REVIEW OF LITERATURE
CHAPTER 2

REVIEW OF LITERATURE

2.1 Background of DFU

The human foot is functionalized by 26 bones, 29 joints, 42 muscles and a multitude of tendons and ligaments. The connection between diabetes and foot ulceration was established as early as 1887 by the surgeon T.D. Pryce who in an article published in Lancet reported that "diabetes itself might play an active part in the causation of perforating ulcers". Diabetes associated foot problem has been a common problem in developing nation as well as in Western countries. It is estimated that every thirty seconds a person loses a limb somewhere in the world due to diabetes related foot problems. In India, about forty thousand leg amputations are performed every year because of diabetes related foot complications. The amputation rate among diabetics in India is 18.7% (Morbach et al., 2003). The term “diabetic foot” was coined to distinguish the specific qualities about the feet of diabetic patients that distinguish this disease from other conditions that target lower extremities of humans. Infection, ulceration and destruction of deep tissues associated with neurological abnormalities and various degrees of PVD in the lower limb define the diabetic foot (Singh et al., 2005). Although both neuropathy and PVD as individual entities may co-exist and interact in a diabetic foot, substantial clinical and histological differences can be distinguished between neuropathic and ischemic wound beds. Both neuropathy and lower limb tissue hypo-perfusion are typical long-term complications of hyperglycemia, which along with wound dimension, and the host’s incapability to control local infection contributes to the disease.

2.2 Pathophysiology of DFU

Numerous observational studies have indicated that DFU is multifactorial in nature. It is well established that insulin deficiency (absolute or relative) is the basis of the biochemical abnormalities that lead to the organic complications of
DFU (Hoogwerf et al., 2006). DFU is characterized by a classical triad of neuropathy, ischemia, and infection (Pendsey et al., 2010). Due to the impaired metabolic mechanisms in DM, there is an increased risk of infection and poor wound healing due to a series of mechanisms which include decreased cell and growth factor response, diminished peripheral blood flow and decreased local angiogenesis (Brem et al., 2007). Thus, the feet are predisposed to neuropathy, PVD, damage of peripheral nerves, deformities, ulcerations and gangrene. Neuropathy, both symmetric and bilateral, plays the main role with varying degrees of alterations in autonomic, sensory, and motor functions while PVD resulting from atherosclerosis plays a secondary role (Fig. 9). Signs or symptoms of vascular dysfunction are observed in 40 to 50% of all patients with the vast majority having neuroischemic ulcers, and only minority of patients have purely ischemic ulcers (Apelqvist et al., 2008).

![Fig. 9. Pathophysiology of DFU](image)

Adapted from Mendes et al., 2012

DFU results from a complex interaction of a number of risk factors. Neuropathy (with alterations in motor, sensation, and autonomic functions) plays the central role and causes ulcerations due to trauma or excessive pressure in a deformed foot without protective sensibility. Once the protective layer of skin is broken, deep tissues are exposed to bacterial colonization. Infection is facilitated by
DM-related immunological deficits, especially in terms of neutrophils, and the infection rapidly progresses to the deep tissues.

2.3 Neuropathy associated DFU

Neuropathy causes more than 60% of the foot ulcers (Clayton et al., 2009) and affects patients with T2DM. Rise in blood glucose levels leads to increased enzyme production such as aldose reductase and sorbitol dehydrogenase. These enzymes convert glucose into sorbitol and fructose. As these sugar products accumulate, the synthesis of nerve cell myoinositol is decreased, affecting nerve conduction (Clayton et al., 2009). Furthermore, hyperglycemia induced microangiopathy leads to reversible metabolic, immunologic and ischemic injury of autonomic, motor and sensory nerves (Younger et al., 1998). This causes a decrease in peripheral sensation and damages the nerve innervations of small muscles of the foot and fine vasomotor control of the pedal circulation (Jeffcoate et al., 2003). When the nerve gets injured, the patient is at a higher risk of getting a minor injury without noticing it until it becomes an ulcer. The risk of developing foot ulcers in patients with sensory loss is increased up to seven-fold, compared to non-neuropathic patients with diabetes (Wild et al., 2004). T2DM also affects the autonomic nervous system, leading to dryness and fissuring of skin, making it prone to infection. Autonomic system also controls the microcirculation of skin. These changes ultimately contribute to the development of ulcers, gangrene, and limb loss (Vinik et al., 2003). The movements of the foot like flexion and extension are affected due to the damage to innervations of the foot muscles. Gradually, it leads to an alteration of the anatomical framework of the foot and formation of deformities. The deformities in turn create abnormal bony prominences and pressure points eventually predisposing to ulcers. Metatarsal fat pads are displaced distally, reducing the cushioning effects of the metatarsal heads and increase the pressure points which lead to callus formations that cause skin breakdown and ulceration (Bowering et al., 2001). Peripheral neuropathy promotes callus formation. The callus (callosity) contributes to high pressure areas and ulcer formation. In the words of (Duckworth et al., 1985) “abnormally high pressures are more common in patients with diabetic neuropathy and almost all patients with a
history of ulceration show high pressure areas which correlate well with the site of previous ulceration.” Usually, ulcers occur on the plantar aspect of great toe and heel. However, ill-fitting shoes (which are the most common source of trauma) can cause ulcers on the dorsal aspect (Peters et al., 2007). Hence neuropathic foot ulcer formation in patients with diabetes has a complex multifactorial aetiopathogenesis wherein areas of high pressure complimented by peripheral neuropathy and associated skin changes lead to ulcer formation. Peripheral neuropathy has also been implicated in Charcot neuroarthropathy (CN) (Perrin et al., 2010). CN is a chronic painless progressive degenerative arthropathy resulting from the disturbance in sensory innervations of the affected joint. The impairment of the autonomic nervous system due to T2DM causes an increase in local blood supply and the blood flow is much higher than in the normal patient. The sudden increase in blood flow causes calcium to dissolve, leading to osteoclastic activity of the bone and thus damaging the bone (Rogers et al., 2011). Another theory is that the repetitive minor trauma to the insensate joints leads to fracture and disintegration (Madan et al., 2013). The production of pro-inflammatory cytokines leads to uncontrolled osteolysis in CN. The cytokines such as TNF-α, IL-6 and IL-1β increase the expression of receptor activator of nuclear factor-κβ (RANKL), which in turn causes maturation of osteoclasts by triggering production of nuclear factor-κβ (Madan et al., 2013). The hallmark deformity associated with this condition is midfoot collapse, also known as “rocker-bottom” foot.

2.4 PVD associated DFU

Hyperglycemia causes endothelial cell dysfunction and smooth cell abnormalities in peripheral arteries. Endothelial cells synthesize nitric oxide which causes vasodilation and protects the blood vessels from endogenous injury. Hence, in hyperglycemia, there is perturbation of the physiological properties of nitric oxide which usually regulates the endothelial homeostasis, anticoagulation, leukocyte adhesion, smooth muscle cell proliferation and antioxidant capacity. Endothelium-derived vasodilators and nitric oxide are decreased hence leading to constriction of the blood vessels (Creager et al., 2003) and propensity for atherosclerosis (Dokken et al., 2008) eventually leading to ischemia. Ischemia can
also occur even in the presence of palpable pedal pulses (Jeffcoate et al., 2003). The microcirculation is also disturbed due to arteriolar-venular shunting, reducing the blood circulation (Jeffcoate et al., 2003). Hyperglycemia in T2DM is also associated with increase in thromboxane leading to plasma hypercoagulability (Paraskevas et al., 2008). Clinically the patient may have signs of vascular insufficiency such as claudication, night pain or rest pain, absent peripheral pulses, thinning of skin, loss of limb hair (Armstrong et al., 1998). Compared to a healthy person’s immune system, that of a patient with diabetes is much weaker. Thus, foot infection in a patient with diabetes is a limb threatening and debilitating condition. The hyperglycemic state causes an elevation of pro-inflammatory cytokines and impairment of polymorphonuclear cell functions like chemotaxis, adherence, phagocytosis and intracellular killing (Gupta et al., 2007). Besides that, high blood glucose is a good medium for the growth of bacteria. The predominant organisms in diabetic foot infections are mainly aerobic gram positive cocci like \textit{S. aureus} and \textit{\beta-hemolytic streptococci} (Lipsky et al., 2004) but in one research conducted in India, gram-negative aerobes were the common microorganisms in diabetic foot (Gadepalli et al., 2006). The soft tissues of foot like plantar aponeurosis, tendons, muscles sheaths and fascia cannot resist infections. Furthermore, several compartments in the foot are interconnected and could not limit the spread of infection from one into another. This soft tissue infection can rapidly spread to the bones, causing osteomyelitis. Thus a simple ulcer on the foot can easily result in complications such as osteomyelitis and gangrene without appropriate care.

2.5 Classification, causes, diagnosis, management and prevention of DFU

Foot ulcers can be classified into various grades. One of the oldest and probably the most well-known classification which was proposed by Wagner’s and Meggitt (Wagner et al., 1981) and is the most commonly known as “Wagner’s Classification” and uses five grades in classifying diabetic foot lesions (Table-3).

Ulceration of the diabetic foot, either neuropathic or ischemic, does not occur spontaneously. It usually follows some form of extrinsic or intrinsic trauma (Reiber et al., 1999). While extrinsic trauma may include any kind of thermal (e.g.,
scalding from hot water), chemical (e.g., abrasion from callus treatment solutions), or localized mechanical (e.g., puncture wounds from foreign objects) injuries, the most common injury leading to ulceration is continuous low-pressure trauma, typically from ill-fitting shoes, and injuries due to chronic repetitive trauma from walking or day-to-day activity (Rathur et al., 2007). Intrinsic traumas are also easily understood as they result from foot deformities (foot drop, equinus, hammertoes, and prominent plantar metatarsal heads) and consequent altered foot biomechanics (Jeffcoate et al., 2003).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>No ulcer in a high risk foot</td>
</tr>
<tr>
<td>Grade 1</td>
<td>Superficial ulcer involving the full skin thickness but not underlying tissues.</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Deep ulcer, penetrating down to ligaments and muscle, but no bone involvement or abscess formation.</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Deep ulcer with cellulitis or abscess formation, often with osteomyelitis.</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Localized gangrene.</td>
</tr>
<tr>
<td>Grade 5</td>
<td>Extensive gangrene involving the whole foot.</td>
</tr>
</tbody>
</table>

A proper investigation should be carried out in all patients with diabetes. History should include the duration of T2DM, neuropathic or PVD symptoms, previous ulcers or amputations and any other complication like retinopathy or nephropathy (Lavery et al., 1998). Foot examinations are reported to be effective in reducing the risk of amputations (Mayfield et al., 2000). The foot should be carefully inspected for abnormalities like dry skin, fissures, deformities, and callosities. Ulcerations, prominent veins, and nail lesions should be looked out for. Changes in the foot temperature must be noted. An increase in temperature might suggest inflammation (Armstrong et al., 2007) while a decrease may indicate ischemia. Capillary refilling time should be assessed. All peripheral pulses must be examined. Pain, redness and swelling of the foot/ankle should be examined carefully, which can be easily confused with septic or gouty arthritis.
A sterile stainless steel probe is used for assessing the ulcer to determine the depth and if there is sinus tracts present (Grayson et al., 1995). The location, size, shape, depth, base and margins of the ulcer should be examined clinically. Presence of granulation tissue or slough should be looked for in the floor of the ulcer to determine subsequent management (Fig.10). Diagnosing a soft tissue infection in patient with T2DM is sometimes difficult, as the signs of inflammation of the overlying ulcer may be absent. The infection is mainly diagnosed based on presence of clinical signs and symptoms such as redness, warmth, tenderness, purulent secretions and fever. Palpation of the bone at the base of the ulcer with a sterile, blunt stainless steel probe has been suggested as positive predictor of underlying osteomyelitis (Grayson et al., 1995).

![Infected ulcer](image1)

![Superficial ulcer with healthy granulation tissue](image2)

![Infection with redness in appearance](image3)

![Infection with pus secretion](image4)

**Fig.10.** Appearance and examination of DFU

Sensory neuropathy can be tested by using monofilaments and biothesiometer. Semmes-Weinstein monofilaments are reported to be easy to use and help in predicting the risk of ulceration and amputation (Mayfield et al., 2000).
Caputo et al., suggested annual testing of all patients with diabetes with a nylon monofilament to detect peripheral neuropathy (Caputo et al., 1994). A 128 Hz tuning fork can also be used to test for vibratory sensation over the tip of the great toe bilaterally since metabolic neuropathies are more severe distally. Pain sensation should be tested as well. The Heart Rate Variability (HRV) with deep breathing or orthostatic blood pressure is measured to detect autonomic neuropathy (Rogers et al., 2008) and any decrease or absence of HRV is considered the earliest sign of autonomic neuropathy in T2DM (Unger et al., 2007). Specialized tests for sudomotor dysfunction include thermoregulatory sweat testing, quantitative sudomotor axon reflex testing, silicone impressions, the Sympathetic Skin Response (SSR) and the quantitative direct and indirect axon reflex testing (Illigens et al., 2009). These tests can be used in various combinations to localise the lesion of autonomic dysfunction (pre-ganglionic or post-ganglionic) (Illigens et al., 2009). Laboratory investigations involve measuring blood glucose level and urine for glucose and ketones. Other investigations like full blood count, blood urea, electrolytes, and creatinine levels should be recorded. HbA1c is important to monitor the patient’s overall glycemic control as HbA1c shows the mean blood sugar concentration best over previous weeks to months (Koenig et al., 1976). Hepatic and renal function tests are necessary for monitoring the patient’s metabolic status. ESR can be done to assess the presence and response to treatment of infections like osteomyelitis (Rabjohn et al., 2007). In the presence of invasive infection, cultures from the deeper tissue are required to identify the causative microorganisms. In case of diabetic foot, it is hard to assess the depth of the ulcer especially when there is pus and slough covering it. Also, it is hard to determine the extent of deep infection as the rubor of inflammatory response is minimal in subfascial sepsis (Naraynsingh et al., 2011). X rays are helpful to determine the depth of foot ulceration and to assess presence of bone infection or neuroarthropathy and also used to evaluate the extent of foot infection by revealing the depth of ulceration, edema and localized fluid collections in the soft tissues, joints and tendon sheaths.
Ankle brachial index (ABI) or toe-brachial index can be used to determine the extent of the vascular problem. A value below 0.9 suggests an obstruction (Doobay et al., 2005) while ABI less than 0.4 is associated with tissue necrosis and a significant risk for amputation (Reiber et al., 1992). Screening ABI every 5 years in patients with diabetes without any signs/symptoms of vascular insufficiency has been recommended. The transcutaneous oxygen tension (TcPO$_2$) method is a reliable indicator of skin perfusion as periwound cutaneous perfusion is the critical physiological determinant of ulcer healing. TcPO$_2$ less than 20mmHg have been associated with early wound healing failure. Other investigations to detect vascular insufficiency include measuring absolute toe pressure, continuous-wave Doppler ultrasonography, duplex ultrasonography, pulse volume recordings and angiography (CT, MRI or contrast). Pedobarography is a study of foot pressure and has been widely used in the research of diabetic foot (Lobmann et al., 2001).

Management for DFU is ideally provided by a multidisciplinary team by ensuring glycemic control, adequate perfusion, local wound care and regular debridement, off-loading of the foot, control of infection by appropriate antibiotics and management of comorbidities. Educating patients helps in preventing ulcers and their recurrence.

Debridement

Ulcers heal faster when the wound is clean as the devitalized necrotic tissues hinder cell migration and predispose it to infection and prohibit healing. Debridement of the wound may hasten healing by removing the dead necrotic tissue, particulate matter, or foreign materials, and reducing bacterial load (Pai et al., 2013). The conventional way is to use a scalpel and excise all unwanted tissues including callus and eschar (sharp debridement). Since the necrotic tissue often extends beyond the ulcer bed. Another approach is to completely excise the chronic ulcer and the underlying bony prominences and convert it to a fresh ulcer (Armstrong et al., 2003). The limiting factors of sharp debridement include inadvertent bleeding, poor pain tolerance by the patient and lack of any objective markers to differentiate impaired and healthy tissue to ascertain the extent of
debridement (Pai et al., 2013). Other methods of wound debridement include enzymatic debridement using enzymes like collagenase and papain as ointment preparations, and biological debridement with use of larvae of common green bottle fly (Lucilia sericata).

**Dressings**

Dressing materials used include saline-moistened gauze dressings (wet-to-dry); moisture retaining dressings (hydrogels, hydrocolloids, hydrofibres, transparent films and alginates) that provide physical and autolytic debridement respectively; and antiseptic dressings (silver dressings, cadexomer). Medicated honey has anti-inflammatory, antiseptic and osmotic properties and has been used as such or in combination with sterile dressings.

**Offloading**

Total contact cast (TCC), removable cast walkers, custom shoes, half-shoes, soft heel shoes, padded socks, and shoe inserts, wheelchairs, crutches etc. have been used for offloading the foot to prevent and treat the DFUs. The aim is to reduce the plantar pressure by redistributing it to a larger area, to avoid shear and friction, and to accommodate the deformities. A randomized control trial compared the efficacy of a TCC, removable cast walker and half-shoe in patients with DFUs found TCC to be the most effective modality. TCC was also found to be superior to traditional dressings in treatment of plantar DFUs. Removable cast walkers such as Aircast walkers allow for surveillance of skin and dressing changes. A recent systematic review found non-removable offloading devices (for example TCC) to be more effective for ulcer healing than removable off-loading devices (for example, removable cast walker) (Morona et al., 2013).

**Medical treatment**

Strict glycemic control should be maintained with the use of diabetic diet, oral hypoglycemic agents and insulin. Infections of the soft tissue and bone are the leading cause of hospital admissions in patients with DFUs. It is well documented
that the diagnosis of infection in DFUs is primarily clinical. Culture from the deeper tissues aids in selecting appropriate antibiotics. Antibiotics are preferably given intravenously for limb threatening infections. Gabapentin and pregabalin have been used for symptomatic relief for painful neuropathy in T2DM (Backonja et al., 1998). Aldose reductase inhibitors are being studied and have shown to be effective in inhibiting progression of peripheral neuropathy (Hotta et al., 2012). Autonomic dysfunction may require the use of beta-blockers. Medical management of symptoms of vascular insufficiency like intermittent claudication includes Cilostazol or Pentoxifylline besides exercise therapy.

**Prevention**

Patient education and self-care practices like maintaining foot hygiene and nail care should be promoted. Skin is kept moisturized with the application of topical moisturizers after washing the feet gently with soap and water (Armstrong et al., 1998). Harsher measures like hot soaks, heating pads and topical agents such as hydrogen peroxide, iodine and astringents must be avoided (Armstrong et al., 1998). There is a direct correlation between glycemic control and ulcer formation. Neuropathic feet are warmer and temperature differences of 2-7°C have been noted between neuropathic and non neuropathic feet. Hence self-monitoring may reduce the risk of ulceration. Smoking and alcohol consumption should be minimized, though the direct link between them and DFUs is weak. Offloading and appropriate footwear to relieve focal high pressure areas is recommended for foot at-risk. Other co-morbidities like hypertension and hyper lipidemia which predispose to vascular occlusion should be treated.

### 2.6 Mechanism of wound healing

The normal healing response begins the moment the tissue is injured. As the blood components spill into the site of injury, the platelets come into contact with exposed collagen and other elements of the extracellular matrix (ECM). This contact triggers the platelets to release clotting factors as well as essential growth factors and cytokines such as platelet-derived growth factor (PDGF) and TGF-β.
Following hemostasis, the neutrophils then enter the wound site and begin the critical task of phagocytosis to remove foreign materials, bacteria and damaged tissue. As part of this inflammatory phase, the macrophages appear and continue the process of phagocytosis as well as releasing more PDGF and TGF-β. Once the wound site is cleaned out, fibroblasts migrate in to begin the proliferative phase and deposit new extracellular matrix. The new collagen matrix then becomes cross-linked and organized during the final remodeling phase. In order for this efficient and highly controlled repair process to take place, there are numerous cell-signaling events that are required.

2.6.1 Inflammation phase

The healing cascade begins immediately following injury when the platelets come into contact with exposed collagen. As platelet aggregation proceeds, clotting factors are released resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing (Clark et al., 2001). Platelets not only release the clotting factors needed to control the bleeding and loss of fluid and electrolytes but they also provide a cascade of chemical signals, known as cytokines or growth factors, that initiate the healing response. The two most important signals are PDGF and TGF-β (Kim et al., 1998). The PDGF initiates the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts. In addition it also stimulates the mitogenesis of the fibroblasts and smooth muscle cells.

TGF-β adds another important signal for the initiation of the healing cascade by attracting macrophages and stimulates them to secrete additional cytokines including FGF (fibroblast growth factor), PDGF, TNF-α and IL-6. In addition, TGF-β further enhance fibroblast and smooth muscle cell chemotaxis and modulate collagen and collagenase expression. The net result of these redundant signals is a vigorous response of the matrix producing cells to ensure a rapid deposition of new connective tissue at the injury site during the proliferative phase that follows the inflammatory phase.
Neutrophils are the next predominant cell marker in the wound within 24h after injury. The major function of the neutrophil is to remove foreign material, bacteria and non-functional host cells and damaged matrix components that may be present in the wound site (Sylvia et al., 2003). Bacteria give off chemical signals, attracting neutrophils, which ingest them by the process of phagocytosis. Neutrophils will engorge themselves until they are filled with bacteria and constitute what is called "laudable pus" in the wound. The mast cell is another marker cell of interest in wound healing. Mast cells release granules filled with enzymes, histamine and other active amines and these mediators are responsible for the characteristic signs of inflammation around the wound site. The active amines released from the mast cell causes surrounding vessels to become leaky and thus allow the speedy passage of the mononuclear cells into the injury area. In addition fluid accumulates at the wound site and the characteristic signs of inflammation begin.

After 48h of injury, fixed tissue monocytes become activated to become wound macrophages. These specialized wound macrophages are perhaps the most essential inflammatory cells involved in the normal healing response. Inhibition of macrophage function will delay the healing response. Once activated these wound macrophages also release PDGF and TGF-β that further attracts fibroblasts and smooth muscle cells to the wound site. The presence of wound macrophages is a marker that the inflammatory phase is nearing an end and that the proliferative phase is beginning.

2.6.2 Proliferative phase

As the proliferative phase progress, the TGF-β released by the platelets, macrophages and T-lymphocytes become a critical signal. TGF-β is considered to be a master control signal that regulates a host of fibroblast functions. Other cytokines considered to be important are interleukins, FGF and TNF-α. As healing progresses several other important biological responses are activated. The process is stimulated by the presence of EGF (epidermal growth factor) and TGF-α that are produced by activated wound macrophages, platelets and keratinocytes (Schultz et
Once the epithelial bridge is complete, enzymes are released to dissolve the attachment at the base of the scab resulting in removal. Due to the high metabolic activity at the wound site, there is an increasing demand for oxygen and nutrients. Local factors in the wound microenvironment such as low pH, reduced oxygen tension and increased lactate actually initiate the release of factors needed to bring in a new blood supply. This process is called angiogenesis or neovascularization and is stimulated by vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF) and TGF-β (Tonnesen et al., 2000). Epidermal cells, fibroblasts, macrophages and vascular endothelial cells produce these factors.

2.6.3 Remodeling phase

Fibroblasts begins to produce collagen and get released into the extracellular space, undergoes further processing by cleavage of the pro-collagen N and C-terminal peptides. In the extra-cellular spaces an important enzyme, lysyl oxidase, acts on the collagen to form stable cross-links. As the collagen matures and becomes older, more and more of these intra molecular and intermolecular cross-links are placed in the molecules. This cross-linking gives collagen its strength and stability. Finally, in the process of collagen remodeling, collagen degradation also occurs. Specific collagenase enzymes in fibroblasts, neutrophils and macrophages clip the molecule at a specific site through all three chains, and break it down to pieces. These collagen fragments undergo further denaturation and digestion by other proteases.
Fig. 11. Biology of wound healing
Adapted from Mendes et al., 2012

Fig. 12. Pathways involved in hyperglycemic state
2.7 Impaired wound healing in diabetes

Various pathways like polyol pathway, formation of AGE (Ojima et al., 2012), hexosamine pathway (Riedl et al., 2011), protein kinase C pathway (Juan et al., 2012), growth factors, cytokines (Titan et al., 2012) and free radicals (Zhou et al., 2012). MAPK activation, PARP activation have been reported to play an important role in diabetic complications.

The delayed wound healing in diabetes is caused by complex factors such as diminished keratinocyte and fibroblast migration, proliferation, differentiation, apoptosis, and vascularization. Several of these cellular deficits have been linked to greater inflammation and pro-inflammatory cytokine production (Fig. 13). Inflammation, immunodeficiency, peripheral neuropathy and ischemia from PVD and subsequent infection are underlying factors that contribute to unhealed chronic wounds in DFU (Sibbald et al., 2008).

![Fig.13. Mechanisms of impaired diabetic wound healing](image)

Adapted from Fanxing Xu et al., 2013

In contrast to normal wound healing, in diabetes the inflammatory cytokines and chemokines are elevated, such as TNF-α, IL-1, IL-6, CCL2, CCL3,
CCL4, CXCL1, CXCL5 and CXCL8. Cellular processes affected by diabetes include abnormal keratinocyte and fibroblast migration, proliferation and enhanced apoptosis, abnormal macrophage polarization (increased pro-inflammatory M1 macrophages and decreased anti-inflammatory M2 macrophages), impaired recruitment of mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) and decreased vascularization.

The impaired healing that occurs in individuals with diabetes involves hypoxia, dysfunction in fibroblasts and epidermal cells, impaired angiogenesis and neovascularization, high levels of metalloproteases, damage from ROS and AGEs, decreased host immune resistance and neuropathy. The influence of these factors on wound healing is summarized in (Fig. 14).

![Fig. 14. The potential effects of diabetes on wound healing](image-url)
2.7.1 Inflammation and impaired immunity

The inflammatory stage of wound repair occurs shortly after tissue damage. After acute injury, platelets and neutrophils are released passively from disrupted blood vessels. The formation of a fibrin clot provides a temporary scaffold for infiltration of inflammatory cells. A large number of growth factors are important in stimulating and coordinating cellular events that occur during normal wound healing (Gurtner et al., 2008). Among them, cytokines and chemokines are especially important because of their roles in promoting inflammation, angiogenesis, leukocyte recruitment, recruitment of stem cells, and epithelialization. Pro-inflammatory cytokines that are elevated shortly after wounding both in human wounds and animal wound models include IL-1α, IL-1β, IL-6, IL-12 and TNF-α (Barrientos et al., 2008). Some pro-inflammatory cytokines and chemokines are essential for normal skin wound-healing process. Delayed wound healing is observed in IL-6 deficient mice (Lin et al., 2003). The CXC chemokine family of chemotactic cytokines CXCL1, CXCL5 and CXCL8 is expressed in keratinocytes and up regulated in wounding by stimulation of pro-inflammatory cytokines such as IL-1 and TNF-α, bacterial products and hypoxia. The induced expression of chemokines stimulates recruitment of leukocytes and monocytes, neutrophils and macrophages to the wound site to remove foreign material, bacteria, dead cells and damaged matrix. Chemokines also induce recruitment of stem cells to sites of injury and include epithelial stem cells from hair follicles or sweat glands, endothelial progenitor cells and mesenchymal stem cells (Arwert et al., 2012). Impaired wound healing in diabetic patients is accompanied by decreased early inflammatory cell infiltration but increased numbers of neutrophils and macrophages in late stages. These changes in inflammatory cell recruitment occur in conjunction with alterations in chemokine and growth factor expression. An increase in inflammatory cytokines is observed in T2DM mediated wounds includes IL-1α, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CCL4. In diabetic models, increased levels of the pro-inflammatory cytokines such as TNF-α and IL-6 and decreased levels of anti-inflammatory IL-10 are observed (Khanna et al., 2010).
### Table-4. Inflammatory cells, their functions and mediators released in tissue repair

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Functions</th>
<th>Mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN</td>
<td>Phagocytosis of infectious agents</td>
<td>ROS, cationic peptides, eicosanoids, Proteases (elastase, cathepsin-G)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>Macrophages activation through phagocytosis</td>
<td>TNF-α, IL-1β, IL-6, VEGF, IL-8</td>
</tr>
<tr>
<td></td>
<td>Amplify inflammatory response &amp; stimulate repair response</td>
<td>IL-10, TGF-β1</td>
</tr>
<tr>
<td></td>
<td>Phagocytosis of PMN and fragments of tissue degradation</td>
<td>PDGF, VEGF, bFGF, TGF-α, and TGF-β</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory function</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stimulate repair response: angiogenesis, Fibroplasias</td>
<td></td>
</tr>
<tr>
<td>Mast cells (MC)</td>
<td>Control vascular permeability</td>
<td>Histamine</td>
</tr>
<tr>
<td></td>
<td>Control influx of PMN</td>
<td>Chymase, tryptase</td>
</tr>
<tr>
<td>T cell; Th1/Th2</td>
<td>Regulate tissue remodeling</td>
<td>CD40 ligand; IL-2, TNF-α/ IL-4, IL-10</td>
</tr>
</tbody>
</table>

#### 2.7.2 Impaired Neovascularization

The endothelial cells (ECs) and fibroblasts (FBs) are major cell types participating in angiogenesis and extra cellular matrix deposition and wound remodeling (Lerman et al., 2003). Cultured fibroblasts from diabetic wounds of human patients as well as genetically diabetic (db/db) mice show impaired
functions \textit{in vitro}. Fibroblasts from db/db murine models exhibited selective impairments (Blakytny \textit{et al.}, 2006; Lerman \textit{et al.}, 2003; Loot \textit{et al.}, 2002) in cellular migration, VEGF production and enhanced matrix metalloproteinases (MMPs) production compared to normal FBs \textit{in vitro}. Endothelial dysfunction and impaired angiogenesis is considered one of the major factors affecting diabetic ulcer healing (Falanga \textit{et al.}, 2005; Blakytny \textit{et al.}, 2006). One of the mechanisms responsible for this dysfunction is due to the reduced expression of vascular endothelial growth factor (VEGF), Angiopoietin 1 receptor Tie-2, platelet-derived growth factor (PDGF) and FGF (Lerman \textit{et al.}, 2003; Galiano \textit{et al.}, 2004). Another mechanism suggested for impaired angiogenesis in diabetic wounds is due to a fundamental deficiency in recruitment of endothelial cells and endothelial progenitor cells (EPCs) to the wound site (Keswani \textit{et al.}, 2004), which contribute up to 25\% of the cells in newly formed vessels in animal models (Liu \textit{et al.}, 2008). In response to trauma or ischemia/hypoxia, which is observed during wound healing, EPCs are released from the bone marrow niche. Chemokines and cytokines trigger stem/progenitor cell release by induction of matrix metalloproteinase-9 (MMP-9) in bone marrow. However, a local increase of SDF-1\(\alpha\) is required for proper trafficking of the EPCs to the ischemic or wound healing site (Liu \textit{et al.}, 2008). Therefore, when designing new treatments for diabetic wound healing, it is important to establish how the treatment modality regulates the chemokine responses of cells that participate in wound healing. Increased matrix degradation (Lerman \textit{et al.}, 2003) and hyperglycemia-induced apoptosis associated with diabetic wounds may create an unfavorable environment preventing attachment and survival of endothelial and progenitor cells, further contributing to the neovascularization deficit. Studies of wound stimulation with angiogenic growth factors showed that both topical applications of VEGF, bFGF and PDGF (Galiano \textit{et al.}, 2004) and gene transfer of PDGF-B and Angiopoietin-1 (Keswani \textit{et al.}, 2004; Cho \textit{et al.}, 2006) significantly improved recruitment of vascular cells to the diabetic wounds and significantly enhanced neovascularization and healing. These results suggest that strategies focused on enhancing vascular cell survival and angiogenesis hold promise for development of new therapies to treat chronic diabetic ulcers.
Fig. 15. Model of multifactorial molecular and cellular mechanisms deleterious in tissue repair

Adapted from Eming et al., 2007

Chronic wounds fail to progress through the normal pattern of wound repair, but instead remain in a state of chronic inflammation predominantly characterized by abundant PMN and MF infiltration. Persisting inflammatory cells play a major role in the generation of pro-inflammatory cytokines (IL-1, TNF-α and IL-6) and a protease rich and pro-oxidant hostile microenvironment. Increased proteolytic activity (neutrophil elastase, MMP-8 and gelatinase) leads to degradation of growth factors and structural proteins of the extracellular matrix crucial for repair. Increased ROS (H₂O₂, O₂⁻) can lead to direct damage of cells or extracellular matrix molecules or contribute to increased expression of MMPs. Bacterial components (extracellular adherence protein (Eap), formyl methionyl peptides, N-acetylmuramyl-L-alanyl-D-isoglutamine) may contribute to impaired repair mechanisms of the host by interference with cell–matrix interactions or promoting the inflammatory response.
2.7.3 Redox stress in DFU

Increased oxidative stress in diabetes, including through the formation of ROS plays a major role in diabetic pathogenesis including DFU formation (Fig. 16). Elevated levels of such free radicals will lead to poor wound healing. These molecules damage proteins, lipids, and DNA causing disruption to cellular function and even leading to cell death. ROS can affect platelet aggregation and the release of growth factor, as well as inducing increased expression of MMPs that will degrade extra cellular matrix (ECM). Free radicals possess the property to directly degrade ECM components. ROS can arise directly from high glucose and increased advanced glycation end products (AGEs) associated with DFU. Increased levels and thus activities of both iNOS and arginase in DFU will increase their competition for their mutual substrate the amino acid L-arginine. This results in NOS synthesizing ROS, including superoxide. The increased NO produced by elevated NOS activity can react with superoxide to generate the even more toxic free radicals OH• and NO₂ the free radicle spot. Apart from generating greater amounts of ROS in diabetes, the ability to remove them is compromised in DFU. The universal reducing agent reduced glutathione (GSH) together with the reducing amino acid cysteine are diminished in DFU. In addition to its direct action, GSH acts as a co-substrate for the reducing enzyme glutathione reductase.

![Fig. 16. Redox stress in DFU](image-url)
2.8 Genetics of DFU

Chronic hyperglycemia, hypertension, dyslipidemia, insulin resistance and uremia are thought to be the key factors that contribute to their pathogenesis, as they cause changes in gene expression, molecular transport, inflammation, and oxidative stress (Fig. 17). Genetic susceptibility and environmental factors are also known to interact and contribute significantly to disease onset and progression, which is evident in observations of twin studies and familial clustering, and in the variation of the prevalence and presentation of these complications among individuals, and among different ethnic groups (Wahlgren et al., 2011; Ciccacci et al., 2013). Recent advances in the application of genome-wide association studies (GWAS) and next-generation sequencing (NGS) are expected to expedite and enhance the ability to identify genetic variants associated with complex diseases, such as T2DM and its associated complications such as DFU. Studies conducted to identify genetic risk factors of DFU still remain scarce. Despite this effort, a significant amount of missing heritability remains to be explained as the majority of studies published in this field are limited by a lack of reproducibility and insufficient statistical power. While the search continues for novel rare variants that may help explain this missing heritability, it is evident that diabetes and its associated complications are the result of the combined effect of numerous genetic variants that may interact with each other and the environment to define disease risk, progression and severity. Complex interactions between risk factors contributing to the various DFU have been difficult to assess though, as the scope of diabetes-related genetic studies has often been limited to one particular candidate gene or one type of complication. GWAS conducted in this field so far have either focused on the risk of developing T2DM or investigated risk factors of diabetes-associated complications (Hindorff et al., 2014). In diabetes, it has been observed that all stages of wound healing are affected in DFU. It has been shown that a large number of growth factors, cytokines and chemokines, which are released by keratinocytes, fibroblasts, endothelial cells, macrophages and platelets, have their serum levels changed in diabetes. These factors are responsible for initiating the wound repair process and its maintenance, which results in gradual
decrease in inflammatory response (Eubank et al., 2010). Diabetic wounds are characterized by a reduced level of the following growth factors and receptors: KGF (keratinocyte growth factor), TGF-β1 (transforming growth factor beta), NGF (nerve growth factor), PDGF, TNF-α, SDF-1, VEGF, IL-6, IL-10, IL-15, neurotrophin-3 (NT-3). Decreased levels of these growth factors might contribute to poor tissue regeneration and impaired wound healing. Some of the molecular factors involved in DFU formation are listed in Table-5. Some of the genetics of vital markers involved in DFU from different population are listed in Table-6. In western population, the prevalence of PVD is high but neuropathy associated foot ulcer is low, whereas in our population it is vice versa. Therefore, the genetics of some of the cytokines/chemokines such as TNF-α, IL-6, SDF-1, HSPA1B and HSPA1L are very scarce in DFU (both DN and PVD) especially in our population.

Fig.17. Multifactorial etiology of the DFU
Table-5. Molecular factors involved in DFU formation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Status in T2DM</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF</td>
<td>decreased</td>
<td>possible defects in general wound healing</td>
<td>Tyndall et al., 2003</td>
</tr>
<tr>
<td>KGF</td>
<td>decreased</td>
<td>healing rate decreased</td>
<td>Werner et al., 1994</td>
</tr>
<tr>
<td>VEGF</td>
<td>decreased</td>
<td>healing rate decreased</td>
<td>Bitto et al., 2008</td>
</tr>
<tr>
<td>IL-6</td>
<td>increased</td>
<td>enhanced immune response, sustained inflammation state</td>
<td>Asrar et al., 2012</td>
</tr>
<tr>
<td>IL-8</td>
<td>decreased</td>
<td>decrease observed in diabetic mice fetuses, possible implications in pancreatic cell development</td>
<td>Shao et al., 2008</td>
</tr>
<tr>
<td>Igf-2</td>
<td>decreased</td>
<td>cell granulation defects</td>
<td>Yu et al., 2007,</td>
</tr>
<tr>
<td>Igf-1</td>
<td>decreased</td>
<td>enhanced immune response and sustained inflammation state</td>
<td>Xu et al., 2012</td>
</tr>
<tr>
<td>mir-146</td>
<td>decreased</td>
<td>slower healing rate, defects in angiogenesis</td>
<td>Bermudez et al., 2011</td>
</tr>
<tr>
<td>SDF-1</td>
<td>decreased</td>
<td>possible marker of B-catenin hyperactivation, resulting in inhibition of keratinocyte migration and EGF response</td>
<td>Stojadinovic et al., 2005</td>
</tr>
<tr>
<td>Hif1-α</td>
<td>decreased</td>
<td>possible defects in angiogenesis</td>
<td>Botusan et al., 2008</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Increased</td>
<td>enhanced fibroblasts apoptosis</td>
<td>Siqueira et al., 2010</td>
</tr>
<tr>
<td>TNF-α</td>
<td>decreased</td>
<td>increased osteoclast activation and bone resorption and cartilage removal</td>
<td>Kayal et al., 2009</td>
</tr>
<tr>
<td>TRAIL</td>
<td>decreased</td>
<td>abnormal ECM decomposition, possible TGFβ degradation</td>
<td>Liu et al., 2009</td>
</tr>
<tr>
<td>MCF</td>
<td>increased</td>
<td>abnormal ECM decomposition, possible TGFβ degradation</td>
<td>Liu et al., 2009</td>
</tr>
</tbody>
</table>
### Table-6. Genetic variants studied globally in DFU

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP/variant</th>
<th>Risk variant</th>
<th>Ethnic group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>I/D (287 bp, intron 19)</td>
<td>D</td>
<td>Caucasians and Asians</td>
<td>Li et al., 2014</td>
</tr>
<tr>
<td>Alpha 2B-AR</td>
<td>I/D</td>
<td>D</td>
<td>Greek</td>
<td>Papanas et al., 2007</td>
</tr>
<tr>
<td>APOE</td>
<td>T/C, C/T</td>
<td>C/C</td>
<td>Japanese</td>
<td>Tsukui et al., 1998</td>
</tr>
<tr>
<td>GPx-1</td>
<td>C/T</td>
<td>T</td>
<td>Caucasian</td>
<td>Tang et al., 2012</td>
</tr>
<tr>
<td>IL-4</td>
<td>VNTR (P1/P2 allele)</td>
<td>P1 allele</td>
<td>Turkish</td>
<td>Basol et al., 2013</td>
</tr>
<tr>
<td>IL-10</td>
<td>−1082 G/A</td>
<td>G</td>
<td>South Indian</td>
<td>Kolla et al., 2009</td>
</tr>
<tr>
<td>AKR1B1</td>
<td>−106 C/T (CA) n repeat (Z allele)</td>
<td>T, Z-2</td>
<td>Finnish</td>
<td>Sivenius et al., 2004</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>874 A/T</td>
<td>A</td>
<td>South Indian</td>
<td>Kolla et al., 2009</td>
</tr>
<tr>
<td>MTHFR</td>
<td>677 C/T</td>
<td>T</td>
<td>Turkish</td>
<td>Yigit et al., 2013</td>
</tr>
<tr>
<td>NOS3</td>
<td>27VNTR (4a/b) −786 T/C</td>
<td>4a, C</td>
<td>North and South Indian</td>
<td>Shah et al., 2013</td>
</tr>
<tr>
<td>TLR4 246</td>
<td>896 A/G, 1196 C/T</td>
<td>A, C</td>
<td>German Caucasian</td>
<td>Rudofsky et al., 2004</td>
</tr>
<tr>
<td>UCP2</td>
<td>−866 G/A</td>
<td>A</td>
<td>Japanese</td>
<td>Yamasaki et al., 2006</td>
</tr>
<tr>
<td>VEGF</td>
<td>I/D (−2549) C242T</td>
<td>D, T</td>
<td>Romanian</td>
<td>Stoian et al., 2014</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>C/T, T/C, C/T</td>
<td>T, C, T</td>
<td>North Indians</td>
<td>Singh et al., 2012</td>
</tr>
<tr>
<td>TCF-α</td>
<td>−308 A/G</td>
<td>A</td>
<td>Serbian</td>
<td>Nikolic et al., 2013</td>
</tr>
<tr>
<td>MMP-9</td>
<td>−1562C&gt;T</td>
<td>T</td>
<td>North Indians</td>
<td>Singh et al., 2013</td>
</tr>
<tr>
<td>TNF-α</td>
<td>−308G/A, +874A/T, −1082G/A</td>
<td>G, A, G</td>
<td>South Indians,</td>
<td>Kolla et al., 2012</td>
</tr>
</tbody>
</table>
2.9 Role of TNF-α in DFU

One aspect of wound healing that has recently received attention is the enhanced and prolonged expression of TNF-α. It is a potent pro-inflammatory cytokine produced as a membrane-bound 26 kDa molecule from which the soluble 17 kDa active TNF-α molecule is released by the TNF-α converting enzyme (TACE) (Elahi et al., 2009). The circulating TNF-α level in serum/plasma are highly variable (Aguillon et al., 2001). TNF-α is involved in several biologic processes such as tissue remodeling, epithelial cell barrier permeability, macrophage activation, recruitment of inflammatory cells, effectiveness of the local and systemic inflammation, and amplification of other pro-inflammatory cytokine actions (Bradley et al., 2008). In normal wound healing the highest levels of TNF-α are seen from 12 to 24h after wounding. After the completion of the proliferative phase of wound healing, TNF-α returns to basal levels. During the early phase of wound repair, it is predominantly expressed in polymorphonuclear leukocytes and later by macrophages. It is also expressed in the hyper proliferative epithelium at the wound edge. TNF-α contributes to the stimulation of fibroblasts and keratinocytes the expression of growth factors and upregulation of antimicrobial defenses. TNF-α levels are elevated in diabetes through increased oxidative stress that promotes inflammation. Other factors may contribute to this elevation including the down regulation of CD33 that inhibits cytokine production. TNF-α is found threefold higher in diabetic mouse wounds than wounds in normal mice and threefold higher found in wound fluid from non healing venous leg ulcers (Wallace et al., 2006).

The TNF-α gene is located on human chromosome 6p21.3 within the major histocompatibility complex (MHC) (Fig. 18) (Hajeer et al., 2000). It lies in the so called class I region, between the genes encoding the MHC class human leukocyte antigen (HLA) class II cell surface molecules (HLA-DP, DQ, and DR) and the MHC class I antigen (HLA-A, B, and C). The 5’ flanking region of the TNF-α gene contains multiple potential regulatory sites that respond to inflammatory stimuli.
Genetic factors may affect TNF-α level as shown by in vitro and in vivo studies. Differences in cytokine production may be partly attributed to the presence of single nucleotide polymorphisms (SNP) within or outside the gene. At least 12 SNP have been identified in the TNF-α locus, some of which have also been shown to influence the rate of transcription and production of TNF-α cytokine (Warle et al., 2003). The most commonly studied TNF-α polymorphism is the -308A/G, also known as TNF 1/2 (rs1800629) (Smith et al., 2009). The function of this SNP has been suggested by conflicting disease association studies rather than in vivo/vitro analysis (Bayley et al., 2004). The presence of the minor -308A allele has been found to be correlated with spontaneous or stimulated TNF-α production (Mira et al., 1999). Several studies suggested that the protein preferentially binding to the -308 promoter region of TNF-α is likely to be a transcriptional activator, although it has yet to be characterized. The -308A allele is strongly associated with the MHC haplotype HLA-A1-B8 and DR3, which is in turn associated with high TNF-α production. This genetic propensity to produce elevated TNF-α levels, due to the presence of the -308A polymorphism, may alter the course of an immune response (Kroeger et al., 1997; Abraham et al., 1999). In vitro studies using
different techniques (transfection with two variant construct cell lines, allele specific TNF-α transcript quantification, -308 tagging SNP within the TNF-α primary mRNA transcript) failed to demonstrate function in vitro for the -308 TNF-α SNP (Kaijzel et al., 2001). *In vivo* studies have demonstrated that the -308A TNF-α allele had higher transcriptional activity compared with the -308G allele (Louis et al., 1998). Other differences may be related to the type of cells and of stimuli used in these studies (Hajeer et al., 2000). Another possible functional promoter SNP is the -238G/A (rs361525) that is located within the TNF-α repressor site, but it has shown contradicting function (Fong et al., 1994). Earlier works demonstrated that the -238A allele is associated with higher TNF-α production with respect to the -238G allele in juvenile idiopathic arthritis (Louis et al., 1998), but this data has not been confirmed by other studies (Ozen et al., 2002). Moreover, another study demonstrated a faster radiological damage in -238 GG genotype with respect to the GA genotype (Brinkman et al., 1997).

2.10 Role of IL-6 in DFU

IL-6 is a pleiotropic cytokine with a key impact on both immuno-regulation and non-immune events in most cell types and tissues outside the immune system (Kamimura et al., 2003). Through a vast number of *in vitro* studies, epidemiological and genetic studies, the putative role of IL-6 in the pathogenesis of obesity, insulin resistance, β-cell destruction, T1DM and T2DM have been reported.

IL-6 belongs to the family of cytokines, including IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin-1, and cardiotrophin-like cytokine. These cytokines are characterized by their common use of the gp130 receptor, also known as IL-6R or CD130 as a signaling subunit. The human *IL-6* gene maps to chromosome 7p21 and has a high degree of sequence homology with the murine *IL-6*, in particular, in regulatory proximal promoter sequences (Fig. 19) (Kristiansen et al., 2003). There are several polymorphisms in and close to *IL-6* (Kristiansen et al., 2003; Terry et al., 2000). Genetic association between SNPs in the *IL-6* promoter viz., the *IL-6* -174G/C,
**IL-6 -572A/G** and **IL-6 -597A/G** has been investigated in T1DM, T2DM, insulin resistance and metabolic syndrome. The human **IL-6** protein comprises 212 amino acids with a signal peptide of 27 amino acids and two potential NH$_2$-linked glycosylation sites (Kamimura et al., 2003). The molecular weight ranges from 21 to 28 kDa.

![Schematic presentation of promoter region of IL-6 gene](image)

**Fig.19.** Schematic presentation of promoter region of IL-6 gene

In the text box above, the nucleotide sequence of the IL-6 gene promoter region from -398 to +1 with the A$_A$(n)T$_A$(n) promoter region polymorphism, the binding site for IL-6 5’ primer (underlined sequence), NlaIII restriction sites (black dot), AP-1 binding site (TGAGTCA), the site of -174G/C polymorphism (G/C), CAMP responsive element (CRE,ACGTCA), the binding site for IL-6 3’ primer (underlined sequence) are shown.

Thus, the biological outcome resulting from IL-6 exposure is complex and may result in a variety of physiological events such as cell proliferation, differentiation, survival, and apoptosis. The cells that can produce IL-6 include cells of the immune system, endothelial cells, skeletal and smooth muscle cells, adipocytes, islet β-cells, hepatocytes, microglial cells, astrocytes and a number of other cell types (Kamimura et al., 2003). IL-6 was initially thought to be a proinflammatory cytokine mainly with effects within the immune system, but this understanding of IL-6 was soon found to be too simplistic (Kamimura et al., 2003). In the adaptive and innate immune systems, IL-6 is involved in both amplification and protection against inflammation (Jones et al., 2001). Thus, inappropriate
regulation of IL-6 may be deleterious role in both auto immune diseases and in diseases where IL-6 or other inflammatory factors cause a low-grade inflammation (as seen in obesity and T2DM), which is likely to be involved in the pathogenesis of these diseases. The IL-6 gene is polymorphic in both 5’ and 3’ flanking regions (Fishman et al., 1998). Using luciferase reporter vector transiently transfected to HeLa cells, the biological function of the promoter region -174G/C base exchange polymorphism was deciphered. It was found that the G allele construct has a higher spontaneous IL-6 gene transcriptional activity and higher inducible IL-6 transcriptional response to LPS or IL-1 stimulus than the allele C construct (Fishman et al., 1998). Also the GG genotypes and G/C heterozygotes were found to have higher plasma IL-6 levels than CC homozygotes. This polymorphism was first observed in 92 patients with systemic onset juvenile rheumatoid arthritis when compared with 383 healthy controls. Results showed a reduced frequency of the potentially protective CC genotype (low IL-6 producer) among patients with an early onset of the disease. Subsequently the GG genotype (high IL-6 producer) has been found to be associated with plasma lipid profile like high triglycerides, very low density lipoproteins and free fatty acids (Fernandez-Real et al., 2000). The exact mechanism for these phenotypic differences linked to IL-6 genotypes is not known, but as the promoter region of IL-6 gene has binding sites for transcription factors like AP-1 and NF-Kb, it is likely that these differences in IL-6 production are caused by differences in the transcription factor binding capacities.

IL-6 is an important pathogenic factor in the development of diabetic microvascular complications such as retinopathy and diabetic neuropathy (Cameron et al., 2007; Funatsu et al., 2002; Haslbeck et al., 2004). Previous publications demonstrated that the -174G/C polymorphism is associated with the development of diabetes, insulin resistance and macrovascular complications (Kristiansen et al., 2005; Targher et al., 2001). One of these is up to now the largest association studies on the genetics of T2DM, which disclosed a modest association of the G allele of the -174G/C variant with the development of T2DM (Huth et al., 2006). IL-6 -174G/C SNP was found to be in linkage disequilibrium with the other IL-6 promoter polymorphisms. The IL-6 -174G allele is associated with
T2DM in the majority of studies investigating this SNP in populations informative for the SNP. The G allele of IL-6 -174 is found to be part of the haplotype that is associated with T2DM. Moreover, the haplotype was comprised of a rare composite genotype, AGC/GCG, conferring modest (odds ratio ~1.7) but with a high risk to T2DM (Hamid et al., 2005). The IL-6 -174G allele is strongly associated with risk (odds ratio with each additional G allele ~18) of T2DM in non-Pima Native Americans (Vozarova et al., 2003). The two studies of prospective risk of converting from impaired glucose tolerance to T2DM in Germans and Finns, respectively, are in striking contrast to the cross-sectional genetic studies. There is no simple genetic explanation for the observed discrepancies. Studies investigating the association between the IL-6 gene variants and T2DM have been contradictory. Of note, no overall association was found between -174G/C and T2DM in high-powered studies (Hamid et al., 2005). -174C allele was associated with risk of developing T2DM in subsets (Illig et al., 2004). -174G was associated with features of the metabolic syndrome in glucose-tolerant subjects (Hamid et al., 2005). It is unclear if this association can be explained by low IL-6 promoter activity in analogy to IL-6 -/- mice that develop maturity-onset obesity probably due to CNS effects on appetite and regulation of energy expenditure. Up to date, association analysis of the -174G/C polymorphism appears to be ambiguous. Numerous studies with contradictory results have not provided clear evidence for or against an influence of the C allele promoter variant on IL-6 transcriptional activity, pathogenesis of diabetes and/or insulin resistance (Festa et al., 2002; Mohlig et al., 2004). This could be due to different haplotypes with respect to other possible variants in the IL-6 promoter interfering in the promoter activity. Alternatively, it is possible that the studied SNP might be of no major functional relevance in influencing the development and progression of diabetic complications.

2.11 Role of SDF-1 in DFU

SDF-1/CXCL12 (Stromal Derived Factor-1) is a chemokine initially identified in bone marrow derived stromal cells and now recognized to be expressed in stromal tissues in multiple organs. SDF-1 is located on chromosome...
10q 11.1 (Fig. 20). The two isoforms of SDF-1 are (SDF-1α and SDF-1β) that arise from a single gene by alternative splicing (Winkler et al., 1998). Studies from diabetic patients revealed a common polymorphism in the 3’-untranslated region, implicated in mRNA turnover regulation of the SDF-1β gene transcript, which contains a G to A transition at position 801, designated SDF-1, 3’UTR-801G-A, abbreviated as SDF1-3’A (Kawasaki et al., 2004). The A allele is probably a target of cis-acting factors, and it is assumed to upregulate the expression of SDF-1 (Luan et al., 2010). It has been reported that SDF1-3’A genotype action involves up-regulation of the quantity of SDF-1 protein available to bind CXCR4 (C-X-C chemokine receptor type-4). The association of this polymorphism is well documented in T1DM (Ide et al., 2003) HIV-1 infection (Dean et al., 2002), cancer, acute myeloid lymphoma, leukemia, and chronic myeloproliferative disease and acute hepatitis (Colobran et al., 2007). To our knowledge, there are no reports describing any association between SDF-1 G801A SNP and pathogenesis of diabetic foot ulcer.

![Fig. 20. 801G/A region of SDF-1 gene](image)

As a chemo-attractant, SDF-1 attracts various kinds of immune cells to the site of inflammation. It is also essential for normal hematopoietic progenitor cell movement and adherence within the bone marrow microenvironment. Roles for SDF-1 have been indicated in vascular remodeling by recruiting smooth muscle cells, regulation of pituitary function, and neuronal generation (by promoting neuronal migration and axonal path finding). In addition, SDF-1 has been
implicated in inflammatory diseases and atherosclerosis. Differential roles for SDF-1 have been implicated in the development of diabetes. Elevated expression of SDF-1 in thymocytes may play a role in the development of autoimmune diseases in non-obese diabetic (NOD) mouse, a T1DM model (Mendes-da-Cruz et al., 2008). Neutralization of SDF-1 function in NOD mice with antisera against SDF-1 reduces insulitis and significantly delays the onset of diabetes (Matin et al., 2002). In contrast, transgenic over expression of SDF-1 in pancreas significantly increase the survival of β-cells and render normal mice more resistant to streptozotocin-induced β-cell inflammation and diabetes probably by promoting the survival and migration of progenitor cells in the pancreas (Yano et al., 2007). Furthermore, blockade of SDF-1 function with an antagonist of CXCR4 (AMD3100) leads to programmed cell death of insulin producing MIN-6 cells (Yano et al., 2007). These reports support the notion that SDF-1 directly protects pancreatic β cells from apoptosis.

![Fig.21. Wound healing in diabetic subjects](image)
Adapted from Brem et al., 2007
In healthy individuals, the acute wound healing process is guided and maintained through integration of multiple signals (in the form of cytokines and chemokines) released by keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. During wound-induced hypoxia, VEGF released by macrophages, fibroblasts and epithelial cells induce the phosphorylation and activation of endothelial nitric oxide synthetase (eNOS) in the bone marrow, resulting in an increase in nitric oxide levels, which triggers the mobilization of bone marrow EPCs to the circulation. The chemokine SDF-1 promotes the homing of these EPCs to the site of injury, where they participate in neo-angiogenesis. Gallagher et al., 2007 showed that, in a murine model of diabetes, eNOS phosphorylation in the bone marrow is impaired, which directly limits EPC mobilization from the bone marrow into the circulation. They also show that SDF-1 expression is decreased in epithelial cells and myofibroblasts in the diabetic wound, which prevents EPCs homing to wounds and therefore limits wound healing. Establishing hyperoxia in wound tissue via hyperbaric oxygen therapy (HBOT) was shown to activate many NOS isoforms, increase NO levels, and enhance EPC mobilization. (Fig. 21)

Derakshan et al., had shown that the frequency of the mutant AA allele to be increased in healthy controls when compared to T2DM patients among Iranian population (Derakshan et al., 2012). Another study from the northern part of Indian population had investigated the association of SDF-1 in high risk seronegative and HIV-1 positive patients (Chaudharya et al., 2008). The frequency of wild GG allele is found to be increased in HIV-1 positive patients when compared to healthy individuals, showing GG allele to be the risk factor for HIV-1 patients. On the other hand the frequency of mutant AA allele to be decreased in HIV-1 positive patients when compared to healthy individuals, showing AA allele to be the protective for HIV-1 patients. Another study on Greek population by Vairaktaris et al., had shown the prevalence of mutant AA allele frequency to be 25.3% in controls, and 23.2% and 12.5% in patients with cancer stages I &II and patients with cancer stages III & IV respectively (Vairaktaris et al.,2008). Djuric et al., had reported that AA allele of SDF-1 801G/A SNP are more frequent in T2DM subjects with proliferative retinopathy suggesting a possible role of this genotype in
diabetic retinopathy (Djuric et al., 2010). It has been reported that individuals with SDF-1 “A” allele have higher levels of the SDF-1 protein due to the up-regulating effect of this allele (Tashiro et al., 1993). Moreover, the “A” allele has also been shown to increase mobilization of CD34+ progenitor cells into peripheral blood in humans (Benboubker et al., 2001). SDF-1 “A” allele has been demonstrated to play a role in the microvascular manifestation in multiple sclerosis and thus may be one of the susceptibility factors that lead to disease. SDF-1 “A” allele variants have also been shown to play a role in metastasis of breast cancer (Hassan et al., 2008).

2.12 Role of HSP-70 in DFU

Oxidative stress, through the production of reactive oxygen species, has been proposed as the root cause underlying the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, and T2DM. Oxidative stress has also been implicated in the progression of long-term diabetes associated complications (Wright et al., 2006). Heat shock proteins (HSPs) are molecular chaperones synthesized under stress conditions; they are induced by denatured proteins during heat shock, ischemia, and other types of cellular stress. HSPs are important in physiological and pathological processes and are highly active within the immune system (Macario et al., 2000). HSPs help restore protein homoeostasis and assist cellular recovery from stress by repairing damaged proteins through refolding or by degrading. HSPs have also been reported to modulate insulin sensitivity and thus play a role in diabetes (Macario et al., 2000). The HSP-70 (70 kDa HSP) family is the most abundant in eukaryotic cells and is essential for cell survival under stress conditions (Daugaard et al., 2007). In human, 3 genes encoding members of the HSP-70 class are mapped within the MHC class III region (6p21.3): HSP-70-1 (HSPA1A) (OMIM: 140550), HSP-70-2 (HSPA1B) (OMIM: 603012) and HSP-70-Hom (HSPA1L) (OMIM: 140559). These genes are polymorphic, with some variants potentially accounting for a change in function and susceptibility to stress tolerance (Wu et al., 2004). HSP-70 SNPs are found to be major risk factors in several human diseases (Vargas-Alarcon et al., 2002; Bogunia-Kubik et al., 2006; Nam et al., 2002).
HSPs are among the most abundant intracellular proteins. Although expressed at low levels under normal physiological conditions, HSPs show dramatically increased expression in response to cellular stress. HSPs function primarily as molecular chaperones, facilitating the folding of cellular proteins, preventing protein aggregation, translocation of proteins or targeting improperly folded proteins to specific pathways for degradation (Morimoto et al., 1998). In case of severe damage, HSPs direct damaged proteins for degradation within the proteasome system (Morimoto et al., 1998). HSPs play a key role in facilitating immune responses, because they can bind antigenic peptides and transport them to antigen-presenting cells and T lymphocytes (Srivastava et al., 2002). HSPs are also capable of binding to adjacent cells, initiating signal transduction. The heat shock protein attenuates pro-inflammatory mechanisms and inducible nitric oxide synthase activity. While intracellular HSP induction in response to pro-inflammatory stimuli can exert anti-inflammatory effects, extracellular HSPs may signal danger, activating immune cells (Chen et al., 2007). Because of the overlap in their functions, HSPs have been classified into families according to their rough molecular weight and homology like HSP-100, HSP-90, HSP-70, HSP-60, HSP-40 and small HSPs.

The HSP-70 family comprises eight isoforms, namely different forms of HSP-72, HSPA-2, Grp-78, HSP-70B, HSP-73 and Grp-75 (Daugaard et al., 2007; Tavaria et al., 1996). The isoforms of HSP are located mainly in the cytosol and nucleus, but they have also been detected in the lysosomes and endoplasmic reticulum (Daugaard et al., 2007). Grp-75 has also been detected in the mitochondria (Tavaria et al., 1996). HSP-72 is the major inducible HSP found in the nucleus and cytosol. HSP-72 requires ATP for its chaperone activity and minimizes aggregation of newly synthesized proteins. Moreover, it is also capable of inhibiting stress induced apoptosis (Mosser et al., 2000), even after the activation of effector caspases. In chronic wounds, such as ulcers and in post-traumatic wounds, the expression of HSP-70 protein is found to be lower. In contrast, in healing wounds with rich granulation tissue the expression of HSP-70 is high. Of note, there is an inverse correlation between HSP-70 levels and the
latency of the healing process, and HSP-70 and essential wound healing related
growth factors are expressed in a co-ordinated manner during the initial phase of
healing at 7-14 days (Shukla et al., 1998). HSP-70 is passively released after cell
death or plasma membrane wounding and also actively secreted via the exosome
system. After skin wounding there are significant differences in the expression
pattern of different HSPs (Keagle et al., 2001). Whereas HSP-72 and HSP-32 are
expressed only in the epidermis, HSP-47 is expressed in both the epidermis and
dermis and only after skin wounding has occurred (Keagle et al., 2001).

The regulation of HSP synthesis is controlled mainly by a major
transcription factor heat shock factor-1 (HSF-1) which binds to the heat shock
elements (HSE) present in promoter region of the specific genes.
Post-transcriptional mechanisms are also implicated in the regulation of HSP
synthesis. Under physiological conditions, HSF-1 monomers are co-localized with
HSP-70 in the nucleus. HSF-1 is activated by cellular stress (Anckar et al., 2007).
The activation process involves trimerization of HSF-1 monomers, translocation of
the trimers, hyper phosphorylation and binding to the promoter of heat shock
genes. The end-products of this process, such as HSP-70, exert negative feedback
regulation. The post transcriptional mechanism involves stabilization of HSP-70
mRNA. HSF-1 and NF-kB signaling are tightly linked because in certain
conditions, HSF-1 inhibits NF-kB activation. In addition to protein denaturation,
stress signals may also originate from cell membranes. Recently it has been
proposed that the lipid composition and the architecture of membranes act as
membrane sensors and modulate HSP response through the activation of HSF-1
(Vigh et al., 2007).

Both T1DM and T2DM are characterized by an increased risk for the
development of microvascular and macrovascular complications. In diabetes,
endogenous defense systems are overwhelmed, causing various types of stress.
Among the other important conditions related to diabetes are dyslipidemia,
modification of proteins and lipids, and perturbations in the tissue antioxidant
defense network (Gul et al., 2002). These disturbances are exacerbated in diabetes
with microvascular complications such as nephropathy, retinopathy and
neuropathy. The antioxidant functions of HSP should therefore prove to be helpful in fighting diabetic complications. Indeed, the crucial role of HSPs in diabetes is highlighted by their ability to counteract denaturation of tissue proteins and facilitate cellular repair and defense mechanisms. A number of factors contribute to wound healing deficiencies in individuals with diabetes. Hyperglycemia leads to increased glycosylation of immune cells, such as neutrophils and macrophages by inhibiting their normal function and predisposing both to chronic inflammation and increased susceptibility to infection. Glycosylation of erythrocytes increases their rigidity, which may predispose to sludging and local ischemia in the microvasculature. Susceptibility to ulceration and impairment of wound healing in diabetes increases dramatically when accompanied by diabetic peripheral neuropathy and PVD. These complications predispose to microtrauma, foot deformities and ischemia (Rathur et al., 2007). Little is known about the role of HSPs in the abnormal wound healing in diabetes. The diabetic state results in delayed expression of HSP-70 at the protein level, despite mRNA up regulation in the epithelial cells and inflammatory cells during the wound healing process (McMurtry et al., 1999). On the other hand, HSP-70 expression in the wound bed in diabetic mice increases after a delay, suggesting that the poorly healing, chronically open wound may result in a more potent HSP induction than a normally healing wound. Similarly, in skin fibroblasts isolated from a patient with diabetic nephropathy, the levels of HSP-70, HSP-60 and HSP-27 are found to be increased (Tessari et al., 2007). This effect was prevented by administration of insulin and normalization of blood glucose level. According to these results, it seems clear that the expression of HSP-70 is altered in diabetes and its later complications.