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Wound Healing and the Role of Biomarkers and Biofilms

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Wound Healing And The Role of Biomarkers And Biofilms

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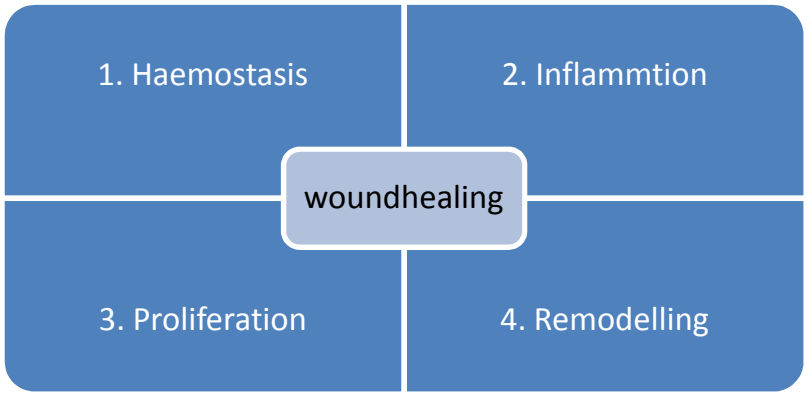
Abstract

An injury to the skin alters the integrity of underlying tissues and microcirculation and thus certainly culminates in a wound. Wound healing is a highly complex, dynamic, interactive and well regulated physiological process involving blood cells, extracellular matrix, parenchyma cells and soluble mediators. It starts with alteration in integrity of tissues and ends with the formation of scar. The process of wound healing is distinguished into Haemostasis, Inflammation, Proliferation and Remodeling. Various cells like platelets, neutrophils, macrophages, lymphocytes, angiocytes, keratinocytes, fibroblasts and factors like platelet derived growth factor, transforming growth factor, platelet derived epidermal growth factors, fibroblasts growth factor, albumin, fibrinogen, fibronectin, anti hemophilic factor, pro accelerin etc play a significant role in wound formation and its amelioration at some crucial points for a brief period of time. Management of chronic wounds requires the use of antimicrobial application so as to eliminate the bacterial colonial biofilm formation at the injury which hinders the recovery. Different pathological parameter serves as biomarker for the evaluation and assessment of wound severity, measure to take care and the alternatives to be needed. Thus timely accurate and precise wound care may prevent the bio burden in the wound and the various sufferings to the patient.

Introduction

Wound healing is an extremely organized and complicated event. An injury to the skin changes the integrity of underlying tissues and microcirculation and thus culminates in a wound[1]. In order to replace and restore the function of damaged tissues, wound healing is required[2]. Wound healing is a highly complex, dynamic, interactive and well regulated physiological process involving blood cells, extracellular matrix, parenchyma cells and soluble mediators. It starts with alteration in integrity of tissues and ends with the formation of scar. [3] The process of wound healing is distinguished into the following phases.



Haemostasis can be defined as spontaneous arrest of blood loss from damaged blood vessels. It is an essential and brief process of wound healing which not only prevents bleeding but also supplies active biomolecules that promotes healing[4]. It occurs within a few minutes of injury, trauma or other similar destruction to tissue results in the disruption of blood vessels. Following tissue injury, the blood vessels immediately constricts around the site of injury by releasing vasoactive amines to reduce bleeding[9]. It involves two processes, platelet aggregation and blood clotting[8]. The platelets adhere themselves to the exposed sub-endothelial of the injured vessels. This result in their activation and subsequent release of factors such as platelet derived growth factor (PDGF), transforming growth factor –β (TGF β), platelet derived epidermal growth factors (EGFs), fibroblasts growth factor- 2 (FGF -2), albumin, fibrinogen, fibronectin, anti-hemophilic factor, and pro accelerin, etc[4, 9].

Inflammation is the second phase which involves the formation of stable fibrin clot called Coagulation. This process involves both intrinsic and extrinsic cellular pathways. Activated platelets stimulate pro-coagulant enzymes to produce thrombin. Thrombin then acts on the

circulating fibrinogen to form a stable fibrin clot[4]. This fibrin clot mainly contains fibrin mesh aggregated platelets and certain RBCs. This clot prevents the loss of fluids and electrolytes from the site of injury and reduces contamination from external environment.[6] Aggregated platelets present within the fibrin clot releases various cytokines and growth factors which directs subsequent events of wound healing.[7] Inflammation phase is essential prerequisite for wound healing process. It can last up to 4 days after injury. It is characterized by redness, swelling, pain, warmth, and loss of function. After initial vasoconstriction, vasodilation occurs due to the release of endothelial products and mast cell derived factors such as histamine, prostaglandins and leucotrienes from damaged cells. Simultaneously the surrounding endothelial cells develop gaps in order to exudate plasma from intravascular space to extravascular compartment. This in turn leads to the generation of pain associated with edema. Inflammatory response enhances vascular permeability and this result in the migration of neutrophiles (PMN's), followed by monocytes, lymphocytes, basophils and esinophils. Migration of neutrophils peaks within 48 hours.

Europhiles engulfs debris and bacteria and thus provides first line of defense against infection when neutrophils begins to wane, monocytes migrates to the injured sites and matures into tissues macrophages. These are considered as the vital regulatory cells in the inflammatory process. They phagocytise bacteria by releasing biologically active oxygen intermediate and enzymatic proteins and thus provides second line defense. In addition, macrophages produce various chemotactic and growth factors (EGF's, TGF β, FGF and IL 1) that effectively promote cell migration, proliferation and production of tissue matrix[1]. Lymphocytes also play an important role in immune response with very similar functions[4]. Macrophages and lymphocytes exist nearly for seven days and then disappear[9].

The third phase is the proliferation phase which starts after 2- 3 days of injury and continues for about 21 days. The events involve in this phase includes re-epithelialization which includes migration and proliferation of epidermal keratinocytes and re-establishment of basement membrane zone. The re-epithelialization can be defined as replacement of dermal as well as sub-dermal tissues following an injury. It occurs within 24 hours of injury. Following events like migration and proliferation of epidermal keratinocytes are involved in re-epithelialization process. The initial event in re-epithelialization is migration of epidermal keratinocytes from skin appendages like hair follicles, sweat glands and wound edges into the wound. After 24 – 48

hours of injury, epidermal cells at the wound margins start proliferating so as to ensure supply of sufficient cells to the healing monolayer. Migration of epithelial cells into the wound requires formation of actin filaments at the edges of their cytoplasm, development of pseudopodia like projections, disappearance of desmosomes which links cells together and hemi desmosomes which links cells to the basement membrane with associated elongation of the keratinocytes. Integrin receptors present on the epidermal cells helps them to interact with the extracellular matrix proteins like vironectin and fibronectin. These proteins assist keratinocytes to migrate across the wound and separate desiccated eschar and debris from viable tissues[3]. In the re-establishment of basement membrane zone, the basement membrane zone which connects epidermis and underlying dermis layer is destroyed following an injury, then the epithelial cells regenerates a new basement membrane zone within 7 – 9 days of re epithelization so as to restore integrity and function of skin. It involves angiogenesis which is a complex process involving formation of new blood vessels from pre-existing vessels to ensure supply of oxygen and nutrients to the newly formed granulation tissues. Production of fibroblast growth factor (FGF) and endothelial cell derived growth factor by macrophages and endothelial cells is mainly responsible for induction of angiogenesis. The former directs angiogenesis during first 3 days of wound healing while the latter sets the stage for angiogenesis from day 4–7 (i.e during the formation of granulation tissue). This process stops after the wound is filled with granulation tissue[3, 8].

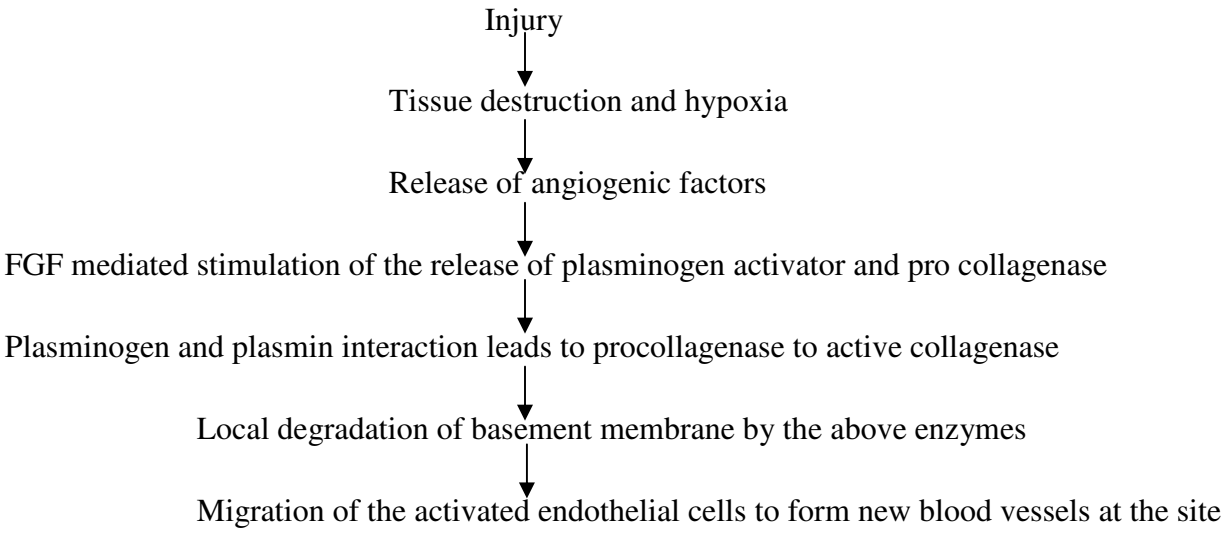


Figure 1: Schematic representation of the events involved in Angiogenesis.

Granulation tissue contains numerous capillaries and a support matrix enriched with collagen fibroblasts, inflammatory cells, endothelial cells, pericytes and myofibroblast. Granulation tissue formation commences 2- 4 days after injury. Chemical mediators released from the macrophages during the inflammatory phase causes fibroblast to activate. This process usually occurs from the 4th day of injury. Activated fibroblast releases proteolytic enzymes such as MMP-1, MMP-2, and MMP-3 to facilitate migration into the provisional matrix of the wound clot. Fibrin, fibrinonectin, and hyaluronic acid molecules present in the matrix contribute to the formation of granulation tissue by providing scaffold for migration of cells. After migrating into the wound, fibroblast replaces the provisional matrix with collagenous matrix releasing collagen, elastin and proteinoglycans. Once the large amount of collagenous matrix is formed, the production of collagen will come to a halt[6, 8, 9,11]. Wound contraction is another complex interactive process involving extracellular matrix and cytokines. This process commences after 4-5 days of injury and continues for about 2 weeks. After 4-6 days of injury, when the defect is covered with epithelial cells, fibroblast modifies into myofibroblasts which are characterized by the presence of actin rich filaments within their cytoplasm, desmsomes, hemidesmsomes, multilobular nucleus, and surplus rough endoplasmic reticulum. Since myofibroblasts are connected to each other as well as to margins of wound, they draw the edges f the wound together and thus contract the complete granulating bed. At the same time, collagen is released to form a rigid scaffold to hold wound in place.[1, 3, 8, 11.] Finally the last process is called Remodeling which involves transition of granulation tissue to a scar. A scar is a relatively acellular and avascular mass of collagen which functions to restore continuity, function and strength of tissue. During this phase, collagen fibres are recognized into a highly organized lattice structure with more tensile strength. Such remodeling gradually replaces type – I collagen with type –III until normal skin ratio of 4: 1 is reached. After 3 months, wound regains 80% of its original strength. The remodeling process continues upto 1 year, however there is no further increase in its tensile tissue strength.

Table: 1

Summary of all four wound healing phases. Major cells involved and days incurred in each phase are listed.

Wound healing stage	Major Cells involved	Days post injury
1. Haemostasis	Platelets	Immediate

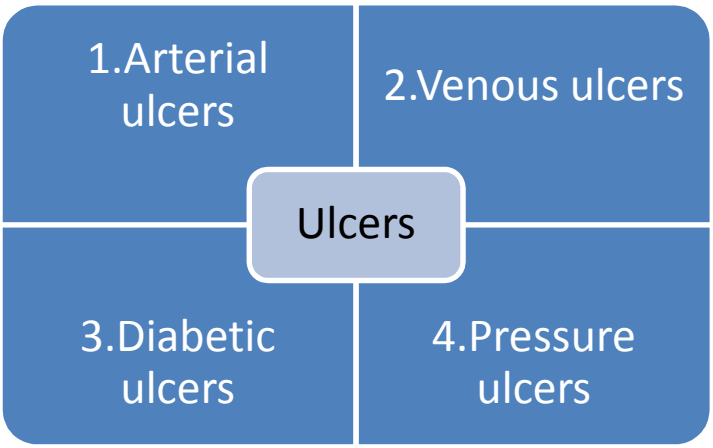
2. Inflammation	Neutrophils	1-4
3. Proliferation granulation contraction	Macrophages, lymphocytes, angiocytes, Fibroblasts, keratinocytes,	4-21
4. Remodeling	Fibroblasts	21 days – 2 years.

Prevention and treatment of chronic wounds

Chronic wound is a wound which fails to restore the anatomic and functional integrity of the injured site in an orderly and timely manner despite optimum and appropriate medical care [15,20,29]. They persist for weeks, months and even years[23]. The degree of wound ranges from superficial lesion to deep tissue destruction affecting muscles, tendons and bones[30]. Chronic wounds are common problem throughout the world and are usually seen in elderly and bed bound patients[27]. Around 3 - 6 million Americans are affected with chronic wounds with estimated treatment cost up to $25 billion annually[29].

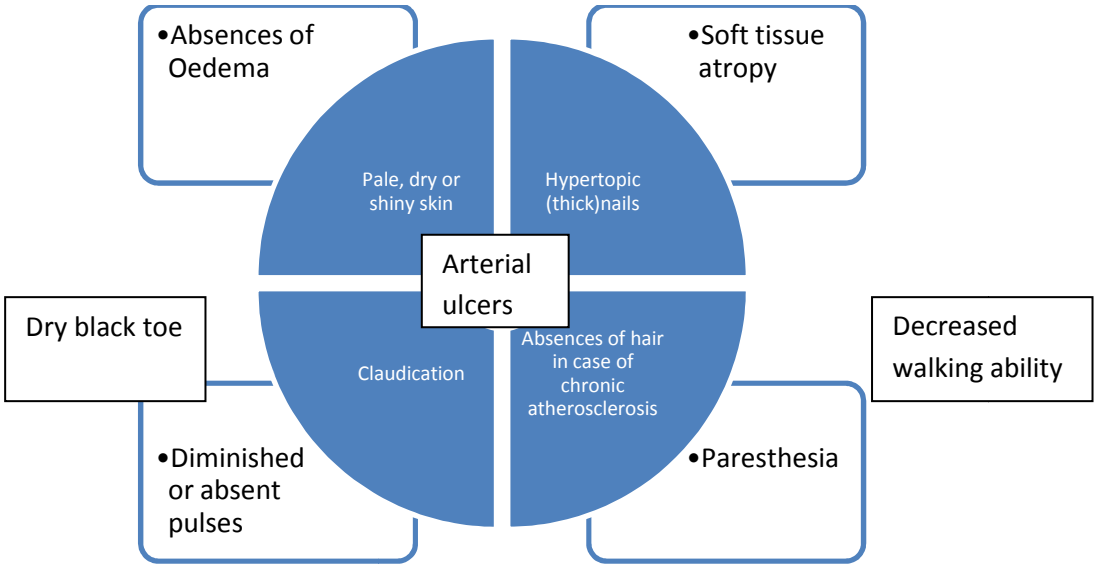
Chronic wounds are characterized by prolonged inflammatory phase, no initial bleeding to induce production of fibrin and secretion of growth factors, altered migration of epithelial keratinocytes, failure or delayed re-epithelialization due to absence of required extracellular matrix, no decrease in wound size with time, increased levels of matrix degrading proteases, increased levels of matrix metalloproteases (MMP-1(collagenase), MMP-2 (Gelatinase A) and MMP-3 (Gelatinase B). reduce levels of protease inhibitors, reduction in surface growth factors, increased degradation of fibronectin, vitronectin, decreased mitotic activity, reduced fibroblastic response to growth factors, enhanced proportion of senescent (unresponsive) cells restricting ECM production, presence of necrotic tissue in wound bed, excess exudates and slough (yellow fibrinous tissue containing fibrin, pus and proteinacious materials) on wound surface, poor blood supply, persistent pain. frequent wound breakdown, lack of healthy granulation tissue (ECM), Clinical /subclinical infection.[15, 17, 28]

Various factors do hamper wound healing and they can be categorized into local factors such as poor blood supply, enhanced skin tension, wound dehiscence, inadequate venous drainage, presence of foreign body in the depth of wound, infection, tissue maceration, ischemia etc, Systemic factors such as advanced age, poor nutrition, obesity, smoking, shock, diseases (diabetes, hepatic and renal disease, cancer), medications (Corticosteroids, immunosuppressant, anticoagulants), chemotherapy and radio therapy, malacoplakia (an inflammatory condition caused by bacteria or fungi) etc. Improper or delayed wound healing may result in several complications like anaemia, contractures and deformity in nearby joints, microbial colonization, fistula, heterotopic calcification, malignant changes in the ulcer bed, osteomyelitis, septicemia, sinus formation, systemic amyloidosis and unrecognized malignancy have been reported [13b, 14.] In the management of wounds it is better to understand types of chronic wounds / ulcers [16,26,27]

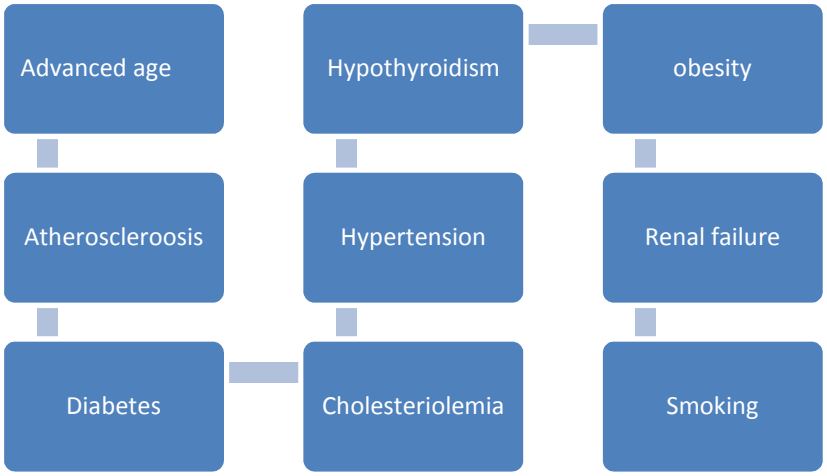


Arterial ulcer

It is a round shaped wound caused due to inadequate blood supply to the feet or legs[27]. They account for 10 – 20 % of all leg ulcers. Patients with arteriosclerosis are more likely to have arterial ulcers15. It is regarded as the first symptom of peripheral arterial occlusive disease (PAOD).



Risk factors



Diagnosis of arterial ulcers is based on the evaluation of the various aspects such as clinical history, where patients with PAOD will describe nocturnal claudication and decreased ability to walk distances. Skin color is indicative of arterial perfusion. A pale, dry, thin hairless skin indicates ischemia. Skin is also evaluated for temperature changes. Ischemic tissue is cooler than the surrounding skin. Capillary refilling where compression of great toe between forefinger and thumb gives capillary refill. Capillary refill > 3 seconds indicates decreased perfusion. Dependant rubor (reactive hyperthermia) in which the foot becomes ruddy (dark red) when the affected extremity is placed below the knee level. This is known as dependent rubor. The presence of palpable pulses at dorsalis pedis artery and posterior tibial artery is checked.

Diminished or absent pulse represents arterial insufficiency. Non-invasive as well as invasive arteriogram tests are performed to diagnose arterial ulcers. With the aid of droppler machine, arterial blood flow to the leg and arterial blood pressure of the leg is assessed. B.P of the foot is then compared with the B.P of the arm. This comparison gives the Ankle brachial Index (ABI), which indicates the extent of diminution of blood flow to the foot. An ABI < 0.4 indicates poor arterial blood flow to the foot. Arteriogram is employed to determine the exact location of arterial blockage of the leg.

Treatment: The general principle of treatment are wound bed preparation which is an essential paradigm in the management of chronic wounds. The goal is to eliminate all barriers to healing process so that wound repair can progress in an orderly and timely fashion. The principal components of wound healing forms a framework that offers practitioners a systematic approach to wound management. This framework has been named TIME.

T: Tissue management

I: Inflammation and infection control

M: Moisture balance

E: Epithelial management

In tissue management chronic wounds are contaminated and colonized by bacteria, fungi and other pathogens. These pathogens hinder the healing process by competing for nutrients and oxygen present within the wound and releasing toxic metabolites. Therefore wound debridement is carried out. Debridement removes devitalize tissue, reduces the number of pathogens, toxins and substances that impairs healing process and thereby promotes buildup of healthy tissue. In inflammation and infection control, the infected wound is characterized by erythema, swelling, warmth and pain. If the wound is infected, devitalized tissue and excess exudate should be cleaned off and an appropriate antimicrobial agent should be applied. Antimicrobials reduce the infection and the associated pain. They keep the wound moist. Examples include povidone iodine, cadexomer iodine and honey. Cadexomer iodine is being increasingly employed as it kills bacteria without developing resistance and toxicity. In mild-moderate infections, oral antibiotics like amoxicillin, cephalexin should be given. In limb threatening infections, patients should be

hospitalized and treated with I.V antibiotics. In Moisture Balance, wound cleansing is followed by application of dressings so as to remove the exudate, protect wound from contaminants and to keep wound moist. They are left on for prolonged periods to provide an environment conducive to natural healing process. Highly advanced wound dressings contain polymers that facilitate better absorption control and fluid management. In addition they also alleviate the risk of maceration at wound edges. Silver containing dressings are appropriate for heavily colonized wounds and for those at high risk of infection.

Table: Wound type and necessary dressing technique

Wound type	Dressing technique
Dirty /Infected wound	➢ Wet to dry technique should be done until wound is cleared.
Clean wound	➢ Wet to wet ointment based dressing

Dressing should be changed at least twice a day and with each dressing change, wound should be cleaned with a mild soap and saline or water. In Epithelial management, the aim is to restore the damaged skin and its function. Various adjuvant therapies are used such as Hyperbaric oxygen therapy (HOT), which is applied to accelerate the healing of non-healing wounds. It enhances oxygen concentration in all tissues including those with diminished blood flow. It stimulates angiogenesis in areas with poor blood supply and thereby improves blood circulation to blocked arteries and compromised organs. It increases SOD levels. It assists in treating infection by restoring leucocytes action and enhancing their phagocytic effect. It also exhibits anti-inflammatory and bactericidal potential. The Growth factors application factors plays a pivotal role in controlling all phases of wound repair. They promote growth and migration of endothelial cells, fibroblasts and keratinocytes into the wound bed. Chronic wounds are deficient in growth

factors. Therefore to compensate for this deficiency and allow normal healing to progress, topical application of these factors is recommended. Eg: Bacaplermin. Gene therapy is being developed to deliver growth factors more effectively. The Stem cell therapy have proliferating capacity and the ability to differentiate into different cell types and form various cytokines and growth factors. This therapy is applied to introduce new cells into damaged tissue . Eg: Endothelial progenitor cells.

The Vacuum assisted wound closure/Negative pressure wound therapy (NPWT) involves application of negative pressure (suction) under a bandaged, sealed tightly over the wound bed. The suction effect withdraws bacteria and exudates away from the wound bed, pulls the edges together and fills the wound with granulation tissue. Skin substitutes involve a human tissue or a bioengineered tissue is used to support wound closure. They are used to treat partial and full thickness venous ulcers of more than one month and full thickness diabetic ulcers. The Electrotherapy causes muscles to contract, augments muscles strength and promotes blood circulation to the affected area. The Ultrasound therapy degrades mast cells and causes the release of histamine and other mediators and thereby prolongs acute inflammatory phase. It also stimulates fibroblasts to secrete collagen leading to increased wound contraction and tensile strength. Malnutrition is directly correlated with impaired wound healing. It results in reduced fibroblast proliferation, cellular and humoral immunity and impairs neovascularisation.

Prevention: Management of the underlying diseases not only helps to prevent future occurrence but also reduce overall mortality. Smoking cessation, management of hypertension, diabetes and hypercholesterolemia decreases long term ischemia and ulcer development. External pneumatic compression and passive warming of the extremity with a boot improves arterial flow in ischemic limbs and thus helps to avoid arterial ulcers. Arterial insufficiency also arises as a result of trauma to the legs and feet of the patients. Hence, leg and foot protection with soft, comforting and properly fitting shoes is the key to ulcer prevention. Foot protection is particularly important in patients with coexisting neuropathy. In patients with PAOD, neuropathy, toe nail trimming should be performed by a podiatrist.

Venous ulcers

Venous ulcers are irregular sores that develop when the valves of the leg veins (that prevents the back flow of blood) becomes damaged. As a consequence, pressure in the venous circulation increases and this causes the fluid to ooze out of the veins below the skin resulting in swelling, thickening and damaged to the skin. Damaged skin breaks open to form ulcers. They account for 7 – 9% of the chronic wounds[30].

Signs, Symptoms and Risk factors

S.No	Signs and symptoms	Risks factors
1	Edema	Deep venous thrombosis
2	Pain	Previous vein surgery
3	Skin discoloration	Lipodermatosclerosis
4	Leg tiredness	Lower extremities trauma
5	Venous dermatitis	Cellulitis
6	Itching	Venous hypertension
7	Wet and weeping wounds	Immobility
8	Burning	Obesity
9	Palpable pulse	Calf muscle pump dysfunction

Diagnosis of venous ulcers is based on clinical histology, physical examination and lab tests. Clinical history involves the duration of wound, location prior treatment, history of phlebitis, deep venous thrombosis (DVT), Venous hypertension, factors that exacerbates wound, its reoccurrence pattern etc are thoroughly reviewed. Reoccurrence of ulcers in the same area is indicative of venous ulcers. Physical examination involves ulcer distribution which is typically found over malleoli, appearance of shallow irregular margin, swelling, lipodermatosclerosis, along with fibrosis of normal adipose tissue. The capillary refill is normally < 3 seconds and the surrounding skin is hyper-pigmented and oedematic. The diagnostic tests performed are Doppler ultrasonography, phlebography, duplex ultrasonography and venography aids to diagnose venous ulcers. Measurement of ABI using Doppler study rules out presence of arterial disease.

Ultrasound based duplex is standard for the assessment of venous system. It allows determination of location and the extent of disease and also assists to differentiate between reflux and obstruction.

Treatment includes compression/support therapy which is regarded as standard of care. Methods include inelastic compression, elastic compression and intermittent pneumatic compression. It improves venous reflex, ameliorates pain associated edema and accelerated healing process. Another treatment involves Leg elevation which is the elevation of the leg above the heart level reduces edema, improves microcirculation and increases oxygen delivery to ischemic tissues by pulling fluid and blood in right direction towards heart. The third treatment is the dressings which promote healing process and prevents adherence of bandages to wounds when used as an adjuvant to compression therapy. Another treatment is the negative pressure wound therapy which decreases the depth and volume of wound. Treatment with medications such as pentoxyfilline and oral zinc are given along with compression therapy to accelerate healing. Antimicrobials are given to control bioburden, povidone iodine, ethacridine, mupirocin etc are applied. In case of suspected cellulitis, antibiotics are administered orally. The hyperbaric oxygen therapy is used as an adjuvant therapy due to its antibacterial and anti-inflammatory potential. Also Debridement is done of the Necrotic or devitalized tissues by removing them by sharp mechanical or autolytic means. Skin grafting is done in the case of refractory venous ulcers

Prevention of venous ulcers should focus on the management of its risk factors. Continual use of compression stockings by individuals with venous hypertension and or phlebitis helps prevents formation of venous ulcers. Calf muscles exercised have been shown to improve calf function and this can help avoid ulcer occurrence. Prophylactic therapy in patients with deep vein thrombosis prevents reoccurrences of disease. SEPS (sub facial endoscopic perforator surgery) is the method of choice for decreasing ambulatory venous pressure in the leg. Treatment with suitable systemic antibiotics in case of cellulitis helps prevent infection.Venous surgery, if performed with compression therapy helps to prevent occurrence of venous ulcers.

Diabetic Ulcers

It is a wound or sore commonly found on the foot, heals or toes of diabetics. Diabetics with ulcers are at a greater risk (15 %) for amputation. Diabetes causes neuropathy which in turn causes loss of protective sensation and coordination of muscles in legs and feet. It is the leading cause for most foot and leg amputations. The signs and symptoms are high blood glucose levels, infections, swelling, erythema, pus generation, fever etc. The risk factors involves diabetic neuropathy, structural foot deformity, ulceration, infection, callus thick toe nail, obesity, smoking, retinopathy, peripheral arterial occlusive disease, poor glycaemic control, advanced age, alcohol. The clinical history of previous trauma, neuropathy, revascularization and ulcers are vital informative pieces in developing wound care plan. The inspection involves meticulous examination of patient's skin covering top, sole and sides of both feet, areas between toes and back of the heels for the signs of edema and cellulitis. Hard, callused and ruddy skin is indicative of high pressure and worsening infection. The vascular assessment involves palpitation of both the dorsalis pedis and posterior tibialis artery. Diminished pulses illustrate ischemia. If pulses are non-palpable, ABI is performed to quantify the information. Transcutaneous oxygen measurement serves as an alternative to ABI. Hematological assessment is done in diabetic patients by complete blood cell count, serum creatinine and plasma hemoglobin should be monitored. The latter parameter is standard for long term monitoring of blood glucose level. Increased HbA1C is suggestive of coronary heart diseases, neuropathy, nephropathy and retinopathy. In neurological assessment, Semmes – Weinstein monofilaments (log) have been used to assess protective sensation. Inability to sense the presence of monofilament on wound signifies neuropathy which may render a patient susceptible to foot ulceration.

The principal components of diabetic ulcer treatment includes Off loading which is the primary component in treatment of diabetic ulcers. Crutches, walkers, wheel chairs are acceptable methods to halt weight bearing on affected foot , total contact protective caste, custom shoes standard shoes are also used. Another therapy called Wound therapy involves adjuvant therapy like NPWT (Negative pressure wound therapy) and HOT (Hyperbaric oxygen therapy) which are opted when wound fails to heal with above measures.

Prevention includes meticulous attention to foot care and appropriate management of minor injuries to foot is the key to ulcer prevention and subsequent amputation. Foot care involves regular washing of the feet with mild soap and luke warm water followed by topical application of moisturizer helps maintain healthy skin. Regular trimming of toe nails and use of protective and properly fitting shoes reduces the incidence of ulcers. Proper foot care is the mainstay of prevention and is recommended in patients with history of amputation, ulcer neuropathy and ulcerative callus. Also 60 % reoccurrence risk in patients with healed ulcers has been reported. Therefore it is highly recommended to educate patients at risk of ulceration on daily foot care and signs of infection. Diabetic patients should have annual foot inspection for sores, cuts, bruises, arterial insufficiency, and loss of pain sensation, fungal toe nails and callus formation. Increased pressure on the foot of diabetics leads to callus formation which eventually results in foot ulcers. Removal of callus reduces the incidence of ulceration. Ceasing smoking and decreasing alcohol consumption also reduces incidence and reoccurrence of ulcers.

Pressure ulcers / Bed sores

Pressure ulcers are caused due to ischemia which occurs when pressure on the tissue is more than pressure in capillaries, thereby restricting blood supply to an area of skin and eventually resulting in bed sores. Ankles, heels, knees, elbow, shoulder blades and sacral regions are highly susceptible to pressure ulcers. They usually occur in unconscious, fatigue, cadaverous, paralyzed or bed ridden patients. Approximately 3 million Americans are affected with pressure ulcers. European pressure ulcer advisory panel (EPUAP) has defined pressure ulcer in 4 stages which are outlined below. Stage 1 involves intact skin with erythema over a bony prominence. The affected area is firm, soft and painful to touch. Stage 2 involves open wound in which epidermis and a portion of underlying dermis is lost. Appears as a shallow abrasion or blister, surrounding area is pinkish to red. Slough is absent. The Stage 3 is deep wound where Epidermis and dermis are fully lost. Subcutaneous fat is exposed. It appears as a crater and slough is present. In Stage 4 extensive damage occurs. Muscles, bones and tendons are visible. Slough is present.

Treatment involves pressure removal from the site of ulceration which accelerates the healing process. Basically there are two types of pressure relieving devices, static and dynamic devices.

Static devices include foam, gel, and air mattresses. Dynamic devices are beneficial in patients who cannot reposition independently and in slowly healing wounds. Example includes low air loss beds and air fluidized beds. Other aids include chair cushions, wedge pillows etc. Friction and shear reduction is done through repeated turning and repositioning helps to reduce friction and shear. Elevation and maintenance of the head of bed at 30° reduces shear and prevents complications like CHF. The wound therapy is done through surgical care. It is required only when wounds fail to heal despite optimum and appropriate medical care. Wound closure by surgical approach includes direct closure, skin grafts and musculo- cutaneous flaps.

Prevention of pressure ulcers involves Pressure ulcer risk screening. The risk factors are best identified immediately after a patient is admitted to the health care setting by health care professionals or trained nurses. Braden scale is most widely used tool to predict development of pressure ulcers. It consists of six parameters namely sensory perception, activity, skin moisture, mobility, nutrition and friction and shear. Each parameter is assigned a score ranging from 1-4. Summing up of all risks gives overall risk ranging from 6 – 23. Lower the score greater is the risk potential.

a. Table : Braden scale

S.No	Score	Risk
1	15 – 18	Mild
2	13 – 14	Moderate
3	10 – 12	High
4	≤ 9	Very high

The Norton scale is a tool that assesses five risk areas for development of pressure ulcers. Areas include physical state, mental state, activity, incontinence and mobility. Each risk area is assigned a score varying from 1 – 4. Sum of all scores yields total score ranging from 5 - 20.

i. Table : Norton scale

S. No	Score	Risk
1	14	Mild
2	13	Moderate
3	12	High

Pressure Ulcer risk assessment involves assessment of physical appearance, history of previous ulcers, onset and duration of ulcers, factors that accelerates / impede healing, behavioral and cognitive status, nutritional status, use of tobacco and alcohol immobility and incontinence, location, size and stage of each ulcer, presence of exudates, odor, eschar, infection and wound margin, nutritional evaluation (Nutritional evaluation is an integral part of general screening of patients with pressure ulcers. In malnourished patients, adequate intake of nutrients is recommended), preventive interventions, pressure relief to preserve microcirculation is the key to ulcer prevention., to minimize shear, head of the bed is elevated and maintained upto 30°. This intervention also reduces complications like aspiration and CHF.

Biofilms

A Biofilm can be defined as a structured community of microbial cells, adherent to living or inert surfaces by a self produced complex matrix of extracellular polymeric substances[1]. It is characterized by complex community interactions, extracellular matrix of polymeric substances, genetic diversity and structural heterogeneity. The thickness of a biofilm ranges from a single cell layer to 6-8cm. On an average, each biofilm is about 100µm in thickness. Slippery layer inside the sewage pipes, slimy green stuff surrounding a pebble in a pound dental plaque, outer layer of chronic wounds are a few examples of biofilms. Biofilms are the common mode of microbial growth that occurs in various forms in natural, industrial and hospital settings. They are commonly seen in hoses, plants, ponds, rocks in water, soil particles, plastic and metal items, kitchen counters, walls of hot tub, swimming pools, medical implants like ocular lenses, intrauterine devices, I.V catheters, heart valves, vascular grafts, reverse osmosis membrane filters and medical apparatuses like dialysis units, water filter systems, storage vessels, gas tubes,

ventilator pipes etc. A biofilm consists of a sessile mushroom shaped microbial colonies of which each colony consists of small groups of different species of bacteria and the other pathogens. Extracellular polymeric matrix which protects the microbes from external environment and strengthens attachments of microbes to a hospitable surface. Fluid (water) channels helps to supply nutrients and water to each of the bacterial and living cell. They also facilitate the disposal of bacterial metabolites. Communication system (quorum sensing system) which helps bacterial cell to communicate with other cells usually through biochemical signals in the community.

A biofilm is formed when free floating and individual cell bacteria adhere to wet or moist surfaces such as foam filters, medical implants, human skin or any other hospitable surface and become sessile by producing a protective, slimy glue like substance. It can be formed from a single bacterial species but more commonly, it is formed by multiple species of bacteria as well as algae, fungi, protozoa and yeasts. Formation of a biofilm involves three stages, attachment or adhesion, growth and development and dispersal or detachment. A biofilm commences to form when freely moving mobile bacterial cells identifies and adheres to a hospitable surface.[3] This process is known as Twitching. Attraction towards such a surface is brought about by various modes like Brownian motion, chemo attraction, gravity and surfaces charge[2]. Attachment of bacterial cells to the surface depends upon the amount and type of cells present at a specific time, amount of nutrients available at the surface, temperature and pH, flow rate of the fluid or water. Initially, they adhere to the surface through weak vanderwaals forces and once they stick, they start producing slimy glue like extracellular polymeric substances (EPS) also called as Glycocalyx. EPS is made up of complex combination of DNA, glycoproteins, polysaccharides, lipids and proteins.

During growth and development stage, a biofilm grows through the cell division of attached bacteria and changes its size and shape. As the bacterial cells within the microcolony divide, a critical population density known as quorum is reached[32,33,34]. The bacteria in the quorum produces signalling molecules which diffuse across the cell membranes and interact with DNA receptors, thus altering the phenotype of bacteria[35]. This process of transmittance of signals from cell to cell is known as quorum sensing. Consequently the microcolony further grows and develops into a mature polymicrobial biofilm . A mature biofilm comprises of three regions, the

lower region mainly comprises of metabolically dormant inactive cells which are capable of persisting even in the most hostile environment. Removal of these cells is necessary to prevent their regeneration. The mid region consists of cells that regulate DNA systems, which facilitate horizontal gene transfer and genetic diversity within the biofilm and the top most region contains metabolically active cells which disperse to new locations to exert their toxicity[32]. Once the biofilm is established, fragments are programmatically released from the biofilm into the environment to spread and colonize new surfaces. Enzymes such as deoxyribonucleases and dispersin B were found to play a role in dispersal of biofilms.

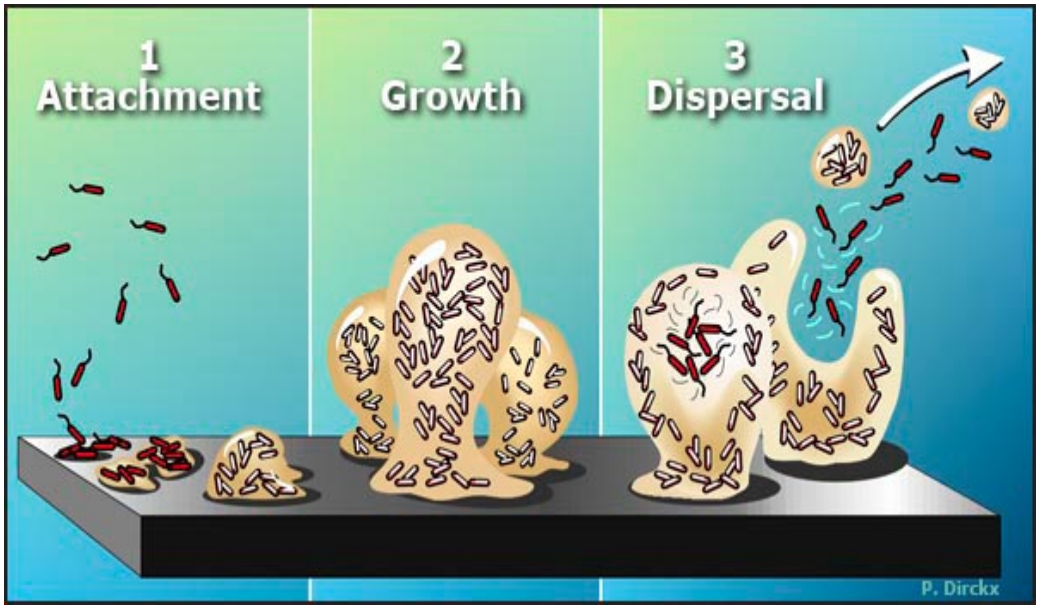


Figure 1 – BIOFILM FORMATION

Biofilms increases the resistances of pathogens against antimicrobial agents, high temperature and host immune responses. They provide easy access to water and food. They facilitate exchange of metabolite and nutrient and even genetic material among organism of same and different species. They are useful in sewage treatment wherein they breakdown harmful compounds and thereby purifies water. They are used in treating petroleum contaminated water of marine systems (pseudomonas putida) and nitrification. They protect the soil and water by forming the barriers.

Biofilms damage the surfaces on which they grow. This type of damage caused by them is called Bio-fouling. Biofilms growing on the surfaces of teeth can cause tooth decay and gum disease. They cause clogging and corrosion of sewage pipes. They decrease the productivity of equipments and they corrode the metal surfaces. They decrease the heat transfer in cooling and heating water systems. They also contaminate the products and make sanitation difficult in manufacturing areas. They cause biofouling of computer chips and marine vessels. They contribute to a number of health problems like atherosclerosis, chronic sinusitis, gingivitis, chronic wounds, cystic fibrosis, leptospirosis, osteomyelitis, otitis media, tooth decay, urolithiasis, urinary tract infections, and heart valve infections.

A wounded tissue serves as a unique microbial niche. A wide variety of microorganisms are known to infiltrate a wounded tissue and interfere with the healing process[9]. Anaerobes, yeasts and filamentous fungi are recognized as potential wound pathogens. Biofilms have been implicated in less than 10% of acute wounds and in more than 70% of chronic wounds. They have been reported to be associated with a number of chronic infections ranging from dental plaque to prostatitis[36].

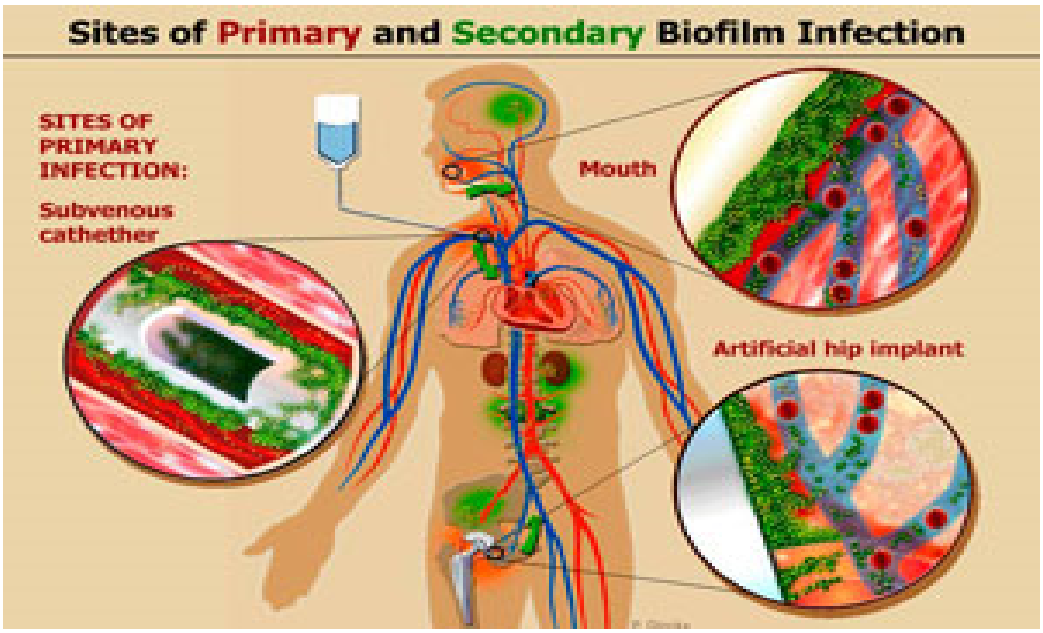


Figure 2 – COMMON SITES OF BIOFILM INFECTION

In acute wounds, bacteria are easily identified and destroyed by the host defense systems[11]. Chronic wounds are associated with compromised hosts that demonstrate an impaired inflammatory phase. Inability of the immune system to destroy bacteria effectively results in the establishment of a biofilm which helps to overcome host's natural defenses and other environmental stresses. This in turn results in prolonged inflammatory phase and subsequent release of free oxygen radicals and various proteases in large amounts. In the normal wound healing process, proteases break down the damaged extracellular matrix proteins and thus facilitate their removal so that a new tissue is formed and the wound heals in an orderly manner. But due to the excessive release of proteases, the newly formed ECM and other essential proteins and immune cells also get degraded. As a result, healing is impaired due to the unusual prolongation of the inflammatory phase that prevents wound from entering into proliferative phase of healing.

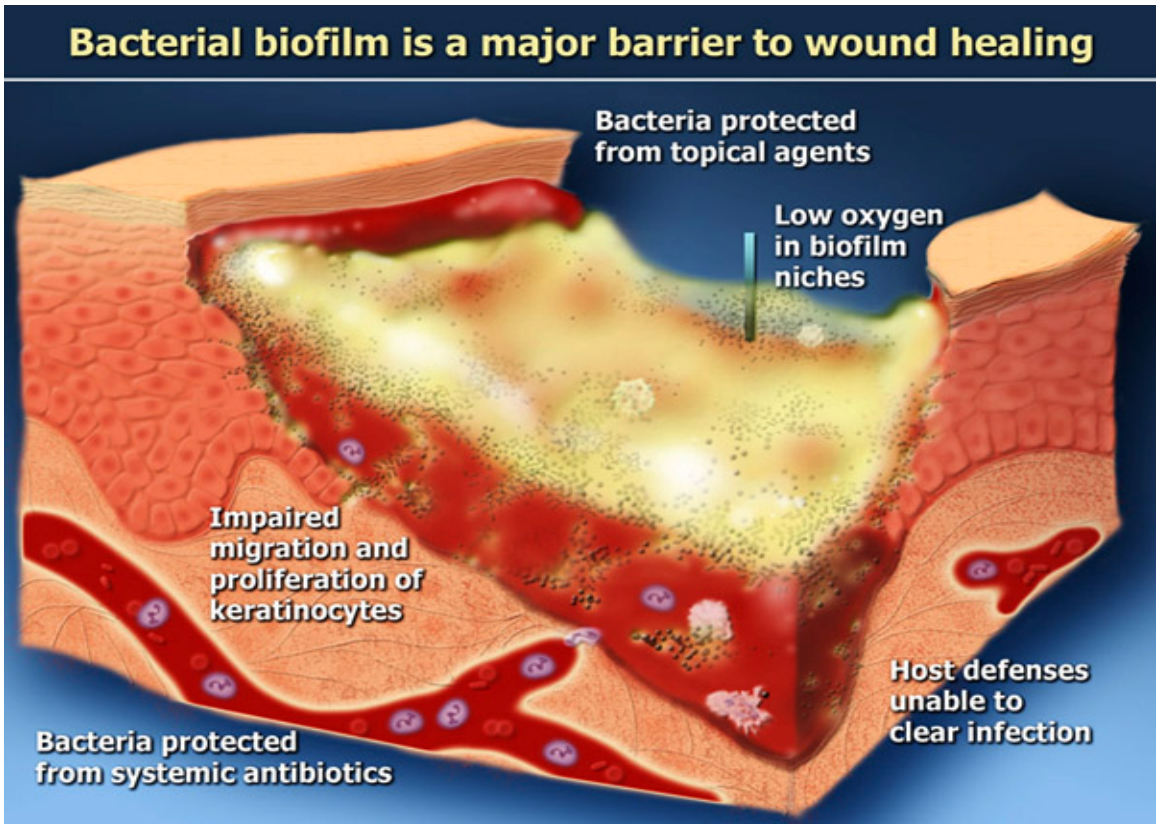


Figure 3 – BIOFILM PREVENTING HEALING PROCESS

The microbial community living within a biofilm is known to possess properties greater than sum of its constituent members[31]. Bacteria present in biofilms are 1000 times more resistant to the effect of anti-microbial agents. EPS plays a pivotal role in making the biofilms resistant against antimicrobial therapies, host immune responses, drying, over-hydration and other environmental stresses. It prevents phagocytosis of the resident microbiota and allows them to persists for a longer period of time by one of the following mechanisms such as reduced permeability or uptake of antibiotic into the biofilm, diminished metabolic activity of resident bacteria, altered gene expression, altered microenvironment within the biofilm and increased production of protective enzymes via quorum sensing[37]. As conventional antimicrobial killing methods are ineffective in case of biofilm microbes, which causes an immense damage to equipment, drugs, man power and to life there is a need of development of strategies and devices to interrupt with the biofilm formation and control their growth.

Biomarkers

In the year 2001, National institute of health (NIH) appointed biomarkers definitions working group (BDWG) to define biomarkers and related terms. BDWG has defined biomarker as " characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, pharmacologic responses to a therapeutic intervention"[39,40]. The term biomarker has been used in a broad sense to measure biochemical components in tissue and fluids and as well as imaging, chemical and physiological measures. It includes tools and technologies which assist in comprehending cause, diagnosis, progression, regression or outcome of treatment[39]. It can also be described as anything which can be employed as an indicator of particular disease. It can be enzymes, genes, gene products, hormones, specific cell or molecules**.** Specific changes in biological structure also serve as biomarkers. Example blood pressure is a biomarker of stroke, C-reactive protein(CRP) is a biomarker of inflammation and the body temperature is a biomarker of hyperthermia. In molecular terms, biomarker is a subset of markers found using imaging, genomics or proteomic technologies[3]. They are produced by the diseased organ or by the body in response to disease[42].

An ideal biomarker should be safe and easy to assess. It should provide rapid, reliable and robust measure. It should be specific for the biological process/organ. It should be consistent across ethnic groups and genders. It should be analytically stable. It should be quantifiable in a readily obtainable sample. Cost of follow up test should be low. There should be an established treatment to alter the biomarker. There should be little or no overlap in biomarker levels in treated and untreated patients. Their levels should not vary widely in general population (i. e between patients). Levels should correlate with the total disease burden but they should be influenced by unrelated conditions and associated co-morbidities. Levels should alter quickly in response to specific treatments. Levels should correlate closely with all effects of therapy [43].

Biomarkers are classified into Type-0/Natural history markers which measure the natural history of a disease and correlates longitudinally with known clinical indicators. The Type-1/drug activity markers indicate intervention and downstream effects of a drug. Type-2/surrogate markers are regarded as surrogate end point because a change in these markers indicates

therapeutic effect. According to USFDA, a surrogate marker is "a laboratory measurement or physical sign that is used in therapeutic trials as a substitute for a clinically meaningful end point that is a direct measure of how a patient feels, functions, or survives and is expected to predict the effect of the therapy[44.] According to BDWG, a clinical end point is an "a characteristic or variable that reflects how a patient feels or functions or how long a patient survives". A surrogate end point is "a biomarker intended to substitute for a clinical end point".

The characteristics related biomarkers are Imaging biomarkers and Molecular biomarkers. The Imaging biomarkers are quantifiable characteristics which make use of various technologies to visualize anatomical and physiological changes occurring in the body. X-ray, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) is widely used imaging techniques. In clinical settings, MRI is most commonly use. It is a promising biomarker in rheumatoid arthritis. It detects cartilage erosion and soft tissue inflammation. PET plays a pivotal role in determining concentration of drug in target organs and tracking how and where drugs bind to target which might act as a biomarker. It is also useful in visualizing targets like amyloid plaque which are believed to trigger Alzheimer disease. CT is currently employed in all stages of cancer management including early detection, diagnosis of suspected lesions and determination of clinical benefit[3]. They have many advantages like they are non-invasive, yields quantitative as well as qualitative information. They are highly sensitive, accelerates drug development process, reduces time and cost involved in drug development process. They assist in selecting proper candidates for treatment, allows serial data collection, reduces inter patient variability and provides complete drug profile (pharmacologic effects, side effects, drug interactions)

Molecular biomarkers are characteristic biomolecules which can be detected and quantified in body parts, fluids like tissue, blood. They can be genes mutations associated with acute myeloid leukemia, special type of cells like CD4 cells in HIV or molecules. They possess biophysical properties which facilitate their measurements in clinical samples, they help identify individuals at disease risk, aids in monitoring disease progression and predicts disease reoccurrence and possible therapeutic benefit. Eg: p16 is a putative molecular biomarker of cervical adenocarcinoma.

The second category of biomarkers is the disease related biomarkers which includes diagnostic/screening biomarkers, prognostic biomarker and the predictive/stratification biomarkers. The diagnostic biomarkers are promising tools for early detection of diseases. These biomarkers are used to confirm diagnosis of a disease. They furnish the means to define a population with specific disease. Certain diseases are free of initial symptoms (Alzheimers disease and Rheumatoid arthritis). In such cases diagnostic biomarkers are helpful in predicting the presence of a disease at an early phase.

Biomarker	**Disease**
TNF-α and IL-6	Rheumatoid
BRCA 1 and 2	Breast and ovarian cancer
Saliva and gingival crevicular fluid (GCF)	Periodontal disease
α- fetoprotein	Neural tube defects(downs syndrome)

The prognostic biomarker predict possible course of the disease and responses to specific therapy in patients receiving standard therapy or no treatment. They also envisage disease reoccurrence and associate this risk with duration of progression-free survival. They form the basis for defining patient population for clinical trials. They identify patients for clinical trials. They identify patients who have poor prognosis on standard chemotherapy and who have good prognosis on standard treatment and who do not require intensive regimen.

Biomarker	**Disease**
C-erbB-2	Breast cancer
HbA1c	Diabetes
Lactate dehydrogenase	Advanced metastatic melanoma
Procalcitonin	Sepsis

TIMP-4 and CD63	Astocytoma

The predictive/ stratification biomarkers define subpopulation of patients who are likely to respond better to a particular treatment or most at risk of adverse effects. In brief they are predictors of efficacy/ toxicity of a drug .They save patient from unnecessary toxicity and increase their chance of receiving most suitable treatment. They improve success rate of drug development. They reduce medical cost. They help choose a therapy with the highest probability of being effective in a particular patient.

Biomarker	**Disease**
HER-2	Predicts response to herceptin treatment against breast cancer
KRAS	Predicts resistance to EGFr antibody therapy against metastatic colorectal cancer
C-KIT	Indicator of response to gastrointestinal stromal tumor(GIST)

The third category of biomarkers are the Exposure assessment related biomarkers[45,46.] They include biomarkers of susceptibility, biomarkers of exposure and biomarkers of effect. The biomarkers of susceptibility are indicators of natural or acquired ability of an individual to respond to the effects of exposure to a particular xenobiotic. In other words, they help to understand degree of response to exposure shown in individuals. They help identify susceptible individuals and critical times when exposure becomes most harmful. The biomarkers of exposure are unaltered parent chemicals or their metabolites or a product of their interaction with the target which can be measured within the body. They are employed to support and determine the exposure of individual to a specific substance providing a correlation between external exposure and internal dosimetry. They enable timely prevention of exposure associated damage. The biomarkers of effect are quantifiable biochemical, behavioral, physiological or other changes

within an individual indicating exposure to a compound and possible health impairment .They are used to document adverse effects elicited by external exposure and absorbance of a chemical. Thus the correlation of biomarker between exposure and effect contributes to dose-response relationship.

At present, there are no effective diagnostic tools to determine the biological activities occurring within the wound [40]. Three types of tests are commonly employed in the management of wound. Nonspecific tests are employed to provide data specific to the patient and clinical context. Example: ESR values[47]. Diagnostic tests are employed in predicting and managing wound outcomes, assessing the cause of a wound and its healing status, identifying complications which delay wound healing and to develop effective wound management plans using established wound protocols[2]. Example: wound tissue culture and sensitivity values. Theranostic tests are employed to indicate the necessity for a particular therapy and the form of treatment that must be chosen for a specific condition. Example: selection of monoclonal antibody trastuzumab to treat breast cancer [47].

Biomarkers are currently being investigated for use as either diagnostic tool or therapy for treating chronic wounds[45]. Nitric oxide and proteases are promising biomarkers that play a critical role in wound repair [40,47,48]. L-arginine in wounds get metabolized by three isoforms of nitric oxide synthase (NOS) to citrulline and nitric oxide. All these NOS isoforms are present in the skin and are produce during wound healing process by inflammatory cells[47,49] .

Environmental as well as biological factors influence wound healing process and NO is one such factor which regulates wound inflammation, angiogenesis, collagen deposition, epidermal cell migration, and wound tensile strength[40]. It serves as an antimicrobial, vasodilator, antiplatelet aggregator and promoter of vascular permeability and thereby plays a pivotal role in inflammatory phase. It hastens angiogenesis by stimulating factors which in turn induces endothelial cell migration, adhesion and eNOS expression. It indirectly promotes re-epitheliazation by acting as a chemo-attractant of IL-1 which is an efficient modulator of keratinocyte proliferation, recruitment and differentiation. It enhances production of collagen in wound derived as well as skin derived fibroblasts and also assists in fibroblasts activation by transferring an inactive TGF-β1 to an active form[49]. Generation of nitric oxide is known to increase in wounded tissue due to activation of cNOS and increased production of iNOS[50].

Consequently, the bioactivity of NO also increases. NO gets metabolized to nitrate (NO_3^-) and nitrite (NO_2^-) which are together termed as NOx. These metabolites are present within the plasma, urine and tissue . Wound fluid NOx is immensely employed as a surrogate marker for wound NO bioactivity[48]. Determination of NO bioactivity of wound aids to predict wound outcomes for chronic wounds, regulate the effectiveness of chosen treatments, and generate unconventional dressings or treatment on the basis of their ability to improve NO bioactivity of wound [2]. Matrix metalloproteinases (MMPs) and human neutrophil elastase (HNE) are one of the major proteases involved in the wound healing process[48]. They are essential factors for all phases of wound healing. They annihilate degraded protein, assists migration to center of the wound, damage the ECM, remodel the granulation tissue , regulate tissue angiogenesis and activity of growth factors (GFs). During normal wound repair , protease levels initially increases and then falls to very low levels by one week whereas in case of chronic wounds, protease activity becomes too high as a result of which ECM and newly formed tissue gets damaged and the healing process gets impaired. Thus high protease activity is a promising biomarker to predict impaired healing of both acute and chronic wounds[48]. Novel thera[pies are needed to initiate effective wound repair process, one such therapy is 3M ™ Tegaderm ™ matrix which helps to diminish total MMP production and thereby helps hastens wound healing[49]. Reduction in wound area by 2-4 weeks is another indicator of the ability of wound to heal by 3 months. For diabetic foot ulcers, 50% reduction in wound area by week 4 is predictive of good wound healing whereas for venous leg ulcers, a reduction of 20- 40 % within 2-4 weeks is predictive of healing. Wounds that fail to show these levels of healing within these times need to be re-assess and re-evaluated for the care regimen[48].

Bibliography:

1. Wayne K. S, Alexander G.D, Gordon R.T, Physiology and healing dynamics of chronic cutaneous wounds, The American journal of surgery, vol 179 (suppl 2A) August 24, 1998 26 – 3

2. Schreml S, Szeimies R.M, Prantl L, Karrer S, Landthaler and Babilas P, Oxygen in acute and chronic wound healing, British journal of Dermatology, 2010 1- 12.

3. Adam J.S, Richard A.F, Clark, Cutaneous wound healing, The new England journal of medicine, vol 341 Sept 02, 1999 738-746.

4. Maureane H, Anna H, Angela L, Ulla H, Harold R, R and Dugald M.M, Cutaneous wound healing is impaired in hemophilia B, Blood vol 108 november 2006 3053- 3060.

5. Pascal M, Anthony C. D, Mechanism involved in wound healing, The biomedical scientist, July 2008.

6. Wound healing , wound healing in midwifery

7. Douglas M, Alan L. M, nutritional support for wound healing, Alternative Medicine Review, Vol 8, 2003, 359-377.

8. Jie. L, Juan, C, Robert. K, Pathophysiology of acute wound healing, Clinics in Dermatology, vol 25 2007, 9-18.

9. Joan. L. M, Thomas W. L, Acute wound healing an overview, Clinics in Plastic surgery, vol 3 2003 1-12.

10. Mohit K, Ian A, Wound healing: Abnormalities and future therapeutic targets, Current Anesthesia and critical care, vol 16 2005, 88-93.

11. Watson T, Soft Tissue wound healing review, Sportex-medicine 2006 1-7.

12. David K, The basic principles of wound healing .

13. Daniela. N, Valentina M, Georgeta C, Raluca C, Cristina I, Cristina S, Anisara C, Dana I, Skin wound healing in a free floating fibroblast populated collagen lattice model, Romanian Journal of Biophys, vol 16(3), 2006 157-168.

14.Shinsuke K, Satoshi Y, Hiroto T, Masumi H, Takahide K, Epithelization in Oral Mucous wound healing in terms of energy metabolism, Kobe jurnal of medical sciences, vol 55(2), 2009 E5 - E15.

15. Templeton S, Management of chronic wounds; the role of silver containing dressings, Primary intension, 2005; 13(4); 170 – 179

16. Nwomeh B.C, Yager D.R, Cohen I.K, Physiology of chronic wounds, Clinical journal of plastic surgery 1998; 25; 407- 414

17. Stadelmann W.K, digenis A.G, Tobin G.R, Impediments to wound healing, American journal of surgery, 1998;176(2A); 39s – 47s

18. Enoch S, Harding K, wound bed preparation, the science behind the removsl of barriers to healing, Wounds; 2003; 15; 213 – 229

19. Sibbald R, Ossted H, Schultz G, Coutts and Keast D, Preparing the wound bed, 2003; fcus on infection and inflammation; wounds; 2003, 49; 24 – 51

20. Chin G,Shultz G and Starey M Principles of wound bed preparation and their application to treatment of chronic wounds; Primary intension; 2003;11;171- 182

21. Cukjati D, Rebesek S, Karba R, Miklavic, Modelling of chronic wound healing dynamics, Medical and biological engineering and computing, 2000; 38; 339-347

22. Nadine B, Semer M D, The help guide to basis of wound care, Global help publication; 2003 1 – 16

23. Simonsen H, Coutts P, Bogert Jansen S, Knight S, Assessing and managing chronic wounds, wound care reference guide, coloplast; 2007; 1 – 12

24. Harding KG, Morris HL, Patel GK, Healing chronic wounds; British medical journal; 202; 324; 160 – 163

25. Andrian Barbul, President; Chronic wound prevention guidelines; The wound healing society; 2009; 1 – 33

26. Gethin G; The significance of surface PH in chronic wounds, Wounds; UK; 2003; 3(3); 52 – 56

27. Joseph E G; Enoch S, Harding K G, ABC of wound healing, British medical journal, 2006; 332, 285 – 288

28. Faucher L D, Angela G L, Micheal JS, management of chronic wounds, ACS, Principles and practice, 2011; 10, 1 – 17

29. Nutrition and wound healing, pharmacy assistant Cubitan The wund care SIP FEED

30. www.wikipedia.com

31. Steven L Percival, John G Thomas, David W Williams. Biofilms and bacterial imbalances in chronic wounds: anti koch. Int Wound J 2010; vol 7:169-175.

32. D. Rhoads, R.W. Wolcott, K.F. Cutting and S.L. Percival. Evidence of Biofilms in Wounds and the Potential Ramifications. 1-15

33. Thomson CH. Biofilms: do they affect wound healing? Int Wound J 2011; vol 8:63–67

34. Widgerow AD. Persistence of the chronic wound – implicating biofilm**.** Wound Healing Southern Africa 2008;vol 1:05-07.

35. Rhonda cornell, How Biofilms Affects Wound Healing In Diabetic Foot Wounds.Podiatry Today April 2010; vol 23:20-24.

36. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284(5418): 1318-22. Steven L. Percival, Philip G. Bowler, Biofilms and Their Potential Role in Wound Healing. Wounds. Nov 2004;vol 16: 234-240.

37. Phillips PL, Wolcott RD, Fletcher J, Schultz GS .Biofilms Made Easy .Wounds International .may 2010 vol 1: 1-6.

38. Randall Wolcott, Keith F Cutting, Scot E . Dowd. Surgical site infections: biofilms, dehiscence and delayed healing .Wounds UK, 2008, Vol 4: 108- 113.

39. Rose Cooper, Keith Cutting, Marco Romanelli. Biofilms and the role of debridement in chronic wounds. Wounds uk, 2010, Vol 6: 160-166.

40. Richard Mayeux, Biomarkers: Potential Uses and Limitations, The Journal of the American Society for Experimental NeuroTherapeutics , April 2004Vol. 1, 182–188.

41. Joseph Boykin, The Future of Wound Care Diagnostics:Biomarkers, 20 Ostomy Wound Management September 2009,20-21.

42. Pradeep Sahu1, Neha Pinkalwar, Ravindra Dhar Dubey, Shweta Paroha, Shilpi Chatterjee and Tanushree Chatterjee, Biomarkers: An Emerging Tool for Diagnosis of a Disease and Drug Development, Asian J. Res. Pharm. Sci. 1(1): Jan.-Mar. 2011; Page 09-16.
43. Manoj Kumar And Shiv K Sarin, Biomarkers of diseases in medicine,current trends in science ,pages:403-417.
44. TH Ward, J Cummings, E Dean, A Greystoke, JM Hou1, A Backen, M Ranson and C Dive, Biomarkers of apoptosis, British Journal of Cancer (2008) 99, 841 – 846
45. Richard Franks and Richard Hargreaves,Clinical Biomarkers in Drug Discovery and Development, Nature publishing group, july 2003,2;566-580.
46. Z. Gârban, Adina Avacovici, Gabriela Gârban, G.D. Ghibu, Ariana – Bianca Velciov, Cristina – Ioana Pop, Biomarkers: Theoretical Aspects And Applicative Peculiarities**,** Scientifical Researches. Agroalimentary Processes and Technologies, Volume XI, No. 1 (2005), 139-146
47. http://en.wikipedia.org/wiki/Biomarkers_of_exposure_assessment.
48. Joseph V. Boykin Jr, Wound Nitric Oxide Bioactivity :A Promising Diagnostic Indicator for Diabetic Foot Ulcer Management, J Wound Ostomy Continence Nurs. 2010;37(1):25-32.
49. http://www.systagenix.com/cms/uploads/International-Consensus-Role-Of-Proteases-in-Wound-Diagnostics.
50. Amit Soneja, Magdalena Drews, Tadeusz Malinski, Role of nitric oxide, nitroxidative and oxidative stress in wound healing, pharmacological reports ;2005,57 suppl, 108-119.
51. Anatoly B Shekhter, Vladimir A Serezhenkov, Tatiana G Rudenko, Alexander V Pekshev, Anatoly F Vanin, Beneficial effect of gaseous nitric oxide on the healing of skin wounds, Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2005 Jun; 12(4): 210-19.