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Positional differences in the gene expression profile of human alveolar mucosa, buccal attached gingiva and palatal tissue-derived fibroblasts in the early phases of wound healing and the role of chlorhexidine digluconate and hyaluronic acid as modifiers of oral gingival-derived cells behaviour.
A clinical, histological, immunohistochemical and biomolecular analysis

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To the memory of my beloved grandfather Alfonso

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"The secret of happiness is not always doing what you want, but always wanting what you do."

Lev Tolstoj

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Mariana Rojas

ABSTRACT

Wound healing is a complex process orchestrated by a variety of known and unknown factors. Oral wound healing presents peculiar characteristics as accelerated wound closure and reduced scar formation when compared with cutaneous wound repair, and it has been recognized the key role of fibroblasts in this concern. In fact, oral-derived fibroblasts represent a resource of great interest for regenerative approaches due to their self-renewal capacity and plasticity. The biomolecular basis of the differences between oral and skin repair have been described by several studies, showing the main differences in transcriptional changes between the first 12-24 hours after injury. Nevertheless, variations in wound healing also exists inside the oral environment. The evidence we have nowadays is that wounding in the alveolar mucosa leads to scarring, while in the gingival tissue does not, but the literature lacks clear evidence concerning comprehensive *in vivo* biomolecular data on the differences between alveolar mucosa, buccal attached gingiva and palatal tissue-derived fibroblasts behaviour.

The course of wound repair process can be modified by different factors. It is well know how the presence of biofilm jeopardizes the healing process. To counteract this during the post-surgical period, chlorhexidine digluconate (CHX) mouthrinse is commonly indicated. Although several *in vitro* studies have reported cytotoxicity effects on fibroblasts, , *in vitro* assays cannot fully represent the oral environment as a whole and this could be a limitation. Nevertheless, there are no *in vivo* data on the effect of post-surgical CHX use in oral cells behaviour in the early phases of wound healing.

Improving wound healing through the use of bioactive substances that can influence cells behaviour, supporting tissue repair/regeneration, is of major clinical interest.
Hyaluronic acid (HA) is the major endogenous component of ECM, involved in cell proliferation, migration and tissue remodeling. *In vitro* and animal studies have demonstrated the ability of exogenous HA to improve wound healing, enhancing proliferative and migratory ability of oral-derived fibroblasts. However, *in vivo* human

studies evaluating its specific mechanisms on cellular activation and gene expression modulation in the early phases after oral surgical wounding are lacking.

The present thesis was organized and divided in the following sections:

Chapter I

The first chapter is intended to give evidence on the peculiar characteristics of oral soft tissues in the early phases of wound healing process from a clinical and biomolecular point of view.

In addition, evidence available concerning the role of post-surgical chlorhexidine digluconate (CHX) mouthrinse and exogenous hyaluronic acid (HA) in oral wound repair is presented.

The aim of this chapter is to provide current knowledge on these concerns, pointing out the missing evidences that lead the research topic during the course of the doctorate and presenting the rationale of the topic that allowed to conclude in the three main branches of this research:

- Differences in the gene expression profiles between human alveolar mucosa, buccal attached gingiva and palatal tissue-derived fibroblasts in the early phases of oral wound repair;
- Effect of post-surgical CHX mouthrinse on gingival tissue features and oral gingival-derived cells behaviour in the early phases of oral wound repair;
- Effect of intra-surgical HA application on gingival tissue features and oral gingival-derived cells behaviour in the early phases of oral wound repair.

Chapter II

The second chapter is intended to provide evidence on the behaviour of fibroblasts derived from different oral soft tissues in the early phases of the wound healing process. The main aim was to analyse and compare the gene expression profiling of fibroblasts from human alveolar mucosa (M), gingival (G) and palatal (P) tissues in the early phases following surgical wounding, correlating it with the clinical response, autophagy activation and

fibrotic markers expression. M, G and P biopsies were harvested from six patients at baseline and twenty-four hours after surgical procedure. Clinical response was evaluated through Early wound Healing Score (EHS). Fibrotic markers expression and autophagy activation were assessed on fibroblasts isolated from those tissues by Western blot and quantitative real time PCR analysis (qRT-PCR). Fibroblasts from two patients were subjected to RT2 profiler array, followed by network analysis of the differentially expressed genes. The expression of key genes was validated with qRT-PCR on all patients. At twenty-four hours after surgery, EHS was higher in P and G than in M. In line with the clinical results, no autophagy and myofibroblast differentiation were observed in G and P. Significant variations in mRNA expression of key genes were observed: RAC1, SERPINE1 and TIMP1, involved in scar formation; CDH1, ITGA4 and ITGB5, contributing to myofibroblast differentiation; and IL6 and CXCL1, involved in inflammation. Some genes involved in the oral soft tissue differential clinical wound healing outcome were identified, providing novel insights into the molecular mechanisms of oral repair and allowing to develop new approaches of essential impact in periodontal surgery.

Chapter III

The present chapter focuses on the study of the *in vivo* effect of post-surgical chlorhexidine digluconate (CHX) mouthrinse on the gingival tissue features and oral gingival-derived cells behaviour in the early phases of wound repair. G biopsies were obtained in three patients twenty-four hours after surgery with the indication of post-surgical 0.12% CHX use and were compared with those obtained from the same patients without any antiseptic use.

Each gingival biopsy was divided in two parts: one for histological-immunohistochemical (IHC) analysis and one for gene expression analysis in order to carry out a morphological and molecular analysis. For the first one, epithelial tissue/chorion features and collagen fibers organization/content were evaluated through Hematoxylin-Eosin and Masson trichrome staining, respectively. The expressions of proteins related to cell proliferation (ki67) and apoptosis (p53) were examined by IHC analysis. Fibrotic markers expression (Vimetin, Col1a1 and αSMA) were also analysed by IHC in order to evaluate collagen

deposition and myofibroblasts differentiation. For the molecular analysis, qRT-PCR was carried out: fibrotic markers expression (Col1a1 and αSMA) and proapoptotic protein (BAX) were analysed in all the patients, and to evaluate the re-epithelialization and collagen turnover, RAC1, SERPINE1 and TIMP1 gene expression were analysing in two patients.
Twenty-four hours after surgery, CHX was able to reduce cellular proliferation and to increase collagen deposition, proapoptotic protein and fibrotic markers expression, and myofibroblast differentiation. In addition, a reduction in the expression of RAC1 and triggering in the expression of SERPINE1 and TIMP1 were observed, showing a "scar wound healing response" pattern. The demonstration of CHX-induced fibrotic transformation, leading to scar repair, could support the need for new post-surgical clinical protocols based on a strategic and personalised use of CHX.

Chapter IV

The present chapter focuses on the study of the *in vivo* effect of exogenous hyaluronic acid (HA) on the gingival tissue features and oral gingival-derived cells behaviour in the early phases of wound repair.
G biopsies were obtained in eight patients twenty-four hours after surgery with intra-surgical application of HA and were compared with those obtained from the same patients without HA application (no treatment group - NT). Clinical response was evaluated through EHS. Each gingival biopsy was divided in three parts: one for histological-IHC analysis, one for protein analysis and one for gene expression analysis in order to carry out a morphological and molecular analysis. For the first one, tissue structure and inflammatory infiltrate, extracellular matrix (ECM) organization and microvascular density (MVD), and collagen fibers organization/content were evaluated through Hematoxylin-Eosin, Sinus red and Masson trichrome staining, respectively; whereas cellular proliferation was evaluated by immunohistochemical detection of Ki67. For the molecular analysis, collagen turnover was evaluated through MMP-1, MMP-2 and MMP-9 protein analysis by Western Blot and LOX, MMP-1, TIMP-1, TGF-β1 genes expression by real time PCR. Since ECM remodeling is also influenced by mechanical stimuli, and fibroblasts are mechanoresponsive cells, the expression of key mechanosensors paxillin (PAX), focal

adhesion kinase (FAK) and vinculin (VNC) were also analysed.

Twenty-four hours after surgery, EHS was significantly higher in HA than in NT group. In line with the clinical results, gene expression analysis showed that mRNA levels of LOX-involved in collagen maturation-, resulted significantly up-regulated in HA-treated gingiva compared to NT group but this was independent of TGF-β1 since no difference was found its expression. MMP-1/TIMP-1 balance was modified: significant increase of MMP-1 protein and TIMP-1 gene expression in HA compared to NT group were revealed. No significant differences were observed in MVD, key mechanosensors expression, collagen content and cell proliferation.

Intra-surgical HA application enhance wound healing properties such as ECM remodeling and collagen maturation, and improve the clinical repair response in human *in vivo* gingival wounds 24 hrs after injury. HA might be an important component in future regimens aiming to accelerate and improve the wound healing after periodontal surgery.

Chapter V

The last chapter aims to provide general conclusions concerning the three branches of the research performed during the course of doctorate, integrating the results obtained and pointing out some key points and recommendations. In addition, future perspectives in the wound healing research field are mentioned.

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LIST OF ABBREVIATIONS

BP	Biological process
CC	Cellular process
CDH1	Cadherin 1
CHX	Chlorhexidine digluconate
COL1 a1	Collagen, tipo I alpha 1
CXCL1	C-X-C motif chemokine ligand 1
CT	Control group
DEGs	Differentially expressed genes
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EHS	Early wound Healing Score
FDR	False discovery rate
G	Buccal attached gingiva
GO	Gene ontology
HA	Hyaluronic Acid
HGFs	Human gingival fibroblasts
hrs	Hours
IHC	Immunohistochemistry
IL6	Interleukin 6
IQR	Interquartile range
ITG14	Integrin subunit alpha 4
ITGB5	Integrin subunit beta 5
kDa	Kilodalton
LOX	Lysyl oxidase
M	Alveolar mucosa
MCC	Maximal clique centrality
MCODE	Molecular Complex Detection
MF	Molecular function

MMP	Matrix metalloproteinase
MVD	Microvascular density
MW	Molecular weight
NT	No treatment group
P	Palate
PANTHER	Protein Analysis Through Evolutionary Relationships
PGA	Polyglycolic acid
PPI	Protein-protein interaction
qRT-PCR	Quantitative real- time PCR
RAC1	Ras-related C3 botulinum toxin substrate 1
s	Seconds
SERPINE1	Serpin family E member 1
TIMP1	Tissue inhibitor of metalloproteinase 1
VRIs	Vertical released incisions

I. Review of the literature

1. OVERVIEW OF WOUND HEALING

The proper course of wound healing needs several specific processes that occur in a particular order. The first phase is initiated by mesenchymal cells migrating to the wounded area. In the next steps, angiogenesis and epithelial processes are required. Then, regeneration and fibrosis processes take place. Regeneration, the most desirable healing outcome, is defined as the complete functional and structural restoration of the damaged tissue, in which damaged cells are replaced by the same type of cells (Politis et al., 2016). Fibrosis is a process in which damaged tissue is replaced by non-specialized connective tissue that can lead to a scar formation (Politis et al., 2016). These processes occur in epithelial cells as well as in fibroblasts, which can replicate at a high rate under stimulation (Enoch et al., 2010).

Irrespective of the type of wounded tissue, the process of wound healing follows four partially overlapping phases: hemostasis, inflammation, proliferation, and remodelling (Kirsner & Eaglstein, 1993).

The hemostasis and inflammation phase is marked by platelet accumulation, coagulation, and leukocyte migration.

The hemostasis phase begins when tissue damage, allows blood to leak into the exposed wound site, triggering the extrinsic clotting cascade and releasing mediators that cause localized vasoconstriction, such as serotonin. Platelets subsequently aggregate and activate the subendothelial collagen, leading to formation of a hemostatic plug through the release of cytokines and growth factors (Palta et al., 2014; Ellis et al., 2018).

The inflammatory phase overlaps considerably with initial hemostasis, occurring during the first 72 hours after tissue injury (Eming et al., 2014). This phase is principally represented by a complex series of molecular signals that ultimately facilitates neutrophil and monocyte infiltration into the wound bed in order to prevent unnecessary tissue damage and eliminate pathogenic organisms and foreign debris (Eming et al., 2014; Ellis et al., 2018).

The aim of the proliferative phase is to diminish the lesioned tissue area by contraction and fibroplasia, establishing a viable epithelial barrier to activate keratinocytes. This stage is responsible for the closure of the lesion itself, which includes angiogenesis, fibroplasia, and re-epithelialization (Ellis et al., 2018). These processes begin in the microenvironment of the lesion within the first 48 hours and can unfold up to the 14th day after the onset of the lesion (Chen & Kirsner, 2007).

Remodeling is the final phase of the healing process in which the granulation tissue matures and tissue tensile strength is increased. This phase involved the production of collagen and matrix proteins by fibroblasts, regulating the extracellular matrix synthesis. Inthis final stage of the lesion's healing, an attempt to recover the normal tissue structure occurs, and the granulation tissue is gradually remodeled, forming scar tissue that is less cellular and vascular. exhibiting a progressive increase in its concentration of collagen fibers (Eming et al., 2014; Ellis et al., 2018).

Each stage specifically involves unique cell types and (signaling) molecules. Dysregulation at any phase, for example wound infection, may result in delayed wound healing and/or hypertrophic scar formation (Guo & Dipietro, 2010).

2. TOWARDS UNDERSTANDING SCARLESS ORAL SOFT TISSUES WOUND HEALING

2.1. Intrinsic differences between oral mucosa and skin

Healthy skin and oral mucosa share many features, but also present several intrinsic histological differences. The oral epithelium is generally thicker compared to skin, as both palate and buccal mucosa consist of considerably more cell layers and a higher proliferation rate in the basal lamina compared to skin (Gibbs & Ponec, 2000). Whereas the epidermis is entirely keratinized, there is a clear differentiation within the oral cavity between the keratinized epithelium of the hard palate and of gingiva, which have to withstand the mechanical forces during mastication versus the nonkeratinized epithelium of the buccal

mucosa that has the flexibility to stretch and withstand compression (Turabelidze et al., 2014). More blood vessels are present in the oral mucosa compared to skin (Szpaderska et al., 2005; Glim et al., 2015) and, in nonkeratinized oral epithelia, the extracellular matrix (ECM) has a looser structure and contains more elastin compared to skin, hard palate, or gingiva (Hsieh et al., 2010; Glim et al., 2014). In skin and oral mucosa, subjacent to the connective tissue, there is a layer of adipose tissue layer containing adipocytes and progenitor cells, whereas in the gingiva and hard palate, the lamina propria is directly attached to the periosteum of the underlying bone without submucosa, trough the mucoperiostium, which provides a firm, inelastic attachment (Squier & Kremer, 2001; Squier & Finkelstein, 2003).

The effect of microenvironmental factors such as saliva and microbes during the different phases of wound healing also represent an important difference between skin and oral tissues. Although wound infection caused by colonization of pathogenic microbes greatly delays wound healing (both in oral and skin), a positive effect of microbes (healthy oral biofilm) on oral wound healing has been demonstrated (Karin & Klevers, 2016; Zheng et al., 2020)

In addition to the intrinsic properties of resident cells and the differential expression of cytokines and growth factors, multiple external factors have been identified that contribute to a better oral wound healing response when compared to the skin: (1) the presence of saliva, (2) a more rapid immune response and, (3) increased ECM remodelling. All these characteristics contribute to an improved tissue repair of the oral tissues, showing a reduction in scar formation compared to the skin.

2.2. Differences between oral mucosa and skin in the early phases of wound healing

Wound healing consist in a highly developed series of 4 phases partially overlapping: hemostasis, inflammation, proliferation, and tissue remodeling.

Transcriptomes of oral mucosal and skin wounds have been compared using microarray analysis in order to identify critical differences in the wound healing response at these two sites (Chen et al., 2010). Using an animal wound model, the healing of skin and mucosal

(tongue) wounds was evaluated at different defined intervals (6, 12, 24 hours and 3, 5, 7 and 10 days). Between the results, the authors founded that skin and mucosal wounds had a comparable degree of transcriptional changes except at 12 and 24 hours after injury where skin wounds have shown to be significantly more reactive than tongue wounds. This time period accounted for 85% of the overall changes in skin and 95% of the difference between skin and tongue.

In the first 24 hours after injury, two phases of the wound healing process are mainly involved: the hemostasis and inflammatory phase.

In a recent study, (Iglesias-Bartolome et al., 2018) the molecular and histological aspects of wound healing in paired samples of oral mucosa and skin in healthy human subjects were evaluated 48 hours and 5 days after injury. Analysis of the healing time course revealed that oral wounds resolved significantly faster that skin wound. When the molecular mechanisms were evaluated, although a differential gene regulation between oral mucosa and skin was observed during wound healing, most of these differences were already evident at starting basal conditions. This raising the possibility that transcriptional regulatory networks responsible for the accelerated healing in oral mucosa are already present in the unwounded state.

Noteworthy, is the fact that when each tissue was analysed, oral tissues showed few significant gene expression changes 48 hrs after injury and no changes after 5 days, while a large number of genes differentially regulated were observed in the skin at both time points. This is in agreement with the aforementioned study (Chen et al., 2010).

- ***Hemostasis phase***

Directly upon tissue damage and vascular rupture, within the first minutes to hours, the coagulation cascade is activated to prevent blood loss, providing a temporary seal to the wound (Smith et al., 2015). The plasma portion of blood contains a collection of soluble proteins that act together in a cascade of enzyme activation events, culminating in the formation of a fibrin clot (Smith et al., 2015). Platelet activation not only results in hemostasis, but also in the release of growth factors such as platelet derived growth factor (PDGF) as well as immune mediators that are responsible for activation of the immune

system and transition to the inflammatory phase of wound healing (Golebiewska & Poole, 2015).

There are two main mechanisms for triggering the blood clotting, termed the tissue factor (TF) pathway and the contact pathway. Only one of these pathways (the first one) functions in normal hemostasis (Mackman, 2004).

The TF pathway (McVey, 1994) is named for the protein that triggers it—a cell-surface integral membrane protein known as tissue factor also called thromboplastin, coagulation factor III, or CD142,(Morrissey & Broze, 2013). This pathway is also known as extrinsic pathway, since it requires that plasma come into contact with something "extrinsic"—i.e., TF—to trigger it (Mackman, 2004). TF is known as the key initiator of the coagulation cascade to arrest bleeding (Butenas et al., 2009).

One of the major differences between skin and oral mucosa which regulates the tissue wound healing response is the presence of saliva in the oral cavity (Brand et al., 2014; Dawes et al., 2015). In fact, although there are no comparative studies evaluating hemostatic phase of wound healing in oral and dermal tissues, saliva is thought to be one of the main contributing factors (Rodrigues Neves et al., 2019). Already in the 1930s, it was observed that saliva reduces clotting time when added to blood samples (Glazko & Greenberg, 1938). The apparent reason could be the fact, as has previously been shown, saliva contains extracellular vesicle-derived TF (Fareed et al., 1995; Yu et al., 2018) capable of triggering coagulation, as evidenced from a shortened clotting time of autologous plasma and whole blood when saliva was added (Berkcmans et al., 2011).

Platelet-derived growth factor (PDGF) is a potent stimulator of fibroblast cell migration, mitogenesis, proliferation, and matrix synthesis with an important role in wound healing (Seppä et al., 1982; Heldin et al., 1998; Rosenkranz & Kazlauskas, 1999). It has been studied for gene delivery to successfully promote soft tissue repair (Liechty et al., 1999; Tyrone et al., 2000). Animal oral wounds showed higher levels of PDGF as compared to skin wounds, which may indicate increased platelet activation in the oral tissues (Kong et al., 2019). Furthermore, it has been demonstrated that PDGF gene delivery stimulates *ex vivo* gingival repair (Anusaksathien et al., 2003).

Activated platelets and surviving keratinocytes and fibroblasts secrete chemokines to rapidly initiate the inflammatory phase by attracting immune cells to the wounded area

(Singer & Clarck, 1999; Martin & Leibovich, 2005; Szpaderska & DiPietro, 2005; Eming et al., 2007). It has been demonstrated that mucosal wounds exhibited decreased levels of pro-inflammatory genes including cytokines and chemokines when compared with dermal tissue (Chen et al., 2010). Therefore, decrease pro-inflammatory cytokines and chemokines levels expression may support the rapid healing and reduced scar formation observed in oral wounds.

- ***Inflammation phase***

The inflammation phase of wound healing is aimed at removing debris from the injured site and prevent infection by pathogens. Inflammatory cell recruitment into the wound site occurs secondary to local stimuli. Injured host cells die and release cellular contents that serve as danger signals (damage-associated molecular patterns -DAMPs) (Kono & Rock, 2008; McDonald et al., 2010). When a wound is contaminated by a pathogen, pathogen associated molecular patterns (PAMPs) are also released (Kaisho & Akira, 2006) trigger toll-like receptor (TLR), receptor for advanced glycation end-products (RAGE), and inflammasome signaling, leading to a cytokine and chemokine cascade released by resident cells that marks the onset of the inflammation phase within hours after wounding, which leads to local inflammatory cells activation (neutrophils, monocytes, macrophages, mast cells, and T cells)(Mogensen, 2009). Subsequently, these cells express numerous genes that code for important chemical mediators that will propagate the inflammatory response (Kaisho & Akira, 2006; Takeuchi & Akira, 2010).

Many immune cells and inflammatory mediators also interact with resident cells throughout the course of wound healing, driving fibrotic responses (Koh & DiPietro, 2011). Therefore, the inflammatory response influences the entire healing process, and its misbalance potentially leads to excessive tissue destruction and scar formation, wound infection, and delayed wound healing (Eming et al., 2007). The pro-inflammatory mediators regulates the conditions of persistent inflammation (Serhan, 2011).

Although low inflammation is associated with scarless fetal wound healing (Lietchy et al, 2010; Yates et al., 2012) and increased inflammation is generally associated with (hypertrophic) scar formation (Satish & Kathju, 2010), some recent studies have also

suggested that reduced inflammation in homeostasis and in the early wound-healing stages could be biomarker for hypertrophic scar formation (Butzelaar et al., 2016; de Bakker et al., 2021).

- ***Phagocytosis***

Four hours post injury, keratinocytes, fibroblasts, Langerhans cells, and resident macrophages start to phagocytose the resulting debris (Koh & DiPietro, 2011). It was observed (in an animal wound model) that phagocytic activity in epithelium and connective tissue of oral mucosa (tongue) reached higher levels than in skin (Sciubba et al., 1978) .

- ***The inflammatory infiltrate into the wound***

Neutrophils

Polymorphonuclear leukocytes (PMNs or neutrophils) are the most abundant leukocyte in humans and essential to innate immune response against invading pathogens. PMNs are the first immune cells to enter into the wounded area (Kobashayi & DeLeo, 2009). They are the most active cells in the early stage of the inflammatory response, playing a crucial role in arrest microbial invasion through degranulation and phagocytosis of microorganisms products, foreign particles and cellular debris (Christoffersson & Phillipson 2018; Phillipson & Kubes, 2019).

Only a few hours after the lesion, a quantity of neutrophils transmigrate through the endothelial cells present in the blood capillary walls, which are activated by pro-inflammatory cytokines, such as IL-1 β, TNF-α (tumor necrosis factor alpha), and IFN-γ (interferon gamma) at the location of the lesion. Such cytokines promote the expression of many classes of adhesion molecules, essentials for the diapedesis of neutrophils, including selectins and integrins which interact with those already present on the membrane surface of endothelial cells (de Oliveira, 2016).

Based on 2 mm excisional wounds in mice, significantly lower levels of PMNs, macrophages and T-cells infiltration were observed in oral vs. dermal wounds. Infiltration of neutrophils starts as early as 4 hours after injury and peaks after 24 hours in both skin and oral mucosa (Szpaderska et al., 2013).

In a human study in which a 3 mm excisional wound model was used, the quantity of PMNs observed were similar in oral mucosa and skin wound during the first 6 days after injury. Nevertheless, at 3 days after injury followed by resolution, PMNs reached its peak in oral mucosa while in skin the number of PMNs continued increasing up to 6 days after injury, suggesting a more rapid influx of neutrophils in oral wound healing (Iglesias- Bartolome et al., 2018).

Macrophages

PMNs secrete chemokines to attract monocytes into the wounded area about 24 hours after injury (Tecchio & Cassatella, 2016). Monocytes mature into macrophages, which contribute to phagocytosis of debris and replace the neutrophil population, becoming the predominant immune cell type in the wounded area. This takes approximately 2–4 days after wounding. Macrophages orchestrate the healing response via secretion of cytokines and growth factors that are initially proinflammatory (M1 phenotype), and in later stages of wound healing, anti-inflammatory and profibrotic (M2 phenotype)(Mantovani et al., 2013; Novak & Koh, 2013). The differences between macrophages subtypes in skin and oral mucosa have not been compared but it has been observed that, following reach its peak (3 days after injury), while a reduction is observed in oral wound, the number remains high in skin wounds, extending the inflammatory phase (Mak et al., 2009).

Macrophages release biochemical mediators which stimulate fibroblasts, collagen synthesis and angiogenesis and regulate the degree of innate inflammatory response, creating an environment in which it can take place the connective tissue (Krzyszczyk et al., 2018).

Among the cytokines and other regulatory factors macrophages release, the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factor-β 1 (TGF-β1) and transforming growth factor α

(TGF-α) are the main regulatory factors released by macrophages, which seem to have a highly significant role in tissue repair (Arango Duque & Descoteaux, 2014).

Mast cells

Mast cells have been shown to stimulate proliferation, angiogenesis, and ECM deposition and remodeling via releasing pro-inflammatory mediators such as histamine , leukotrienes, cytokines and growth factors (Ng, 2010).

It was reported that in pigs, mast cells infiltrate skin and oral wounds at the same time after injury but while remains in the skin tissue, disappear gradually in oral wounds (Mak et al., 2009). In an animal wound model, it was observed that mast cells modulate the recruitment of neutrophils into sites of injury, but are unlikely to exert a major influence on the proliferative response within healing wounds (Egozi et al., 2003).

T Cells

Infiltrated T cells produce cytokines and growth factors that drives immune responses and wound healing (Landén & Ståhle, 2016) and a direct effect of T cells to wound healing has recently been recognized (Boothby et al., 2020)

In the above-mentioned human study in which a 3 mm excisional wound model was used (Iglesias-Bartolome et al., 2018), T cells behaviuor in oral and skin wounds was the same as that observed in PMNs, suggesting that the influx of T cells is delayed in skin compared to oral mucosa. Although comparative data on T cell and associated cytokines in skin and oral wound healing are not available, different chemokines are expressed by those cells in oral and skin tissues and it has been suggested a higher influence of TH1 in oral wounds (Viola et al., 2006; Strazza, & Mor, 2017).

- *Cytokines*

Cytokines have a key role in wound healing ***by*** determining immune cell responses and wound healing outcome. (Barrientos et al., 2008). Have been classified as pro- or anti-inflammatory (or pro- or anti-fibrotic) (Cavaillon, 2001).

Comparative studies, through transcriptional analysis, have demonstrated more pro-inflammatory cytokines in skin wounds respect to oral wounds (Szpaderska et al., 2003; Chen et al., 2010; Iglesias-Bartolome et al., 2018).

Szpaderska et al (2003), demonstrated that oral wounds contained significantly less IL6 than skin wounds. Similarly, the level of the pro-fibrotic cytokine TGF-β1was lower in mucosal than in skin wounds. No significant differences between skin and mucosal wounds were observed for the expression of the anti-inflammatory cytokine IL-10 and theTGF-β1 modulators, fibromodulin and LTBP-1.

Iglesias-Bartolome et al., (2018) reported that oral mucosa showed minimally up-regulated inflammatory pathways during the healing process. In contrast, in the cutaneous microenvironment, inflammatory responses were less active at steady state but became up-regulated throughout the healing process and did not resolve by day 6, suggestive of a chronic inflammatory response when compared to the oral mucosa. These findings demonstrate that decreased inflammation is a key feature of the privileged repair of oral mucosa and thus, the persistent and more prolonged inflammatory response in skin could explain the phenomena of scar formation, fibrosis and delayed wound closure observed in this tissue (Eming et al., 2007).

Furthermore, the contribution of immune cells to wound healing appears to be prolonged in skin compared to oral wound healing. This trend for faster resolution of the inflammation in the oral wound compared to the skin was also reflected in the number of T cells and monocytes, which were more frequently observed in skin compared to oral tissues at the later stages of wound healing, and this might be related to microbial clearance (Szpaderska et al., 2003; Mak et al., 2009; Iglesias-Bartolome et al., 2018).

Since the studies shown in the literature have been performed using different wound sizes and different species, they are not exactly comparable. However, all studies conclude that the inflammatory phase of wound healing is more intense and longer in skin compared to oral wounds.

3. EXPLORING SCAR AND SCARLESS WOUND HEALING IN DIFFERENT ORAL SOFT TISSUES

The oral cavity has many structurally different tissues that likely heal in different ways. Periodontists and oral surgeons are well aware that a differential outcome in terms of scarring between oral mucosa and attached gingiva is a common finding in clinical practice, with gingival repair resulting in a clinically scar-free healing (Wong et al., 2009, Larjava et al., 2011; Vescarelli et al., 2017).

To date, the influence of tissue site, patient age and other patient-related factors on oral wound healing is unclear.

Larjava et al., (2011) reported that, palatal wounds heal with minimal scars and rapid resolution of inflammation. The expression of about 1000 genes was either up-regulated (≥ 2-fold change) or downregulated (≥ 50% change) 1 day after wounding and about half of that number in 3- and 7-day-old wounds and that. Therefore, the main changes were observed in the first day after injury.

In a recent study, Wang & Tatakis (2017) analysed the gene expression profile of human gingiva following experimental surgical wounding (palatal biopsies) in a split mouth model. The significance of differentially expressed genes (DEGs) was evaluated comparing the healthy and healing tissue 5 days after the intervention. The results indicated that there are several hundred significant DEGs in healing gingiva, compared to normal gingiva. Functionally, most of the DEGs were linked to cytokines, chemokines, immunoglobulins, ECM, cytoskeleton, and angiogenesis. Of all the DEGs identified, several have not been previously reported to be expressed in normal human gingiva. These include highly up-regulated genes, for example, SPP1, C2CD4A, and TREM2, as well as significantly down-regulated genes, such as COCH and SYT16. The functional significance of many of these

genes in the course of gingival healing remains unknown.

Vescarelli et al., (2017) in a human study compared the wound healing in oral mucosa and gingiva, investigating the role of α-smooth muscle actin (αSMA)-expressing myofibroblasts and autophagy. In this study, biopsies were obtained from seven patients immediately before and 24 hrs after vertical releasing incision in oral mucosa and attached gingiva. Both whole biopsies and primary cultures of fibroblasts derived from the same tissues were subjected to immunofluorescence, Western blot and quantitative real-time PCR analyses. The results showed that, in oral mucosa, characterized by partially fibrotic outcome during repair, the activation of autophagy determined an increase in αSMA and collagen 1a1 production and that; conversely, wound healing did not stimulate autophagy in attached gingiva, and subsequently, no increase in myofibroblast differentiation and collagen deposition could be seen, thus justifying its scar less outcome.

A very recent human study (Kabakov et al., 2021) have compared the gene expression profiles and proliferation rates of fibroblasts from the oral lining and masticatory mucosa (palate). It was observed that lining mucosa derived-fibroblasts exhibit significantly higher expression of the principal structural collagens while masticatory mucosa fibroblasts showed greater expression of genes related with cell proliferation. In fact, palate-derived fibroblasts showed a 10%-30% higher proliferation rate.

Although palatal and gingival tissue are similar in their structure and characteristics, palatal tissue presents peculiar features (Squier & Kremer, 2001; Squier & Finkelstein, 2003), and a faster clinical wound healing is observed when compared with buccal attached gingiva.

The current literature on soft tissues wound, comparing the response between different oral soft tissues, mainly from a biomolecular point of view, remains scarce and unclear. Therefore, comparative studies evaluating differences between alveolar mucosa, gingival and palatal tissues in the wound healing process are lacking.

4. THE IMPORTANCE OF POST-SURGICAL CARE IN ORAL WOUND HEALING

After a surgical procedure, mechanical plaque control cannot be performed. Since microbial post-surgical infection inhibits normal tissue healing process (Powell et al., 2015), plaque accumulation control using antimicrobial agents during early post-surgical period is of fundamental importance (Sanz et al., 1989; Newman et al., 1989).

4.1. Role of clorhexidine digluconate mouthrinses

The use of antiseptics has generated a lot of controversy as studies have shown that they are detrimental to wound healing (Drosou et al., 2003). Many of the studies were carried out on tissue culture and have had conflicting results with those done on live animals (Tatnall et al., 1991).

Chlorhexidine (CHX), widely used as antiseptic, especially as post-surgical indication, has a toxic effect both *in vivo* and *in vitro* and their influence on wound healing has been studied for a long time. One of the first animal studies, in 1980,(Bassetti & Kallenberger, 1980) concluded that intensive rinsing with high concentrations of chlorhexidine after oral surgical operations, could result in delay and disturbance of wound healing. Another more recent animal study (Faria et al., 2009), concluded that CHX induces apoptosis or necrosis in the fibroblasts, in a concentration-dependent manner.

Mariotti and Rumpf, in 1999, carried out an *in vitro* studied incubating human gingival fibroblasts in CHX. The results have suggested that chlorhexidine could induce a dose dependent reduction in cellular proliferation and that concentrations of CHX that have little effect on cellular proliferation can significantly reduce both collagen and non-collagen protein production of human gingival fibroblasts. Hence, the introduction of commercially available concentrations (0.12%) or diluted commercial concentrations (as low as 0.00009%) of chlorhexidine to surgical sites for short periods of time prior to wound closure can conceivably have serious toxic effects on gingival fibroblasts and may negatively affect

wound healing (Mariotti & Rumpf, 1999).

Therefore, several *in vitro* studies have demonstrated that the CHX is not harmless to the oral tissues (Kenney et al., 1972; Helgeland et al., 1971; Knuuttila & Söderling, 1981; Mariotti & Rumpf, 1999; Chang et al., 2001; Faria & Celes, 2007; Faria et al., 2009). Taking into account that the main changes in the oral wound healing process occurs in the early phases, understanding the CHX effect at this time could be relevant to the repair process. However, *in vivo* CHX effect in the early phases after oral surgical wounding is not entirely clear.

5. THE USE OF BIOACTIVE SUBSTANCES TO IMPROVE ORAL WOUND HEALING

Wound healing is often prolonged or extremely difficult in patients with comorbidity (for example with diabetes or vascular disease) and represent a challenge for the treating physician. In cases of initially normal wound healing, a change in the progress of wound closure is sometimes observed and often depends on individual circumstances. Intra-surgical or topical applications of bioactive substances are commonly used to improve/accelerate the wound healing process or to treat badly healing wounds.

5.1. Role of exogenous hyaluronic acid

HA is a key element in the soft periodontal tissues, gingiva, and periodontal ligament, and in the hard tissue, such as alveolar bone and cementum (Dahiya & Kamal, 2013). It has many structural and physiological functions within these tissues.

It can play a regulatory role in inflammatory response: the high molecular weight HA synthesized by hyaluronan synthase enzymes in the periodontal tissues, gingiva, periodontal ligament, and in alveolar bone (Ijuin et al., 2001) undergoes extensive degradation to lower molecular weight molecules in chronically inflamed tissue, such as gingival tissue inflammation (Bartold & Page, 1986) or in the postoperative period after implant or sinus lift surgery.

As a consequence of the many functions attributed to HA, advances have been made in the development and application of HA-based biomaterials in the treatment of various inflammatory conditions (Laurent, 1998).

To date, topical therapies guarantee a better delivery of high concentrations of pharmacologic agents to the soft periodontal tissue, gingiva, and periodontal ligament as well as to the hard tissue such as alveolar bone and cementum. Topical hyaluronic acid (HA) has recently been recognized as an adjuvant treatment for chronic inflammatory disease in addition to its use to improve healing after dental procedures (Casale et al.,2016). Data obtained from a systematic review of 20 clinical studies demonstrate that, due to its positive action on tissue repair and wound healing, topical administration of HA could play a role not only in postoperative dental surgery, but also in the treatment of patients affected by gingivitis and periodontitis, with a significant improvement in their quality of life (Casale et al.,2016).

However, to date, the studies describing the role of exogenous HA in the wound healing process are *in vitro* or using animal models (West et al., 1985; Scully et al., 1995; Pilloni & Bernard, 1998; Pilloni et al., 2003; Fujioka-Kobayashi et al., 2017). Human studies reported the results only in terms of clinical response (Romeo et al., 2014). The main problem of *in vitro* experiments is that during the post-surgical period, HA would undergo degradation to molecules of lower molecular weight (MW) due to the hyaluronidase activity, thus exerting additional or even opposing effects on the wound repair process (Asparuhova et al., 2019). Therefore, *in vivo* exogenous HA effect in the early phases after oral surgical wounding is not entirely clear.

6. PERSONALISED MEDICINE: APPLIYING MOLECULAR UNDERSTANDING. TRANSLATIONAL GAP?

Personalised medicine (PM) refers to an emerging approach to medicine that uses scientific insights into the genetic and molecular basis of health and disease to guide decisions in regard to the prediction, prevention, diagnosis and treatment of disease, to offer the "right treatment for the right person at the right time"(Scholz, 2015).

6.1. Tools for molecular understanding: omics technologies and biomarkers

- Omics technologies (OT) allow define and explain the molecular mechanisms of the human body. Genomics (the study of genes and their functions) and proteomics (the study of proteins) are examples of OT. Omics platforms are capable of analysing the functions of different classes of molecules in a high-throughput manner. These analyses can provide information on the molecular and cellular processes that have an impact on altered normal biological processes or disease.

Translating omics into clinical applications is thought to play a crucial role not only in diagnosis and treatment, but also in prevention of illness, and enable a better understanding of human health and disease in general (Scholz, 2015).

- Biomarkers are measurable indicators of healthy and pathological processes in the body, and may be used to identify and diagnose a disease as early as possible, detect risk of developing a disease, assess the response to a treatment (Scholz, 2015).

With a better understanding of the molecular basis of both normal and altered biological process it will become easier to select better-adapted treatments and also to develop new approaches (Di Sanzo et al., 2017). Nevertheless, it has been pointed out that progress in translating molecular-biology into clinical practice is slow ("translational gap") (Scholz, 2015).

6.2. PM in dental practice

The key to the successful implementation of personalised medicine in dental practice will depend on the identification of clinically validated biomarkers that can be reliably linked to a specific altered normal biological processes or diseases, providing reliable targets for therapy (Ballman, 2015)

Biomarkers will revolutionize the diagnosis and treatment of dental diseases (Polverini, 2018). In the dental field, PM could be relevant to reduce the negative and detrimental

effects that systemic conditions or chronic diseases have on oral health and wound healing. In fact, that the integration of molecular diagnostics and PM has been shown to provide a directed and targeted approach to wound care (Dowd, 2011).

For this reason, the identification of biomarkers that could be useful to reveal new therapeutic targets is of fundamental importance. Greater knowledge about gene expression and cellular signalling networks enables the design of drugs and therapeutic approaches with greater precision, with the aim of improving diseases /altered conditions (Godman, 2013).

7. CONCLUSIONS

Proper repair after trauma surgery, or illness serves to maintain the structural and functional integrity of organs and tissues. Normal wound healing, is critical for successful surgical outcomes.

A different clinical wound healing response is commonly observed between the different oral soft tissues (alveolar mucosa, buccal attached gingiva and palatal tissue). Recognizing the differences in the behaviour of fibroblasts (key players cells in tissues repair process) derived from these tissues should facilitate the understanding of the physiology and physiopathology of the oral wound repair process, allowing the identification of new molecules/genes and helping to promote cell therapies for diseases or injuries treatment involved both oral and extra-oral tissues. Therefore, the aim of the first part of this research is focused on comparing the behaviour of fibroblasts derived from different oral soft tissues in the early phases of wound healing process from a biomolecular point of view.

Since the presence of biofilm can alter the normal course of the wound healing, antimicrobial agents should be considered during the post-surgical period. Several *in vitro* studies have reported that CHX (considered the gold standard for antiseptic treatment of the oral cavity) compromises the repair process, showing a high cytotoxicity. Thus, the second part of this project assesses the *in vivo* effect of post-surgical CHX on gingival tissue in the early phases of the wound healing process.

Finally, improving wound healing through the use of bioactive substances has been of great interest in the last years. The role of exogenous HA has been described, mainly through *in vitro* and animal studies. The last part of this investigation evaluates the *in vivo* effect of intra-surgical HA applicaiton on gingival tissue in the early phases of the wound healing process.

As reported through Personal Medicine applied to dental practice, a better understanding of the molecular basis of both normal and altered biological process will become easier to select better-adapted treatments and to develop new approaches. For this reason, in the three parts of the present investigation a biomolecular analysis is carried out, in order to detect new gene markers and cellular signalling networks involved in molecular process of

oral tissues repair and how this can be modified after post-surgical CHX and intra-surgical HA treatment. These results are correlated with a histological, immounohistochemical and clinical evaluation.

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II. A comparative *in vivo* analysis of gene expression profiles of different human oral soft tissue-derived fibroblast from healing wounds. Correlation with clinical outcome, autophagy activation and fibrotic markers expression

1. INTRODUCTION

Wound healing is a complex process orchestrated by a variety of known and unknown factors, divided into four phases (haemostasis, inflammation, proliferation and remodeling) that occur in both skin and oral tissues (Hämmerle & Giannobile, 2014). However, oral tissues present special features as rapid wound closure and reduced scar formation (Zuckerman & DiPietro, 2003; Szpaderska et al., 2005; Chen et al., 2010; Iglesias-Bartolome et al., 2018; Simões et al., 2019).

In order to provide a better understanding of the complexity of the repair process, previous studies have examined the wound transcriptome through a microarray analysis comparing oral and dermal wound repair responses (Chen et al. 2010; Iglesias-Bartolome et al., 2018; Simões et al., 2019).

Chen et al., (2010) compared tongue wounds and dermal wounds gene expression at different healing time intervals in an animal model, and concluded that oral and skin wounds had a comparable degree of transcriptional changes, except for 12 and 24 hrs.

In a recent human study (Iglesias-Bartolome et al., 2018), epithelial tissues from oral and dermal wounds were analysed, showing a keratinocyte-driven wound repair in healthy individuals and raising the possibility that the transcriptional regulatory networks responsible for the accelerated healing in oral mucosa are already present in the unwounded state.

Recently, Simões et al. (2019) evaluated differential microRNA profiles in dermal versus hard palate oral wounds on an animal model, showing an intrinsic genetic response that accelerates repair in oral tissues.

Nevertheless, the above-mentioned studies used different oral wound models (cheek, tongue, buccal gingiva, palate) without taking into account their anatomical and functional differences. Moreover, two of them were performed in animals. However, while the biomolecular basis of the differences between oral and skin repair have been described, this is less understood regarding the different oral tissues.

To date, a global and comprehensive comparative profiling of the differently expressed genes in the human oral soft tissues after injury has not been reported. Nevertheless, a different clinical repair outcome between alveolar mucosa and attached gingiva is a common finding, with a scar-free gingival repair (Wong et al., 2009; Larjava et al., 2011). In a previous human study (Vescarelli et al., 2017), we demonstrated an increase in αSMA expression and autophagy activation in alveolar mucosa 24 hrs after injury, but not in gingiva, resulting in a scarless outcome. Although a similar behaviour could be expected in the palatal tissue (Chen et al., 2019; Simões et al., 2019), its peculiar characteristics (Squier & Finkelstein, 2003) could account for a different outcome.

Fibroblasts are mesenchymal cells essential for wound healing and the repair processes, since they are responsible for the production of most of the extracellular matrix (ECM) in connective tissues. In latest years, the role of fibroblasts and myofibroblasts as key players in tissues repair has been extensively reported and it has become clear that fibroblasts from different tissues present several distinct features (Chiquet et al., 2015). In periodontal wound healing, the regeneration of connective tissues involves cellular activities driven by fibroblasts populations, such as secretion of matrix molecules and the organization of these matrix components into functionally active fibers that finally restore the periodontium (Chiquet et al., 2015; Smith et al., 2019).

A better understanding of the role of these cells in the wound healing of periodontal soft tissues, through a genetic profile analysis, could be of fundamental importance, in order to identify selected pathways and molecules that could open the way to the development of targeted approaches directed to the mesenchymal component of periodontal tissues, optimizing the wound microenvironment.

2. AIM

The main aim of this project is to analyse and compare the gene expression profiling of oral fibroblasts from human alveolar mucosa (M), gingival (G) and palatal (P) tissues in the early phases following surgical wounding, in order to identify critical differences in the healing response, correlating it with the clinical response, autophagy activation and fibrotic markers expression.

The second aim is to evaluate and compare gene expression profiling, autophagy activation and fibrotic markers expression of M, G, and P-derived fibroblasts in unwounded tissues.

3. HYPOTHESIS

There is a difference in the gene expression between human alveolar mucosa, buccal attached gingiva and palatal tissue derived-fibroblasts in the early phases of woundhealing. The differences observed are correlated with the clinical response, autophagy activation and fibrotic markers expression: (1) palatal tissue present the highest clinical wound healing score value (clinical scarless repair), in agree with a reduced autophagy activation and fibrotic markers expression; (2) alveolar mucosal tissue present the lowest clinical wound healing score value (clinical scar repair) in agree with an increase in autophagy activation and fibrotic markers expression.

Some of the observed differences are already present in the unwounded tissues, since their peculiar features could be partially predetermined.

4. MATERIALS AND METHODS

4.1. Ethics statements

The study protocol (ClinicalTrial.gov-NCT04202822) was approved by Sapienza University of Rome Ethics Committee (Ref.5315-Prot.2018/19). Each participant signed an informed consent in accordance with the Declaration of Helsinki (1975, revised in 2013).

4.2. Study design and patient selection

The present pilot study included six healthy adult patients (mean age 42.83±13.28, Table 1). Inclusion criteria were: (1) patients who underwent periodontal surgery to treat residual periodontal pockets at completion of non-surgical periodontal therapy; (2) patients with periodontal healthy status (Full-mouth Plaque Score and Full-Mouth Bleeding Score <15%, Lang & Bartold, 2018); (3) patients who agreed to be "volunteer" for biopsy collection procedures by signing an informed consent. Patients who underwent antibiotic or anti-inflammatory drugs consumption during the previous six months, patients in pregnancy or lactation period and smokers were excluded from the study. The subjects were enrolled at the clinical center of the Section of Periodontics, Sapienza University of Rome, Department of Oral and Maxillo-Facial Sciences.

TABLE 1 Study population

Patient	Demographic data	
	Age	**Sex**
1	33	F
2	50	F
3	55	F
4	22	M
5	37	M
6	60	F
Mean ± SD	42.83 ± 13.28	
Range	22-60	

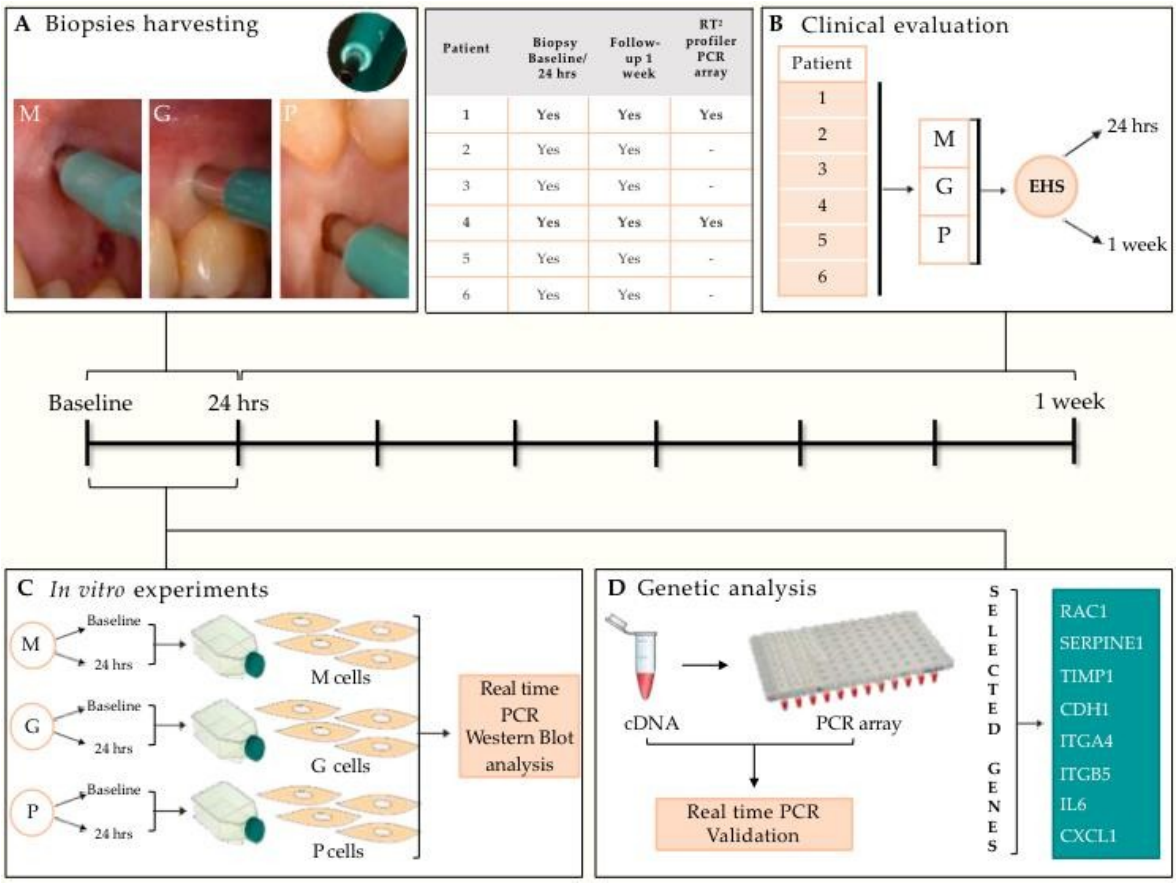
F, female; M, male; SD, standard deviation

Biopsies from alveolar mucosa (M), buccal attached gingiva (G) and palatal tissue (P) were harvested from vertical releasing incisions (VRIs) at baseline and 24 hrs after surgical procedures, replicating the wound model used in our previous study (Vescarelli et al., 2017). Clinical response was evaluated at 24 hrs and 1 week after surgery by means of the Early Wound Healing Score (EHS; Marini et al., 2018; Marini et al., 2019).

Primary cultures of fibroblasts derived from biopsies of the three tissues were subjected to Western blot and qRT-PCR analyses to assess the expression of fibrotic markers and autophagy activation. Cells obtained from biopsies of two patients were processed for gene expression profiling and a network analysis of the differentially expressed genes (DEGs) was performed. The expression of the following key genes was then validated through qRT-PCR on cDNAs from cells obtained from the biopsies of each patient and on pooled cDNAs of all the enrolled patients: ras-related C3 botulinum toxin substrate 1 (RAC1), serpin family E member 1 (SERPINE1), TIMP metallopeptidase inhibitor 1 (TIMP1), cadherin 1 (CDH1), integrin subunit alpha 4 (ITGA4), integrin subunit beta 5 (ITGB5), interleukin 6 (IL6) and C-X-C motif chemokine ligand 1 (CXCL1).

The experimental design is presented in Figure 1.

FIGURE 1 Experimental design



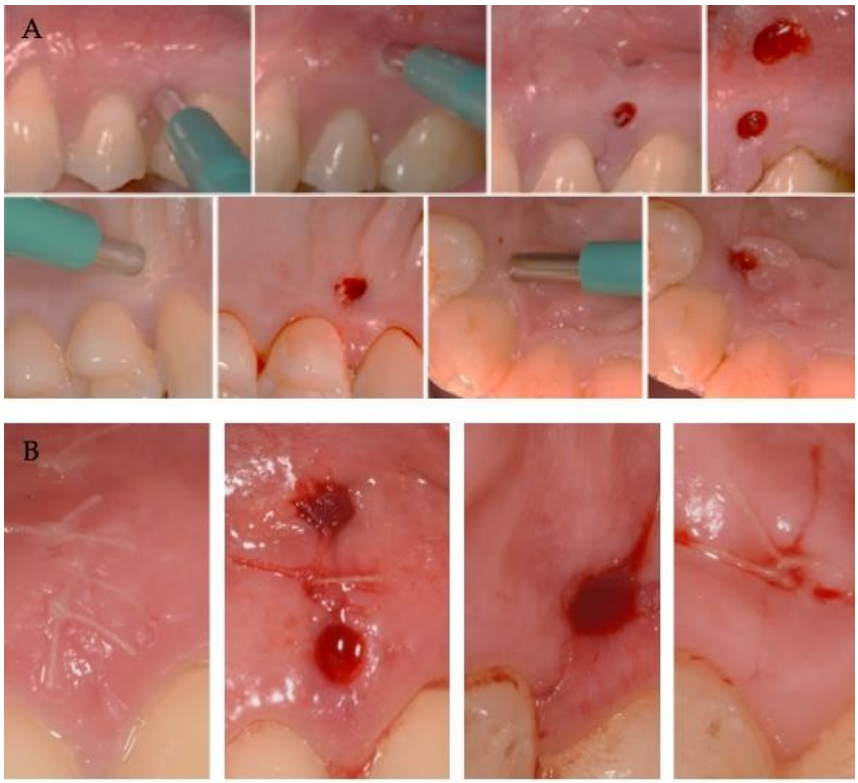
(A) Alveolar mucosa (M), buccal attached gingival (G) and palatal (P) biopsies were harvested at baseline and 24 hrs after surgery from six patients. **(B)** Clinical evaluation was performed at 24 hrs and 1 week after surgery by means of Early Healing Score (EHS). **(C)** *In vitro* experiments were performed on primary cultures of human fibroblasts derived from M, G and P tissue established at baseline and 24 hrs. In a pool of six patients, quantitative real-time PCR analysis of αSMA expression was performed to evaluate the extent of myofibroblast differentiation. Autophagic activation was evaluated through Western blot analysis. **(D)** Total RNA was extracted from primary cultures of human fibroblasts derived from M, G and P tissues biopsies. Gene expression profiling of wound healing genes was performed in M, G and P cells obtained from two different patients, using the Human Wound Healing RT² Profiler™ PCR Array. Then, the expression of eight selected genes (*RAC1, SERPINE1, TIMP1, CDH1, ITGA4, ITGB5, IL6* and *CXCL1*) was validated by means of qRT-PCR analysis on total RNA from M, G an P cells pooled from six patients, as well as from each patient separately.

4.3. Surgical procedures and biopsy collection

All the surgical procedures and biopsies were performed by the same operator (MR). Following local anesthesia, M, G, and P baseline biopsies were harvested immediately prior to the surgical procedure at the level of the VRIs with a biopsy punch of 2.0 mm diameter (Figure 2A). At the end of the surgical procedure primary closure was obtained with interrupted sutures (polyglycolic acid-PGA, 6-0 monofilament). During this period, patients were instructed not to use mouth-rinses.

The final M, G and P biopsies were harvested at the level of VRIs 24 hrs after surgery (Figure 2B). These areas healed by second intention and sutures were removed at 1 week.

FIGURE 2 Alveolar mucosa, buccal attached gingiva and palatal tissue biopsies collection at baseline and 24 hrs after surgery



M,G and P biopsies harvested with 2mm dimeter punch at baseline **(A)** and 24 hrs after the surgical procedure **(B).**

4.4. Cell cultures

Primary cultures of human fibroblasts from M, G and P biopsies were established as previously described (Vescarelli et al., 2017). Briefly, the biopsy samples were cut into small pieces. All fragments were transferred into a centrifuge tube and subjected to enzymatic dissociation. Fragments were gently pipetted until disintegration into a single cell suspension. Cells were then seeded onto collagen IV (10 mg/ml)-coated culture plates and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich, MI, Italy), supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and antibiotics (Penicillin/Streptomycin). Experiments were performed at the same time for the three tissues, so M, G and P cells of each patient were analysed at the same cell passage (3-8).

4.5. RT² Profiler PCR array

Total RNA of M, G and P cells from two patients was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). cDNAs obtained were used for gene expression profiling using the Human Wound Healing RT² Profiler™ PCR Array (Qiagen, MI, Italy), according to the manufacturer's instructions. Fold changes in expression between baseline and 24 hrs or between the three tissues at baseline were determined with the $2^{-\Delta\Delta CT}$ method. Heatmap and Venn diagrams were generated using the web-based tools Morpheus (https://software.broadinstitute.org/morpheus/ and Venn (http://bioinformatics.psb.ugent.be/webtools/Venn/), respectively.

4.6. Clinical analysis

A blinded examiner evaluated the clinical wound healing response at the level of the VRIs in M, G and P tissue 24 hrs after surgery (before harvesting the biopsies) using the EHS. The same evaluation was performed 1 week after the surgical procedure, immediately before suture removal.

4.7. Bioinformatics analysis

Gene ontology (GO) analysis was performed using Protein Analysis Through Evolutionary Relationships (PANTHER) classification system software (http://www.pantherdb.org). The protein-protein interaction (PPI) network was constructed from STRING database (https://string-db.org/) then visualized and edited with Cytoscape software (version 3.8.0). The APP plug-ins Molecular Complex Detection (MCODE) and cytoHubba were used to cluster densely connected genes and to identify important hub genes of the entire network, respectively.

4.8. Quantitative real- time PCR (qRT-PCR)

qRT-PCR was performed on RNA from cell cultures of all the patients, as previously described (Nodale et al., 2014a). Total RNA from cell cultures obtained from M, G and P biopsies of six patients at baseline and 24 hrs after surgery was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and was reverse transcribed using High Capacity RNA to cDNA Kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). cDNAs from M, G and P cells of single patients as well as pooled cDNAs of all patients were then used for amplification of αSMA, RAC1, SERPINE1, TIMP1, CDH1, ITGA4, ITGB5, IL6 and CXCL1 using the appropriate TaqMan gene expression assay kits (Applied Biosystems). A total of 2 µl/well of template was added to the sample wells along with TaqMan Universal PCR master mix at a concentration of 1x

and water to a volume of 25 μl/well. Assays were conducted in triplicate on an ABI 7500 Real Time instrument (Applied Biosystems) using the following conditions: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min, repeated 40 times. Relative quantification was performed using GAPDH mRNA as an endogenous control.

4.9. Western blot analysis

Cells were lysed in RIPA buffer and processed for Western blot analysis as previously described (Nodale et al., 2014b). Densitometric analysis was performed with Quantity One Program (Bio-Rad Laboratories S.r.l., Segrate, MI, Italy) as previously described (D'Amici et al., 2013).

Total proteins (50 μg) were resolved under reducing conditions by 8–15% SDS-PAGE and transferred to Immobilon-FL membranes (Millipore). Membranes were incubated overnight at 4°C with the following primary antibodies: αSMA (1: 1000 dilution; Sigma-Aldrich), Col1a1 (1:200 dilution; Santa Cruz Biotechnology), p-AKT (1:1000 dilution; Cell Signaling Technology), LC3 (1:2000 dilution; Sigma-Aldrich) and P62 (1:1000 dilution; BD Biosciences), followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich). β-actin (1:2000 dilution; Santa Cruz) was used as internal control. Bound antibody was detected by enhanced chemiluminescence detection reagents (Pierce Biotechnology Inc., Rockford, IL, USA) according to manufacturer's instructions.

4.10. Statistical analysis

Data were analysed on Prism 8.0 (GraphPad Software, La Jolla, USA) and are shown as mean ± SD from three independent experiments conducted in triplicate. Two-tailed unpaired Student's t test was used for statistical analysis. For continuous variables (EHS score), median and the interquartile range (IQR) were calculated, and the nonparametric Mann-Whitney U test was used for statistical analysis. P values < .05 was considered

statistically significant. DEGs were identified via fold change filtering using $p < .05$ and a cut-off of absolute fold change > 2.

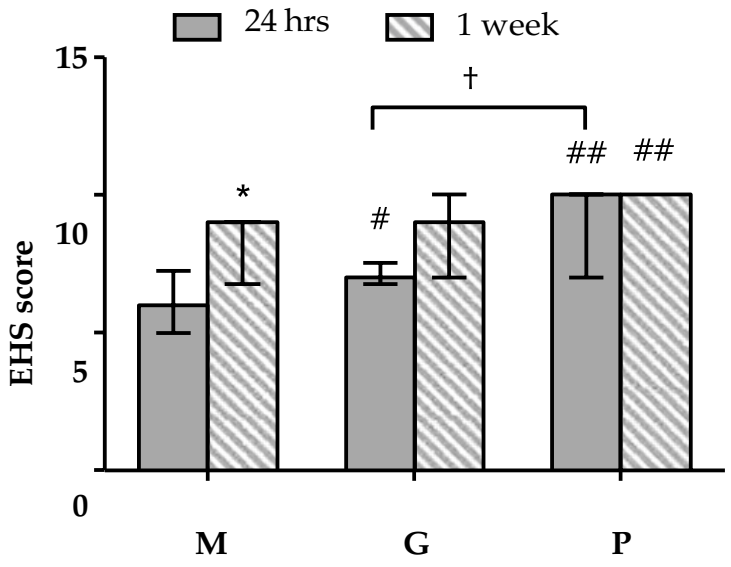
5. RESULTS

5.1. Clinical wound healing response

All the surgical procedures were uneventfully and successfully completed.

24 hrs post-surgery, the median EHS value in P (10, IQR 3) and in G (7, IQR 0.75) were significantly higher than in M (6, IQR 1.25). At this time, P showed the highest EHS value and the difference was significant with the G and M tissues. At 1 week, no significant differences were found between M and G tissues, while *P* values were still significantly higher than M (Figure 3, Table 2). Clinical photographs of patients 4 and 6 are presented in Figure 4.

FIGURE 3 Differential clinical wound healing response after vertical releasing incision (VRI) in M, G and P tissues



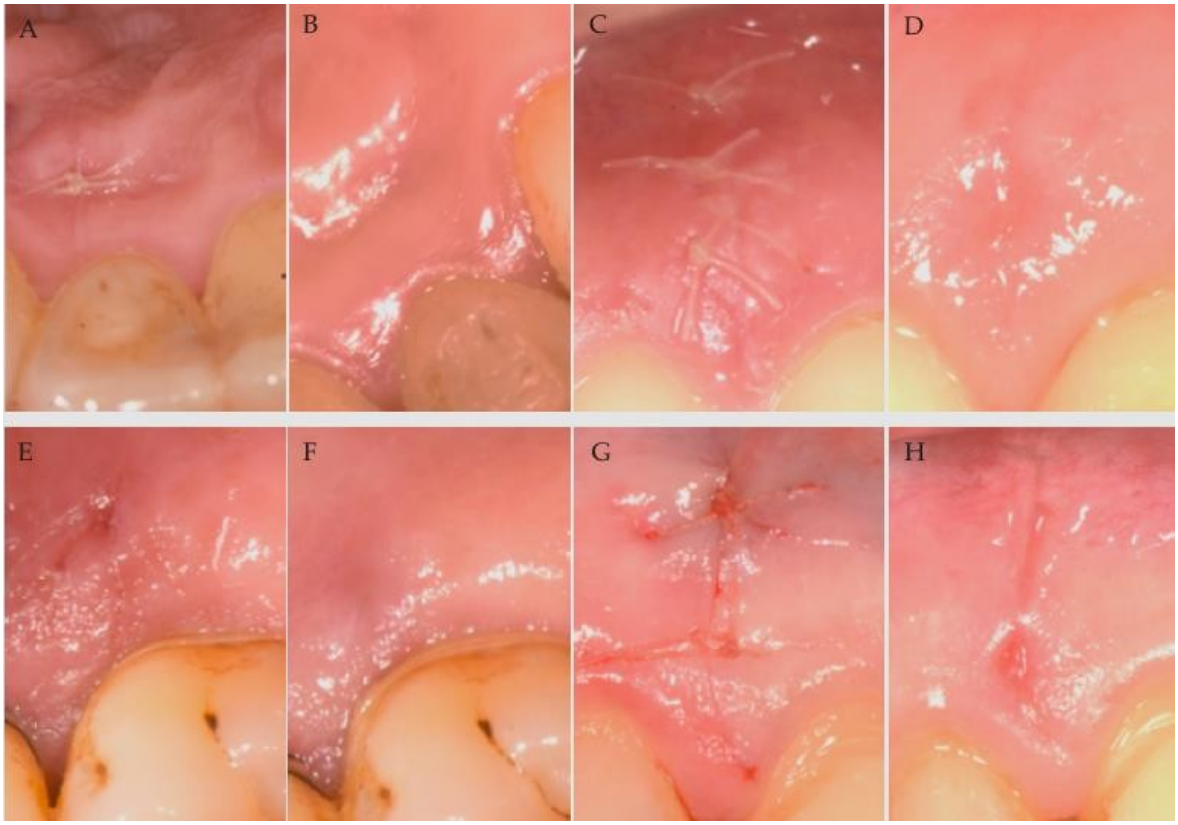
Clinical wound healing response was evaluated through assessment of Early Wound Healing Score (EHS) at 24 hrs and 1 week after surgery in M, G and P tissues. The median values of EHS were reported. Error bars represent interquartile range (IQR). $^{*}p < .05$ *vs* 24 hrs; $^{\#}p < .05$ and $^{\#\#}p < .005$ *vs* M; $^{\dagger}p < .05$ *vs* G.

TABLE 2 Clinical wound healing response 24 hrs and 1 week after injury

Patient	EHS					
	24 hrs			1 week		
	M	G	P	M	G	P
1	5 (R3,HO,I2)	6 (R3,H1,I2)	10 (R6,H2,I2)	9 (R6,H2,I1)	9 (R6,H2,I1)	10 (R6,H2,I2)
2	6 (R3,H2,I1)	7 (R3,H2,I2)	10 (R6,H2,I2)	9 (R6,H2,I1)	9 (R6,H2,I1)	10 (R6,H2,I2)
3	7 (R3,H2,I2)	9 (R6,H2,I1)	10 (R6,H2,I2)	9 (R6,H2,I1)	10 (R6,H2,I2)	10 (R6,H2,I2)
4	6 (R3,H2,I1)	7 (R3,H2,I2)	10 (R6,H2,I2)	9 (R6,H2,I1)	10 (R6,H2,I2)	10 (R6,H2,I2)
5	6 (R3,H2,I1)	7 (R3,H2,I2)	7 (R3,H2,I2)	6 (R3,H2,I1)	7 (R3,H2,I2)	10 (R6,H2,I2)
6	5 (R3,HO,I2)	7 (R3,H2,I2)	7 (R3,H2,I2)	7 (R3,H2,I2)	7 (R3,H2,I2)	10 (R6,H2,I2)
Median (IQR)	6 (1.25)	7 (0.75)	10 (3)	9 (2.25)	9 (3)	10 (0)

EHS, Early Wound Healing Score; M, alveolar mucosa; G, buccal attached gingiva; P, palate; R, clinical signs of re-epithelialization; H, clinical signs of haemostasis; I, clinical signs of inflammation; IQR, interquartile range

FIGURE 4 Clinical wound healing of alveolar mucosa, buccal attached gingiva and palatal tissue 24 hrs and 1 week after surgery



Clinical wound healing at the level of VRIs **(A-D)** Patient 4: (A) P at 24 hrs (EHS=10), (B) P at 1 week (EHS=10), (C) M and G at 24 hrs (EHS=6; EHS=7), (D) M and G at 1 week (EHS=9; EHS=10). **(E-H)** Patient 6: (E) P at 24 hrs (EHS=7), (F) P at 1 week (EHS=10), (G) M and G at 24 hrs (EHS=5; EHS=10), (H) M and G at 1 week (EHS=7; EHS=7)

5.2. Myofibroblasts differentiation and autophagy activation in palatal wound healing

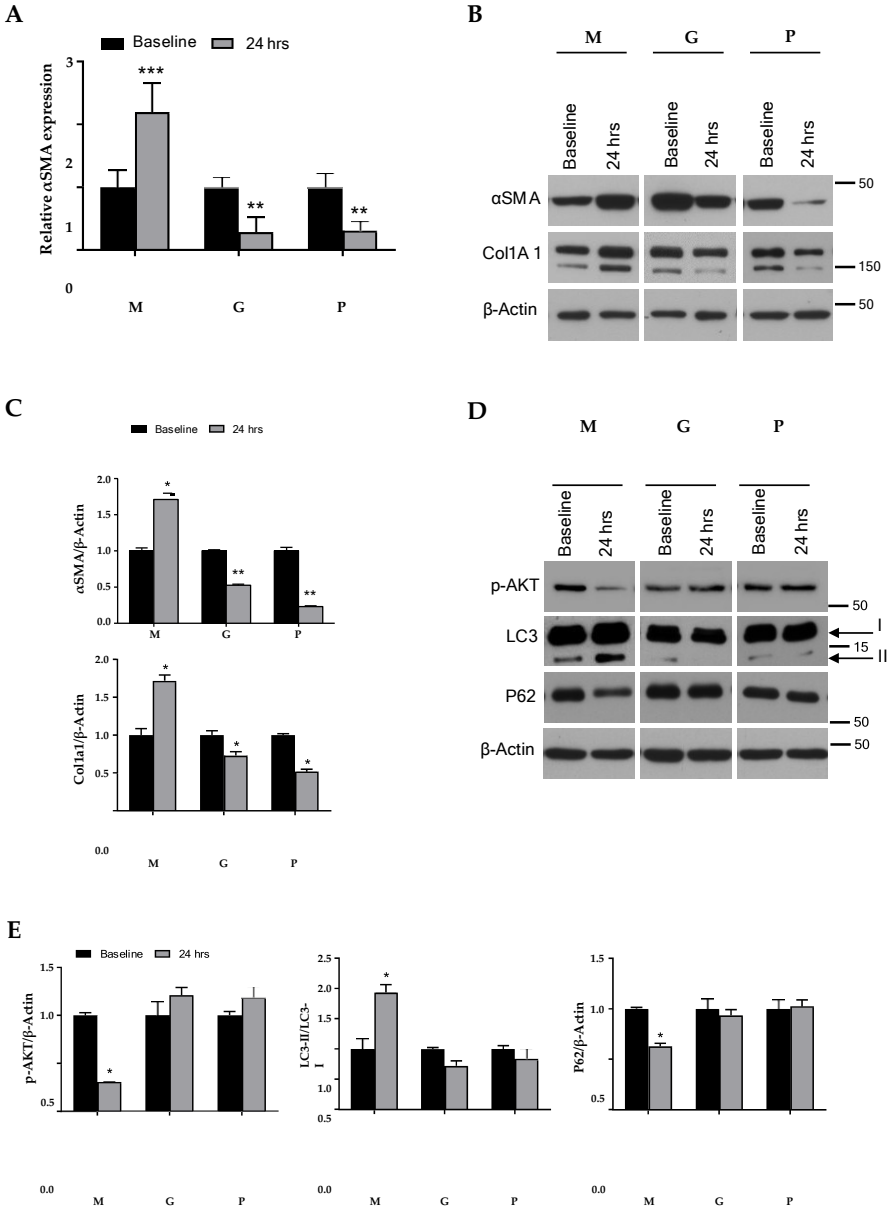
Fibroblast-like cells were isolated to analyze αSMA expression at both mRNA and protein level. qRT-PCR analysis in a pool of six patients confirmed αSMA increase in M (2.2-fold) and decrease in G (0.3-fold) at 24 hrs. P behaved like G, with a significant decrease in αSMA expression at 24 hrs (0.3-fold) (Figure 5A). The results of qRT-PCR analysis were highly consistent on an inter-individual basis: αSMA was upregulated in M at 24 hrs in all the six patients, while for G and P a decrease in αSMA at 24 hrs was observed in four and five out of six patients, respectively (Figure 6).

Such data were also confirmed at protein level, and the same trend was observed for the fibrotic marker Collagen 1a1 (Col1a1) (Figure 5B, C). Noteworthy, we also observed a differential expression of both αSMA and Col1a1 between the three tissues at baseline, with higher levels in G and P than in M (Figure 7).

We then analysed the activation of AKT, a key mediator of cell survival and differentiation and a well-known inhibitor of autophagy (Lotti et al., 2007). We observed a reduction of AKT phosphorylation at 24 hrs in M (0.4-fold), with no significant variations in G and P (Figure 5D, E).

Autophagy was not active in G and P, since no modulation of LC3-II/LC3-I ratio and P62 expression between baseline and 24 hrs was observed. However, it was active in M, which displays increased LC3-II/LC3-I ratio (2.0-fold) and P62 degradation (0.6-fold) (Figure 5D, E).

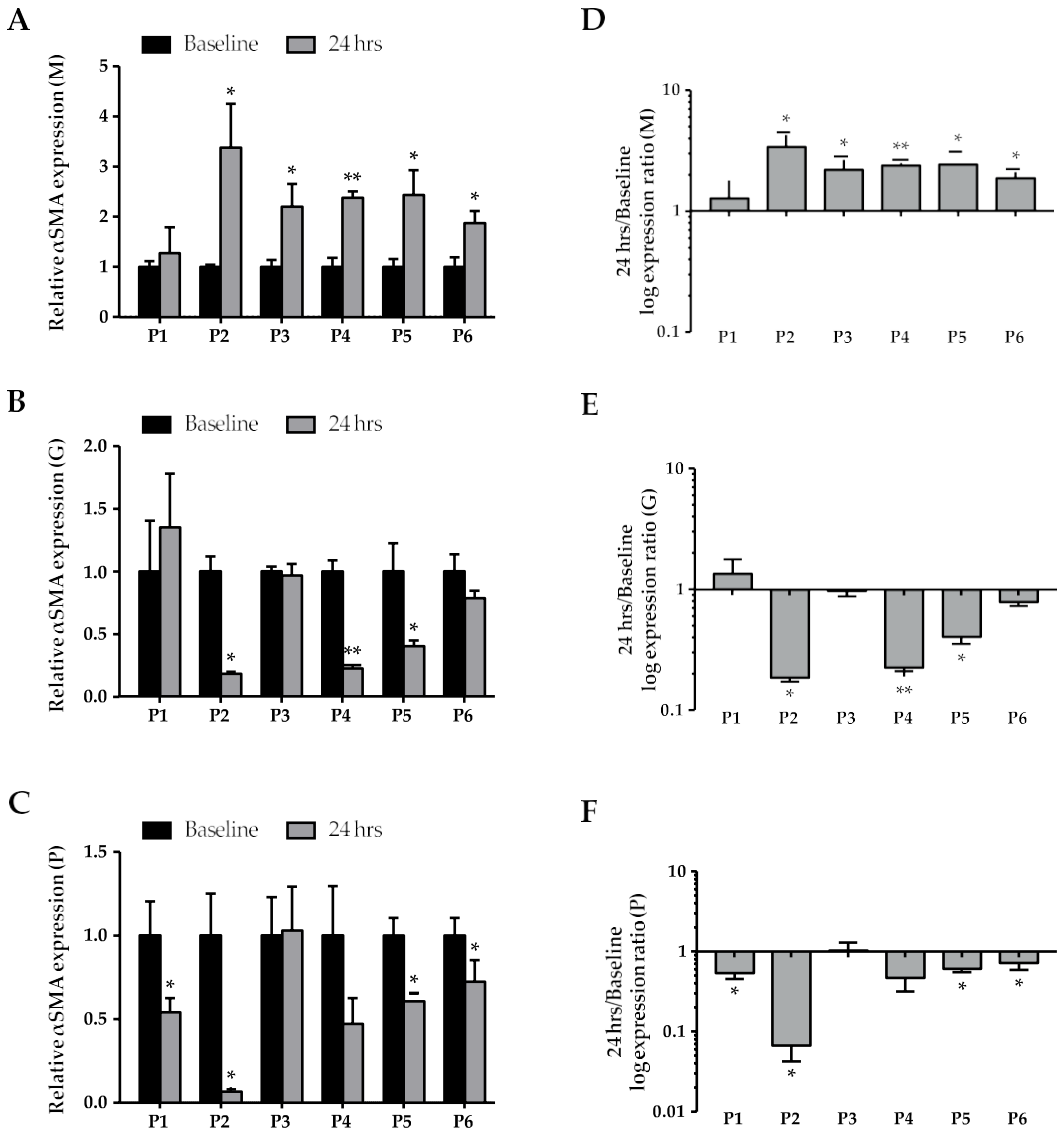
FIGURE 5 Fibrotic markers expression and autophagy pathway activation in M, G and P cells at baseline and 24 hrs after vertical releasing incision



(A) Quantitative real-time PCR analysis of αSMA mRNA expression in a pool of six patients. Relative mRNA levels are shown as fold value of the levels at baseline. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. $^{**}p < .005$ and $^{***}p < .0005$ vs baseline. **(B)** Western blot analysis of αSMA and Collagen 1a1 (Col1A1) protein expression. β-Actin served as loading control. The images are representative of at least two independent experiments for each patient. **(C)** The intensity of the bands in (B) was evaluated by densitometric analysis, normalized and reported as relative expression with respect to baseline. $^{*}p < .05$ and $^{**}p < .005$ vs baseline. **(D)** Western blot analysis of p-AKT, LC3 and P62 in M, G and P cells at baseline and 24 hrs. β-Actin was used as loading control.

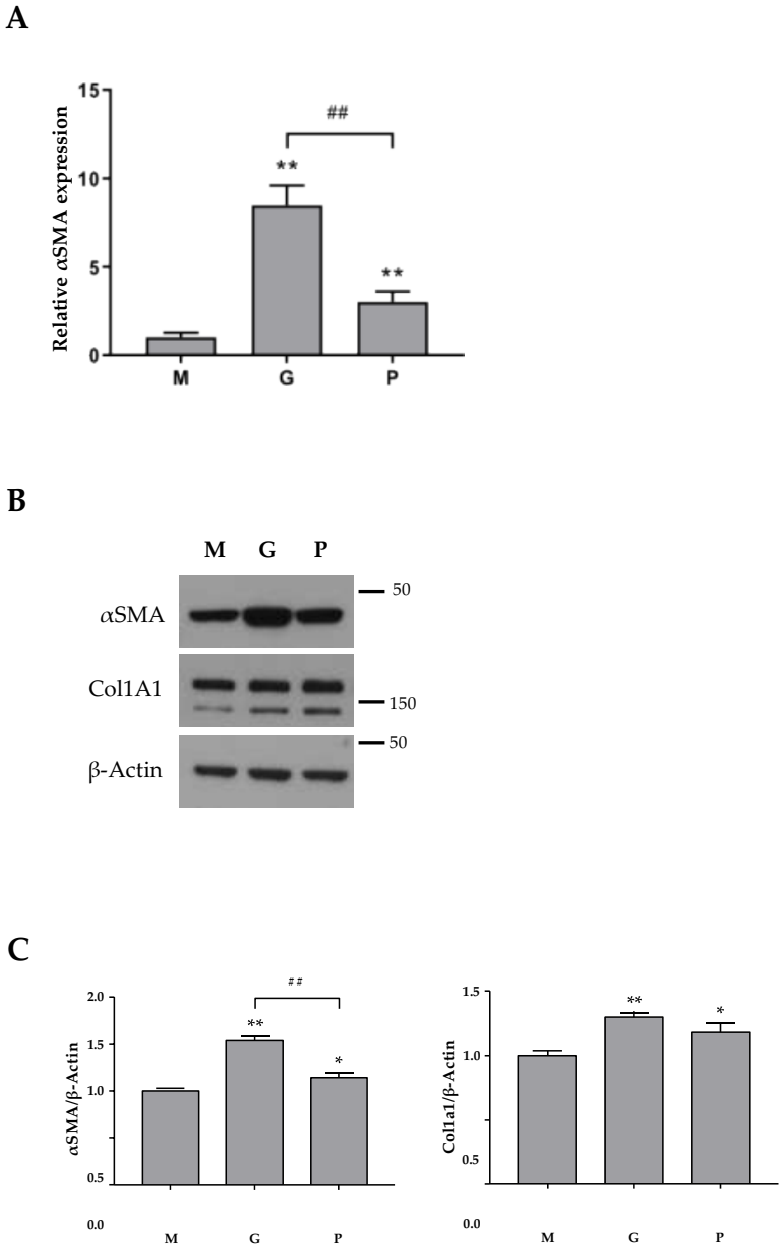
The images are representative of at least two independent experiments for each patient. **(E)** Densitometric analysis of p-AKT/β-Actin, LC3-II/LC3-I and P62/β-Actin was reported as relative expression with respect to baseline. Error bars represent standard deviations. *$p < .05$ and **$p < .005$ *vs* baseline.

FIGURE 6 Inter-individual expression of αSMA in M, G and P cells at baseline and 24 hrs after vertical releasing incision (VRI)



(A-C) Quantitative real-time PCR analysis of αSMA mRNA expression in M (A), G (B) and P (C) cells obtained from each of the six patients (P1-P6). Relative mRNA levels are shown as fold value of the levels at baseline. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$ and **$p < .005$ vs baseline. (**D-F**) Ratio of αSMA expression at 24 hrs/Baseline in M (D), G (E) and P (F) cells obtained from each of the six patients (P1-P6), expressed in logarithmic scale, where values >1 represent αSMA increase at 24 hrs and values <1 represent αSMA decrease. Error bars represent standard deviations. *$p < .05$ and **$p < .005$ *vs* baseline.

FIGURE 7 Fibrotic markers expression in M, G and P cells at baseline



(**A**) Quantitative real-time PCR analysis of αSMA mRNA expression in a pool of six patients. Relative mRNA levels are shown as fold value of the M levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$ and **$p < .005$ *vs* M, $^{\#\#}p < .005$ *vs* G. **(B)** Western blot analysis of αSMA and Collagen 1a1 (Col1A1) protein expression. β-Actin served as loading control. The images are representative of at least two independent experiments for each patient. **(C)** The intensityof the bands in (B) was evaluated by densitometric analysis, normalized and reported as relative expression with respect to M. *$p < .05$ and **$p < .005$ *vs* M; $^{\#\#}p < .005$ *vs* G.

5.3. Differentially expressed genes (DEGs) associated with early wound healing

Of the 84 examined genes (raw data can be found in Appendix A), 52 showed a > 2-fold differential expression at 24 hrs *vs* baseline, in at least one of the three tissues in both patients (Table 3). In unwounded tissues (baseline), 39 of the total examined genes showed differential expression between at least two of the three tissues (Table 4).

Table 1 in Appendix B present the fold changes between baseline and 24 hrs in the expression of the 84 wound healing related examined genes in patient 1 and 4.

Scatter plots (Figure 1, Appendix B) showing up-regulated, unchanged and down-regulated genes between baseline vs 24 hrs in both patients, display how, in the three studied tissues, many genes remain unchanged between baseline and 24 hrs.

TABLE 3 Expression of wound healing mediators differentially modulated in M, G and P cells obtained from Patient 1 and Patient 4 between baseline and 24 hrs after vertical releasing incision (VRI), identified by RT² Profiler PCR Array

Gene symbol	*Gene name*	Patient	Fold expression difference (24 hrs *vs* baseline)†					
			M		G		P	
			Up	Down	Up	Down	Up	Down
ACTC1	*actin, alpha, cardiac muscle 1*	**1**	-	663.8	-	-	-	-
		4	-	64.4	-	-	-	-
ANGPT1	*angiopoietin 1*	**1**	12.5	-	-	-	-	-
		4	-	-	-	-	-	2.7
CCL2	*C-C motif chemokine ligand 2*	**1**	3.0	-	-	12.1	-	-
		4	-	2.0	-	8.2	-	10.8
CCL7	*C-C motif chemokine ligand 7*	**1**	50.7	-	-	12.9	4.0	-
		4	-	-	-	34.8	-	45.7
CDH1	*cadherin 1*	**1**	-	-	-	3.0	-	4.0
		4	2.2	-	-	4.0	-	2.6
COL14A1	*collagen type XIV alpha 1 chain*	**1**	-	5.2	-	6.0	8.3	-
		4	2.0	-	-	4.1	-	10.3
COL1A1	*collagen type I alpha 1 chain*	**1**	-	2.6	-	-	2.1	-
		4	2.0	-	-	-	-	-
COL4A1	*collagen type IV alpha 1 chain*	**1**	-	5.5	-	12.7	-	2.1
		4	-	-	-	-	-	42

COL4A3	*collagen type IV alpha 3 chain*	**1**	3.0	-	2.7	-	8.1	-
		4	-	-	-	2.0	-	-
COL5A1	*collagen type V alpha 1 chain*	**1**	-	2.6	-	-	2.0	-
		4	-	-	2.1	-	-	2.7
COL5A3	*collagen type V alpha 3 chain*	**1**	-	2.6	-	6.0	-	-
		4	-	2.0	-	2.0	-	85.3
CTSK	*cathepsin K*	**1**	6.1	-	5.5	-	2.0	-
		4	-	-	2.0	-	-	-
CTSV	*cathepsin V*	**1**	6.1	-	-	5.8	-	4.1
		4	-	-	-	4.1	-	-
CXCL1	*C-X-C motif chemokine ligand 1*	**1**	48.9	-	-	23.8	-	-
		4	-	16.3	-	32.0	419.0	-
CXCL11	*C-X-C motif chemokine ligand 11*	**1**	-	-	-	-	2.0	-
		4	2.2	-	-	-	-	-
CXCL2	*C-X-C motif chemokine ligand 2*	**1**	49.4	-	-	24.3	-	-
		4	-	16.1	-	32.4	-	10.5
EGF	*epidermal growth factor*	**1**	-	5.3	-	3.0	-	-
		4	-	-	-	-	-	5.3
F13A1	*coagulation factor XIII A chain*	**1**	-	2775.1	-	-	-	-
		4	-	62.4	-	-	-	-
FGF10	*fibroblast growth factor 10*	**1**	6.6	-	-	-	4.0	-
		4	8.5	-	-	-	-	5.4

FGF7	*fibroblast growth factor 7*	**1**	-	-	-	-	2.0	-
		4	-	-	-	8.3	-	2.7

HBEGF	*heparin binding EGF like growth factor*	**1**	-	5.1	-	3.0	-	-
		4	-	-	-	2.0	-	-
HGF	*hepatocyte growth factor*	**1**	3.1	-	2.7	-	4.1	-
		4	-	-	-	-	-	5.3
IFNG	*interferon gamma*	**1**	-	-	-	-	3.9	-
		4	2.1	-	-	-	-	-
IGF1	*insulin like growth factor 1*	**1**	-	-	-	3.1	31.6	-
		4	-	4.0	-	2.1	-	2.8
IL1B	*interleukin 1 beta*	**1**	6.0	-	-	5.9	-	16.0
		4	-	-	-	2.2	-	-
IL2	*interleukin 2*	**1**	-	-	-	-	4.0	-
		4	4.4	-	-	-	-	-
IL4	*interleukin 4*	**1**	-	-	-	-	8.1	-
		4	2.2	-	-	-	-	-
IL6	*interleukin 6*	**1**	3.1	-	-	-	-	-
		4	4.1	-	-	-	-	-
IL6ST	*interleukin 6 signal transducer*	**1**	-	-	-	-	2.0	-
		4	2.0	-	-	-	-	-
ITGA1	*integrin subunit alpha 1*	**1**	-	-	2.7	-	-	-
		4	-	-	-	-	-	2.7
ITGA2	*integrin subunit alpha 2*	**1**	-	2.7	5.5	-	-	2.0
		4	-	2.0	-	2.0	-	5.5
ITGA3	*integrin subunit alpha 3*	**1**	-	2.7	-	3.1	-	4.1
		4	-	-	-	-	-	5.4

ITGA4	*integrin subunit alpha 4*	**1**	-	-	2.7	-	-	2.2
		4	-	-	4.1	-	3.0	-
ITGB3	*integrin subunit beta 3*	**1**	3.1	-	-	-	-	4.1
		4	-	-	-	4.0	-	2.6
ITGB5	*integrin subunit beta 5*	**1**	-	2.6	-	-	2.0	-
		4	-	2.1	-	-	-	-
ITGB6	*integrin subunit beta 6*	**1**	-	10.5	-	11.8	-	4.0
		4	-	2.0	-	2.1	-	-
MMP9	*matrix metallopeptidase 9*	**1**	13.1	-	-	-	-	7.9
		4	-	2.0	-	-	-	-
PLAT	*plasminogen activator, tissue type*	**1**	-	5.3	21.5	-	-	-
		4	-	-	-	-	-	2.7
PLAU	*plasminogen activator, urokinase*	**1**	-	2.6	2.7	-	-	-
		4	-	-	-	4.1	-	2.7
PLAUR	*plasminogen activator, urokinase receptor*	**1**	-	-	2.7	-	-	2.1
		4	-	-	-	2.0	-	-
PTGS2	*prostaglandin-endoperoxide synthase 2*	**1**	3.0	-	-	6.0	-	-
		4	-	2.0	-	4.0	3.0	-
RAC1	*ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)*	**1**	-	2.7	-	-	-	-
		4	-	2.0	-	-	-	-
RHOA	*ras homolog family member A*	**1**	-	-	-	-	-	2.1
		4	-	2.1	-	-	-	-

SERPINE1	*serpin family E member 1*	**1**	-	5.3	-	-	-	-
		4	-	4.1	-	4.1	-	-
STAT3	*signal transducer and activator of transcription 3*	**1**	-	-	-	-	4.1	-
		4	-	-	2.0	-	-	-
TGFA	*transforming growth factor alpha*	**1**	-	-	-	3.2	-	3.9
		4	2.2	-	-	-	-	-
TGFB1	*transforming growth factor beta 1*	**1**	-	2.6	-	-	-	-
		4	-	-	-	-	-	2.7
TGFBR3	*transforming growth factor beta receptor 3*	**1**	3.2	-	2.7	-	4.0	-
		4	2.1	-	-	-	-	-
TIMP1	*TIMP metallopeptidase inhibitor 1*	**1**	-	5.1	-	-	-	-
		4	-	2.3	-	-	-	-
VTN	*Vitronectin*	**1**	-	10.8	-	-	8.1	-
		4	-	-	-	2.1	-	10.6
WISP1	*WNT1 inducible signaling pathway protein 1*	**1**	-	2.6	-	-	-	4.1
		4	-	2.0	-	2.0	-	2.6
WNT5A	*Wnt family member 5A*	**1**	6.3	-	2.6	-	-	2.0
		4	4.1	-	-	-	-	-

M, alveolar mucosa; G, buccal attached gingiva; P; palate

†Minimum cut-off expression difference > 2-folds

TABLE 4 Expression of wound healing mediators differentially modulated between M, G and P cells obtained from Patient 1 and Patient 4 at baseline, identified by RT² Profiler PCR Array

Gene symbol	*Gene name*	Patient	Fold expression difference†					
			G *vs* M		P *vs* M		P *vs* G	
			Up	Down	Up	Down	Up	Down
ACTA2	*actin, alpha 2, smooth muscle, aorta*	**1**	-	-	-	-	-	2.3
		4	-	-	-	-	2.3	-
ACTC1	*actin, alpha, cardiac muscle 1*	**1**	-	2672.4	-	3009.1	-	-
		4	-	289.5	-	257.3	-	-
ANGPT1	*angiopoietin 1*	**1**	12.6	-	5.3	-	-	2.4
		4	13.4	-	32.1	-	2.4	-
CCL2	*C-C motif chemokine ligand 2*	**1**	-	-	5.3	-	3.5	-
		4	-	-	16.1	-	2.4	-
CCL7	*C-C motif chemokine ligand 7*	**1**	13.0	-	44.3	-	-	-
		4	28.2	-	32.4	-	-	-
CDH1	*Cadherin 1*	**1**	-	-	21.3	-	-	-
		4	-	-	2.1	-	-	-
COL14A1	*collagen type XIV alpha 1 chain*	**1**	-	-	-	3.0	-	-
		4	-	-	-	7.9	-	-
COL4A1	*collagen type IV alpha 1 chain*	**1**	-	2.7	-	-	-	-
		4	-	2.3	-	-	-	-
COL5A1	*collagen type V alpha 1 chain*	**1**	-	2.6	-	-	-	2.4
		4	-	2.3	-	-	2.3	-

COL5A3	*collagen type V alpha 3 chain*	**1**	-	2.7	-	-	-	-
		4	-	4.6	-	-	-	-
CSF2	*colony stimulating factor 2 (granulocyte-macrophage)*	**1**	-	-	10.4	-	13.7	-
		4	-	-	2.1	-	2.5	-
CTGF	*connective tissue growth factor*	**1**	-	10.7	-	-	-	-
		4	-	4.6	-	-	-	-
CTSK	*cathepsin K*	**1**	3.0	-	-	-	-	-
		4	-	2.3	-	-	-	-
CXCL1	*C-X-C motif chemokine ligand 1*	**1**	-	-	170.1	-	28.2	-
		4	-	-	-	559.4	-	948.9
CXCL2	*C-X-C motif chemokine ligand 2*	**1**	6.1	-	83.3	-	13.6	-
		4	3.5	-	8.0	-	2.3	-
EGF	*epidermal growth factor*	**1**	-	-	-	3.0	-	2.3
		4	-	-	2.0	-	2.4	-
F13A1	*coagulation factor XIII A chain*	**1**	-	2801.6	-	3210.4	-	-
		4	-	153.5	-	133.0	-	-
FGF10	*fibroblast growth factor 10*	**1**	6.5	-	22.6	-	3.5	-
		4	7.3	-	68.8	-	9.3	-
FGF7	*fibroblast growth factor 7*	**1**	-	-	2.7	-	-	-
		4	-	-	-	4.0	-	-
HBEGF	*heparin binding EGF like growth factor*	**1**	-	2.6	-	-	-	-
		4	-	2.2	-	-	-	-

HGF	*hepatocyte growth factor*	**1**	-	-	5.4	-	3.5	-
		4	-	-	7.9	-	9.0	-
IGF1	*insulin-like growth factor 1*	**1**	-	-	-	12.1	-	9.3
		4	-	-	-	15.9	-	6.9
IL1B	*interleukin 1 beta*	**1**	3.0	-	42.7	-	-	-
		4	-	4.5	-	3.9	-	-
IL6	*interleukin 6*	**1**	-	-	5.3	-	-	-
		4	-	-	8.2	-	-	-
ITGA2	*integrin subunit alpha 2*	**1**	-	2.7	-	-	3.6	-
		4	-	4.5	-	-	4.6	-
ITGA6	*integrin subunit alpha 6*	**1**	-	11.1	-	3.1	-	-
		4	-	4.7	-	4.0	-	-
ITGB3	*integrin subunit beta 3*	**1**	-	-	21.8	-	21.8	-
		4	-	-	15.7	-	15.7	-
MMP1	*matrix metallopeptidase 1*	**1**	3.0	-	21.3	-	7.0	-
		4	-	4.8	2.0	-	9.7	-
MMP9	*matrix metallopeptidase 9*	**1**	-	-	178.5	-	-	-
		4	-	-	-	2.1	-	-
PDGFA	*platelet-derived growth factor alpha polypeptide*	**1**	-	-	-	3.0	-	-
		4	-	-	2.0	-	-	-
PLAT	*plasminogen activator, tissue type*	**1**	-	10.7	-	3.1	-	3.1
		4	-	4.7	2.0	-	2.0	-

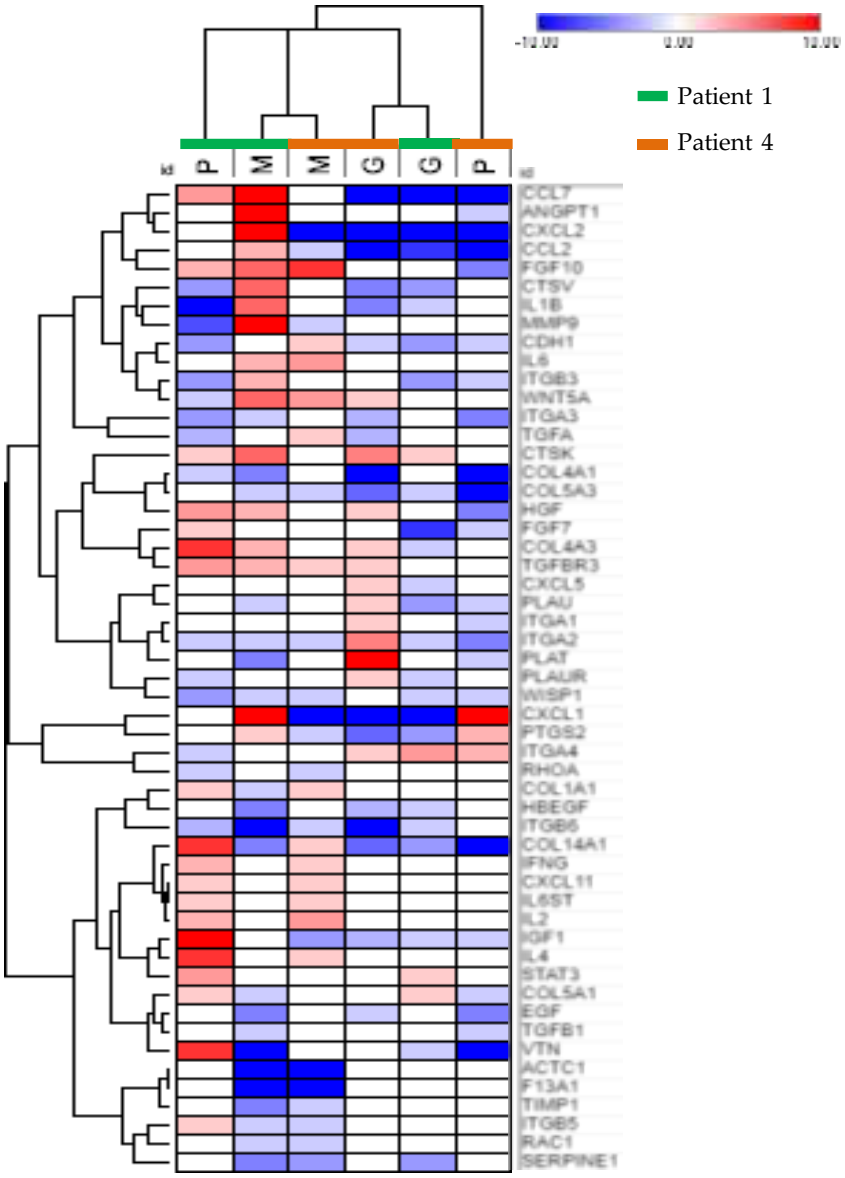
PLAU	*plasminogen activator, urokinase*	**1**	-	2.7	-	-	3.5	-
		4	3.5	-	-	-	2.3	-
SERPINE1	*serpin family E member 1*	**1**	-	5.3	-	6.0	-	-
		4	-	4.6	-	7.9	-	-
STAT3	*signal and activator of 3*	**1**	-	-	-	-	-	2.3
		4	-	-	-	-	2.4	-
TAGLN	*transgelin*	**1**	-	-	-	3.1	-	2.3
		4	-	-	2.0	-	2.3	-
TGFBR2	*transforming growth factor, beta receptor*	**1**	-	-	2.7	-	-	-
		4	-	-	2.0	-	-	-
TIMP1	*TIMP metallopeptidase inhibitor 1*	**1**	-	5.1	-	5.9	-	-
		4	-	4.6	-	7.9	-	-
VTN	*Vitronectin*	**1**	-	10.6	-	12.3	-	-
		4	-	2.2	2.0	-	-	-
WNT5A	*Wnt family member 5A*	**1**	6.3	-	5.5	-	-	-
		4	3.6	-	3.9	-	-	-

M, alveolar mucosa; G, buccal attached gingiva; P; palate

[†]Minimum cut-off expression difference > 2-folds

Hierarchical heatmap showed that M and G samples of Patient 1 and 4 presented similar patterns, clustering together on the column side, while P samples varied between the two patients (Figure 8).

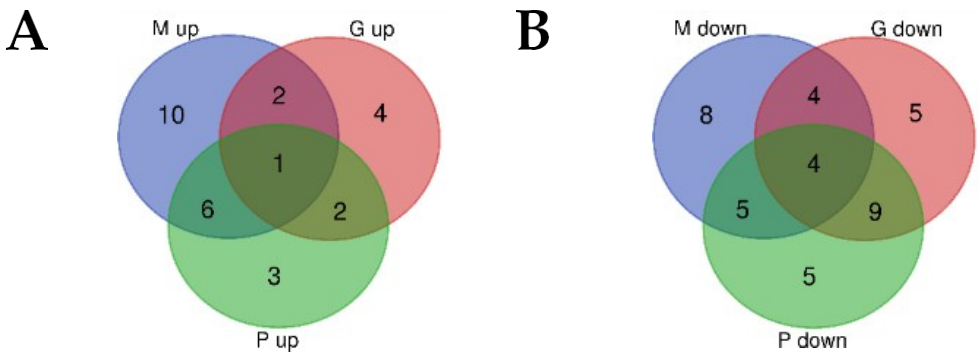
FIGURE 8 RT² Profiler PCR array to detect the expression of genes associated with wound healing and fibrosis in M, G and P cells



Hierarchical clustering of 52 differentially expressed genes (DEGs) with a > 2-fold modulation in M, G and P cells of Patient 1 (green) and Patient 4 (orange). Blue and red indicate under- and over-expression at 24 hrs *vs* baseline.

Through the intersection of M, G and P datasets in Venn diagrams, we found the highest number of overlapping DEGs between M and P for the upregulated genes (Figure 9A), while in the downregulated datasets, this was observed between G and P (Figure 9B).

FIGURE 9 Venn diagrams to detect overlapping DEGs between M, G and P cells

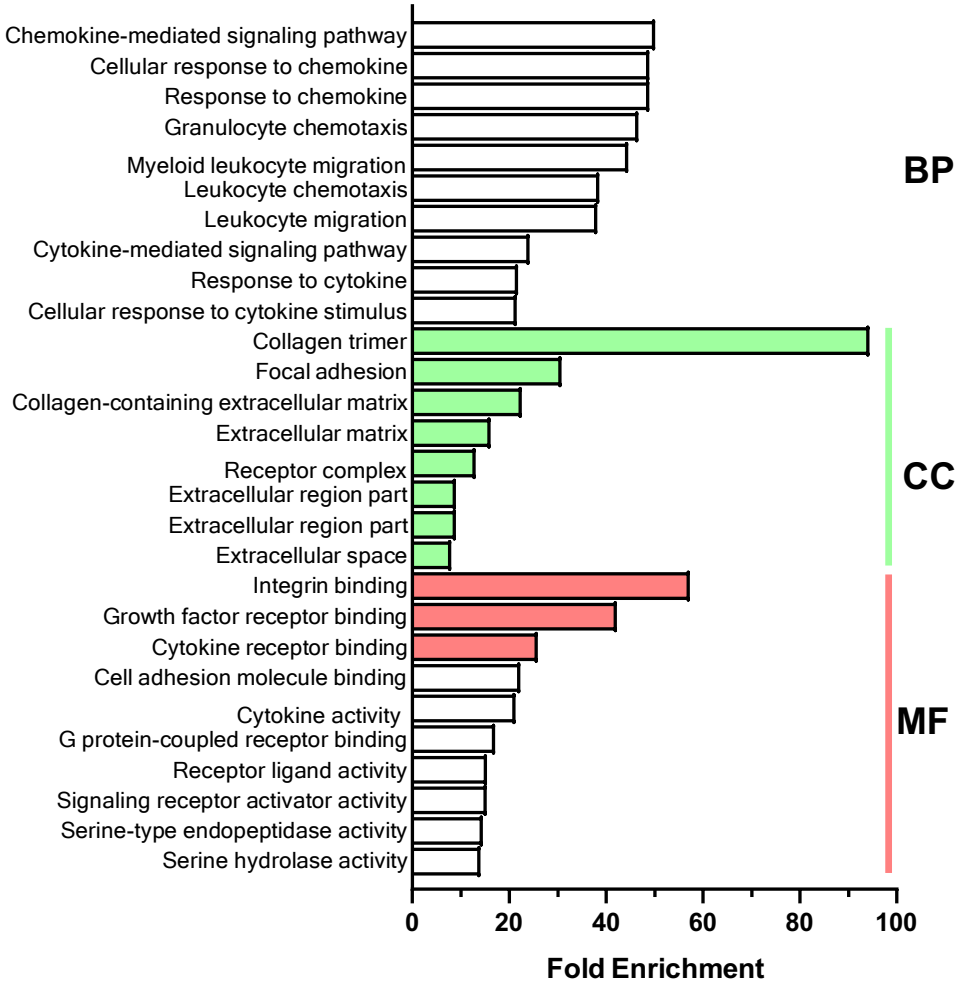


Total number of up-regulated **(A)** or down-regulated **(B)** transcripts identified as statistically significant in the three sample groups (M, blue; G, pink; P, green) at 24 hrs *vs* baseline. Overlapping genes among the sample groups are represented in the areas of intersection between the three circles.

5.4. Gene enrichment analysis and PPI network

The Gene Ontology (GO) analysis of DEGs (Figure 10), showed the most enriched biological process (BP, Table 5.1) cellular component (CC, Table 5.2) and molecular function (MF, Table 5.3) GO terms (FDR < 0.05).

FIGURE 10 Gene Ontology (GO) analysis for biological process (BP), cellular components (CC) and molecular function (MF) obtained by PANTHER software



A false discovery rate (FDR) < 0.05 was settled as a threshold. Significantly overrepresented GO categories were visualized in the bar chart reporting the fold enrichment.

TABLE 5.1 Top 20 list of significantly enriched GO Biological Processes

PANTHER GO-Slim Biological Process	GO ID	# Genes	Fold Enrichment	Raw *P* value	FDR *q* values
Cell surface receptor signaling pathway	GO:0007166	16	7.7	1.17E-10	2.41E-07
Cell migration	GO:0016477	10	15.5	1.00E-09	1.03E-06
Cell motility	GO:0048870	10	13.8	2.97E-09	1.53E-06
Response to cytokine	GO:0034097	8	21.8	4.35E-09	1.80E-06
Localization of cell	GO:0051674	10	13.8	2.97E-09	2.04E-06
Myeloid leukocyte migration	GO:0097529	6	44.5	7.95E-09	2.73E-06
Cytokine-mediated signaling pathway	GO:0019221	7	24.2	2.21E-08	5.07E-06
Leukocyte migration	GO:0050900	6	38.2	1.87E-08	5.52E-06
Locomotion	GO:0040011	10	11.1	2.18E-08	5.64E-06
Cellular response to cytokine stimulus	GO:0071345	7	21.6	4.66E-08	9.61E-06
Signal transduction	GO:0007165	19	4.0	6.37E-08	1.10E-05
Response to stimulus	GO:0050896	23	3.3	6.24E-08	1.17E-05
Movement of cell or subcellular component	GO:0006928	11	8.0	1.03E-07	1.33E-05
Response to chemokine	GO:1990868	5	48.9	9.92E-08	1.37E-05
Chemokine-mediated signaling pathway	GO:0019221	5	50.1	8.86E-08	1.41E-05
Cellular response to chemokine	GO:1990869	5	48.9	9.92E-08	1.46E-05
Granulocyte chemotaxis	GO:0071621	5	46.6	1.24E-07	1.50E-05
Cell communication	GO:0007154	19	3.7	2.02E-07	2.20E-05
Signaling	GO:0023052	19	3.8	1.96E-07	2.25E-05
Leukocyte chemotaxis	GO:0030595	5	38.6	2.97E-07	3.06E-05

GO, Gene Ontology; ID, identifier; FDR, False Discovery Rate

TABLE 5.2 Significantly enriched GO Cellular Components

PANTHER GO-Slim Cellular Component	# Genes	Fold Enrichment	Raw *P* value	FDR *q* values
Extracellular region part	21	9.0	3.07E-15	7.98E-13
Extracellular region	21	9.0	3.07E-15	1.60E-12
Extracellular space	17	8.0	1.53E-11	2.64E-09
Collagen trimer	4	94.3	1.97E-07	2.57E-05
Extracellular matrix	6	16.1	2.32E-06	2.41E-04
Collagen-containing extracellular matrix	4	22.6	3.63E-05	3.15E-03
Receptor complex	5	13.1	4.52E-05	3.36E-03
Focal adhesion	3	30.8	1.56E-04	1.01E-02
Intracellular part	6	0.4	9.37E-04	4.87E-02
Intracellular	6	0.4	9.17E-04	5.30E-02

GO, Gene Ontology; FDR, False Discovery Rate

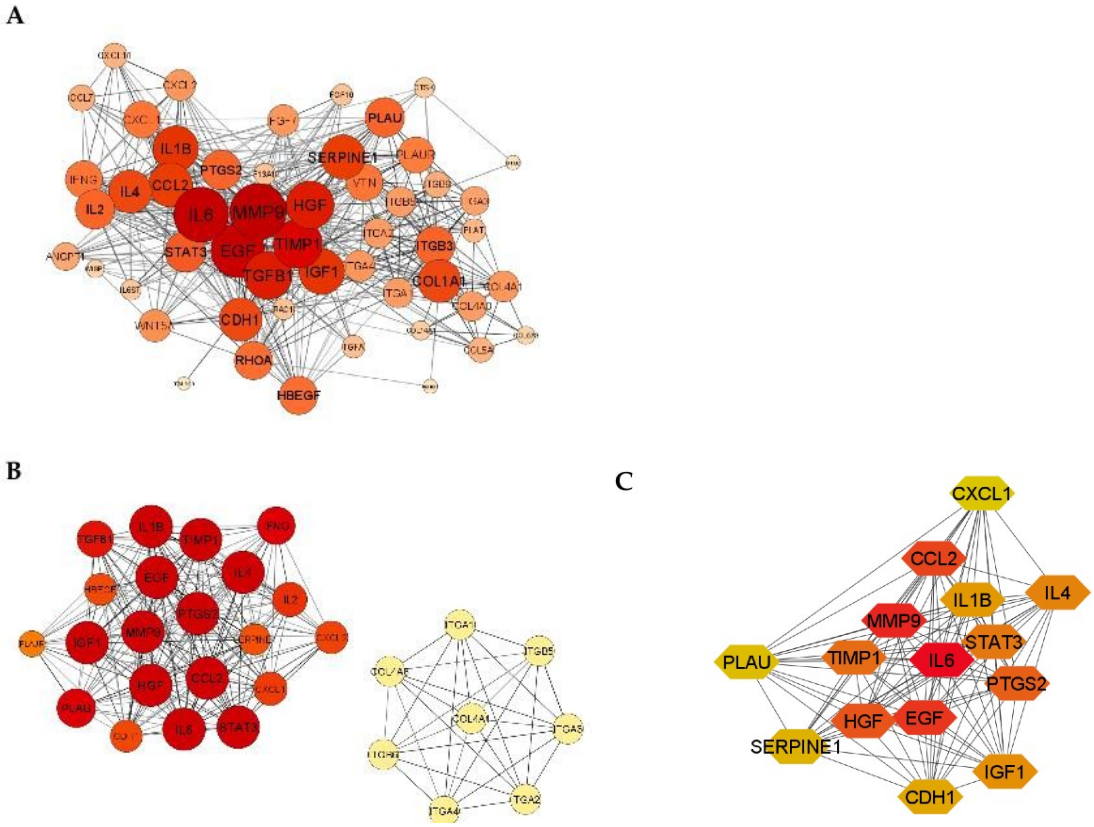
TABLE 5.3 Significantly enriched GO Molecular Functions

PANTHER GO-Slim Molecular Function	# Genes	Fold Enrichment	Raw *P* value	FDR *q* values
signaling receptor binding	13	8.3	4.05E-09	2.15E-06
signaling receptor activator activity	8	15.3	6.03E-08	1.07E-05
receptor ligand activity	8	15.4	5.81E-08	1.55E-05
cytokine receptor binding	6	25.9	1.65E-07	1.76E-05
receptor regulator activity	8	13.6	1.49E-07	1.98E-05
cytokine activity	6	21.3	4.93E-07	4.37E-05
protein binding	20	3.3	7.13E-07	5.42E-05
endopeptidase activity	8	10.4	1.03E-06	6.84E-05
integrin binding	4	57.3	1.16E-06	6.87E-05
G protein-coupled receptor binding	6	17.1	1.70E-06	9.06E-05
molecular function regulator	10	5.8	6.92E-06	3.35E-04
peptidase activity, acting on L-amino acid peptides	8	7.9	7.91E-06	3.50E-04
peptidase activity	8	7.7	9.08E-06	3.72E-04
cell adhesion molecule binding	4	22.3	3.83E-05	1.45E-03
serine-type endopeptidase activity	4	14.6	1.86E-04	6.58E-03
hydrolase activity, acting on acid phosphorus-nitrogen bonds	4	14.1	2.12E-04	6.63E-03
serine hydrolase activity	4	14.1	2.12E-04	7.05E-03
binding	22	1.9	1.13E-03	3.34E-02
growth factor receptor binding	2	42.2	1.24E-03	3.46E-02

GO, Gene Ontology; FDR, False Discovery Rate

A protein-protein interaction (PPI) network including 52 nodes and 258 edges was obtained by applying STRING data to Cytoscape software (Figure 11A). The enriched number of interactions among these DEGs is due to their biological connections as involved in wound healing. By using MCODE plug-in, we found two clusters with 21 nodes and 189 edges (score=18.9) for the first one and 8 nodes and 28 edges (score=8) for the second (Figure 11B). Applying the cytoHubba plug-in, we detected 15 hub genes of the network using the MCC method (Figure 11C, Table 6).

FIGURE 11 Protein-protein (PPI) interaction network of DEGs, constructed using Cytoscape software



(A) Nodes and font size are positively related to connectivity degree, which is further underlined by color gradient. Edges color gradient is associated with STRING combined score, computed by combining the probabilities from the different evidence channels and corrected for the probability of randomly observing an interaction. **(B)** The two most relevant clusters visualized by MCODE in Cytoscape. Filters were based on the default parameters (Degree Cutoff =2; Node Score Cutoff =0.2; K-Core =2; Max.Depth =100). Nodes and font size are positively related to MCODE score, which is further underlined by color gradient. **(C)** Hub genes screened through the maximal clique centrality (MCC) algorithm from cytoHubba. Color gradient is positively related to MCC score.

TABLE 6 Top 15 hub genes in the PPI network (ranked by MCC method)

Rank	Gene Symbol	Score
1	*IL6*	2.32108765644E11
2	*MMP9*	2.32108754582E11
3	*EGF*	2.3210401227E11
4	*CCL2*	2.3206002624E11
5	*HGF*	2.32059241038E11
6	*PTGS2*	2.3204760768E11
7	*TIMP1*	2.31490269722E11
8	*STAT3*	2.3148514464E11
9	*IL4*	2.3092631441E11
10	*IGF1*	2.2483148832E11
11	*IL1B*	2.1852062076E11
12	*CDH1*	2.00406377064E11
13	*SERPINE1*	1.94131825104E11
14	*PLAU*	1.75366921488E11
15	*CXCL1*	1.2558224448E11

Eight genes were subsequently selected: RAC1, SERPINE1, TIMP1, CDH1, ITGA4, ITGB5, IL6 and CXCL1, based on their differential modulation between M, G and P in the two patients (Figure 12), their inclusion in the most relevant clusters, their identification as hub genes and their potential role in wound repair (Kuwahara et al., 2001; Romagnani et al., 2004; Simone & Higgins, 2015; Basso et al., 2016; Buskermolen et al., 2017; Jakhu et al., 2018). The fold expression of the selected genes in Patient 1 and Patient 4 is reported in Table 7. PCR array substantially showed a differential expression between M on one side, and G and P on the other. In M we observed down-regulation of RAC1, SERPINE1, TIMP1, ITGB5, and up-regulation of IL6. In G and P, CDH1 resulted to be down-regulated, and ITGA4 up-

regulated. Some discordance between the two patients was observed, especially for CXCL1 (Figure 12).

FIGURE 12 Schematic representation of the differential expression of the eight selected genes in M, G and P cells of Patient 1 (P1) and Patient 4 (P4) at 24 hrs *vs* baseline by PCR array

GENE SYMBOL	MODULATION					
	M		G		P	
	P1	P4	P1	P4	P1	P4
RAC1	↓	↓	-	-	-	-
SERPINE1	↓	↓	-	↓	-	-
TIMP1	↓	↓	-	-	-	-
CDH1	-	↑	↓	↓	↓	↓
ITGA4	-	-	↑	↑	↓	↑
ITGB5	↓	↓	-	-	↑	-
IL6	↑	↑	-	-	-	-
CXCL1	↑	↓	↓	↓	-	↑

↓ blue squares indicate down-regulated genes; ↑ red squares indicate up-regulated genes

TABLE 7 Differential expression of selected wound healing mediators in alveolar mucosal (M), buccal attached gingival (G) and palatal (P) cells derived from Patient 1 and Patient 4 between baseline and 24 hrs, identified by RT² Profiler PCR Array system

Gene Symbol	*Gene Name*	Patient	Fold expression difference (24 hrs *vs* baseline)†					
			M		G		P	
			Up	Down	Up	Down	Up	Down
RAC1	*ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)*	**1**	-	2.7	-	-	-	-
		4	-	2.0	-	-	-	-
SERPINE1	*serpin family E member 1*	**1**		5.3	-	-	-	-
		4	-	4.1	-	4.1	-	-
TIMP1	*TIMP metallopeptidase inhibitor 1*	**1**	-	5.1	-	-	-	-
		4	-	2.3	-	-	-	-
CDH1	*cadherin 1*	**1**	-	-	-	3.0	-	4.0
		4	2.2	-	-	4.0	-	2.6
ITGA4	*integrin subunit alpha 4*	**1**	-	-	2.7	-	-	2.2
		4	-	-	4.1	-	3.0	-
ITGB5	*integrin subunit beta 5*	**1**	-	2.6	-	-	2.0	-
		4	-	2.1	-	-	-	-
IL6	*interleukin 6*	**1**	3.1	-	-	-	-	-
		4	4.1	-	-	-	-	-
CXCL1	*C-X-C motif chemokine ligand 1*	**1**	48.9	-	-	23.8	-	-
		4	-	16.3	-	32.0	429.0	-

M, alveolar mucosa; G, buccal attached gingiva; P, palate. †Minimum cut-off expression difference was considered > 2-folds.

5.5. qRT-PCR Validation

Significant alterations in mRNA expression at 24 hrs vs baseline in at least one of the three tissues were confirmed (Figure 13A).

RAC1 confirmed to be down-modulated at 24 hrs in M (0.5 -fold) and no significantly modulated in P, as in PCR array, while by qRT-PCR it resulted up-regulated in G (1.7-fold, Figure 13B).

As for SERPINE1 and TIMP1, we observed a discrepancy between PCR array and qRT-PCR, since in the latest these two genes appeared to be up-modulated in M (1.8-fold and 2.6-fold, respectively) and down-modulated in P (0.7-fold and 0.2-fold respectively), with no significant changes in G (Figure 13C, D).

CDH1 confirmed to be decreased in G and P (0.1 and 0.2-fold, respectively), while the up-regulation in M detected in Patient 4 was not confirmed, since we observed no significant variations, as in Patient 1 (Figure 13E).

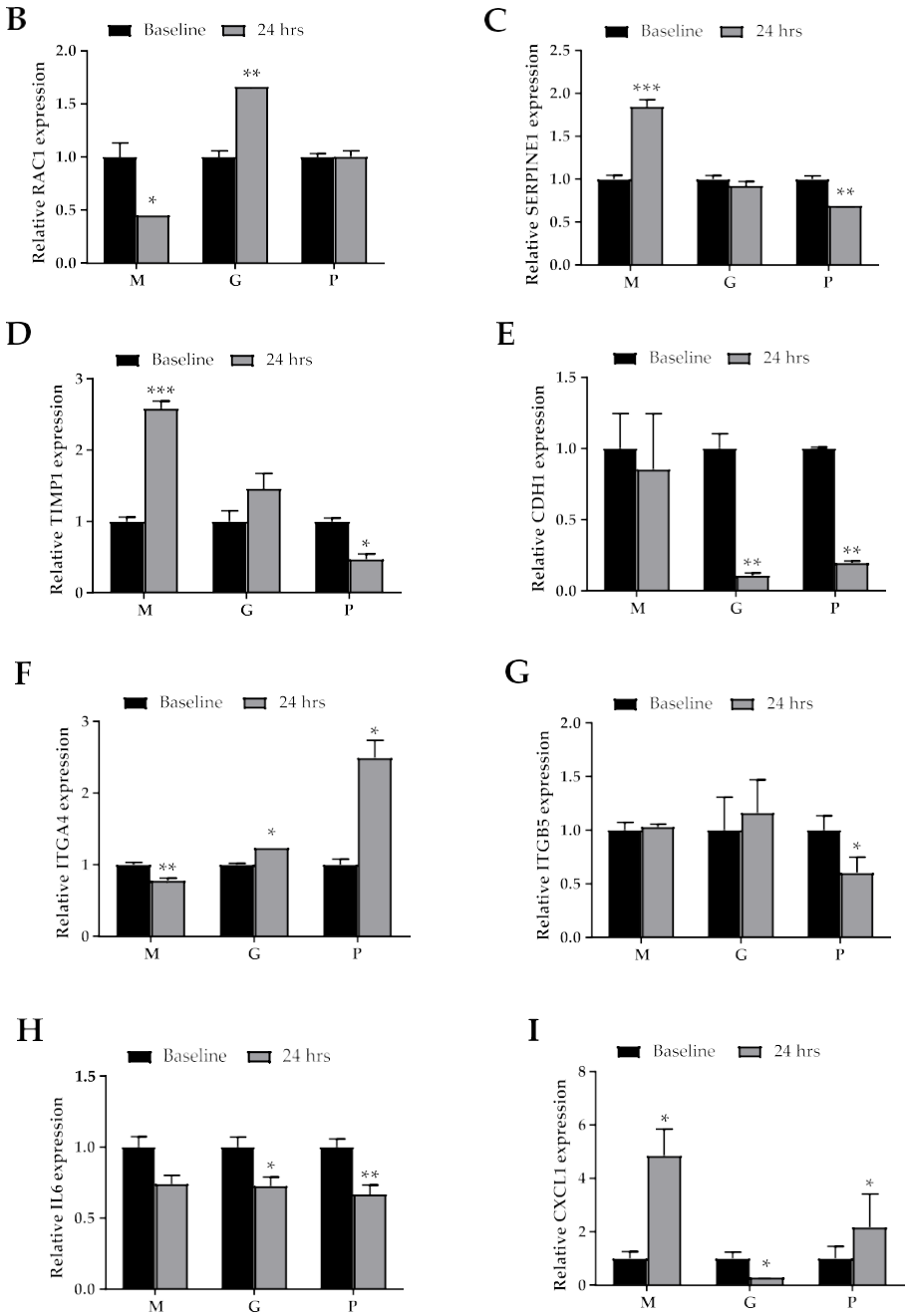
ITGA4 and ITGB5 displayed an opposite behaviour in P, with a significant increase for the first (2.5-fold) and a decrease for the latest (0.6-fold) (Figure 13F, G). This modulation was contrary to PCR array results, in which we observed variability between the two patients. Regarding M and G, ITGA4 resulted decreased (0.8-fold) and increased (1.2-fold), respectively, while no variations were observed for ITGB5.

qRT-PCR validation for IL6 showed discrepancy with PCR array, with no variations in M and down-modulation in G and P (0.7-fold) (Figure 13H). Finally, CXCL1 confirmed the opposite modulation in G (0.3-fold decrease) and P (2.2-fold increase). An up-modulation (4.9-fold) revealed in M (Figure 13I) have been observed in PCR array only for Patient 1.

FIGURE 13 Validation of differential gene expression by qRT-PCR in M, G and P cells.

A

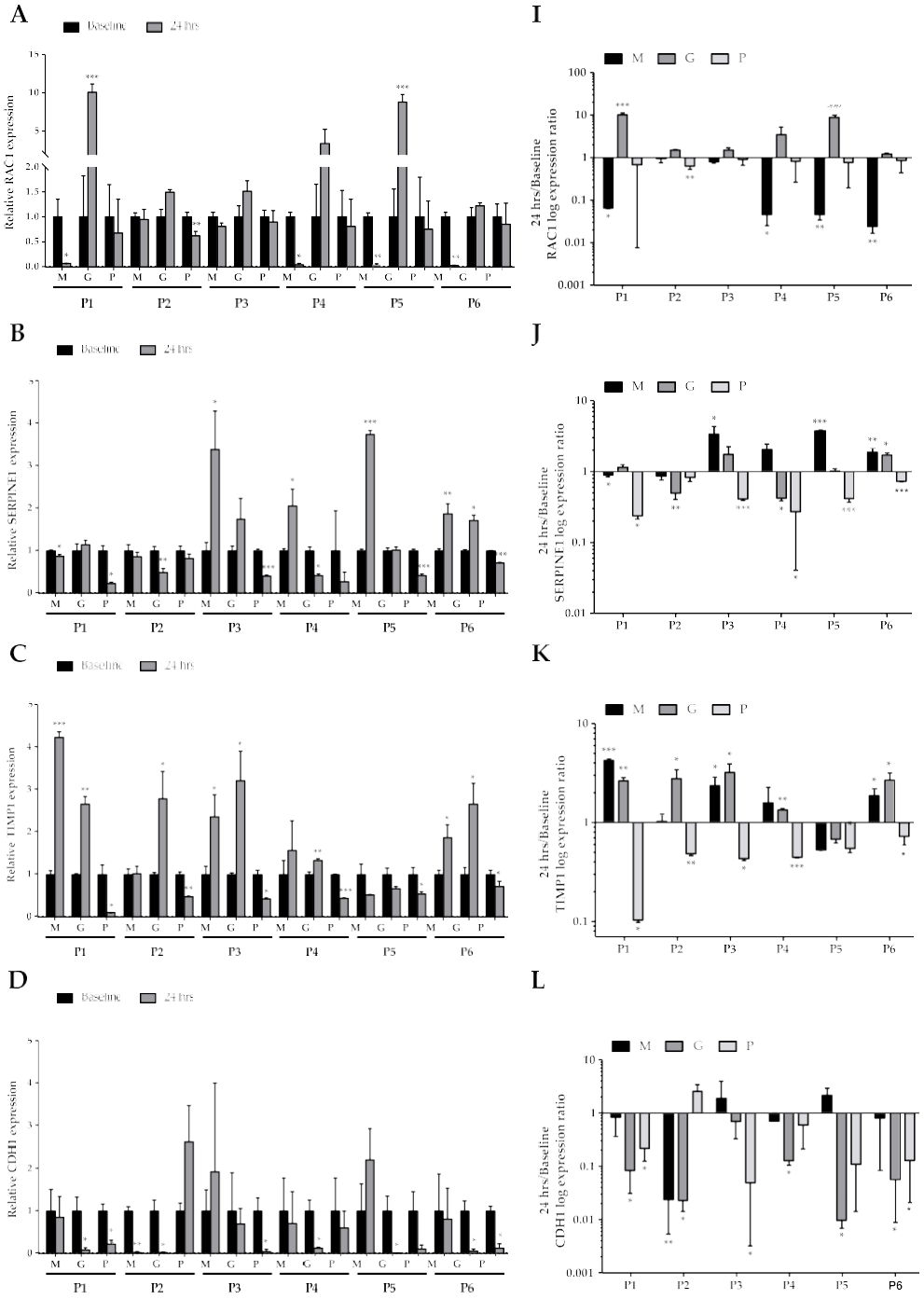
GENE SYMBOL	MODULATION		
	M	G	P
RAC1	↓	↑	-
SERPINE1	↑	-	↓
TIMP1	↑	-	↓
CDH1	-	↓	↓
ITGA4	↓	↑	↑
ITGB5	-	-	↓
IL6	-	↓	↓
CXCL1	↑	↓	↑

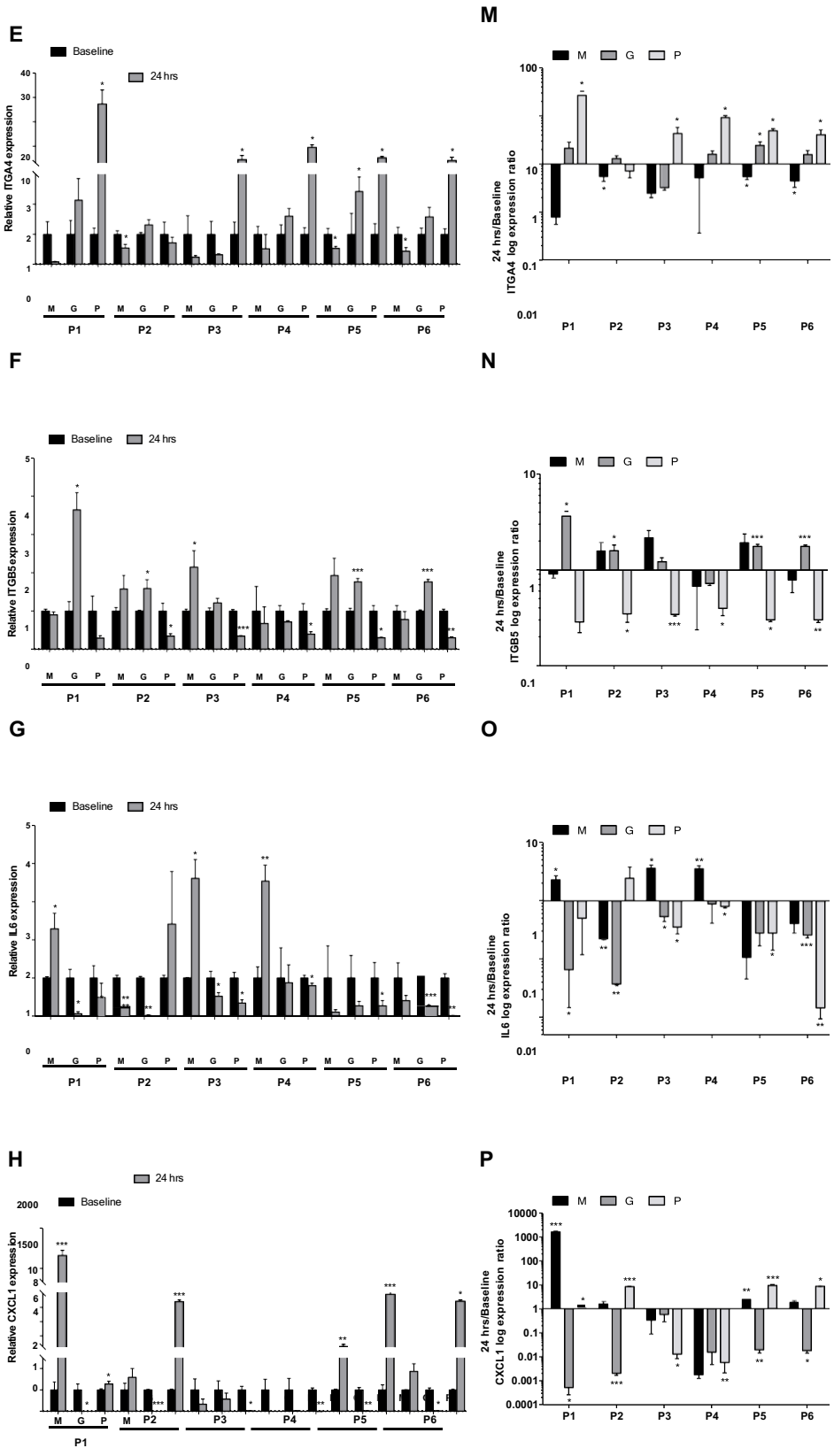


(A) Schematic representation of the differential expression of the eight selected genes in M, G and P cells at 24 hrs *vs* baseline by qRT-PCR. ↓ blue squares indicate down-regulated genes; ↑ red squares indicate up-regulated genes. **(B-I)** Quantitative real-time PCR analysis of mRNA expression levels of RAC1 (B), SERPINE1 (C), TIMP1 (D), CDH1 (E), ITGA4 (F), ITGB5 (G), IL6 (H) and CXCL1 (I) in a pool of six patients. For each gene, relative mRNA levels are shown as fold value of the levels at baseline. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$, **$p < .005$ and ***$p < .0005$ *vs* baseline

The significant variations observed by qRT-PCR on pooled cDNAs were highly consistent on an inter-individual basis: in particular, at 24 hrs RAC1 was down-modulated in M and up-regulated in G in all the patients (Figure 14A, I); SERPINE1 down-modulation in P was observed in all the patients, while M resulted to be up-regulated in four out of six patients (Figure 14B, J); TIMP1 up-modulation in M was confirmed in five out of six patients, and down-modulation in P was found in all the six patients (Figure 14C, K); CDH1 confirmed to be decreased in G and P in six and five patients, respectively (Figure 14D, L); ITGA4 down-modulation in M was observed in all the patients, while increase in G and P was detected in five out of six patients (Figure 14E, M); as for ITGB5, the significant down-modulation in P was confirmed in all the patients (Figure 14F, N); IL6 showed some inter-individual variations in M, while down-modulation in G and P was confirmed in six and five patients, respectively (Figure 14G, O); CXCL1 showed up-modulation in M and P in four out of six patients, while down-modulation in G was confirmed in all the patients (Fig. 14H, P).

FIGURE 14 Inter-individual gene expression by qRT-PCR in M, G and P cells





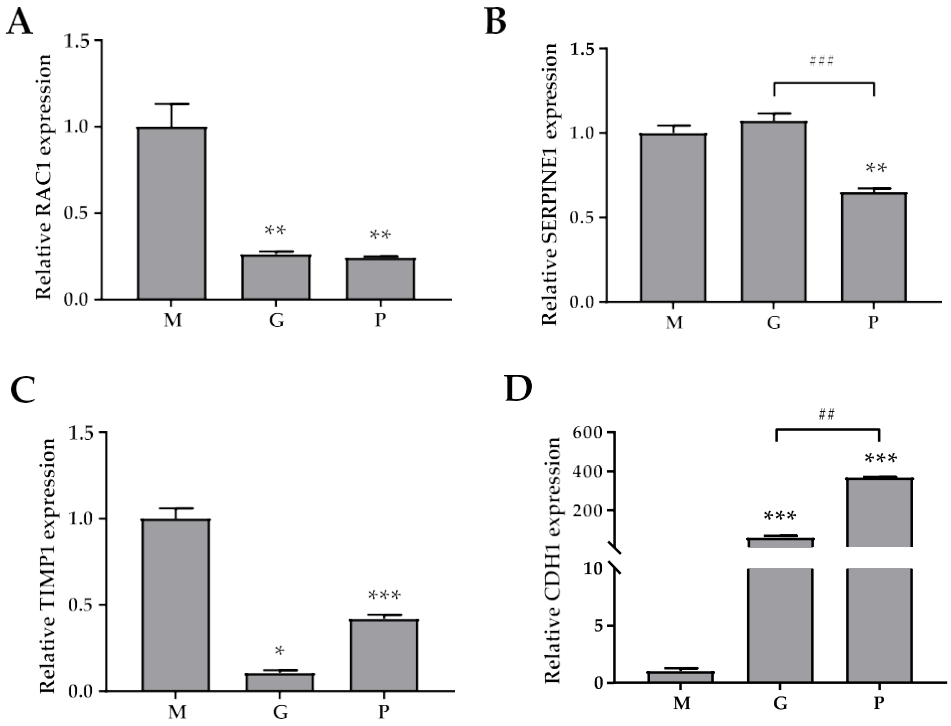
(A-H) Quantitative real-time PCR analysis of mRNA expression levels of RAC1 (A), SERPINE1 (B), TIMP1 (C), CDH1 (D), ITGA4 (E), ITGB5 (F), IL6 (G) and CXCL1 (H) in M, G and P cells obtained from each of the six patients separately (P1-P6). Relative mRNA levels are shown as fold value of the levels at baseline. mRNA levels were normalized to GAPDH mRNA expression. Each

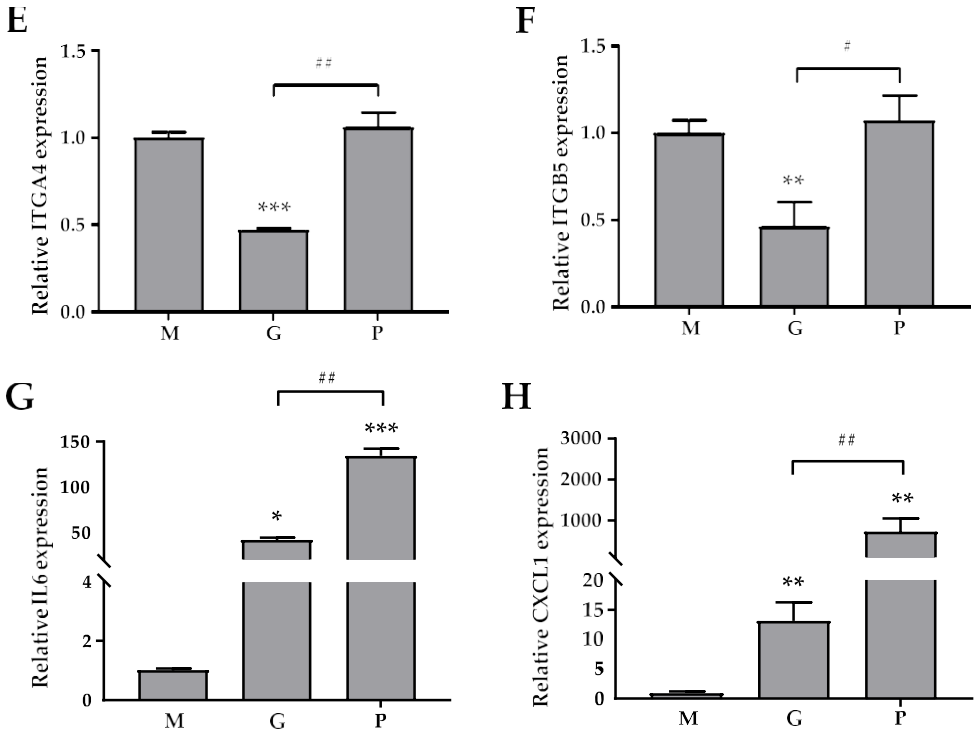
experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$, **$p < .005$ and ***p < .0005 *vs* baseline. **(I-P)** Ratio of RAC1 (I), SERPINE1 (J), TIMP1 (K), CDH1 (L), ITGA4 (M), ITGB5 (N), IL6 (O) and CXCL1 (P) expression at 24 hrs/Baseline in M, G and P cells obtained from each of the six patients (P1-P6), expressed in logarithmic scale, where values >1 represent gene expression increase at 24 hrs and values <1 represent decrease. Error bars represent standard deviations. *$p < .05$, **$p < .005$ and ***$p < .0005$ *vs* baseline.

5.5.1. Differential expression of selected genes in unwounded tissues

As concerning differences between the three tissues at baseline, with respect to M, P showed lower basal levels of RAC1, SERPINE1 and TIMP1, and higher basal levels of CDH1, IL6 and CXCL1. This partially agrees with PCR array data relative to M vs P at baseline (Table 4), reporting a down-modulation of TIMP1 and an up-modulation of CDH1 and IL6. As for CXCL1, PCR array showed an opposite behaviour between Patient 1 and Patient 4. Similarly, G cells showed lower basal levels of RAC1 and TIMP1 (the last also confirmed by PCR array), and higher basal levels of CDH1, IL6 and CXCL1. Moreover, in G we observed lower ITGA4 and ITGB5 levels than in M (Figure 15).

FIGURE 15 Validation of differential gene expression by qRT-PCR in M, G and P cells at baseline. (A-H)





Quantitative real-time PCR analysis of mRNA expression levels of RAC1 (A), SERPINE1 (B), TIMP1 (C), CDH1 (D), ITGA4 (E), ITGB5 (F), IL6 (G) and CXCL1 (H) in a pool of six patients. For each gene, relative mRNA levels are shown as fold value of the M levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$, **$p < .005$ and ***$p < .0005$ *vs* M; $^{\#}p < .05$, $^{\#\#}p < .005$ and $^{\#\#\#}p < .0005$ *vs* G.

6. DISCUSSION

Oral wound healing presents an accelerated rate with respect to cutaneous wounds (Iglesias-Bartolome et al., 2018). However, wound healing response varies between the different oral sites, ranging from absence to extensive scar formation (Larjava. et al., 2011). Multiple cells types are involved in the wound repair process. Nevertheless, during wound healing, fibroblasts have a fundamental role, since they are primarily responsible for synthesis of the replacement ECM (Sandulache et al., 2005; Buskermolen, 2017; Smith et al., 2019).

Currently, the intrinsic characteristics that mediate healing at different oral soft tissues are poorly understood, mainly in humans.

In the present study, EHS, a score assessing clinical signs of re-epithelialization (CSR), haemostasis (CSH) and inflammation (CSI) (Marini, et al., 2018; Marini et al., 2019), was used to evaluate the clinical healing response of M, G and P tissues 24 hrs and 1 week after injury.

We found higher mean EHS values in G and P with respect to M at 24 hrs. Noteworthy, the highest value was observed in P, raising the possibility of a better wound healing capacity of this tissue. However, at 1 week less significant differences were found, confirming the need to investigate the peculiar characteristics of oral repair preferentially the early phases.

To our knowledge, this is the first human study investigating gene expression profiling of fibroblasts from three different oral soft tissues.

Using paired human M, G and P biopsies samples, we assessed myofibroblasts activation and autophagy. In accordance with our previous study (Vescarelli et al., 2017), we confirmed αSMA and Col1a1 increase and autophagic activation in M at 24 hrs and the opposite situation in G. Here, we demonstrate for the first time that in P cells the autophagic pathway is not active, and both αSMA and Col1a1 are downregulated 24 hrs after injury, suggesting that P behaves like G, with low expression of fibrotic markers. This is in line

with the observation of reduced scar formation in this tissue and with our clinical evaluation (EHS).

The low αSMA expression found in P at 24 hrs is in line with the suppression of wound contraction, mainly mediated by myofibroblasts, in this tissue, due to the tight attachment of the connective tissue to the palatal bone. Since wound contraction induces substantial scarring (El Ayadi et al., 2020), our findings are in line with the better clinical response observed in P, in which connective tissue remodeling via cellular response might be more important than myofibroblast differentiation (Jinno et al., 2009).

In the last years, gene profiling analysis has gained clinical importance aiming to develop new approaches for non-healing or impaired wounds treatment (Peake et al., 2014). Therefore, we performed a gene expression profiling of fibroblasts from oral soft tissues using the Wound Healing RT^2 Profiler PCR array, demonstrating differential gene modulation between M, G and P 24 hrs after injury.

Among the 84 genes examined, 52 showed a > 2-fold differential expression at 24 hrs *vs* baseline, in accordance with previous studies that reported the greatest cellular changes at 12-24 hrs post-injury (Chen et al., 2010).

The biological roles of the DEGs were studied using GO enrichment analyses. It is known that cell migration is the basis of re-epithelialization, playing a primary role in angiogenesis (Torres et al., 2018). Consistent with this notion, our GO analysis showed that most of the enriched biological processes were related to cell movement, cell migration, extracellular matrix (ECM) organization and angiogenesis. Functionally, most of the DEGs are linked to chemokines, cytokines, integrins, collagen, but also to the inflammatory response, suggesting that inflammatory cytokines influence the wound healing response of different oral tissues. In fact, a direct correlation between reduced inflammation and scarless healing was previously demonstrated (Mak et al., 2009).

The 52 DEGs identified were subjected to PPI analysis, selecting the top 2 modules and 15 hub genes. On the basis of bioinformatic analysis and of the above-mentioned results indicating a different healing response between M on one side and G and P on the other, we selected some genes with differential modulation between M and G/P (RAC1, TIMP1,

CDH1 and IL6). As PCR array revealed some similarities between M and P, we evaluate some genes with differential regulation between G and P (SERPINE1, ITGA4, ITGB5 and CXCL1) to investigate potential divergences between them.
Validation of the selected genes in a pool of six patients confirmed a differential regulation of RAC1, TIMP1, SERPINE1 and ITGB5 in P with respect to both M and G, while CDH1, IL6 and ITGA4 expression was similar between G and P, and only CXCL1 showed a similar regulation in M and P.
The role of these genes in wound healing has been investigated in previous studies.

Deletion or inhibition of RAC1 -a member of the Rho family of small GTPases with an essential role in cell migration, adhesion, proliferation and spreading - causes delayed oral wound healing by impairing the re-epithelialization process (Liu et al., 2009; Castilho et al., 2010). Conversely, increased RAC1 promotes healing of oral mucositis lesions (Han et al., 2013) and RAC1-based biologic products have been proposed for impaired cutaneous wound healing (Fan et al., 2018). Consistent with these findings, our analysis showed RAC1 down-modulation in M and up-regulation in G, with higher CSR values for G, confirming the correlation between increased RAC1 expression and increase basal cells proliferation. The re-epithelialization regulatory mechanism could be different in G and P, since although P showed the highest CSR values, no RAC1 modulation was observed.
Noteworthy, is the fact that while in the cutaneous tissue repair it has been demonstrated how RAC1-deficiente cells possessed a reduced mRNA expression for αSMA and type I collagen -associated with delayed wound repair- (Liu et al., 2009), in oral tissues this was not observed, since M cells showed an increase in αSMA expression, suggesting different signaling pathways for myofibroblasts activation between dermal and oral tissues.

Transforming growth factor beta 1 (TGFβ1) is involved in tissue fibrosis regulating collagen production (Schrementi et al., 2008). Plasminogen activation induces dose- and time-dependent fibroblast apoptosis in association with pericellular fibronectin proteolysis. Autocrine TGFβ1 production in the wound microenvironment increases SERPINE1 expression (also called plasminogen activator inhibitor type-1, PAI-1), blocking the activation of exogenous plasminogen, decreasing fibronectin proteolysis and increasing

myofibroblasts differentiation. This creates an anti-proteolytic cellular microenvironment that would favor the accumulation and stabilization of the extracellular matrix, collagen deposition and increased wound contraction, key features of progressive tissue fibrosis and hypertrophic scar (Horowitz et al., 2008; Simone & Higgins, 2015).

TGFβ1 also promotes collagen deposition by inhibiting the matrix metalloproteinases (MMPs) that mediate collagen degradation and inducing TIMP1 expression (Barrientos et al., 2008). In our previous study (Vescarelli et al., 2017), we observed a persistent activation of myofibroblast induced by TGFβ1-stimulated autophagy, resulting in scar wound repair in M. In agreement with such findings, here we show up-regulation of both SERPINE1 and TIMP1 at 24 hrs in M. In addition, this tissue presented the lowest CSI value consistent with a higher inflammatory response, and this agree with the results of a previous study carried out in an oral animal model that has shown an increase in the expression of SERPINE1 in inflammatory cells and fibrin clot 1 day after injury (Xiao et al., 2001). Moreover, a study performing a microarray analysis of adult oral mucosal, normal skin, and chronic wound fibroblasts concluded that SERPINE1 is within the "dysfunctional wound healing gene set"(Peake et al., 2014).

Interestingly, and in line with the above-mentioned, both SERPINE1 and TIMP1 are down-modulated in P, in line with our clinical results showing the highest CSR and CSI values. We believe that, together, SERPINE1 and TIMP1 might play a role in regulating scar formation in oral tissues. However, a recent human study reported SERPINE1 up-modulation in P 5 days after excisional injury (Wang & Tatakis, 2017). This discrepancy could be due to differences in evaluation timing or in the wound repair model.

Noteworthy, no changes were observed for both SERPINE1 and TIMP1 in G. Such results led us to hypothesize a differential myofibroblasts regulation between P and G, through SERPINE1-dependent and -independent pathways, respectively. In fact, when uninjured tissues were evaluated we observed that αSMA and Col1A1 expression are lower in M than P and G, in which baseline values are very high. This means that an up-regulation mechanism develops in oral mucosal tissues and a down-regulation mechanism in palatal and gingival tissues. We could even infer from our results a different mechanism between G and P, since αSMA expression at baseline is significantly higher in the former. This is in accordance with our results regarding SERPINE1 gene expression, since P and G seems to

have different down-regulation pathways to reach the same αSMA expression values observed at 24 hrs.

Since control of SERPINE1 expression/activity is critical to repair outcomes and deficient or elevated levels has been reported as etiologic factors in different healing anomalies (Simone et al., 2013; Simone & Higgins, 2015), a elucidation of individual cascade pathways in the different tissues could provide the rational design for targeted therapies, with translational implications for the treatment of fibrotic tissue repair.

During wound healing, epithelial cells adopt a more migratory mesenchymal phenotype in order to spread rapidly and cover the wound area through epithelial-mesenchymal transition (EMT) process (Thiery & Sleeman 2006). EMT requires a complex orchestration of multiple signaling pathways, including TGF-β , fibroblast growth factor, Wnt/β -catenin, epidermal growth factor (EGF) and others. Loss of E-cadherin is considered to be a fundamental event in EMT (Hill et al., 2019). Autophagy inhibition might induce EMT, revealed by down-regulation of the epithelial marker E-cadherin (CDH1). In fact, it has been demonstrated in a recent study (trough a wound scratch assay) that 20 hours after creating the scratch wound, cells in which autophagy was inhibited had more completely repaired the wound (Hill et al., 2019). Our results agree with this, since we observed CDH1 reduction where autophagy is absent (G and P). Instead, CDH1 did not change in M, where autophagy is active.

Integrins are critical components of the cell attachment machinery, promoting myofibroblasts differentiation and αSMA stress fibers assembly. Myofibroblasts present the ability to activate TGF-β1 from self-generated deposits in the ECM by means ITGB5, which transmits the highly contractile forces of these cells to the latent complex of TGF-β1.Thus, ITGB5 is of particular significance in wound healing since may participate in fibroblast transformation to myofibroblasts and its interaction with CCN1/Cyr61 mediatesfibroblasts migration (Larjava et al., 1993, Koivisto et al., 2014). In chronic human wounds, the expression of CCN1/Cyr61 is increased (Minhas et al., 2011) and ITGB5 increase has been correlated with fibrosis in many tissues (Jakhu et al., 2018). Therefore, our findings of

ITGB5 down-modulation only in P are consistent with the observation of lower myofibroblasts activation in this tissue, resulting in a scarless healing.
In addition, it has been reported that ITGB5 expression may depend on the severity of the trauma, as it is expressed in deep human and porcine excisional wounds (Asano et al., 2006), but not in smaller incisional skin or oral mucosal wounds (Clarck et al., 1996). Our results agree with this since no changes were observed in M and G. However, the changes observed in P could reinforce the concept of the peculiar response of this tissue to injury.

ITGA4 has been shown to have important physiological roles, especially in regulating immune system function, such as homing ability of T-cells (Arroyo et al., 1999). Furthermore, is involved in cell attachment to the ECM, fibroblast and keratinocyte proliferation, TGF-β1 processing and tissue remodeling (Koivisto et al., 2014, Jakhu et al., 2018). In the present study, a down-modulation in M, slight up-modulation in G and consistent overexpression in P has been observed. In this light, a proper ITGA4 stimulation during early wound healing could be essential to ensure integrin-dependent migration and leukocytes recruitment, providing efficient tissue repair. Indeed, this gene could serve as therapeutic target. A recent study reported as a treatment with a biomaterial that trigger the expression of ITGA4 could improve the wound repair process (Sivasubramanian et al., 2017).

Since inflammation is a key determinant of fibrosis (Mak et al., 2009), we must consider the role of chemokines and cytokines as modulators of the initial inflammatory phase. Previous studies have demonstrated that chronification of oral wounds, caused by maintenance of the inflammatory phase, can impair or at least delay complete healing of damaged oral mucosal tissue and that, besides persistent pathologic inflammation, the wound-healing delay may also be the result of increased synthesis of local inflammatory cytokines (Muller et al., 2008; Gethin, 2012). This was corfirmed by Basso et al. (2016), in an *in vitro* study in which human oral mucosal epithelial cells and fibroblasts were exposed for 24 hours to IL6 and IL8. The results demonstrated that the presence of high concentrations of inflammatory cytokines not only inhibited cells migration but also enhanced the expression of TNF-a and IL-1b , creating a continuous positive inflammation feedback and an increase in apoptosis

rates. Moreover, it has been observed that IL6 indirectly favouring collagen production through induction of TGF-β1 gene expression.

It has also been widely reported that fetal wound healing is characterized by minimal inflammation and scarless repair. IL-6 stimulates inflammation in postnatal wound healing. Lietchy et al. (2010), showed that fetal fibroblasts produced less IL6 protein that adult fibroblasts. The authors concluded that decreased production of inflammatory cytokines such as IL-6 may be responsible for the lack of inflammation seen during fetal wound healing and, thus, diminished inflammation may provide a permissive environment for scarless wound healing. Therefore, IL6 deficiency reduces collagen deposition and its attenuation leads to decrease of inflammatory cells recruitment and scar formation (Lietchy et al, 2010). All the aforementioned allows us to understand how modulation of inflammatory reactions is essential to allow adequate tissue regeneration.

In our patients, IL6 -one of the hub genes with the highest score in our network- , showed a significant reduction in both G and P. Such observation is consistent with the faster wound healing and reduced scarring observed in these tissues with respect to M. However,in M IL6 expression was not significantly modified, in contrast to the results presented by Chen et al., (2010), reporting significant increase in IL6 expression in the oral mucosa compared to skin. This difference could be due to the differences in the tissue and cells evaluated (tongue wound model and keratinocytes culture). Furthermore, the study was conducted *in vitro* and this could also lead to discrepancies in the results.

Finally, we analysed CXCL1, a pro-inflammatory chemokine that stimulates epithelial cell migration and promotes angiogenesis (Simone & Higgins, 2015), identified within the "enhanced wound healing set" (Peake et al., 2014). 24 hrs after injury, a significant increase in M and in P was observed, although to a lesser extent in the latter. Thus, our data suggest that the slight increase in P could account for a better re-epithelialization, as confirmed by higher values of CSR in this tissue than in G, in which CXCL1 is down-modulated. Conversely, a more consistent increase, as that reported in M, might result in an excess of inflammatory signals, thus leading to scar formation.

The unwounded tissues gene expression evaluation in the present study showed relevant differences between the three tissues in both, DEGs and fibrotic markers expression, raising the possibility that the regulatory networks involved in the better oral wound repair capacity are already present, albeit partially, in the uninjured state. This is in agreement with previous studies (Chen et al. 2010; Iglesias-Bartolome et al. 2018; Simões et al. 2019).

Our research aimed at identifying the differential mechanism of early wound repair in fibroblasts derived from three oral soft tissues. We chose not to analyze total RNA isolated from the biopsies, although it could be more representative of the 'in-situ' situation, but to focus on the role of the mesenchymal component in wound healing, and especially of fibroblasts, the principal cell type present in the connective tissues (Sriram et al., 2015; Smith et al., 2019). Since their primary functions are to differentiate into myofibroblasts, to synthetize and maintain the ECM and to promote an inflammatory response (Kendall & Feghali-Bostwick, 2014; Häkkinen et al., 2014), fibroblasts can be considered to be key players in the wound healing process.
Moreover, in the latest years, translational research focused on oral fibroblasts, aiming to develop oral cell-based therapy that takes advantage of the potential regenerative properties of this cells to improve the wound healing of other tissues, such as the skin (Jiang & Rinkevich, 2020). Therefore, deepening into the knowledge of biomolecular mechanisms that regulate fibroblasts and myofibroblasts behaviour in the repair process is of interest for future wound healing and regenerative therapies. For this purpose, an analysis of the genetic profile was carried out in the present study through primary cultures of human fibroblast obtained from M, G and P biopsies.

It is known that many cell types alter their morphology and gene expression profile when grown on chemically equivalent surfaces with different rigidities (Yeung et al., 2005). In particular, culturing on stiff substrates and passaging might affect fibroblast phenotype in vitro (Landry et al., 2019). In this regard, it is important to clarify that in our previous study (Vescarelli et al., 2017) we have analysed αSMA expression of cells derived by M and G at various passages (2 to 8), confirming no significant variability due to cell culture. In the present work, the experiments were performed at the same time for the three tissues in each

patient. Some variability of passage number occurred between patients, since some patients required a further cell expansion to reach an adequate cell number for experimental setting. However, independent experiments (triplicate) were repeated also using cell at different passages, with reproducible results.

We demonstrated concordance between the results of the present work and our previous study, showing that P tissue behaviour is similar to G when myofibroblasts differentiation and autophagic activation were evaluated. In a very recent study evaluating palatal wound healing with primary intention in a rat model, the authors demonstrated that αSMA was not influenced by surgical trauma at 7 days (Chaushu et al., 2020). The results of this in vivo study are consistent with our observation of αSMA modulation at 24 hrs from the incision, thus contributing to support the hypothesis that main changes in the wound healing process occur in very early phases.
However, we cannot exclude that αSMA expression in the three tissues could change in a later time period, since it has been also demonstrated that myofibroblasts differentiation (during tissue remodeling phase) may last for several days after wounding and this time is highly variable depending on several factors, including the wound size and whether the injury has healed by primary or secondary intention (Smith et al., 2019). So, we believe that it could be interesting -replicating this experimental model-, to extend the evaluation period allowing a dynamic myofibroblasts differentiation evaluation.

Some discrepancies between the PCR array and qRT-PCR validation experiments results were observed in the present study. The potential impact of inter-individual variations of the selected genes on pooled qRT-PCR results was evaluated by performing qRT-PCR validation in each patient. The results obtained indicated a good consistency between patients; indeed, the significant up-regulation or down-modulation of the eight genes at 24 hrs assessed in the pooled cDNAs were observed in the majority of patients, thus confirming the trend of each gene in the three tissues. In particular, qRT-PCR validation on Patient 1 and Patient 4 confirmed the discrepancies with PCR array, demonstrating that they are not due to inter-individual variations. The differential expression of some genes between PCR array and qRT-PCR could be explained by technical differences in probe

locations, by cross-hybridization of the probes on the array with other targets, or simply by variations in normalization, since PCR array use five different housekeeping genes while qRT-PCR expression is normalized only with respect to GAPDH.

This is the first study comparing gene expression profiles of fibroblasts derived from three different oral soft tissues in the healing process. Nevertheless, some limitations of our study should be addressed. The RT2 profiler array was performed in fibroblasts derived from the tissues of only two patients and this could generate a variation in the results. Furthermore, the tissue evaluation was limited to one cell type, and future studies assessing genetic profiles of whole periodontal tissues are encouraged.

7. CONCLUSION

In conclusion, in the present study we focused on specific genes involved in the early wound healing process, showing different regulation pattern between the three periodontal soft tissues, which could account for their differential clinical outcome after surgery. A deeper gene analysis will require further studies to confirm these results, potentially including more patients, which was not possible here due to the strict enrolment conditions. Nonetheless, we think that our findings may contribute to elucidate the mechanisms behind the differential clinical repair outcomes of alveolar mucosa, buccal attached gingiva and palatal tissue, providing the basis for further investigations focused on deepening the knowledge about specific molecular pathways correlated with the most relevant DEGs here reported, thus facilitating the identification of novel molecular-targeted strategies aimed at improve oral tissues wound repair.

8. CLINICAL RELEVANCE

Scientific rationale for the study: Clinical practice indicates differential healing between periodontal soft tissues, whereas literature provides scarce information comparing oral tissues repair in humans.

Principal findings: Twenty-four hours after injury, clinical healing score was higher in the palate and gingiva with respect to oral mucosa. Accordingly, palate and gingiva showed lack of fibrotic markers and autophagy activation, explaining their scarless healing. Gene expression profiling in oral tissues demonstrated differential gene modulation after injury.

Practical implications: The discovery of key genes implicated in oral soft tissues differential healing can provide insights into the molecular mechanisms involved, allowing to develop new approaches of essential impact in periodontal surgery.

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III. Effect of post-surgical chlorhexidine digluconate on early wound healing of human gingival tissues. A histological, immunohistochemical and biomolecular *in vivo* analysis

1. INTRODUCTION

Microbial infection of post-surgical area inhibits normal tissue healing process. Meticulous plaque control during early post-surgical period has been correlated to lower incidence of post-operative infection of the wounded area (Powell et al., 2005). For this reason, especially after surgical procedures in which mechanical plaque control cannot be performed, is extremely important the reduction of plaque accumulation by means of antimicrobial agents (Sanz et al., 1989; Newman et al., 1989)

Chlorhexidine digluconate (CHX), a bisbiguanide broad-spectrum antiseptic with antibacterial action, is widely used as therapeutic agent in periodontology. Numerous studies have demonstrated the ability of CHX in reducing oral biofilm deposition (Löe & Schiott ,1970; Davies et al., 1970; Addy & Moran, 1983). Moreover, by penetrating biofilms, CHX shows a bactericidal action (Denver, 1995), reaching a substantivity of 12 hours (Schiott et al., 1970).

Although different effects have been reported based on a variety of available concentrations, a study conducted by Jones in 1997 (Jones, 1997) concluded that twice daily rinses with 15 ml of 0.12% CHX are enough for effective plaque control in the oral cavity. However, side effects of CHX mouthrinses, such as desquamation of the oral mucosa, soreness, increased calculus formation and tooth discoloration have already been reported in the literature, suggesting a strict control in their use and recommending it only for short periods (Flötra, 1971).

In a recent systematic review (Solderer, 2019), the authors concluded that CHX helps in reducing biofilm formation and gingival inflammation after periodontal and implant surgery and that less concentrated formulations (e.g., 0.12%) should be indicated in order to reduce the adverse effects.

Due to the above-mentioned bactericidal and bacteriostatic activities (Löe & Schiott ,1970; Davies et al., 1970; Addy & Moran, 1983) and to the absence of toxic systemic effects reported (Houri-Haddad et al., 2018), CHX has been considered the gold standard for antiseptic treatment of the oral cavity (Jones, 19997). Nevertheless, a recent *in vitro* study evaluating the impact of CHX use in controlling oral biofilms showed an initial drop in biofilm bacterial cell concentration followed by a quick recovery after its use. Therefore, the authors concluded that CHX can be ineffective in maintaining oral health since it presents a temporal effect and, as a broad-spectrum antiseptic, it can also affect the endogenous oral microbiota, increasing the risk of microbial dysbiosis, leading in turn to the development of oral diseases (Chatzigiannidou et al., 2020).

Furthermore, since 1970s, several studies have reported noxious effects on many different cells as macrophages (Kenney et al., 1972), leucocytes (Knuuttila & Söderling, 1981) and skin epithelial cells (Helgeland et al., 1971). Bassetti and Kallenberger in 1980 through an animal experimental model have demonstrated that intensive post-surgical rinsing with high concentrations of CHX could delay and impair the wound repair process. In addition, many recent studies showed cytotoxic effects in human periodontal tissues cells, such as gingival epithelial cells (Babich et al., 1995), gingival fibroblasts (Mariotti & Rumpf, 1999; Faria & Celes, 2007; Faria et al., 2009), bone (Cabral & Fernandes, 2007) and periodontal ligament cells (Chang et al., 2001).

Faria et al. (Faria & Celes, 2007), observed that CHX induces apoptosis of cultured fibroblasts at lower concentrations and necrosis at higher concentrations. Mariotti and Rumpf (Mariotti & Rumpf, 1999) postulated that CHX can reduce both collagen and non-collagen proteins production and proliferation of human gingival fibroblasts (HGFs), even at very low concentrations, and this negatively affects the wound healing process. This was confirmed in a recent *in vitro* study in which cells were exposed to a concentration diluted 100-fold when compared to their current uses in clinical practice (Fujioka-Kobayashi et al., 2020).

Another recent *in vitro* study using HGFs showed that a CHX concentration ≥0.04% inhibits cell proliferation, affects cells morphology and induces apoptosis. These effects are concentration and time-dependent. The authors concluded that post-surgical applications of CHX should be limited (Wyganowska-Swiatkowska et al., 2016).

All the above-mentioned *in vitro* studies allow to understand that CHX is not harmless to oral tissues, mainly in the wound healing process. However, it is important to highlight that *in vitro* assays cannot fully represent the oral environment as a whole and this could be a limitation (Chen et al., 2016).

Chen et al. (2010), have demonstrated that the main transcriptional changes in the wound healing process occur in the first 12-24 hours. In fact, we have observed significant changes in myofibroblast differentiation, fibrotic markers and wound healing genes expression of oral soft tissues derived-fibroblasts 24 hours after surgery when compared to baseline (Vescarelli et al., 2017; Rojas et al., 2021). In addition, it has been demonstrated that until the first 24 hours the biofilm is primarily populated by gram-positive cocci, and gram-negative anaerobic bacteria rapidly increase and predominate after 48 hours (Kolenbrander et al., 2006; Wake et al., 2016).

Considering all the aforementioned, immediate post-surgical use of CHX might not be necessary. This could be of beneficial effect on the healing process, since the most important changes in tissue repair occur in the early phases.

To date, no *in vivo* study has been conducted evaluating the CHX effects on gingival tissue behaviour in the early wound healing process.

2. AIM

The aim of the present study is to evaluate the *in vivo* effect of post-surgical CHX mouthrinse on the gingival tissue features and oral gingival-derived cells behaviour in the early phases following surgical wounding in terms of (1) collagen deposition and content, (2) cell proliferation, (3) cell apoptosis, (4) fibrotic markers expression and myofibroblasts differentiation and (5) collagen turnover and re-epithelialization; through a histological, immunohistochemical and biomolecular analysis of human G biopsies obtained 24 hours after injury.

3. HYPOTHESIS

CHX impairs the wound healing potential of oral gingival-derived cells 24 hours after injury by: (1) increasing collagen deposition and content, (2) reducing cell proliferation ability, (3) increasing cell apoptosis, (4) increasing fibrotic markers expression and myofibroblasts differentiation and (5) modifying the expression of genes related with collagen turnover and re-epithelialization.

4. MATERIALS AND METHODS

4.1. Ethics statements

The study protocol (ClinicalTrial.gov-NCT04276129) was approved by Sapienza University of Rome Ethics Committee (Ref.5315-Prot.1066/19). Each participant signed an informed consent in accordance with the Declaration of Helsinki (1975, revised in 2013).

4.2. Study design and patient selection

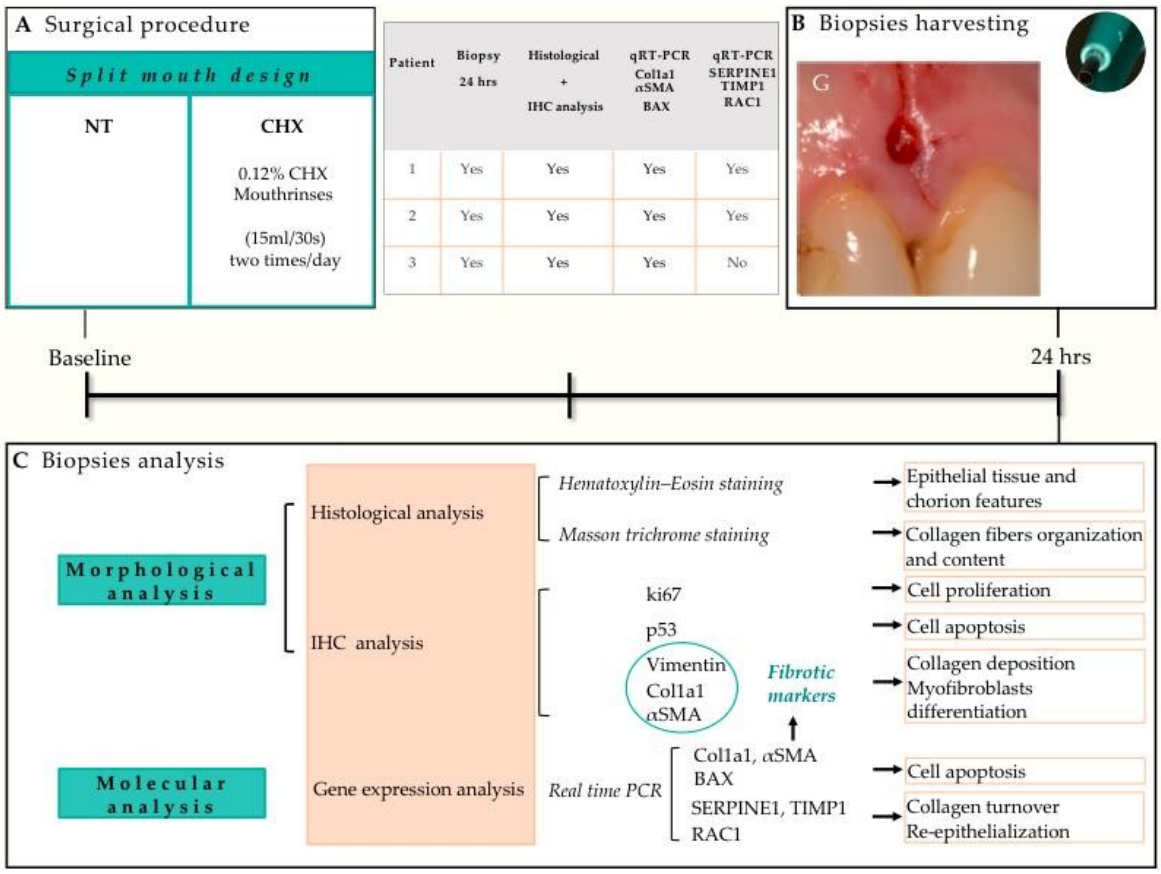
The present pilot study involved three systemically healthy adult patients (mean age 39.3 ± 5.44) who undergone at least two periodontal surgery procedures and who agreed to be "volunteer" for biopsy collection procedures by signing an informed consent. Patients who underwent antibiotic or anti-inflammatory drug consumption during the previous six months, patients in pregnancy or lactation period and smokers were excluded from the study. The subjects were enrolled at the clinical center of the Section of Periodontics, Sapienza University of Rome, Department of Oral and Maxillo-Facial Sciences.

Each patient underwent two surgical procedures and was treated in split mouth design to either post-surgical CHX mouthrinses indication (treatment group - CHX) or non-post-surgical mouthrinses indication (no treatment group - NT).

Biopsies from buccal attached gingiva (G) were harvested 24 hours after surgical procedures.

The experimental design is presented in Figure 1.

FIGURE 1 Experimental design



(A) Three patients underwent two surgical procedures were treated in split mouth design to either post-surgical CHX mouthirinses indication (treatment group – CHX) with 0-12% CHX (15ml/30s) 2 times/day or non-post-surgical mouthrinses indication (no treatment group-NT). **(B)** Buccal attached gingival (G) biopsies were harvested at 24 hrs after surgery. **(C)** Each gingival biopsy was divided in two parts: one for histological-IHC analysis and one for gene expression analysis in order to carryout a morphological and molecular analysis. For the first one, epithelial tissue/chorion features and collagen fibers organization/content were evaluated through Hematoxylin-Eosin staining and masson trichrome staining, respectively. Cell proliferation and apoptosis were examined by ki67 and p53 IHC analysis, respectively. Fibrotic markers expression (Vimetin, Col1a1 and αSMA) were also analysed by IHC in order to evaluate collagen deposition and myofibroblasts differentiation. For the molecular analysis, real time PCR was carried out: fibrotic markers expression (Col1a1 and αSMA) and proapoptotic protein (BAX) were analysed in all the patients. To evaluate the collagen turnover and re-epithelialization, SERPINE1, TIMP1 and RAC1 gene expression were evaluated in two patients.

4.3. Surgical procedures and biopsy collection

All surgical procedures and biopsies were performed by the same operator (MR). At the end of the surgical procedure, primary closure was obtained at the level of VRIs with interrupted sutures (polyglycolic acid-PGA, 6-0 monofilament). Patients were randomized (by a coin toss) during the first surgical procedure to received or not post-surgical CHX mouthrinses indication. In the treatment group, 0.12% CHX mouthrinses (15ml/30s) were indicated two times/day. Therefore, at the time the biopsy collection, the patients had already performed two mouthrinses with CHX. In the NT group, patients did not perform any mouthrinse after surgery. Twenty-four hours after the surgical procedure, gingival biopsies were harvested at the level of the VRIs with a biopsy punch of 2.0 mm diameter. The biopsy areas healed by second intention and sutures were removed at 1 week.

4.4. Histological analysis

Gingival biopsies were fixed in 10% neutral buffered formalin and processed for paraffin embedding. Blocks of paraffin were cut at 3 μm thickness using a Leica microtome. Sections were deparaffinized in xylene, rehydrated through graded alcohol series and stained with Hematoxylin–Eosin and Trichrome Masson according to standard protocols.

4.5. Immunohistochemistry

Immunohistochemistry (IHC) was performed using the automated BOND system (BOND-MAX Fully automated IHC and ISH system, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), according to manufacturer's instructions. Heat induced epitope retrieval was performed through incubation with BOND Epitope Retrieval Solution (BOND Epitope Retrieval Solution 2 (Cat# AR9640), Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK) for 20 minutes at 100°C. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 minutes at room temperature. Slides were then incubated with the

following primary antibodies for 15 minutes at room temperature: vimentin (BOND™ Ready-To-Use Primary Antibody Vimentin (V9) (Cat# PA0640), Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), Col1a1 (Mouse monoclonal antibody (clone 3G3) (cat# sc-293182), Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), αSMA (BOND™ Ready-to-Use Primary Antibody Smooth Muscle Actin (alpha sm-1) (Cat#PA0943), Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), Ki67 (BOND™ Ready-to-Use Primary Antibody Ki67 (MM1) (Cat# PA0118), Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), p53 (BOND™ Ready-to-Use Primary Antibody p53 (DO-7) (Cat# PA0057), Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK). The detection was performed using BOND Polymer Refine Detection System (Cat# DS9800, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK)according to the automated IHC protocol. Negative control slides were obtained by omitting the primary antibody.

Sections were analysed using a Leica microscope coupled to a digital camera. Two independent pathologists, blinded to the treatment, observed the immunostaining and, subsequently, images were captured. The staining intensity for αSMA, vimentin and Col1a1 was determined using a semi-quantitative score (0, no staining; 1, low staining; 2, moderate staining; 3, strong staining) [32,33]. This evaluation was performed by two independent investigators blinded to the treatment, who observed five microscopic fields for each of the three sections randomly selected for each case using the objective ×20.

Immunohistochemical staining for the nuclear proliferation-associated antigen Ki67 and for p53 was estimated as the percentage of stained nuclei among all nuclei visible in the field. The analysis was performed by two blinded examiners. The number of cells with Ki67/p53-positive nuclei was evaluated in 10 random microscopic fields in each cell preparation and expressed as percentage of Ki67/p53-positive nuclei per optical field.

4.6. Quantitative real- time PCR (qRT-PCR)

Total RNA from CHX and NT gingival biopsies of the three enrolled patients wereextracted using TRIzol reagent (Cat# 15596026, Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer's instructions, and was reverse transcribed using High

Capacity RNA to cDNA Kit (Cat# 4387406, Thermo Fisher Scientific, Carlsbad, CA, USA). cDNAs were then used for amplification of BAX, Col1a1, αSMA, RAC1, SERPINE1 and TIMP1, using the appropriate TaqMan gene expression assay kits
(Assay IDs: Hs00180269_m1 (BAX); Hs00164004_m1 (Col1a1); Hs00559403 (αSMA); HS00167155-M1 (SERPINE1); HS01902432_S1 (RAC1); HS01092512_ G1 (TIMP1); Thermo Fisher Scientific, Carlsbad, CA, USA). A total of 2 µl/well of template was added to the sample wells along with TaqMan Universal PCR master mix (Cat# 4305719, Thermo Fisher Scientific, Carlsbad, CA, USA) at a concentration of 1x and water to a volume of 25 µl/well. Assays were conducted in triplicate on an ABI 7500 Real Time instrument (Thermo Fisher Scientific, Carlsbad, CA, USA) using the following conditions: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min, repeated 40 times. Relative quantification was performed using GAPDH mRNA as an endogenous control.

4.7. Statistical analysis

Data were analysed on Prism 8.0 (GraphPad Software, La Jolla, USA) and are shown as mean ± SD from three independent experiments conducted in triplicate. Two-tailed unpaired Student's t test was used for statistical analysis. *P* values < .05 were considered statistically significant.

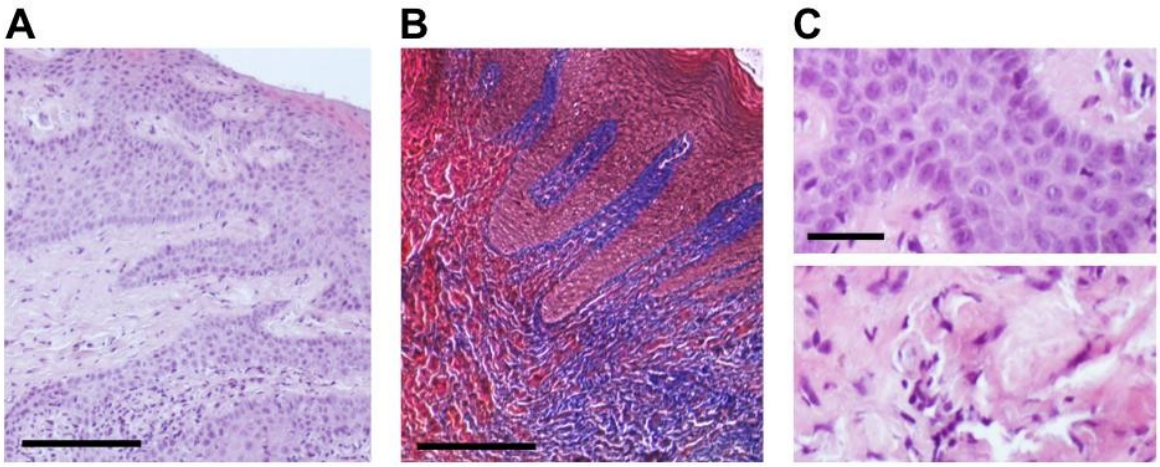
5. RESULTS

5.1. CHX post-surgical mouthrinse increases fibrotic markers expression and myofibroblasts differentiation

Myofibroblasts activation and collagen deposition are key events in physiological and pathological tissue repair.

To identify the effect of CHX treatment on the phenotype of fibroblasts involved in collagen synthesis, we analysed gingival biopsies of three patients subjected or not to CHX mouthrinses in the 24 hours between surgical intervention and biopsy collection. HE staining revealed in both NT and CHX group a thick gingival mucosa, with deep and branching epithelial ridges partly joined by epithelial bridges. Subjacent chorion was full of collagen bundles, appearing as a dense and homogeneous structure (Figure 2A). Collagen deposition was further revealed with Masson's Trichrome staining (Figure 2B). As for CHX group, HE staining showed the presence of enlarged, polymorphic and polymetric nuclei, indicative of activated cells, in the epithelial layer (Figure 2C, upper panel), and a more extensive fibrosis in the chorion (Figure 2C, lower panel).

FIGURE 2 Histological characterization of gingival biopsies 24 hrs after surgery in NT and CHX group



(A) Representative photomicrograph of sections of gingival biopsies showing elongated and branched epithelial ridges and subjacent chorion full of a dense and homogeneous structure of collagen bundles. HE staining, scale bar 100 μm. **(B)** Representative photomicrograph of sections of gingival biopsies showing collagen bundles in the deep chorion (blue). Trichromic Masson staining, scale bar 100 μm. **(C)** Representative photomicrographs of histological alterations observed in CHX biopsies, such as enlarged and polymorphic nuclei in the epithelial layer (upper panel) and enhanced fibrosis in the deep chorion (lower panel). HE staining, scale bar 25 μm.

Afterwards, the expression levels of fibrosis markers were analysed with IHC staining. We incubated serial sections of each biopsy belonging to the two groups (NT and CHX) with the following antibodies: anti-αSMA, anti-Col1a1 and anti-vimentin. For αSMA, normal vessels' smooth muscle immunoreactivity was used as an internal positive control, while αSMA-positive stromal cells, showing cytoplasmic immunostaining, were considered to be myofibroblasts. NT samples showed an extremely weak positivity in the mesenchymal cells, while cells of blood vessels were labeled. In the CHX group, we noted a higher number of blood vessels in the chorionic papillae and the deep chorion compared to NT samples (Figure 3A), and we also observed the presence of cells with cytoplasmic positivitylocalized in the basal epithelial layer, particularly in the deep and prickle cell layers (Figure3B).

As for the fibrotic marker Collagen 1a1 (Col1a1), its expression was localized in the subepithelial layer, and it was significantly higher in CHX biopsies with respect to NT group (Figure 3C).

Immunostaining for vimentin, specific for cells of mesenchymal origin, showed few positive cells concentrated mainly in the subepithelial layer. We observed no significant differences both in the amount of positive cells and in their location between samples from NT and CXH group.

The semiquantitative evaluation for αSMA, Col1a1 and vimentin staining intensity is reported in Table 1.

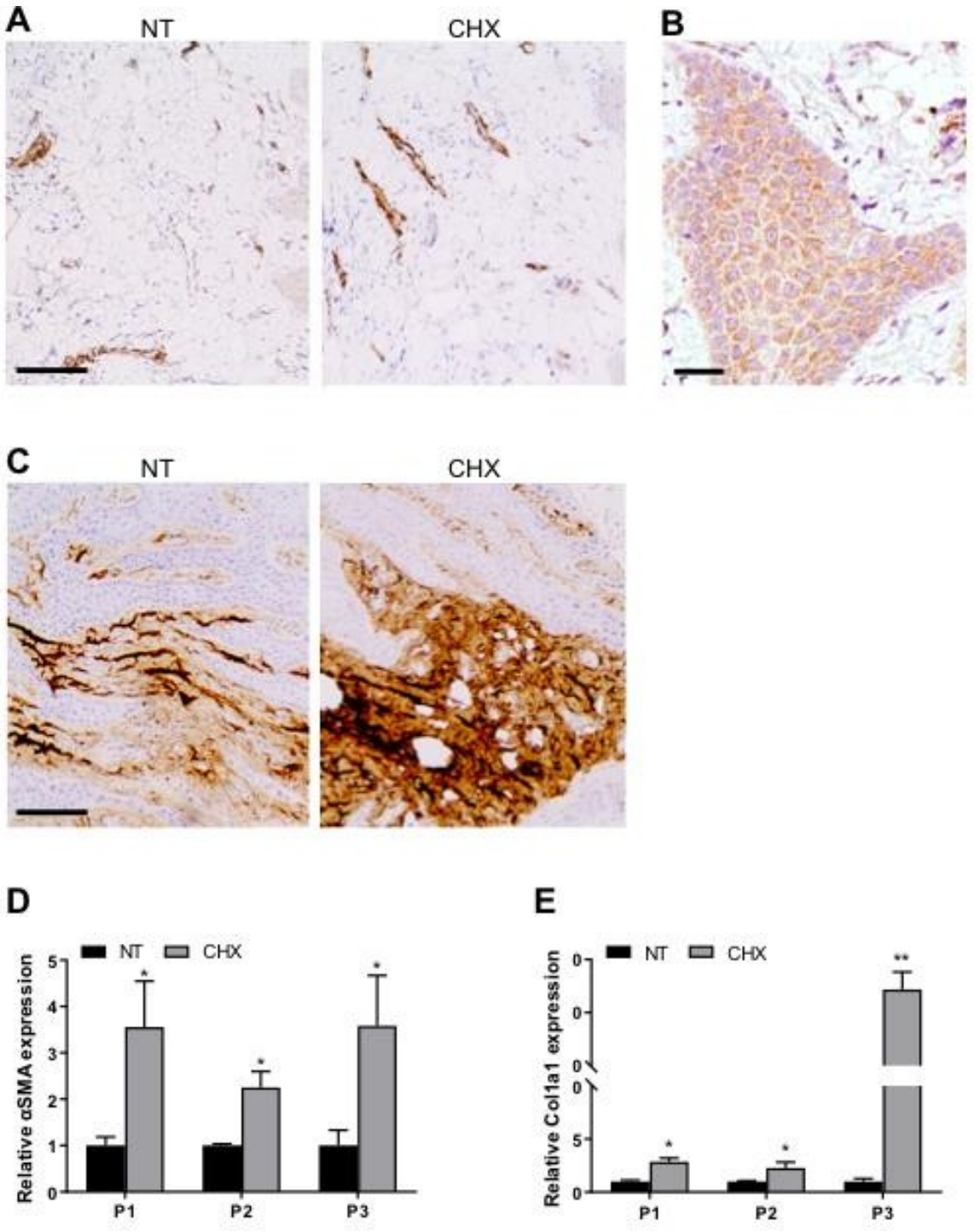
TABLE 1 Immunohistochemical scoring of staining intensity for αSMA, Col1a1 and Vimentin

Patient	IHC score[a]					
	αSMA		**Col1a1**		**Vimentin**	
	NT	**CHX**	**NT**	**CHX**	**NT**	**CHX**
1	0	1	2	3	1	1
2	0	1	1	2	1	1
3	0	1	1	3	1	1

IHC, immunohistochemistry; NT, no treatment group; CHX, chlorhexidine mouthrinses group.
[a] Staining intensity scores were as follows: 0, no staining; 1, low staining; 2, moderate staining; 3, strong staining (Allred et al., 1990; Keiner et al., 2013).

The expression of αSMA and Col1a1 was also assessed at mRNA level by qRT-PCR analysis in gingival biopsies of three patients subjected or not to CHX mouthrinses in the 24 hours between surgical intervention and biopsy collection. Our results confirmed a significant increase in αSMA expression in the CHX biopsies of all the three patients (3.6, 2.3 and 3.6-fold, respectively) (Fig. 3D). The same trend was observed for Col1a1, with a consistent increase in the CHX biopsies of all patients (2.9, 2.3 and 34.4-fold, respectively) (Figure 3E)

FIGURE 3 IHC and qRT-PCR analysis of fibrotic markers αSMA and Col1A1 expression in gingival tissues 24 hrs after surgery in NT and CHX group



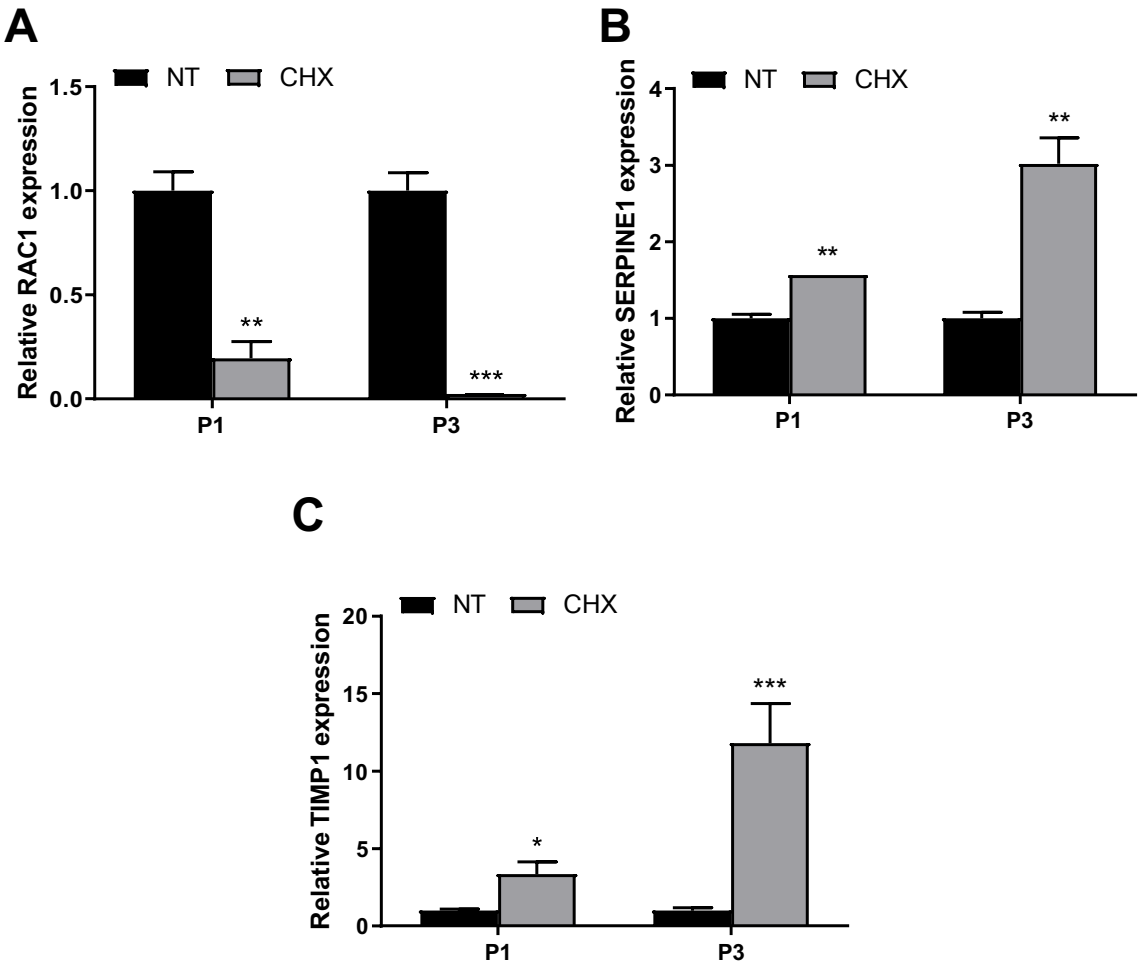
(A) Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-αSMA. Scale bar 100 μm. **(B)** Representative photomicrograph of cytoplasmic staining for αSMA in

the epithelial layer observed in CHX biopsies. Scale bar 25 μm. **(C)** Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-Col1a1 antibodies. Scale bar 100 μm. **(D, E)** Quantitative real-time PCR analysis of αSMA (D) and Col1a1 (E) mRNA expression in NT and CHX biopsies of three patients. Relative mRNA levels are shown as fold value of the NT levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$ and **$p < .005$ vs NT.

5.2. CHX influences the expression of key genes involved in early wound healing

We then investigated the effect of CHX on the expression of some previously shown genes to play a role in the early wound healing process (Rojas et al., 2021), in two out of the three enrolled patients (since in one of the patients the material obtained with the biopsy was not enough to carry out the analysis). We first evaluated RAC1, a member of the Rho family of small GTPases that promotes healing and that has been previously shown to increase in gingival tissue 24 hours after injury (Rojas et al., 2021). Interestingly, we observed a significant downmodulation of RAC1 expression at 24 hours in CHX biopsies of both patients (0.2 and 0.02-fold, respectively) (Figure 4A), thus suggesting that CHX might impair gingival wound healing. Other two genes that play a role in regulating scar formation in oral tissues, SERPINE1 and TIMP1, were evaluated. Such genes, involved in collagen deposition and fibrosis, were previously shown to remain stable in gingival tissue at 24 hours after injury (Rojas et al., 2021). In our study, we observed an increase of SERPINE1 and TIMP1 in CHX biopsies of both patients (1.6 and 3.0-fold for SERPINE1; 3.4 and 11.8-fold for TIMP1, respectively; Figure 4B, C).

FIGURE 4 qRT-PCR analysis of mRNA expression levels of early wound healing-related genes in gingival tissues 24 hrs after surgery in NT and CHX group

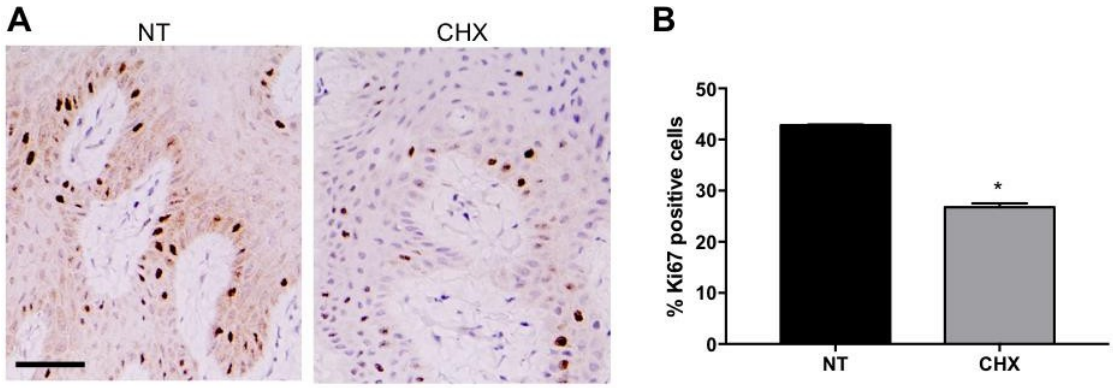


Quantitative real-time PCR analysis of RAC1 **(A),** SERPINE1 **(B)** and TIMP1 **(C)** mRNA expression in NT and CHX biopsies of two patients. Relative mRNA levels are shown as fold value of the NT levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$, **$p < .005$ and ***$p < .0005$ vs NT.

5.3. CHX increases the expression of apoptotic markers and reduces the proliferative ability of gingival cells

In order to understand the molecular events underlying the effect of CHX on early gingival wound healing, the expressions of proteins related to proliferation and apoptosis were examined by IHC analysis. As compared with NT group, the Ki67 proliferation marker was significantly downregulated in the CHX group (Fig. 5A), as indicated by the percentage of stained nuclei reported in Figure 5B (26.8% vs 42.8% of NT, $*p < .05$).

FIGURE 5 IHC analysis of Ki67 expression in gingival tissues 24 hrs after surgery in NT and CHX group

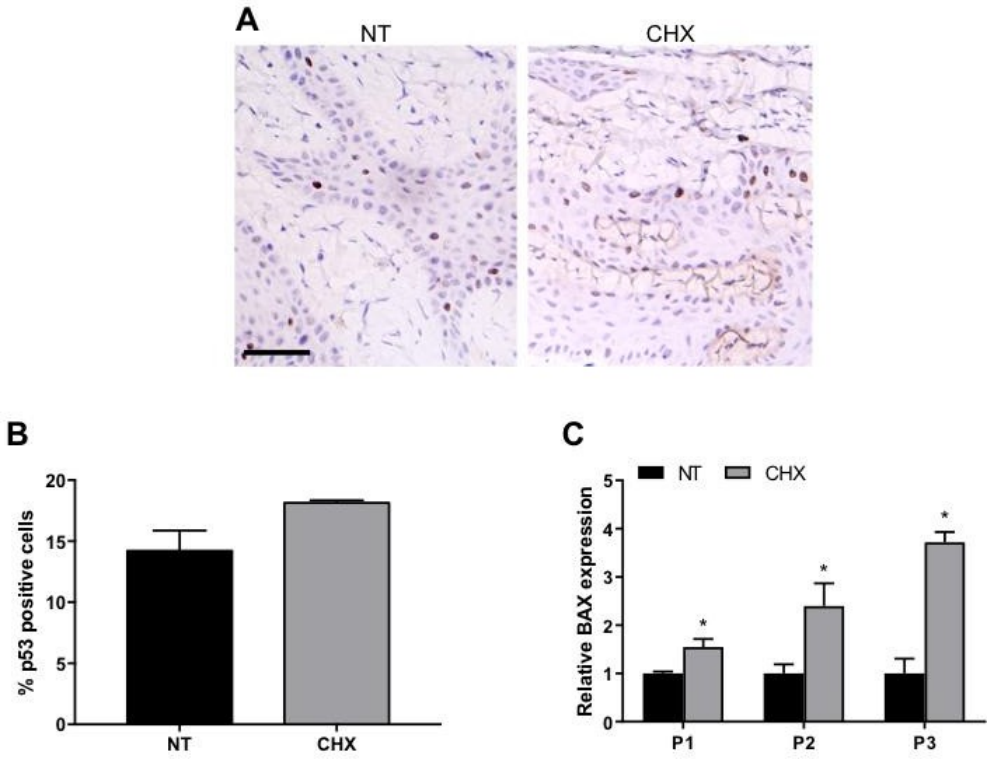


(A) Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-Ki67 antibodies. Scale bar 50 μm. **(B)** Mean percentage of Ki67 immunopositive cells. Error bars represent standard deviations. $*p < .05$ vs NT.

So, we assessed if the reduced proliferation could be accompanied by an induction of apoptosis. To this aim, we evaluated the expression of the tumor suppressor gene p53, a key regulator of cell death under multiple physiological and pathological conditions. In our *in vivo* model, IHC analysis showed that p53 expression was slightly higher in the CHX group (Figure 6A), with a modest but not statistically significant increase of the percentage of stained nuclei in CHX samples (18,1% vs 14.2% of NT, Figure 6B).

Interestingly, when analysing the expression of the proapoptotic BAX protein in gingival tissue by Real Time PCR, we found a significantly higher expression of BAX in the CHX biopsies of all the enrolled patients (1.5, 2.4 and 3.7-fold, respectively) (Figure 6C), thus indicating a potential p53-independent proapoptotic effect of CHX post-surgical treatment on gingival tissue.

FIGURE 6 IHC analysis of p53 expression and qRT-PCR analysis of BAX mRNA expression in gingival tissues 24 hrs after surgery in NT and CHX group



(A) Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-p53 antibodies. Scale bar 50 μm. **(B)** Mean percentage of p53 immunopositive cells. **(C)** Quantitative real-time PCR analysis of BAX mRNA expression in NT and CHX biopsies of three patients. Relative mRNA levels are shown as fold value of the NT levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < .05$ vs NT.

6. DISCUSSION

Chlorhexidine is considered as the gold standard in the antiseptic treatment of the oral cavity (Jones, 1997). Nevertheless, time and dose-dependent cytotoxic effect of CHX in human fibroblasts has been demonstrated in previous *in vitro* studies (Mariotti & Rumpf, 1999; Chen et al., 2016), delaying wound healing or increasing wound dehiscence rates (Thomas et al., 2009; Hirsch et sl., 2010).

The present study was designed to investigate the *in vivo* effect of post-surgical 0.12% CHX mouthrinse in the early phases of gingival tissue repair to understand its role on cell behaviour in terms of (1) collagen deposition and content, (2) cell proliferation, (3) cell apoptosis, (4) fibrotic markers expression and myofibroblasts differentiation and (5) collagen turnover and re-epithelialization through a histological, immunohistochemical and biomolecular analysis of human gingival biopsies.

Our findings demonstrate that, 24 hours after injury, CHX is able to (1) increase collagen deposition and content, (2) reduce cell proliferation and increase the expression of proapoptotic molecules, (3) increase fibrotic markers expression and myofibroblasts differentiation, (4) reduce expression of RAC1 gene, characterizing keratinocytes migration and proliferation and (5) trigger expression of SERPINE1 and TIMP1, related with collagen turnover.

In our *in vivo* experimental setting, we observed that Ki67 proliferation marker was significantly downregulated in the CHX group compared with NT group, confirming the anti-proliferative effects of CHX in gingival tissue *in vivo*, in agreement with those obtained *in vitro* by other authors (Mariotti & Rumpf, 1999; Wyganowska-Swiatkowska et al., 2016; Chen et al., 2016; Coelho et al., 2020). Many citotoxic agents modulates the balance between cell proliferation and cell death (Müller & Kramer, 2008). Cell death can occur through different pathways that can culminate in autophagy, necrosis or apoptosis (Wyganowska-Swiatkowska et al., 2016).These mechanisms may play an important role in the scarring

response. In fact, it has been reported the ability of apoptotic cells to induce myofibroblasts differentiation and proliferation (Laplante et al, 2010; Johnson et al., 2014).

Gianelli et al. (2008), reported that after 1 min treatment, nearly 50% of fibroblastic and endothelial cells treated with 0.12% CHX exhibited apoptotic nuclei. Regarding this concern, some clarifications need to be pointed out: in the present work, our goal was not to study the amount of apoptotic cells, since the *in vivo* response of gingival tissue 24 hours after CHX mouthrinse could be influenced by compensation mechanisms aimed to rescue cells from death. Instead, we were more interested in exploring the potential pathways activated by CHX *in vivo.* As for apoptosis we chose to evaluate the involvement of p53/BAX pathway. In fact, previous findings demonstrated that BAX is a p53 downstream effector (Johnstone et al., 2002). Some data reported the centrality of BAX in this pathway, demonstrating that BAX-deficient cells were protected from p53-induced apoptosis (Cregan et al., 1999). On the other hand, although caspase 3 has been also defined as an enzyme with an important role in the initiation of apoptosis (Nichani et al., 2020), it has been reported the occurrence of BAX-mediated apoptosis in an caspase-independent manner (Cregan et al., 2004). Therefore, BAX expression seems to be more relevant that caspase 3 activation. Moreover, while activated caspase 3 could have been assessed only by IHC, more accurate qRT-PCR methods can be used for the evaluation of BAX expression.

In our results, we did not observe a significant increase in the percentage of stained nuclei after CHX treatment through IHC analysis using p53 as a marker of apoptosis. However, we can infer a proapoptotic potential of CHX since we demonstrated a consistently higher expression of the proapoptotic gene BAX in the three enrolled patients. p53 is known to accumulate in the nucleus following death stimuli, such as oxidative stress and genotoxic injury, and to induce activation of downstream proapoptotic gene expression, e.g., PUMA, Noxa, and/or BAX, to induce cell death. Nevertheless, it has been suggested that other kind of injuries can also produce BAX activation members, thus initiating a p53-independent apoptosis (Villunger et al., 2003).

Thus, our results confirmed *in vivo* the detrimental effect of CHX in reducing cell viability, and led us to hypothesize that CHX mouthrinse could trigger a p53-independent apoptosis. It is known that the mechanism of apoptosis derives from the local environment of preapoptotic cells and it has been reported that in oral wound healing predominates the

intrinsic apoptotic pathway, generally initiated by DNA damage, growth factors levels or cytokines reduction (Laplante et al., 2010). Interestingly, a study demonstrated that the timing of the peak of gene expression related to intrinsic apoptosis in oral wound healing was most commonly seen at 24 hours, and the authors also suggested a correlation between the apoptosis peak and the resolution of the inflammation, both occurring at the same time (Johnson et al., 2014). Thus, it would be expected to observe P53 positive stained nuclei in both groups in our work, as it may be related to the normal intrinsic apoptotic response. However, as mentioned above, this is not correlated with BAX gene upregulation observed in CHX group, suggesting a different pathway activation.

The increase of cell proliferation during early wound healing is thought to be regulated by a decrease of apoptosis. Instead of, cellularity reduction during final wound maturation may be controlled by an increase of apoptosis (Vollmar et al., 2002). CHX treatment induce this latter response, but at a very early phase, in which cell proliferation and viability are required for rapid tissue repair.

Fibroblasts become activated upon wounding, as evidenced by expression of αSMA, proliferation and migration to the wound area, and ECM deposition (Eming et al., 2014).

In our previous studies (Vescarelli et al., 2017; Rojas et al., 2021), we demonstrated a downregulation of αSMA and Col1a1 in gingival tissue 24 hours after injury, in line with clinical observation of reduced scar formation in this tissue. Instead of, the alveolar mucosal (M) tissue showed the opposite response, according to the clinical observation of scar tissue repair. We observed that CHX-treated G tissue present similar behaviour to M tissue suggesting that it could induce a "fibrotic response".

The effect of CHX on collagen production was reported by Mariotti e Rumpf (1999). The authors postulated that, at concentrations which have little effect on cellular proliferation, it can significantly reduce both HGFs collagen and non-collagen protein production. Consistent with these findings, a very recent study showed decreased COL1 expression after CHX treatment (Fujioka-Kobayashi et al., 2020). Here, we observed the opposite response, and this could be related with the differences between *in vitro*/*in vivo* analysis (Chen et al., 2016). It is noteworthy that these features are similar to those reported in adult skin fibroblasts, which show a reduction in genes associated with proliferation and an

enrichment for GO terms ECM production and remodeling-related with increasing age (Rognoni et al., 2016). Additionally, it is interesting to mention that CHX intraperitoneal injection has been reported as the most commonly used method to create a peritoneal fibrosis animal model showing increased expression of transforming growth factor β1 (TGF- β1), αSMA, type I collagen, and vascular endothelial growth factor (VEGF) (Lee et al., 2012).

Based on our group's previous results (Rojas et al., 2021), it was still interesting to further investigate the findings based on previously assessed genes related to scar wound healing. Through qRT-PCR analysis, we evaluated the expression of RAC1, TIMP1 and SERPINE1 genes. Noteworthy, we observed that gingival tissue after CHX treatment present the same pattern observed in alveolar mucosal-derived fibroblasts (Rojas et al., 2021), showing RAC1 downmodulation and TIMP1 and SERPINE1 upregulation. These results are in line with the evidence of an increase in collagen deposition mediated by CHX mouthrinses. Moreover, we have previously hypothesized that myofibroblasts differentiation in gingival tissues is independent of SERPINE1 and TIMP1 expression, and that other pathways could be involved, since HGFs did not show significant changes in the expression of these genes 24 hours after injury (Rojas et al., 2021). One of the more interesting findings to emerge from this study is that after CHX treatment, these genes present changes in their regulation, with similar characteristics to "fibrotic response" tissues (such as alveolar mucosal tissue). Therefore, CHX appear to induce mechanisms related to impaired wound healing, which are not present in gingival tissues under normal conditions.

Regarding the bactericidal effects of CHX, although it was not the aim of the present study to evaluate it, we considered important to point out that several studies have reported a lack of dramatic bacterial reduction after the use of CHX mouthrinse (Eberhard et al., 2008; Matesanz-Pérez et al., 2013; Bowen et al., 2015). In fact, the strongest data supporting CHX as an antimicrobial agent is from *in vitro* studies, while human clinical trials failed to consistently demonstrated a positive effect (Lang et al., 2008). For example, in a well-designed comparative study, 30 seconds of 0.12% CHX treatment failed to produce significant change in bacteria load *in vivo*, while it produced significant toxic effects on

gingival fibroblasts *in vitro*, with a reduction of 94% in the number of cells (Bowen et al., 2015). Therefore, finding the balance between bactericidal effects, without cellular toxicity could be important for home based oral treatment.

In the present study, we indicated CHX alcohol free-formulation to patients, as several studies have reported similar effectiveness in controlling plaque and reducing gingival inflammation compared with alcohol-based CHX (Todkar et al., 2012; Olsson et al., 2012; Papaioannou et al., 2016). Accordingly, its use has been recommended in all periodontal patients, but especially in those in whom alcohol may produce side effects (Todkar et al., 2012). However, a recent study concluded that the presence of alcohol may increase the effectiveness of CHX in early wound healing and that an alcohol-based 0.12% CHX mouthwash was more effective than an alcohol-free 0.12% CHX on plaque control in the absence of mechanical oral hygiene (Gkatzonis et al., 2018). The authors proposed that alcohol serves as a stabilizing agent for solutions of oral use, but this concern remains unclear. Therefore, taking into account the side effects of alcohol (Winn et al., 1991; Poggi et al., 2003; Lachenmeier et al., 2009) and the results of the above-mentioned studies (Todkar et al., 2012; Olsson et al., 2012; Papaioannou et al., 2016), we preferred to perform our *in vivo* analysis using CHX alcohol-free mouthwashes.

IHC analysis presented in this study was performed through a semiquantitative evaluation measuring staining intensity (Allred et al., 1990; Keiner et al., 2013). Although it has been reported a variety of IHC scoring methods, including computer-based plans, no generally accepted protocols for scoring the immunostaining results are available. The objective accuracy of computer-based programs did not significantly improve conventional analysis by pathologists, since the former lack of standardized IHC scoring algorithms (Koo et al., 2009). Nonetheless, in the present study, we wanted to confirm the results obtained through the IHC analysis regarding α-SMA and Col1a1, assessing their expression at mRNA level using qRT-PCR analysis and these results were consistent with the differences observed between CHX and NT group in the IHC assessment.

It is important to highlight that, although it has been demonstrated a higher tolerance of human tissues for antiseptic solutions *in vivo* compared to *in vitro* tissue culture (Liu et al., 2018), in the present study we demonstrated that even after only two mouthrinses for 30 seconds with 0.12% CHX, gingival tissue behaviour is modified, altering the normal wound healing repair response 24 hours after injury.

Finally, it is interesting to point out that, the international Society of Oral Oncology and Multinational Association of Supportive Care in Cancer, in a review of management of oral health, concluded that antimicrobials such as CHX should not be prescribed for cancer patients with mouth sores (Hong et al., 2019), since the cell death occurs due to damage to DNA and proteins, which can result not only in oral mucositis, but it also decreases the body's natural defence against further bacterial invasion (Hans & Hans, 2014).

Undoubtedly, our study presents some limitations, since the evaluation was carried out in only three patients and in a single period-time. Moreover, the data obtained here should be paralleled with a clinical evaluation through an accurate assessment of the healing characteristics (Marini et al., 2018; Marini et al., 2019). Although our results should be extended to solve the aforementioned issues, the *in vivo* data obtained in the present work confirms previous *in vitro* findings and provide additional *in vivo* evidence to understand the potential of CHX to negatively interfere in the early phases of human gingival tissue wound healing. However, because of a small sample size, the results should be cautiously interpreted.

One of the main strengths of this study is that the effect of CHX was evaluated *in vivo*, through a human biopsy wound model. Although through an *in vitro* assay a better quantitative analysis can be achieved, without the interference of other *in vivo* factors (Tripton et al., 1995), surgical wounds present particular conditions to consider, such as vascularization, local and systemic inflammatory responses after injury, mechanical forces affecting tissue repair process, multiple cell layers and presence of saliva and crevicular fluid. All these features are not present in a monolayer culture and this could produce relevant changes in the oral tissue response. In fact, we observed some differences between

our results and the *in vitro* performed studies and many similarities with *in vivo* animal studies performed in other medical fields. Therefore, *in vivo* evaluations appear to be critical to elucidate the mechanisms impairing the wound healing process after the post-surgical use of CHX mouthrinses.

7. CONCLUSION

The present research was designed to evaluate the *in vivo* effect of post-surgical CHX mouthrinse in the gingival tissue wound repair 24 hours after injury. The results of this investigation showed significant changes in the expression of BAX, Col1a1, αSMA, RAC1, SERPINE1 and TIMP1 in CHX-treated gingival biopsies when compared with NT group. These findings further support that features such as increased collagen deposition, myofibroblasts differentiation and cell apoptosis, as well as reduced cell proliferation, could be relevant for a CHX-induced fibrotic transformation, leading to scar tissue repair. Nevertheless, due to the present pilot study was performed in three patients, further investigation is needed to confirm the data obtained and to define a post-surgical clinical protocol that provides a strategic and personalized use of CHX during the first hours after surgery.

8. CLINICAL RELEVANCE

Scientific rationale for the study: Several *in vitro* and animal studies have reported cellular toxicity and delayed wound repair after CHX use in the post-surgical period. However, *in vitro* assays cannot fully represent the oral environment. To date, there are no *in vivo* studies reporting CHX effect on the gingival tissue during the early phases aftersurgical wounding.

Principal findings: : Twenty-four hours after injury, CHX was able to (1) reduce cellular proliferation, (2) increase collagen deposition and myofibroblasts differentiation, (3) increases the expression of proapoptotic molecules and fibrotic markers expression, and (4) modify early would healing-related genes expression; showing a "scar wound healing response" pattern.

Practical implications: The demonstration of a CHX-induced fibrotic transformation, leading to scar repair, could support the need for new post-surgical clinical protocols based on strategic and personalized use of CHX.

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IV. Effect of exogenous hyaluronic acid on early wound healing of human gingival tissue. A clinical, histological, immunohistochemical and biomolecular *in vivo* analysis

1. INTRODUCTION

Hyaluronic acid (HA) is a major endogenous component of the extracellular matrix (ECM) in almost all tissues with a fundamental role in maintaining their hydration and structural and homeostatic integrity (Dahiya & Kamal, 2013). It is active through the entire process of wound healing being involved in cell proliferation, migration and tissue remodeling (Chen & Abatangelo, 1999; Aya & Stern, 2014).

Wound healing is a dynamic process that requires a new collagen matrix deposition and efficient vascularization. New vascular network formation is a critical step in the proliferative phase wound healing process (Boerckel et al.,2011; DiPietro, 2016)). The growth of new vessels determines traction that induces remodeling of the ECM, regulating neovessel responses (Sottile, 2014). Nevertheless, excessive angiogenesis in the early phases of wound repair could directly stimulate scar formation (DiPietro, 2016).

Fibroblasts, endothelial cells and macrophages are key players in the tissue repair process and the activation of specific functions of these cells may significantly improve the wound healing (Prosdocimi & Bevilacqua, 2012). In particular, in oral wound repair, fibroblast and mesenchymal cells are essential since they are responsible for the production of the most of ECM components in connective tissues (Chiquet et al., 2015). HA, as well as its degradation products, present the ability of activate specific responses in all the cells involved in the process; in particular, fibroblast proliferation and new vessel formation (Prosdocimi & Bevilacqua, 2012). In periodontal wound healing, HA degradation products are able to induce pro-inflammatory cytokine production by fibroblasts, keratinocytes, cementoblasts

and osteoblasts, promoting inflammatory response and stimulating hyaluronan synthesis by endothelial cells (Larjava et al., 1989).

In vitro and animal studies have demonstrated that HA significantly increases granulation tissue tensile strength (Scully et al., 1995), stimulates clot formation (West et al., 1985), induces angiogenesis (Pilloni & Bernard, 1998), increases osteogenesis (Pilloni et al., 2003), and improve ligament cell viability (Fujioka-Kobayashi et al., 2017). All these aforementioned properties are essential for tissue regeneration and wound healing.

HA also has been claimed to be a potent anti-inflammatory agent, which is able to modulate wound healing as a consequence of its ability to scavenge the inflammatory cell-derived reactive oxygen species (Moseley et al., 2002). Due to the numerous beneficial effects (Casale et al., 2016), HA has been extensively used in the periodontics field, specifically in gingivitis (Jentsch et al., 2003) and periodontitis non-surgical treatment (Johannsen et al., 2009; Eick et al., 2013; Pilloni et al., 2021), mucogingival surgery (Pilloni et al., 2018), and inthe surgical treatment of periodontal intra-bony defects (Shirakata et al., 2021; Pilloni et al., 2021). Furthermore, the effect of topical application of HA in the wound repair has been studied. Romeo et al., (2014), showed that the use of a gel containing amino acids and 1.33%HA, topically applied three times per day for 1 week, was able to promote faster healing by secondary intention in oral soft tissue biopsy created wound. Casale et al., (2016) in a systematic review, concluded that the topical HA application can be useful to accelerate healing during the post-operative period after implant placement and sinus lift procedures, also reducing patient discomfort.

Nevertheless, although several studies have described the role of exogenous HA in non-surgical and surgical periodontal therapy, the potential beneficial effects were reported in terms of clinical response but the specific *in vivo* cellular mechanisms involved from a morphological and molecular point of view in the early phases of surgical wounding remains unclear.

Asparuhova et al., (2019) evaluated *in vitro* the effects of two HA formulations on human oral palatal and gingival derived-fibroblast. The authors demonstrated that the

investigated HA formulations maintained the viability of oral fibroblasts and enhance the proliferative, migratory and wound healing properties of cell types involved in soft tissue wound healing. However, the authors pointed out the limitations of *in vitro* experiments: during the post-surgical period, HA would undergo degradation to molecules of lower molecular weight (MW) due to the hyaluronidase activity, thus exerting additional or even opposing effects on the wound repair process (Asparuhova et al., 2019).

Recently, Canciani et al., (2021) have carried out an *in vivo* study using a human oral wound model, analysing biopsies obtained 10 days after injury. The authors reported higher microvascular density (MVD) and collagen fibers organized in closely packed and well-oriented bundles in sites treated with HA containing amino acids.

However, it has been recognized the importance of the first 24 hours in the wound healing process (Chen et al., 2010). In fact, in our previous *in vivo* study evaluating gene expression profiles of the different oral soft tissues we demonstrated as different genes modulation can be observed at this time and also how this is correlated with the clinical response (Rojas et al., 2021). Furthermore, a recent *in vivo* study have demonstrated that, 24 hrs after injury, exogenous intradermal HA accelerates re-epithelization and alters protein expression in human deep dermal skin wounds (Nyman et al., 2019). Thus, elucidation of the mechanisms of cellular activation and gene expression modulation in the early phases of oral wound repair after HA treatment could allow an optimal use of hyaluronan and its derivatives in the wound care environment.

2. AIM

The main aim of the present study is to evaluate the *in vivo* effect of exogenous HA on the gingival tissue features and oral gingival-derived cells behaviour in the early phases following surgical wounding in terms of (1) ECM organization, (2) MVD, (3) collagen deposition and content, (4) collagen turnover and (5) cell proliferation; through a histological, immunohistochemical and biomolecular analysis of human G biopsies obtained 24 hours after injury.

The second aim is to evaluate the clinical response at 24 hrs and 1 week after injury, correlating it with the results obtained from the G biopsy analysis.

3. HYPOTHESIS

HA improves the wound healing potential of gingival-derived cells 24 hours after injury by (1) increasing ECM remodeling, (2) modifying MVD, (3) potentiating collagen turnover and (4) increasing cell proliferation.

There is a difference in the clinical response of gingival tissue 24 hrs after injury between HA and NT group: HA treated group present higher clinical wound healing score value (clinical scarless repair) when it was compared with NT group, and this is correlated with the results obtained from the G biopsy analysis. The differences observed are not significant 1 week after injury.

4. MATERIALS AND METHODS

4.1. Ethics statements

The study protocol (ClinicalTrial.gov- NCT04865952) was approved by Sapienza University of Rome Ethics Committee (Ref.5315-Prot.0640/2020). Each participant signed an informed consent in accordance with the Declaration of Helsinki (1975, revised in 2013).

4.2. Study design and patient selection

A split mouth design study was conducted to evaluate the *in vivo* effect of exogenous HA on early wound healing of human gingival tissue.

The following inclusion criteria were applied: (1) patients between 18-50 years, (2) patients who undergone at least two periodontal surgery procedures, (3) patients who agreed to be "volunteer" for biopsy collection procedures by signing an informed consent. Patients who underwent antibiotic or anti-inflammatory drug consumption during the previous six months, patients reporting a history of poor wound healing, patients in pregnancy or lactation period and smoking patients were excluded from the study.

The subjects were enrolled at the clinical center of the Section of Periodontics, Sapienza University of Rome, Department of Oral and Maxillo-Facial Sciences.

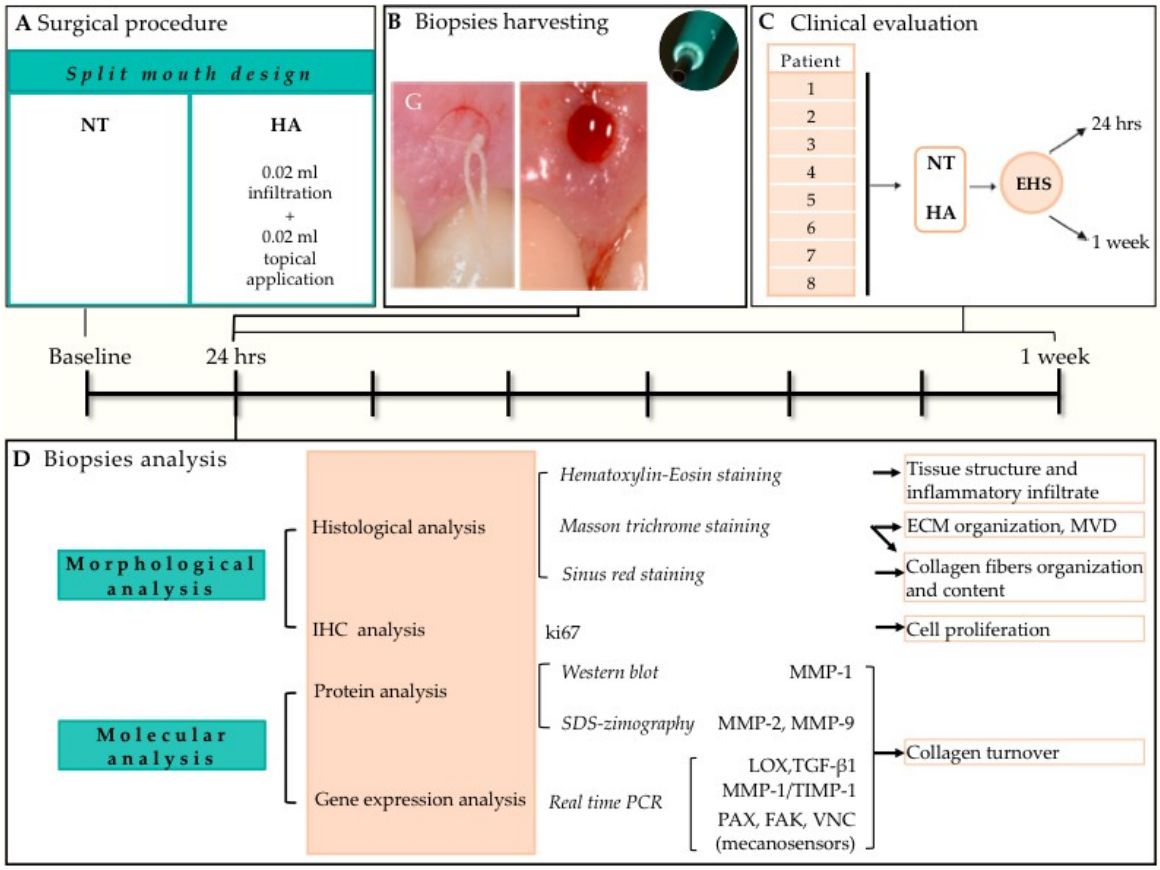
Each patient underwent two surgical procedures and was treated in split mouth design to either intra-surgical HA application (treatment group - HA) or non HA application (no treatment group - NT). Biopsies from buccal attached gingiva (G) were harvested 24 hours after surgical procedures, replicating the model used in our previous studies (Rojas et al., 2021; Pilloni et al., 2021).

The effect of exogenous HA on cell proliferation, collagen deposition and maturation, ECM organization, MVD, *MMPs* /*TIMPs* balance and *TGF-β1* expression was investigated. In addition, since ECM remodeling is influenced also by mechanical stimuli and fibroblasts are mechanoresponsive cells (Wang et al., 2012; Jansen et al., 2015; Burridge & Guilluy,

2016)., the expression of key mechanosensors paxillin (PAX), focal adhesion kinase (FAK) and vinculin (VNC) were also analysed to understand whether HA exerts any mechanical effect on gingival repair and collagen turnover pathways.

The experimental design is presented in Figure 1.

FIGURE 1 Experimental design



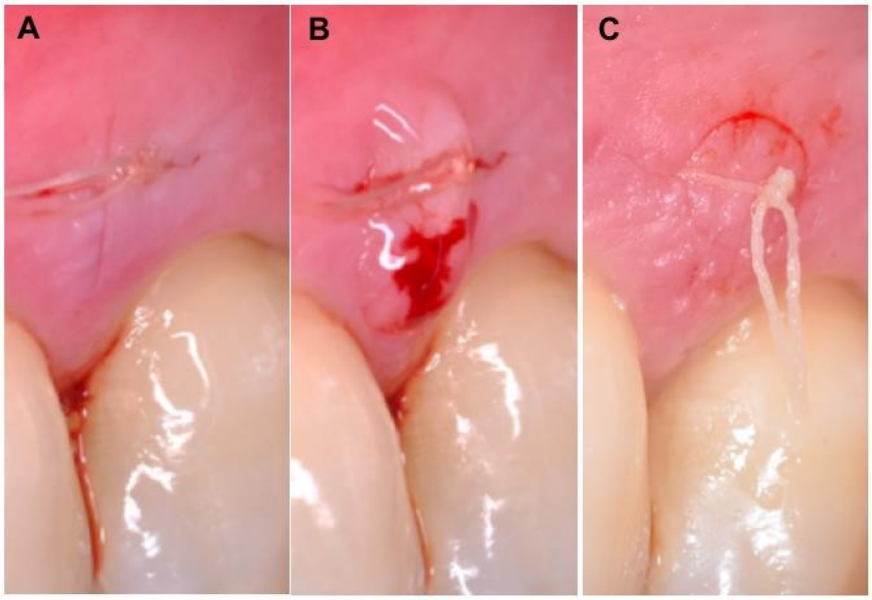
(A) Eight patients underwent two surgical procedures were treated in split mouth design to either intra-surgical HA application (treatment group – HA) with 0.02 ml HA infiltration + 0.02 ml HA topical application or non HA application (no treatment group - NT). **(B)** Buccal attached gingival (G) biopsies were harvested at 24 hrs after surgery from eight patients. **(C)** Clinical evaluation was performed at 24 hrs and 1 week after surgery by means of Early Healing Score (EHS) in NT and HA group. **(D)** Each gingival biopsy was divided in three parts: one for histological-IHC analysis, one for protein analysis and one for gene expression analysis in order to carry out a morphological and molecular analysis. For the first one, tissue structure and inflammatory infiltrate, extracellular matrix (ECM) organization and microvascular density (MVD), and collagen fibers organization/content were evaluated through Hematoxylin-Eosin, Sirius Red and Masson Trichrome staining, respectively, while cellular proliferation was evaluated by immunohistochemical detection of Ki67. For the molecular analysis, the collagen turnover was evaluated through MMP-1, MMP-2 and MMP-9 protein analysis by Western Blot and LOX, MMP-1, TIMP-1, TGF-β1 gene expression by real time

PCR. Gene expression of key proteins playing a role as mechanosensors (PAX, FAK, VNC) were also assessed.

4.3. Surgical procedures and biopsy collection

All the surgical procedures and biopsies were performed by the same operator (MR).
At the end of the surgical procedure, primary closure was obtained at the level of VRIs with interrupted sutures (polyglycolic acid-PGA, 6-0 monofilament, Figure 2A). Patients were randomized (by a coin toss) during the first surgical procedure to received or not HA application. 0.04 ml of cross-linked HA with a combination of three different molecular weights (MW) (2000, 1000 and 500 kDa) were applied in the treatment group as follows: 0.02 ml were infiltrated around the VRI with a gentle massage for 2 minutes, avoiding saliva contamination (Galli et al., 2008), and 0.02 ml were applied topically over the VRI (Figure 2B). No post-operative chlorhexidine mouthrinse was prescribed (Rojas et al., 2021).Twenty-four hours after the surgical procedure, gingival biopsies were harvested at the level of the VRIs with a biopsy punch of 2.0 mm diameter (Figure 2C).
The biopsy areas healed by second intention and sutures were removed at 1 week.

FIGURE 2 HA application and gingival biopsy collection



(A) VRIs suture immediately after surgical procedure. **(B)** HA topical application over VRIs. **(C)** 2 mm diameter punch biopsy collection 24 hrs after injury.

4.4. Clinical analysis

A blinded examiner evaluated the clinical wound healing response at the level of the VRIs in the G tissue 24 hrs after surgery (before harvesting the biopsies) using the Early Wound Healing score (EHS) (Marini et al., 2018; Marini et al., 2019). The same evaluation was performed 1 week after the surgical procedure, immediately before the sutures removal..

4.5. Morphological analysis

Each gingival biopsy was divided in three parts: one for histological analysis, one for gene expression analysis and one for protein analysis. For morphological analysis G biopsies were fixed in 10% neutral buffered formalin and processed for paraffin embedding. 4 μm thick sections were cut using a Leica microtome, routinely dewaxed, rehydrated and

processed for histological staining, histochemistry and immunohistochemistry. All the slides were acquired using scanner at high resolution (NanoZoomer S60, Hamamatsu, Japan) and the digital slides were managed and evaluated using NDP.view2 (Hamamatsu, Japan) image dedicated software. Sections were analysed to study morphological features, MVD, ECM organization, collagen content and cellular proliferation.
Qualitative histological analysis was performed by Hematoxylin–Eosin staining to analysed tissue structure and the eventual presence of inflammatory infiltrate.

4.5.1. Masson Trichrome staining

Masson Trichrome with Anylin staining was performed following manufacture protocol (Bio Optica, Milan), and the sections were used to analyze ECM organization and to calculate MVD. The staining highlights in black the nuclei, cytoplasm in red, erythrocytes in yellow and collagen fibers in blue. Microvessels in the connective tissue were evaluated by stereology-based method on the slides scanned with an Aperio Scan Scope System CS2 (Leica Biosystem, Milan, Italy). A customized digital counting grid was employed to evaluate the MVD of each tissue slide by means of histomorphometric analysis. More specifically, the intersection points that fell on the vessels were manually counted, and the ratio between test points and total points of the grid that fell on the overall connective tissue was calculated and expressed as a percentage value (Pellegrini et al., 2014; Canciani et al 2021):

4.5.2. Sirius Red staining

Sirius Red/ Picric Acid 0.1% staining (Sigma Aldrich, Italy) was performed to specifically stain and analyze interstitial collagen fibers organization and content (Canciani et al., 2021). To obtain specific stain for fibrillary collagen, slides were deparaffinized and immersed for 30 minutes in saturated aqueous picric acid containing 0.1% Sirius Red F3BA (Sigma, Milan, Italy). Newly deposited collagen was observed under polarized light (Nikon Eclipse 80i, Japan) in order to describe collagen maturation and evaluate fibers orientation

4.5.3. *Immunohistochemistry*

The expression of Ki67 in gingival lining epithelium was analysed by immunohistochemistry using an anti-Ki67 (clone B56) and a secondary antibody conjugated with HRP (Mach 4 Universal HRP-polymer, Biocare Medical, USA). The evaluation of Ki67 positive cells was quantified by Image J software. Stained cells were evaluated and results were expressed as a percentage relative to the considered total area of the epithelium.

4.6. Molecular analysis of collagen turnover pathways on gingival homogenates

4.6.1. *Gene expression analysis*

Gene expression analysis was performed on gingival homogenates. Total RNA was isolated using Tri-Reagent. One μg of total RNA was reverse-transcribed in 20 μL final volume of reaction mix (Biorad, Segrate, Milan, Italy). mRNA levels for lysyl oxidase (LOX), matrix metalloproteinase 1 (MMP-1), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), and TGF-β1 were assessed by real time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene, and gene expression levels were normalized on its expression (Francetti et al. 2019). Each sample was analysed in triplicate in a Bioer LineGene 9600 thermal cycler (Bioer, Hangzhou, China). The cycle threshold (Ct) was determined and gene expression levels relative to that of GAPDH were calculated using the ΔCT method.

4.6.2. *Western blot analysis*

Gingival fragments were homogenized in Tris-HCl 50 mM pH 7.6, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1% SDS, and centrifuged at 14,000 xg, for 10 min at 4°C to remove cell

debris. For analysis, samples (40 μg of total proteins) were diluted in SDS-sample buffer, loaded on 10% SDS-polyacrylamide gel, separated under reducing and denaturing conditions at 80 V, and electro-blotted transferred at 90 V for 90 min to a nitrocellulose membrane in 0.025 M Tris, 192 mM glycine, 20% methanol, pH 8.3. After electro-blotting, membranes were air dried and blocked for 1 h in 5% skimmed milk in TBST.

Membranes were blocked for 1 h in 5% skimmed milk in TBST and incubated with the mouse anti-MMP-1 (1:2000) (Millipore) o.n. at 4 °C. After washing, membranes were incubated with an anti-mouse HRP-conjugated secondary antibody (1:20000 in TBST). To confirm equal loading, membranes were re-probed by monoclonal antibody to actin (1:7500 in TBST). Immunoreactive bands were revealed by the Amplified Opti-4CN substrate (Bio Rad) and quantified by densitometric scanning (UVIBand, Eppendorf).

4.6.3. SDS-zymography

Gingival homogenates (10 μg of total protein per sample) were run under non-reducing conditions without heat denaturation on 10% polyacrylamide gel (SDS-PAGE) co-polymerized with 1 mg/mL of type I gelatin at 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each and incubated overnight in a substrate buffer at 37°C (Tris-HCl 50 mM, $CaCl_2$ 5 mM, NaN_3 0.02%, pH 7.5). MMPs gelatinolytic activity, detected after staining the gels with Coomassie brilliant blue R250 as clear bands on a blue background, was quantified by densitometric scanning (UVBand, Eppendorf).

4.7. Molecular analysis of mechanotransduction pathways

To understand whether HA exerts any mechanical effect on gingival repair and collagen turnover pathways, the mRNA levels of some proteins acting as mechanosensors were also analysed. For this purpose, mRNA levels for PAX, FAK and VNC were assesses by real time PCR and normalized on GAPDH gene expression.

4.8. Sample size

Sample size calculation was performed using $\alpha = 0.05$ and the power of sample $(1 - \beta) = 95\%$.

Considering the absence of previous studies providing data on the *in vivo* effect of HA on the early wound healing of gingival tissue, the sample size was calculated based on data from a study of Canullo et al., (2011), in which the average microvascular density (MVD %) was from 8% to 12% with a standard deviation of 3%. Therefore, the population of the study was calculated to be $n > 7.35$. (Canullo et al 2011; Canciani et al 2021).

Considering possible dropouts, the number of the patients was also increased by of 15%. On the basis of the data and these assumptions, 8 patients were required to be entered in this study.

4.9. Statistical analysis

Data was expressed as mean ± standard deviation (SD). HA and no-HA histology, viability, molecular and proteomic data were analysed by GraphPad Prism v 9.0 software using the non-parametric Wilcoxon test or paired samples with a level of significance of $p < .05$.

For continuous variables (EHS score), median and the inter-quartile range (IQR) were calculated, and the non-parametric Mann-Whitney U test was used for statistical analysis. *P* values < .05 were considered statistically significant.

5. RESULTS

5.1. Study population

Eight systemically healthy adult subjects were included in the present study. The study population consisted of 4 females and 4 males, aged 21 to 47 years with mean age of 35 years ± 9.4 (Table 1). All patients completed the study without drop-outs.

TABLE 1 Study population

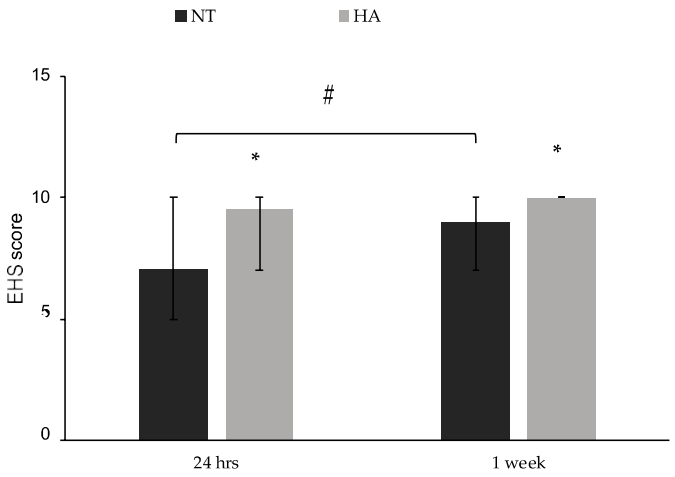
Patient	Demographic data	
	Age	**Sex**
1	47	M
2	41	M
3	21	F
4	28	F
5	26	F
6	35	F
7	39	M
8	45	M
Mean ± SD	35.3 ± 9.4	
Range	21-47	

F, female; M, male; SD, standard deviation

5.2. Clinical wound healing response

No relevant intra-operative or post-operative complications occurred in any of the patients. Twenty four hours post-surgery, the median EHS value for HA and NT group was 9.5, IQR: 1 and 7, IQR: 1; respectively. At 1 week, the values observed were 10, IQR: 0 for HA and 9, IQR: 2 for NT group. Significant differences between groups were founded at both period times evaluated ($p < .05$). When each group was analysed, EHS value in HA group showed no significant differences between 24 hrs and 1 week, while in NT group this difference was statistically significant ($p < .05$), (Figure 3, Table 2). Clinical photograph of patient 5 is presented in Figure 4.

FIGURE 3 Differential clinical wound healing response between NT and HA group at 24 hrs and 1 week after surgery



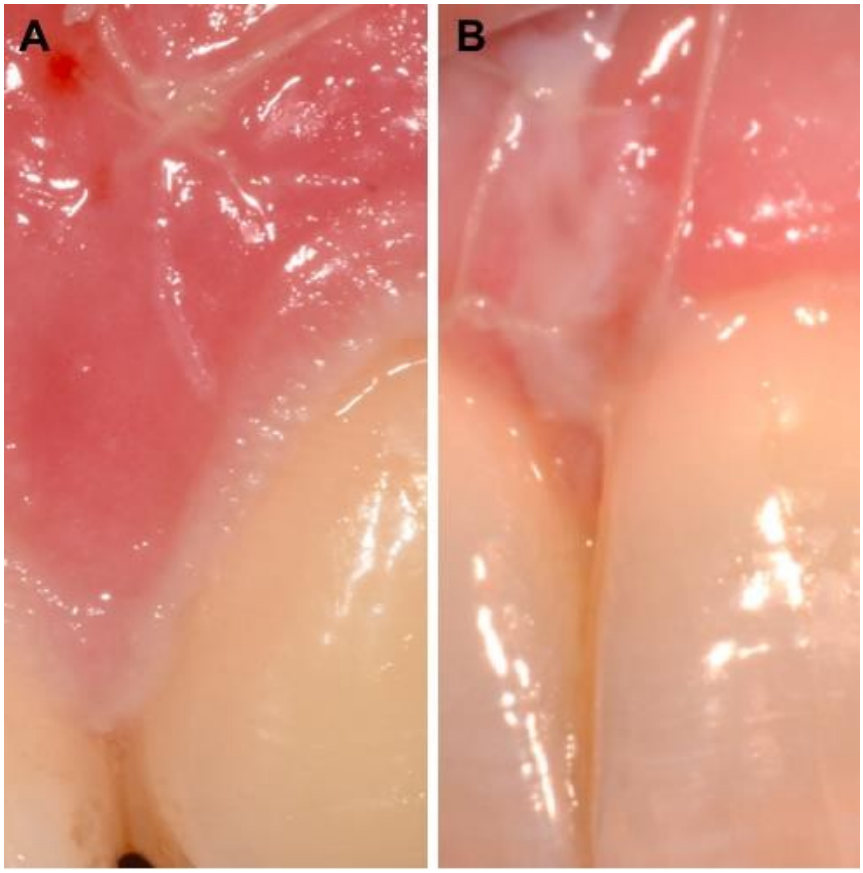
Clinical wound healing response was evaluated through assessment of Early Wound Healing Score (EHS) at 24 hrs and 1 week after surgery in NT and HA group. The median values of EHS were reported. Error bars represent interquartile range (IQR). *$p < .05$ NT *vs* HA; $^{\#}p < .05$ 24 hrs *vs* 1 week

TABLE 2 Clinical wound healing response 24 hrs and 1 week after injury in HA and NT group

	EHS			
	24 ore		1 week	
Patient	**NT**	**HA**	**NT**	**HA**
1	7 (R3, H2, I2)	10 (R6, H2, I2)	9 (R6, H2, I1)	10 (R6, H2, I2)
2	6 (R3, H2, I1)	10 (R6, H2, I2)	9 (R6, H2, I1)	10 (R6, H2, I2)
3	6 (R3, H1, I2)	7 (R3, H2, I2)	7 (R3, H2, I2)	9 (R6, H2, I1)
4	10 (R6, H2, I2)	10 (R6, H2, I2)	10 (R6, H2, I2)	10 (R6, H2, I2)
5	5 (R3, H1, I1)	10 (R6, H2, I2)	7 (R3, H2, I2)	10 (R6, H2, I2)
6	7 (R3, H2, I2)	9 (R6, H2, I1)	9 (R6, H2, I1)	10 (R6, H2, I2)
7	7 (R3, H2, I2)	9 (R6, H2, I1)	9 (R6, H2, I1)	10 (R6, H2, I2)
8	7 (R3, H2, I2)	9 (R6, H2, I1)	7 (R3, H2, I2)	10 (R6, H2, I2)
Median (IQR)	**7 (1)**	**9.5 (1)**	**9 (2)**	**10 (0)**

EHS, Early Wound Healing Score; HA, Hyaluronic acid; NT, no treatment; R, clinical signs of re-epithelialization; H, clinical signs of haemostasis; I, clinical signs of inflammation; IQR, interquartile range.

FIGURE 4 Clinical wound healing of buccal attached gingiva 24 hrs after surgery in NT and HA group

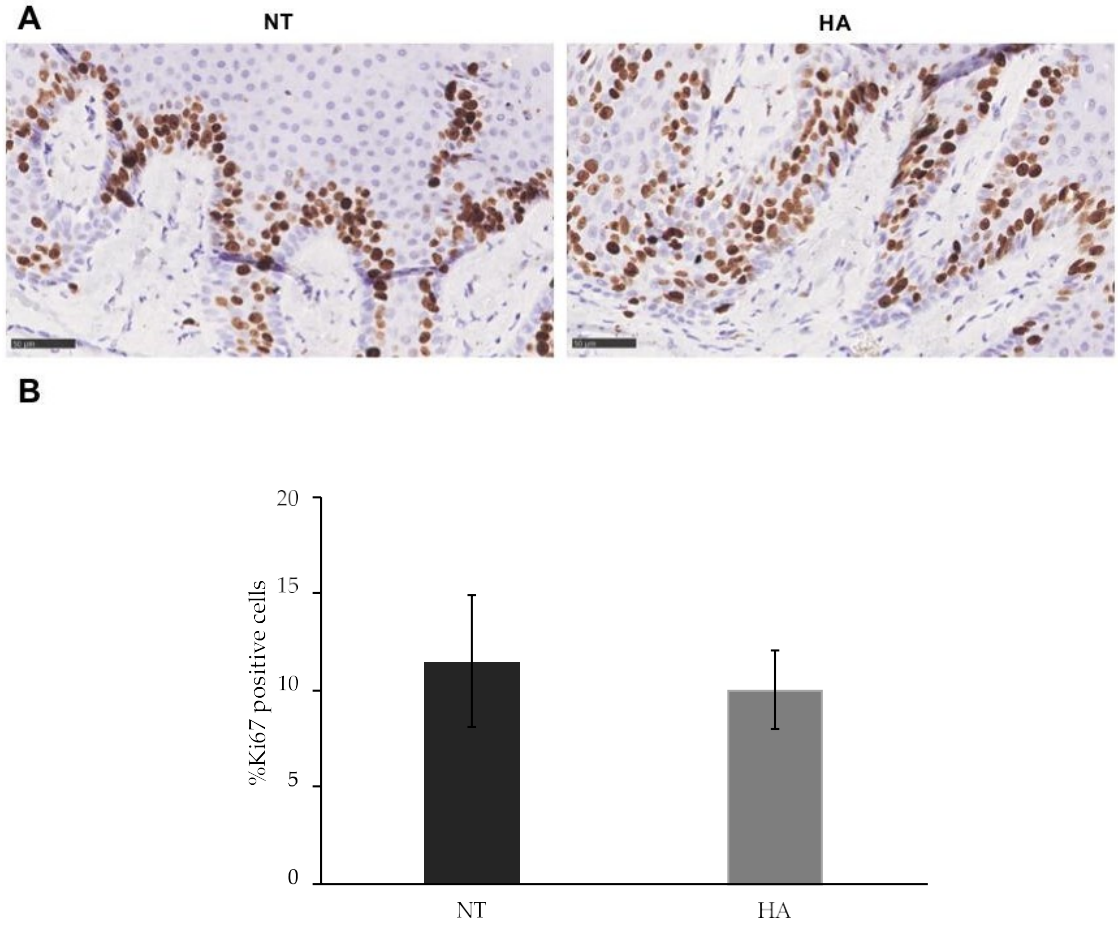


Clinical wound healing at the level of VRIs in Patient 5: **(A)** HA: EHS=10. **(B)** NT: EHS=5.

5.3. Histological analysis and cell proliferation

Histological analysis of Hematoxylin-Eosin stained sections revealed that gingival structure was not affected by HA treatment and that no signs of inflammatory infiltration was evident (data not shown). Immunohistochemistry analysis of Ki-67 expression in gingival epithelial cells showed that cell proliferation was similar in NT and HA-treated patients (Figure 5A). In fact, the quantification of Ki-67 expressed as a percentage relative to the total area suggested a similar proliferation rate in NT and HA groups (Figure 5B).

FIGURE 5 IHC analysis of Ki67 expression in gingival tissue 24 hrs after surgery in NT and HA group

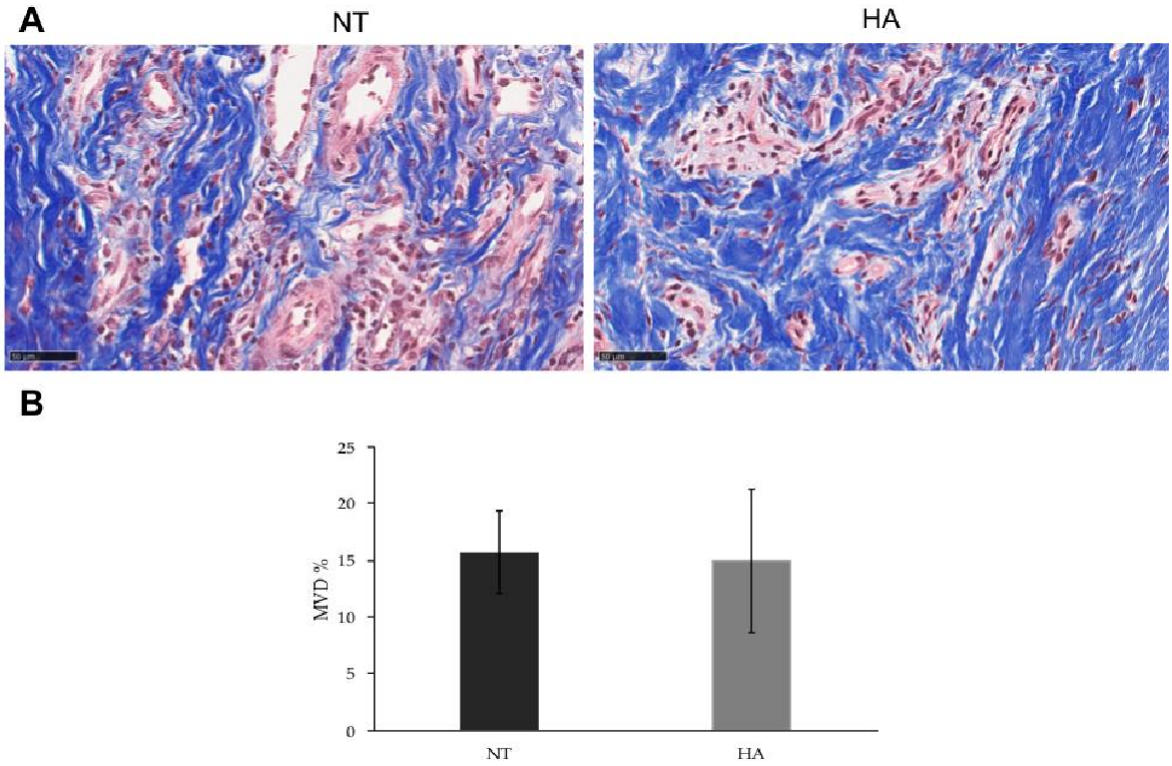


(A) Representative photomicrographs of sections of NT and HA gingival biopsies stained with anti-Ki67 antibodies. Scale bar 50 μm. **(B)** Mean percentage of Ki67 immunopositive cells. Error bars represent standard deviations.

5.4. Microvascular density

Sections stained with Masson Trichrome showed that no differences in microvascular distribution between two groups was present. MVD was similar in both groups (15.63% ± 3.64 in NT group compared with 14.94% ± 6.32 in the HA group (p >.05). (Figures 6 and 7)

FIGURE 6 Histomorphometrical analysis of MVD in NT and HA group at 24 hrs after surgery



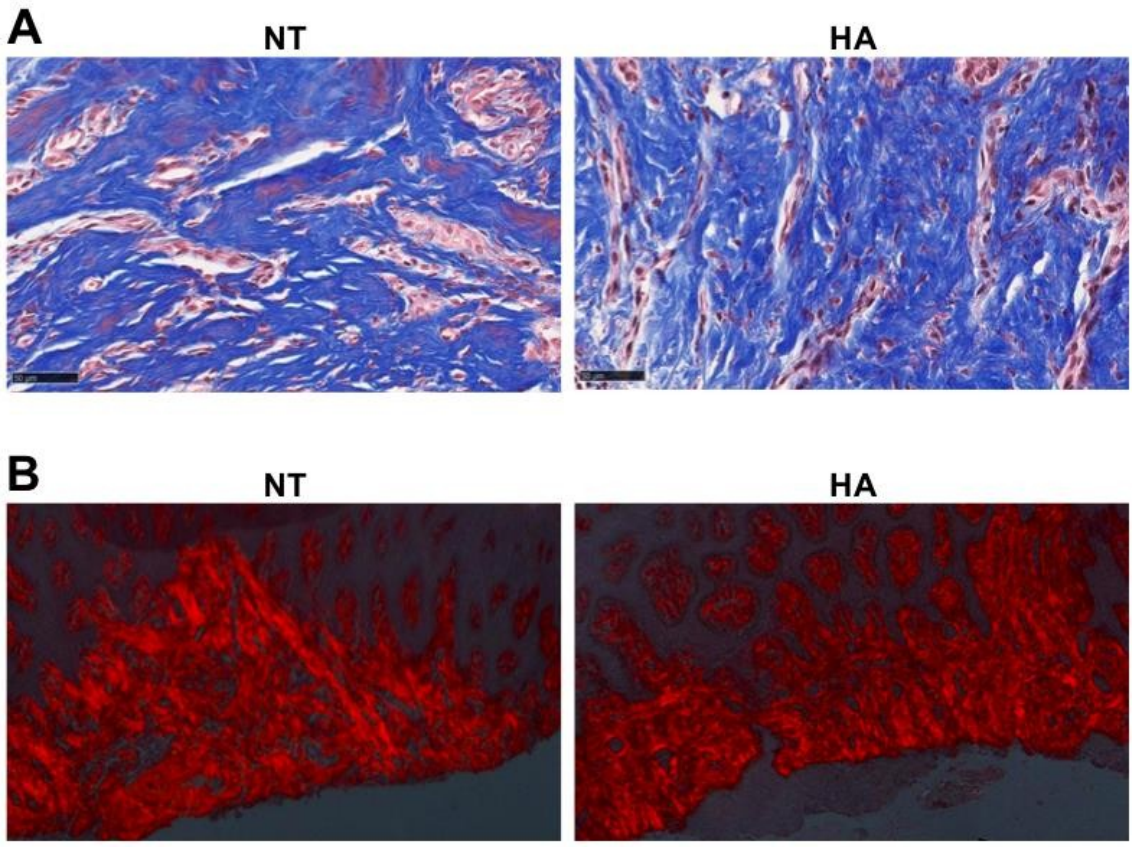
(A) Representative photomicrographs of sections of NT and HA gingival biopsies stained with Masson Trichrome. Scale bar 50 μm. **(B)** Mean percentage of MVD values (%) in NT and HA group. Error bars represent standard deviations.

5.5. Collagen content and turnover pathways

Collagen content was analysed by morphological and molecular methods. Light microscopy analysis of Masson Trichrome stained sections revealed that ECM organization, microvascular distribution and collagen content were similar in NT and HA-treated samples (Figure 7A). In both experimental conditions, collagen fibers were arranged in dense bundles extending in all directions, having the typical organization of the irregular dense connective tissue of the gingiva. This finding was confirmed by the analysis of Sirius Red stained sections (Figure 7B). Moreover, at polarized light observation in both NT and

HA-treated samples the staining was similarly dark orange-red, consistent with the presence of mature collagen fibers, while newly deposited collagen was not detected.

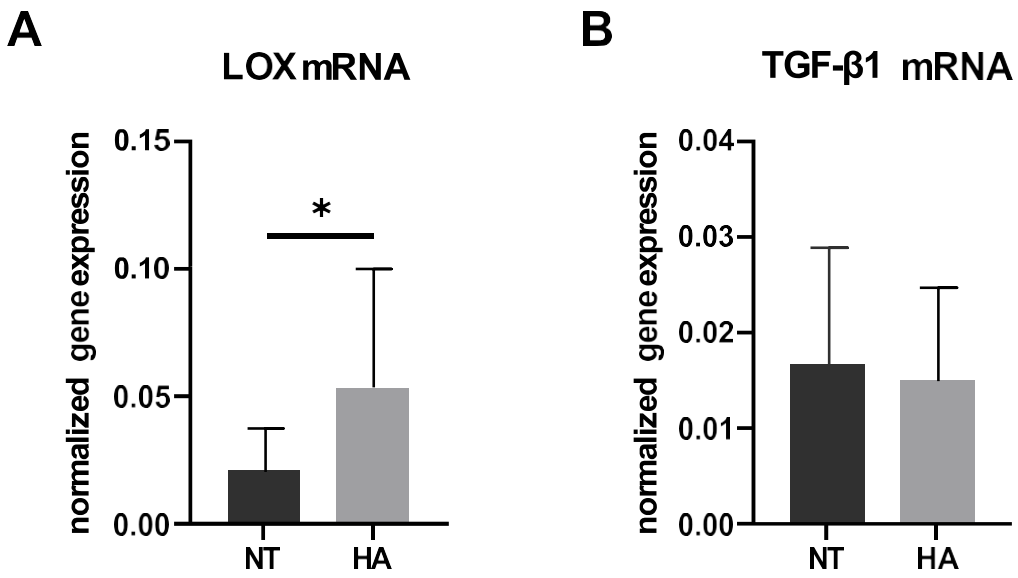
FIGURE 7 Collagen content, ECM organization and microvascular distribution in NT and HA group at 24 hrs after surgery



Representative photomicrographs of sections of NT and HA gingival biopsies: **(A)** Sections stained with Masson Trichrome showing dense bundles of collagen fibers in multiple directions for both groups, without evident differences in ECM components and microvascular distribution **(B)** Sirius Red stained gingiva observed at the polarized light microscope showing collagen fibers having a similar content in NT and HA treated gingiva. No new deposited collagen was evidenced. Scale bar 50 μm.

Gene expression analysis showed that mRNA levels of LOX, involved in collagen maturation, resulted significantly up-regulated in HA treated gingiva compared to NT ($p < .05$) (Figure 8A). Since collagen turnover pathways can be modulated by TGF-β1, mRNA levels for this factor were also investigated. TGF-β1gene expression was not influenced by HA and was similar in NT and HA treated samples (Figure 8B).

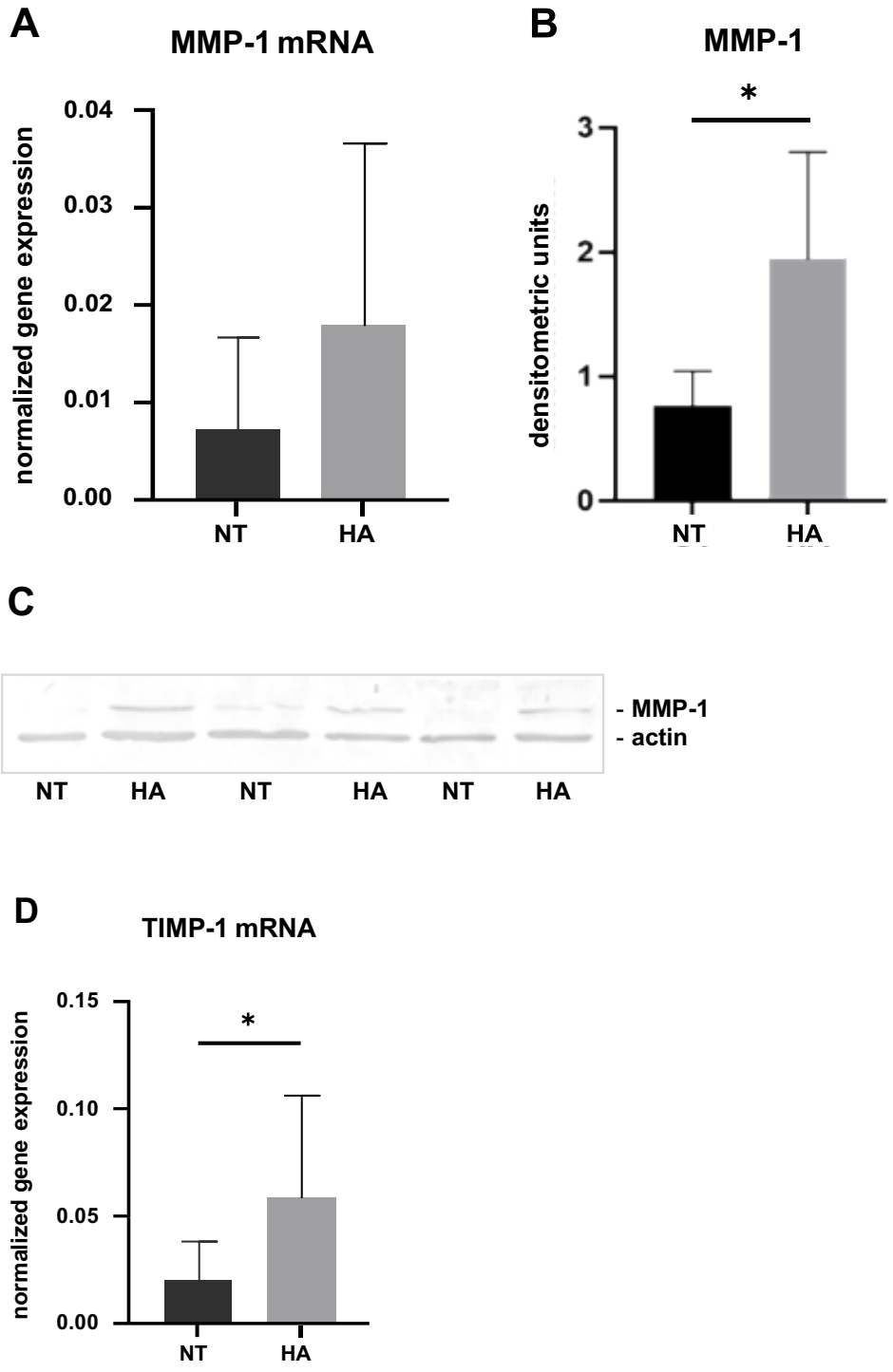
FIGURE 8 Real time-PCR analysis of mRNA expression levels of collagen turnover-related genes in gingival tissues 24 hrs after surgery in NT and HA group



Real time PCR analysis of mRNA expression levels of (A) LOX and (B) TGF-β1 in NT and HA- treated patients. Error bars represent standard deviations. *$p < .05$

Collagen degradation was assessed at the mRNA and protein level. MMP-1 gene expression was higher in HA compared to NT, even if the difference was not statistically significant (Figure 9A). Western blot analysis of MMP-1 revealed significant increase in HA compared to NT patients ($p < .05$) (Figure 9B, C). Also TIMP-1 gene expression was significantly induced by HA ($p < .05$) (Figure 9D).

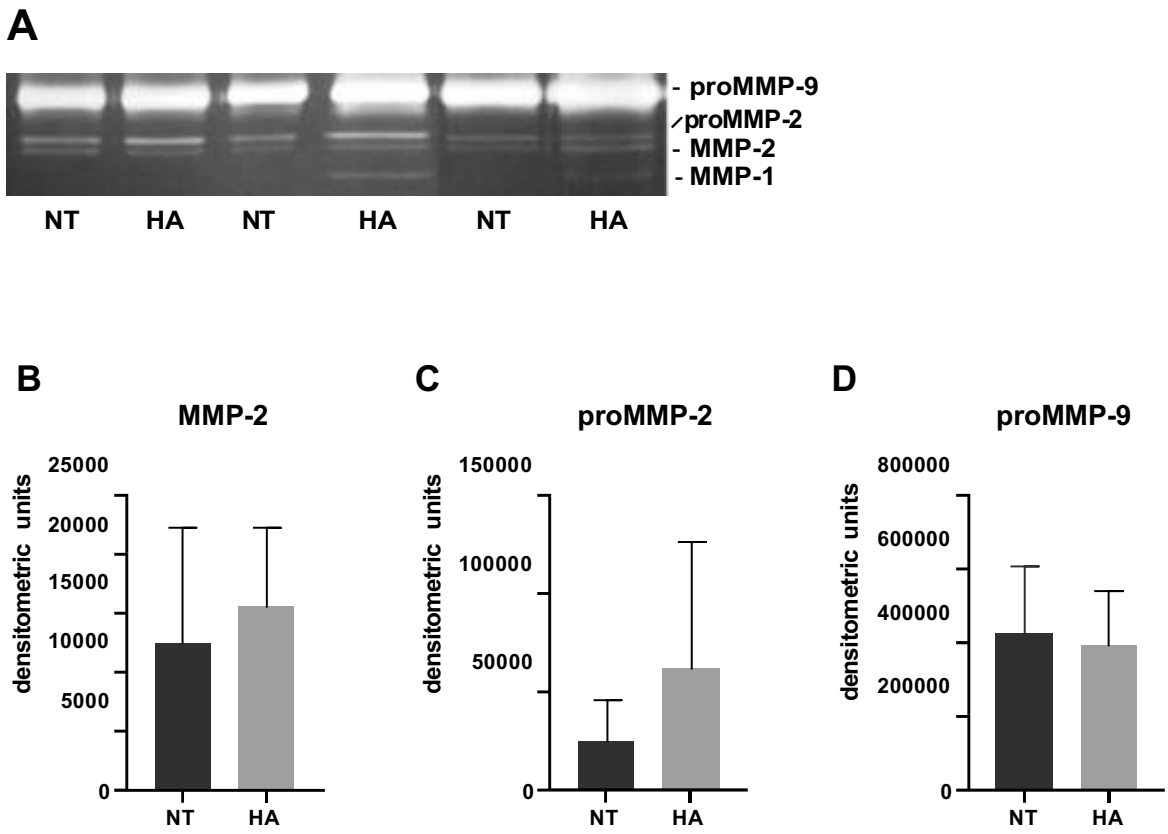
FIGURE 9 MMP-1 and TIMP-1 gene and protein expression analysis in gingival tissues 24 hrs after surgery in NT and HA group



(A) Real time PCR analysis of mRNA expression levels of MMP-1 in NT and HA-treated patients. Densitometric (B) and Western blot (C) analysis of MMP-1 protein levels expression in NT and HA group. (D) Real time PCR analysis of mRNA expression levels of TIMP-1 in NT and HA-treated patients. Error bars represent standard deviations. *$p < .05$

MMP-2 and MMP-9 expression and activity was assayed by SDS-zymography, showing that they were not affected by HA treatment (Figure 10A-D).

FIGURE 10 MMP-2 and MMP-9 protein expression analysis in gingival tissues 24 hrs after surgery in NT and HA group

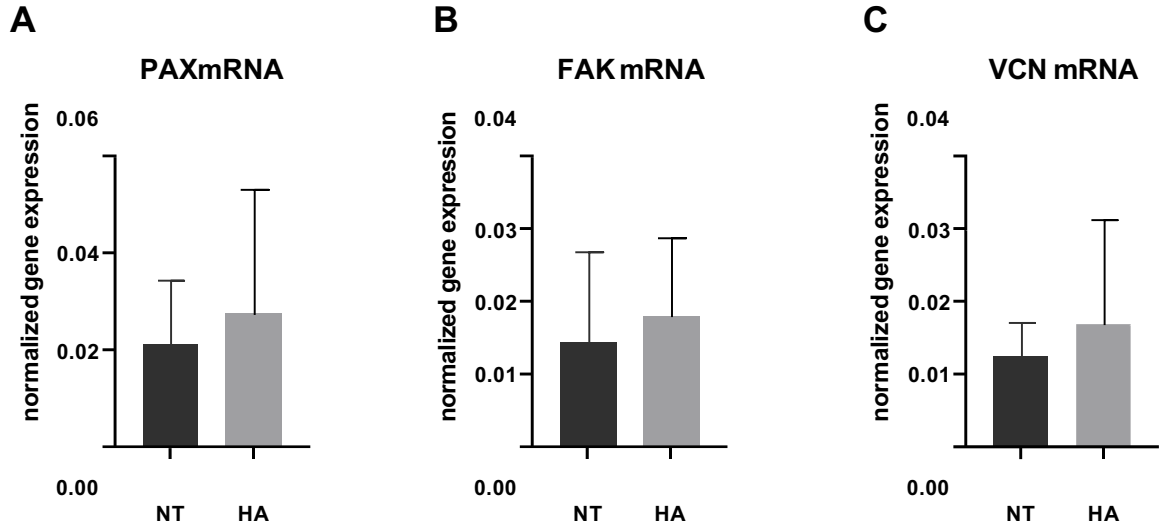


(A) SDS-zymogram analysis of proMMP-9, proMMP-2, MMP-2 and MMP-11 in NT and HA-treated patients. The intensity of the bars was evaluated by densitometric analysis **(B-D).** Error bars represent standard deviations

5.6. Expression of FAK, PAX and VNC as mechanosensors

The expression of FAK, PAX and VCN was assessed at the mRNA levels. mRNA levels codifying for these proteins acting as mechanosensors resulted unaffected by HA treatment (Figure 11A-C)

FIGURE 11 Real time-PCR analysis of mRNA expression levels of mechanosensory proteins in gingival tissues 24 hrs after surgery in NT and HA group



Real time PCR analysis of mRNA expression levels of mechanosensors PAX **(A),** FAK **(B)** and VCN **(C)** in NT and HA-treated patients. Error bars represent standard deviations.

6. DISCUSSION

Gingival tissue-derived cells play an important role in oral wound healing (Smith et al., 2019). The use of biologically active agents that influence their behaviour, and therefore, promote oral soft tissue wound healing, is of significant interest. In the present study, we were interested in investigating whether exogenous HA use was able to affect the mechanisms responsible for the homeostasis of gingival connective tissue to favor gingival repair, since although several *in vitro* and animal studies have reported beneficial effects (Scully et al., 1995; West et al., 1985; Pilloni & Bernard, 1998; Pilloni et al., 2003; Fujioka-Kobayashi et al., 2017), *in vivo* human data is unclear. Therefore, the aim of this project was to evaluate the *in vivo* effect of intra-surgical HA application in the early phases of gingival tissue repair in terms of in terms of (1) ECM organization, (2) MVD, (3) collagen deposition and content, (4) collagen turnover and (5) cell proliferation by means of a histological, immunohistochemical and biomolecular analysis of human G biopsies obtained 24 hours after injury, correlating it with a clinical evaluation performed at 24 hrs and 1 week post-surgical.

In the present study, we used the EHS score (Marini et al., 2018; Marini et al., 2019) to assess the clinical healing response. We found significantly higher median EHS values in HA-treated group when compared to NT in both evaluated periods. However, at 1 week, the difference observed between groups are lower and, although it was statistically significant ($p < .05$) may not be clinically relevant, which confirms the importance of investigating the HA effect on the wound healing response in the early phases of tissue repair process. Interestingly, G tissue clinical response in the HA-treated group at 24 hrs was very similar to NT G tissue at 1 week, and this could us to hypothesize that HA might accelerate the clinical wound healing response. These results are consistent with clinical studies reporting HA's ability to promote faster healing after topical application (Romeo et al., 2014; Casale et al., 2016).

Noteworthy, is the fact that if we compare EHS values obtained in the present work with those obtained in our previous study (Rojas et al., 2021) two interesting correlation can be

observed: (1) although inter-individual variation in the early wound repair response has been observed (Rojas et al., 2021), the median EHS values obtained at 24 hrs and 1 week after injury in the NT group in the present study are very similar to those obtained in G tissue in our previous study in both periods; (2) the values obtained in HA-treated G tissue in both time periods are very similar to those observed in the palatal tissue in the previous work, which present the highest clinical healing score when compared with G and M tissues, supporting the hypothesis that HA improves and promote faster clinical wound healing.

Morphological analysis of Hematoxylin-Eosin, Sirius Red and Masson Trichrome stained sections showed that tissue structure, inflammatory infiltrate, MVD, ECM and collagen fibers organization, and interstitial collagen content were not affected by HA 24 hrs after injury. Furthermore, no differences between groups were founded when epithelial gingival cells proliferation was evaluated by immunohistochemistry analysis of Ki-67. However, a recent *in vitro* study (Asparuhova et al., 2019) reported an increase in the proliferative and migratory abilities of human oral gingival and palatal derived-fibroblasts.

The stimulatory or inhibitory effect of HA on cell proliferation *in vitro* is known to be closely related to its MW and concentration (Zhao et al., 2016), the cellular context and the method of delivery HA to the cell culture (Asparuhova et al., 2019). Low MW HA was reported to increase cell proliferation (Pilloni et al., 1998; Huang et al., 2003; Gu et al., 2010; Zhao et al., 2015) , while the effect of high MW on cell proliferation are controversial (Zhao et al., 2016). Nevertheless, generally, high MW HA is considered to promote cell quiescence because HA break down product signals injury (David-Raoudi et al., 2008; Jiang et al., 2011; Prosdocimi & Bevilacqua, 2012; Litwiniuk et al., 2016). Regarding this, it is important to highlight the fact that HA used in the present study is composed by three different MW; and that, based on the literature, little is known about *in vivo* HA MW distribution in wound repair settings (Monslow et al., 2015).

The differences between the above-mentioned study (Asparuhova et al., 2019) and our results could be due to several reasons, such as the different method used to evaluate cell proliferation, the specific characteristics of HA used, and mainly, the discrepancies between *in vitro* and *in vivo* analysis. In fact, it has been reported that *in-vitro* wound healing assays

using monocultures of human keratinocytes or fibroblasts (Kramer et al., 2013; Justus et al., 2014) , although allow to investigate cell migration and proliferation changes, are simplified models that involve fewer cellular interdependencies which, on one hand, might enable the dissection of the various mechanisms in wound healing but, on the other hand, makes them less comparable to the *in-vivo* situation (Ueck et al., 2017).

A recent *in vivo* study, (Nyman et al., 2019) has analysed biopsies obtained from human dermal incisional wounds. The histologic results showed that, 24 hrs after injury, low MW HA (500-730 kDa) stimulates keratinocytes migration and proliferation . Nevertheless, the authors reported that immunohistochemical analysis is required to deepen and confirm their results.

From the immunohistochemical data obtained in our study, we can hypothesize that, hyaluronidase activity during the post-surgical period could be a possible reason for the opposite effect observed on cell proliferation in our results, but further *in vivo* research is needed to identify if HA activates signaling pathways that promote gingival cells proliferation in the first 24 hrs after injury.

Regarding MVD, no differences were observed between NT and HA-treated group in the present study. However, the role of HA in enhance angiogenesis has been reported (Pilloni & Bernard, 1998; Prosdocimi & Bevilacqua, 2012) and; a recent study (Canciani et al., 2021) has reported an increase in MVD in human oral wounds treated with HA containing amino acids in biopsies collected after 10 post-surgical days. Although the method used was IHC analysis with CD31 marker and, in the present study, we used Masson´s Trichromestaining, the literature has not reported differences in effectiveness to quantify MVD in oralmucosa when both methods were compared. (Caceres et al., 2017). Nevertheless, the different composition in the HA used and in the biopsy collection time (10 days vs 24 hrs) might be the reasons for the differences observed with the results presented here.

It should be noted that our results could be beneficial since although it is well known that new blood vessels growth is a key element in wound repair (Boerckel et al.,2011; Sottile, 2014), this is mainly relevant for the proliferative healing phase (DiPietro, 2016) in which an increase in MVD could be of interest to improve wound healing, while in the very early period, this might not be strictly necessary (Polimeni et al., 2006). In fact, wounds that heal

faster and with less scar formation exhibit reduced inflammation and capillary growth and a more rapidly maturing capillary network (DiPietro, 2016). Therefore, both decreased inflammation an decreased angiogenesis are features of optimal healing and reduced scar formation. This idea is supported by studies demonstrating that hypertoric scar formation is linked to increased microvascular content (Amadeu et al., 2003; van der Veer et al., 2011). Agree with this, in our previous study (Rojas et al., 2021), we observed that CXCL1, a pro-inflammatory chemokine that stimulates epithelial cell migration and promotes angiogenesis (Simone & Higgins, 2015), significantly increased in M (scar wound repair) while is down-regulated in G (scarless wound repair).

The mechanism by which excessive angiogenesis influences fibrosis and fibroblasts is currently unknown (DiPietro et al., 2016), although one of proposed mechanisms is the following: pericytes have been proposed to transition to myofibroblasts (Dulmovits & Herman et al., 2012; Greenhalgh et al., 2015) and, in a situation of abundant angiogenesis, increased pericyte recruitment to wounds may provide a large population that can adopt a myofibroblast phenotype (DiPietro et al., 2016). We have demonstrated in our previous studies how the increase in myofibroblasts differentiation 24 hrs after injury is related with a scar wound repair response (Vescarelli et al., 2017; Rojas et al., 2021; Pilloni et al., 2021)

Noteworthy, whereas morphological analysis regarding ECM organization and collagen organization/content in the present study showed no differences between NT and HA groups, significant differences have been found when collagen turnover was evaluated from a molecular point of view. This confirms the need to deepen our research through gene and protein expression analysis to better understand the effects of HA on early wound healing of oral gingival-derived cells behaviour.

Collagen fibril stabilization is influenced by maturation of newly synthesized collagen leading to the formation of cross-links needed to provide tensile strength (Shoulders et al., 2009). The lysyl oxidase (LOX) is a secreted copper-containing amine oxidase playing a key role in the formation of covalent cross-linking of collagen and elastin in ECM (Lucero & Kagan, 2006). The essential role of LOX in the early period of the healing response, i.e., inflammation phase, has been described (Cai et al., 2017). In fact, some approaches to

accelerating healing of injured tissues through up-regulating LOX expression in the acute phase after injury has been reported (Olaso et al., 2011; Pathi et al., 2012)

The significant up-regulation of LOX induced by HA in the gingiva is consistent with the hypothesis that collagen maturation enhanced by LOX is an early response after injury to favor gingival repair. This suggestion is supported by the results related to collagen degradation. Collagen breakdown is driven by MMP-1, which cleaves the intact collagen triple helix, allowing further degradation by other proteases such as MMP-2 and MMP-9 (Sakai et al., 1967; Woessner et al., 1991). TIMP-1 is the main inhibitor of MMP-1, binding MMP-1 in a 1:1 stoichiometric ratio (Murphy et al. 1994; Brew et al. 2001). The interaction among the MMPs and TIMPs, plays a key role in maintaining the balance between ECM synthesis and degradation (Giannandrea & Parks, 2014; Robert et al., 2016).

While MMP-1 gene expression was not significantly affected by HA, our results show that MMP-1 protein levels were strongly induced in HA-treated gingiva, suggesting an increased collagen degradation. This results agree with those report in a previous *in vitro* study, in which an increase in MMP-1 was observed in oral gingival and palatal derived-fibroblasts after HA treatment (Asparuhova et al., 2019).

However, also TIMP-1 mRNA levels were significantly induced by HA, leading to the hypothesis that HA increased the overall ECM remodeling to favor tissue repair, but the increase of MMP-1 is paralleled by TIMP-1 up-regulation. As a consequence, the collagen turnover is stimulated, but collagen content is not modified. MMP-2 and 9, having a gelatinolytic activity, are not primarily involved in interstitial collagen breakdown (Amar et al., 2017) and they remain unchanged in NT and HA-treated samples. On contrary, the study of Asparuhova et al., (2019) showed an indirectly increase in MMP-2 expression, that reflects the HA-induced expression of proinflammatory citokines. These contradictions might be related to the specific HA formulations as well as the cellular context and tissue/cells analysis methodology used (*in vitro vs in vivo*).

Excessive amount of MMPs acting for a long time on healing tissue are deleterious for the wound healing process (Gill et al., 2008). The present findings suggest that HA-induced interstitial collagen degradation is balanced by the concomitant TIMP-1 up-regulation.

In our previous study we demonstrated that, 24 hrs after injury, the expression of TIMP-1 in G increases in five of the six examined patients, accompanied by a significant down-

regulation of Col1a1, which implies that another pathway counteracts the increase of TIMP-1 avoiding excessive collagen deposition. This is consistent with clinical scarless healing response observed in G. In contrast, in M we observed an up-regulation of TIMP-1 but accompanied by an increase in Col1a1, consistent with a major collagen accumulation and a fibrotic response (clinical scar tissue repair) (Rojas et al., 2021).

It seems that HA maximizes the "normal" response of G at 24 hrs after injury, i.e., TIMP-1 up-modulation without collagen deposition increment, since although in the present study we have not evaluated the expression of Col1a1, we have not observed differences in collagen content, but rather an increase in the collagen turnover stimulation with a significant up-modulation in MMP-1 protein expression. However, remains controversial the fact that, in the above-mentioned study (Rojas et al., 2021) we showed a significant reduction of TIMP-1 expression in palatal tissue (P) biopsies 24 hrs after injury, and this tissue showed the better clinical wound healing response (represented by the highest EHS value). Anyway, both un-treated P tissue (Rojas et al., 2021) and HA-treated G tissue in the present study showed scarless clinical wound healing response. Further research is needed to clarify the role of TIMP-1 and the networks related to its modulation in the early phases of oral wound healing.

TGF-β1 is a pleiotropic cytokine having a central role in the regulation of collagen turnover as well as in fibrogenesis (Schrementi et al., 2008). Increased ECM protein expression in response to TGF-β1, especially interstitial collagen, is accompanied by attenuation of collagen degradation**,** through increased expression of TIMPs and an inhibition of MMPs expression (Barrientos et al., 2008); thus facilitating ECM protein accumulation (Schiller et al., 2004; Kim et al., 2018). TGF-β1 also modulates LOX, increasing its expression and activity, therefore contributing to the collagen stabilization in the ECM (Xie et al., 2012; Cai et al., 2017). Our results show that TGF-β1 gene expression is unchanged in HA-treated G compared to NT after 24 hrs. This is in agreement with the results observed in a previous *in vitro* study (Asparuhova et al., 2019).

Since TGF-β1 is generally known as pro-fibrotic (Kim et al., 2018), and has previously been shown to induce autophagy activation and myofibroblasts differentiation (scar tissue repair) in oral gingival fibroblasts (Vescarelli et al., 2017), the unchanged TGF-β1 gene

expression together with the results indicated above regarding TIMPs/MMPs balance and LOX expression would appear to indicate that HA favors the expression of genes characteristic of improved wound healing. In addition, it can be assumed that the increase in LOX expression could be due other activation pathway, TGF-β1-independent.

The correct regulation of cell function *in vivo* requires the integration of numerous biological and mechanical signals arising from the surrounding cells and the ECM. The elucidation of the molecular mechanisms by which the cell perceives and transforms the mechanics of the ECM has become the subject of intense investigation and a number of intracellular molecules has been identified that can react to mechanical stimulation and - in turn – modify cell function (Martino et al., 2018). Cells perceive mechanical stimuli through diverse mechanosensitive molecules at the cell membrane activating different mechanotransduction pathways (Martinca, 2014; Luis Alonso & Goldman, 2016).

To understand if HA could elicit a mechanical effect influencing collagen turnover pathways in the early phases of gingival connective tissue repair, we analysed the gene expression of key proteins playing a role as mechanosensors such as FAK, PAX and VNC. In fact, fibroblasts in connective tissues, including gingival fibroblasts, respond to mechanical forces that can influence gene expression, cell morphology and cell fate, thus adapting their activity in ECM by remodeling (Wang et al., 2012; Jansen et al., 2015; Burridge & Guilluy, 2016). Mechanotransduction, the ability of cells to translate mechanical stimuli into biochemical signals, can be studied investigating the expression of mechanosensors (Randelli et al., 2020).

A recent *in vitro* study (Canciani et al., 2021) reported that the presence of HA used to charge polycaprolactone nanofibers downregulate PAX and VNC mRNA levels expression in human gingival fibroblasts. To explain this effect, the authors hypothesized that HA interaction with its CD44 receptor could likely modulate fibroblast adhesion to the substrate, thus influencing fibroblast behaviour (Price et al., 2005)

Our results showed that mRNA levels for FAK, PAX and VNC are not influenced by HA treatment, suggesting that the events triggered by HA in the early wound healing are not dependent on mechanotransduction mechanisms.

Nonetheless, few studies addressed the modalities of activation of the mechanosensitive genes so far. An important task for future research will be to elaborate integrated strategies aimed at unraveling the interactions between different mechanobiology pathways, which at the moment appear to be intertwined in a complex network (Hansen et al., 2015)

7. CONCLUSION

The present research was designed to evaluate the *in vivo* effect of intra-surgical HA application in the gingival tissue wound repair 24 hours after injury. The results of this investigation showed significant changes in the expression of LOX, TIMP-1 and MMP-1 in HA-treated gingival biopsies when compared to the NT group. These findings further support that features such as the induction of collagen maturation, increased ECM remodeling and collagen turnover could be relevant for an HA-accelerate wound healing response, as we have observed in the clinical results in the present study. Since we did not detect a modification of gene expression for mechanosensors proteins, we can hypothesize that HA does not trigger an evident mechanical response in gingival fibroblasts at the considered time point, and that the effect on LOX and MMP-1 and TIMP-1 is not primarily based on a mechanical response in our experimental setting. Further studies should focus on extending the follow-up period, in order to understand the role of exogenous HA in the different phases of the oral wound healing process, allowing the development of new therapies for accelerated wound healing.

8. CLINICAL RELEVANCE

Scientific rationale for the study: Several *in vitro,* animal and clinical studies have described the role of exogenous HA in improving the wound healing process. However, *invitro* assays represent a limitation due to hyaluronidase activity during the post-surgical period. Furthermore, although the potential beneficial effects of HA in surgical periodontaltherapy have been widely reported in terms of clinical response, the specific *in vivo* cellular mechanisms involved during the early phases after surgical wounding from a biomolecular point of view remains unclear.

Principal findings: : Twenty-four hours after injury, HA was able to (1) increase LOX gene expression, involved in collagen maturation (2) increase ECM remodeling and collagen turnover and (3) improve clinical wound healing response.

Practical implications: Intra-surgical HA application enhance wound healing properties in human *in vivo* gingival wounds 24 hrs after injury. HA might be an important component in future regimens aiming to accelerate and improve the wound healing after periodontal surgery.

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V. General conclusions and future perspectives

1. PALATAL TISSUE-DERIVED FIBROBLASTS MAY BE AN ATTRACTIVE ALTERNATIVE TO IMPROVE WOUND HEALING THERAPIES

Improving wound healing resolution is an important medical priority due to the increase in the incidence of chronic and scarring wounds. Effective wound healing depends on the action and interaction of thousands of genes that are involved in several biological processes.
Increase our understanding of the mechanism involved in wound repair is needed to improve wound care.

Although several studies characterize in detail the mechanisms and pathways altered deficient wounds, a different approach that defines factors involved in accelerated wound healing would allow the identification of novel therapeutic targets to improve tissue repair. Research has started to identify gene expression profiles associated with wound healing through *in vitro* and *in vivo* studies, generating insight into the wound healing process, to identify potential prognostic indicators and also to aid clinical decision making.

Oral and fetal wound healing have long been considered models of optimal wound resolution characterized by their rapid and scarless response after injury. The deepening of the knowledge of the different molecular events driving wound healing resolution in oral mucosa compared to those of the skin has allowed to define why oral lesions heal more efficiently, providing a basis from developing strategies to treat deficient healing processes. Nonetheless, although the molecular networks that drive the different phases of cutaneous repair have been characterized, the unique environment of the oral cavity represents a different wound healing paradigm that remains poorly understood, mainly if we

considered that many of the studies comparing fibroblasts within the oral mucosa have not clearly defined the anatomic origin, making comparisons between different population challenging. However, it is well know that variations also exists inside the oral environment and that the peculiar repair in oral mucosal tissues involve intrinsic characteristics.

To close this gap, in the first part of this work, we have deepened previous investigations, characterizing the molecular aspects of wound healing in the oral soft tissues, aiming al elucidating the shared and unique features of fibroblasts derived from different anatomical sites, and correlating it with the different clinical response observed. Human oral fibroblasts are cells with desirable phenotype characteristics that can adopt a variety of alternative destinations in response to various extrinsic factors. These conditions make these cells an interesting resource for tissue regeneration trough different advanced therapies. In fact, human gingival fibroblasts have been used in the periodontal tissue regeneration field due to their proliferative, migratory, and anti-fibrotic capabilities. Our investigation has identified, for the first time, positional differences in the gene expression profile of human alveolar mucosa (M), buccal attached gingiva (G) and palatal (P) tissue-derived fibroblasts in the early phases of wound healing.

Our results suggest that palatal derived-fibroblasts may be more attractive for cell therapies since we have observed the fastest and scarless clinical healing response in this tissue, in agreement with the biomolecular data, related mainly with fibrotic markers and early wound healing-related genes expression. Although also buccal attached gingiva showed characteristics related with a scarless wound repair pattern, biomolecular differences were observed when compared with palatal tissue. Further more detailed comparative analysis of the expression of some of these genes can help to determinate the healing potential of the cells under investigation. However, genes and networks characterized in this study may lead to novel approaches with improved clinical and patient outcomes after periodontal and peri-implant surgery, and could provide useful information to enhance wound healing in systemically compromised patients. Moreover, it could be considered for therapeutic application also to non-oral mucosal sites.

Since fibrosis and scarring involve an imbalance of various mediators throughout wound healing, efficacious therapies should include a multidimensional approach targeting all three phases of wound healing, starting by moderating the inflammatory response, limiting the excessive myofibroblasts proliferation, and applying physical and biological interventions during wound remodeling.

Therefore, although the purpose of the first part of our investigation was to specifically assess gene expression in wound healing outcomes 24 hrs after injury, future analysis at different time periods through microarray analysis, monitoring the changes in the gene expression of palatal and buccal attached gingiva tissue-derived fibroblasts over time, would add a more interesting dimension to this evaluation.

2. IMPROVING WOUND HEALING

2.1 Post-surgical CHX mouthrinses could induce fibrotic transformation, leading to scar tissue repair. Should new post-surgical protocols be defined?

Improving wound healing also means not disturbing the tissue repair process during the post-surgical period, allowing the cells to carry out their functions.
Since the presence of biofilm can alter the normal course of the wound healing, antimicrobial strength needs to be considered when choosing an antiseptic agent, mainly during the post-surgical period when mechanical plaque control cannot be performed.
CHX mouthrinses, the gold standard for antimicrobial oral treatment, appear to induce *in vivo* a fibrotic response in the gingival tissue-derived fibroblasts during the early post-surgical phase, confirming its detrimental effect reported in the literature through *in vitro* studies. In the second part of our research, assessing some of the most relevant genes differentially expressed 24 hrs after surgery in the gingival tissue (involved in collagen

turnover and fibrotic response) we observed relevant differences when comparing with patients without antiseptic use. Furthermore, the increased expression of pro-apoptotic proteins and the reduction in cell proliferation reinforce the need to reconsider the use of CHX , at least, according to our results, during the first post-surgical day.

Due to the pilot nature of our research, future investigations that (1) include more patients, (2) evaluate the expression of differentially expressed genes over time through a microarray analysis, and (3) compare different antiseptics evaluating *in vivo* the effects on cellular behaviour and gene expression and also the antimicrobial strength; could allow to define new post-surgical protocols with the aim of controlling the biofilm deposits accumulation without cytotoxic effects that could jeopardize the normal course of the wound healing process.

2.2 SERPINE1 and TIMP1: key role genes in regulating scar formation in the initial phases of oral soft tissue repair?

SERPINE1 and TIMP1 present a relevant role in collagen turnover regulation. Noteworthy, in the first part of our research, through a protein-protein interaction (PPI) network, we have assessing the number of interactions between the 52 differentially expressed genes (DEGs) involved in the wound healing process and we detected that SERPINE1 and TIMP1 are included into the 15 hub genes of the network, which confirms their preponderant role in the early phases of oral soft tissues wound healing.

In this regard, three key points should be considered:

- in the first part of our research, different modulation of both genes was observed between M, G and P tissues at baseline and 24 hrs after injury: M and P tissues showed opposite response;
- in the second part of our research, G tissue after CHX treatment showed the same modulation pattern as M tissue (scar healing response) and an opposite response to that observed in palatal tissue (scarless and faster wound healing response);
- without any external stimulus, we have observed that TIMP1 and SERPINE1 expression in G tissue shows high inter-individual variability, however when we

performed qRT-PCR validation no changes were observed in the modulation of both genes in G tissue.

Considering all the aforementioned, we could hypothesize that, together, SERPINE1 and TIMP1 play a role in regulating scar formation in oral tissues. Both genes have a bidirectional regulatory function: their elevated expression is related with hypertrophic/keloid scar, while their reduced expressions is related with hard-to-heal chronic wounds. Therefore, we believe that in both pathological scenarios, SERPINE1 and TIMP1 expression modulation by genetic ablation/small-molecule antagonism or recombinant amplification, respectively, may constitute attractive therapeutic options.

2.3 Intra-surgical HA application improves the intrinsic biological activity of gingival-derived cells

Improving wound healing through the use of bioactive substances that can influence cells behaviour and thus, supporting tissue repair/regeneration, is of major clinical interest.

In the third part of our research, we confirm the *in vivo* ability of HA to modify gingival cells behaviour -mainly with regard to collagen turnover-, favouring the wound healing process.

Two key points should be highlighted:

- HA appears to "accelerate" the clinical wound healing response (since the clinical response observed at 24 hrs in HA-treated group is the same observed at 1 week in NT group);
- HA appears to significantly improve the clinical wound healing response (since HA-treated G tissue at 24 hrs showed the same response that those observed in P tissue in the first part of our research).

The beneficial effect of HA in the wound healing process can be of great interest mainly in patients with chronic and metabolic conditions such as diabetes and rheumatoid arthritis or unhealthy lifestyles including smoking in which the tissue repair process is altered.

Since many aspects are still unclear when considering the effects of exogenous HA on the oral cells behaviour, further studies should be conducted, focusing on the followingaspects: (1) the influence of molecular weight, cross linking and concentration of HA in itsbiological properties, (2) the influence of hyaluronidase activity during the post-surgical period on the *in vivo* effect of HA on cells behaviour (3) the effect of HA over time, extendingthe evaluation performed in our study.

Appendix A - RAW DATA

Patient 1

Alveolar mucosa

HF		M	
Position	**Target Name**	**Baseline**	**24 hrs**
A01	ACATA2	21,93458176	21,93538094
A02	ACATA1	21,92478943	30,91374588
A03	ANGPT1	27,93291664	23,90822029
A04	CCL2	23,9142189	21,92393303
A05	CCL7	35,91851807	28,95096588
A06	CD40LG	36,88492203	35,90611267
A07	CDH1	31,89546776	31,94105339
A08	COL14A1	22,91856194	24,90009117
A09	COL1A1	17,9148674	18,91396713
A10	COL1A2	17,89935684	18,94195366
A11	COL3A1	19,97759056	19,92106056
A12	COL4A1	25,90114784	27,96140862
B01	COL4A3	32,88463974	30,89776611
B02	COL5A1	20,94905853	21,92139244
B03	COL5A2	20,89492416	20,912323
B04	COL5A3	25,92174911	26,93278694
B05	CSF2	34,9176178	34,94384384
B06	CSF3	34,00242233	28,90148926
B07	CTGF	19,89773941	22,9049015
B08	CTNNB1	25,92728806	25,94013786
B09	CTSG	Undetermined	36,95663071
B10	CTSK	25,89164352	22,88860321
B11	CTSV	33,89364243	30,89569283
B12	CXCL1	26,87217522	20,87460518
C01	CXCL11	Undetermined	35,88347244
C02	CXCL2	27,90229416	21,88972473
C03	CXCL5	25,88495255	24,89275169
C04	EGF	30,9129734	32,92324448
C05	EGFR	25,93562317	24,92514992
C06	F13A1	23,94723511	35,9078331
C07	F3	24,90855789	24,88995552
C08	FGA	36,90140533	36,90159225
C09	FGF10	36,96826172	31,89395523
C10	FGF2	23,92428398	23,90612984
C11	FGF7	23,98463058	22,98630142

C12	HBEGF	28,94585228	30,90090942
D01	HGF	31,93183327	29,9237709
D02	IFNG	36,92662811	35,93400574
D03	IGF1	30,92604065	30,93526077
D04	IL10	36,92107391	35,91860962
D05	IL1B	33,90421677	30,92435646
D06	IL2	Undetermined	37,52600479
D07	IL4	35,89492035	36,95019531
D08	IL6	24,91268158	22,91353226
D09	IL6ST	22,96606636	21,9382267
D10	ITGA1	25,89550209	25,93384361
D11	ITGA2	28,92159653	29,95035172
D12	ITGA3	25,94002151	26,96870422
E01	ITGA4	26,90447044	25,88314629
E02	ITGA5	24,9294796	24,93691444
E03	ITGA6	23,89509392	26,92902184
E04	ITGAV	22,911726	23,93181801
E05	ITGB1	20,92497444	19,89263535
E06	ITGB3	29,94243813	27,9262886
E07	ITGB5	23,9237709	24,89330482
E08	ITGB6	28,9058075	31,9091835
E09	MAPK1	24,89870834	25,90388298
E10	MAPK3	25,90776443	26,93911362
E11	MIF	22,93650627	23,97852325
E12	MMP1	25,95797157	21,89970016
F01	MMP2	22,9335537	20,90686417
F02	MMP7	Undetermined	34,90851974
F03	MMP9	36,94489288	30,90497017
F04	PDGFA	26,93399239	26,90078926
F05	PLAT	27,89970589	29,91584778
F06	PLAU	25,91235924	26,90825844
F07	PLAUR	26,91161728	26,90008163
F08	PLG	36,91669846	36,96820831
F09	PTEN	23,86972618	23,86573219
F10	PTGS2	28,94641304	26,98165703
F11	RAC1	22,89494896	23,91700363
F12	RHOA	21,92910576	21,89931297
G01	SERPINE1	20,89607811	22,92124367
G02	STAT3	24,96531105	23,93702316
G03	TAGLN	20,90621758	20,91493988
G04	TGFA	35,9219017	33,95739746
G05	TGFB1	24,93076897	25,94327354
G06	TGFBR3	27,95805168	25,91424561
G07	TIMP1	19,94966888	21,91949654
G08	TNF	Undetermined	36,90450287

G09	VEGFA	23,9280262	24,97010422
G10	VTN	27,91898155	30,96967697
G11	WISP1	25,88465118	26,89128876
G12	WINT5A	24,95814514	21,909132
H01	ACTB	17,88727379	17,9618454
H02	B2N	20,9158268	19,92825127
H03	GAPDH	20,94685173	19,91737366
H04	HPRT1	27,93484306	27,94871521
H05	RPLP0	19,90167809	19,90255737
H06	HGDC	36,92622375	37,10421753
H07	RTC	37,05003738	38,55473709
H08	RTC	38,6074791	37,02571106
H09	RTC	37,02940369	36,92848587
H10	PPC	21,75414848	21,89583015
H11	PPC	21,93799591	21,92573357
H12	PPC	21,96952629	21,7848587

HF, human fibroblasts; M, alveolar mucosa

Buccal attached gingiva

HF		G	
Position	**Target Name**	**Baseline**	**24 hrs**
A01	ACATA2	20,93652916	20,90259361
A02	ACATA1	32,90945816	32,88872528
A03	ANGPT1	23,87592697	24,9293766
A04	CCL2	22,92066956	26,92617607
A05	CCL7	30,8962841	36,91833115
A06	CD40LG	36,8865242	35,91070557
A07	CDH1	31,92245865	33,90678406
A08	COL14A1	23,87574768	26,88511848
A09	COL1A1	17,91173744	17,91591263
A10	COL1A2	17,90583992	17,92283249
A11	COL3A1	20,96621323	21,95796585
A12	COL4A1	26,91026688	30,9896946
B01	COL4A3	33,9184227	32,90355682
B02	COL5A1	21,92508507	22,9738884
B03	COL5A2	21,94729996	21,90730667
B04	COL5A3	26,92871666	29,93550491
B05	CSF2	34,91801834	34,94133759
B06	CSF3	32,95618057	34,97388458
B07	CTGF	22,92292404	21,91692734
B08	CTNNB1	25,93620491	25,92098236
B09	CTSG	36,90402222	35,90952301
B10	CTSK	23,9322834	21,89066315
B11	CTSV	30,93411636	33,88393784
B12	CXCL1	23,88208008	28,87046051
C01	CXCL11	35,89054871	38
C02	CXCL2	24,89156342	29,91155434
C03	CXCL5	26,99306297	25,88929367
C04	EGF	30,89280891	32,88830185
C05	EGFR	25,92764282	24,92941284
C06	F13A1	35,9132576	36,92005539
C07	F3	25,88086891	26,87983894
C08	FGA	36,98454666	35,89720917
C09	FGF10	31,90540504	32,89377594
C10	FGF2	23,91384697	23,92283058
C11	FGF7	21,93251991	22,9525795
C12	HBEGF	29,92495155	31,92613602
D01	HGF	30,88966942	29,89334106
D02	IFNG	35,89801025	35,89259338
D03	IGF1	30,91502571	32,94857407
D04	IL10	36,97932053	35,93080139

D05	IL1B	31,92074966	34,89722824
D06	IL2	35,90176392	34,95031357
D07	IL4	34,89749527	35,88893127
D08	IL6	23,93107796	24,89702034
D09	IL6ST	21,91460228	21,95503235
D10	ITGA1	25,93410683	24,89948273
D11	ITGA2	29,93717575	27,89298058
D12	ITGA3	24,89513588	26,930336
E01	ITGA4	26,88614273	25,88673782
E02	ITGA5	24,94055367	24,92135429
E03	ITGA6	26,96740913	25,92486382
E04	ITGAV	23,90943909	22,90707207
E05	ITGB1	20,94561768	20,95598221
E06	ITGB3	26,90266609	26,91388893
E07	ITGB5	23,89662552	23,95094872
E08	ITGB6	30,9119091	34,88687897
E09	MAPK1	24,88220024	24,90155792
E10	MAPK3	26,96685982	25,9269886
E11	MIF	22,92992592	23,96901321
E12	MMP1	23,95811272	22,8886795
F01	MMP2	21,91282082	20,91484261
F02	MMP7	35,9103508	35,89333725
F03	MMP9	34,94186401	36,96538162
F04	PDGFA	27,94091225	27,90533829
F05	PLAT	30,92340469	26,909132
F06	PLAU	26,93581581	25,91131592
F07	PLAUR	26,93300056	25,89790535
F08	PLG	35,91311646	36,90220261
F09	PTEN	23,90065765	23,89459229
F10	PTGS2	26,95107269	29,95953178
F11	RAC1	22,88769722	22,89208221
F12	RHOA	21,89187622	21,92043114
G01	SERPINE1	22,90029526	22,90750122
G02	STAT3	23,92046356	23,92353821
G03	TAGLN	20,95394135	20,92010498
G04	TGFA	32,9051857	35,9008522
G05	TGFB1	24,90042877	24,90835381
G06	TGFBR3	26,92552948	25,91742134
G07	TIMP1	21,91351891	21,96137238
G08	TNF	34,9092865	36,92213821
G09	VEGFA	22,91921997	23,9095993
G10	VTN	30,92849541	30,93248177
G11	WISP1	25,89095116	25,89133263
G12	WINT5A	21,89864731	20,92703438
H01	ACTB	17,95103836	17,95328712

H02	B2N	19,90151405	19,8982029
H03	GAPDH	19,90044594	20,94920158
H04	HPRT1	27,93550301	27,91750336
H05	RPLP0	19,90172005	20,94614029
H06	HGDC	35,95201874	35,91279984
H07	RTC	34,08726883	37,12679291
H08	RTC	37,07036972	36,39855957
H09	RTC	36,52507019	37,0255394
H10	PPC	21,86345673	21,82496452
H11	PPC	21,95708084	21,84715843
H12	PPC	21,91463089	21,93588638

HF, human fibroblasts; G, buccal attached gingiva

Palate

HF		P	
Position	**Target Name**	**Baseline**	**24 hrs**
A01	ACATA2	21,96449089	21,90349579
A02	ACATA1	32,88414383	32,90270233
A03	ANGPT1	24,92406654	25,89554405
A04	CCL2	20,91691208	21,93036461
A05	CCL7	28,93632889	27,94102097
A06	CD40LG	35,89658737	33,88817215
A07	CDH1	26,88908958	29,89569855
A08	COL14A1	23,90341568	21,85407639
A09	COL1A1	18,94373512	18,91751289
A10	COL1A2	17,90332413	18,94961548
A11	COL3A1	19,90652084	19,93590164
A12	COL4A1	25,90526009	27,98428726
B01	COL4A3	32,89501953	30,88728714
B02	COL5A1	22,98332024	22,9646225
B03	COL5A2	20,88070297	21,90302849
B04	COL5A3	25,95792961	26,92301559
B05	CSF2	30,94272423	33,92719269
B06	CSF3	29,8944397	29,96730804
B07	CTGF	21,90942001	22,91532516
B08	CTNNB1	25,94953156	26,94618607
B09	CTSG	36,90242386	34,90225983
B10	CTSK	23,92703819	23,917696
B11	CTSV	26,89801407	29,93388557
B12	CXCL1	18,86655235	19,88035583
C01	CXCL11	33,88739014	33,87698364
C02	CXCL2	20,92593575	21,93689156
C03	CXCL5	24,8893013	25,90320015
C04	EGF	31,90859604	32,88580704
C05	EGFR	24,92490387	25,94514084
C06	F13A1	35,91670609	35,92395401
C07	F3	25,87312889	28,8890152
C08	FGA	37,00977325	34,90583038
C09	FGF10	29,90517426	28,92221642
C10	FGF2	22,90597153	23,90623856
C11	FGF7	21,93925667	21,94185448
C12	HBEGF	28,93636894	30,91066551
D01	HGF	28,90336609	27,88521767
D02	IFNG	35,88494873	34,92590332
D03	IGF1	33,92723846	29,95349312

D04	IL10	35,9264679	34,91717911
D05	IL1B	27,89283562	32,90280151
D06	IL2	34,90333557	33,91684341
D07	IL4	35,89230728	33,88990784
D08	IL6	21,91621208	22,90720749
D09	IL6ST	21,95645523	21,93840981
D10	ITGA1	24,91566849	25,89225197
D11	ITGA2	27,91022301	29,92136383
D12	ITGA3	24,90404892	27,95823479
E01	ITGA4	26,89334869	28,91680336
E02	ITGA5	23,91965866	24,91919708
E03	ITGA6	24,93723869	26,92817497
E04	ITGAV	23,92237091	24,96487236
E05	ITGB1	20,94799232	21,940979
E06	ITGB3	24,90077591	27,92462349
E07	ITGB5	23,89866447	23,89084625
E08	ITGB6	27,93132973	30,91761971
E09	MAPK1	24,89225197	25,89099121
E10	MAPK3	25,92830276	25,91752434
E11	MIF	22,97000313	23,95256996
E12	MMP1	20,94736862	21,89011574
F01	MMP2	21,92152405	21,91874695
F02	MMP7	34,94894409	33,92960739
F03	MMP9	26,92445755	30,90996742
F04	PDGFA	27,92063522	28,95597076
F05	PLAT	28,91829872	29,90032196
F06	PLAU	24,92292595	26,90182686
F07	PLAUR	25,89844704	27,94415665
F08	PLG	36,91730499	33,9068222
F09	PTEN	23,88048363	23,87292671
F10	PTGS2	23,9634304	24,92383957
F11	RAC1	22,89254189	23,89989471
F12	RHOA	20,88011551	22,92589569
G01	SERPINE1	22,89294815	24,89377785
G02	STAT3	24,95267868	23,92195129
G03	TAGLN	21,9417572	22,96865273
G04	TGFA	29,95546722	32,9331398
G05	TGFB1	24,92793465	25,92121696
G06	TGFBR3	25,90708351	24,90107536
G07	TIMP1	21,9171505	21,97332954
G08	TNF	31,95532608	34,97512054
G09	VEGFA	23,91688538	23,91104507
G10	VTN	30,94301605	28,92818069
G11	WISP1	24,88426208	27,91494942
G12	WINT5A	21,90221596	23,92947769

H01	ACTB	16,91708946	18,9522934
H02	B2N	20,92377472	20,89862251
H03	GAPDH	19,92861366	20,93972778
H04	HPRT1	26,93075752	27,91587448
H05	RPLP0	19,90743828	20,93592453
H06	HGDC	35,88556671	34,9186058
H07	RTC	37,94775009	Undetermined
H08	RTC	37,12779236	37,6966629
H09	RTC	34,83483124	37,11392593
H10	PPC	21,57902145	21,85396957
H11	PPC	21,79006386	21,8884964
H12	PPC	21,93244553	21,93902779

HF, human fibroblasts; P, palate

Patient 4

Alveolar mucosa

HF		M	
Position	**Target Name**	**Baseline**	**24 hrs**
A01	ACATA2	23,909338	23,95762444
A02	ACATA1	24,90550423	30,91942787
A03	ANGPT1	28,88411331	27,90041351
A04	CCL2	25,92098427	26,93617439
A05	CCL7	34,90390396	34,93795013
A06	CD40LG	35,90966034	35,88269806
A07	CDH1	35,89684296	33,88846588
A08	COL14A1	25,89543152	24,87217712
A09	COL1A1	18,93510628	17,91604614
A10	COL1A2	17,89296532	17,92961693
A11	COL3A1	19,94514084	19,92591095
A12	COL4A1	27,96151543	27,94678497
B01	COL4A3	32,91636658	32,91075516
B02	COL5A1	21,96813774	21,96781158
B03	COL5A2	21,90672112	21,92988396
B04	COL5A3	26,92278671	27,93045235
B05	CSF2	35,90910721	35,9524498
B06	CSF3	32,96339798	32,94922638
B07	CTGF	20,91851997	20,94796181
B08	CTNNB1	25,90702248	25,92746162
B09	CTSG	36,98009491	34,90354538
B10	CTSK	23,89402962	23,92765808
B11	CTSV	31,93068504	31,8964138
B12	CXCL1	25,8588295	29,88689995
C01	CXCL11	37,14419556	33,87398148
C02	CXCL2	26,89320564	30,90974617
C03	CXCL5	25,88239098	25,89149857
C04	EGF	32,9179306	32,88612747
C05	EGFR	24,93560982	24,93115425
C06	F13A1	27,93126297	33,89894485
C07	F3	23,88478661	23,88048172
C08	FGA	36,90273285	34,91439819
C09	FGF10	35,94301224	31,92363739
C10	FGF2	22,92984772	22,93967438
C11	FGF7	21,96181107	22,95309448
C12	HBEGF	29,93682861	30,90371704
D01	HGF	29,88847542	30,88274956

D02	IFNG	36,88013458	33,90906525
D03	IGF1	29,90599251	31,90977097
D04	IL10	36,96644211	35,94337463
D05	IL1B	31,91706276	32,90016174
D06	IL2	36,90022659	32,88050079
D07	IL4	35,92622757	33,89898682
D08	IL6	28,93300629	26,90846634
D09	IL6ST	22,92210197	21,91187859
D10	ITGA1	26,93688393	26,93640327
D11	ITGA2	26,9277668	27,93946075
D12	ITGA3	26,92337418	26,8979435
E01	ITGA4	25,89946747	26,89972496
E02	ITGA5	24,923069	24,9161644
E03	ITGA6	24,92357063	25,88604736
E04	ITGAV	23,94785881	23,96738243
E05	ITGB1	20,95131302	20,9346714
E06	ITGB3	29,89982033	30,89871788
E07	ITGB5	23,9039135	24,94169807
E08	ITGB6	33,90293884	34,91584778
E09	MAPK1	24,89174271	24,90429878
E10	MAPK3	25,91369247	25,91192818
E11	MIF	22,95268822	22,92474365
E12	MMP1	20,8959198	20,94921112
F01	MMP2	21,91279221	21,91509819
F02	MMP7	37,15855408	34,95503616
F03	MMP9	33,91571808	34,95105362
F04	PDGFA	27,89701462	27,91049576
F05	PLAT	27,89364815	27,88880539
F06	PLAU	26,90971375	26,94004822
F07	PLAUR	25,92387199	25,8956604
F08	PLG	36,92464828	34,93948746
F09	PTEN	23,87200546	23,87665176
F10	PTGS2	27,94417	28,9495163
F11	RAC1	21,89141655	22,89636993
F12	RHOA	20,8927269	21,93400383
G01	SERPINE1	19,89749146	21,92045975
G02	STAT3	24,92908096	24,94779015
G03	TAGLN	21,90683365	20,90373993
G04	TGFA	35,9359436	33,89686203
G05	TGFB1	24,91797447	24,90383148
G06	TGFBR3	26,94569397	25,91353416
G07	TIMP1	18,91074753	19,93905067
G08	TNF	35,90475845	36,00785446
G09	VEGFA	24,94274139	24,95066071
G10	VTN	29,96334267	30,92812347

G11	WISP1	26,91731644	27,92213249
G12	WINT5A	23,91992188	21,88801003
H01	ACTB	17,91669083	17,93354225
H02	B2N	20,90399742	20,90124512
H03	GAPDH	20,92469406	20,92103577
H04	HPRT1	26,92453384	27,96180725
H05	RPLP0	20,93519974	19,90838623
H06	HGDC	35,90907669	32,91872406
H07	RTC	37,16161346	34,78556061
H08	RTC	Undetermined	37,08101273
H09	RTC	Undetermined	36,9955864
H10	PPC	21,94297981	21,89727592
H11	PPC	21,84191513	21,94371033
H12	PPC	21,89285851	21,93910027

HF, human fibroblasts; M, alveolar mucosa

Buccal attached gingiva

HF		G	
Position	**Target Name**	**Baseline**	**24 hrs**
A01	ACATA2	22,91837502	21,91858101
A02	ACATA1	32,88911057	32,92121506
A03	ANGPT1	24,9434166	24,90671921
A04	CCL2	22,91332626	25,94011879
A05	CCL7	29,89262199	35,91139603
A06	CD40LG	35,88129807	36,89713287
A07	CDH1	32,89486313	34,8925705
A08	COL14A1	24,85599327	26,86123657
A09	COL1A1	17,91687012	17,91980553
A10	COL1A2	17,91426277	17,93588638
A11	COL3A1	19,88781166	20,9650135
A12	COL4A1	28,99574471	29,9638176
B01	COL4A3	32,90951538	33,91120529
B02	COL5A1	22,97660065	21,93174744
B03	COL5A2	21,95757484	20,89057159
B04	COL5A3	28,93442154	29,92782974
B05	CSF2	35,93156815	35,94775391
B06	CSF3	32,97669601	32,94229126
B07	CTGF	22,92054749	22,9054718
B08	CTNNB1	25,95896721	25,92301178
B09	CTSG	35,90122223	Undetermined
B10	CTSK	24,9314518	23,89450645
B11	CTSV	31,90397644	33,93876648
B12	CXCL1	24,90280151	29,88910675
C01	CXCL11	35,87189865	36,87284088
C02	CXCL2	24,88913727	29,89594841
C03	CXCL5	25,90081024	26,94529343
C04	EGF	32,94537735	32,90024948
C05	EGFR	25,94244576	25,93791962
C06	F13A1	Undetermined	36,91361618
C07	F3	25,88518906	24,89517212
C08	FGA	35,89955139	Undetermined
C09	FGF10	31,92566681	31,88113785
C10	FGF2	23,94850922	23,91791344
C11	FGF7	21,93899918	24,98134995
C12	HBEGF	30,90260696	31,91805649
D01	HGF	29,88837433	29,89583015
D02	IFNG	Undetermined	37,0118866
D03	IGF1	30,92530251	31,95576477

D04	IL10	36,92320633	36,9637413
D05	IL1B	33,89768982	36,90014267
D06	IL2	34,90719986	37,06265259
D07	IL4	34,89405441	35,88302612
D08	IL6	25,93462753	25,90362358
D09	IL6ST	21,91151237	21,95241547
D10	ITGA1	25,90174294	25,91729546
D11	ITGA2	28,8997364	29,90341949
D12	ITGA3	25,9399662	26,92240143
E01	ITGA4	26,92313576	24,89090729
E02	ITGA5	24,91920662	24,91894531
E03	ITGA6	26,96011162	27,93279266
E04	ITGAV	23,92520523	23,91832161
E05	ITGB1	19,89581299	20,89857674
E06	ITGB3	28,91319656	30,90513992
E07	ITGB5	24,93560028	24,8963356
E08	ITGB6	33,91456604	35,88244247
E09	MAPK1	24,8861599	24,89133644
E10	MAPK3	25,91823196	25,9160614
E11	MIF	22,93474388	22,92826653
E12	MMP1	22,95505333	22,92569733
F01	MMP2	21,9133625	21,91251373
F02	MMP7	36,95568848	35,90433502
F03	MMP9	35,90948868	Undetermined
F04	PDGFA	27,91518211	26,90349007
F05	PLAT	29,93788147	29,90013504
F06	PLAU	24,90639305	26,91776657
F07	PLAUR	25,90584564	26,92122269
F08	PLG	Undetermined	36,95311356
F09	PTEN	23,87199402	23,88983536
F10	PTGS2	27,96568298	29,96590042
F11	RAC1	22,88829231	22,88568497
F12	RHOA	21,88217735	21,90045547
G01	SERPINE1	21,8911171	23,92044067
G02	STAT3	24,95532227	23,92413139
G03	TAGLN	21,91492653	21,94478607
G04	TGFA	34,90314865	36,92302704
G05	TGFB1	24,91706848	24,90917778
G06	TGFBR3	25,91153717	25,92120934
G07	TIMP1	20,93140602	21,91280365
G08	TNF	35,90616989	36,91529083
G09	VEGFA	23,91669083	23,91235733
G10	VTN	30,92292595	31,96270943
G11	WISP1	25,89200783	26,8936882
G12	WINT5A	21,89950371	20,90105247

H01	ACTB	17,93348503	17,93153191
H02	B2N	20,92124367	20,92291832
H03	GAPDH	19,91900253	19,90184784
H04	HPRT1	27,96165085	27,91419983
H05	RPLP0	19,90172577	19,90953636
H06	HGDC	36,93289948	Undetermined
H07	RTC	37,08670044	37,3323555
H08	RTC	36,658638	35,92355347
H09	RTC	Undetermined	37,22751617
H10	PPC	21,76464653	21,88846016
H11	PPC	21,9382267	21,86188889
H12	PPC	21,92430878	21,9281311

HF, human fibroblasts; G, buccal attached gingiva

Palate

HF		P	
Position	**Target Name**	**Baseline**	**24 hrs**
A01	ACATA2	21,94267082	21,89780998
A02	ACATA1	32,92599106	32,88602066
A03	ANGPT1	23,89475441	24,93656731
A04	CCL2	21,92213821	24,94232368
A05	CCL7	29,89967346	35,92685318
A06	CD40LG	34,88717651	35,89562607
A07	CDH1	33,92115784	34,8878479
A08	COL14A1	22,93378258	25,88224602
A09	COL1A1	18,93793869	18,93908119
A10	COL1A2	17,90968323	17,90914154
A11	COL3A1	18,89617729	20,89836884
A12	COL4A1	24,96277428	29,94066238
B01	COL4A3	34,92133331	33,90619278
B02	COL5A1	21,97214508	22,98347664
B03	COL5A2	20,9395237	20,89508629
B04	COL5A3	24,9337368	30,93454742
B05	CSF2	33,91490173	32,95511627
B06	CSF3	32,95304871	32,93101883
B07	CTGF	21,91503525	21,90776062
B08	CTNNB1	25,9460907	25,93742561
B09	CTSG	36,90243149	35,90840912
B10	CTSK	23,91327286	22,8910408
B11	CTSV	32,9385376	32,95447159
B12	CXCL1	Undetermined	25,87481689
C01	CXCL11	35,8744545	36,88246536
C02	CXCL2	23,90796089	26,88920212
C03	CXCL5	25,90277672	25,89765358
C04	EGF	31,90560722	33,89031982
C05	EGFR	24,92889023	24,93174553
C06	F13A1	36,93264008	Undetermined
C07	F3	24,89529419	23,8847084
C08	FGA	Undetermined	36,89411926
C09	FGF10	28,90877151	30,93357468
C10	FGF2	23,89655685	22,90147781
C11	FGF7	23,96009636	24,95183182
C12	HBEGF	28,97776794	28,9058075
D01	HGF	26,92095375	28,92398643
D02	IFNG	35,88982391	36,86709595

D03	IGF1	33,91218185	35,9125824
D04	IL10	36,92066956	36,91716385
D05	IL1B	33,89379883	33,91181564
D06	IL2	35,96047592	34,93871689
D07	IL4	35,92385483	Undetermined
D08	IL6	25,91379547	24,89642715
D09	IL6ST	21,9044838	21,93649673
D10	ITGA1	24,90788269	25,91242981
D11	ITGA2	26,89903259	28,94927597
D12	ITGA3	23,90361023	25,92651176
E01	ITGA4	27,90180588	25,89185143
E02	ITGA5	24,95809364	24,92669487
E03	ITGA6	26,94553185	25,92727661
E04	ITGAV	23,92817116	23,95515251
E05	ITGB1	20,93388557	20,90983582
E06	ITGB3	25,94273758	26,90517616
E07	ITGB5	23,92811012	23,89453316
E08	ITGB6	35,88911819	35,91384125
E09	MAPK1	24,9026165	24,910429
E10	MAPK3	25,93994141	25,92922783
E11	MIF	22,95532036	22,93791008
E12	MMP1	19,89106178	19,91259193
F01	MMP2	22,92193794	21,92270279
F02	MMP7	35,89902496	36,89422607
F03	MMP9	36,92076111	36,95359039
F04	PDGFA	26,89623451	27,936306
F05	PLAT	26,88736153	27,89190483
F06	PLAU	23,9433136	24,94271088
F07	PLAUR	25,90415764	25,90700531
F08	PLG	35,91973877	36,9042511
F09	PTEN	23,8988266	23,90436935
F10	PTGS2	28,92016411	26,90532875
F11	RAC1	22,89966774	22,88765717
F12	RHOA	21,90034103	21,92251396
G01	SERPINE1	22,88678551	22,95191193
G02	STAT3	23,91993713	23,94225693
G03	TAGLN	20,89967537	20,91000557
G04	TGFA	35,91260147	35,91841507
G05	TGFB1	24,91654396	25,9342308
G06	TGFBR3	25,95887184	25,90191078
G07	TIMP1	21,91360474	21,91203308
G08	TNF	35,9086647	36,91272736
G09	VEGFA	23,94146538	23,91954041
G10	VTN	28,97074127	31,96452713
G11	WISP1	25,92705727	26,90289688

G12	WINT5A	21,96208	20,9194622
H01	ACTB	17,93735695	16,88797379
H02	B2N	20,92769623	20,94172287
H03	GAPDH	19,91893578	19,90619469
H04	HPRT1	27,96473503	27,96366692
H05	RPLP0	20,92371941	19,90116692
H06	HGDC	37,42192459	35,93130493
H07	RTC	7,253512859	39,37491608
H08	RTC	37,00256729	37,10013962
H09	RTC	36,00099182	35,82800674
H10	PPC	20,95776749	21,7448616
H11	PPC	21,12501717	21,93452454
H12	PPC	22,96660233	21,92808723

HF, human fibroblasts; P, palate

Appendix B – GENE EXPRESSION PROFILING

TABLE 1 Expression of 84 wound healing related genes in M, G and P cells obtained from Patient 1 and Patient 4, identified by Human Wound Healing RT² Profiler™ PCR Array. Fold changes between baseline and 24 hrs

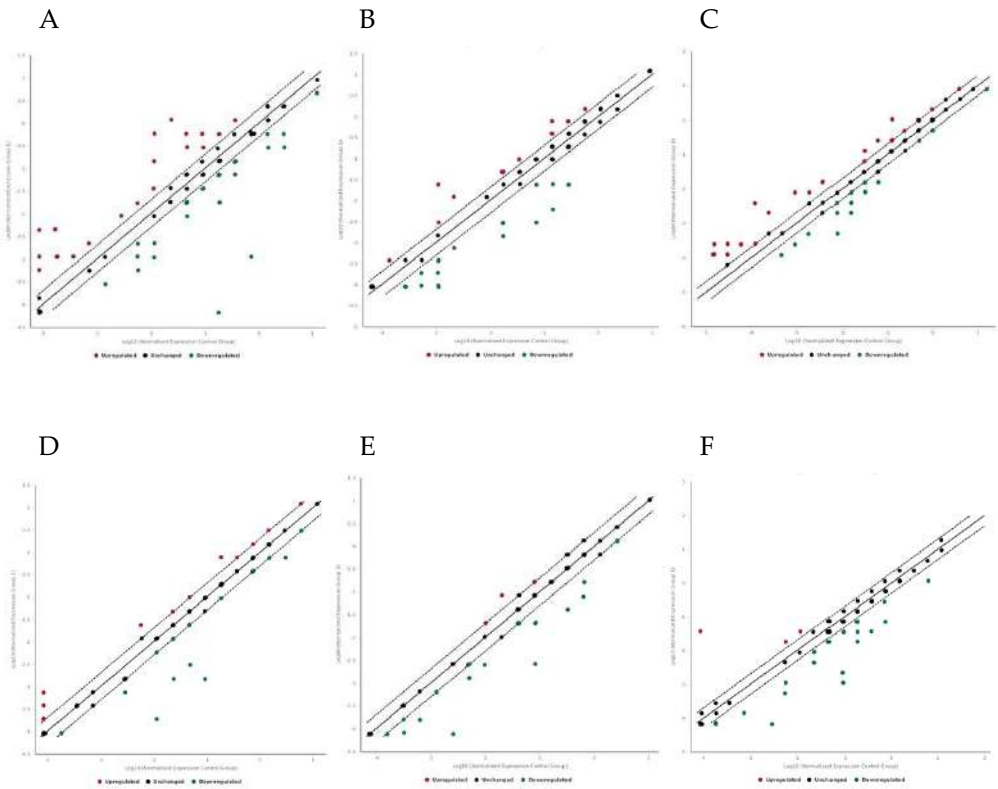
		PATIENT 1			PATIENT 4		
		M	G	P	M	G	P
A01	ACTA2	2,1					
A02	ACTC1			-663,75			-64,43
A03	ANGPT1			12,46	-2,74		
A04	CCL2		-12,05	3,04	-10,81	-8,21	-2,02
A05	CCL7	4,01	-12,9	50,69	-45,72	-34,75	
A06	CD40LG	8,09					
A07	CDH1	-4	-2,97	=	-2,6	-4,03	2,17
A08	COL14A1	8,32	-6,04	-5,16	-10,29	-4,05	2,04
A09	COL1A1	2,05		-2,61			2,03
A10	COL1A2			-2,69			
A11	COL3A1				-5,34	-2,13	
A12	COL4A1	-2,1	-12,68	-5,45	-42		
B01	COL4A3	8,08	2,69	3,03		-2,02	
B02	COL5A1	2,04		-2,56	-2,69	2,05	
B03	COL5A2					2,08	
B04	COL5A3		-6,03	-2,63	-85,34	-2,01	-2
B05	CSF2	-3,94	NO	NO			
B06	CSF3	=	-3,04	26,27	=		
B07	CTGF		2,68	-10,5			
B08	CTNNB1						
B09	CTSG	8,04	NO	NO			
B10	CTSK	2,02	5,49	6,14		2,04	
B11	CTSV	-4,08	-5,8	6,12	=	-4,13	
B12	CXCL1		-23,81	48,91	419,01	-31,95	-16,27
C01	CXCL11	2,02	NO	NO			2,19
C02	CXCL2	=	-24,34	49,42	-10,52	-32,41	-16,14
C03	CXCL5		2,87			-2,08	
C04	EGF		-2,99	-5,26	-5,27		
C05	EGFR		2,66				
C06	F13A1	NO	NO	-2775,07			-62,4
C07	F3	-4,02					
C08	FGA	8,64	NO	NO			
C09	FGF10	3,97		6,59	-5,42		8,46
C10	FGF2						
C11	FGF7	2,01			-2,65	-8,3	
C12	HBEGF	=	-3	-5,07	=	-2,04	=
D01	HGF	4,07	2,66	3,08	-5,34		
D02	IFNG	3,91	NO	NO			2,14

D03	**IGF1**	31,57	-3,07		-2,83	-2,06	-4
D04	**IL10**	4,05	NO	NO			
D05	**IL1B**	-16,03	-5,9	6,04		-2,16	
D06	**IL2**	3,98	NO	NO			4,36
D07	**IL4**	8,05	NO	NO			2,15
D08	**IL6**			3,06			4,08
D09	**IL6ST**	2,03					2,02
D10	**ITGA1**		2,73		-2,67		
D11	**ITGA2**	-2,01	5,5	-2,67	-5,52	-2,02	-2,01
D12	**ITGA3**	-4,13	-3,07	-2,67	-5,42		
E01	**ITGA4**	-2,02	2,67		3,02	4,06	
E02	**ITGA5**						
E03	**ITGA6**		2,75	-10,7			
E04	**ITGAV**		2,67	-2,65			
E05	**ITGB1**					-2,02	
E06	**ITGB3**	-4,05		3,1	-2,6	-4,01	
E07	**ITGB5**	2,02		-2,56			-2,05
E08	**ITGB6**	-3,94	-11,8	-10,48	NO	-2,14	-2,01
E09	**MAPK1**			-2,62			
E10	**MAPK3**	2,02	2,74	-2,67	=	=	=
E11	**MIF**			-2,69			
E12	**MMP1**		2,8	12,75			
F01	**MMP2**	2,01	2,66	3,12			
F02	**MMP7**	4,07	NO	NO			
F03	**MMP9**	-7,88	NO	13,08			-2,04
F04	**PDGFA**				-2,74	2	
F05	**PLAT**		21,54	-5,28	-2,67		
F06	**PLAU**		2,71	-2,61	-2,66	-4,06	
F07	**PLAUR**	-2,05	2,73			-2,04	
F08	**PLG**	16,19	NO	NO			
F09	**PTEN**	2,02					
F10	**PTGS2**		-6,04	2,99	3,03	-4,03	-2
F11	**RAC1**			-2,65			-2
F12	**RHOA**	-2,05					-2,05
G01	**SERPINE1**			-5,32		-4,11	-4,05
G02	**STAT3**	4,11	=	=	=	2,03	=
G03	**TAGLN**	=	=	=	=	=	2,01
G04	**TGFA**	-3,92	-3,2	NO	NO		2,15
G05	**TGFB1**	=	=	-2,64	-2,7	=	=
G06	**TGFBR3**	4,04	2,68	3,16			2,05
G07	**TIMP1**			-5,12			-2,03
G08	**TNF**	-4,04		NO	NO	NO	NO
G09	**VEGFA**	2,02		-2,69			
G10	**VTN**	8,12		-10,82	-10,62	-2,07	
G11	**WISP1**	-4,07		-2,62	-2,62	-2,02	-2
G12	**WNT5A**	-2,03	2,61	6,34	=	=	4,1

M, alveolar mucosa; G, buccal attached gingiva; P; palate

Red color indicate up-regulated genes; green color indicate down-regulated genes

FIGURE 1 Scatter plots showing up-regulated, unchanged and down-regulated genes between baseline vs 24 hrs in Patient 1 and Patient 4.



(A-C) Patient 1 (A) alveolar mucosa, (B) buccal attached gingiva, (C) palatal tissue
(D-F) Patient 4 (D) alveolar mucosa, (E) buccal attached gingiva (F) palatal tissue