIMFA on human dermal fibroblasts appear very encouraging, both for the lack of toxicity of the treatment, which also does not demonstrate an (unwanted) hyper-proliferative effect on the cells both, and above all, for the pro-stimulatory effect of the production of procollagen I. This is occurred despite that, due to the lack of important cofactors present instead live (such as Vitamin C) and of the intrinsic characteristics of the culture conditions cell phone in vitro, the formation of stable collagen in vitro is very difficult (Chen CZ, e Raghunath M. Fibrogenesis Tissue Repair. 2009 Dec 15;2:7. doi: 10.1186/1755-1536-2-7). There normalizing our data for changes in the number of viable cells, one item appears relevant, not only because it confirms the statistical significance of the pro-secretory effect of LIMFA treatment, but also because it is in line with what is reported in the literature, according to which the Cell density is a relevant aspect for production in vitro of collagen. Very interesting

would investigate the production of other collagen molecules (e.g. type III collagen), either, after extraction and dialysis, the release of insoluble collagen in the pericellular matrix.