Skin ulcers-microbiota-infected ulcers

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ABSTRACT

It is nowadays established that the modifications of the skin barrier and/or the skin's immune system are the inevitable cause of an alteration of the host-microbiome relationship followed by a proliferation of the opportunistic and/or pathogenic flora and an uncontrolled immune response which, in turn, might amplify the pathological case itself. The ulcer allows the skin and the environmental microbiome to penetrate the tissues and find optimal conditions to grow; it is believed that the interaction between host, microbiome and the skin might have a positive role in modulating the immune response in the wound healing process or that, at least, its composition might significantly affect it. Wound healing is a complex process that includes many interconnecting and overlapping mechanisms, including cell migration and proliferation, the release of pro- and anti-inflammatory cytokines, growth factors, synthesis, and degradation of the extracellular matrix. An imbalance of these complex mechanisms, regulated by many different signaling pathways, strongly affects the result which, in turn, is correlated with the inflammatory status and the immune response. The relationship microbe-microbe and microbe-skin; the role of the biofilm, defined as an organized aggregate of microorganisms and their communication system through Quorum-Sensing; the progression of a colonized wound towards infection; the role of the host; the microenvironment's impact on the host and on the microbiota and an overview of future applications are the objects of this scientific review which aims at discussing wound healing as a perfect, inseparable balance between host, skin and microbiota.

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Introduction

The skin is the largest organ of the human body, approximately 1.8 m2, with multiple, irreplaceable functions, among which that of a physical barrier against exogenous substances and protection from the invasion of pathogenic germs stands out.[1,2] Each square cm hosts approximately one million microorganisms of at least a thousand different species and each adult individual provides them with approximately 30m2 of habitat in relation to the presence of approximately five million invaginations, including hair follicles and sweat ducts which increase the colonizable surface area significantly.[3] In addition to the skin, every surface of the human organism that is in contact with the external environment: intestine, oral cavity, airways, genitals, urinary tracts, is colonized by bacteria, fungi, viruses, and other unicellular eukaryotic organisms that constitute the microbiota.[4] These are at least 100,000 billion microorganisms, ten times more numerous than the cells of the human organism and which belong to hundreds of different species, with a genetic complement of 3 to 8 million genes (the DNA of the 10,000 billion cells that make up the human organism has a set of 20,000 genes).[4] Approximately 70% of the human microbiota is located in the intestine.

For the purposes of understanding the microbiota, the technique of ribosomal RNA sequencing has proven to be fundamental: the sequence of the gene for the 16s subunit shared by all bacteria- but not by humans- is determined, a sort of "barcode" to identify and quantify the microor-

ganisms present. For fungi, the 18s RNA subunit is encoded. This technique has made it possible to overcome the very obvious limitations of traditional culture tests (think, for example, of the difficulty in isolating and cultivating anaerobic germs). With the latest shotgun technique metagenomic, furthermore, sequencing makes it possible today to reconstruct the genome of viruses and microeukaryotes and thus identify, over time, other new microorganisms.[5] The set of microbes commonly present on the skin is defined as resident or commensal because it is not recognized as aggressive; this means, and is of fundamental importance, that the skin immune system is able to distinguish the resident flora from the pathogenic one.[3] In a specific time window in neonatal age the skin immune system is able to establish a tolerance to comparisons of the microbiota present through the recall of specific T cells, the regulatory T cells which mediate the suppression of the inflammatory response only towards commensals and not towards pathogens. Proof of this is that they recognize *S. epidermidis* as commensal, but not *S. aureus,* producer of an alpha-toxin which, through the activation of IL-1, is able to inhibit its function and guarantee the immune response against the pathogen.[6] The resident flora is not simply a host of the skin but is able to oppose the continuous attempts to colonize it by different organisms, first of all by occupying it in a stable way and competing with every other non-commensal microorganism, for example by acidifying it through some products of its metabolism and therefore making it inhospitable to other strains, or through the synthesis of antimicrobial molecules (AMPs). An example above all: *S. epidermidis* coagulase negative (CoNS) produces a specific antimicrobial peptide (AMP) that counteracts the attempted colonization by the pathogen *S. aureus*. Some strains of *S. epidermidis* are also able to synthesize modules capable of destroying the membrane of some pathogens or producing a lipopeptide to support host defense or, again, producing lipoteithoic acid, a Toll-like receptor 2 ligand involved in the mechanism of skin inflammation.[6]

There is therefore a network of microbe-microbe interactions that act in favor of the host, confirming that the skin microbiota is actively involved and interacts with the complex immunological control system carried out by the skin organ. In fact, it modulates the expression of various innate factors, *e.g.*, IL-1a, complement components, and antimicrobial peptides (AMPs) produced by keratinocytes and sebocytes; interacts with the nonclassical major histocompatibility complex of commensal derived antigen promoting protective barrier immunity, immunoregulation, and tissue repair; activates Mucosal associated invariant T cells, also present in the skin which has a protective action against pathogens. Modifications of the skin barrier and/or of its immune system, inevitably determine an alteration of the host-microbiota balance with the proliferation of opportunistic and/or pathogenic flora and a consequent uncontrolled immune response which, in turn, may amplify the pathological picture itself. This is the premise for understanding how a skin commensal can transform, in favorable situations, into a pathogen and cause an infection.

The skin microbiota: its characteristics and its plasticity

It is now agreed that microbial species exist in a continuum between mutualism and pathogenicity, closely related to the immunological and metabolic situation of the host and the presence of some microbial partners rather than others.[3,7] As proof of this, *S. aureus*, oriented towards pathogenicity as already highlighted, is among the main causes of community-acquired and nosocomial infections, but at the same time asymptomatically colonizes 20-40% of the general population, confirming the variability of its pathogenicity range. It is hypothesized that this variability may be related to microbe-microbe interactions, for example the presence of some species of *Corynebacteria* capable of interacting with a communication mechanism between bacterial cells, Quorum Sensing, using a transcriptional signal; in this way, the *Corinebacteria* would antagonize its virulence. The *Cutibacterium Acnes* would be able to induce the aggregation of *S. aureus* in the form of biofilm through the production of coproporphyrin III, but also to produce short-chain fatty acids that suppress a particular strain of *S. aureus,* resistant to methicillin, called USA 300.[7] In summary: the skin microbiota is intimately correlated with the state of wellness and/or skin pathology; the constant and specific dialogue between the commensals and the skin cells, both endowed with immunological competence, regulates their homeostasis and contributes to the restoration of the damaged barrier.[8] The composition of the skin microbiota is correlated to multiple factors such as ethnicity, age, gender, geography, lifestyle, hygiene, profession, and skin thickness. It also varies in the different body areas: in humid areas, the growth of *Corinebacteria* and *Staphylococci* is favored, in dry areas exposed to wide temperature fluctuations *Proteobacteria (e.g.*, *E. coli*) are favored, in sebaceous areas the growth of obligate and facultative anaerobes such as *Cutibacteria, Staphylococci* and fungi of the *Malassezia genus* are favored. Regardless of these variations, the skin commensals typically present belong to the genera *Corinebacterium, Cutibacterium, Staphylococcus,* and *Prevotella.*[6]

In summary: the skin microbiota is composed of a vast range of microorganisms including fungi, viruses, arthropods, as well as bacteria, the best known and most studied. The fungal species of the microbiota, in addition to the aforementioned *Malassezia genus* – the most represented – approximately 80% of the entire mycobiota, include

Cryptococcus spp., *Rhodotorula* spp., *Aspergillus* spp., *Epicoccum* spp., and *Candida* spp.; they play a role of primary importance both in maintaining homeostasis and in pathological situations. In recent times, with the application of functional metagenomic methods, the skin virome, a complex resident flora, has also been, identified essentially represented by various species of *betapapillomavirus, and gammapapillomavirus, polyomavirus,* and *circovirus*. Further studies are needed on the dynamics, the variations in different anatomical sites, and the potential variations in pathological conditions to fully understand their role, for example in proliferative skin pathologies. Finally, it is believed that small arthropods, usually located on the skin of the face, on the periphery of the pilosebaceous glands and hair follicles, such as *Demodex* mites, belong to the commensal skin microbiota because they are present in between 23 and 100% of healthy individuals; it should not be forgotten, however, that they are also associated with pathologies such as rosacea, chronic blepharitis, demodecicosis.[9]

Microbiota and skin: a double bond

The skin microbiota is also present in the dermis and this localization plays an important role in immune defense.[6,10] The Toll-like receptors expressed by the keratinocytes of the epidermis, for example, are also expressed by fibroblasts and by adipocytes present in the dermis; they actively participate in the immunosurveillance mechanism because they recognize specific microbial components and instruct the responses of the innate and adaptive immunity.[6] The immune system and the skin microbiota interact stably in order to guarantee a healthy immunological function, in close correlation with the host's inflammatory and metabolic situation; as a consequence of skin barrier or immune system modifications, the host-microbiota balance breaks down with the consequent proliferation of a microbial flora that is no longer commensal but rather opportunistic and/or pathogenic and with an uncontrolled immune response.[6,9] The ulcer allows the skin and environmental microbiota to penetrate the tissues and find optimal conditions for growth; it is believed that the interaction between resident flora and skin has a positive role in modulating the immune response in the wound healing process or that, in any case, its composition significantly conditions it.[11,12] Wound healing is a complex process that involves multiple interconnecting and overlapping mechanisms of cell migration and proliferation, recall and release of pro- and anti-inflammatory cytokines, growth factors, synthesis and degradation of the extracellular matrix which, to simplify, can be divided into three phases: inflammatory, proliferative and remodeling.[12] The inflammatory phase begins with the activation of platelets to achieve hemostasis and the formation of a transitory fibrin scaffold and pathogens and foreign material present through the recall of neutrophils and monocytes to the site of injury. Platelet degranulation releases damage-associated molecular patterns, cytokines, chemokines, and growth factors which, accumulating within the scaffold, generate a chemotactic gradient with recall of the cells of the innate immune system. In particular, macrophages, derived from monocytes, are fundamental in the transition from inflammatory to proliferation phases including the transition from M1: inflammatory macrophages to M2: anti-inflammatory wound healing cells. At the same time, they guarantee the debridement of necrotic tissue, the phagocytosis of harmful antigens, and the secretion of growth factors and cytokines essential for the subsequent wound healing mechanisms.[13-15] The proliferative phase is supported by migration and proliferation of M2 macrophages, endothelial cells and fibroblasts which secrete growth, endothelial proliferation, angiogenesis, keratinocyte proliferation and differentiation factors, essential for the re-epithelialisation of the lesion. Fibroblasts deposit collagen for the construction of granulation tissue or the extracellular matrix which will replace the transitory fibrin scaffold already present. In this phase, the role of keratinocytes is essential for re-epithelialization.[13-15] In the final remodeling phase, there is a decrease in granulation tissue, degradation of the proteins of the temporary extracellular matrix by metalloproteases (MMP), and their replacement with the definitive one, in order to guarantee greater resistance and flexibility to the regenerated skin.[13,14]

It is evident how the imbalance of these complex mechanisms, regulated by a large variety of signaling pathways, can affect wound healing and how this is closely correlated with inflammation and the immune system.[12] The cutaneous commensal microbiota, which we know to be closely involved in the countless, complex pathways that regulate inflammatory responses, is also able to influence every phase of tissue repair, both in advantageous and disadvantageous terms. It means that there is the possibility that commensals can assume the role of pathogen and *vice versa*.[12] Some examples: *S. epidermidis* stimulates AMP production by host keratinocytes, induces TCD8+ and IL-17 cells, improves innate barrier immunity, and limits pathogen invasion in the absence of inflammation, but occasionally, it is implicated as a pathogenic microorganism, in the production of biofilms.[12] *Group A Streptococcus*, when present as a commensal, stimulates the production of AMPs, promotes epithelial differentiation, and activates plasminogen which promotes the chemotaxis of keratinocytes in order to obtain re-epithelization of the lesions. When present as a pathogen, it expresses proteases that prevent the recruitment of neutrophils, produces a hyaluronidase that allows the migration of bacteria through the host's extracellular matrix and is the cause of common

superficial and deep skin infections such as impetigo, erysipelas, cellulitis.[12] *S. aureus* , usually pathogenic, implicated in the production of biofilms and in the chronicity of lesions, is however capable of producing superantigens that decrease IL-17 favoring the healing of lesions; it can also amplify the innate skin immune response through the production of specific AMPs.[12] And again: *P. aeruginosa,* usually pathogenic and biofilm producer, is also able to suppress pathogenic *staphylococci* in polymicrobial colonized ulcers and to accelerate the reepithelialization and neovascularization through TAK1 transforming growth factor-activated kinase 1 signaling.[12]

The biofilm

All wounds host microorganisms from the commensal microbiota or the environment; it is known that bacteria can be present as single planktonic cells, capable of moving freely in their environment or, instead, in a sessile state, *i.e.*, adhered to surfaces (urinary catheters, vascular catheters, implants, prostheses, contact lenses, *etc*.) or, in particular, to each other to form multicellular aggregates that lead to the formation of biofilms. This is a multi-step process in which heterogeneous communities of microorganisms (bacteria and/or fungi) are embedded in a self-produced matrix of extracellular polymeric substance (EPS). EPS contains proteins, glycoproteins and polysaccharides and gives the biofilm the ability to adhere to any surface, biological or otherwise. Within the aggregated state, microorganisms have the ability to create an environment favorable to their protection and longevity.[15,16] The cells included in the biofilm can develop an intracellular communication mechanism: the aforementioned Quorum-Sensing (QS) which controls bacterial pathogenicity and the formation of biofilm itself. Bacterial density influences biofilm production. However, microbial cells within a biofilm are able to leave it and spread into the wound environment. The behavior of the released bacteria may differ from that of the colonizing bacteria due to the adaptation and transformation that occurred within the biofilm itself. Dowd *et al.*, after observing that different bacterial species can collaborate and interact with each other within the biofilm, proposed the concept of Functionally equivalent pathogroups (FEP) responsible for the chronicity of the infection and the maintenance of the pathogenic biofilm.[16] In non-resolving lesions, most bacteria are present as biofilms that come correlated with the chronic inflammatory state of the lesion and therefore of delayed repair,[13,15-17] but since all microorganisms are able to adhere to any surface, it is believed that biofilms, in themselves present in all chronic wounds, are not always responsible for delayed healing, but only pathogenic ones are.[18] A biofilm has the characteristics of a multicellular organism; although each is unique, it has some common characteristics with each other. For example, it has channels of fluid running through it, similar to a circulatory system; responds to external and internal stimuli, such as a nervous system and shows responses that can be defined as “altruistic”. We can actually consider the skin microbiota as a "benign biofilm" as it is protective, (the same goes for the microbiota of the gastrointestinal tract) however able, in favorable situations, to transform into a pathogenic or virulent one. Compared to commensal biofilms, pathogens have a significantly higher number of up-regulated genes, responsible for the excessive development of degradation enzymes, such as matrix metalloproteinases, a greater development of EPS which constitutes the physical barrier against antimicrobial agents and the immune system itself, greater generation of QS molecules, greater proliferation and capacity for microbial dissemination. Enhanced genetic and biochemical effects within a pathogenic biofilm lead to up-regulated immune responses that are in turn responsible for chronic inflammation.[18] While it is now well established that the wound microbiota and biofilm formation are involved in delaying healing, the causal relationship between microbiota composition, biofilm formation, unregulated innate immune activation and persistent inflammation in chronic wounds remains to be explored. It is unclear whether bacteria and biofilm formation drive innate immune dysfunction or innate immune dysfunction itself makes the wound microenvironment more susceptible to biofilm formation. Understanding *in vivo*, as well as verifying *in vitro*, the mechanisms underlying the actions and reactions of a single bacterium when it interacts with other nearby bacterial cells, other microorganisms and the host, will be of great help in restoring healing processes and, therefore, in the therapeutic field.[15] All open wounds contain microorganisms from endogenous (the patient’s flora) or exogenous (the surrounding environment) sources. The immune system takes action to guarantee an adequate response to the situation of altered barrier, but the adhesion of microbes to the surface of the wound and their proliferation could give rise to the formation of a biofilm which, once consolidated, will become mature and more difficult to eradicate, with an increased risk of the wound becoming chronic and clinically infected. (Consequently, preventing a biofilm is critical for faster and more effective treatment of chronic wounds).[18] The mechanism of QS that regulates various bacterial physiological processes, including virulence, motility, luminescence, biofilm formation, sporulation, development of genetic competence, synthesis of antimicrobial peptides, production and secretion of proteolytic enzymes and its important implications on the characteristics of the biofilm, which have already been mentioned, plays a fundamental role in understanding that the ability control of the growth of microorganisms in a wound by the host de-

creases as the biofilm community matures.[18,19] Within a stable biofilm, interactions occur between aerobic, anaerobic bacteria and fungi which likely increase the pathogenic effect of these microorganisms and delay healing by promoting a chronic inflammatory state. This results, for example, in the release of free radicals and lytic enzymes that damage the cellular processes responsible for wound healing. It is proven that the proteases released by some microbes negatively affect growth factors and other tissue proteins necessary for the repair process. The increased exudate production that often accompanies increased microbial load has been associated with the degradation of growth factors and matrix metalloproteinases which in turn influence cell proliferation and wound healing.[18] Wounds expose tissues to the environment that under normal circumstances would be free of microbial contamination. However, as happens with healthy skin, the wound will naturally be colonized by microflora and the compromised tissue, particularly necrotic, will encourage its proliferation. In the presence of tissue hypoxia, frequent in skin ulcers, the commensal aerobic and anaerobic flora (coming from the skin, oral cavity, intestine and genitourinary tract) will establish itself in this unusual but favorable environment, where its strategies of survival will be able to transform it into a pathogen. The unique microbial interactions of this particular environment will significantly influence wound pathogenesis and healing.[20]

The colonization of ulcers

The bacteria that first enter a wound, colonize it and predominate, are Gram-positive bacteria, in particular of the *Staphylococci genus coagulase negative* (CoNS), commensals of healthy skin.[21] In the following period, days or weeks depending on the immunological competence of the patient, the gram-negatives, coming from the urogenital tract or from the environment close to the patient, will invade the field and compete with the resident species. Enterobacteriaceae, such as: *E. coli , Klebsiella pneumoniae* spp*., Enterobacter* spp. from the urogenital district, frequently *Pseudomonas, Acinetobacter* or yeasts from the environment. This colonization only causes a localized immune reaction. In this phase the bacterial load increases and negatively affects the healing process;[21] acute colonization is typically linked to the onset of an inflammatory reaction and, from a clinical point of view, to the increase in local pain.[22] Microbial invasion of deep tissues will result in an intense host immune response characterized clinically by local and systemic reactions such as diffuse and marked erythema, purulent collection, or symptomatic cellulitis. Most wounds, therefore, host a polymicrobial flora. Anaerobic bacteria, already present in approximately one-third of colonized lesions, increase in number up to at least 50% of the microflora present in clinically infected and/or non-healing lesions, also in relation to the rapid consumption of oxygen by the aerobic bacteria present: synergic effect with which aerobic and anaerobic microorganisms support the persistence and multiplication of each other. Anaerobic species can hinder the phagocytosis of other microorganisms by producing short-chain fatty acids, and the flow of nutrients from one bacterium can support the evolution and proliferation of another. This implies that antimicrobial treatment of these wounds should cover a variety of potentially synergic obligate or facultative aerobic and anaerobic microorganisms and should not simply target specific pathogens that are often considered the only causal agents (*e.g.*, S. *aureus* and P. *aeruginosa*) only because they are commonly isolated in the wound bed. Since no chronic wound is colonized by a single species but by a multitude of aerobes and anaerobes that increase with the age of the wound, the biological effects exerted by microbes must be considered as the result of the "Microbial Network" and cannot be predicted by described effects of a single specific species.[20-22] This means that the progression of a colonized wound towards an infected state cannot be predicted by the presence of a specific type of bacterium or a specific pathophysiological condition, because it is likely that a multitude of factors simultaneously influence the pathogenesis. Microbiological factors such as population density, the type of microorganisms present, microbial interactions and host factors, such as the effectiveness of the immune response and the condition of the tissue, are all critical elements and must be considered collectively as factors that predispose to infection.[20] Regarding the type of microorganisms present, biopsies taken from different points of chronic wounds highlight not only highly variable numbers and types of bacteria, but also that their distribution is heterogeneous: for example, *S. aureus* prefers to settle closer to the surface of the lesion than *P. aeruginosa*. This most likely occurs due to local environmental differences such as the possibility of nutrition, oxygen concentration and the type of host response. Gradients of these factors are also observed within bacterial biofilms which, according to some authors, will most likely be single species due to their competitive nature. In the presence of an abundant local supply of nutrients, such as in wound areas with necrotic tissue, bacteria could evolve side by side or even within the same biofilm. These findings suggest that different bacteria prefer different environments and/or that they compete with other bacteria and find colonization niches in chronic wounds where they have the best opportunities for survival. Conventional culture cannot identify all bacteria as some are difficult to isolate and others demand culture or are present only in certain areas of the wound; this could lead to its underestimation, for example found in the case of

Pseudomonas.[23] Regarding the importance of further aspects in the process of wound infection, for example, take into account the condition of the tissue. Think of oxygen, a critical component of respiratory activity in polymorphonuclear leukocytes (PMN) responsible for the production of highly potent antimicrobial metabolites. With a pO_2<30 mmHg the antimicrobial action of PMNs is significantly reduced, which is why poorly perfused wound tissue is considered much more susceptible to infection than wounds involving well-perfused tissue.[20] Oxygen tension values lower than 30 mmHg are frequently found in infected tissues or chronic wounds, which constitutes the fundamental requirement for active cell division. Therefore, cell death and tissue necrosis caused by tissue hypoxia or anoxia can create ideal growth conditions for members of the wound microflora, including anaerobes that proliferate as residual oxygen is consumed by facultative bacteria.[20] Aerobic-anaerobic polymicrobial interactions are known to contribute significantly to disease progression and severity in acute soft tissue infections.[20] In 2008, two articles on Wound Repair and Regeneration changed the clinical perspective on chronic wounds by stating that the bacterial biofilm contained in them was possibly responsible; however, to date, there is no complete clarity on the exact role of biofilm in hindering the healing process, but above all, there is no availability of treatments with guaranteed efficacy. One reason could be that *in vivo* biofilms differ significantly from those *in vitro* from which most of the current knowledge derives and that the approximation of *in vitro* models is currently high. Furthermore, very little is known about the microenvironment surrounding bacteria in chronic infections.[23]

The zone model

To better understand bacterial behavior and the impact of the microenvironment on the host and microbiota, some authors have proposed the "Zone Model" (Figure 1).[23] Until now, bacterial identification, gene expression, and postscript regulation have looked at bacteria as a whole and averaged their behavior. The Zone Model, however, starts from the assumption that each individual bacterium reacts specifically to its own microenvironment and aims to understand its behavior both as an individual, and in interactions with other bacteria and microbial aggregates of different species, and in the involvement of the mechanisms of the local response of the host, of the surrounding tissue and of the systemic context of the host itself.[23] It is known that many chronic wounds with biofilm heal with adequate traditional treatment: for example, venous leg ulcers require compression therapy, pressure ulcers and DFUs require offloading. Many wounds become chronic mainly due to insufficient traditional treatment and not due to the presence of biofilm. Since even chronic wounds with biofilm can resolve, it is necessary to consider what its role is. The Zone Model considers it more likely that the extracellular matrix does not influence healing, but that the phenotype of the bacteria is instead the obstacle to healing as it produces virulence factors that modify the surrounding microenvironment. The microenvironment is important for the phenotype of bacteria and is the one most referred to in this model which identifies five zones and characterizes them.

Zone 1 is specific to the single bacterium as it is adjacent to it: it is exocapsular. It determines its physiological state and behavior based on the level of oxygen, carbon sources, antimicrobial factors and the presence of any other element that characterizes it. The entire genome and transcriptome of the individual bacterium, together with the microenvironment, determines its phenotype. Zone 1 is small and difficult to study. If the bacteria are located in the center of the biofilm, it is likely that substances such as oxygen, iron and other nutrients are lacking and that, in such conditions, the bacterium may be dormant, waiting for environmental changes favorable to it. Zone 2 is made up of the aggregate of several bacteria incorporated into a self-produced matrix (EPS) and mixed with polymers, DNA, proteins and polysaccharides coming from the host. The oxygen concentration decreases towards the central area of the biofilm as well as, probably, also some antimicrobial substances, nutritional factors, *etc*. Zone 3, the exobiofilm zone, is the environment that encapsulates the aggregate. Here host/bacteria and bacteria/host interactions occur which can be reflected in the evolution of the lesion and which can achieve, among others, also a stalemate sit-

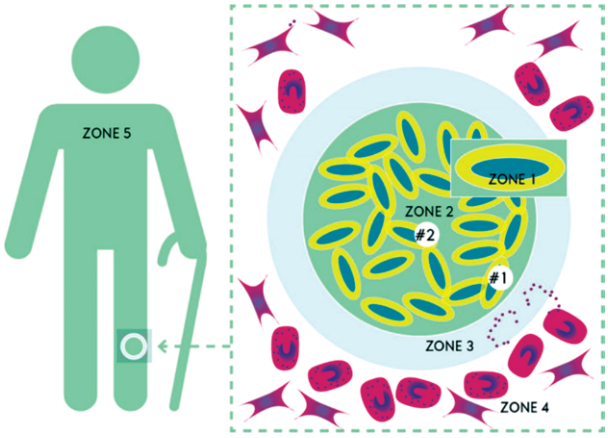

Figure 1. The zone model: a conceptual model for understanding the microenvironment of chronic wound infection. Retrieved from Wound Repair Regen 2020;28:593-9 (https://creativecommons.org/licenses/by/4.0/).[23]

uation in which the host physically contains the pathogen without determining its complete elimination and the pathogen persists in a state of decreased activity and invasiveness, but survives inside the host for a long period of time (this possibility is more favorable to bacteria). Zone 4 includes the tissue immediately surrounding zone 3 and reflects the results of host/bacteria and bacteria/host interactions. It does not have the same characteristics for all biofilms present as in the superficial layer of the wound the density of bacteria is high, but in the deeper parts, the distance between the aggregates of the biofilm is greater. Furthermore, if, as verified in many studies, each microbial aggregate – within the biofilm – contains a single species, there may be multiple separate and distinct aggregates of different species capable of interacting with each other and characterizing different areas of zone 4 for example with mechanisms of collaboration, mutualism and commensalism. In zone 4 the host's immune cells are oriented towards establishing wound healing itself: PMNs, macrophages, lymphocytes, and keratocynocytes free the tissue from debris, necrotic host cells and bacteria and produce collagen as a scaffold for the repair phase. Zone 5 is represented by the host himself: a healthy person is extremely unlikely to develop chronic wounds. Concomitant pathologies such as diabetes, vasculopathies, immunological disorders interfere with the physiological mechanisms of repair; the increase in tissue damage related to systemic pathologies may favor microbial infection, further tissue damage and the chronicity of the condition. In summary: within a specific biofilm, each bacterium has a unique zone 1 that determines its activity, so two bacteria within the same biofilm could have markedly different physiological states. Oxygen, a critical element for the growth of many microorganisms, is shown as an example to illustrate the interaction between the zones. Under normal conditions, the influx of oxygen can be regulated by vasodilation in order to keep the tissue oxygenated in zone 4. In zone 3, the immune response is mainly driven by PMNs which consume oxygen for the production of antimicrobial metabolites, decreasing that available for zone 2. Due to the uneven distribution of PMNs in the wound, the different microbial aggregates may have different levels of access to oxygen. If the host has ischemia, zone 3 will have less oxygen available, the functionality of the PMNs will be affected and zone 2 will have even less oxygen. If the blood supply is restored it will affect all compartments; hyperbaric oxygen therapy can increase the availability of diffuse oxygen in zone 4 and, consequently, in every other zone which, in turn, will influence the adjacent ones.[23] The Zone Model can help understand the proposed "window of opportunity" by Wolcott *et al.*,[24] for which surgical debridement of chronic wounds can open a temporal window of therapeutic opportunity during which bacteria are more susceptible to the action of antibiotics and the host's immune defense system following physical disruption of the biofilm. Through debridement, slough, debris and granulation tissue are removed and, at least in part, also the bacteria of the superficial layer. In the context of the zone model this induces changes in the environment of zone 4 with a knock-on effect on all the others and on the bacteria themselves which will modify phenotype or expression. During this phase of adaptation to the new environment, bacteria - even in the biofilm - can be sensitive to antibiotics and accessible to the immune defense system. The treatment of chronic wounds, based on molecular diagnostics of biofilm and with personalized treatment of topical and systemic antibiotic therapies, has shown significantly better results than those obtained with standard care groups who were prescribed systemic antibiotics on the basis of empirical and traditional methodologies based on culture.[23]

Diabetes and microbiota

Among the host comorbidities that modulate the wound microbiota, glycemic control and the duration of diabetes mellitus play a prominent role. Diabetes causes persistent low-grade inflammation, local hypoxic conditions, and impaired cellular responses to hypoxia and infection that significantly affect wound healing. Zone 5 of a diabetic patient, therefore, will condition the response of all the others, determining different results on the wound healing, compared to those of a non-diabetic patient.[23] Diabetic patients have a 15-25% incidence of foot ulceration (DFU); infection is the most common and serious complication, it affects recovery times and, together with ischemia, is the cause of amputation. 80% of lower limb amputations in diabetic patients are preceded by infected foot ulcers which lead to an increased risk of death within eighteen months.[16] The host-microorganism interface plays a critical role in both the development and healing of DFU. The observed number of pathogenic microbial species in this interface is lower than in the presence of many commensal bacteria. Furthermore, many of the species present in chronic wounds are commensal in healthy skin and there are clear differences in the composition and diversity of the microbiota in diabetic foot ulcers (DFU) and healthy skin microbiota. And again: recent-onset diabetes and not-too-high AbA1c levels are associated with a greater diversity of the microbiota; long-standing diabetes and a high level of AbA1c are associated with the predominance of some genera with greater abundance of *Actinobacteria* or *Streptococcus*.[17,25] This metagenomic comparison study of foot bacterial microbiota in diabetic and non-diabetic men and quantification of clinically relevant species highlighted that in diabetic patients the staphylococcal species were reduced with an increase, however, in the *S. aureus species* and a greater

diversification of the bacterial population.[26] Compared to the contralateral healthy skin, the lesion microbiota was characterized by a decreased bacterial diversity with higher levels of opportunistic pathogens. It is believed that these changes may be precursors to diabetic foot infection and ulcer development.[26] Although studies on the microbiota in other body sites have demonstrated that pathological states are associated with its lower stability, this study highlighted surprisingly, delayed-healing or amputation-responsible DFUs were associated with greater stability of the microbiota itself while the opposite is the case for faster-healing DFUs. One way to interpret these results suggests that there is no "normal" DFU community. A wound is by definition an abnormal and transitory state; colonizing bacteria should be considered opportunistic, in a state of instability with the host. Instability in the microbiota is an expression of effective immune control that prevents any community structure from stabilizing. In contrast, a DFU with stable growth of some bacteria reflects a stalled healing state in which colonizing bacteria have overcome host defenses. The microbiota in DFUs can be divided into 4 community types (CTs): CT1-CT2, in which a highly heterogeneous flora is represented and no dominant species; CT3, in which streptococcal species are highly represented, closely associated with HbA1C levels and the presence of anaerobes; CT4, in which *S. aureus* is highly represented. In DFUs with a tendency to heal, CT1 and CT2 show a tendency to keep their composition unchanged while CT3/CT4 show a tendency to transition into CT2; in chronic DFUs TC3/CT4 are very stable. It is clear how the possibility of transition favors healing and how the stability of the pathogenic microbiota hinders it; information on the specific characteristics of the microbiota of a DFU could have a significant impact on the prognostic capacity precisely based on the correlation with the transition phenomenon.[27] Persistent hyperglycemia, chronic inflammation, hypoxia, peripheral neuropathy, modified angiogenesis and altered immune response in diabetes are factors that compromise wound healing and predispose them to chronicity and infection: at least 60% of DFUs become infected.[28] Biofilm formation plays a substantial role in the chronicity of DFUs, in the development of antibiotic resistance and in delayed healing; it is the biofilm that modulates bacterial virulence and communication between the microorganisms themselves.[18] Microorganisms residing within a biofilm are phenotypically different from their free-floating or planktonic counterparts; for example, *P. aeruginosa* expresses approximately 73 additional genes when grown as a biofilm compared to the planktonic situation. Modulation of gene expression is considered important for bacterial survival and maintenance once a microorganism is adhered to a surface. This critical control of gene expression is believed to allow biofilm microorganisms rapid adaptation during adverse external conditions, a concept referred to as "Biological insurance".[29] As a biofilm matures the synthesis of EPS – which has the role of scaffolding – is up-regulated and sequesters and retains nutrients, enzymes and metal ions, for example, iron, necessary for the maintenance and stability of the biofilm itself and, therefore, for microbial survival.[29] Unlike biofilms found physiologically in the human body, such as the skin, teeth, gastrointestinal and vaginal mucosa, the presence of biofilms in a chronic wound is not considered "natural" and, therefore, has never been taken into consideration the existence of an "indigenous microbiota" for wounds which, however, could be hypothesized in a state of commensalism or mutualism with the host: for example in immunocompromised patients.[29] A microbial population in a homeostatic situation can be defined as a "Climax Community" in which the biofilm has reached a state of equilibrium as a result of synergistic, antagonistic and mutualistic interactions between the different microorganisms present in the wound. The relationship between facultative and strictly anaerobic species within the biofilm could be an exemplification of this. It is likely that as a biofilm develops toward its "Climax Community," the activity of facultative anaerobes may create anaerobic regions within the biofilm that support the growth of strictly anaerobic organisms. Clearly, this synergistic relationship between species influences the composition of the "Climax Community" which is further supported by communication systems between bacteria – QS – which coordinates gene expression and the function and activities of biofilm organisms, promoting microbial stability and long-term survival.[29] The microorganisms within the biofilm are randomly distributed, but functionally organized into niches; each niche with its specific function and role. Heterogeneity within the biofilm is considered critical to its stability. Exogenous and endogenous factors, such as pH, temperature, and host immune response are able to act on microbial competition within the biofilm, favoring the growth, for example, of less predominant but more pathogenic species or the decrease in the growth of bacteria non-pathogenic suppressants (competitors). Consequently, better control measures are needed to prevent this shift towards a "predominantly pathogenic biofilm" that could delay wound healing.[29] The most studied bacterial interaction in DFUs is the cooperation between *S. aureus and P. aeruginosa*. Many substances produced by *P. aeruginosa* can play a protective role for *S. aureus* – synergic cooperation – which increases its tolerance to antibiotics, the ability to form biofilms and to secrete species-specific virulence factors. These interactions can also be competitive towards some nutrients, for example iron, or inhibit the unidirectional growth of *S. aureus*. At the same time, *P. aeruginosa* can suppress the growth of *S. aureus* and improve its resistance to aminoglycosides.

The coexistence of synergi(sti)c and antagonistic mechanisms between the two species is evident, in full agreement with what has already been illustrated. Among the bacteria most frequently identified in DFUs: Gram-positive bacteria, such as *S. aureus* (methicillin-susceptible and methicillin-resistant), *β-hemolytic Streptococcus* and *C. striatum*, but also Gram-negative, such as *P. aeruginosa*, *E.coli, A. baumannii* , *Proteus* spp., *Enterobacter* spp., and *Citrobacter* spp., as well as some anaerobes deeper in the wound bed, such as *Bacteroides* spp., *Prevotella* spp., *Clostridium* spp., and *Peptostreptococcus* spp.[28] Diabetic patients have an increased risk of fungal infections; more than a quarter of DFUs that fail to resolve, exhibit necrosis, and demonstrate poor therapeutic outcomes are associated with fungal infection. Furthermore, the mycobiome constitutes a scaffold for bacterial adhesion and provides additional protection to pathogens against the host's immune system, promoting the formation of multi-kingdom biofilms: bacterial/fungal. The most commonly isolated fungi are *Candida* spp., *Trichophyton* spp., *Aspergillus* spp., *Trichosporon* spp., and *Cladosporium herbarum*.[28] There are two main interpretative hypotheses regarding the type of bacterial organization in patients with DFU: i) the specific bacterial hypothesis according to which, despite a complex microbial diversity present in a wound, only a few bacterial species are actually involved in delayed healing and therefore contribute to the infection process (V zone theory); ii) The non-specific hypothesis instead considers that the entire, complex heterogeneous microflora present on the wound plays a role in the infection and does not consider the pathogenic bacteria individually responsible for the chronicity of the wound. This concept has led to the use of the term, already mentioned, of FEP for which some bacterial species – pathogenic or commensal – can co-aggregate symbiotically in a pathogenic biofilm and act synergistically to support chronic infections.[25,29]

A look at the future

In order to identify new and better therapeutic strategies, research must take into account the bacterial organization in the biofilm of chronic DFUs.[30] Some studies are aimed at the treatment of individual bacteria, the most pathogenic, and others at the biofilm as a whole, however starting from the assumption that microbial colonies that form biofilms are 10 to 1000 times more resistant to antimicrobial agents than planktonic ones.[29,30] There are numerous new, possible, encouraging therapeutic strategies; some of them are listed: i) antimicrobial peptides -AMP- associated with new delivery systems in order to increase their stability, reduce their toxicity, enhance their antimicrobial activity and improve their targeting and prolonged administration to the wound site; ii) biodegradable vehicles for the transport and transfer of ABT in high concentration in the biofilm in order to increase its effectiveness and minimize its side effects; iii) guanylated polymethacrylates, effective both on the formation of biofilms of single species and of polymicrobial films, even inter-kingdom; iv) nutraceuticals (*e.g.*, blueberry extracts, polyphenolic compounds, cinnamon essential oil, propolis) with purposes similar to those mentioned above and with the possibility of sometimes increasing the sensitivity of the biofilm to ABT; v) probiotics with activity on specific biofilms; vi) bacteriophages; vii) substances effective in inhibiting initial bacterial adhesion in order to prevent the formation of biofilms by some species. Starting from the assumption that bacterial growth requires the presence of metals, in particular, calcium, iron and magnesium, ionic chelators could be used to limit initial adhesion and bacterial growth itself; viii) modulation of quorum sensing of some microbial species: inhibition of the QS signal inhibits biofilm formation; ix) enzymes that increase bacterial dispersion by breaking down the biofilm; x) new generation dressings and grafts with action on some species of *S. aureus* and *P. aeruginosa* resistant to ABT, capable of preventing the formation of biofilms or promoting their removal.[30]

Conclusions

The microbiota as an Organ/Organism plays an active role in the "Wound", but also in the "Healing"; at the moment still little known by the majority of professionals, but with realistic prospects of attracting the attention of the vulnological world as well as many other areas of medicine. Possibility and future prospects of synergy with the therapeutic strategies currently available in the full awareness that the microbiota – the regulator of skin homeostasis and promoter of the restoration of the damaged barrier – is intimately correlated with skin health and disease through the constant and specific dialogue between commensals and skin cells because it is mediated by the immunological competence of both. The Microbiota intervenes through a series of complex mechanisms/interactions/regulatory patterns between healthy tissue and damaged tissue; commensal flora/opportunistic flora/pathogenic flora; injured tissue/repair mechanisms/characteristics of colonization; microbe/microbe and microbe/host interaction at any time in the history of a skin ulcer.[3]

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