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From Tissue Repair to Tissue Regeneration

Aragona Salvatore Emanuele, Mereghetti Giada,
Ferrari Alessio and Giorgio Ciprandi

Additional information is available at the end of the chapter

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Abstract

In Regeneration 3.0, the priority is to combine the anti-inflammatory activity of the nine proteins acting as growth factors in the bovine colostrum, the homeostatic, angiogenic and reorganizational activities of the matrix, the modulation of collagen synthesis and the remodeling of the epithelium. The choice of bovine colostrum and its associated properties was the basis for the design of devices that could also offer those properties: barrier action, anti-inflammatory action and pain reduction, reduction and absorption of exudates, combating of bacterial and fungal proliferation, antioxidant action and hydration and protection against skin diseases and dermatosis. We now know the key players in the wound healing process and we have new molecules available to act on them, but the future must necessarily lie in the transfer of molecules and information between the endothelium, ECM and cell membrane, which can be directed toward tissue regeneration if the resident stem cells have the chance of communicating and interacting with new therapeutic models, all this without forgetting that the human being is at the center of research and scientific evolution.

Keywords: wound healing, tissue regeneration, bovine colostrum, stem cells, aimed protocol

1. Introduction

The complexity of the wound healing process is increasingly understood and characterized. Until recently, the wound healing mechanism was interpreted as a fibroproliferative response with the aim of producing a cicatricial reaction (repair), with different mechanisms than those seen in a fetal environment, in which the scope of the healing process is tissue regeneration. However, recent awareness of the biological pathways and cell classes

characterizing the various phases of the wound healing process and current attention toward biomaterials and possible new applications for stem cells, together with the use of bioengineered tissues, have led to a reinterpretation of this process from the perspective of regenerative medicine, intended as the possibility of recreating a tissue as similar as possible to the original.

Current understanding of some of the tissue repair mechanisms enables the application of therapeutic models that have become an established part of everyday practice. The healing process takes place over three complex phases: management of the inflammatory process, cell proliferation (excessive or impaired), and extracellular matrix (ECM) remodeling. Wound repair is characterized by the incomplete regeneration of the original tissue with hyperproduction of organized collagen, which can lead to the production of new tissue with an 80% similarity to the original tissue. Impaired host management of this process leads to an abnormal fibroproliferative response, causing the production of hypertrophic or keloid scars. While some of the mechanisms are already known, new discoveries in the field of molecular and cellular mechanisms enable us to hypothesize other tissue healing management mechanisms and to apply new therapeutic models to achieve results beyond those currently possible. As far back as 2013, Aragona and Marazzi and colleagues [1] published an article in the Italian edition of *Surgical Tribune* discussing new research in the treatment of skin lesions in regenerative medicine, establishing the bases for the interpretation of the inflammatory process that directly involve some of the cell classes naturally involved in the inflammatory process underlying wound regeneration.

Current gains in knowledge of tissue regeneration, and above all of stem cells and their behavior, open new ground and suggest new future therapeutic models. Research into the effects of electromagnetic fields on stem cells in particular indicates that a paradigm shift is within our grasp. With a little imagination, we can visualize ourselves as an avatar observing nature and the universe: we can close our eyes and listen to the sounds and energy emitted by cells, or open them to observe phenomena that yesterday, we thought a world away. We begin this journey through the study of the inflammatory process by taking a look at in-vitro experimental models, fundamental above all in understanding the molecular, genetic and cellular patterns of the various tissues.

2. The inflammatory model of skin lesions. From experimental model to humans

“Why, sir, his hide is so tanned with his trade that he will keep out water a great while, and your water is a sore decayer of your whoreson dead body.”

W. Shakespeare, Hamlet, Act 5, Scene 1.

Understanding the inflammation process is enabled by the design and creation of in vitro and in vivo cellular models, with the primary objective of establishing the effect of the molecules of biological agents on the inflammatory process associated with the tissue repair and

regeneration process. This innovation over animal models makes it possible to establish the role and concentration of all the humoral factors (cytokines and growth factors), genetic factors (genes expressed in the various phases of the process), cellular factors and, above all, the fibroblasts and collagen associated with the role of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), fundamental for extracellular matrix (ECM) remodeling. It also enables the concept of tissue regeneration to take over from tissue repair as the primary objective of research and clinical practice.

The literature is full of studies meticulously describing the inflammatory process of wounds. It should be remembered that modulation of this process and its impact on the proliferation, differentiation and function of inflammatory cells is aimed at controlling that very inflammation. Our research had the objective of framing all these observations and this scientific knowledge in the biological context in which the process takes place, within its specific individual reactivity.

The shift of perspective from inflammation to regeneration involves the systemic treatment of patients with skin and mucosal wounds and local anti-inflammatory treatment involving modulation of the endothelial and ECM inflammation, the anti-inflammatory cytokines and the MMPs.

Figure 1 highlights the switch between the wound repair and regeneration pathway and the chronic inflammation pathway that feeds the chronicity of the skin lesions. Initially, platelet activation leads to the release and activation of TGF-beta 1, PDGF, TNF-alpha and IL1, with the recruitment of neutrophils deputized to natural debridement and to regulation of the expression of the adhesion molecules. The neutrophils are followed by macrophages, the cell population now at the center of attention in wound healing research. These ensure sustained debridement, the release of proinflammatory cytokines, and the potentiation of the fibroproliferative response in the context of chronic inflammation.

The role of macrophages deserves a chapter of its own in relation to their division into the M1 population, which can eliminate invading microorganisms and promote the inflammatory response during the initial inflammatory process, and subsequently the M2 population, during the resolution of the inflammatory process. By losing its reactivity to inflammatory stimuli, the increasingly studied M2 population becomes capable of eliminating damaged cells and tissues, promoting neoangiogenesis and tissue remodeling.

This all takes place within the dynamic structure of the ECM, which can be defined as “the submerged world where everything is possible” thanks to the equilibrium between the glycoprotein, molecular and cellular components assured by the aqueous component that, in light of the latest knowledge, seems to enable communication between the cells and the ECM and could explain some amplification mechanisms of the healing process. The authors had the opportunity to test an *in vitro* model on both epidermal and Dry Eye Syndrome cell cultures. In both trials, the primary objective was to have a cellular, ultrastructural and biochemical model similar to real life in which to cause an injury similar to that under investigation. The biological injury, whether damage to the epidermis and dermis or creation of an area of dehydration on the eye surface, recreated the natural pathophysiological environment, and the *in vitro* model offers a faithful reflection. *“Efficacy of a New Ocular Surface Modulator in Restoring*

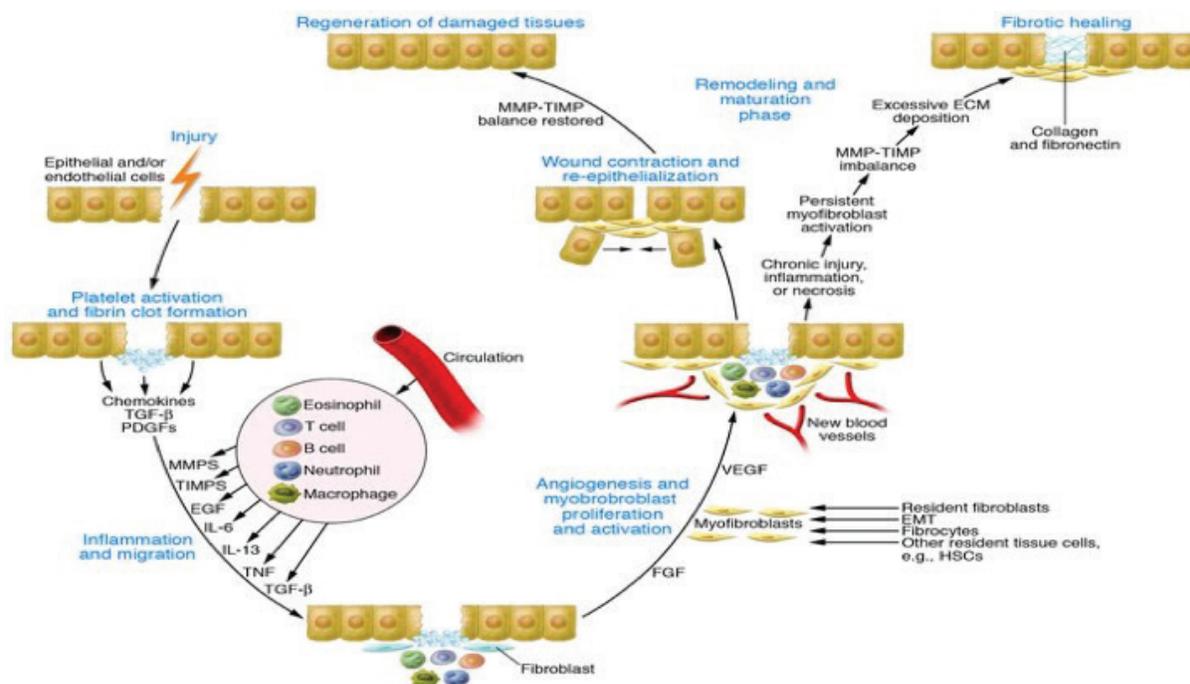


Figure 1. Highlights the switch between the wound repair and regeneration pathway and the chronic inflammation pathway that feeds the chronicity of the skin lesions (M. Meloni- Vitroscreen Milano Italy 2017).

Epithelial Changes in an in Vitro Model of Dry Eye Syndrome Barabino and colleagues, Current Eye Research, in press [2].

The process of learning about the biological reality examined in vitro and the scientific observation necessary to recreate the metabolic, structural and ultrastructural conditions of a cell/tissue/organ model make it possible to understand and reproduce the biological mechanism involved. Above all, however – and this is the revolutionary part for the translation of research to clinical practice – they make it possible to predict quantitatively the model's response, and hence the natural response of the disorder under study, following treatment with a given biological substance. This means understanding if that biological substance – that drug – actually works.

For skin lesions, the in vitro model has an absolute value, because animal models suffer from important biological interferences. For investigation of the mechanism of action, the in vitro method opens up:

“the marvelous opportunity to discover something different, something unknown before now, because using a biological model guarantees the predictive value of the generated data and permits us to measure what cannot or should not be measured in vivo.

“Measure what can be measured, and make measurable what cannot be measured” (Galileo).

“The in vitro model, therefore is not a test, but an experimental model, and in skin lesions it enables a better understanding of the mechanisms of action, the collection of quantitative information that could not be obtained in any other way, and the creation of a body of evidence, case by case.” Marisa Meloni, CEO Vitroscreen, Milano, Italy.

It enables us to approach the real target and model, human beings (relevance), and to determine and distinguish between differences in the response (reliability) and to reproduce the response *in vivo* (predictivity). The model is reproducible (the tests can be repeated with similar results) and the biological response of the trial products can be confirmed at different times (reproducibility).

For researchers working with skin lesions every day, the *in vitro* experimental model makes it possible to develop a clinical intuition and investigate the hypothesized role of a therapeutic substances or agents, while the use of data from *in vitro* models and the testing of potential medications enables a decision on whether or not to develop them and use them in subsequent *in vivo* studies to add substance and support to the clinical studies.

As noted above, the repair and regeneration process consists of three phases (modulation of the inflammatory process, modulation of cell proliferation, and modulation of extracellular matrix remodeling), and regenerative medicine must be based on cell therapies, engineered tissues and biological products formulated by clinical research and able to mimic and reproduce the natural repair and regeneration process.

Before entering into the merit of the *in vitro* model, it is worth asking what diagnostic factors are predictive of an evolution toward healing. Clinical factors are the first to be considered: underlying conditions, comorbidities, nutritional status, and medical treatments. In relation to humoral factors, can proinflammatory cytokines, MMPs, growth factors and macrophages be measured or tested in a recalcitrant skin ulcer? Can they be made the diagnostic target of an inflammation that develops toward healing or toward chronicity? All these targets may be measurable and are – or could be – predictive, but we are still far from their use in clinical practice to establish the most suitable treatment.

In the author's opinion, it should not be forgotten that humans are variable individuals: we know more than ever how humans become ill, but we also know that the repair, regeneration and healing process is highly individual.

In vitro models of skin lesions enable us to establish the behavior of the innate immune system in the first, inflammatory phase, which lasts from 2 hours to 5 days. Cell migration and inter-cellular and cell–cell matrix adhesion have been observed (and are discussed below), and the following markers have been quantified: IL-1a, IL-8, TNF-alpha, IFN-A1, HSP-70 (Heat protein shock 70), MAP3K8, NK, CD68, MMP-2, MMP-9, ADAM15, ADAMTS8, ITGA1, ITGB2, and RPSA.

After 1 week, in both the experimental model and *in vivo*, the cell proliferation phase begins. This involves granulation of the newly formed tissue and modulation of the cell population (transition from M1 to M2, blockage of monocytes, increase in fibroblasts and deposition of type III and VII collagen), with a process defined as wound retraction and re-epithelialization. In this phase we can measure VEGF-C, CTNNA1 (gene expression of cadherin production), FLNB, TMP2, BPI, FN1, and DECORIN.

The remodeling phase involves an increase in collagen deposition and in its tensile strength, with the substitution of collagen type III by type I; this should not necessarily be encouraged, as it leads to cicatricial retraction and repair rather than regeneration of the original tissue.

We can thus determine the presence and role of IL-1a, IL-8, HPS70, NK and BPI in the injured tissue in the inflammatory phase, of IL1a-IL-8, TNF-alpha, MMP2, MMP9, ADAM15 and ADAMTS8 in the re-epithelialization phase, of ITGA1 and ITGbeta2 in the cell migration and adhesion phase, and of VEGF-C, CTNNB1, DNAI1, FLNB, and TPM2 in the subsequent phase, in which remodeling begins.

The biological processes involved in the various phases of cicatrization are complex and often concomitant. The inflammatory phase, the keratinocyte migration phase, the proliferation phase, the formation of new tissue, and remodeling are all associated with a number of morphological and biochemical changes that can be quantified in the various skin layers through the choice of relevant markers.

The observation of these pathophysiological phenomena and processes in nature leads us to hypothesize new therapeutic procedures. The ability to detect and quantify the biological agents involved in the process, and to study their behavior, could enable the development of new therapeutic strategies.

Innovation means searching and researching.

Researching means having intuitions that can be tested.

Testing means using scientific processes that can validate the research.

Skin lesions, precisely because they are an expression of a systemic pathological status, lead us to use the experimental model to validate future therapies based on awareness of the fine molecular and cellular mechanisms. Understanding the phenomena inherent to the acute and chronic inflammatory process as a defense mechanism activated by the body leads to an understanding of how people can become ill and recover. Reproducing these phenomena in vitro and validating them and comparing them with in vivo data enables the evaluation of any identified therapeutic agent and its possible use in clinical practice.

It can today be asserted that the skin is a sophisticated immune surveillance system acting through its network of epithelial cells, lymphocytes and AgP cells as well as its resident microbiota, the alteration of which can trigger serious consequences. Future experimental models will be characterized by studies in this sense, with great benefits for medical research, pharmaceuticals and cosmetics.

3. The inflammatory process in healing tissue in vivo. Molecular and cellular components involved

3.1. Changes in the healing process

Wound repair involves the partial regeneration of the original tissue, with hyperproduction of organized collagen, which can lead to the production of new tissue with an 80% similarity to the original tissue. Abnormal host management involves a fibroproliferative response that

causes the production of hypertrophic scars or keloids. New scientific knowledge in the fields of molecular and cellular mechanisms allows us to hypothesize other healing tissue process management procedures and apply new therapeutic models.

Chronic non-responding skin lesions are incurred by a defect in ECM remodeling (third stage of the regeneration process). Abnormal collagen deposition blocks the action of fibroblasts and re-epithelialization is halted, resulting in an inflammatory process that becomes chronic due to the humoral (cytokines) and cellular components present that prevent the lesion from healing. The proinflammatory cytokines produced by the cell populations involved in wound healing trigger, promote and regulate the process by stimulating these cells (macrophages) to act. Any disruption of this combined and synergistic action between cytokines and M1 and M2 macrophages lays the basis for non-healing. In fact, M1 macrophages secrete IL1, IL6, IL12, TNF-alpha and MMPs, which in turn stimulate, amplify and regulate the proinflammatory phase preparatory to the next phase, where the switch to M2 produces TGF-beta and IL10.

In the chronic lesion, proteases alter the granulation tissue, stopping cell migration for the purpose of scarring. Histologic data demonstrating the altered regulation of synthesis and collagen synthesis is salient.

3.2. Scientific background of the operating rationale

Under the microscope, the endothelium-ECM-cell complex resembles a dynamic world in continuous movement, with genetically encoded interactions and biological pathways aimed at recovering the normal physiology of the damaged tissue (ES Aragona). This is the basis for the work method studied and applied in our Centre.

Attention is given to the role of MMPs, the enzymes that degrade the extracellular matrix, and to their balancing by their inhibitors (TIMPs) and cytokines, especially in local arterial and venous diseases. The cytokine TNF-alpha and gelatinase MMP-9, which are significantly over-expressed in both endothelial inflammation conditions and damaged venous vascular walls, are investigated in particular.

Endothelial cells play an important role in early wound repair, thanks to their ability to stimulate the inflammatory process. They produce large amounts of TNF-alpha, which can be quantified through intracellular mobility receptors for hyaluronic acid (ICAM). At the same time, endothelial function restoration begins with the restoration of endothelial glycosaminoglycans linked to the reduction of MMP9 and the block of MMPs through the action of natural or organic derivatives of the hydrodynamic substance hyaluronic acid. This increases the water level, thus enabling the zinc at the core of the MMPs to be blocked, triggering phase repair and regeneration.

TNF-alpha is an important mediator during the inflammatory phase and, with TGF-beta, activates the expression of MMP9. In in-vitro cell cultures, TNF-alpha is over-produced in the first 24 hours, and the proinflammatory function is preparatory to the repair process. TNF-alpha inhibits the collagen-alpha-1 gene in fibroblasts, stimulates the fibroblasts to produce collagen and promotes angiogenesis. (**Figure 2**).

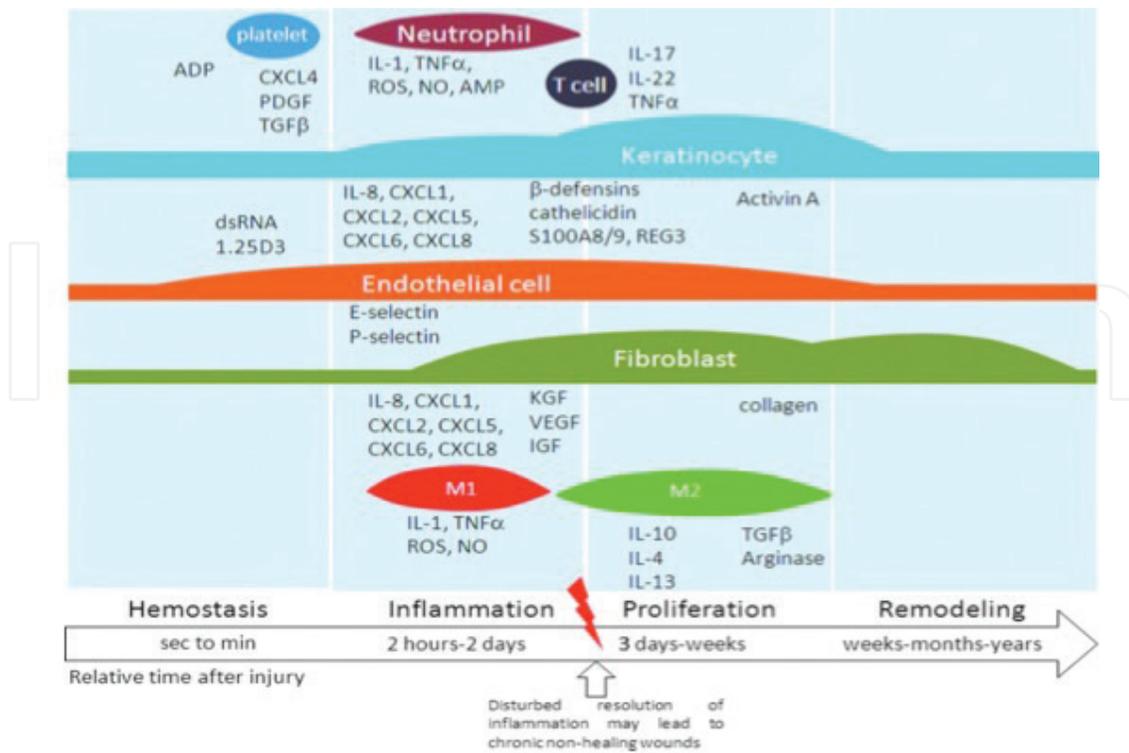


Figure 2. The phases of tissue regeneration and the involvement of cell types, micromolecules and extracellular matrix proteins.

The reduction in M1 macrophages and hence in TNF-alpha, associated with the block of monocytes and the increased production of fibroblasts and hence of collagen, creates the foundation for the M1-M2 switch and the start of the repair and regeneration process in an ever-dynamic and constantly changing cytokine and cellular pool (ES Aragona).

4. Macrophage regulation in wound healing

The role of M1 macrophages in the inflammatory process and of M2 macrophages in the repair and regeneration process is still debated, especially in the event of a disrupted M1/M2 ratio, which produces various pathological effects related to the chronification of inflammation and associated disorders. Naturally, in this case our focus is on non-healing.

It is worth repeating that M1 macrophages are proinflammatory cells to all effects, producing proinflammatory cytokines (IL1, IL6, IL12, TNF-a and oxidative metabolites (NO and SAD) (3, 4) which are involved in defense of the host and in the debridement process. (Figures 3 and 4).

The M2 population is stimulated above all by the drop in M1, IL4 and IL13; this is the key for the remodeling process, which follows or accompanies the switching-off and termination of the inflammatory process.

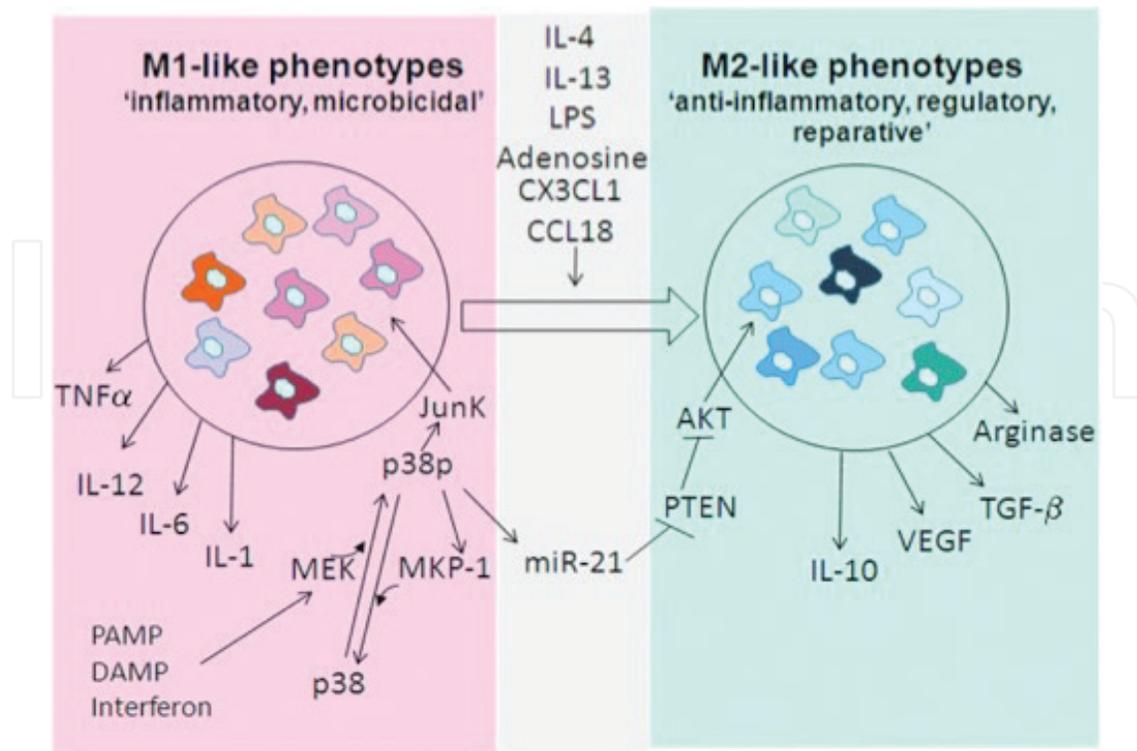


Figure 3. Cytokines pathways in M1-like and M2-like phenotypes.

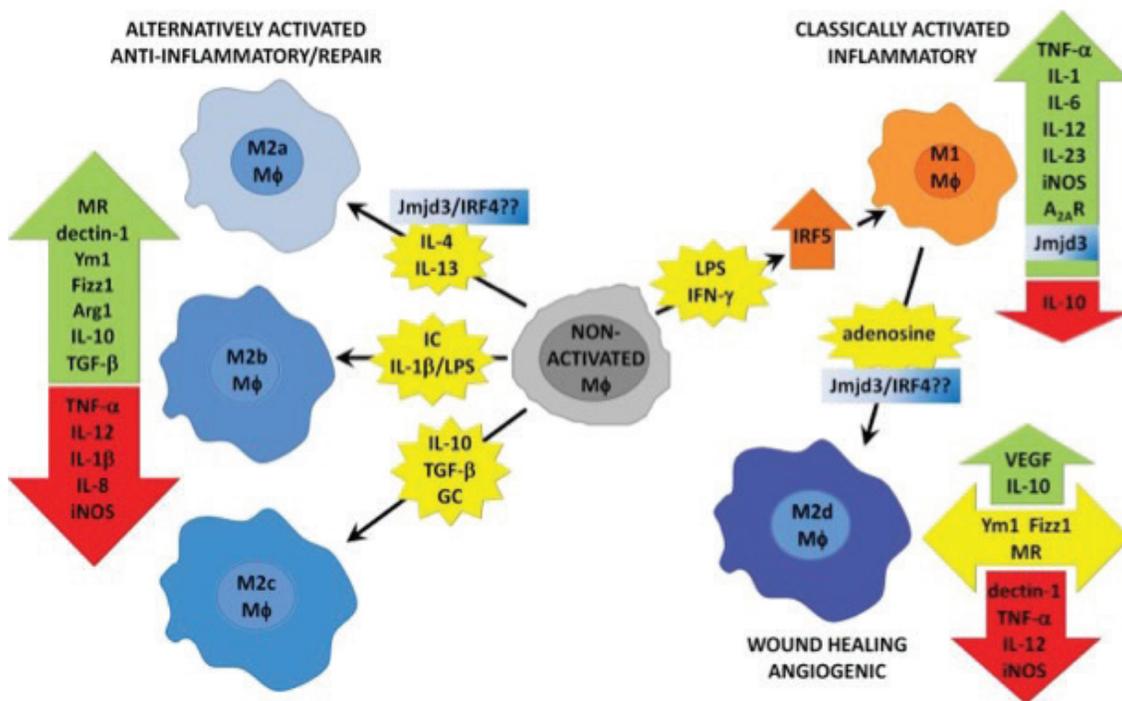


Figure 4. The type of macrophages, their differentiation and the role in tissue repair.

One of the keys for interpreting M1/M2 switching is the genotyping and receptor typing of the two populations and how they react to both chemical and energy stimuli, a concept we will return to below. The M2 population is divided into three subclasses, M2a, M2b and M2c. M2a is stimulated by the cytokines IL4/IL13 and IL4Ra. Leibovich and colleagues [3] also characterized a fourth group, M2d. This is involved in the diminishment of inflammation and the upregulation of IL10 and VEGF, like subtype M2a, which is associated with low levels of TNF α and IL12.

M1/M2 polarization seems to depend on two transcription regulators, the interferon regulatory factors IRF5 and IRF4. A correlation has been demonstrated between IRF5, high levels of M1 and inflammation and between IRF4 and M2, both linked to specific gene expressions and modulated by various substances. The latter include adenosine, which modifies the membrane's response to the M1-M2 switch, hence modifying the intracellular and ECM information signal [3].

5. A new approach to the treatment of skin lesions with regenerative medicine

5.1. The anti-inflammatory regenerative medicine (AIMED) protocol: the importance of inflammatory process modulation in triggering the tissue regeneration phase

The care and treatment of non-healing wounds is a major challenge for specialized centers. These wounds have a significant impact on health expenditure and a profound effect on the wellbeing of both patients and their families.

The skin is an important barrier. It protects us from numerous agents that would otherwise cause more frequent and more severe damage to our bodies. The skin system is a defense mechanism that aims to maintain a balance and involves various molecular, cellular, immune, endocrine, and neurological mechanisms. The understanding of these mechanisms has led to the development of numerous new drugs and medical devices for skin diseases.

Numerous authors have investigated the phases of wound repair processes and regeneration. The perfect picture is that of a complex system of humoral, cellular, molecular and ultra-structural regulation, described as a cohesive orchestra. This is the basis of the regeneration process, but it often stalls and impairs healing.

The Centre for Regenerative Medicine (now RMC) was set up by the Istituto Clinico Humanitas Mater Domini in July 2015 to focus on chronic wounds and high morbidity (infections, pain, complications), given their social implications. It has created a working group, now promoted by the Multidisciplinary Association for Wellbeing and Regeneration (AMbeR), involving numerous professionals working on the treatment and care of people with chronic skin and mucous membrane diseases. The team's research was the first step in collaboration and cooperation with other specialized centers, universities and organizations throughout Italy. Its attention is focused on the most important and decisive area of skin lesion management: modulation of the inflammatory process.

The RMC's data on the etiological causes of skin lesions confirm the etiology of such a lesion, as reported in the studies provided by the various centers involved. The success of the guidelines and protocols will only be maintained if we carry on managing the inflammatory process with a view to regeneration (**Picture 1**).

The chronic skin wound outpatient clinic involves experts in various disciplines for the treatment of:

Vascular lesions; (**Picture 2**)

Pressure lesions; (**Picture 3**)

Diabetic foot lesions; (**Picture 4**)

Autoimmune and rheumatic lesions; (**Picture 5**)

Post-surgical skin lesions; (**Picture 6**)

Burns and scars. (**Picture 7**)

Current knowledge of gross and ultrastructural skin anatomy enables the stages of the regeneration process to be followed and highlights the importance of the extracellular matrix. This



Picture 1. Case report. A 82-year-old man patient with cerebral and peripheral and diabetic circulatory pluripathologies. Appearance of internal perimalleolar lesion right with clear infection and with positivity to E. Coli and Pseudomonas healing process in 3 months.



Picture 2. Case report. A 71-year-old man patient with vascular lesion present for 6 years and resistant to therapies. Healing process in 5 months.



Picture 3. Case report. A 69-year-old patient with IV stage decubitus injury. Healing process in 35 days.

has the appearance of a semi-fluid gel, and contains enzymes, hormones and vitamins and a dense network of macromolecular complexes (GAGs, proteoglycans and glycoproteins). The cells are immersed in this active substance, whose electromagnetic properties are fundamental for the life of the cells themselves as well as the water in the human body. The coherence of the electromagnetic diffusion is essential for correct intercell and cell-ECM harmony.

The other aspect highlighted by research into the modulation of the inflammatory process is that the ECM determines the process of differentiation, proliferation and cell migration,



Picture 4. Case report. A 63-year-old patient with infected diabetic food. . Healing process in 2 months.



Picture 5. Case report. A 78- year-old patient with arthritis, left hand with inflammation and abscess. Healing process in 3 months.

ensuring the balance of all the components involved in the life cycle of the regenerative phase. The regeneration of skin that has been damaged by multiple etiological factors is made possible by its ability to interact with the outside, and especially the essence of the tissue, that is constantly renewed and capable of repairing and reacting to lesions due to the presence of epidermal stem cells in the dermis and epidermis. In patients with recalcitrant skin lesions, the presence of comorbidities such as chronic disease, diabetes, vascular insufficiency, peripheral edema secondary to heart failure, malnutrition, bedsores, and infections can affect the body's ability to respond to treatment, but can also have a negative effect on the inflammatory modulation process itself, triggering a chronic phase that feeds the non-healing of the lesions.



Picture 6. Case report. A 57-old-patient with dehiscence of the surgical wound. Application of NPWT therapy and Healing process in 2 months.



Picture 7. Case report. A 24 old-patient with II and III degree burns on the face and upper limb.

These stages are always present in all types of lesions and physiological process phases, and their timescales, interaction, genetic, humoral, cellular, and ultrastructural mechanisms (which are the basis for the regeneration of injured tissues, in both in vitro models and live models) are all understood. In this article, the authors underline some aspects of the repair and regeneration process in relation to the rationale of the proposed AIMED Model and cohesion between clinical research activities and their translation to methodology. Key roles are played by fibrin deposition and hydration of the matrix by hyaluronic acid, stimulating the production of fibroblasts, while other cell types (granulocytes, monocytes, M1 macrophages and cytokines) play a role in the shutdown of the inflammatory proliferative phase (endothelial cells, fibroblasts and keratinocytes) and the ECM remodeling phase.

5.2. Personal experience; materials and methods

The regenerative medicine outpatient clinic (RMC) for the treatment of recalcitrant lesions was established in Castellanza in the summer of 2015. In 2017–2018, 869 patients (286 men and 583 women) were treated at the RMC, for a total of 1718 treatments. Even before applying national and international guidelines on the management of acute and chronic wounds the main activity was the formation of a multidisciplinary team sharing the same philosophy of care: to put patients, their inner world and their families at the heart of the process. The clinical research already practiced by some members of the RMC team was the driving force for the development of hospital treatment and home care models as a therapeutic continuum and for the transferral of the results of this research to clinical practice.

5.3. The foundation of the RMC

“There are skills and abilities, and then there’s a subtle strength that the patients transmit to you, to say that everything we are doing has given them a better quality of life (Giada Mereghetti, RMC Coordinator).”

The Regenerative Medicine Centre (RMC) was set up in June 2015 in Castellanza, a town in the province of Varese in Lombardy, near the border with the province of Milan, on the basis of the skills and motivation of a group of professionals with a common objective. The RMC forms part of a much larger project, which aims to create a close-knit network of professionals to act as national and international spokespersons for a new way of looking at skin wounds. This ambitious project initially brought together professionals with different skills but who were united by a single mission: to confront and photograph the world of difficult wounds, broadening the objective beyond the usual common goal of mere treatment, and involving patients and their families.

Motivation is the main characteristic common to all project members: the same members who (initially only through ideological discussions) later actively contributed to the RMC’s construction, putting an ideal and an objective into practice. Each of the individual professionals, with their important personal experience of the medical world and with years upon years of study in their various scientific disciplines, decided to contribute their knowledge, experience and abilities to the construction of a new organizational model and the creation of a close-knit network of activities, which they hope will spread through the entire country. Each of the members has embraced the philosophy of caregiving, associating respect for the clinical priorities of the patients and their families with the use of national and international methods and guidelines for clinical research and the continuous evolution of the field of wound management. From this perspective, all the professionals making up the RMC outpatient team, with their individual duties and respect for the shared guidelines and protocols, have opened up their perspective of wounds in relation to constant interaction with the university and hospital research institutes with which they work.

The RMC is close to achieving its main research objective in relation to the treatment of wounds: namely, gaining knowledge of the inflammatory process resolution mechanisms in tissue repair and regeneration. Its ambition is an organizational model which takes its professionals outside the hospital walls to enable their scientific and human knowledge to be transferred to and shared with the key players in this project: the patients and their families.

5.4. Main objectives of the RMC

- To respect the mission of managing patients with any acute or chronic lesion affecting the skin or mucous membranes of any etiology (vascular, diabetic, rheumatological, traumatic) while respecting their humanness as a whole.
- To standardize the mechanisms for stimulating regenerative and reparative biochemical and cell processes leading to the healing of lesions, making use of the abilities of the team's clinicians alongside the public and private institutes with which RMC works.
- To create new therapeutic models at the base of regenerative medicine to exploit synthetic biological molecules, engineered tissues and cell therapies that could reproduce the body's own wound repair mechanisms in everyday clinical practice.
- To remain part of a much wider project that focuses attention on skin, cartilage and mucous membrane lesions and places patients at the center of an innovative, barrier-free patient journey.
- To take care of the patient from diagnosis through treatment and follow-up, providing services as needed on the basis of clinical indications and integrating them with innovative complementary treatments.

5.5. Clinical care journey at the RMC

The patient journey in the RMC thus requires careful management involving numerous professionals with distinct areas and responsibilities. Patients go through a well-defined process that should give them a sense of competence, humanity and harmony, making them feel an active part of the group alongside the medical workers treating them. These workers act through the application of protocols and guidelines detailing the treatment and use of the main advanced dressings in relation to the different wound types, which are drawn up on the basis of the main clinical studies found in the literature and others, published more recently, followed directly by the RMC. The RMC makes use of both its own professionals and those employed by the institutes in which it works, as well as of partners and disciplines whose goal is to channel their energies toward the better treatment of patients with skin, cartilage and mucous membrane lesions.

5.6. Care and assistance in the RMC

Given the main objective and mission of the RMC, which can be summarized as the management of patients with any acute or chronic lesion affecting the skin or mucous membranes and who have non-healing ulcers or wounds of different etiologies (vascular, diabetic, rheumatological, traumatic), the RMC's activities can be classified in the following areas:

- Assessment of the lesion;
- Overall assessment of the patient;
- Management and removal of the cause leading to the formation of the lesion;
- Application of validated, shared protocols;

- Prevention and management of complications;
- Ultrasound treatment of skin lesions to remove necrotic and fibrinous tissue, acting as a bactericide and stimulus for tissue regeneration;
- Surgical procedures for biopsies, surgical debridement, and removal of lesions with reconstruction and/or skin grafts;
- Documentation of the assessments and procedures through shared records;
- Creation of protocols on the basis of technological innovations and monitoring of markers;
- Consultancy and cooperation with all in-house services (Inpatient wards and Accident & Emergency Department);
- Consultancy and cooperation with community services (GPs, integrated home care (ADI), residential care homes (RSA));
- Cooperation with the training department to create a network of consistent, competent professionals;
- Promotion of community outreach initiatives;
- Health education.

The Castellanza RMC has a 360° structure, in which the specialists taking charge of care injuries collaborate in important phases of patient assessment and management of both the injuries and the patients as a whole. After this phase of diagnostic classification, the patients are provided with multilevel treatment that applies all the steps detailed in international guidelines,

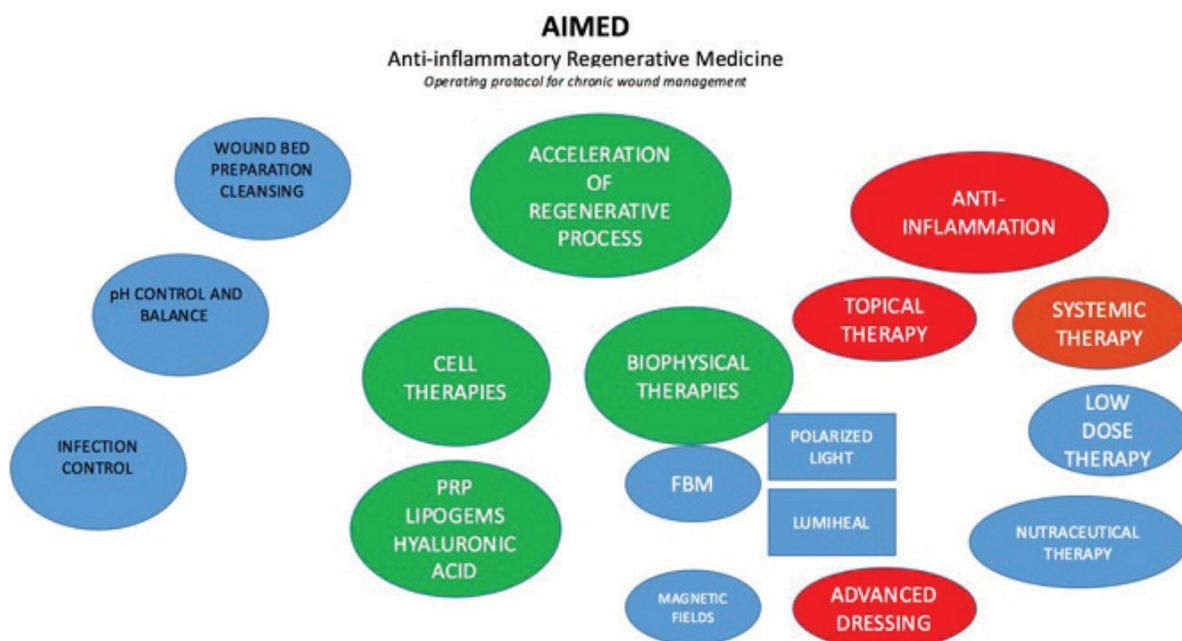


Figure 5. AIMED - Anti Inflammaging regenerative medicine: operating protocol for chronic wound management (E.S. Aragona – 2017).

but with an additional, innovative perspective that gives importance to anti-inflammatory and regenerative activity.

The AIMED operating protocol provides local treatment of skin lesions and a general evaluation of the patient, with particular attention to the preliminary assessment of the causes of lesion). (Figure 5). The model enables the dynamic partnership of all professionals working with RMC specialists to ensure a simple, interruption-free patient journey in the Institute. An example of this is the in-house cooperation with the Cardiology and Hemodynamics Dept. for vascular lesions of arterial origin, which are evaluated within a multidisciplinary team where, from their first visit to the clinic, patients are guided through a diagnostic angiography journey involving a vascular rehabilitation process. In these revascularized patients the care model also focuses on the risks of reperfusion and the production of a proinflammatory state with increased peripheral oxidation and potential necrosis of the tissues affected by the critical ischemia.

6. Description of the lesion model under the AIMED protocol

The lesions are classified by type and stage in accordance with international guidelines, and the various types of advanced treatment are assessed and selected in relation to the type of lesion and the operating protocol, with attention to pharmacoeconomics.

Five basic operating protocol steps.

Step 1: Preparation of the lesion for treatment. This includes cleansing and combating infections using current methods [4].

Step 2: Topical and systemic treatment protocols to modulate the inflammatory phase and trigger the regenerative phase.

Step 3: Biophysical therapies to stimulate regeneration.

Step 4: Cell therapies.

Step 5: Surgical therapies.

These steps are discussed below in detail.

STEP 1—Wound Preparation

The first step is deep cleansing of the wound and modifying its pH. This important step requires careful management: cleaning the wound of nonviable tissue, fibrin, protease, bacteria and biofilm or cellular debris can eliminate potential causes of non-healing. This is followed by modification of the lesion's pH.

The pH value within the microenvironment of the wound directly and indirectly influences all biochemical reactions that take place in the healing process. It has been shown that the pH of the surface of a wound plays an important role in wound healing and helps to control the infection and increase antimicrobial activity, the release of oxygen, angiogenesis, protease activity and bacterial toxicity. The pH value influences cellular events that regulate the healing process of wounds. It was observed that

the acute and chronic wounds with an alkaline pH have a lower rate of cure than the wounds with a pH close to neutral. The wound healing process slows down when the pH is high, under alkaline conditions. (Levine).

Intact skin has a pH between 4.8 and 6, depending on the area in question. The pH of wounds cannot be easily measured, but literature data demonstrate that a wound pH of around 4 can trigger a more rapid wound healing process.

In *The effects of pH on wound healing, biofilms, and antimicrobial efficacy* published in *Wound Repair Regen.* 2014 (March) [5], Percival et al. attribute wound pH with an important role in the activity of MMPs, TIMPs and fibroblasts and in collagen production. pH also interferes with bacterial proliferation and the patient's immunological response, and its monitoring and control is one of the strategies used to trigger the healing process. In *The effect of pH on the Extracellular Matrix and Biofilms*, published in *Adv. Wound Care*, Jul 1,4 [6], Jones, Cochrane and Percival provide an overview of the role of pH and its effect on the ECM and biofilm in connection with wound healing. Chronic lesions have an alkaline pH, while the pH tends toward acidity during the healing process.

The model involves the use of commercially available products chosen on the basis of their properties, their contact time and the duration of their action on the wound.

Polyhexanide with betaine surfactant.

Hypochlorous acid.

The authors of the present article have started an observational study of a class III medical device following a study of the bacterial load of the lesion and of certain bacterial strains (*Staphylococcus aureus*, *Staph. epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa*) that associated a pH of 4.5–5.00 with the mechanical removal of bacteria and protease due to the presence in its composition of d-mannose, copper sulfate, zinc and other components that are part of the authors' know-how.

STEP 2A—Topical Treatment

The topical treatment involves two phases. The first, enzymatic and mechanical debridement (the technique preferred by the authors), is the crucial moment in wound management, eliminating the mechanical and biochemical causes that can perpetuate the inflammatory process and preparing the wound for the action of biological and physical substances that can trigger a local anti-inflammatory action and catalyze the proliferation of cell populations, ensuring good hydration in the wound bed.

The lesions are always treated with the local application of medical devices, biophysical therapies and cell therapies.

STEP 2B—Systemic Treatment

Even though it is described as part of step 2, systemic treatment is applied from the very start of the wound's management under a 360°, polyvalent protocol depending on the type and severity of the lesion.

Administration of a dietary supplement containing a well-balanced mix of serratio-peptidase, escin, bromelain and selenium. This supplement has proteolytic, fibrinolytic, anti-edema and draining properties, as well as an antioxidant action.

The rationale for the prescription of this supplement is:

- Anti-inflammatory action (proteolytic and fibrinolytic);
- Bacteriostatic action in uninfected lesions and antibiotic therapy in infected lesions;
- Anti-edema and draining action;
- Reduction of secretions;
- Pain relief;
- Promotion of healing [7].

Administration of low-dose cytokines to combat the inflammatory process.

Patients are prescribed with 3–6 months of treatment with low-dose cytokines, formulated with a kinetic system called Sequential Kinetic Activation (SKA), and containing:

1. Anti IL-1 - regulation and suppression of the inflammatory response.
2. IL-10 - adjustment of the anti-inflammatory process in chronic diseases with reduction of IL-6.
3. IL-4 - Th1-Th2 switch control.

Low dose therapy is an important part of wound management because it introduces into clinical practice a strategic concept for future therapies: the disease may be the result of an altered concentration of messenger or signal molecules (hormones, cytokines, neurotransmitters) for cellular activity, and in this case the modulation of these molecules can restore the disrupted balances, enabling healing [8].

STEP 3—Biophysical Therapies

Photobiomodulation

During the process the wound is subjected to dual-type light frequencies for 8–20 minutes.

1. Polarized light

RMC is the first center in Italy to use a unique light source that is:

- Polarized, propagating in parallel planes;
- Polychromatic, with a wavelength ranging from 480 to 3400 nm;
- Incoherent, with out-of-phase waves delivering low-intensity light;
- Low-energy, reaching the wound with a constant intensity, producing bio-stimulating effects.

It has been demonstrated that polarized light at 590 nm stimulates angiogenesis and growth factors, while at 830 nm it activates the cells involved in wound healing; a dose of 20 J/cm² stimulates increased collagen deposition, an increase in myofibroblasts and a better ultra-structural organization of the wound healing process [9–14].

2. *LumiHeal*

The Lumiheal protocol involves the use of broadband wavelengths (blue, green, yellow/orange from 450 nm to 610 nm) from a light emitting diode to amplify the physical effect of stimulation of the regenerative processes in the injured area due to the emission of photons in the form of fluorescence. The LumiHeal Protocol has been applied over the last 3 months in 8 patients with complicated treatment-resistant infected wounds. The improvement in the wounds is documented by photographic evidence [15–20].

Pulsed electromagnetic fields (PEMF)

High intensity, variable frequency magnetic fields for outpatient and home treatment. As already demonstrated in our first patients, they:

- Are anti-inflammatory, through modulation of the profile of cytokines produced by proinflammatory cells (IL-1, NGF, ROS, IL8);
- Are angiogenic, with increased proliferation of endothelial cells and FGF-2 (fibroblast growth factor), improving microcirculation;
- **Improve the microcirculation**, with increased collagen production [21, 22].

STEP 4—Cell Therapies

Platelet-rich plasma (PRP)

PRP is a powerful concentrate of growth factors that stimulate tissue regeneration and is used to treat damaged tissues. Under current Italian legislation, the PRP used by the RMC must be prepared in a local transfusion center (**Picture 8**) [23–26].



Picture 8. Case report. A 74-old-patient with vascular ulcers of the lower limbs treated with PRP.

Lipogems

Method for obtaining, through adipose liposuction, micro-fractured tissue for autologous use, which is reapplied to patients with skin lesions to further stimulate the cell regeneration process [27–30].

STEP 5—Surgical Therapies

At the RMC, surgical therapies for the repair of skin lesions are standardized. They involve the use of skin substitutes that promote the production of a structured collagen matrix, enabling better angiogenesis. When monitoring the application of skin substitutes, the focus is on the skin's reparative capacity as well as the risk of tissue rejection. The removal of any necrosis when cleaning the wound is essential to prepare it for autologous or heterologous grafts that fully integrate into the patient's dermis, and signs of rejection must be managed promptly.

7. From repair to regeneration: Regeneration 3.0

7.1. The Prometheus project

The RMC's clinical experience in cell therapies is based on the traumatic extraction methods that underlie the preparation of platelet-rich plasma and the extraction and centrifugation of adipose tissue (lipoaspiration) with the aid of modern technology. The results of their use in a treatment pathway that accompanies patients in their management are remarkable.

In relation to the use and performance of mesenchymal cells (immunomodulatory, paracrine and regenerative activity) in the RMC's clinical research, the multidisciplinary team were united by the realization that mesenchymal cells cannot be used in therapy and there is a need to optimize a daily therapy that has a similar effect on cell and tissue biostimulation. The Prometheus Project - Alfabjn Wound Care has been embraced by the RMC because it is *innovative* and because it anticipates the next frontier for regenerative medicine: *specific, individualized cell therapies*. Growth factors have made an explosive breakthrough into clinical practice, and the decision to focus on the quality and efficacy of the therapies containing them is strategic for the near future.

A careful analysis of the components of and claims made for the medical devices trialed by the RMC for the treatment of superficial and deep wounds and lesions through a clinical research joint venture has highlighted the need for a scientific value to be attributed to the rationale for using these devices, within the framework of a multidisciplinary activity intended to give added value to the device's action in regenerative medicine. The objective of the RMC's study was to offer an innovative solution to the current difficulties in managing nonhealing skin lesions. To do this, we first tried to answer a question: Do difficult wounds exist, or is it simply that we do not know how to treat them?

Our research is based on two articles, published in 2016 (Zhao R et al.: Inflammation in Chronic Wounds. *Int J Mol Sci*) and 2017 (Han G et al.: Chronic Wound Healing: A Review of current Management and Treatments. *Adv. Ther*) [12, 13].

These articles affirm what we wrote in the introduction: inflammation has a major role in the wound healing process, in which disabling chronic diseases add to local systemic effects such as tissue hypoxia and pH changes, post-revascularization damage, cell aging and infections. Therapeutic resources take account of the numerous techniques and resources available, with particular attention to growth factors.

In this research process we began with a definition: **Repair 1.0**, signifying a dressing process involving the use of advanced dressings. This type of dressing is required to maintain an adequate wound moisture level, to be partly or totally occlusive, and to passively absorb the exudate, with a function determined by the patient's metabolism and biological "performance".

Repair 2.0, in contrast, involves the use of bioactive dressings with a biological action on the wound (hyaluronic acid, collagen, silver, etc.). From this perspective, the RMC investigated a sterile gauze dressing in which the role of the bioactive substances (hyaluronic acid, carnosine) is specifically defined in the literature, and involves mechanical protection of the lesion (gauze) combined with a direct anti-inflammatory action.

Zhao et al. examine the causes of nonhealing wounds, and attribute the greatest responsibility to the inflammatory process. That study's relevance to the present article is its affirmation of the role of nitric oxide in the repair process and the well-known harm caused by ROS that, through systemic or topical treatment with antioxidants (carnosine), can be turned around in non-responding lesions [31].

In this context, we began working with bioactive substances with innovative properties (in relation to both composition and biological action) in comparison with their competitors. This potential innovation lies in the use of bovine colostrum, that, when stabilized through industrial processes to a pH of 6.8, assures the compound's stability and its action against the tissue acidosis found in damaged tissues. This has positive consequences for the modulation of the inflammatory process and the tissue repair process as well as on the ability to stimulate the cellular and ultrastructural regenerative process. (Bagnara G: *Le cellule staminali*, Cap 11. Ed Esculapio 2017).

In **Regeneration 3.0**, the priority is to combine the anti-inflammatory activity of the nine proteins acting as growth factors in the bovine colostrum, the homeostatic, angiogenic and reorganizational activities of the matrix, the modulation of collagen synthesis and the remodeling of the epithelium. The choice of bovine colostrum and its associated properties was the basis for the design of devices that could also offer those properties: barrier action, anti-inflammatory action and pain reduction, reduction and absorption of exudates, combating of bacterial and fungal proliferation, antioxidant action and hydration and protection against skin diseases and dermatosis.

This is the culmination of an in-vitro and an in-vivo test.

7.2. In Vitro comparative evaluation of wound healing activity of medical

The purpose of this test is to compare the efficacy of two medical devices in the repairing of wounds simulating this situation in vitro by making a cut on cell monolayer of human

Campione / Sample	Controllo negativo / negative control	Pr 1 mg/ml	Pr 0,25 mg/ml	Pr 0,15 mg/ml
O.D. Valore medio / Mean value	1,9	1,292	1,659	1,755
pg/ml Valore medio / Mean value	3064,727	1959,970	2627,693	2838,670
% IL8	100	63,95	85,74	92,62
% riduzione IL8 / % reduction of IL8	0	36,05	14,26	7,38

A % of reduction of IL8 is observed at tested concentrations of 1 and 0.25 mg/ml

Campione / Sample mg/ml	1	0,25	0,15	Controllo negativo / Negative control	CQ
O.D. Media / Medium	1,419	1,473	1,648	1,586	2,566
% Vitalità cellulare / % of cell vitality	89,50	92,87	103,89	100,00	161,78

The viability of fibroblasts in the plates treated with P1003 AIM Gel Herpes (AIM3-B03D) Lotto 11/2014 has proved to be comparable to that of untreated cells, indicating the total absence of cytotoxicity of the tested medical device and to demonstrate a modulation of the metabolism of the cells that resulting healthy and viable and able to reconstruct the monolayer where we have made the cut.

Figure 6. In vitro comparative evaluation of wound healing activity of medical devices, Alfakjn ResearchCenter Milano 2018 by Bio Basic Europe SRL. Via A. Panizzi,10 Milano Italy.

fibroblasts (Hude) and then evaluating the approximation of the edges of the cut in cells treated with the two medical devices, in comparison to untreated cells. In order to select the concentrations of 2 medical devices to be used for the test (not cytotoxic concentrations for the cells), a preliminary MTT cell cultures of fibroblasts was performed. The cells were treated with scalar concentrations of the two medical devices (as low as 1 mg/ml and subsequent dilutions 1: 2) and untreated cells were used as negative control. Based on the obtained results concentrations of 2 medical devices of 1–0.25–0.15 mg/ml were chosen to continue the test. After making a cut on the cell monolayer of confluent fibroblasts (simulation of a wound), the cells were treated with the chosen concentrations of the two medical devices, as negative control untreated cells were used and, as internal quality control one standard with known activity of wound healing activity. Therefore, we have performed a morphologic evaluation of the monolayer by microscopy and a measurement of IL-8 levels. From the morphological evaluation a net approach of the flaps of the monolayer was observed in the plates treated with the various concentrations of Colostrum Gel. The dosage dell'IL8 showed significant decrease in the% of IL8 to concentrations of 1 and 0.25 mg/ml by both tested medical devices, showing therefore a comparable anti-inflammatory action on fibroblasts (**Figure 6**). These results indicate that the effectiveness of the active ingredients present in the product have a different target than the reduction of the inflammatory response. The obtained results have showed the effectiveness in wound healing of the medical device Colostrum Gel, compared to the medical device Gel no active. The medical device Gel Herpes no active is in fact shows a “nutrient” activity on cells but it is not able to stimulate the repair of the damage (cutting), this latter activity is due to the presence of actives ingredients present in the formula of Colostrum Gel. Colostrum Gel reduced IL8 production by fibroblasts and contains active ingredients. Those stimulate wound healing (simulation in vitro by cutting the monolayer of cultured fibroblasts and evidence of the approximation of the edges of the cut and almost total closure of the same) (**Figure 7**).

7.3. Topical use of Colostrum AIM 4% fluid cream in a murine model of pressure ulcers

This study reports the development of a murine model of pressure ulcers by using externally placed magnets to create the ischemic events of ischemia reperfusion (IR) injury.

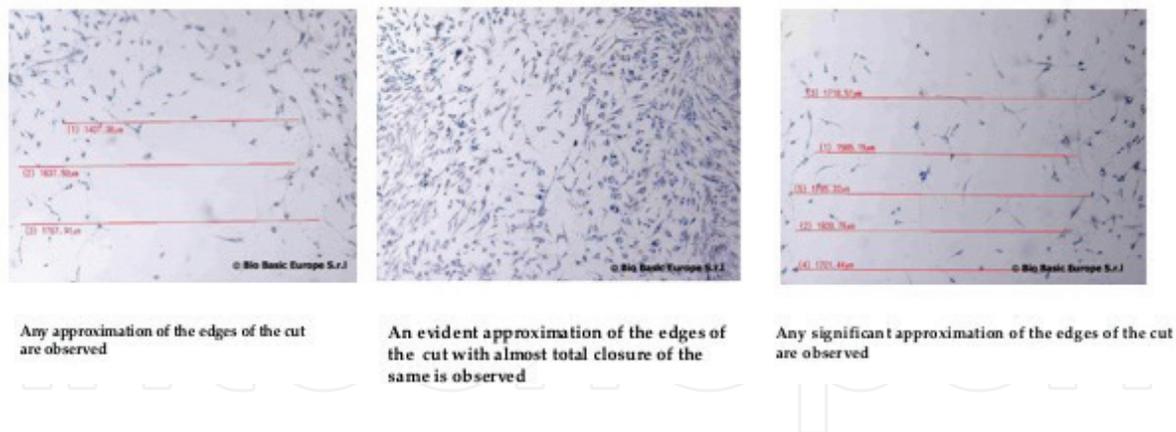
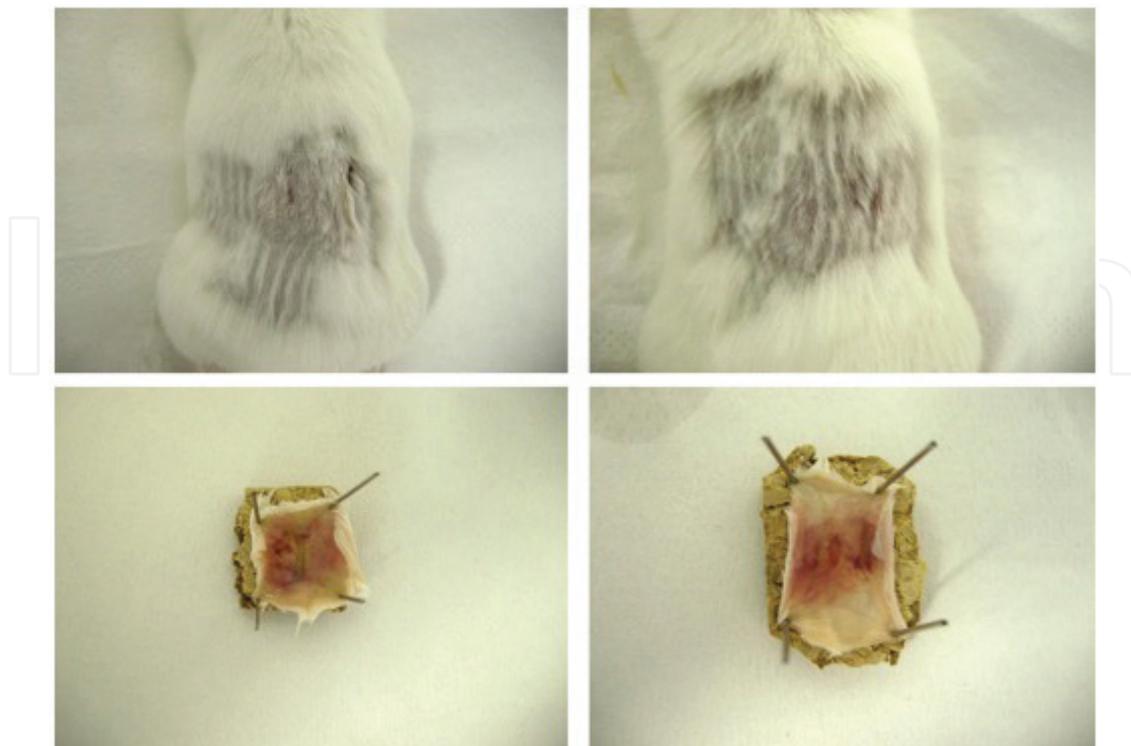


Figure 7. In vitro comparative evaluation of wound healing activity of medical devices - Cell Growth . Alfakjn ResearchCenter Milano 2018 by Bio Basic Europe SRL. Via A. Panizzi,10 Milano Italy.

The animals were individually housed, their backs have been shaved, cleaned with alcohol, the skin has been gently pulled up and placed between two round ceramic magnetic plates which have a 12-mm diameter (113 mm²) and are 5 mm thick, with an average weight of 2.4 g and 1000G magnetic forces; this process creates a compressive pressure of 50 mmHg between the two magnets. Then the animals have been divided into two groups as follows:

Control group: three IR cycles have been performed in 3 mice to initiate decubitus ulcer formation. A single IR cycle consists of a 12-hour period of magnet placement, followed by a release of rest period of 12 hours. After the 3 IR cycles, the animals have been sacrificed.



Picture 9. Initiate decubitus ulcer formation in mice to test colostrum derivative therapy.

Group B: three IR cycles have been performed in each mouse to initiate decubitus ulcer formation. A topical administration of 200 mg of AIM LIFEIN- SIDE 4% (AI13-002-B) has been applied on the backs of each mouse the day before the first IR cycle and at the end of each compressive cycle. After the 3 IR cycles, the animals have been sacrificed. Skin samples of each mouse have been collected from the treated area, fixed in 10% phosphate-buffered formalin and wax embedded. 2 μ m thickness sections were obtained and collected on silanized slides and stained by hematoxylin- eosin. The samples were then observed with an optical microscope Nikon 80i, fitted with a digital camera (**Picture 9**).

8. Results

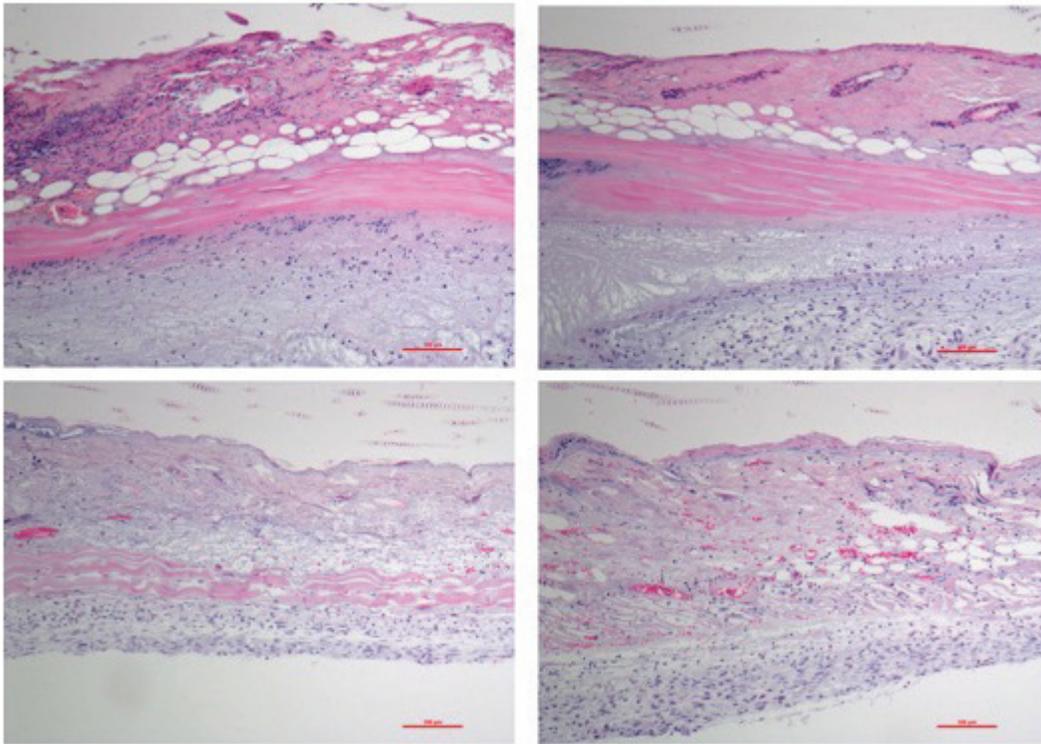
1. Control group: 3 IR cycles Skin macroscopic analysis Macroscopic analysis of dorsal skin revealed the presence of mild skin lesions, edema and signs of necrosis of the epidermis. (Photo 10). Histological analysis of dorsal skin samples stained by hematoxylin–eosin showed: wide ulcerative area of the epidermis; marked spongiosis of basal layer (inter-cellular bridges appear very prominent); marked diffuse inflammatory infiltrate; conjunctival edema; vascular hyperemia; presence of extravasal erythrocytes (**Picture 10**).
2. Group B: 3 IR cycles+Colostro AIM at the end of each compressive cycle and the day before the first IR cycle. Skin macroscopic analysis. Macroscopic analysis of dorsal skin revealed: skin ulcers with necrosis areas and edema (**Picture 11**). Histological analysis of dorsal skin samples stained by hematoxylin–eosin showed: wide ulcerative area of the epidermis; when present epidermis is hyperplasic, with not regular thickness, marked spongiosis of basal layer and presence of lymphocytes; marked diffuse inflammatory infiltrate; conjunctival edema; vascular hyperemia; presence of extravasal erythrocytes (**Picture 12**).

The most significant aspect with a view to new tissue regeneration therapies and hence the control of the inflammatory process were the results in relation to the disappearance of the wound and the reduction of the inflammatory process. This requires the future consideration of the action time of the medical device and its contact time with the damaged tissue.

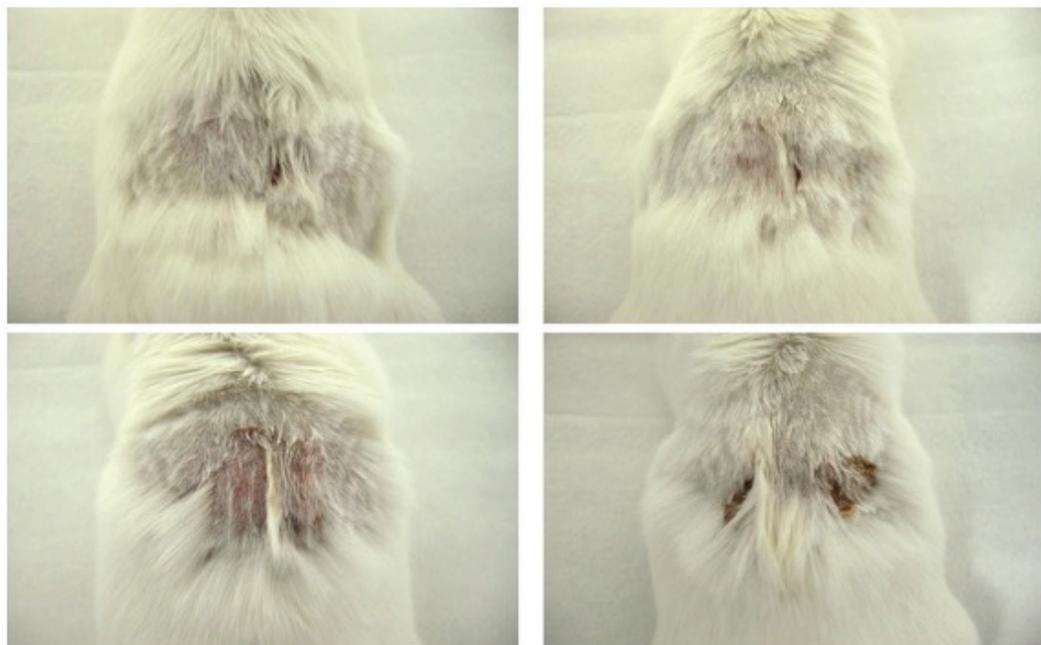
The results in relation to clinical healing take account of chronic conditions defined as non-responders and their good management from the time of diagnosis. The data on patient compliance with the use of the medical device reveal the absence of any symptoms or side effects, ensuring the patient's safety and boosting the device's reliability and efficacy.

8.1. Analysis of results and validity of the protocol

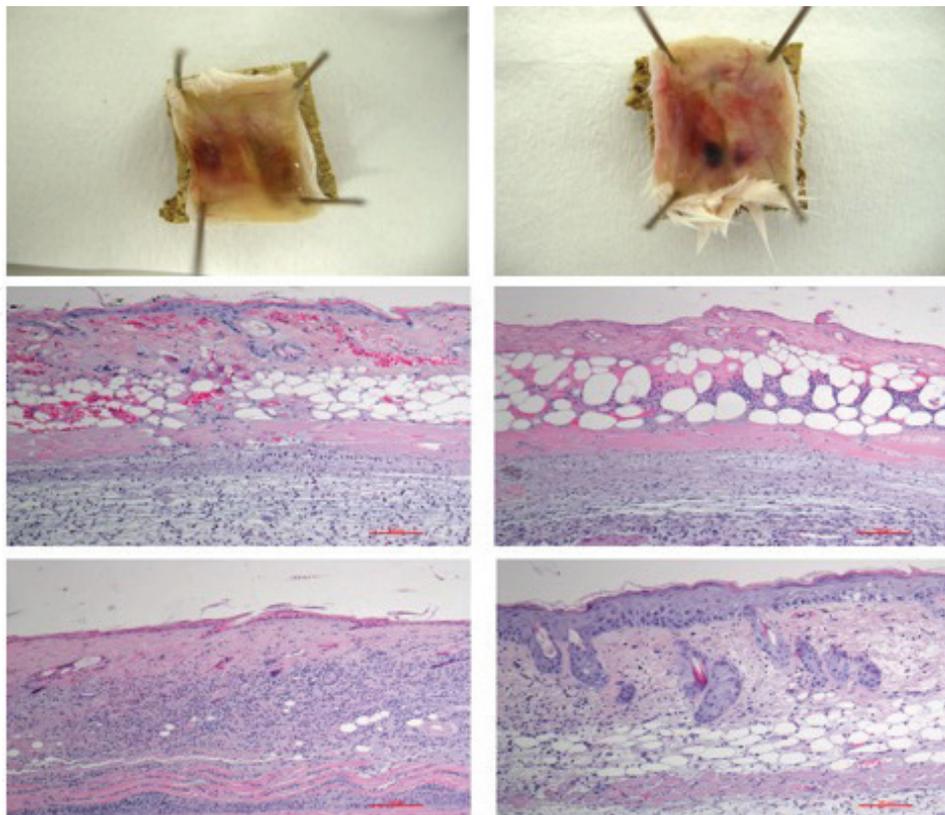
The RMC treats patients with chronic wounds of various etiologies. The AIMED model treatment has been applied in 85% of cases. Analysis of the 360° wound management process has revealed remarkable results in relation to:



Picture 10. Wide ulcerative area of the epidermis; marked spongiosis of basal layer (intercellular bridges appear very prominent); marked diffuse inflammatory infiltrate; conjunctival edema; vascular hyperemia; presence of extravasated erythrocytes.



Picture 11. Macroscopic analysis of dorsal skin revealed: skin ulcers with necrosis areas and edema.



Picture 12. Wide ulcerative area of the epidermis; when present epidermis is hyperplasic, with not regular thickness, marked spongiosis of basal layer and presence of lymphocytes; marked diffuse inflammatory infiltrate; conjunctival edema; vascular hyperemia; presence of extravasal erythrocytes.

- Best wound management with a greater awareness of dressing protocols and the use of existing medications on the market, integrated into the AIMED model;
- Rapid healing;
- Pain reduction;
- Reduced complications and signs of comorbidity;
- Reduced health expenditure;
- Better compliance and patient and family satisfaction;
- Reduced rate of recurrence.

Another important aspect of the RMC's activity is the constant back-and-forth between the results obtained through clinical observation and the analysis of the collected data through clinical research, in the light of a possible reinterpretation in a future scenario focusing on two key aspects of the healing of chronic wounds: anti-inflammatory action and regenerative action [31, 32].

In this article, the authors lay down the scientific basis for a chronic wound healing process involving an appropriate sequence of the modulation of the inflammatory and proliferative



Picture 13. A 73-year-old patient with heart failure and lower limb ulcers. Healing process with the AIMED method in 3 and a half months.

processes and remodeling of the ECM. At the same time, they highlight how the abnormal evolution of the inflammatory process to a chronic condition involves abnormal cellularity, inappropriate collagen deposit and the presence of protease, preventing re-epithelialization and regeneration of the lesion. (Picture 13).

Knowledge of the biological pathways at an ultramolecular and cellular level enables the identification of various areas where clinical research could intervene with biological drugs or biophysical therapies to influence the healing pathways of non-responding chronic wounds or stimulate the metabolic or regenerative processes, blocking the mechanisms leading to chronicity and, in particular, intervening in the chronic inflammatory process. In this case, in vitro tests could help by enabling new biological compounds to be tested on cellular models of skin damage. We have demonstrated that colostrum is paradigmatic of the therapeutic philosophy adopted by the RMC, in the sense that it is capable of reducing levels of proinflammatory cytokines and protease (MMP-9), blocking M1 activity and stimulating the activity of fibroblasts, resulting in the production of type III and VII collagen to aid regeneration.

Analysis of the results obtained with low dose therapy and the effects of biophysical therapies (photobiomodulation and PEMF) could provide guidance on aspects that the authors consider to be of current and future interest: modulation of nitric oxide in vasodilation and the provision of regenerative molecules (PEMF), and the reduction of the inflammatory component (IL-6 and C-reactive protein). Analysis of the current literature suggests that reduction of the inflammatory component is the key to regenerative recovery of chronic nonresponding wounds, now that we have a better understating of their pathogenesis and pathophysiological processes. The adoption of a 360° rather than sequential wound management model is based on the authors' choices and experience, and has a firm scientific basis. We believe that this model ensures that patients receive the best possible care and attention.

9. Conclusions

The molecular pond is in a state of constant agitation and turbulence, with the molecules spinning and vibrating and bouncing off one another...

Life on the Edge – J. McFadden and J. Al-Khalili

10. The future of regeneration

The study of biological molecules has enabled a glimpse of a possible new key for the interpretation of biological phenomena linked to management of the inflammatory process, and some such molecules could be prototypes for others still to come. The greater availability of water molecules around more hydrophilic molecules and the better organization of the body's water seem to produce a greater and better biological response.

“We could interpret the disease as a loss of some levels of cohesive hierarchy between the domains, with a consequent loss of the electromagnetic control exerted over the biomolecules” (E. Del Giudice).

In the study of the hydrodynamic behavior of numerous molecules, it always comes back to the endothelium, the ECM and the cells themselves. The role of the cell membrane seems worthy of attention, as its structure enables substances to travel or be transported into the cell, but the functional properties of the membrane that we know today make it the protagonist of a new biological culture, in which chemistry meets anatomy and anatomy is subject to physical stimuli that can modify its essence [33–36].

The inner cell is packed with a thick, intricate network of microtubules formed by well-known proteins (tubulin). This network, called the **cytoskeleton**, has a complicated and constantly changing dynamic structure and function: some branches form, others break down and disappear, others extend in multiple directions.

Most intracellular metabolic reactions take place along the branches of the cytoskeleton. Its structure is thus fundamental for biochemical functionality (a new functional concept), which marks a continuum between the cell nucleus, the membrane and the outside of the cell, protected by another important structure, the glycocalyx. When the cell dies, its cytoskeleton breaks down. This highly dynamic behavior is difficult to understand, but there have been a number of studies of both the biochemistry involved and the energy and charge transport capacity along the microtubules (Davydov, 1982 and references reported therein).

The cytoskeleton is a system of canals (microtubules) in which substances are transported and information is transmitted. This is made possible because all its molecules, including water (which makes up 80% or more of the cytoskeleton by weight), have a dipole moment – in other words, an electrically charged spatial distribution involving a positively charged pole and a negatively charged pole. **All macromolecules become biologically active only if they are immersed in an aqueous matrix.** This demonstrates the predominant role of water in living beings. Intuition is transformed into scientific data and becomes reality.

Quantum medicine returns to that concept of electromagnetic fields, the energy of which can change the very essence of nature. The concept that emerges is that the cell can undergo a self-healing process if it receives the right information: this is the new advance.

Stem cells will be mentioned only briefly, as it will be left to other authors to present the latest data. Stem cells deriving from adipose tissue have now become part of everyday clinical practice. However, their results in the treatment of skin wounds are not yet unequivocal, as the greatest obstacle they encounter, directly after implantation, is the inflammatory reaction of the host. They have a migratory capacity that enables them to reach the target site through the blood, enabling rapid access to the entire body. They are then captured by the target organs through complex interactions with endothelial cells that enable them to leave the circulation for tissue regeneration.

Given this premise, stem cells could offer an opportunity for the regenerative treatment of skin lesions, but only if used according to holistic principles. The authors explain their view by returning to the concept of self-healing of the cell in a context such as tissue regeneration, where cell proliferation and differentiation are specific and fundamental processes. After transplantation, stem cells work only if they can communicate with the stem cells already resident in the tissue, acting as a starter and stimulating the existing cells through the cell membrane. In 2012, Yamanaka won the Nobel prize for a 2006 study on the induction of stem cells from fibroblasts through cell manipulation, observing that adult cells can be reprogrammed to become pluripotent. The limitation of this technique is the low efficiency of the differentiation process and the oncogenic risk caused by the use of viral vectors.

Today, stem cells can be incubated and stimulated with platelet lysate, which stimulates their proliferation, or with biological agents, but they can also be reactivated *in vivo* through low-intensity magnetic fields affecting the matrix, membrane and cytoskeleton. Following external stimulation from receptors, the microtubules immersed in the intracellular water vibrate and transmit information to and from the nucleus through signal molecules, just as a dipole transmits the signal beyond the point where it was generated, amplifying the response and turning the cell membrane into a center for signal processing and communication with the outside world. The microtubule is a “molecular cable” that enables the system to memorize information (C. Ventura). All this is made possible by the presence of water, which is essential to enable the humoral and cellular components to perform their roles and the microtubules to spread a dynamic network sensitive to even the slightest signal alteration: a “conscious” network that modulates recognition and communication through the signification of coded messages.

Pluripotent stem cells differentiate thanks to an epigenetic code comprising a molecular network that turns specific genes on and off. The information carried by the molecules is only a part of all the information that reaches the cells, of which a large part arrives with magnetic and sound fields. Ventura and colleagues differentiated embryonal stem cells from heart tissue cells by subjecting them to low frequency (50 Hertz), low intensity (0.6 mm tesla) electromagnetic fields.

“The cells communicate with each other using information carried by molecules, which act over a short range, or transported by electromagnetic waves and sounds transmitted over a long range and targeted precisely to the molecules” (Pier Maria Biava).

Molecular information comprises a concerto that enables life to begin and maintain its balance. It is a chemical system governed by electromagnetic forces. The body's water enables this electromagnetic regulation of the biochemistry, as described in the studies of Emilio Del Giudice on the dynamics of water. Cohesive water (in which the molecules are held together by smaller energy forms) oscillates at a given frequency and attracts molecules that resonate at the same frequency. These molecules interact chemically and produce a new form of energy that, in turn, "reconditions" the magnetic field, modifying its frequency and causing the emergence of its information content of various levels of complexity, which tells the water molecules what to do.

"We could interpret disease as a loss, by the body's water, of some levels of cohesive hierarchy between the domains, with a consequent loss of the electromagnetic control exerted over the biomolecules (Emilio Del Giudice).

While awaiting the new frontiers and conquests that the use of stem cells will open up in the field of cell regeneration, today it is possible to introduce biological therapies tailor-made for each individual patient. The biological molecules used in the preclinical and clinical phase enable greater communication between the patient's biological components (endothelium, matrix, cell), thanks to their greater hydrodynamic capacity and the formation of cohesive, organized water, which modulates the components of the inflammatory process and directs it toward tissue regeneration and healing of skin wounds.

Later I looked again, and before my eyes a door stood open in Heaven, and in my ears was the voice with the ring of a trumpet, which I had heard at first, speaking to me and saying, "Come up here, and I will show you what must happen in the future."

Revelation 3.4

In conclusion, this article has briefly presented our current knowledge of the modulation of the inflammatory process. It first discussed the possibility of following the process in in-vitro models – a valuable option, both for the knowledge they provide and for the possibility of learning more about the behavior of biological agents in relation to tissue regeneration. It then followed the process from a molecular perspective, delving into the "magma" of the pro- and anti-inflammatory cytokines and concentrating on what needs to be blocked in order to reduce the inflammatory process (TNF-alpha and IL1), without losing sight of the structure of the ECM, which remains the main target and the place in which the newly formed tissue is remodeled. It went on to discuss current results in relation to the possible clinical application of stem cells in regenerative medicine, highlighting the role of biological water as a transducer of molecular and energy information perceived by the stem cells, as well as the role of the cell membrane which, in the presence of water and in concert with the complex of molecular "cables" (the cytoskeleton), becomes a signal and information processing center involving receptors, adhesion molecules, the ECM structure and cell populations, with a "chimera" effect that is subject to both known and undiscovered physicochemical laws.

We would like to end with some practical considerations. The wound healing process is a complex process intertwined with the biological mechanisms causing individuals to become ill. Systemic and local factors combine to cause the process to become chronic and perpetuate

itself in the skin wound, an expression of it all. Bacterial infections, which are difficult to combat due to antibiotic resistance, greatly complicate the roles of our innate immune system and lymphocytes. Reading between the lines of routine blood tests, the patient's discomfort can often be sensed through information on the nitrogen balance and the hemoglobin value. The lack of new antibiotics and the impossibility of treating patients at home with hospital medications mean that new, more biological and more physical pathways must be investigated to interact with and defeat bacteria. The results of Montagnier and colleagues suggest that exposing bacteria to electromagnetic fields and hence altering their genetic code or forcing their membranes to become more water permeable could lead to their implosion.

We now know the key players in the wound healing process and we have new molecules available to act on them, but the future must necessarily lie in the transfer of molecules and information between the endothelium, ECM and cell membrane, which can be directed toward tissue regeneration if the resident stem cells have the chance of communicating and interacting with new therapeutic models; all this without forgetting the human being, at the center of research and scientific evolution [37, 38].

Author details

Aragona Salvatore Emanuele^{1*}, Mereghetti Giada¹, Ferrari Alessio² and Giorgio Ciprandi³

*Address all correspondence to: saadmaswood@gmail.com

1 Center of Regenerative Medicine—Humanitas Mater Domini, Castellanza, VA, Italy

2 Alfakjn Research Center, Valenza Po, Alessandria, Italy

3 Allergy Clinic, Casa di Cura Villa Montallegro, Genoa, Italy

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