Politecnico di Torino

Dottorato di ricerca in
BIOINGEGNERIA E SCIENZE MEDICO-CHIRURGICHE

XXXVI ciclo

Hyaluronic and Synthetic Aminoacid Treatment of the post extraction tooth socket healing in subjects with diabetes mellitus type 2

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Summary

Introduction

Diabetes and prevalence

Diabetes mellitus (DM) is a systemic metabolic disorder characterized by hyperglycemia and glycosuria and causing chronic consequences as a result of impaired insulin, faulty insulin secretion or impaired insulin sensitivity. It is among the top 10 causes of death in adults and was estimated to have caused four million deaths globally in 2021. The three main types of diabetes are type 1 diabetes (T1D), type 2 diabetes mellitus (T2DM), MODY (Maturity Onset Diabetes of Young) and gestational diabetes mellitus (GDM).

The global world diabetes prevalence in 20-79 year olds in 2021 was estimated to be 10.5% (536.6 million people), rising to 12.2% (783.2 million) in 2045. For T2DM, which accounts for approximately 90% of the total, this rising trend can be attributed to aging, rapid increase in urbanization, and obesogenic environments. [1]

Diabetes prevalence was similar in men and women and was highest in those aged 75-79 years. Prevalence (in 2021) was estimated to be higher in urban (12.1%) than rural (8.3%) areas, and in high-income (11.1%) compared to low-income countries (5.5%). The greatest relative increase in the prevalence of diabetes between 2021 and 2045 is expected to occur in middle-income countries (21.1%) compared to high- (12.2%) and low-income (11.9%) countries. [2]

Physiopathology of diabetes

In the past three decades, the prevalence of T2DM has risen dramatically in countries of all income levels. People with diabetes have an increased risk of developing a number of serious life-threatening health problems resulting a reduced quality of life, an increased mortality, and high medical care costs. Persistently high blood glucose levels cause generalized vascular damage affecting the heart, eyes, kidneys and nerves and resulting in various complications. [3]

T2DM: usually is diagnosed at an advanced age. It is strongly influenced by several etiological factors, such as genetic predisposition (several polymorphisms), obesity, sedentary lifestyle, hypertension, dyslipidemia associated with a. [4] It is due to onset of insulin resistance followed by the loss of beta cells ability in secreting adequate insulin amount. [5]

The main role of insulin is the regulation of glucose homeostasis, promoting its uptake from the bloodstream into the cells, storage and utilization as an energy form. Failing the role of insulin, as a consequence there is hyperglycemia, as cells unable to exploit glucose as an energy source resort to metabolizing fatty acids, as cells unable to exploit glucose as an energy source resort to metabolizing fatty acids, resulting in blood accumulation of ketone bodies. [6]

Prolonged exposure to the state of hyperglycemia is the main etiological factor in the complications of diabetic disease:

- polymorphonucleate dysfunction: impaired healing processes, delayed healing, and increased probability of infection. [7]
- microangiopathy: due to inability of fibroblasts to utilize anaerobic metabolism, resulting in deficits in collagen production and endothelial proliferation. This complication also responsible for the typical disorders of diabetic disease such as neuropathy, nephropathy and retinopathy, as well as adversely affecting the wound healing process because of the impediment to peripheral vasodilation, a physiological reaction of the inflammatory process underlying the healing itself. [8]
- macroangiopathy: it is due to an increased extent atherosclerosis, which can lead to myocardial infarction, cerebrovascular diseases and peripheral gangrenes. [9] Atherosclerosis is secondary to endothelial dysfunction, inflammation, thrombosis and oxidative stress, as well as dyslipidemia and hemodynamic stress, plays an important role in the long-term development of vascular disease, especially in patients with T2DM. [10]

The leading cause of death in diabetic patients turns out to be athero-thrombosis, resulting in acute coronary syndrome: these factors contribute to the picture of vascular alterations; the platelet hyperactivity that is typical of diabetes, associated with abnormalities in coagulation and fibrinolysis, may be the triggering event for intravascular thrombus formation. [11]

Diabetes and healing failure

Skin wound healing occurs through a coordinated cellular response to damage and involves the activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. [12] These cell lines support healing also through the release of certain growth factors and cytokines. Reduced immune cell response and impaired recruitment of healing cells and the reduced oxygen/ nutrient availability due to microangiopaty result in deficit healing in the patient with diabetes mellitus. [13]

The healing deficit in the diabetic patient can thus be considered as the result of multiple and complex pathophysiological mechanisms, as listed below. [14]

- Cytokines, monocytes and macrophages: it has been shown by several studies in the periodontal field, that in the crevicular fluid of decompensated diabetic patients, increased levels of Interleukin 1beta (IL-1β) and prostaglandin E2 (PGE2), released by monocytes can be detected. [15]
- Hypoxia: A situation of prolonged hypoxia, which may result from insufficient perfusion or insufficient angiogenesis, is detrimental to healing. Hypoxia can amplify the early inflammatory response and thus prolong damage due to an increased level of free radicals. [16]
- Advanced Glycation End Products (AGEs): AGE formation due to the direct interaction between glucose and long half-life proteins of vessel wall. In case of persistent hyperglycemia, AGE production becomes irreversible and AGEs can accumulate in the vessel wall and act as modulators of several cell functions through the interaction with the specific receptors RAGE. The accumulation of AGEs in tissues alters the function of intercellular matrix components,

including collagen present at the level of the vascular walls, compromising vascular integrity. [17] In fact, a thickening of the vessel wall can impair oxygen diffusion, elimination of metabolic products, migration of polymorphonucleates, and diffusion of antibodies.[18]

- Fibroblasts: Hyperglycemic environment may reduce growth, proliferation, and cell matrix synthesis by fibroblasts and osteoblasts. [19]
- Polymorphonuclear leukocytes: Decompensated diabetes induces a reduction in the function of polymorphonuclear lymphocytes and consequently defects in chemotaxis. [20]
- Platelets: A review of the literature by Natarajan in 2008 showed that several alterations in platelet function can be detected in patients with T2DM, mostly associated with hypersensitivity to platelet agonists, resulting in platelet hyperactivation and increased release of cytokines and chemokines (IL-1β) that contribute to maintaining the inflammatory state and increasing atherogenetic risk. [21] The main alterations noted were:
  - increased production of Thromboxane A2, a potent platelet activator;
  - increased expression of receptors and adhesion molecules;
  - non enzymatic glycation of proteins exposed on the surface of platelets, resulting in altered lipid membrane dynamics and expression of receptors;
  - disorders of calcium homeostasis, which is responsible for platelet activation, secretion and aggregation; [22]
  - Reduction in the number of insulin receptors on the platelet membrane, thus the role of insulin in limiting platelet activation is diminished. [23] in diabetic patients with T2DM, a platelet hyperactivation occurs, due to increased platelet susceptibility from glycation of serum albumin. [24]
- Neuropathy: probably contributes to healing difficulties. Neuropeptides such as nerve growth factor, substance P, and calcitonin gene-related peptide are relevant in wound healing because they promote cell chemotaxis, induce growth factor formation, stimulating cell proliferation . A reduction in neuropeptides has been associated with diabetic foot ulcer. In addition, sensory innervation plays a key role in modulating immune defense mechanisms. [25]

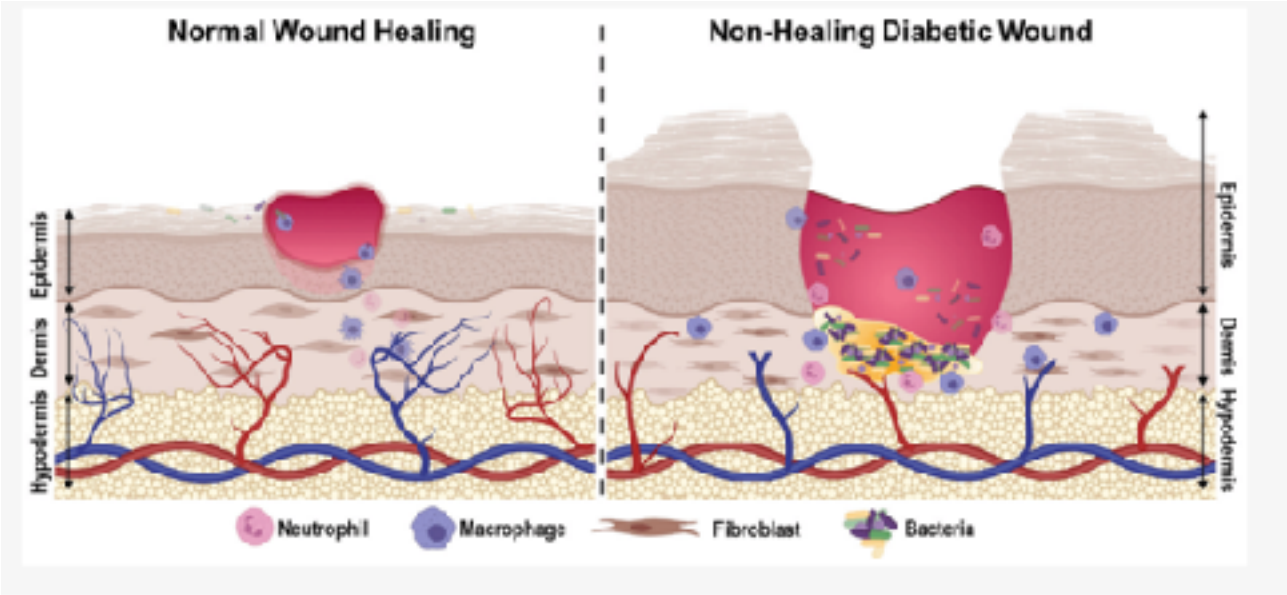


Figure 1. Pathophysiology of diabetic wounds. Diabetic sounds exhibit deregulated angiogenesis, chronically sustained sub-optimal inflammatory responses, increased levels of reactive oxygen species, and persistent bacterial colonization that often develops into a hard to treat biofilm [25]

- Bone tissue: several studies have shown that reductions in bone mineral content can be detected in diabetic patients, resulting in reduced bone mass and delayed healing of bone fractures. [26-27]

A study conducted on rats with spontaneous diabetes, which can be likened to T1DM in humans, demonstrated that the state of metabolic compensation assumes great significance for the healing of bone defects, depending on the extent of the defect itself. Histomorphometric analyses, performed 7-14-24-42 days after bone removal surgery at the level of the femur showed that in the case of small defects there were no statistically significant differences in the degree of bone apposition and remodeling between diabetic animals and healthy controls and between poorly and well-compensated diabetics. As the magnitude of the bone defect increased, the metabolic status became significant, with the appearance of delays and defects in bone apposition and mineralization proportionate to the magnitude of the decompensated state. Conversely, no

significant differences were detected between healthy controls and diabetic animals, even in presence of large bone defects, in case metabolic compensation by insulin administration. [28]

Wound healing physiology

Wound healing is a fundamental biological process in the human body; it is achieved through four precise and highly programmed phases (figure 3): hemostasis, inflammation, proliferation and remodeling. [29] These phases must occur in a correct sequence, last for the necessary time and at the appropriate intensity.

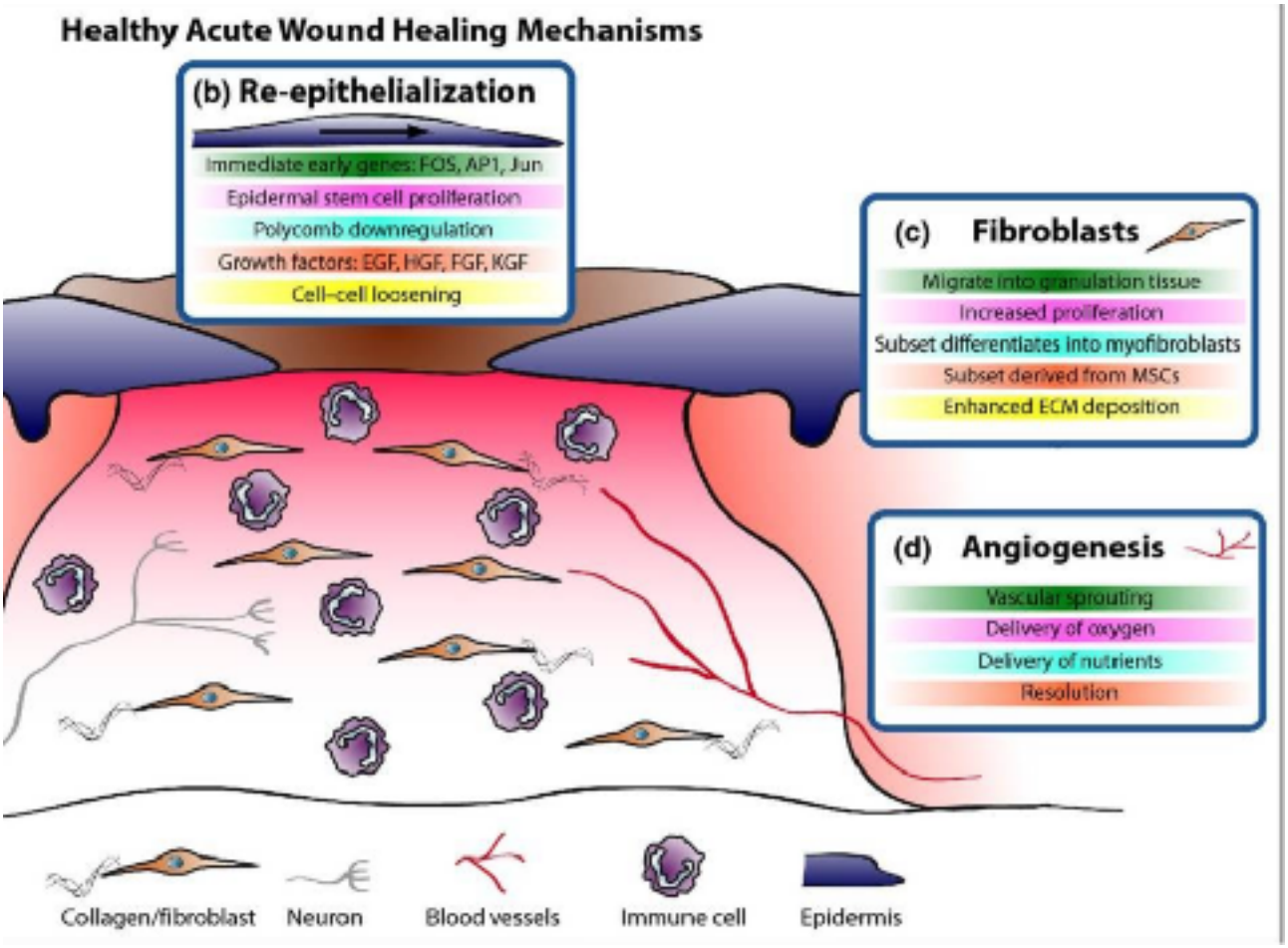


Figure 3. Normal wound healing process [29]

Hemostasis

The hemostasis begins immediately following injury: there is an initial vasoconstriction for the purpose of reducing blood flow and loss and promoting hemostasis. [30]

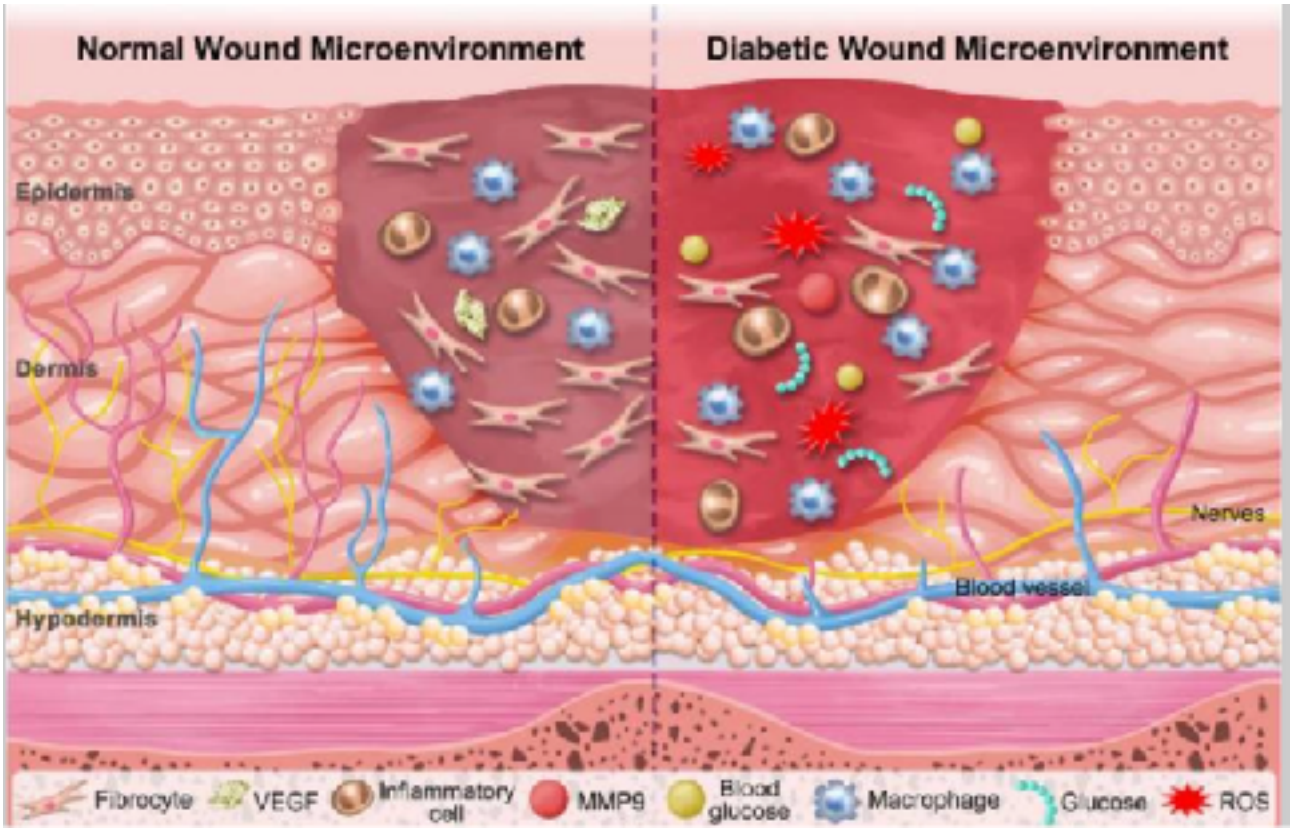


Fig. 4 Typical alterations in diabetic wounds and a summary of the classification and biological functions of smart hydrogels. [30]

The second phase of the blood clot formation process is the phase in which platelets are directly involved: platelets adhere to the exposed collagen of the damaged basement membrane; this binding induces their activation resulting in structural modifications that, in turn, cause degranulation, which is the release of the contents of the platelet granules. Growth factors and enzymesare contained within the granules. [31]

Release of these molecules contribute to the formation of a platelet plug, or primary hemostatic plug, which must then be stabilized by subsequent activation of the coagulation cascade. Also present within the granules are cytokines and growth factors, including TGF-β and PDGF, which stimulate the migration of neutrophil granulocytes and macrophages within the wound.

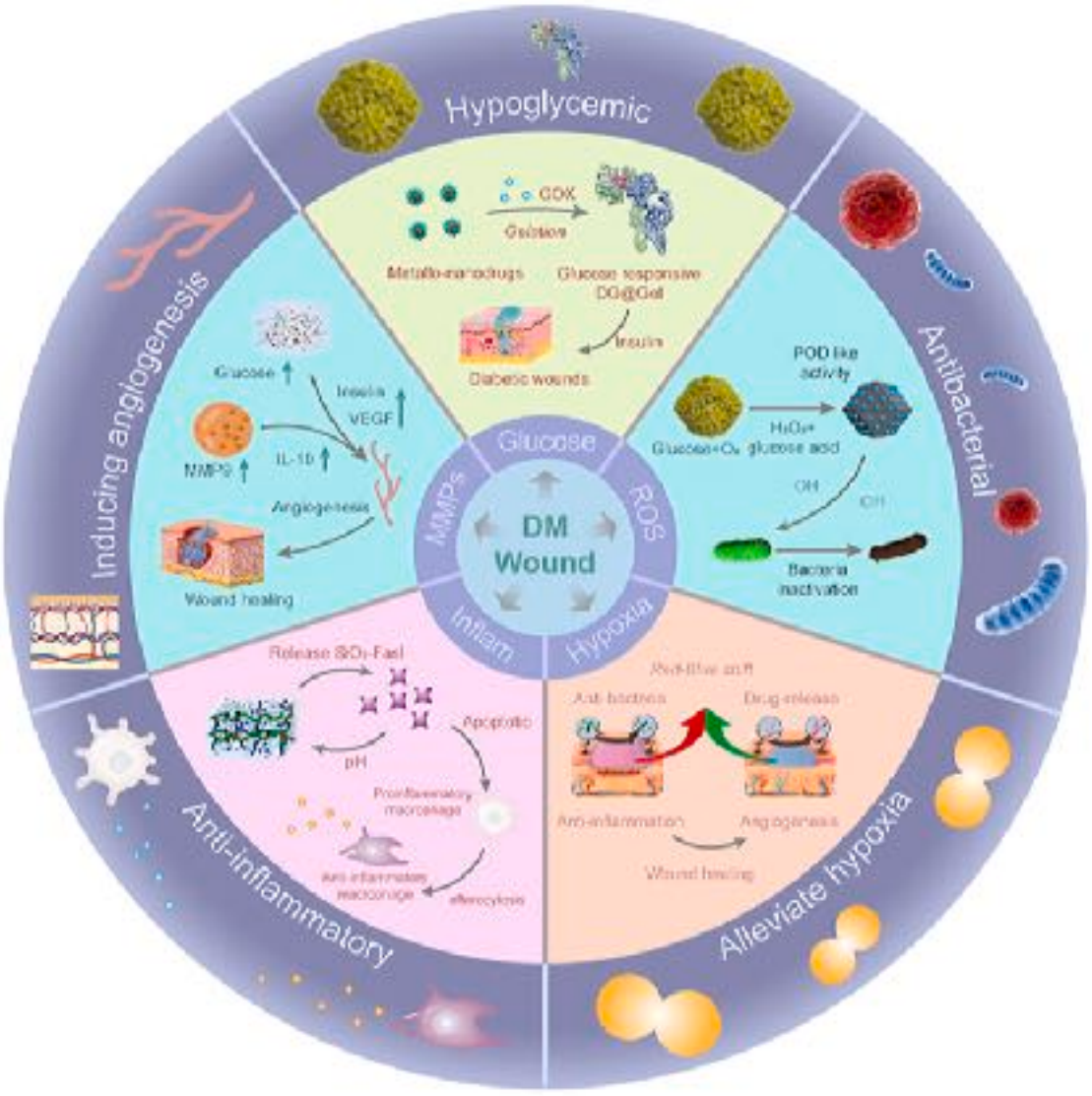


Fig. 5 Differences between Normal wound microenvironment and Diabetic wound microenvironment [30]

The fundamental role of platelets is hemostasis, although they play an essential role in modulating healing and tissue regeneration.

As a consequence of endothelial damage there is exposure of collagen fibers.

The action of platelets is carried out through an initial phase of adhesion to the injured endothelium and this is possible due to the presence on the surface of platelets of several receptors including:

- GPIb receptor, a transmembrane glycoprotein capable of binding to von Willebrand factor exposed on the subendothelial surface;
- α2β1 and GPVI receptors which mediate binding to collagen exposed at the subendothelial surface;
- Integrin α2bβ3 main mediator of platelet aggregation as it can bind multivalently to different proteins-ligand upon platelet activation. [32]

Through such receptors, platelets adhere with stable binding to the endothelium. As a result of such binding, a change in platelet morphology occurs, and this results in degranulation (Figure 4), which is the release of the contents of platelet granules containing enzymes, proteins, growth factors, and chemical mediators that will actively participate in the later stages of healing. [33]

Dense granules: the substances they contain play a minor role in hemostasis, except for calcium and fibrinogen, but they regulate other important biological processes. Among these substances we find signal molecules that can influence cell migration and proliferation by acting at the level of nucleotide receptors; molecules that influence vascular tone; calcium, which is an indispensable cofactor for platelet aggregation and wound healing to occur as it represents Factor IV of the coagulation cascade and thus is indispensable for the consequential activation of subsequent factors involved in the process; Fibrinogen, Factor I of the coagulation cascade, which is indispensable for hemostasis to occur as it is the inactive precursor of fibrin, which will form the meshwork in which platelets and repair cells will be trapped; Serotonin, whose action is exerted at the level of vascular cells resulting in vasodilation and increased vascular permeability; and finally, Histamine, which possesses both pro- and anti-inflammatory effects. [34]

Alpha granules: within them are a large number of proteins, growth factors, chemokines, and cytokines; in particular, adhesive proteins (fibronectin, thrombospondin, laminin) are stored that determine cellular communication and interaction, hemostasis, clot formation, and extracellular matrix formation; coagulation factors and associated proteins (factors V, XI, protein S, anti-thrombin III) that participate in thrombin activation and its regulation; fibrinolytic factors (plasminogen, plasminogen activator inhibitor PAI) are essential for plasmin activation and subsequent clot dissolution after hemostasis has occurred); proteases and anti proteases (MMP, metallo protease) that participate in angiogenesis, vascular remodeling, as well as in the regulation of cell behavior; growth factors (PDGF, TGF-β1, β2, EGF, IGF, VEGF,FGF, HGF,BMP) that because of their importance in the regulation of angiogenesis, osteogenesis and tissue regeneration will be analyzed specifically later; chemokines and cytokines also indispensable for tissue regeneration; anti-microbial proteins; membrane glycoproteins, which are involved in the regulation of platelet aggregation, endocytosis, inflammatory process, interaction between platelets and leukocytes, and thrombin activation. [35]

Lysosomal granules: contain hydrolases, cathepsins, elastases and other degradative enzymes that participate in tissue remodeling resulting from the regenerative or reparative phase. Following degranulation and release of the substances contained within the granules, platelets undergo aggregation, a crucial step in the process of primary hemostasis as it leads to the formation of a hemostatic plug, or thrombus. Platelet aggregation occurs through the interaction between the integrin α2β3 receptor and adhesive molecules, the most important of which is fibrinogen, which acts as a bridge between platelets. The thrombus thus formed is called pro-coagulant, as the platelets it contains release coagulation factors that accelerate the formation of thrombin and the subsequent production of fibrin. Fibrinogen as has been mentioned plays the role of a bridge by creating multiple bonds with the α2β3 receptors of multiple platelets; cofactors secreted by the dense granules such as ADP, or metabolites such as Thromboxane-A2 interact with other receptors thus promoting the formation of the aggregate or thrombus. [36]

Coagulation phase

The third phase of clot formation is the coagulation phase (Figure 5), which occurs by activation of the coagulation cascade. If the trauma is severe the clot begins to form within 15-20seconds, for minor trauma the clot forms within 1-2minutes. This is a cascade of factors whose activation is essential for the activation of the following, and the end result is the activation of fibrinogen into fibrin. During platelet aggregation, as seen above, platelets aggregate due to specific receptors for fibrinogen, which acts as a bridge for such binding to occur; activation of the subsequent coagulation cascade causes fibrinogen to be converted to fibrin, resulting in retraction of the primary clot formed and formation of a stabilized secondary clot. [37]

The factors involved in the coagulation cascade are circulating proteins in the blood produced largely by the liver, which act sequentially by consecutive activation. [38] They are divided into two groups: factors of the Intrinsic pathway and factors of the extrinsic pathway; part of the factors, from factor X converge into a common pathwa. [39]

The end step of the coagulation cascade is the activation of Thrombin, which in turn transforms plasma fibrinogen into monomeric fibrin, an insoluble protein that polymerizes to form a dense network within which platelets and subsequently monocytes, fibroblasts, and endothelial cells are trapped. [40]

The fibrin network, other than to contribute to stop blood loss , is also necessary for the migration of cells involved in the following phases. Because of its role as a supporting matrix, the structure of fibrin itself (fiber thickness, porosity, and permeability of the clot). is an essential structure for the healing process. [41-42]

Based on the importance of this protein, methods have been developed for to obtain autologous fibrin by blood collection to be used as biomaterial supporting tissue healing; these methods include the technique of making PRGF according to Anitua. [43]

Inflammation: after clot formation, the inflammatory phase, which is characterized by the sequential recruitment of neutrophils and macrophages,can take place. [44]

A key function of neutrophils is to remove noxious agents and cellular debris within the wound area, and to produce chemical mediators of angioflogosis. The same functions are also played by macrophages.

Macrophages are also responsible for inducing apoptosis and removing apoptotic cells (in cooperation with neutrophils). Once these apoptotic cells are removed, macrophages undergo a phenotypic transition to a reparative state that stimulates keratinocytes, fibroblasts, and the process of angiogenesis by promoting tissue regeneration. [45]

T lymphocytes migrate to the wound following inflammatory cells and macrophages and come in between the end of the proliferative phase and the beginning of the remodeling phase. The role of T lymphocytes is not completely clear and is currently an interesting area of study. Some studies state that late infiltration of T lymphocytes is associated with impaired healing, while others report that CD 4+ cells (helper T cells) have a positive role in healing and CD8+ cells (the cytotoxic T lymphocytes) have an inhibitory role in healing. [46]

Epidermal dendritic T cells (DETCs) are activated by stress, damage, or keratinocytes and produce fibroblast growth factor-7 (FGF-7), keratinocyte growth factor, and insulin-like growth factor-1 to support keratinocyte proliferation and cell survival. DETCs also produce cytokines and chemokines that help initiate and sustain the inflammatory response during healing. While the interaction between gamma-delta T cells and keratinocytes contributes to healing, in mice with reduced in number or inefficient gamma-delta T cells, altered wound closure and reduced proliferation of keratinocytes in the wound area is noted. [47-48]

The proliferative phase generally follows and overlaps with the inflammatory phase and is characterized by the migration and proliferation of epithelial cells onto the provisional matrix within the wound (re-epithelialization). Within the dermis under repair, fibroblasts and endothelial cells are the most represented cell types that support type III collagen production and

angiogenesis, leading to the granulation tissue formation. In addition to collagen, fibroblasts produce glycosaminoglycans and proteoaminoglycans which are the major components of the extracellular matrix.

Following proper proliferation and synthesis of extracellular matrix, healing enters its remodeling phase, which has a longer duration. In this phase there is regression of a large number of capillaries to a normal density. Other very important events during remodeling are the substitution of type III collagen with type I and final organization of the extracellular matrix . In case of healing by secondary union, the wound also undergoes a process of contraction due to the activity of myofibroblasts and aiming to bring the edges of the wound closer together. [49]

Post extraction alveolar healing

Following avulsion of a dental element, trauma triggers bleeding within the alveolar walls due to injury to the gingival capillaries, periodontal ligament, and bone marrow. Bleeding leads to the processes described earlier with formation of a clot stabilized by fibrin. Following the hemostasis phase, the healing process is triggered, in which the clot is gradually transformed into granulation tissue. [50] Many studies have evaluated the healing of the post-extraction socket by distinguishing the following stages. [51 - 58]

First 24 hours after extraction: Immediately after extraction, blood, leaking from severed blood vessels, and damaged cells trigger the phenomena that will lead to the formation of first a fibrin network and then, thanks to platelet aggregation, the blood clot. It is responsible for stopping bleeding as well as acting as a carrier of substances important for the healing process and as a physical matrix for cell movement. 48-72 hours after extraction. The clot contains growth factors and substances that promote the differentiation and migration of mesenchymal cells and the activity of inflammatory cells. Neutrophils and macrophages migrate into the wound in order to remove debris; neutrophils appear earlier and upon completion of their task undergo apoptosis;

macrophages settle after neutrophils and, in addition to phagocytic activity, carry out production of growth factors and cytokines stimulating tissue regeneration. When all debris has been removed, the apoptotic neutrophil bodies are also phagocytized by macrophages, and new tissue formation (granulation tissue) can begin.

4-5 days after extraction: A noticeable proliferation of epithelial cells can be observed from the free margins of the mucosal flaps surrounding the coronal opening of the socket, so that a covering of the mass of granulation tissue contained within the wound is formed; The clot, which immediately filled the site after surgery, is progressively replaced by newly formed tissue, in which a highly vascularized, cellular, and mineralization-free temporary matrix is formed. Specifically, migration of mesenchymal cells is triggered and they begin to proliferate and deposit matrix components in situ. This forms granulation tissue, which gradually replaces the clot. It contains macrophages, fibroblast-like cells, and numerous blood vessels. The intense synthesis of matrix components is called fibroplasia, while the formation of new vessels is called angiogenesis; through the combination of the two phenomena, temporary granulation tissue is created.

1 week after extraction: Granulation tissue and osteoid matrix can be observed in the healing socket starting from the apical end; in addition, the lamina dura and periodontal ligament appear dissolved.

2 weeks after extraction: activation of the remodeling process was observed. It takes place in a centripetal direction, that is, starting from the residual lateral and apical bone walls and then proceeding toward the center of the socket and along the vascular structures. More specifically, osteoprogenitor cells, derived from the vascular structures (pericytes), congregate near the vessels and differentiate into osteoblasts capable of producing a collagen matrix characterized by an intertwined pattern and indicated as osteoid matrix. Osteoblasts continue with their metabolic deposition activity while also promoting the gradual mineralization of the tissue and sometimes

becoming trapped in the matrix as osteocytes. Osteoid is rather quickly replaced by a more mineralized but immature tissue, termed woven-fiber bone or woven bone, again characterized by the random and intertwined arrangement of collagen fibers, as in osteoid. A thin layer of woven-fiber bone tissue, very rich in osteoblasts, localizes to separate the residual alveolar bone (bundle bone or fibrous bone) from the temporary matrix accumulated in the center of the alveolus.

3 weeks after extraction: no more inflammation is present, but some granulation tissue and osteoid (which now shows signs of ongoing mineralization) are still present.

4 weeks after extraction: immature bone (woven bone) occupies almost the entirety of the socket, leaving a small central remnant of provisional matrix. It provides a solid scaffold, a source of osteoprogenitor cells, and a substantial blood supply, i.e., everything needed to have good cell function and tissue mineralization.

6 to 8 weeks post-extraction: both the presence of bony trabeculae and an high degree of mineralization of the tissue filling the alveolus are evident. Indeed, a progressive and steady maturation of woven bone is observed (6 to 8 weeks after extraction), as well as a formation of medullary spaces to occupy a large part of the post-extraction socket and its coronal closure by a thin layer of lamellar bone (corticalization). The interwoven fiber bone was then gradually replaced by lamellar bone and bone marrow. In this process, definitive osteons are formed to replace the primitive ones in the immature bone.

4-12 months after extraction: the socket appears covered by a new layer of lamellar cortical bone lining a layer of woven bone. The deeper part, is occupied by thin trabeculae, again of lamellar bone, around which adipocyte-rich medullary tissue is arranged.

Platelet role in healing

Platelets are fundamental for hemostasis and inflammatory processes; however, they also have an important role in tissue repair and regeneration. [59]

Platelets are cell fragments resulting from the fragmentation of megakaryocytes, polyploid cells that differentiate and mature in the bone marrow under thrombopoietin stimulation. Being fragments of an original cell, they contain no nucleus or organelles and therefore have a short half-life of about 7-10 days. They are normally present in blood at a rate ranging from 150,000 to 400,000 platelets/µl of blood. The essential functions of platelets are:

- hemostasis: they adhere to continuous vessel solutions and aggregate to form the so-called white platelet thrombus, or platelet plug, which is later stabilized by the activation of the coagulation cascade in which they play a key role by releasing coagulation factors; they also participate in clot retraction.
- inflammation: contained within their granules are vasoactive amines, such as histamine and serotonin, initiators of the inflammatory response.
- phagocytosis: they phagocytose bacteria and toxins that are bound on their surface, thereby detoxifying the plasma.
- repair/regeneration: within their granules they contain more than 30 different bioactive proteins, which depending on their stimulatory activity are called growth factors (PDGF, TGF-beta, IGF, FGF, EGF, VEGF..), as by binding to transmembrane receptors of osteoprogenitor cells, endothelial cells, mesenchymal cells, they induce their activation, proliferation, differentiation, collagen production and angiogenesis. Platelets stimulate the mitogenic activity of bone cells, whose proliferation increases depending on the amount of platelets, the release of microparticles by platelets, and the presence of PDGF. [60-61]

Although it has not yet been possible to elucidate specifically how all these mechanisms interact with each other in physiological processes, the role of platelets is undisputed, and this is the theoretical prerequisite to the use of platelet gels and consequently to the implementation of operational systems and protocols for the purpose of increasing the concentration of platelets at surgical sites, or within wounds, accelerating their healing. [62]

Role of growth factors in healing

Growth factors (Figure 6) are polypeptides that coordinate healing processes by regulating the cellular processes of proliferation, differentiation, cellular activity of synthesis and cell death. [63]

Each growth factor, which can be produced by different cells, has the characteristic of being multifunctional, and activation is determined not only by direct protein-receptor binding but also, and especially, by changes in the cellular microenvironment. [64- 65]

Type	Source	Effect
Transforming growth factor-β (TGF-β)[91]	Platelets, extracellular matrix of bone, cartilage matrix, activated TH1 cells and natural killer cells, macrophages/monocytes and neutrophils	Stimulates undifferentiated mesenchymal cell proliferation, regulates endothelial, fibroblastic and osteoblastic mitogenesis; regulates collagen synthesis and collagenase secretion; regulates mitogenic effects of other growth factors; stimulates endothelial chemotaxis and angiogenesis; inhibits macrophage and lymphocyte proliferation; regulates balance between fibrosis and myocyte regeneration; regulates mitogenic effects of other growth factor
Basic fibroblast growth factor (bFGF)[93,94]	Platelets, macrophages, mesenchymal cells, chondrocytes, osteoblasts	Promotes growth and differentiation of chondrocytes and osteoblasts; mitogenic for mesenchymal stem cells, chondrocytes, and osteoblasts; stimulates angiogenesis; stimulates proliferation of myoblasts
Platelet-derived growth factor (PDGF)[93,95]	Platelets, osteoblasts, endothelial cells, macrophages, monocytes, smooth muscle cells	Mitogenic for mesenchymal stem cells and osteoblasts; stimulates chemotaxis and mitogenesis in fibroblast/glial/smooth muscle cells; regulates collagenase secretion and collagen synthesis; stimulates macrophage and neutrophil chemotaxis, stimulates angiogenesis
Epidermal growth factor (EGF)[96,97]	Platelets, macrophages, monocytes	Stimulates endothelial chemotaxis/angiogenesis; regulates collagenase secretion; stimulates epithelial/mesenchymal/chondrocytes/osteoblasts mitogenesis; promotes growth/differentiation of chondrocytes and osteoblasts
Vascular endothelial growth factor (VEGF)[98,99]	Platelets, endothelial cells	Increases angiogenesis and vessel permeability; stimulates mitogenesis for endothelial cells
Connective tissue growth factor (CTGF)[100]	Platelets through endocytosis from extracellular environment in bone marrow	Promotes angiogenesis, cartilage regeneration, fibrosis, and platelet adhesion

Figure 6: Main growth factors, their source and effects [64- 65]

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming TGFbeta superfamily; they are produced in large quantities by keratinocytes and fibroblasts and participate in the stimulation of pluripotent cells by promoting their differentiation into bone- and cartilage-producing cells. [66] Among the various growth factors and differentiation molecules, some BMPs appear to be the

most potent inducers of osteoblast proliferation. For this reason, , systems containing recombinant human BMPs have been recently commercially introduced for the purpose of promoting bone regeneration, especially in case of bone fractures, or to induce osseointegration following implant placement. [67-68]

Fibroblast growth factors (FGFs) superfamily comprises now 22 structurally and evolutionarily correlated proteins. They are synthesized predominantly by fibroblasts, but it can be also produced by other cells, including, endothelial cells, macrophages, osteoblasts and by platelets. FGF have mitogenic and chemotactic effects on endothelial cells, fibroblasts and vessel smooth muscle cells and is considered one of the most important angiogenetic factorwith VEGF and angiopoietins). [69]

Insulin-like growth factors (IGFs) are present in large quantities in bone; they stimulate the proliferation of osteoblastic cells and the expression of collagen type I, thus participating in the process of bone growth, development and repair. [70]

Platelet-derived growth factors (PDGFs) owe their name as they were first discovered within platelet α granules, but they are also expressed by macrophages, endothelial and epithelial cells. They are released during the platelet aggregation at the beginning of hemostasis process and induce proliferation of fibroblasts, smooth muscle cells, macrophages and neutrophils. Based on their effect on fibroblast and endothelial cells, in wound healing these growth factors are responsible for the formation of granulation tissue.. and contribute to the bone formation. [71]

PDGF also plays an important role in promoting re-epithelialization by positively regulating the production of IGF, which promotes keratinocyte mobilization, and thrombospondin-1, which delays proteolytic degradation and promotes the proliferative response in wounds. [72]

PDGF positively regulates the production of matrix metalloproteases (MMPs) involved in the mechanism of tissue remodeling. A reduction in PDGF levels has been demonstrated in chronic wounds, this further corroborates the importance of this factor in the healing process, and why the use of recombinant PDGF has been introduced to support the healing of diabetic ulcers. [73]

TGF-β is part of a large family of polypeptides with multiple regulatory effects on cell proliferation and differentiation. This family includes the three isoforms TGF-β 1, TGF-β2, TGF-β3, inhibins, activins, and BMPs.

TGF-β are released from activated platelets, fibroblasts, monocytes/macrophages and stimulate chemotaxis, angiogenesis, extracellular matrix deposition, fibroblast proliferation, myofibroblast differentiation, granulation tissue formation, and re-epithelialization. [74]

VEGF (vascular endothelial growth factor) is the most important angiogenic factor. During wound repair it is produced in large amounts by keratinocytes, endothelial cells, fibroblasts, smooth muscle cells, platelets, neutrophils, and macrophages and mainly stimulates proliferation, migration, and differentiation of endothelial cells by binding to specific tyrosine kinase receptors. [75]

VEGF is important in wound healing because it promotes the initial events of angiogenesis, especially the migration and proliferation of endothelial cells, mainly in response to hypoxia in order to restore proper tissue blood perfusion. [76]

In particular, it has been shown that in venous stasis ulcers there is a reduction in the production and levels of VEGF, and its introduction at the level of the lesion appears to promote healing resulting in re-epithelialization. The same effect has been shown in the management of diabetic ulcers, with reduced healing time and prevention to limb amputation. [77] TGF-β 1 and TGF-β 2 isoforms are produced by osteoblasts and then are incorporated into the organic matrix, which then mineralizes, both during bone development and during healing processes. They can both stimulate and inhibit osteoblast proliferation. [78]

Cytokines

The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the proinflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized. Major anti-inflammatory cytokines include interleukin (IL)-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13. [79]

Diabetes could be associated to immunologic abnormalities: T cell abnormalities are believed to be the major cause of autoimmune disease in type 1 diabetes, leading to the destruction of pancreatic islets. In T2DM, inflammation and activation of monocytes are postulated to be important for enhancing insulin resistance and may contribute to the loss of insulin secretory function by islet cells. Increases in inflammation, such as activation of monocytes and increased levels of inflammatory markers, C-reactive protein, plasminogen activator inhibitor-1, and other cytokines, were reported in insulin-resistant states without diabetes. One possible mechanism is that abnormal levels of metabolites, such as lipids, fatty acids, and various cytokines from the adipose tissue, activate monocytes and increase the secretion of inflammatory cytokines, enhancing insulin resistance. Abnormalities in innate immunity might also participate in the development of diabetic complications. [80]

Cytokines are among the most important effector and messenger molecules in the immune system. They profoundly participate in immune responses during infection and inflammation, protecting against or contributing to diseases such as allergy, autoimmunity, and cancer.

Although the precise role of inflammation in the development of diabetic microvascular diseases is still unclear, it is likely that inflammation induced by diabetes and insulin resistance can accelerate atherosclerosis in patients with diabetes. [81]

GM-CSF

The **granulocyte-macrophage colony-stimulating factor** (GM-CSF) serves in inflammation as a communication conduit between tissue-invading lymphocytes and myeloid cells. Even though lymphocytes are the instigators of chronic inflammatory disease, GM-CSF-activated phagocytes are well equipped to cause tissue damage. [82]

GM-CSF can direct the activation, proliferation and differentiation of myeloid-derived cells. It is also responsible for maturation and function of professional antigen presenting cells thereby impacting adaptive immune responses, while assisting to maintain epithelial barrier function. GM-

CSF in combination with other endogenous cytokines and secondary stimuli, such as tumor necrosis factor can modulate pro-inflammatory monocyte priming via chromatin remodeling and enhanced transcriptional responses, a concept termed trained immunity. [83]

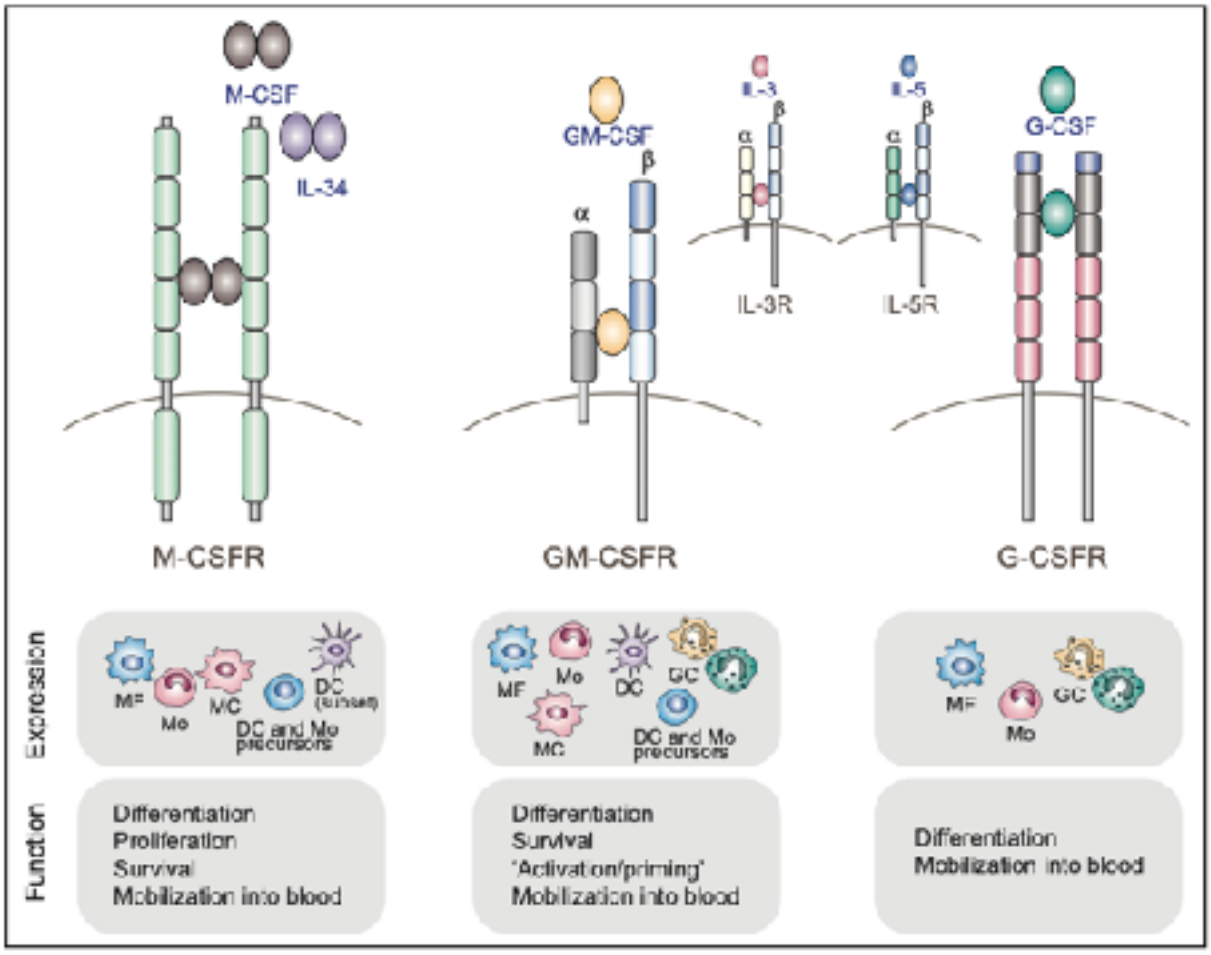


Figure 7. The CSF Super Family [82]

GM-CSF is virtually undetectable in the systemic circulation. [84] Instead, GM-CSF is produced and active locally at sites of tissue inflammation. Increased concentrations of GM-CSF are found in skin biopsies from patients with late-phase cutaneous reactions, in affected joint fluid in rheumatoid arthritis (RA).Circulating GM-CSF can also be rapidly and markedly raised in response to endotoxin.

These observations are concomitantly supported by data arising from several preclinical models, which highlight the importance of GM-CSF in promoting inflammation. [85-86]

The central role of GM-CSF in tissue inflammation has justified the development of clinical trials evaluating GM-CSF blockade in RA and MS; neutralization of GM-CSF in RA is beneficial. There is, however, only a limited understanding of the molecular and cellular underpinnings of the effect GM-CSF has in inflammation and immunopathology.[87]

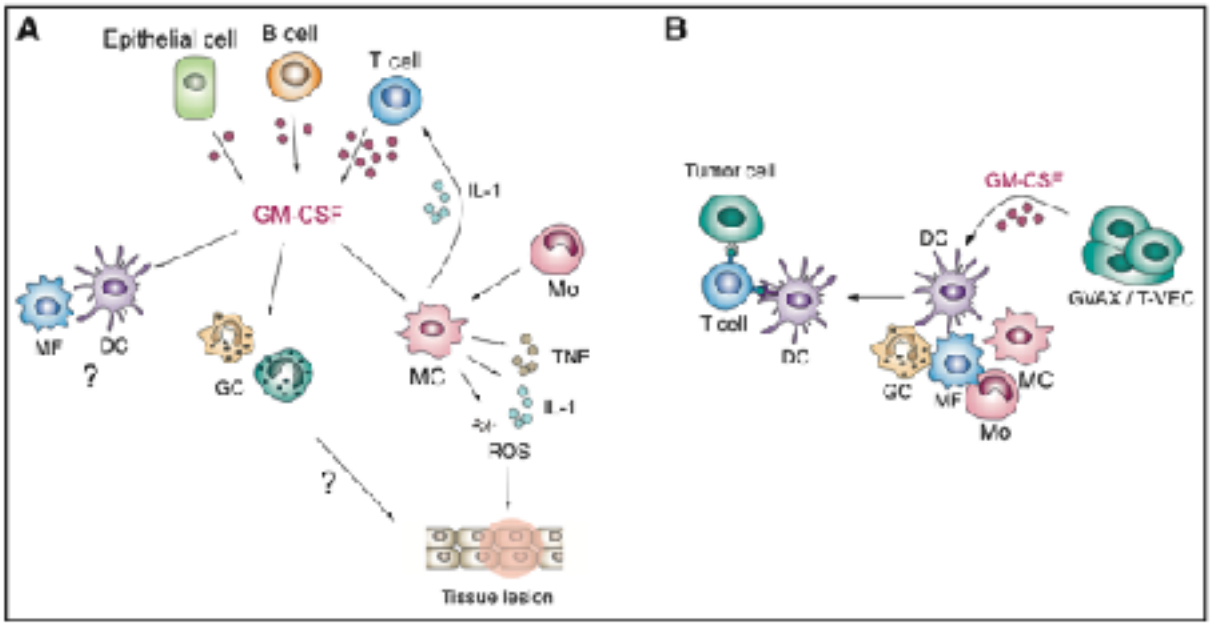


Figure 8. GM-CSF in Inflammation and Cancer [82]

TNF-α

Tumor necrosis factor-alpha (TNF-α) is an inflammatory cytokine generated by macrophages/ monocytes in response to acute inflammation. It initiates a cascade of signaling events within cells, ultimately resulting in necrosis or apoptosis.

The protein is also important for resistance to infection and cancers. TNF-α exerts many of its effects by binding, as a trimer, to either a 55 kDa cell membrane receptor termed TNFR-1 or a 75

kDa cell membrane receptor termed TNFR-2. Both these receptors belong to the so-called TNF receptor superfamily. The superfamily includes FAS, CD40, CD27, and RANK. The defining trait of these receptors is an extra cellular domain comprised of two to six repeats of cysteine rich motifs. Additionally, a number of structurally related decoy receptors exist that act to sequester TNF molecules, thereby rescuing cells from apoptosis. The crystal structures of TNF-α, TNF beta, the extracellular domain of TNFR-1 (denoted sTNFR-1), and the TNF beta sTNFR-1 complex have been defined by crystallography. [88]

TNF-α is ubiquitous in the human body and plays a significant role in various physiological and pathological processes. However, TNF-α-induced diseases remain poorly understood with limited efficacy due to the intricate nature of their mechanisms. N6-methyladenosine (m6A) methylation, a prevalent type of epigenetic modification of mRNA, primarily occurs at the post-transcriptional level and is involved in intranuclear and extranuclear mRNA metabolism. Evidence suggests that m6A methylation participates in TNF-α-induced diseases and signaling pathways associated with TNF-α. [89]

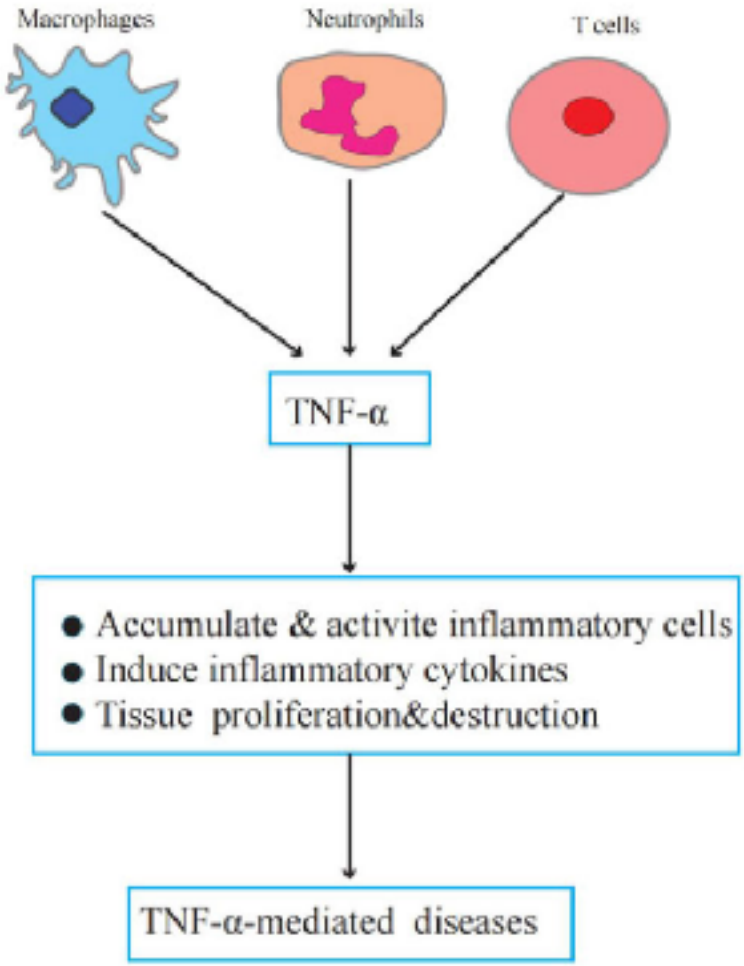


Figure 9. The role of TNF-α in TNF-α-induced diseases. Macrophages, neutrophils, and T cells secrete TNF-α. TNF-α promotes inflammatory cell activation and accumulation induces inflammatory factor production, forms a local inflammatory environment, and leads to tissue proliferation and destruction. [89]

IL-2

Interleukin-2 (IL-2) exerts crucial functions during immune homeostasis via its effects on regulatory T (Treg) cells, and the optimizing and fine-tuning of effector lymphocyte responses. Thus, somewhat, low doses of recombinant IL-2 have been used for Treg cell-based immunosuppressive strategies against immune pathologies, while high-dose IL-2 has shown some success in stimulating anti-tumor immune responses. IL-2 is a 15.5–16-kDa, four-helix-bundle cytokine that exerts its actions via binding to various IL-2Rs, monomeric, dimeric, or trimeric. Interaction of IL-2 with CD25 alone does not induce a signal; hence, isolated membrane-bound or soluble CD25 molecules might serve as scavenger or decoy receptors for IL-2. On triggering of IL-2R, signal transduction occurs via three major pathways, involving: Janus kinase (JAK)–signal transducer and activator of transcription (STAT), phosphoinositide 3- kinase (PI3K)–AKT, and mitogen-activated protein kinase (MAPK). [90]

Several immune cells have been shown to secrete IL-2 when activated, including T cell receptor (TCR) /b^+ and TCRgd^+ T cells, natural killer (NK) cells, NKT cells, dendritic cells (DCs), and mast cells . At resting conditions, $CD4^+$ helper T (Th) cells are the main source of the constant but low levels of IL-2. On immune activation, IL-2 production rapidly rises. [90]

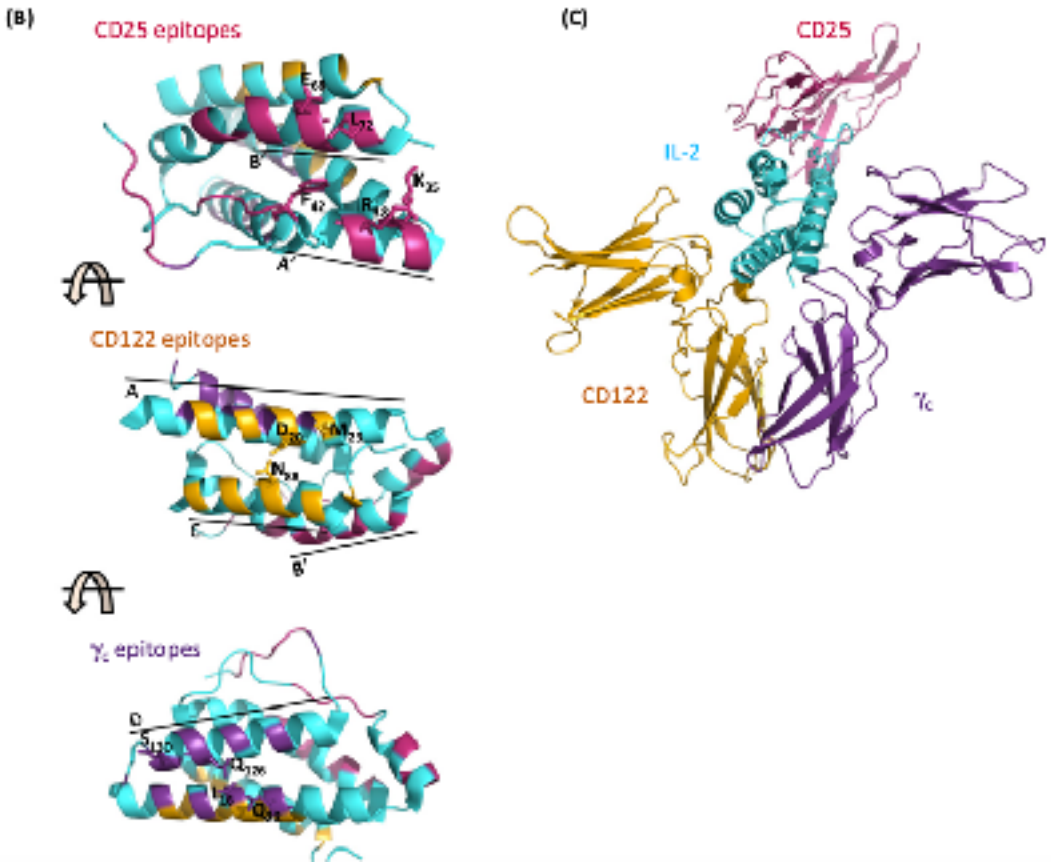


Figure 10. Sequence and Structure of IL-2 in Association with its Receptor Subunits [90]

IL-4

Interleukin-4 (IL4) drives TH2 responses, mediates the recruitment and activation of mast cells, and stimulates the production of IgE antibodies in plasma cells. Also IL-4 has masked inhibitory effects

on the expression and release of the pro inflammatory cytokines, it is able to block or suppress the monocyte derived cytokines, including IL-1, TNF-a, IL-6, IL-8 . It is a pleiotropic cytokine involved in host protection from gastrointestinal nematodes. [91-92]

IL 5

Interleukin-5 (IL-5) is a lineage-specific cytokine for eosinophilisis and plays an important part in diseases associated with increased eosinophils, such as asthma. Human IL-5 is a disulphide-linked homodimer with 115 amino-acid residues in each chain. The crystal structure reveals a novel two-domain structure, with each domain showing a striking similarity to the cytokine fold found in granulocyte macrophage and macrophage colony-stimulating factors, IL-2, IL-4, and human and porcine growth hormones. IL-5 is unique in that each domain requires the participation of two chains. The IL-5 structure consists of two left-handed bundles of four helices laid end to end and two short beta-sheets on opposite sides of the molecule. The C-terminal strand and helix of one chain complete a bundle of four helices and a beta-sheet with the N-terminal three helices and one strand of the other chain. The structure of IL-5 provides a molecular basis for the design of antagonists and agonists that would delineate receptor recognition determinants critical in signal transduction. This structure determination extends the family of the cytokine bundle of four helices and emphasizes its fundamental significance and versatility in recognizing its receptor. [93]
Activation of Th2-pathways is at the core of type 2 inflammation, producing excessive amounts of the cytokines IL-4, IL-5, and IL-13. IL-5 induce eosinophil activation, maturation, and recruitment, while IL-4 and IL-13 are involved in goblet cell metaplasia, airway smooth muscle (ASM) contractility, and airway hyperresponsiveness (AHR) [93-94]

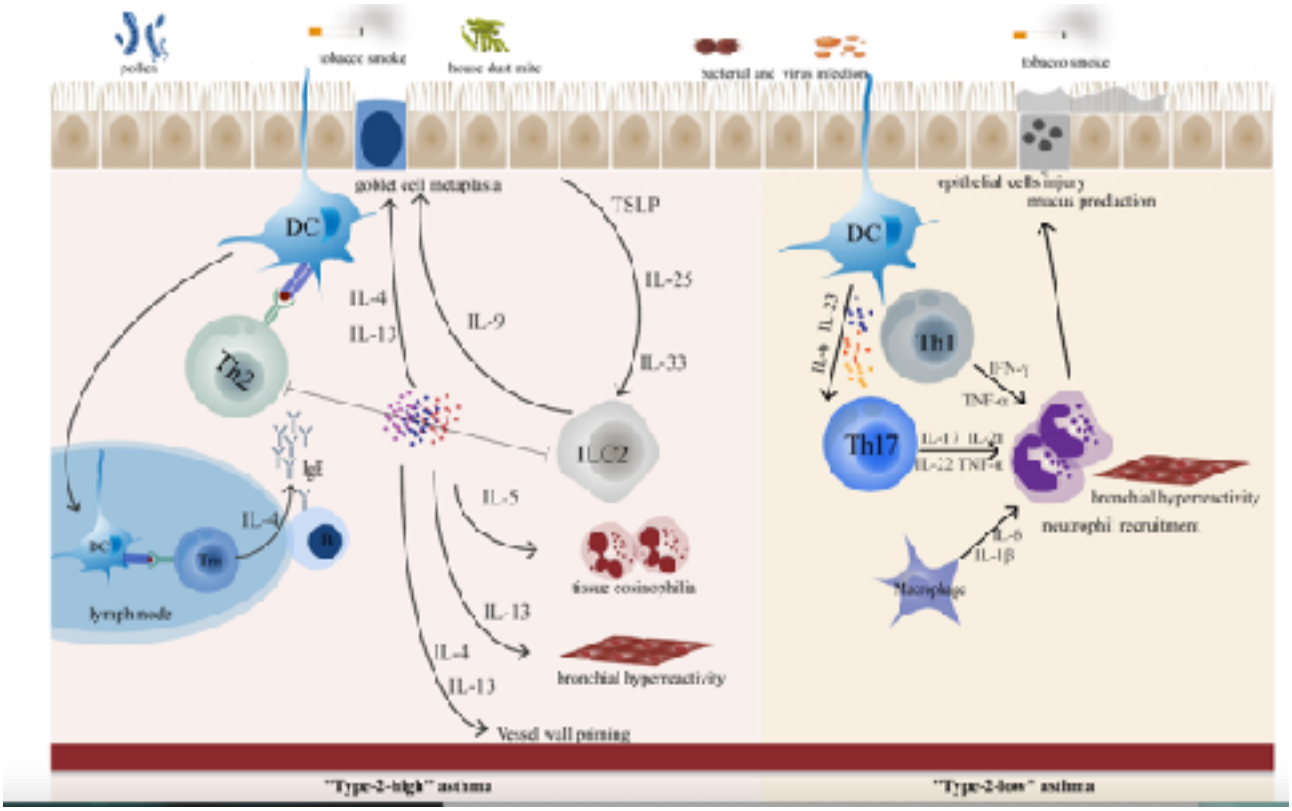


Figure 11. In "Type-2 high" asthma, epithelial-derived TLPS, IL-25, and IL-33 can awaken ILC2. Cytokines produced by epithelial cells promote DC function, polarize CD4+ T cells and promote the polarization of Th2 cells. TFH cells control IgE synthesis by secreting IL-4 to allergen-specific B cells. [94]

IL-3, IL-5, and GM-CSF are important for allergic inflammation. The receptors for human IL-5, IL-3, and GM-CSF are members of the hematopoietin receptor superfamily and are comprised of a cytokine-specific alpha chain and the common beta chain that is shared among these cytokines for signaling. Each of these cytokines contributes to the differentiation and function of leukocyte subpopulations and have clinical importance in protective immunity and in the pathophysiology of a spectrum of immunologic diseases that are as diverse as allergy and asthma, pulmonary alveolar proteinosis, neurodegenerative diseases, and malignancies.[95]

IL-6

Interleukin-6 (IL-6), an important mediator of various pathways, especially in intestine and digestive, participates in the interactions between different kinds of cells and closely correlates

with intestinal physiological and pathological condition. Nearly all of the nutrient digestion and absorption are carried out in the intestine tract and abundant microbes parasitic in the gastrointestinal tract may be involved in the process. Through this complex process microbial metabolites, together with remains in the digestive tract, interact with host cells widely and even a seemingly small dysregulation may lead to the breakdown of intestinal homeostasis. The maintenance of this precise balance requires the control of epithelial cells via different immune mechanisms constituting the intestinal immune barrier. As a polypeptide, IL-6 consists of α and β chains whose structure has been fully elucidated and used for targeted therapy widely. In fact, besides the role in physiological conditions, IL-6 also pathologically promotes the mucosa preservation and facilitates mucosal repairing, relevant to inflammation associated diseases.

The gut wall is protected by a well-developed immune system and an intestinal mucosal barrier composed of epithelial cells and mucus layers secreted by goblet cells, and this contains commensal bacteria that regulate the passage of fluids, macromolecules and antigens. It may help in limiting bacterial colonization by releasing mucus, antimicrobial peptides and immunoglobulins. Normally it assists with the elimination of pathogens but long-term inflammation such as the CD and UC may lead to a serious of pathological manifestation seriously affect the patients quality of life. IL-6 is normally known to aggravate inflammation by both directly driving lymphocytes proliferation as well as differentiation and directly/indirectly through nervous system. Nevertheless, the anti-inflammatory functions like fresh for many people, was gradually unveiled. Like a key hub in the busy transporting net, IL-6 acts as a mediate in many functions in the gastrointestinal immune barrier. [96]

Also, the role of IL-6 in health and disease has been under a lot of scrutiny in recent years, particularly during the recent COVID-19 pandemic. The inflammatory pathways in which IL-6 is involved are also partly responsible of the development and progression of rheumatoid arthritis, opening interesting perspectives in terms of therapy. Anti-IL-6 drugs are being used with variable degrees of success in other diseases and are being tested in RA. Results have been encouraging, particularly when anti-IL-6 has been used with other drugs, such as metothrexate. [97]

IL-9

Interleukin 9 (IL-9) is a cytokine secreted by CD4+ helper cells that acts as a regulator of a variety of hematopoietic cells. IL-9 is a pleiotropic cytokine produced in different amounts by a wide variety of cells including mast cells, NKT cells, Th2, Th17, Treg, ILC2, and Th9 cells. Th9 cells are considered to be the main CD4+ T cells that produce IL-9. IL-9 exerts its effects on multiple types of cells and different tissues. To date, its main role has been found in the immune responses against parasites and pathogenesis of allergic diseases such as asthma and bronchial hyperreactivity. Additionally, it induces the proliferation of hematologic neoplasias, including Hodgkin's lymphoma in humans. [98] However, IL-9 also has antitumor properties in solid tumors such as melanoma. IL-9-producing CD4+ T cells have been considered to represent a distinct T helper cell (TH cell) subset owing to their unique developmental programme in vitro, their expression of distinct transcription factors and their copious production of IL-9. It remains debatable whether these cells represent a truly unique TH cell subset in vivo, but they are closely related to the T helper 2 (TH2) cells that are detected in allergic diseases. In recent years, increasing evidence has also indicated that IL-9-producing T cells may have potent abilities in eradicating advanced tumours, particularly melanomas. [99]

Naïve CD4+ T cells are pleiotropically divided into various T helper (Th) cell subsets, according to their pivotal roles in the regulation of immune responses. The differentiation of Th9 cells, IL-9 producing subset, can be impacted by specific environmental cues, co-stimulation with transforming growth factor β (TGF-β) and IL-4, and other regulatory factors. Although IL-9 has been recognized as a classical Th2-related cytokine, recent studies have indicated that IL-9-producing cells contribute to a group of autoimmune disorders including systemic lupus erythematosus, multiple sclerosis, inflammatory bowel diseases, rheumatoid arthritis and psoriasis. Studies of Th9 cells in autoimmune diseases, although in their infancy, are expected to be of growing interest in the study of potential mechanisms of cytokine regulatory pathways and autoimmune pathogenesis. Several in vitro and in vivo pre-clinical trials have been conducted to explore

potential therapeutic strategies by targeting the IL-9 pathway. Specifically, anti-IL-9 monoclonal antibodies (mAbs) and IL-9 inhibitors may potentially be used for the clinical treatment of allergic diseases, autoimmune diseases or cancers. Here, we review recent research on Th9 cells and IL-9 pertaining to cell differentiation, biological characteristics and pivotal cellular inter-relationships implicated in the development of various diseases. [100]

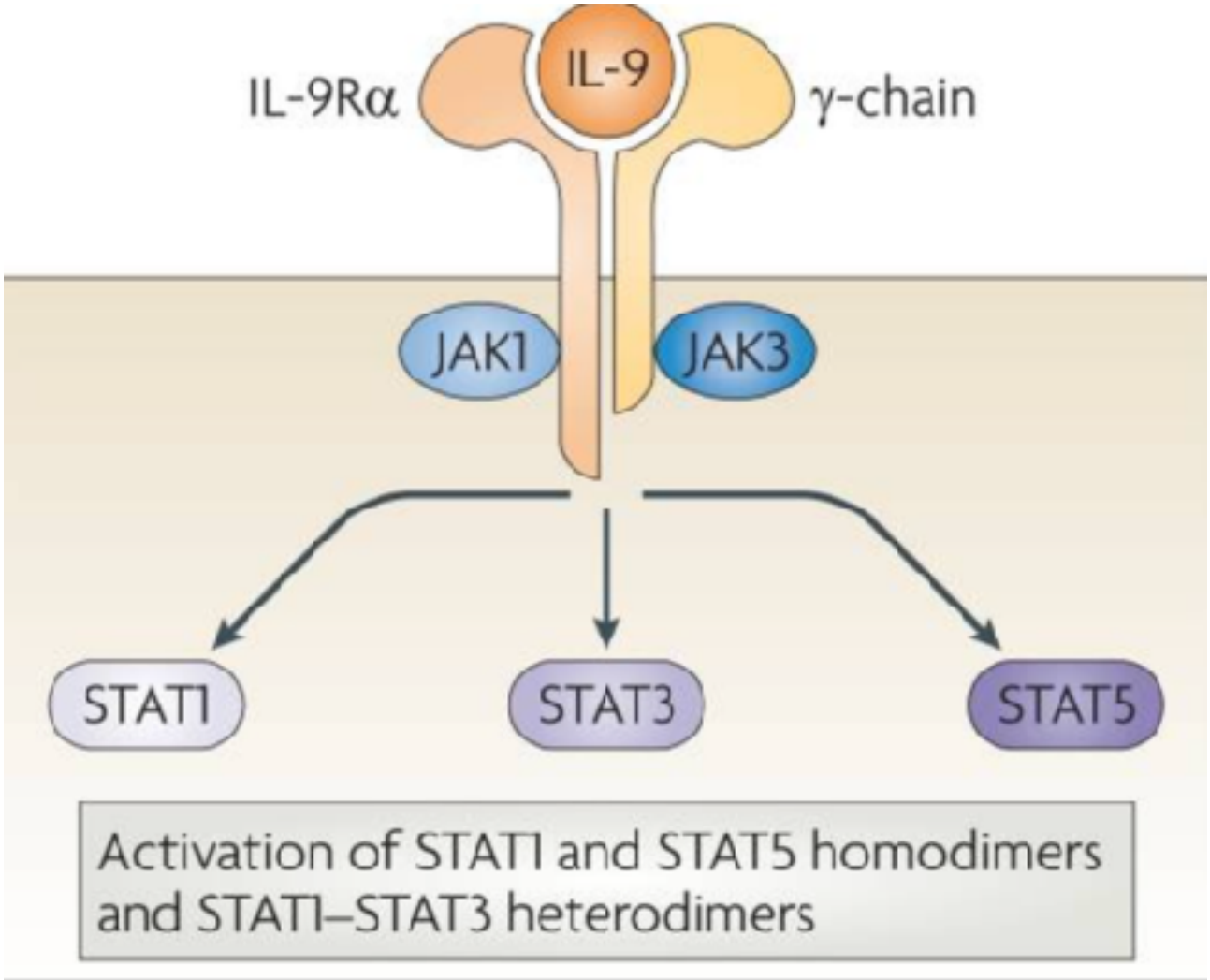


Figure 12. The IL-9 receptor signalling complex

Recent studies have highlighted a crucial regulatory role of the cytokine IL-9 in driving immune responses in chronic inflammatory and autoimmune diseases at mucosal surfaces. IL-9 activates various types of immune and non-immune cells carrying the membrane bound IL-9R. IL-9 signaling plays a pivotal role in controlling the differentiation and activation of these cells by inducing the

Jak/STAT pathway. In particular, IL-9 induces activation of T helper cells and affects the function of various tissue resident cells such as mast cells and epithelial cells in the mucosa. Importantly, recent findings suggest that blockade of IL-9 signaling is effective in treating experimental models of autoimmune and chronic inflammatory diseases such as inflammatory bowel diseases, allergic disorders such as food allergy and asthma. Thus, blockade of IL-9 and IL-9R signaling emerges as potentially novel approach for therapy of inflammatory diseases in the mucosal immune system. [101]

IL-10

The interleukin-10 (IL-10) family of cytokines included nine members: IL-10, IL-20 subfamily members IL-19, IL-20, IL-22, IL-24, and IL-26, and the distantly related cytokines IL-28A, IL-28B, and IL-29, which are more commonly classified as type III interferons (IFNs) and designated as IFN-l2, IFN-l3, and IFN-l1. These cytokines are produced by both innate and adaptive immune cells and function as effector and regulatory molecules of the immune system. Studies on these cytokines have revealed their profound and indispensable functional roles in infection, inflammation, tissue homeostasis, autoimmunity, and cancer.

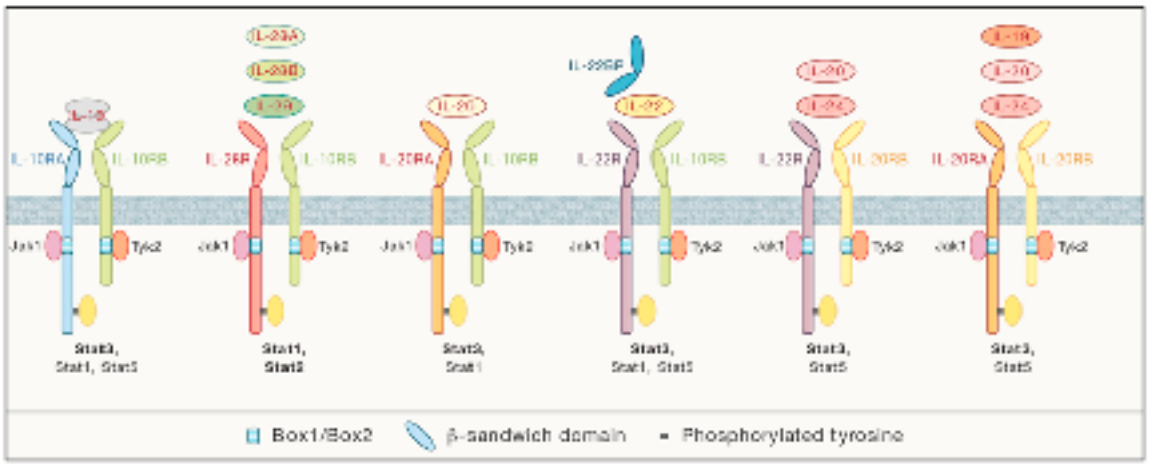


Figure 13. IL-10 Family Cytokines [102]

IL-10 family cytokines can be roughly divided into three sub- groups based on their functions. The first group contains only IL-10, which targets both innate and adaptive immune responses and exerts immunosuppressive functions to reduce tissue damage caused by excess and uncontrolled inflammatory effector responses, especially during the resolution phase of infection and inflammation and to maintain homeostasis to gut microbes. The second group comprises the IL-20 subfamily cytokines, including IL-19, IL-20, IL-22, IL-24 and IL-26. These cytokines primarily act on tissue epithelial cells and stroma cells to induce innate host defense mechanisms that control the invasion of extracellular cellular pathogens. In addition, they play a broad patrolling role to protect barrier integrity and tissue homeostasis through the promotion of proliferation, remodeling, and repair of various tis- sues and organs. Lastly, the IL-28 subfamily of cytokines, including IL- 28A, IL-28B, and IL-29, are commonly classified as type III IFNs and designated as IFN-l2, IFN-l3 and IFN-l1, respectively . They share largely overlapping biology and down- stream signaling pathways with type I IFN family cytokines but preferentially target tissue epithelial cells. IL-10 has both immunosuppressive and stimulatory effects, and IL-22, while promoting tissue repair in the intestine, induces acanthosis during skin inflammation. [102]

IL-12

The interleukin 12 (IL-12) family is unique in having the only heterodimeric cytokines, including IL-12, IL-23, IL-27 and IL-35. This feature endows these cytokines with a unique set of connections and functional interactions not shared by other cytokine families. Despite sharing many structural features and molecular partners, cytokines of the IL-12 family mediate diverse functional effects. [103] The balance of proinflammatory cytokines interleukin IL-12 and IL-23 plays a key role in shaping the development of antitumor or protumor immunity. In particular, have been discussed the mechanism by which IL-23 promotes tumor growth and metastases and how the IL-12/IL-23 axis of inflammation can be targeted for cancer therapy. [104]

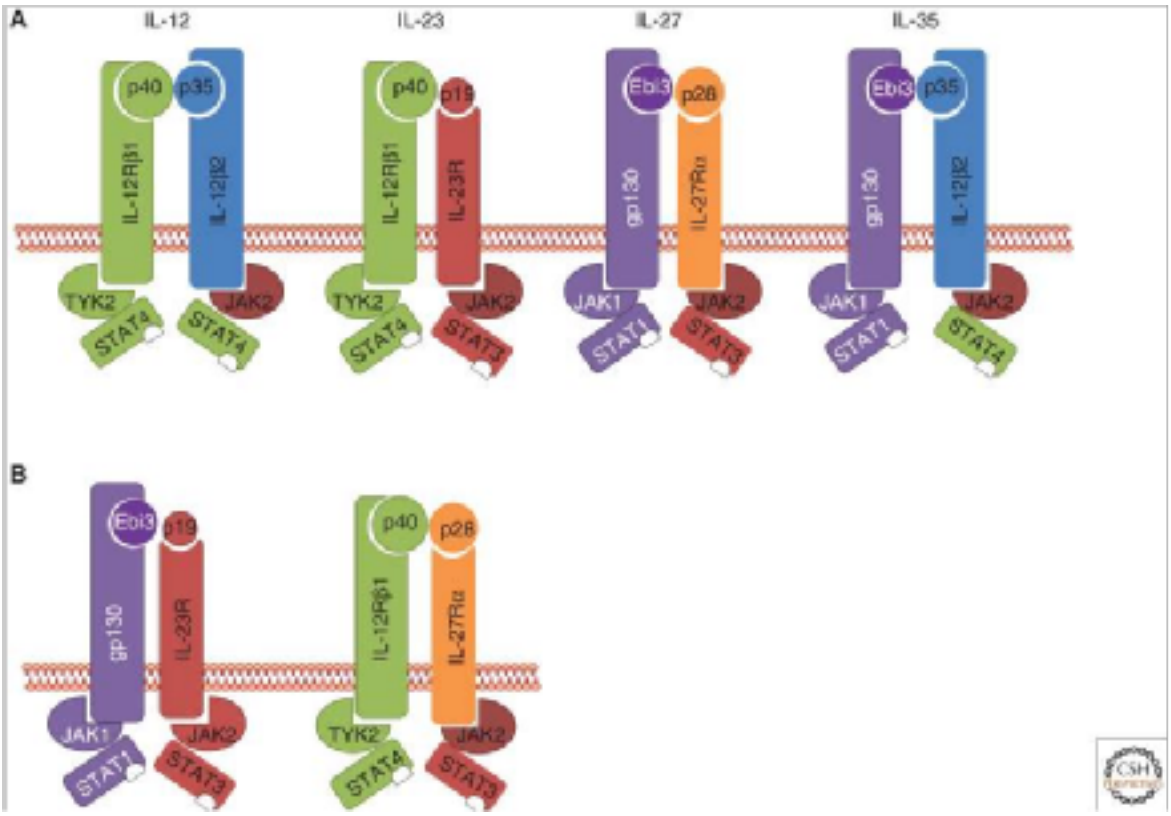


Figure 14. Schematic representation of the interleukin (IL)-12 cytokine family and their receptors, and associated Janus kinase and signal transducers and activators of transcription (JAK-STAT) signaling partners. [104]

Being a pro-inflammatory cytokine, IL-12 has been proven to be effective in inhibiting virus infections and ameliorating the infection symptoms when administered prophylactically and post-infection. Relative to other cytokines (such as IL-18, TNF-α and IFN-α, -β, and -γ), IL-12 is synthesized more significantly in hosts to initiate the antiviral immune response as early as day-1 post-infection. [105]

IL- 17

The IL-17 cytokine family is formed by six members. As numerous other cytokines, IL-17 cytokines are part of the adaptive and innate immunity IL-17A and IL-17F are significantly implicated in immune responses to infectious pathogens and in the pathogenesis of inflammatory autoimmune diseases like psoriasis. Furthermore, evidence indicates an additional proinflammatory activity of IL-17C and IL-17E within the pathogenesis of psoriasis.

Interleukin 17 is implicated in immune responses to infectious pathogens and in the pathogenesis of inflammatory autoimmune diseases like psoriasis. Thus, anti-IL-17A therapy not only improves skin manifestations of psoriasis, but also cardiovascular inflammation as well as metabolic factors and different domains of psoriatic arthritis (PsA) including peripheral arthritis, enthesitis, dactylitis, and axial involvement. [106]

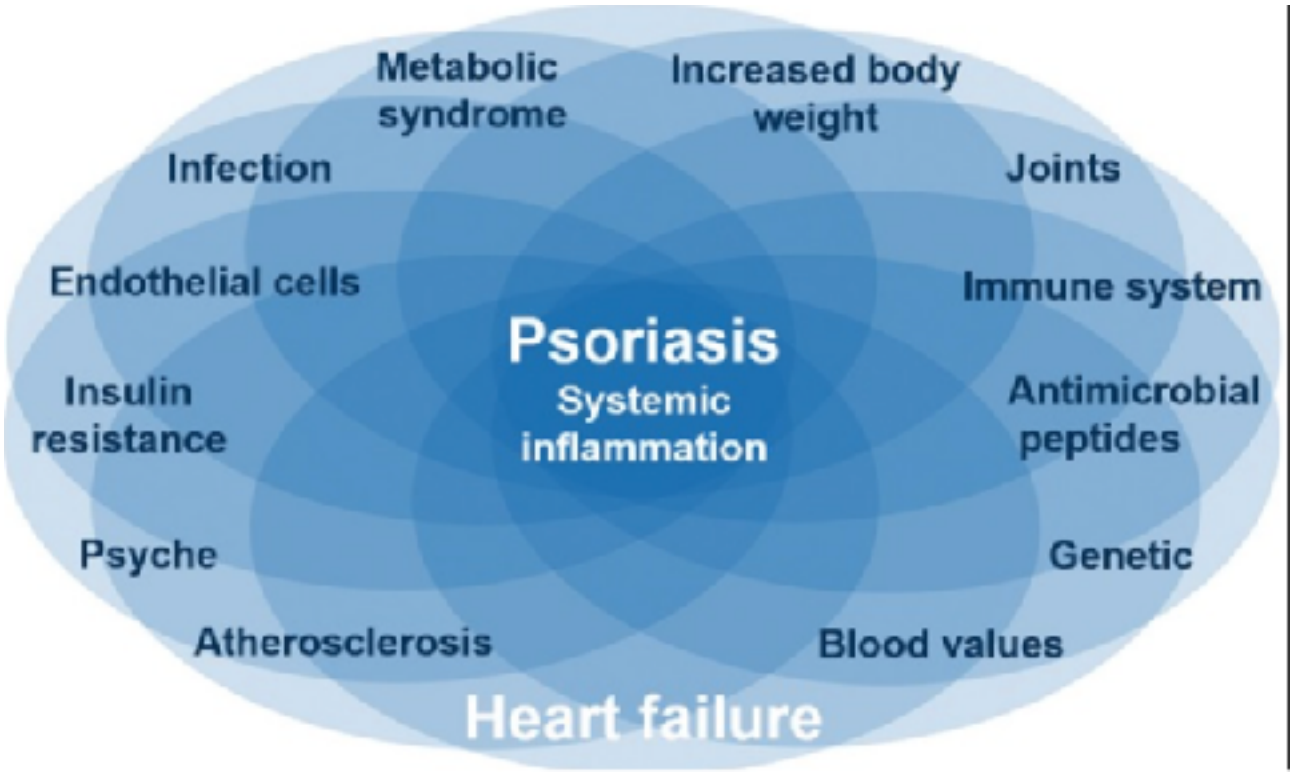


Figure 15. Common pathways for psoriasis, cardiovascular disease, and metabolic syndrome. [106]

Cytokins and Diabetic ulcers

A recent review examines the role of growth factors and cytokines in the management of Diabetic Foot Ulcers, such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and Insulin like growth factor (IGF). Taking this a step further, the role of Hypoxia-inducible factors (HIFs), Transforming growth factor beta 1 (TGF-β-1) and other growth factors have also been examined, with regard to the treatment of diabetic foot ulcers. The roles of these above-mentioned growth cytokines have been analyzed by studying various scholastic articles. The complete process of wound healing is implemented and regulated by numerous cytokines and human growth factors. The findings of the study indicate that wound healing of diabetic foot ulcers is a complex and extremely challenging biological and molecular process that involves coordinated efforts of multiple cell types. The therapeutic effects of various growth factors in the clinical management of wounds are chronic venous ulcers, pressure ulcers, and diabetic foot ulcers. It has been concluded that altercations of various cytokines are found in patients enduring diabetic foot ulcers. In a similar way, changes in the level of cytokines are also found in patients suffering from other diabetic complications such as diabetic nephropathy, retinopathy, and neuropathy. Subsequently, the diabetic wound healing process can be accelerated by regulating the levels of the cytokines. [107]

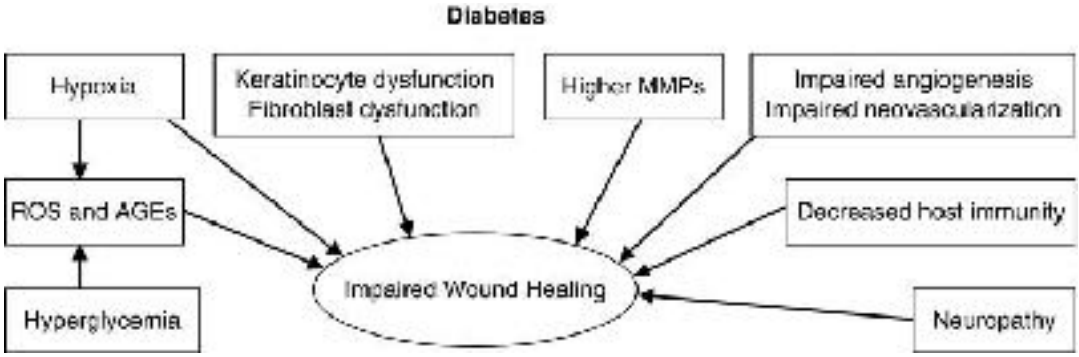


Figure 16. Impaired wound healing in diabetic patients

Diabetes and oral manifestations

More than 90% of diabetic patients were found to have oral manifestations. It is known that DM severely damages oral tissues causing periodontal disease, tooth loss, xerostomia, caries, burning mouth disorder, taste and salivary gland dysfunction, delayed wound healing, lichen planus, geographic tongue, and candidiasis. The evidence is mounting about a strong bidirectional relationship between DM and periodontal disease. [108]

Research has shown a greater prevalence of oral mucosal disorders in patients with diabetes mellitus than non-diabetic population: 45-88% in patients with T2DM compared to 38.3-45% in non-diabetic subjects and 44.7% in type 1 diabetic individuals compared to 25% in the non-diabetic population. [109]

An important problem of DM patient undergoing dental procedures is the delay in wound healing after tooth extraction. The process behind the reason for slow wound healing in DM patients is complex. The elevated glucose microenvironment can prolong the healing of tooth extraction sockets. Therefore, the promotion of healing up tooth extraction sockets is of great clinical importance to the patients with diabetes mellitus. The current evidence indicates the mechanism of the recovery period of extraction sockets in 109 conditions from physiological, inflammation, immune, endocrine and neural aspects. Diabetic alteration of wound healaing involve the alterated expression of all the cells normally involved in the healing as well as a dysregulation in the production of grow factors and cytokines. [110]

Post extractive socket of diabetic patients required more time to health compared to healthy patients with an increased risk of over infection and drugs required income. Commonly, the

diversity of healing between DM and non-DM patients is maximum 7 days after the extraction. Diabetic patients were found to have a larger socket size than the non-diabetic group on postoperative day 7, which suggested a slow healing process than non-diabetic and prediabetic patients. This supported the fact that higher the glycemic levels could delay healing. [111]

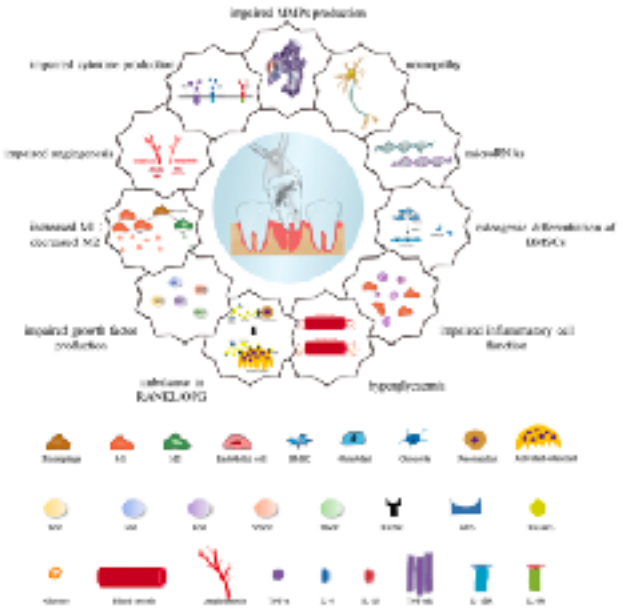


Figure 17. Factors responsible for the healing process of diabetic extraction sockets [110]

DM patients have more complications and longer period of post-extraction wound healing than healthy patients followed by unfavorable post-extraction alveolus changes. Men with T2DM showed a 29% increased risk of periodontitis compared to those without. Data od some studies suggest a strong connection between the presence of periapical radiolucency on root filled teeth amongst diabetics as determined by the pooled OR. [112]

Reports on the wound healing in diabetes shown the mechanism of the protracted wound healing of the oral mucosa in diabetes. Delayed vascularization, reduction in blood flow, decline in innate immunity, decreases in growth factor production, and psychological stresses may be involved in the protracted wound healing of the oral mucosa in diabetics [113]

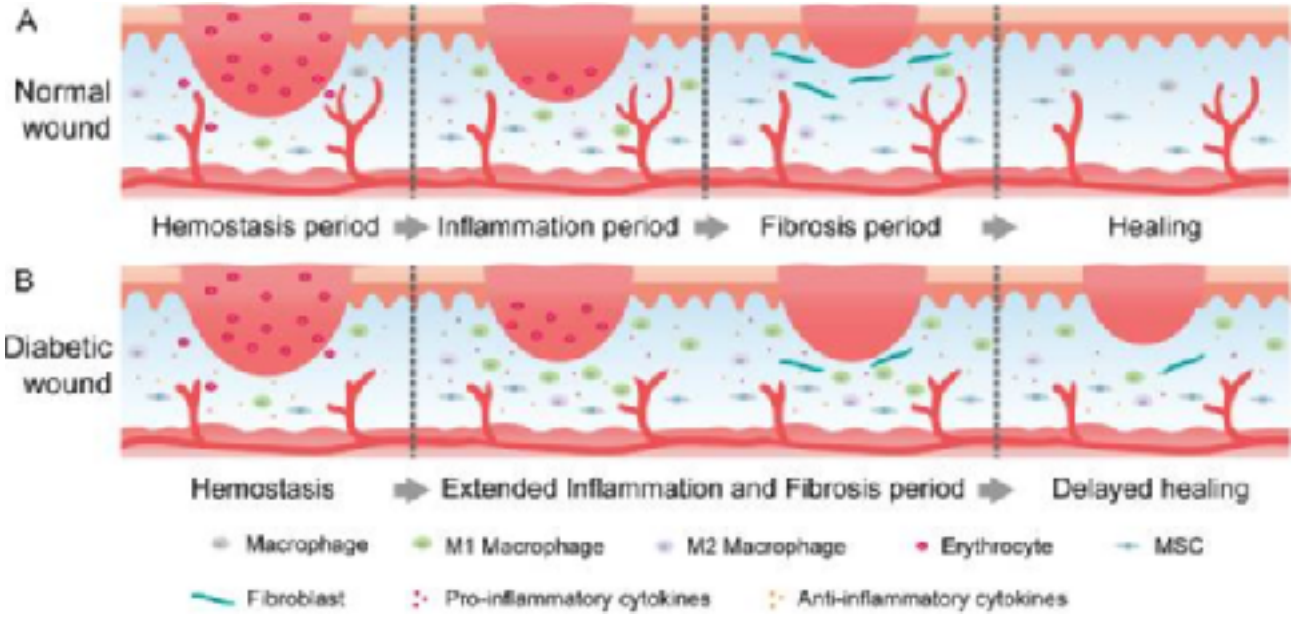


Figure 18. Lower healing process in diabetic patients [111]

Due to bone alteration DM patients with poorly controlled diabetes suffer from impaired osseointegration, elevated risk of peri-implantitis, and higher level of implant failure. [114] Specially, patients with poorly controlled diabetes mellitus suffer more often from peri-implantitis, especially in the post-implantation time. Moreover, these patients show higher implant loss rates than healthy individuals in long term. Whereas, under controlled conditions success rates are similar. Perioperative anti-infective therapy, such as the supportive administration of antibiotics and chlorhexidine, is the standard nowadays as it seems to improve implant success. [115]

Commonly, different materials and strategies are adopted to better seal the post extractive site. Bone grafting in post-extractive site improves tissue regeneration. Soft tissue sealing of the grafted post-extractive alveolus is supposed to limit microbiological contamination from the oral cavity and to stabilize the coagulum [116]

Different procedures were proposed over the years, in DM patients for reuse time of healing including the use of grow factors, synthetic drugs and laser therapy. However, despite positive benefits, the results are sometimes controversial and, therefore, research on the topic remains open. [117-119]

T2DM has a deleterious effect on dental implant integration into alveolar bone, thought to arise from impaired osteoblast function and consequent reduced bone turnover. However, whether controlling blood glucose with antidiabetic drugs is sufficient to improve implant integration is unclear. This study was designed to evaluate implant integration using diabetic rats with/without an antidiabetic drug. [120-121]

Chewing well is essential for successful diet therapy and control of blood glucose level in patients with diabetes. In addition, long-term hyperglycemia is a risk factor for microvascular complications, which are the main cause of morbidity and mortality in these patients. Hence, it is plausible that masticatory disorder may be relevant to diabetic microvascular complications which is caused by long-term hyperglycemia. Patients with T2DM who developed diabetic neuropathy had significantly reduced masticatory efficiency. Effective mastication is an important factor in successful diet therapy for diabetes. To prevent the progression of diabetic complications, especially in patients with diabetic neuropathy, it may be necessary to combine individualized therapies from dentists and nutritionists with consideration for the level of masticatory dysfunction. [122]

Possible treatment for diabetic wounds

In the past, the treatment of diabetic wounds was not unlike traditional wound treatment, it mainly uses dry dressings such as bandages and gauze with antibiotic supplements to absorb wound exudates and prevent infection. However, as a passive intervention strategy, the use of dry dressings fails to adapt to the diabetic wound microenvironment and may cause secondary damage due to adhesion between dressings and wounds [123].

In addition, the lack of knowledge of the most appropriate treatment and management of diabetic wounds by health care professionals contributes to delayed wound healing. Recently, tissue engineering strategies are seeking advanced bioactive platforms that can artificially mimic or fully restore the function of native skin tissue. The healing of diabetic wounds does not follow the

normal phases of wound healing as acute wounds do, and diabetic wound healing may stall at a particular stage of the process, which prolongs wound healing. To address this concern, modern functional wound dressings have attracted great attention in this field.

The significant therapeutic effects of modern functional wound dressings, including nanofibre, hydrophilic colloids, foams, and hydro- gels, have been demonstrated in diabetic wounds.

These new dressings can provide a moist, antibacterial healing environment and carry bioactive molecules or drugs to accelerate wound healing. The capacity of wet dressings to speed up the healing process has been tested [124].

The method of delivering drugs through nanomaterials to reduce the dose of drugs with increased drug utilization efficiency is considered a promising strategy to promote wound healing [125-126]. At present, most nano- materials are widely used as drug carriers. However, some nanomaterials have a low loading capacity and have difficulties in avoiding the toxic effects of the carrier itself. They also have a single function in the treatment of wounds and cannot keep the wound moist and isolated from the external environment. [127] Hydrogels with three-dimensional network structures are soft materials that can be physically and chemi- cally tailored for various applications in the biomedical field. In addition to their strong water absorption and moisturizing properties, hydrogels have many similar physical and chemical properties to the natural extracellular matrix, which provide a more ideal management for diabetic wounds [128].

Similar to a 3D carrier, hydrogels can be easily loaded with drugs or cytokines that specifically target the main difficulties of diabetic wounds, such as blood sugar control agents, antibacterial agents, and angiogenic factors. Many designed strategies of hydrogels have been proposed and developed for wound healing. However, among these strategies, conventional designed hydrogels as a drug delivery system mainly rely on the diffusion of the drug itself or its own solution to release the drug, which is uncontrollable and may not achieve the expected effect of drug delivery. [129].

Hyaluronic Acid

One possible technology to improve soft tissue healing and regeneration lies in the use of hyaluronic acid, which was first introduced in medicine and then in dentistry. In the last years, hyaluronic acid (HA), a naturally occurring glycosaminoglycan, has risen attention due to its properties in improving cell migration and proliferation [130- 131].

HA is a naturally occurring non-sulfated glycosaminoglycan. It is a linear polysaccharide that is part of the extracellular matrix of connective tissue, synovial fluid, embryonic mesenchyme, vitreous humor, skin, and many other organs and tissues of the body. HA is a key element in soft periodontal tissues, such as gingiva and periodontal ligament, and hard tissues, such as alveolar bone and cementum. It has many structural and physiological functions within these tissues.

In healthy tissues HA has a high molecular weight (4000-20,000,000 Da), whereas after trauma or injury it may be represented as fragments with lower molecular weights, derived from neosynthesis or molecular degradation by hyaluronidase or oxygen radicals. [132] It is a molecule with a simple chemical structure, of a polar (hydrophilic) nature, composed of repeated disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, linked by alternating β-1,3 and β-1,4 glycosidic bonds. In its native form, HA appears as a very long polymer, called high- molecular weight HA (HMWHA) . However, under certain conditions, the molecule can be broken down into small fragments, called low-molecular weight HA (LMWHA). [133]

It may also play a regulatory role in the inflammatory response: high-molecular-weight HA, synthesized by the enzyme hyaluronan synthase in periodontal tissues (gingiva, periodontal ligament, and alveolar bone), undergoes massive degradation to low-molecular-weight molecules in tissues with chronic inflammation, such as in gingival inflammation or in the postoperative stages. [134- 135]

Fragmentation of high molecular weight HA is due to reactive oxygen species, which include superoxide radical and hydroxyl radical species, observed within periodontal disease. [136] These radicals are mainly generated by the 'infiltrate of polymorphonuclear leukocytes and other

inflammatory cells involved in bacterial phagocytosis. Low molecular weight fragments are involved in tissue damage signaling and mobilization of immune cells, whereas high molecular weight HA suppresses the immune response by preventing excessive exacerbation of inflammation. [137]

The molecule is a key component in the series of steps associated with wound healing in both mineralized and non mineralized tissues (inflammation, granulation tissue formation, epithelium formation, tissue remodeling). [138 - 139]

The function of HA is related to healing dynamics through the mechanisms of: activation and modulation of the inflammatory response, promotion of cell proliferation in re-epithelialization processes, and inhibition of CD44 receptors (cell adhesion molecules expressed in leukocytes). [140- 141] Due to the many functions attributed to HA, progress has been made in the development and application of HA-based biomaterials in the treatment of various inflammatory conditions.

In recent years, formulations of HA have been developed for topical administration as an adjuvant treatment in dental conditions, acute and chronic, such as in tissue healing after oral surgery, based on the extensive scientific evidence of the role of ha in dentistry in both animal and in vivo models. [142 - 143]

From the international literature, evidence confirms the action and properties of sodium hyaluronate: ability to stop bleeding, reduction of edema, promoted healing of injured tissues. [140- 142]. These characteristics are appreciated in the field of dermatology, [144-145] but can also be applied in diseases and lesions of the oral cavity. [146]

The use of this molecule can give benefits and advantages in wound healing, due to the action of stimulating fibroblasts, remodeling of extra-cellular matrix, increased healing resulting in reduced healing time. [147-148]

HA could be a reliable approach to wound closure. One study investigated the underlying role of HA, a component of extracellular matrix, in promoting TES healing in diabetic patients. In a randomized controlled split-mouth study including 30 patients with poorly controlled T2DM who

required tooth extraction, 0.8% HA placed in post-extraction socket improved the wound healing, in particular on the first days after applying . [149] In addition, sodium hyaluronate is the product of the neutralization of the carboxyl groups of HA, which has been proved to enhance the healing process in the extraction sockets of rats. [150] Diabetic rats gained greater percentage of newly formed trabeculae in the post-extraction wound treated with HA or carbon nanotubes functionalized with HA [151]

To date, different studies have investigated the usage of HA for dentistry application. Fujioka-Kobayashi et al. [152] reported in an in vitro study positive effect of HA in enhancing and maintaining cell proliferation, viability and osteogenic differentiation. Also, HA formulations are capable of enhancing the migration and proliferation of cell types typically involved in soft tissue wounds healing, in in vitro studies. In vivo studies also showed beneficial effect of HA in the soft tissue healing. [153]

HA facilitate angiogenesis and provide anti-inflammatory effects after third molar extraction, while a triple blind randomized controlled trial found positive effect of HA in bone remodeling and repair in dental sockets. [154- 155]

Furthermore, a recent systematic review highlighted a correlation between the application of HA and a reduction of postoperative pain. [156] Moreover, HA is widely used in other branches of medicine and neither contraindications nor interaction with drugs have been reported [157-159]. However, the investigation of the possible benefits of applying HA in the post extractive sockets of diabetic patients is currently lacking.

Aminogam 4 Gel and Aminogam 6 gel formulation

Aminogam Gel is a hyaluronic acid-based gel that promotes rapid healing of wounds and lesions caused by ulcers within the oral cavity and in particular of the orogingival tissues and oral mucosa. The former constitutes the substrate for the activity of fibroblasts and, thanks to their action and

the modulation of the inflammatory process a faster regeneration of the injured oral mucosa, or the re-epithelialization of the injured area in various pathological conditions are favored.

The gel is produced into 2 formulations: Aminogam®4 gel (AG4) and Aminogam®6 Gel, (AG6).

Ingredients and Composition Aminogam®4 gel formulation: purified water; sodium hyaluronate; glycine; l-proline; leucine; 1-lysine hcl, methyleparahydroxibenzoate; propyleparaben; sorbitole; polyvinyl pyrrolidone; sodium hydroxide.

Ingredients and Composition Aminogam®6 Gel, formulation: purified water; sodium hyaluronate; glycine; l-proline; leucine; 1-lysine hcl; L-Valine, L-Alanine, methyleparahydroxibenzoate; propyleparaben; sorbitole; polyvinyl pyrrolidone; sodium hydroxide.

Aim of the study

The aim of the present study was to investigate the effect of Hyaluronic Acid (HA) in improving the post-extraction tooth socket healing in subjects with T2DM.

A comparison between the efficacy of Aminogam®4 gel versus Aminogam®6 Gel has been performed.

The null hypothesis was that HA can significantly improve the post extractive healing of diabetic patients compared with no treatment.

Methods

All the study procedures were carried out according to the World Medical Association's (WMA) Helsinki Declaration and its amendments (Ethical Principles for Medical Research Involving Human Subjects, adopted by the 18th WMA General Assembly Helsinki, Finland, June 1964, and amendments). All patients enrolled in the study were thoroughly informed about the research purpose and signed an informed consent form prior to undergoing the procedures.

The present study was reported following the CONSORT 2010 guidelines.

The study protocol and the research were approved by the local ethical committee of the University of Turin (approval code 0100924 on 15/09/2022). The trial was registered at ClinicalTrials.gov (ID: NCT05896319, Registration date: 09/06/2023).

Study design

The study was designed as a single center randomized controlled trial. Patients requiring extraction of not impacted teeth were visited at the C.I.R. (Interdepartmental Research Center) of Dental School, Section of Oral surgery, Department of Surgical Sciences, University of Turin from September 2022 to July 2023.

The inclusion criteria were:

- age ≥ 18 years old;
- T2DM patients with a positive history for diabetes complications (e.g., nephropathy, neuropathy, retinopathy, cardiopathy, peripheral vascular disease);
- requirement of extractions of not impacted teeth;
- consent for enrollment in the study;
- availability to attend the control visit.

The exclusion criteria were:

- presence of platelet dysfunction;
- presence of thrombocytopenia;
- corticosteroid treatment;
- smokers;
- refusal to participate in the study;

- assumption of drugs possibly interacting with the wound healing;
- extractions requiring the elevation of a flap and teeth requiring separation with burs;
- ankylosed teeth requiring the usage of a burs to allow the extraction;
- apical fractures during extractions.

Patients were randomly assigned through a computer-generated random sequence of numbers (SPSS 24.0; SPSS Inc., Chicago, IL, USA) to the test for Aminogam 4 gel (T4) group, for its control group (C4) or to the test for the Aminogam®6 Gel, (T6) group, and for its control group (C6).

T4 group (treated with Aminogam®4 gel) included: post-operative application of AG4 3 times per day (8 hours distance between each application) for 7 days after oral hygiene and without swallowing, eating or drinking for one hour after the application, as follows: “wash your hands thoroughly before each application, apply a layer of gel on the injured mucosa, massage with a finger in order to facilitate spreading of the product over the treated area and compressing the product with gauze”.

C4 group (untreated) included: no treatment.

T6 group (treated with Aminogam®6 Gel,) included: post-operative application of AG6 3 times per day (8 hours distance between each application) for 7 days after oral hygiene and without swallowing, eating or drinking for one hour after the application, as follows: “wash your hands thoroughly before each application, apply a layer of gel on the injured mucosa, massage with a finger in order to facilitate spreading of the product over the treated area and compressing the product with gauze”.

C6 group (untreated) included: no treatment.

All the surgeries were performed by the same experienced clinicians specialized in oral surgery who were blinded to the group allocation of the sites.

All the pre- and post-operative assessments were performed by two calibrated and trained operators who were blinded to the T and C group allocation.

Pre-operative assessment

Prior to extractions, patients underwent a professional oral hygiene session while the dentist clinically and radiographically evaluated:

- Demographic characteristics of the subjects enrolled in the study. The following data were collected:
  - gender; age; ethnic origin; body mass index (BMI); smoking habits.
- Diabetes-related data by reviewing the medical records. The following data were collected:
  - duration of diabetes; diabetes status (blood sugar level); glycated hemoglobin (HbA1c) level; End-Organ disease score.

- Pre-operative status of the teeth that required extraction. The following information was collected: single or multi-rooted teeth; presence of cavities; pulp vitality and previously endodontic treatments; presence of peri-apical lesion.

- The degree of difficulty of the extraction. Table 1. The operative difficulty was classified according to 3 degrees:

• Low: all low grade parameters, no more than one intermediate grade parameter;

• Intermediate: more than one parameter of intermediate difficulty, no parameter of high difficulty;

• High: one or more high-grade parameters. [160]

Parameters	Low difficulty	Medium difficulty	High difficulty
Patient collaboration	cooperative	suspicious	uncooperative
Space	higher than the MD crown size	Equal to the MD crown size	Smaller than the MD crown size
Crown integrity	Intact crown	Incomplete crown	Crown absent
Root anatomy	Low difficulty	Medium diffulty	High difficulty

Table 1. degree of difficulty of the extraction

- Patient's systemic risk (Table 2). A model derived from a study in which the relationship between

the degree of glycemic control and the results following tooth extraction were evaluated, bearing in mind the diagnosis and management of the diabetic patient [117- 161]

Low/Absent systemic risk	Moderate systemic risk	High systemic risk
End-organ disease score 0	End-organ disease score ≤ 2	End-organ disease score >2
Diagnosis ≤ 5 years	Diagnosis between 6 to 10 years	Diagnosis > 10 years
Usual blood sugar levels < 180 mg/dl	Usual blood sugar levels 180 – 240 mg/dl	Usual blood sugar levels > 240 mg/dl
More than 3 positive parameters between: - no hospitalizations - no episodes of ketoacidosis - no episodes of hypoglycemia - hypoglycemic therapy - controlled diabetes - no changes of therapy	Less than 3 positive parameters between: - no hospitalizations - no episodes of ketoacidosis - no episodes of hypoglycemia - hypoglycemic therapy - controlled diabetes - no changes of therapy	Less than 3 positive parameters between: - no hospitalizations - no episodes of ketoacidosis - no episodes of hypoglycemia - hypoglycemic therapy - controlled diabetes - no changes of therapy

Table 2. Patient's systemic risk

All the above parameters were acquired in order to have a comparison for inter and intra patients to avoid any bias related to different T0 parameters.

Surgical appointments

Before extraction local anesthesia (plexus or alveolar nerve block infiltration) was given using 1.8 ml vials of 3% mepivacaine. All the extractions were performed in a non-traumatic way and without a full thickness mucoperiosteal flap elevation to preserve the bone crest and the soft tissue integrity. After the extractions, the sockets were cleansed (removal of infected tissue) to help the wound healing.

If the patient required sutures for blood dyscrasias, this was applied (non absorbable silk suture, stainless steel Permahand 3/0, Ethicon LLC, Highway 183, km 8.3 San Lorenzo Puerto Rico 00754, USA). The sockets were then compressed with a sterile gauze.

Patients were then instructed with postoperative recommendations including, hygienic instructions and a tube (15 ml) of the test product (AG 4 or AG6) for **T4 group** and **T6 group**.

Visit name	Baseline, V1, V2, V3, V4

Form name: Healing Index	
Tissue color	◯ 100% pink gum ◯ <= 50% of red, hyperemic, moving gum ◯ >= 50% of red, hyperemic, moving gum
Color and consistency of the scar tissue	◯ Fine-grained, pink ◯ Soft, red ◯ Brittle, greenish or grayish
Suppuration	◯ Absent ◯ Quantity of plaque absent but enhanced around the walls of the alveolus ◯ Enhanced
Bleeding	◯ Absent ◯ Induced by palpation ◯ Spontaneous
Total score	__|__|
The total score will be a computed field, calculated as the sum of the previous 4 fields	

Since anti-inflammatories act by inhibiting the production of cytokines and the inflammatory response and they can consequently alter the mechanism of action of HA as well as the perception of pain, no anti-inflammatory drugs or antibiotics were prescribed following the extraction. The need to administer the antibiotic after the extraction was considered a negative post-operative evaluation parameter, as it was a sign of complication due to infection.

Clinical outcomes

Primary clinical outcomes were evaluated.

Healing index

To evaluate the healing of the post extractive sockets, a simplified version of Landry's index [162]

was adopted, considering only 3 possible scores for each of the 4 parameters considered:

- tissue color (1= 100% pink gum; 2=<50% hyperemic gum ; 3=>50% hyperemic gingiva),
- bleeding (1=absent; 2=provoked by palpation; 3=spontaneous),
- granulation tissue (1=pink and firm; 2=red and soft; 3=brittle),
- suppuration (1=no accumulation of plaque on the margins; 2=evident plaque on the margins; 3=suppuration/alveolitis).

In this index, a score of 4 corresponds to excellent healing, conversely a score of 12 corresponds to poor healing.

Figure 19. Healing index evaluation

Socket Closure

Socket Closure was defined as the ratio between the volume of the healing socket at a given time (3, 7, 14, 21 days) and the volume of the socket at T0. It was calculated by measuring (in millimeters) the maximum oral-vestibule (OV) diameter (Figure 20A), the maximum mesio-distal (MD) diameter (Figure 20B), and the maximum socket depth (SD) (Figure 20C).

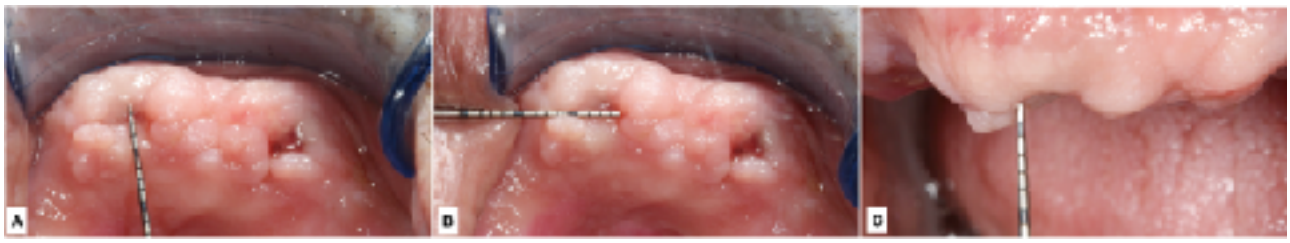


Figure 20. Measurement (in millimeters) the maximum oral-vestibule (OV) diameter (Figure 20A), the maximum mesio-distal (MD) diameter (Figure 20B), and the maximum socket depth (SD) (Figure 20C).

MD diameter was measured in the point of the maximum MD width of the socket both for single-rooted and multi-rooted teeth.

OV diameter was measured in the point of the maximum vestibule-oral width of the socket or of each root (considering only the maximum value for the pluri-rooted teeth).

SD was measured as the distance between the gingival margin and the socket bone in the point of its maximum depth (without forcing the probe).

All the measurements were performed using a HuFriedy PCPUNC 15 probe (HuFriedy, Chicago, IL, USA) at the day of the surgery (D0), and after 3 (D3), 7 (D7), and 14 (D14) days from the surgery. An additional follow-up visit after 21 (D21) days was planned in case the socket was not completely healed after 21 days from the surgery.

Secondary outcomes were:

- **pain measurement** on a Visual Analogue Scale (VAS);
- need to add a **follow-up visit after 21 days** from the surgery;
- need to prescribe the **use of antibiotics** to counteract post-operative symptoms;
- **over-infection** of the granulation tissue;
- need of a **reoperation** in case of complications during the healing process.

Cytokine analysis

During the first follow-up appointment at 3 days after extraction, after removal of the suture by means of sterile scalpel and sterile tweezers, 12 different human cytokines developed during the healing process of the post-extraction socket were quantitatively assessed: GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A, and TNF-α.

Sterile tweezers, sterile alveolar spoons, and/or sterile hand excavators were used to harvest the newly formed healing tissue within the site. The procedure was quick and painless as, where necessary, plexic anesthesia was performed using Scandonest 3% without vasoconstrictor injectable solution.

The harvested material was placed inside a sterile Eppendorf tube containing haeparin to prevent

clotting on which was preliminarily entered the date of collection, the site of collection, and the patient's anonymous identification code.

The MACSPlex Cytokine 12 Capture Beads kit from Miltenyi Biotec (Bologna, Italy) was used for the analysis of the above cytokines. MACSPlex assays are designed to determine concentrations of soluble analytes in a single sample. The analysis is based on MACSPlex Capture Beads, which have defined fluorescence properties and can be identified by standard flow cytometry techniques. MACSPlex Capture Beads contain a cocktail of various populations of fluorescently labeled microspheres, each coated with a specific antibody that reacts with one of the respective analytes present in the sample. Samples containing unknown levels of cytokines were incubated with the antibody-coated MACSPlex microbeads, and the cytokines bind to the specific antibody. A detection reagent, consisting of a cocktail of antibodies conjugated with cytokine-specific APCs, is added. As a result, sandwich complexes are formed between the MACSPlex capture bead, the cytokine and the detection reagent. These complexes can be analyzed based on the fluorescence characteristics of the MACSPlex Capture Bead and the detection reagent using the MACSQuant 10 analyzer located at the Bone and Dental Bioengineering Laboratory of the Department of Surgical Sciences of the C.I.R. Dental School of the University of Turin directed by Dr. Ilaria Roato.

Cytokines are captured by MACSPlex capture beads on samples taken from patients after a 2-hour incubation period. Next, MACSPlex Cytokine 12 Detection Reagent, containing a mixture of 12 anti-cytokine antibodies conjugated with APC, is added to form sandwich complexes during a 1-hour incubation period. The entire procedure was carried out faithfully following the protocol provided by the manufacturer.

Standard curves for each of the 12 cytokines were generated. The median APC fluorescence of each capture bead population gave the concentration of each cytokine (pg/mL) in the samples. The result of the analysis of each cytokine for each patient was entered within a Microsoft Excel spreadsheet for subsequent statistical analysis.

Protocols for assay performance

Reconstitute and dilute MACSPlex Cytokine 12 Standard with MACSPlex Buffer, or use the same media as is used for the dilution of the unknown sample.

The generation of standard curves requires seven samples: six samples ranging from 3.2 to 10,000 pg/mL of the MACSPlex Cytokine 12 Standard, and one blank control. These samples will be measured as duplicates.

1. Thaw one vial containing the lyophilized MACSPlex Cytokine 12 Standard.
2. Open the vial and add 200 µL of MACSPlex Buffer or media to the pellet. Mix gently. This is the stock solution (1:50; 10,000 pg/mL).
3. Label five reagent tubes and arrange them in the following order: 1:5 (1:51; 2,000 pg/mL), 1:25 (1:52; 400 pg/mL), 1:125 (1:53; 80 pg/mL), 1:625 (1:54; 16 pg/mL), and 1:3125 (1:55; 3.2 pg/mL)
4. Pipette 200 µL of MACSPlex Buffer or media into each tube.
5. Perform a 1:5 dilution by transferring 50 µL from the stock solution to the tube labeled 1:5 and mix thoroughly. Continue making 1:5 serial dilutions by transferring 50 µL from the tube labeled 1:5 to the tube labeled 1:25 and so on to the tube labeled 1:3125. Mix each dilution before performing the next transfer.

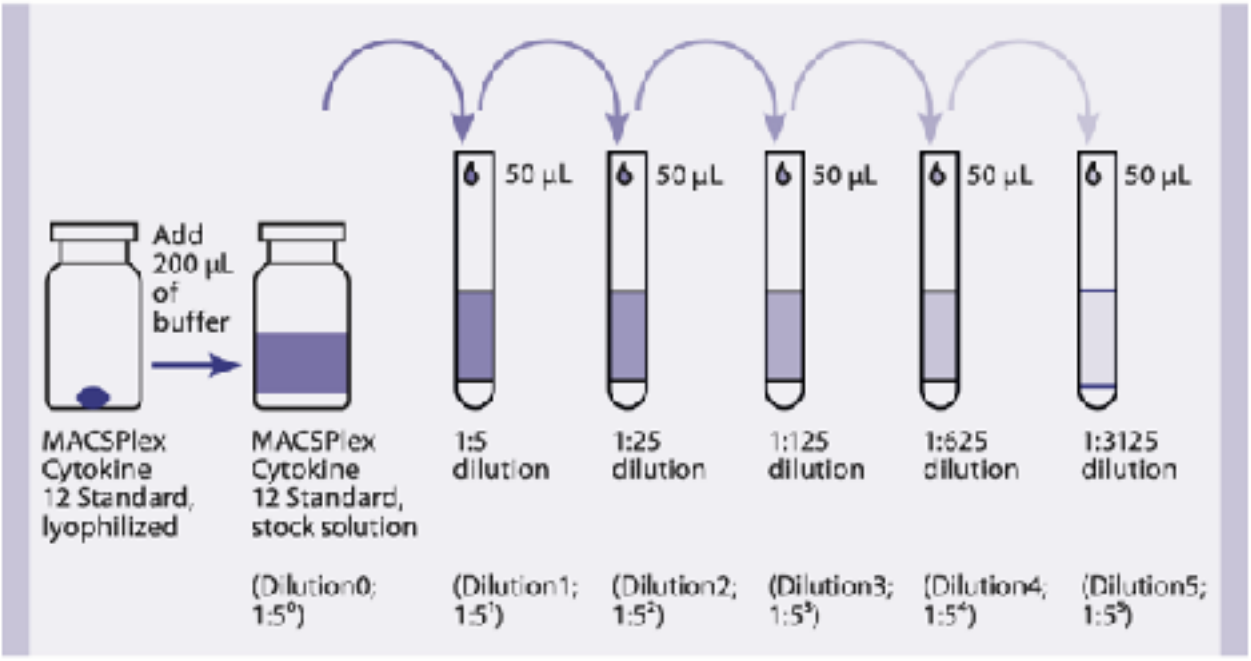


Figure 21. Correct dilutions

Keep 200 µL MACSPlex Buffer or media as blank control (0 pg/mL).

Sample preparation

1. Handle all blood components and biological material as potentially hazardous.
2. If unknown samples are expected or known to contain levels >2000 pg/mL of a certain cytokine, it is recommended to dilute the samples to make sure the fluorescence values are within the dynamic range of the standard curve.
3. Use polypropylene or polystyrene reagent tubes. Do not use glass vials for sample preparation, dilution, or storage.

Frozen samples

1. Thaw the samples completely and mix well by vortexing.

2. Centrifuge at 10,000×g for 10 minutes at 4 °C to remove particulates.

3. Transfer serum or plasma into a new tube and dilute at least 1:8 with MACSPlex Buffer, i.e., add 25 µL of the undiluted sample to 175 µL of MACSPlex Buffer. Cell culture supernatants can be diluted optionally with cell culture medium or MACSPlex Buffer.

MACSPlex Cytokine 12 Assay: Run the assay at room temperature. Work fast and keep samples protected from light, for example, cover plate or tubes with aluminum foil, especially during incubation steps. Unknown samples should be run in replicates, for example, in duplicates or triplicates and in different dilutions to make sure the fluorescence values are within the dynamic range of the standard curve.

Design your assay using two columns of the MACSPlex Filter Plate for the standards. Add each of the seven standard samples in duplicates next to each other. Standards should be run in order from the lowest concentration (blank control: 0 pg/mL) to the highest concentration (stock solution: 10,000 pg/mL). Start with the unknown sample in the next column of the plate.

Place the MACSPlex Filter Plate on a non-absorbent surface during loading steps and incubation, i.e. remove any tissues from the surface, to prevent the wells from running dry. Ensure that residual drops under the plate are completely removed to prevent liquid transfer, by placing the plate briefly on a tissue. Cover unused wells of the filter plate for later use with the adhesive foil provided with the kit.

Washing steps are described for the use of a vaccum manifold. Alternatively, a centrifuge with an adapter for microtiter plates can be used: Put the MACSPlex Filter Plate on top of a conventional 96-flat- bottom microtiter plate without lid and place both into the adapter. Centrifuge at 300×g for 3 minutes at room temperature.

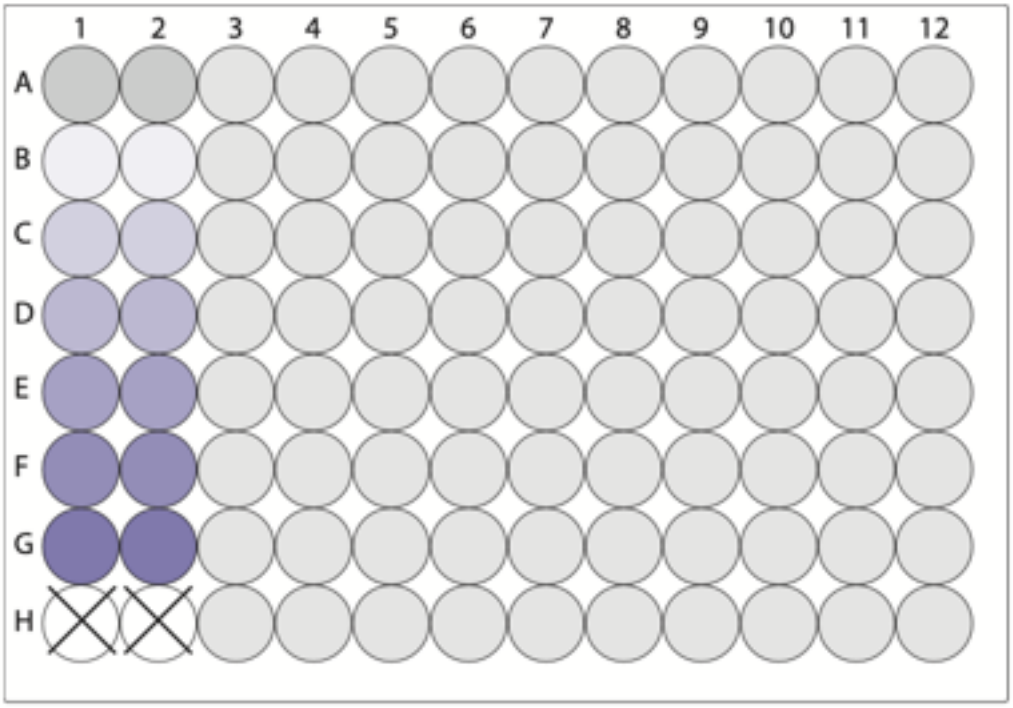
Pre-wet required wells of the MACSPlex Filter Plate with 200 µL of MACSPlex Buffer per well and aspirate off using a vacuum manifold designed to accommodate the filter plate (max. –300 mbar) until the wells are drained. Place the filter plate briefly on a tissue to remove any residual liquid.

Add 50 µL of MACSPlex Buffer or media as a blank control, 50 µL of each dilution, and the stock solution of the MACSPlex Cytokine 12 Standard to the corresponding wells of the filter plate. Add 50 µL of each unknown sample per well. Resuspend MACSPlex Cytokine 12 Capture Beads by vortexing for at least 30 seconds and transfer 20 µL of MACSPlex Capture Beads to each well. Incubate filter plate for 2 hours protect from light on an orbital shaker (450 rpm).

Apply the filter plate to the vacuum manifold and aspirate until wells are drained. Place the filter plate briefly on a tissue to remove any residual liquid.

Figure 22. Setup of the assay

4. Add 200 µL MACSPlex Buffer to each well and apply the filter plate to the vacuum manifold and aspirate off until wells are drained. Place the filter plate briefly on a tissue to remove residual liquid.
5. Repeat step 8.
6. Add 80 µL of MACSPlex Buffer to each well.
7. Add 20 µL of MACSPlex Cytokine 12 Detection Reagent to each well.
8. Incubate filter plate for 1 hour protect from light on an orbital shaker (450 rpm).



Well position	Sample	Dilution
A1/A2	Blank control	
B1/B2	MACSPlex Cytokine 12 Standard (3.2 pg/mL)	1:3125 (Dilution5; $1:5^5$)
C1/C2	MACSPlex Cytokine 12 Standard (16 pg/mL)	1:625 (Dilution4; $1:5^4$)
D1/D2	MACSPlex Cytokine 12 Standard (80 pg/mL)	1:125 (Dilution3; $1:5^3$)
E1/E2	MACSPlex Cytokine 12 Standard (400 pg/mL)	1:25 (Dilution2; $1:5^2$)
F1/F2	MACSPlex Cytokine 12 Standard (2 ng/mL)	1:5 (Dilution1; $1:5^1$)
G1/G2	MACSPlex Cytokine 12 Standard (10 ng/mL)	Stock solution (Dilution0; $1:5^0$)
H1/H2	Leave empty	
A3-H12	Add unknown samples	

9. Repeat washing steps 7 and 8.
10. Add 200 µL of MACSPlex Buffer to each well.
11. For sample acquisition using MACSQuant Instruments and the Express Mode place the filter plate onto the Chill 96 Rack. To prevent liquid transfer from the wells, ensure that residual drops under the plate are completely removed by placing the plate briefly on a tissue.

Flow cytometer set up

Perform the flow cytometric acquisition on the same day, as prolonged storage of samples can result in increased background and reduced sensitivity. Keep samples protected from light by using the protection lid during the flow cytometric acquisition with the MACSQuant Instrument.

The kit includes MACSPlex Setup Beads for flow cytometer set up. MACSPlex Setup Beads are not required when using the MACSQuant Analyzer or MACSQuant Analyzer 10 but for all other instruments.

To setup of the MACSQuant Instrument calibrate the MACSQuant Instrument using MACSQuant Calibration Beads After successful completing the calibration, the MACSQuant Instrument is ready for measurement. No further steps are required as all necessary setup steps are performed automatically during calibration.

Sample size calculation

A total of 80 patients and extractions were calculated as required for this study based on a previously published article. [163] However, based on the usual large flow of patients arriving at the Section of Oral surgery, a minimum sample size of 100 patients and extractions was calculated to increase the study power.

Statistical analysis

Continuous variables are reported as mean ± standard deviation (sd). The Mann-Whitney test or the Student's t-test were used for nonparametric or parametric distributed variables, respectively. Categorical variables (dichotomous or polychotomous) were reported as counts and/or percentages. The statistical analysis of categorical variables was carried out on contingency tables or RxC cross-correlation matrices. The chi-square test (with Yates correction for 2x2 tables) was used if the estimated data in any given cell was > 5, otherwise the Fisher test was used. The Risk Ratio (RR) or the Odds Ratio (OR) was used in the case of 2x2 tables. A $p < 0.05$ was considered statistically significant. For the RR calculation, the 95% confidence interval excluded 1.

Results

In total, 112 patients (n = 112), with a mean age of 69.44 ± 10.08 years, met the inclusion criteria and were enrolled in the study. Demographic and baseline characteristics are reported in Table 3.

		%	n
Sex			
	Male	51%	57
	Female	49%	55
Ethnic origin			
	Caucasian	100%	112
Age		69.44	
Smokers		33%	37
Duration of Diabets			
	1-5 years	18%	21
	6-10 years	35%	40
	>10 years	47%	51
BMI		28.2 ± 4,8	
Preoperative diabetes status			
	<180 mg/dl	82%	92
	>180 mg/dl <240 mg/dl	16%	18
	>240 mg/dl	2%	2
HbA1c		7,3 ±1,2 (mmol/mol)	
End-organ disease score			
	Cardiomyopathy	73%	82
	Nephropaty	23%	26
	Neuropaty	21%	23
	Peripherial vasculopaty	28%	31
	Retinopathy	10%	11
Systemic risk			
	Low	9%	10
	Medium	56%	63
	High	35%	39

Table 3. Demographic and baseline characteristics of all patients enrolled in the study

No drop-outs or patients lost to follow-up were recorded. All subjects were included in the efficacy and safety analysis data set. The tested product was well tolerated. No adverse reactions occurred during the study period. No requirement of antibiotic prescription was recorded for any of the enrolled patients.

No differences were observed in the patient collaboration. Only one subject was not cooperative.

Results of AG4 evaluation

In total, 72 patients (n = 72), 34 males (47%) and 38 females (53%) with a mean age of 67.28 ± 11.22, met the inclusion criteria and were enrolled in the study, for a total extraction volume of 72 teeth (n = 72). T4 group and C4 group comprised of 72 sites of extraction (n = 72 per group). Excellent intra-observer (kappa values of 0.78 and 0.80) and inter-observer (a kappa value of 0.80) agreements were recorded in this study. Demographic and baseline characteristics are reported in table 4.

Sex	
Male, % (n)	47% (34)
Female, % (n)	53% (38)
Ethnic origin	
Caucasian	100% (72)
Age (years)	67.3 ± 11.2
Smokers	25% (18)
Duration of diabetes	11.4 ± 9.9
Body Mass Index (BMI)	28.2 ± 4.8
Preoperative diabetes status:	
< 180 mg/dl[1]	83% (60)
>180 and < 240 mg/dl[2]	17% (12)
> 240 mg/dl[3]	0% (0)
HbA1c (%)	7.41 ± 1.02
End-Organ disease score:	
Cardiomyopathy	86.1% (62)
Nephropathy	19.4% (14)
Peripheral vasculopathy	2.8% (2)
Retinopathy	0% (0)
Systemic risk:	
Low	0% (0)
Medium	86% (62)
High	14% (10)

Table 4. Subjects baseline demographic and clinical characteristics. Data are means (±SD) or percentage (numbers). 1 medium glycemic control with the current therapy, 2 insufficient glycemic control despite the therapy, 3 no glycemic control. HbA1c, Glycated hemoglobin.

No drop-outs or patients lost to follow-up were recorded. All subjects were included in the efficacy and safety analysis data set. The tested product was well tolerated. No adverse reactions occurred during the study period. No requirement of antibiotic prescription was recorded for any of the enrolled patients.

No differences were observed in the patient collaboration. Only one subject was not cooperative.

Table 5 shows the baseline pre-operative data recorded regarding teeth status and surgical difficulty between T and C groups.

	T4 group	C4 group
	Preoperative surgical difficulty	
Low	80.6%	75%
Medium	19.4%	25%
p	0.38	
	Preoperative crown integrity	
Intact	58.3%	61.1%
Broken	27.8%	33.3%
p	0.47	
	Root anatomy	
Low difficutly	80.6%	75%
Medium difficutly	19.4%	25%
p	0.38	
	Pulp vitality	
Vital	38.9%	30.6%
Necrotic	58.3%	63.9%
Endodontically treated	2.8%	5.6%
p	0.67	
	Presence of cavity	
Negative	38.9%	33.3%
Positive	61.1%	66.7%
p	0.40	
	Periodontopathic	
Positive	66.7%	61.1%

p	0.40	
	Precence of radriographically visible periapical osteolysis	
Negative	94.4%	94.4%
Positive	5.6%	5.6%
p	0.9999	

Table 5. Baseline pre-operative data recorded regarding teeth status and surgical difficulty for both T4 and C4 groups. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4.

Based on the results, no statistically significant differences ($p > 0.05$) were highlighted between the two groups for any of the considered baseline variables. Therefore, it is possible to conclude that both the group T4 and C4 were similar, indicating an unbiased randomization and absence of covariates.

Healing index

Table 6 shows data related to the healing index recorded at different time points.

Healing index	D3		D7		D14		D21	
	T4	C4	T4	C4	T4	C4	T4	C4
Mean	6.6	7.6	5.0	5.9	4.1	4.5	4.0	4.0
Standard deviation	1.8	1.9	1.2	1.7	0.5	1.0	0.0	0.0
P	0.08		0.01*		0.02*		1	
% of Excellent healing (score n. 4)	5.6 (n= 4)	0 (n = 0)	44.4 (n = 32)	30.06 (n = 22)	97.2 (n = 70)	72.2 (n = 52)	100 (n = 72)	100 (n = 72)
p	0.23		0.16		0.004*		1	

Table 6. Data related to the healing index recorded at different time points. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4.

Sockets treated with Aminogam 4 gel (T4 group) showed significantly ($p < 0.05$) better Healing index values at D7 ($p = 0.01$) and D14 ($p = 0.02$), while no statistically significant difference was highlighted at D3 ($p = 0.08$) and D21 ($p = 1$).

In regard to the % of sockets that presented with optimal healing (healing index = 4), a statistically significant difference ($p < 0.05$) was highlighted at D14 ($p = 0.004$), were sockets treated with Aminogam 4 gel shows better results.

OV, MD, and SD

Values recorded in regard to OV diameters showed a statistically significant difference ($p = 0.03$) in favor of the T4 group at D3, while no significant difference ($p > 0.05$) was recorded between the 2 groups at the other follow-up times. Regarding the MD diameters, a statistically significant difference was highlighted at D3 ($p = 0.03$) in favor of sites treated with HA (T4 group), while no significant difference ($p > 0.05$) was recorded between the 2 groups at the other follow-up times. In regard to SD, a statistically significant difference was recorded at D14 ($p = 0.04$) in favor of the T4 group, while no statistically significant difference ($p > 0.05$) was recorded between the 2 groups at the other follow-up times.

Socket closure

No statistically significant difference ($p = 30$) was recorded between the two groups at D0, therefore the initial conditions were superimposable.

Socket closure	D0		D3		D7		D14		D21	
Group	T4	C4	T4	C4	T4	C4	T4	C4	T4	C4
Mean	148.44	145.74	0.25	0.45	0.05	0.14	0.00	0.02	0.00	0.00
Standard deviation	125.38	105.59	0.22	0.29	0.06	0.17	0.01	0.04	0.00	0.00
p	0.30		0.04*		0.001*		0.001*		1	
% of socket fully closed (n)	/	/	0	0	27.78 (n= 20)	33.33 (n=24)	77.78(n =36)	61.11 (n=44)	100(n = 72)	100 (n =72)
p	/		/		0.8		0.57		0.33	

Table 7. Values recorded regarding socket closure at different time points. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4

Table 7 shows values recorded regarding socket closure. Sockets treated with HA (T4 group), showed significantly ($p < 0.05$) better socket closure value at D 3 ($p = 0.04$), D7 ($p = 0.001$), and D14 ($p = 0.001$) compared to the C group.

Regarding the % of socket fully closed, no significant difference ($p > 0.05$) was highlighted between the two groups at any time points.

VAS

Table 8 shows data of VAS at different time points and evidences valuesstatistically ($p < 0.05$) more favorable for the T4 Group at D3, D7 and D14.

VAS	D3		D7		D14		D21	
	T4	C4	T4	C4	T4	C4	T4	C4
Mean	3.63	5.38	2.23	3.08	0.15	0.38	0.17	0.17
Standard deviation	1.77	2.39	1.81	1.59	0.38	0.51	0.39	0.39
p	<0.001		0.04		0.03		0.9999999	

Table 8. Data recorded regarding VAS scale at different time points. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4

Results of AG6 evaluation

In this part of the study, a total of 43 patients were recruited and randomly assigned to T6 group (Aminogam®6 Gel) or C6 group (No Aminogam®6 Gel). All data are show in Table 9.

		Group T6 (n21)	**Group C6 (n22)**	**P**
Sex				
	Male	6 (28,6%)	20 (91%)	
	Female	15 (71,5%)	2 (9,1%)	
Ethnic origin				
	Caucasian	21 (100%)	22 (100%)	
Age		78±3.1	70±6	P<0,001
Smokers		13 (62%)	8 (36%)	
BMI		28.3±5.8	33.71	0,04
HbA1c		6,8	7,5	
End-organ disease score				
	Cardiomyopathy	21 (100%)%	22 (100%)	
	Nephropaty	10 (47,6%)	3 (13,6%)	
	Neuropaty	13 (62%)	9 (40,9%)	
	Peripherial vasculopaty	19 (90,5%)	13(59%)	
	Retinopathy	5 (23,8%)	6 (27,3%)	
Systemic risk				
	Low	0	0	
	Medium	0	15(68%)	
	High	21 (100%)	7(32%)	
PSR		3,5	4	0,002

Table 9. Subjects' baseline demographic and clinical characteristics. Data are means (±SD) or numbers(percentage) Legend HbA1c, Glycated hemoglobin. T6 : group treated with Aminogam®6 Gel,; C6: group control of T6. BMI: Body Mass Index, PSR: Periodontal Screening Record

Patients are homogeneously distributed: 21 in the T6 group and 22 in the C6 group. Of the 43 patients examined, 40% were women and 60% were men. In the preoperative evaluation, there was statistical significance ($p<0.001$) between the age of the T6 group (78±3.1) and the C6 group (70±6).

The evaluation of BMI shoved a significant difference($p=0.04$) between T6 group and the C6 group (28.3±5.8 versus 33.8±9.4, respectively). Regarding smoking habit, it was found that 13 patients in the T6 group and 8 patients in the C6 group were smokers.

With regard to the chronic DM-associated complication : 11 patients suffered from retinopathy (5 Aminogam®6 Gel, 6 No Aminogam®6 Gel), 13 from nephropathy (10 Aminogam®6 Gel, 3 No Aminogam®6 Gel), 22 from neuropathy (13 Aminogam®6 Gel, 9 No Aminogam®6 Gel), 43 of Diabetes-induced heart disease (21 Aminogam®6 Gel, 22 No Aminogam®6 Gel), and 32 from vasculopathy (19 Aminogam®6 Gel, 13 No Aminogam®6 Gel).

The combination of the previous characteristics generated the systemic risk: it was observed that in the T6 group, all 21 patients showed a high risk, while in the C6 group 7 showed a the medium risk and 15 a high risk.

Moving on to analyze the oral tissue health prior to the surgical stage, the periodontal PSR index was found to significant different between the two groups ($p=0.002$) with values in the T6 group of 3.5±0.5 and in the C6 group of 4.0±0.5.

Surgery was classified according to surgical difficulty into: low, medium, and high. No patients had high difficulty surgery, 25 had medium difficulty (16 Aminogam®6 Gel and 9 No Aminogam®6 Gel), and 18 had low difficulty (5 Aminogam®6 Gel and 13 No Aminogam®6 Gel).

Postoperative evaluation

Data analysis of the postoperative evaluation was developed in several steps:

- Evaluation of differences in healing index between the T6 group and the C6 group
- Evaluation of RSV differences between theT6 group and the C6 group

- Evaluation of quantitative differences three cytokines taken from the healing site between theT6 group and the C6 group .

D0 is the day the extraction was performed, D3 is the first follow-up at 3 days after extraction, D7 represents the follow-up 7 days after surgery while D14 is 14 days after the surgical act.

Evaluation of differences in Healing Index

Regarding the Healing Index the following parameters were analyzed: bleeding, suppuration, tissue color, and granulation tissue appearance.

It was found that at D3 there was no statistically significant difference between the 2 groups (p=0.7) being the healing index 5.8±2.1 in the T6 group and 6.0±1.3 in the C6 group .

In contrast, statistically significant improved healing was observed at D7 (p<0.001) and D14 (p<0.001) in favor of the T6 group. In fact the mean healing index value of the T6 group was 4.0±0.2 versus 6.0±1.4 for the C6 group . At follow-ups at D14, healing is always in favor of the T6 group (4.0±0.1) compared with the C6 group (5.0±0.9).

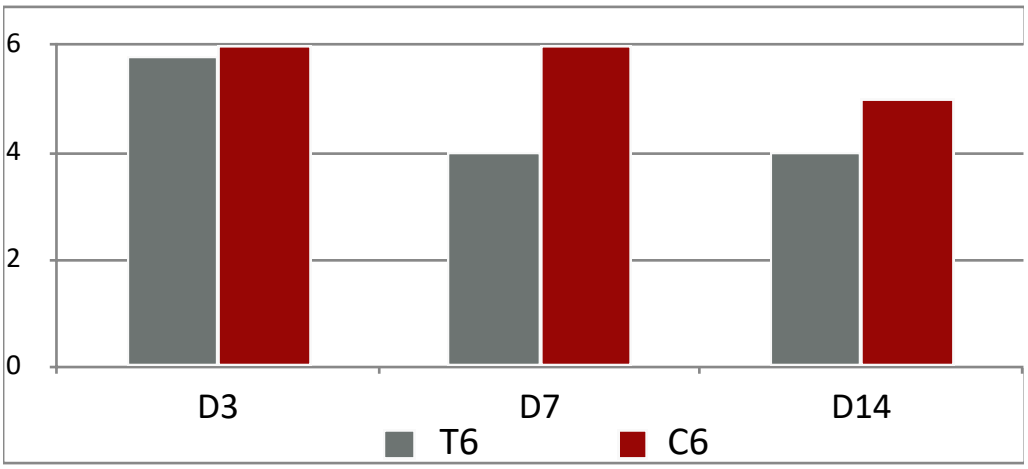


Figure 24. Healing index recorded at different time points. T6: group treated with Aminogam®6 Gel; C6: group control of T6.

Socket closure

The second end-point of the study was the evaluation of the dimensional closure of the alveolus by calculating the Socket closure. Imagining the alveolus similar to a parallelepiped, the 3 dimensions

are measured in order to calculate the volume (RSV: Residual Socket Volume): the maximum mesio-distal distance (MD), the maximum vestibulo-oral distance (VO) and the maximum probing depth (P).

The dimensions measured at D0 already show at baseline a statistically significant difference in MD (p=0.01) and VO (p=0.01) between the two groups, while no statistically significant difference in probing depth. With the periodontal probe, the following measurements were measured for the T6 group: MD measurement of 5.9±2.7mm, VO of 6.0±2.6mm and a P of 10.9±3.3mm. While for the C6 group , the dimensions are: MD 8.0±2.7mm, VO 8.0±2.4mm and P 11.0±3.5mm. (Figure 25)

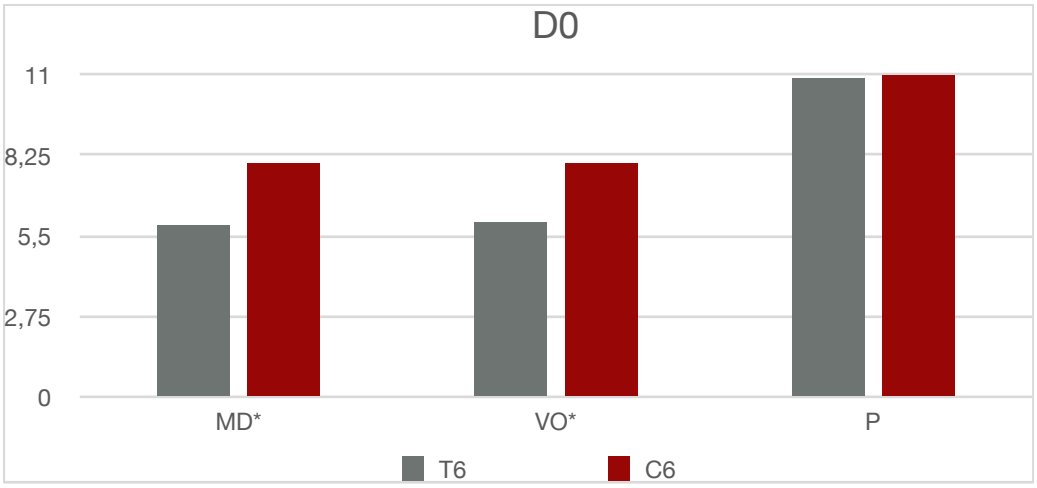


Figure 25. Socket dimension at D0, day of extraction. Legend: maximum mesio-distal distance (MD), the maximum vestibulo-oral distance (VO) and the maximum probing depth (P). T6: group treated with Aminogam®6 Gel; C6: group control of T6. (*) : statistically significant p.

The mean sizes at D3 of the T6 group were: MD 4.7±2.0mm, VO 3.8±1.4mm, P 7.6±2.5mm with an RSV of 0.5±0.5. The mean sizes at D3 of the C6 group were: MD 6.0±2.4mm, VO 5.0±2.1mm, P 9.0±3.7mm with an RSV of 0.4±0.2 (figure 26). At D3 the only statistically difference between the two groups was VO (p=0.03).

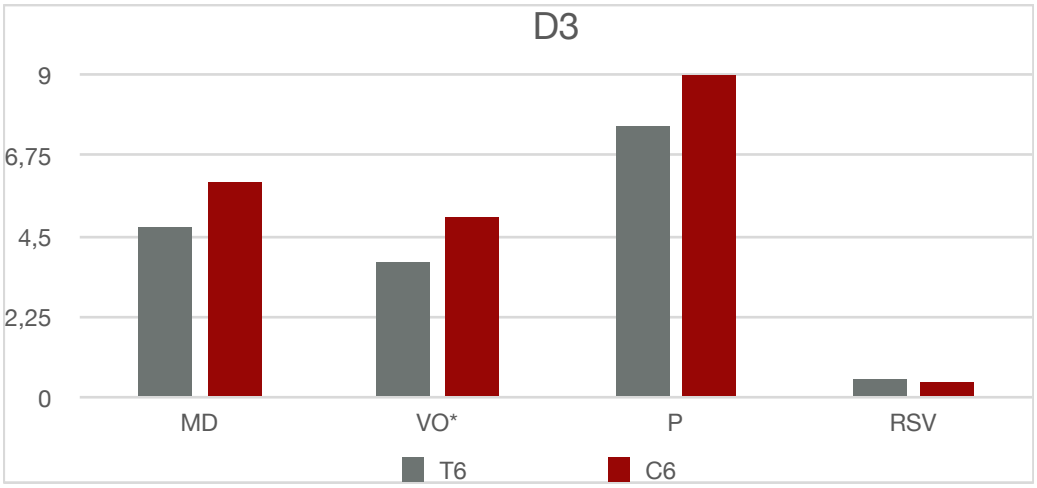


Figure 26. Socket closure at D3, day 3. Legend: maximum mesio-distal distance (MD), the maximum vestibulo-oral distance (VO) and the maximum probing depth (P). T6: group treated with Aminogam®6 Gel; C6: group control of T6. (*) : statistically significant p.

The observed sizes at D7 (figure 27) for the are: MD 3.5±1.5mm, VO 3.1±1.4mm, P 5.8±2.2mm and RSV 0.3±0.3. As for No Aminogam®6 Gel, however: MD 5.0±2.1mm, VO 4.0±2.1mm, P 6.0±2.2mm and RSV 0.2±0.1. At D7 the only statistically significant difference between the two groups was MD (p=0.01)

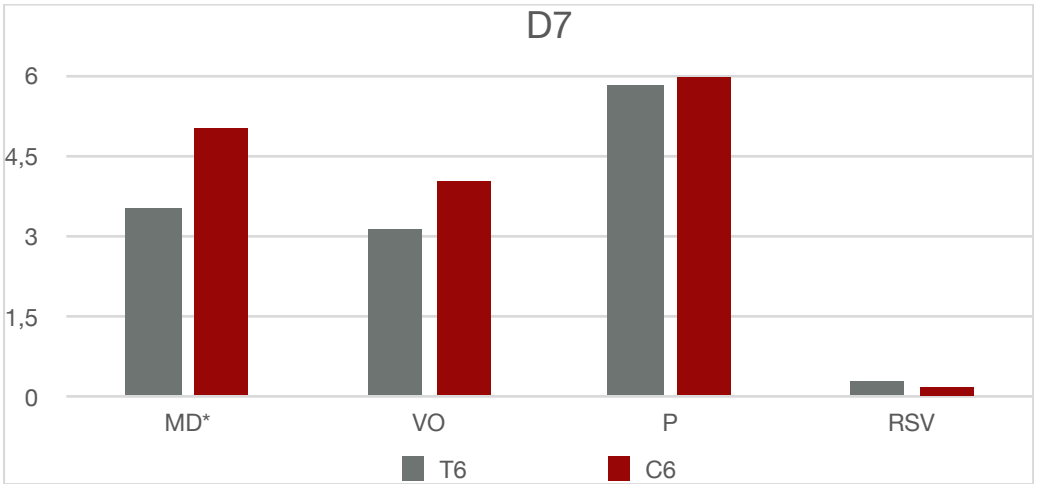


Figure 27. Socket closure at D7, day 7. Legend: maximum mesio-distal distance (MD), the maximum vestibulo-oral distance (VO) and the maximum probing depth (P). T6: group treated with Aminogam®6 Gel; C6: group control of T6. (*) : statistically significant p.

The mean sizes at D14 of theT6 group are: MD 2.8±2.1mm, VO 2.0±1.5mm, P 3.7±2.5mm with an RSV of 0.1±0.1. The mean dimensions at D14 of the C6 group are: MD 3.0±1.3mm, VO 3.0±1.6mm, P 3.0±2.5mm with an RSV of 0.04±0.03. (figure 28) Regarding D14 there are significant differences between the two groups for VO (p=0.04) and RSV (p=0.01).

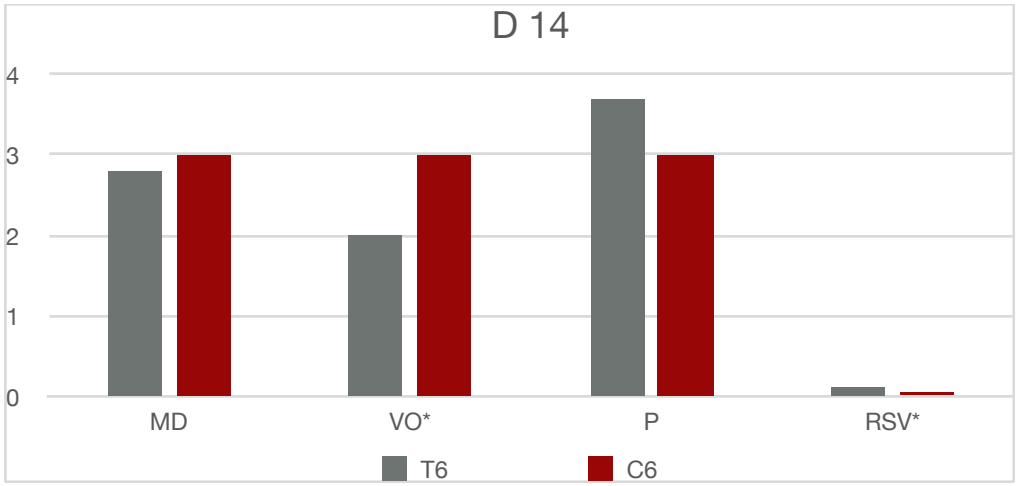


Figure 28. Socket closure at D14, day 14. Legend: maximum mesio-distal distance (MD), the maximum vestibulo-oral distance (VO) and the maximum probing depth (P). T6: group treated with Aminogam®6 Gel; C6: group control of T6. (*): statistically significant p.

Cytokines

During follow-up on day 3, healing tissue was taken and use to quantitatively measure the presence of cytokines. Specifically 3 cytokines were statistically significant between the two groups: GM-CSF (p=0.01), IL-10 (p<0.001) and TNF-α (p<0.001). (Figure 29)

GM-CSF in the T6 group was 3.9±2.2pg/ml while in the C6 group it was 2.6±1.1pg/ml.

IL-10 of the T6 group is 11.5±6.4pg/ml while in the C6 group it is 5.6±3.9pg/ml.

TNF-α in the T6 group was 27.8±12.5pg/ml while in the C6 group it was 14.3±6.5pg/ml.

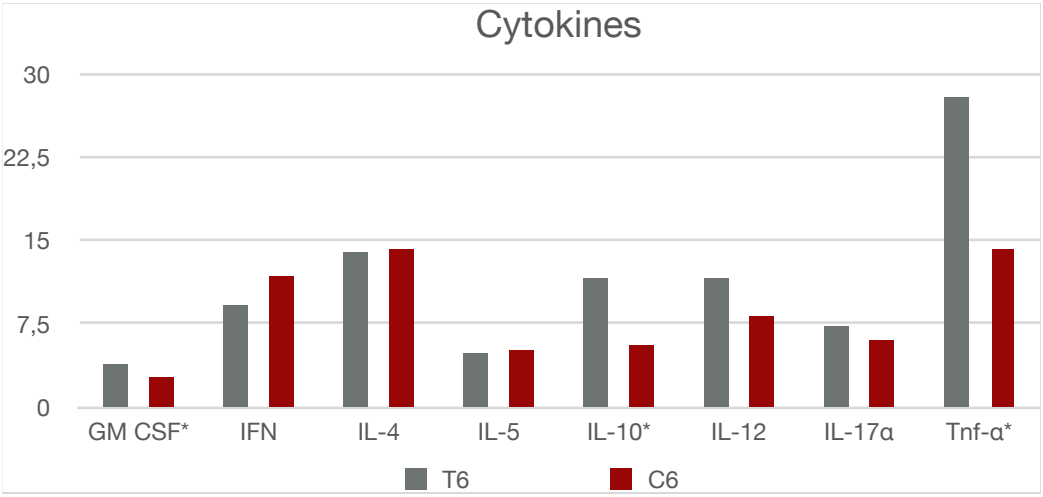


Figure 29. Cytokines analysis. T6: group treated withT6; C6: group control of T6. (*): statistically significant p.

Results of AG4 vs AG6 evaluation

The four study groups were compared with an advanced statistical analysis for a comparison of the efficacy of Aminogam®4 Gel vs Aminogam®6 Gel.

In total, 112 patients (n = 112), with a mean age of 69.44 ± 10.08 , met the inclusion criteria and were enrolled in the study.

In total, 112 patients (n = 112), with a mean age of 69.44 ± 10.08 , met the inclusion criteria and were enrolled in the study. Demographic and baseline characteristics are reported in Table 3. No drop-outs or patients lost to follow-up were recorded. All subjects were included in the efficacy and safety analysis data set. The tested product was well tolerated. No adverse reactions occurred during the study period. No requirement of antibiotic prescription was recorded for any of the enrolled patients.

No differences were observed in the patient collaboration. Only one subject was not cooperative.

When analyzing the variable Age in years in the four groups, (table) it can be seen that the T6 group has older patients (mean 78.08) ANOVA Between Groups p-value **< 0.001.**

Age (years)						
Group	N	Mean	Std. Deviation	Median	Minimum	Maximum
T4	36	66.853	10.9739	67.585	42.8	86.1
C4	36	66.853	10.9739	67.585	42.8	86.1
T6	21	**78.088**	3.3700	78.841	71.8	81.3
C6	19	69.686	5.6491	74.416	61.9	75.7
Total	112	69.440	10.0831	71.844	42.8	86.1
ANOVA Between Groups p-value **< 0.001**						

Table 10 . Descriptive with comparison between groups. Demographic parameters: Age. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Chi Square test p-value = **0.001**			Genre		Total
			Female	Male	
Group	T4	Count	19	17	36
		% Treatment	52.8	47.2	100.0
	C4	Count	19	17	36
		% Treatment	52.8	47.2	100.0
	T6	Count	15	6	21
		% Treatment	71.4	28.6	100.0
	C6	Count	2	17	19
		% Treatment	10.5	89.5	100.0
Total		Count	55	57	112
		% Treatment	49.1	50.9	100.0

Table 11. Descriptive with comparison between groups. Demographic parameters: Genre. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Analyzing the sex between groups, there are no statistically significant differences as shown in the table. Total number of female is 49.1, male is 50.9%

The evaluation of BMI showed a higher value in the C6 group (mean 33.11 ± 9.12), with ANOVA Between Groups p-value = 0.019 (table 12).

BMI						
Group	N	Mean	Std. Deviation	Median	Minimum	Maximum
T4	33	28.2109	4.82285	28.6000	22.00	39.90
C4	33	28.2109	4.82285	28.6000	22.00	39.90
T6	21	28.2386	5.83549	33.1500	19.92	34.19
C6	19	33.1137	9.12739	27.0400	24.20	46.98
Total	106	29.0952	6.20529	28.6000	19.92	46.98
ANOVA Between Groups p-value = **0.019**						

Table 12 . Descriptive with comparison between groups. Demographic parameters: BMI.

Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Table 13 shows the duration in years since the diagnosis of diabetes, with a higher proportion of patients with DM for more than 10 years (47.3%)

Chi Square test p-value = **0.009**			**Years Since Diagnosis of Diabetes**			Total
			1-5 y	6-10 y	> 10 y	
Group	T4	Count	9	13	13	35
		%	25.7	37.1	37.1	100.0
	C4	Count	9	13	13	35
		%	25.7	37.1	37.1	100.0
	T6	Count	0	5	16	21
		%	0.0	23.8	76.2	100.0
	C6	Count	0	9	10	19
		%	0.0	47.4	52.6	100.0
Total		Count	18	40	52	110
		%	16.4	36.4	47.3	100.0

Table 13 . Descriptive with comparison between groups. Demographic parameters: Years of Diabetes.
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Primary endpoint. Healing index

Table 14 shows data related to the healing index recorded at different time points, the descriptive analysis average of scores, with all measures.

Group		Healing Index (Hi)			
		D3 (Day 3)	D7 (Day 7)	D14 (Day 14)	T4 (Day 21)
T4	N	35	35	35	36
	Mean	**6.49**	**5.00**	**4.09**	**4.00**
	Std. Deviation	1.634	1.213	.507	.000
	Median	6.00	5.00	4.00	4.00
	Minimum	4	4	4	4
	Maximum	10	9	7	4
C4	N	35	35	35	36
	Mean	**7.63**	**5.91**	**4.54**	**4.00**
	Std. Deviation	1.750	1.669	1.010	.000
	Median	8.00	5.00	4.00	4.00
	Minimum	5	4	4	4
	Maximum	11	9	7	4
T6	N	21	21	21	21
	Mean	**5.81**	**4.05**	**4.00**	**4.00**
	Std. Deviation	2.112	.218	.000	.000
	Median	5.00	4.00	4.00	4.00
	Minimum	4	4	4	4
	Maximum	10	5	4	4
C 6	N	19	19	19	19
	Mean	**6.05**	**5.42**	**4.47**	**4.00**
	Std. Deviation	1.177	1.170	.841	.000
	Median	6.00	5.00	4.00	4.00
	Minimum	4	4	4	4
	Maximum	8	7	6	4

Table 14 . Data related to the healing index recorded at different time points. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Sockets treated with AG6 (T6 group) showed significantly ($p < 0.05$) better Healing index values at D7 ($p = 0.01$) and D14 ($p = 0.02$), while no statistically significant difference was highlighted at D3 ($p = 0.08$) and D21 ($p = 1$).

Sockets treated with AG6 had optimal healing (healing index = 4) at day 7, C4 and C6 at day 21.

To analyze the statistically difference between group treated with Aminogam 4 (group T4) and group treated with Aminogam®6 Gel, (group T6) it has been conducted a Univariate Analysis (ANOVA) - (T6 vs T4) analysis of the median compared with the mean.

ANOVA		Sum of Squares	df	Mean Square	F	Sig.
Day 3	Between Groups	6.001	1	6.001	1.801	.185
	Within Groups	179.981	54	3.333	-	-
	Total	185.982	55	-	-	-
Day 7	Between Groups	11.905	1	11.905	12.617	**.001**
	Within Groups	50.952	54	.944	-	-
	Total	62.857	55	-	-	-
Day 14	Between Groups	.096	1	.096	.596	.444
	Within Groups	8.743	54	.162	-	-
	Total	8.839	55	-	-	-

Table 15. Univariate Analysis (ANOVA) - (T6 vs T4) analysis of the median compared with the mean. Legend. T4 : group treated with Aminogam 4 gel T6 : group treated with Aminogam®6 Gel.

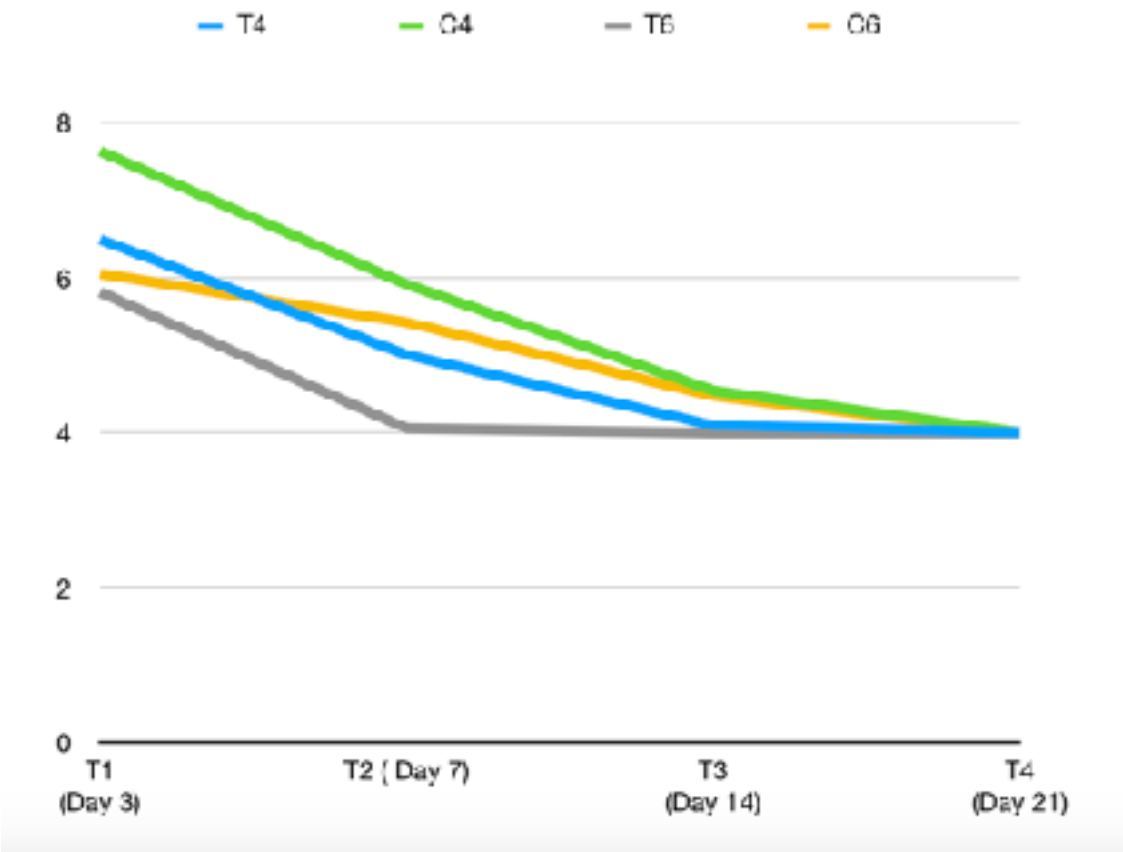


Figure 30. Univariate Analysis (ANOVA) - healing index during time, an evaluation of all 4 groups. Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Multivariate Analysis: GLM Repeated Measures (groups T6 vs. T4) – (Treatment and Age)

To add strength to the statistical analysis and to evaluate potential confounders, Multivariate Analysis were performed. GLM repeated measure is a statistical technique that takes a dependent, or criterion variable, measured as correlated, non-independent data.

Table 16 reported the multivariate analysis of the T6 vs. T4 groups of treatment and age for the healing index. Since age is different in the groups a multivariate analysis is performed; correcting for age and balancing the weight of age, there a significant comparison of 0.019 which shows how healing index varies with respect over time.

Mauchly's Test of Sphericity							
Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Time	.098	120.062	5	.000	.495	.525	.333

Tests of Within-Subjects Effects						
Measure: healing index						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Sphericity Assumed	7.595	3	2.532	2.630	.052
	Greenhouse-Geisser	7.595	1.484	5.118	2.630	.093
	Huynh-Feldt	7.595	1.574	4.825	2.630	.090
	Lower-bound	7.595	1.000	7.595	2.630	.111
Time * age	Sphericity Assumed	9.846	3	3.282	3.410	.019
	Greenhouse-Geisser	9.846	1.484	6.636	3.410	.052
	Huynh-Feldt	9.846	1.574	6.255	3.410	.049
	Lower-bound	9.846	1.000	9.846	3.410	.070
Time *treatment	Sphericity Assumed	16.528	3	5.509	5.724	.001
	Greenhouse-Geisser	16.528	1.484	11.139	5.724	.010
	Huynh-Feldt	16.528	1.574	10.500	5.724	.008
	Lower-bound	16.528	1.000	16.528	5.724	.020
Error (Time)	Sphericity Assumed	153.044	159	.963	-	-
	Greenhouse-Geisser	153.044	78.643	1.946	-	-
	Huynh-Feldt	153.044	83.431	1.834	-	-
	Lower-bound	153.044	53.000	2.888	-	-

Tests of Within-Subjects Contrasts						
Source	Hi	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Linear	.453	1	.453	.276	.602
Time * age	Linear	4.399	1	4.399	2.681	.107

Time * treatment	Linear	9.564	1	9.564	5.828	**.019**
Error (Time)	Linear	86.971	53	1.641		

Table 16 . Multivariate Analysis: GLM Repeated Measures (T 6 vs. T4) – (Treatment and Age)
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Between subjects, healing Index is statistically different; correcting for age and treatment results are: age is not significant (p 0.064), while is treatment that improves healing (p= 0.002).

Tests of Between-Subjects Effects					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	40.034	1	40.034	29.493	.000
Age	4.843	1	4.843	3.568	.064
Treatment	14.411	1	14.411	10.616	**.002**
Error	71.942	53	1.357	-	-

Table 17 .Tests of Between-Subjects Effects (T6 vs. T4) – (Treatment and Age)
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Multivariate Analysis: GLM Repeated Measures [(T6 vs. C6) vs. (T4 vs. C4)] for age

In table 18 and 19 the multivariate analysis is reported, GLM Repeated Measures for T6 group and C6 group (T6 vs. C6) vs. T6 group and C6 group (T4 vs. C4) with own control group, for repeated measures. Treatment of T6 corrected for own control group ,and T4 corrected for own control group.

Data are corrected for age and own control group, and the p value < 0.001, both in subjects and between subjects, demonstrates that the age has a predictive impact toward healing index, corrected for treatment group.

Mauchly's Test of Sphericity							
Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Time	.364	104.854	5	.000	.656	.694	.333

Tests of Within-Subjects Effects						
Measure: healing index						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Sphericity Assumed	2.842	3	.947	.912	.435
	Greenhouse-Geisser	2.842	1.968	1.444	.912	.402
	Huynh-Feldt	2.842	2.082	1.365	.912	.406
	Lower-bound	2.842	1.000	2.842	.912	.342
Time * age	Sphericity Assumed	7.898	3	2.633	2.536	.057
	Greenhouse-Geisser	7.898	1.968	4.013	2.536	.083
	Huynh-Feldt	7.898	2.082	3.794	2.536	.079
	Lower-bound	7.898	1.000	7.898	2.536	.114
Time * treatment	Sphericity Assumed	54.378	9	6.042	5.821	.000
	Greenhouse-Geisser	54.378	5.904	9.210	5.821	.000
	Huynh-Feldt	54.378	6.245	8.707	5.821	.000
	Lower-bound	54.378	3.000	18.126	5.821	.001
Error (Time)	Sphericity Assumed	326.986	315	1.038	-	-
	Greenhouse-Geisser	326.986	206.647	1.582	-	-
	Huynh-Feldt	326.986	218.585	1.496	-	-
	Lower-bound	326.986	105.000	3.114	-	-

Tests of Within-Subjects Contrasts						
Source	Hi	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Linear	8.593E-007	1	8.593E-007	.000	.999
Time * age	Linear	6.390	1	6.390	4.304	**.040**
Time * treatment	Linear	41.750	3	13.917	9.373	**< 0.001**
Error (Time)	Linear	155.904	105	1.485	-	-

Table 18. Multivariate Analysis: GLM Repeated Measures [(T6 vs. C6) vs. (T4 vs. C4)]
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Tests of Between-Subjects Effects					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	100.546	1	100.546	55.819	.000
Età	11.572	1	11.572	6.424	**.013**
Trattamento	74.722	3	24.907	13.827	**< 0.001**
Error	189.137	105	1.801	-	-

Table 19. Tests of Between-Subjects Effects (T6 vs. C6) vs. (T4 vs. C4) – (Treatment and Age) Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Multivariate Analysis: GLM Repeated Measures (AM 6 vs. AM4) - (Treatment and Diabetes) corrected for year of onset of diabetes.

When corrected for the number of years of diabetes onset has no statistical significance. The effect of healing index absorbs power of the study. (Table 20)

Mauchly's Test of Sphericity							
Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Time	.112	111.030	5	.000	.535	.571	.333

Tests of Within-Subjects Effects						
Measure: healing index						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Sphericity Assumed	30.098	3	10.033	10.000	.000
	Greenhouse-Geisser	30.098	1.605	18.756	10.000	.000
	Huynh-Feldt	30.098	1.712	17.584	10.000	.000
	Lower-bound	30.098	1.000	30.098	10.000	.003
Time * Duration of diabetes (years)	Sphericity Assumed	1.391	3	.464	.462	.709
	Greenhouse-Geisser	1.391	1.605	.867	.462	.589
	Huynh-Feldt	1.391	1.712	.812	.462	.601
	Lower-bound	1.391	1.000	1.391	.462	.500
Time *	Sphericity Assumed	8.399	3	2.800	2.791	.042

treatment	Greenhouse-Geisser	8.399	1.605	5.234	2.791	.078
	Huynh-Feldt	8.399	1.712	4.907	2.791	.075
	Lower-bound	8.399	1.000	8.399	2.791	.101
Error (Time)	Sphericity Assumed	156.503	156	1.003	-	-
	Greenhouse-Geisser	156.503	83.445	1.876	-	-
	Huynh-Feldt	156.503	89.006	1.758	-	-
	Lower-bound	156.503	52.000	3.010	-	-

Table 20. Multivariate Analysis: GLM Repeated Measures (AM 6 vs. AM4) – (Treatment and duration of diabetes in years)
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Effect of healing index absorbs the power of the study, when compared with baseline confounders. Between subjects, healing Index is statistically different; correcting for years of diabetes and treatment results are: is treatment that improves healing (p= .032).

Tests of Within-Subjects Contrasts						
Source	Hi	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Linear	21.027	1	21.027	12.373	.001
Time * duration of diabetes (years)	Linear	.006	1	.006	.003	.954
Time * treatment	Linear	3.968	1	3.968	2.335	.133
Error (Time)	Linear	88.370	52	1.699	-	-

Tests of Between-Subjects Effects					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	820.561	1	820.561	567.013	.000
Duration of diabetes (years)	.015	1	.015	.011	.918
Treatment	7.004	1	7.004	4.840	**.032**
Error	75.253	52	1.447	-	-

Table 21.Tests of Between-Subjects Effects (AM 6 vs. AM4) – (Treatment and Duration of diabetes in years)
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel; C6: group control of T6.

Multivariate Analysis: GLM Repeated Measures [(T6 vs. C6) vs. (T4 vs. C4)] for years of diabetes

In table 22 AND 23 multivariate analysis is showed, GLM Repeated Measures for T6 group and C6 group (T6 vs. C6) vs. T6 group and C6 group (T4 vs. C4) with own control group, for repeated measures, years of diabetes. Treatment of T6 corrected for own control group, and T4 corrected for own control group.

Data are corrected for age and own control group, and there is a p value < 0.001, both in subjects and between subjects, showing that years of diabetes have a predictive impact toward healing index, corrected for treatment group.

Mauchly's Test of Sphericity							
Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Time	.375	99.700	5	.000	.670	.710	.333

Tests of Within-Subjects Effects						
Measure: HEALING INDEX						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Sphericity Assumed	80.209	3	26.736	25.425	.000
	Greenhouse-Geisser	80.209	2.009	39.921	25.425	.000
	Huynh-Feldt	80.209	2.129	37.678	25.425	.000
	Lower-bound	80.209	1.000	80.209	25.425	.000
Time * duration of diabetes (years)	Sphericity Assumed	.412	3	.137	.131	.942
	Greenhouse-Geisser	.412	2.009	.205	.131	.878
	Huynh-Feldt	.412	2.129	.194	.131	.889
	Lower-bound	.412	1.000	.412	.131	.718
Time * treatment	Sphericity Assumed	46.747	9	5.194	4.939	.000
	Greenhouse-Geisser	46.747	6.028	7.756	4.939	.000
	Huynh-Feldt	46.747	6.386	7.320	4.939	.000
	Lower-bound	46.747	3.000	15.582	4.939	.003

Error (Time)	Sphericity Assumed	324.942	309	1.052		
	Greenhouse-Geisser	324.942	206.946	1.570		
	Huynh-Feldt	324.942	219.269	1.482		
	Lower-bound	324.942	103.000	3.155		

Tests of Within-Subjects Contrasts						
Source	Hi	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Linear	72.076	1	72.076	47.938	.000
Time * duration of diabetes (years)	Linear	.026	1	.026	.017	.896
Time * treatment	Linear	35.435	3	11.812	7.856	**< 0.001**
Error (Time)	Linear	154.862	103	1.504	-	

Table 22. Multivariate Analysis: GLM Repeated Measures [(T6 vs. C6) vs. (T4 vs. C4)]
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel,; C6: group control of T6.

Tests of Between-Subjects Effects					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	1996.770	1	1996.770	1068.233	.000
duration of diabetes (years)	.004	1	.004	.002	.964
Treatment	63.946	3	21.315	11.403	**< 0.001**
Error	192.530	103	1.869	-	-

Table 23. Multivariate Analysis: Tests of Between-Subjects Effects
Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel,; C6: group control of T6.

Secondary Endpoint - Socket closure

The secondary endpoint, the socket closure did not give significant statistics, it has a closing trend, as all tooth extraction sockets have a tendency for uniform closure however, there is an important standard deviation, as shown in table 24, where are showed also descriptive analysis and statistical analysis.

Group		Socket closure (%)			
		D3 (Day 3)	D7 (Day 7)	D14 (Day 14)	D21 (Day 21)
T4	N	36	36	36	36
	Mean	37.6810	56.7983	78.3100	98.1944
	Std. Deviation	21.12038	22.16839	20.29422	5.62555
	Median	33.3333	53.5714	83.3333	100.0000
	Minimum	.00	.00	20.00	75.00
	Maximum	100.00	100.00	100.00	100.00
C4	N	36	36	36	36
	Mean	23.7163	51.2109	73.1302	94.8148
	Std. Deviation	21.73066	27.91733	21.87552	9.98124
	Median	18.3333	46.4286	77.5000	100.0000
	Minimum	.00	.00	33.33	66.67
	Maximum	80.00	100.00	100.00	100.00
T6	N	21	21	21	21
	Mean	31.6564	41.3069	67.0573	87.6562
	Std. Deviation	27.91825	37.37202	23.96677	20.19810
	Median	40.0000	50.0000	70.0000	100.0000
	Minimum	-25.00	-66.67	.00	50.00
	Maximum	75.00	100.00	100.00	100.00
C6	N	19	19	19	19
	Mean	37.7453	47.2086	69.4272	94.1414
	Std. Deviation	22.63806	25.79535	17.14487	9.64461
	Median	40.0000	55.5556	66.6667	100.0000
	Minimum	-16.67	-25.00	25.00	75.00
	Maximum	80.00	80.00	100.00	100.00

Ranks			
	GROUP	N	Mean Rank
Socket closure % D3	T4	36	64.38
	C4	36	42.79
	T6	21	58.62
	C6	19	65.21
	Total	112	-
Socket closure % D7	D21	36	63.86
	C4	36	54.39
	T6	21	49.45
	C6	19	54.34
	Total	112	-
Socket closure % D14	D21	36	65.60
	C4	36	56.46
	T6	21	47.93
	C6	19	48.82
	Total	112	-
Socket closure % D21	D21	36	63.35
	C4	36	55.44
	T6	21	50.29
	C6	19	52.39
	Total	112	-

Test Statistics				
	Socket closure % D3	Socket closure % D7	Socket closure % D14	Socket closure % D21
Chi-Square	10.041	3.087	5.428	5.103
df	3	3	3	3
Asymp. Sig.	.018	.378	.143	.164

Table 24. Socket closure statistics

Legend. T4 : group treated with Aminogam 4 gel; C4: group control of T4; T6 : group treated with Aminogam®6 Gel,; C6: group control of T6.

Discussion

The aim of the present randomized control trial was to investigate whether the employment of HA gel can provide benefits in the post-extraction tooth socket healing in subjects with T2DM. Patients with this systemic chronic disease experience slowed wound healing and adverse alterations in the three-dimensional remodelling of the socket. [113]

More than half of diabetic wounds in other districts develops into chronic wounds, with increased risks of amputation and death. Additionally, diabetic wounds are 60–70% more likely to recur, which makes their treatment costs significantly more expensive. [164- 165].

The role of growth factors and cytokines in the management of Diabetic Foot Ulcers has been analyzed, such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and Insulin like growth factor (IGF). Diabetic wound healing process can be accelerated by regulating the levels of the cytokines. [71]

Ideal interventions to be used in oral surgery should facilitate the repair of extraction sockets, reducing the postoperative infection, pain and complications. Several investigations have explored pathways to accelerate healing under high-glucose conditions based on molecular regulators of their activity. Directly interacting targets include growth factors, BMPs, parathyroid hormone, and stem cells. A variety of drugs may act indirectly on molecular targets by up- or down-regulating the expression of growth factors, MMP, collagen synthesis/degradation, pro- and anti-inflammatory cytokines, and pro-angiogenic factors. [110]

The present study was designed as a single center randomized controlled trial to assess the evaluation of use of AG4 and AG6 on T2DM post extractive socket healing.

Healing index

The first primary outcome of the study was to evaluate the impact of the different treatments on the healing index. In order to clinically evaluate the healing of the soft tissues following extraction, the Landry index [162] was used, which was modified so that it could be applied to the evaluation of the post-extraction socket. In fact, this index was devised by the authors to evaluate the healing of soft tissues following periodontal surgery, with suturing and closure of the wound by primary intention. Since the healing of the post-extraction socket recognizes a healing mechanism by secondary intention and in order to be able to evaluate the regeneration of the soft tissues, some of the parameters obtained from the original index were adopted in this study, modifying them so as to be able to apply them to healing by second intention.

Evaluating the healing index, a statistically better result was highlighted for the T4 group vs C4 group at D7 and D14, while no statistically significant difference was found at D3 and D21.

Also a strong statistically better result was highlighted for the T6 group vs C6 group at D7 and D14, where a statistically significant improved healing is observed at D7 ($p<0.001$) and D14 ($p<0.001$) in favor of the T6 group.

These results seem to indicate that the application of HA plays a significant role in improving the post extractive wound healing in the first 2 weeks post-surgery. In these time frames the diabetic patient has a greater risk of superinfection and, therefore, it is fundamental to have a fast and predictable wound healing process. The possible role of HA gels in improving the healing was also highlighted by the percentage of ‘‘excellent healing’’ (healing index = 4). A statistically significance difference between groups was highlighted at D7 and D14, with 97.2 % of sites for the T4 group and 72.2% of sites for the C4 group that reached the excellent healing at D14. This result indicate that in the sites not treated a delayed healing of the wound occurs, confirming the difficulties in wound healing typical of diabetic patients.

Still, better results are seen in T6 group, in regard to the % of sockets that presented with optimal healing (healing index = 4), sockets treated with AG6 were performed at day 7 (4.05). At follow-ups

at D14, i.e., 14 days after surgery, healing is always in favor of the T6 group (4.0±0.1) compared with the C6 group (5.0±0.9).

To add strength to the statistical analysis and to evaluate potential confounders, Multivariate Analysis were performed. GLM Repeated Measures for T6 group and C6 group (T6 vs. C6) vs. T6 group and C6 group (T4 vs. C4) with own control group, for repeated measures, were performed for age and years of diabetes. Data were corrected for age and own control group, and there is a p value < 0.001, both in subjects and between subjects, showing that years of diabetes have a predictive impact toward healing index, corrected for treatment group. This analysis shows that age and years of diabetes are not confounders for the study.

Socket closure

The second primary outcome that was analyzed was the Socket closure. Based on the results, a statistically significant difference ($p < 0.05$) was highlighted between the first groups at D3, D7 and D14, with the T4 group presenting a better remodeling of the socket compared to the C4 group. On the opposite, when considering the % of fully closed sockets, no statistically significant difference ($p > 0.05$) was highlighted between the two groups at any time point.

When comparing T6 and C6, there is a statistically significant measurement between the two groups at D3 of VO ($p=0.03$), at D7 of MD ($p=0.01$). T6 has a better closing trend than C6 but not statistically significant; however, there is an important standard deviation.

This result may indicate that, despite the full closure of the sockets visually observed, the quality and timing of the healing could be significantly influenced by the application of the HA gel.

Secondary outcomes

As secondary outcomes, the VAS scale and patient questionnaires were analyzed. For both of the outcomes, positive results in favor of the HA gels were found. All of the patients (100%) found

healing with HA more comfortable and would prefer the product for a future extractions. 89% of patients noted more bleeding at the control site, and 56% reported more pain in the socket not treated with HA.

Following this result, the application of HA gels seems to be promising in improving both the timing, the quality, and patients' experience during the wound healing process.

However, some consideration must be taken into account. The whole number of sockets reach the perfect scores, both considering the healing index and the socket closure, after 21 days from the surgery despite the application or not of the HA gel. No complications or superinfections were noted for either the 2 groups. This result may indicate how the application of HA may represent a way to speed up the healing process and lower the risk of infection by decreasing the timing of wound closure, however, the final healing observed in the longest follow up period (D21) was not influenced by the application of the HA gel.

No data about bone remodeling was collected. This is a limitation inherent to the short follow-up period applied in the study. Further studies with higher follow up periods are necessary to evaluate whether the HA gel may improve the bone healing and the socket remodeling after teeth extraction. Indeed, the socket preservation is sometimes challenging even in healthy patients and it becomes more important for diabetic patients who seek to undergo implant therapy in the future. [116, 166]

Citokines

Citokines detected at D3 were statistically significant between the two groups: GM-CSF ($p=0.01$), IL-10 ($p<0.001$) and TNF-α ($p<0.001$).

This could be explained as GM-CSF was recognized as a central mediator of inflammation bridging the innate and adaptive arms of the immune system. GM. CSF plays a central role in the recruitment of cells of inflammation.

Phagocytes sensing GM-CSF adapt an inflammatory phenotype and facilitate pathogen clearance. However, in the context of chronic tissue inflammation, GM-CSF secreted by tissue-invading lymphocytes has detrimental effects by licensing tissue damage and hyperinflammation. Accordingly, therapeutic intervention at the T cell-phagocyte interface represents an attractive target to ameliorate disease progression and immunopathology. Although GM-CSF is largely dispensable for steady state myelopoiesis, dysregulation, as seen in chronic inflammatory diseases, may however lead to disrupted haematopoiesis and long-term effects on bone marrow output. [167]

The cytokine IL-10 is increased in treated sockets, as it is a key anti-inflammatory mediator ensuring protection of a host from over-exuberant responses to pathogens and microbiota, while playing important roles in other settings as sterile wound healing, autoimmunity, cancer, and homeostasis. IL-10 regulates basic processes of neural and adipose cells and how it promotes CD8 T cell activation, as well as wound healing repair. [168]

The increase of TNF-α its commonly balanced by the increase of IL-10, following the results in the clinical point of view, where it seems to have an improved healing in treated sockets. However, the presence of TNF-α is controversial, as the microenvironment of a chronic wound is characterized by high quantities of pro-inflammatory macrophages, overexpression of inflammatory mediators such as TNF-α and IL-1β. Moreover, chronic wounds are frequently complicated by bacterial biofilms, which perpetuate the inflammatory phase. Continuous inflammation and microbial biofilms make it very difficult for the chronic wounds to heal. Neutrophils, as “first responders” for tissue injury, accumulate at the wound site at an early stage of inflammatory response. Neutrophils can be recruited to the site of injury by DAMPs, pro-inflammatory cytokines, including TNF-α and chemo-attractants, such as CXCL 1–3 and IL-8, anaphylatoxins C3a and C5a and macrophage inflammatory protein-1α. [169]

A recent review showed several chronic disorders including T2DM, obesity, heart disease and cancer can develop after a period of persistent low-level inflammation. Cytokines could be early indicators for these chronic conditions and can be detected in saliva through the bloodstream, and there exists a strong correlation between their levels in saliva and blood serum, specifically for a variety of interleukins (IL-1β, IL-2, IL-4, IL- 6, IL-10 etc.) and TNFα a. Also,IL-1β, a cytokine released from macrophages and non-immune cells in the context of inflammation, participates to innate immune response, whereby promoting the secretion of IL-6 and TNFα. [170]

Conclusion

Based on the results of the present study, HA seem to be promising in improving the timing and the quality of post-extractive wound healing in T2DM. Further research is required to confirm the results.

The results of the present study are in agreement with the randomized control trial who followed 30 diabetic patients treated with and without HA up to 25 days post-extractions. The study results demonstrated significantly improved healing in sockets treated with HA after 10 and 15 days, showing considerable enhancement in socket closure across all observed time points. In contrast to this current study, the HA-treated group exhibited notably higher socket closure rates compared to the control group, extending up to 25 days post-extraction. Nevertheless, the methodology used to assess closure involved overlaying photos taken at various checkpoints, differing from the approach employed in our present study. [149]

To date, different studies analyzed the effect of HA in the post-extractive healing of healthy patients.

A recent study compared HA, applied both as a gel and a spray, with no treatment in the healing of extraction sockets and found a beneficial effect in favor of HA in the immediate post-operative

healing. HA gel was seen to offer the better results when compared to the spray, however, the difference was not found statistically significant. [171]

In agreement, another study investigated the effect of HA in improving wound healing and bone formation after teeth extraction in sockets with chronic pathology in dogs. The authors' findings showed promising results of HA in enhancing the wound healing and the sub-sequential bone formation. [172]

Conversely, a different study presented conflicting findings compared to the aforementioned research. Their investigation focused on applying amino acid and sodium hyaluronate gel following surgical extraction of third molars. The study's results did not reveal any significant disparity in post-extraction healing between the use of the tested product and the control group (no treatment). [173]

In conclusion, research is continuing to focus on the interaction that systemic conditions may have on the dental treatment of the patients. The present study reports positive effects on the employment of HA as an adjunct treatment of DM patients that require tooth extractions. Also, it seems to be interesting to evaluate the efficacy of two different formulations of a HA gel.

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