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CLINICAL STUDY

# Endothelin 1, NF-κB, and ADAM-15 expression in diabetic foot wounds

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**ABSTRACT**

OBJECTIVES: The diabetic foot is an important and destructive complication of diabetes. This study examined 65 individuals (35 males, 30 females) diagnosed with diabetic foot with open wounds on their feet.

METHODS: After washing the foot with isotonic solution, the wound was debrided and the excised tissues (diabetic dermis) were fixed with neutral buffered 10 % formalin solution. Specimens stained with haematoxylin-eosin, endothelin-1 (ET-1), nuclear factor kappa B (NF-κB), and ADAM 15 antibodies were used to examine angiogenesis, cytokine activity, and the extracellular matrix, respectively.

RESULTS: Histopathologically, in the diabetic feet of males, leukocytes, lymphocytes, and macrophages were spread diffusely throughout the lesion, with inflammatory cells invading all layers of blood vessels. In the diabetic feet of females, dilation and congestion of the blood vessels, degenerative changes in the subendothelial layer, and perivascular infiltration by lymphocytes were observed. ET-1 was expressed in inflammatory cells around pre-capillary vessels in the stromal area. NF-κB was expressed in macrophages around blood vessels, and in nodular organised cells distributed throughout the perivascular space. ADAM 15 were expressed in fibroblasts, endothelial cells, and inflammatory cells. Blood parameters differed significantly between diabetic and non-diabetic patients (Significant at $p < 0.005$, $p < 0.001$ and $p < 0.0001$)

CONCLUSION: ET-1 expression in the endothelial cells of diabetic foot ulcers is an important determinant of insulin resistance at the onset of disease and induces macrophages to produce NF-κB, which regulates inflammation. It is thought that ADAM 15 contributes to angiogenic effects as a means of stimulating endothelial cells *(Tab. 2, Fig. 3, Ref. 31).* Text in PDF *www.elis.sk.*

KEY WORDS: diabetic foot, endothelin-1, NF-κB, ADAM-15.

## Introduction

Metabolic complications of diabetes in the lower limb include ulceration of the feet, infection, and the destruction of deep tissues, in turn associated with neurological abnormalities and varying degrees of peripheral vascular disease. Diabetic foot infections are most frequently described as an inflammatory response and tissue injury due to an interaction between the host and microbial pathogens (1, 2). Systemic complications, such as failure to heal, can lead to amputation in diabetic patients as a result of the vicious cycle between wound chronicity and inadequate local infection control; of all non-traumatic lower-extremity amputations, 85 % are performed in diabetics (3). Type 2 diabetes exerts its pathogenic effects via two important pathways. First, as in other organs, there is a pathway involving chronic hyperglycaemia, oxidative stress in joint tissues, overexpression of inflammatory cytokines and advanced end glycation products, and reduced differentiation potential of stem cells. Second, insulin resistance has local and systemic effects, as in the case of low-grade inflammation (4). Osteoarthritis is a degenerative process that affects the joints and is characterised by worn joint cartilage, altered subchondral and peri-cartilage bone, mild-to-moderate synovial inflammation, and pain (5). Non-healing microfractures in diabetics may alter bone mechanics, promote osteoarthritis and contribute to poor arthroplasty outcomes. Many clinical studies have demonstrated an increased fracture risk in post-menopausal women with type 2 diabetes mellitus that is not linked solely to low bone mineral density on clinical densitometry (6, 7). Vascular insufficiency results in decreased neutrophil migration, loss of tissue viability, and delayed wound healing. One consequence of not being able to control blood sugar is that a breakdown in neutrophil function causes impaired wound healing and inadequate colloidal production, ultimately leading to chronic wound healing deficits.

Endothelin signalling is thought to act through two types of signalling cascades, either through a short term action characterized by second messenger signals (involved in vascular contraction and/or secretion) or through a long term action characterized by pathways of cytosolic and nuclear signalling (involved in cell growth). Studies have been done in various tissues and species. Endothelin-1 (ET-1) is produced primarily in the endothelium, al-

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though some ET-1 is also produced in vascular smooth muscle cells, macrophages, leukocytes, cardiomyocytes, and fibroblasts (8, 9).

NF-κB is known to regulate hundreds of genes involved in many important cellular responses such as inflammation, migration, proliferation and apoptosis. So the obvious question is how a single transcription factor family can regulate so many different genes. Apparently, the early cytoplasmic events leading to the release and translocation of NF-κB into the nucleus are not sufficient to explain the complex nature of NF-κB biology (10, 11). Dysregulation of NF-κB-induced inflammation induces inflammatory and neurodegenerative diseases (11). ADAMs are a family of membrane-bound proteinases that bind integrins along the disintegrin domain (a metalloproteinase domain) and are responsible for cell adhesion, cell fusion, proteolysis, and signal transduction (12). ADAM15 plays a role in neovascular diseases, atherosclerotic plaque formation, and new-vessel formation within the rheumatoid synovium (13).

This study examined the relationships among inflammation, angiogenic effects, cell–cell interactions, and the cell membranes of wound tissues in the feet of patients with type 2 diabetes using biochemical and immunohistochemical methods.

## Materials and methods

*Human skin tissue samples*

Diabetic dermis tissue samples were only obtained from 65 patients (females; aged 38–55 years; males; aged 37–56 years) with diabetes. In order to statistically compare blood and biochemical parameters between diabetic and nondiabetic patients, 65 normoglycemic patients (35 females, 30 males) who needed debridement due to trauma in foot were included in this study. All normoglycemic patients had no medical history of diabetes (fasting blood glucose and glycosylated hemoglobin in the normal range) and did not suffer from general infection or cardiovascular or renal diseases. Dermis tissue samples were not obtained from normoglycemic patients. Because the tissues were healthy in terms of diabetes and there was no ethic committee approval. All tissue samples from diabetic patients included a 1-cm margin surrounding the wound. This study enrolled 65 patients (35 females, 30 males) with open wounds on their feet and a diagnosis of diabetic foot, seen in orthopaedic and endocrinology clinics between April 2017 and January 2018. The mean age of the men and women was 62 years (range: 50–67 years) and 56 years (range: 48–67 years), respectively. The study was approved by the hospital ethics committee and the patients provided informed consent (2017/24).

In the supine position, the patients underwent surgical debridement of the open wound after the foot was washed with isotonic saline and the excised tissues were sent for examination. Samples were fixed with neutral buffered 10 % formalin, dehydrated in a graded ethanol series, and embedded in paraffin. Then, 5-mm sections were cut with a microtome (RM 2265 rotatory microtome; Leica, Germany) and mounted on coated slides. The sections were stained with haematoxylin-eosin for light microscopy examination.

*Immunohistochemical technique*

Formaldehyde-fixed tissue was embedded in paraffin for immunohistochemical examination. The sections were deparaffinised in absolute alcohol. Antigen retrieval was performed twice in citrate buffer solution (pH 6.0), first for 7 minutes and then for 5 minutes, and boiled in a microwave oven at 700 W. The specimens were allowed to cool to room temperature for 30 minutes and were then washed twice in distilled water, for 5 minutes each time. Endogenous peroxidase activity was blocked with 0.1 % hydrogen peroxide for 20 minutes. Ultra V block (Cat. no. 85-9043; Invitrogen, Carlsbad, CA, USA) was applied for 10 minutes prior to overnight application of the primary antibodies: ET-1 antibody (1:100; Invitrogen), NF-κB antibody (1:100; Invitrogen), and ADAM-15

**Tab. 1. Blood and biochemical parameters of the control and diabetic males and females.**

| | Males | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Controls (n:30) | | | | Diabetes group (n:30) | | | | p |
| | Mean | Min | Max | SD | Mean | Min | Max | SD | |
| WBCs | 13.01 | 9.36 | 15.82 | 1.92 | 8.91 | 6.78 | 10.30 | 1.09 | <0.005* |
| RBCs | 4.77 | 3.34 | 7.69 | 1.28 | 3.94 | 2.98 | 5.78 | 0.96 | <0.005* |
| Glucose | 87.67 | 81.54 | 100.00 | 4.55 | 116.95 | 81.00 | 294.00 | 47.86 | <0.001** |
| ALP | 82.27 | 74.36 | 92.36 | 5.02 | 110.56 | 63.00 | 192.00 | 29.96 | <0.001** |
| CK | 40.98 | 30.04 | 52.16 | 7.44 | 118.75 | 24.00 | 296.00 | 74.29 | <0.005* |
| CRP | 0.39 | 0.20 | 0.99 | 0.19 | 7.91 | 2.36 | 12.45 | 2.37 | <0.001** |
| | Females | | | | | | | | |
| | Controls (n:35) | | | | Diabetes group (n:35) | | | | p |
| | Mean | Min | Max | SD | Mean | Min | Max | SD | |
| WBCs | 12.59 | 8.73 | 15.99 | 1.78 | 7.98 | 5.17 | 10.11 | 1.09 | <0.0001*** |
| RBCs | 4.74 | 4.76 | 7.55 | 1.19 | 4.02 | 3.01 | 3.84 | 0.94 | <0.0001*** |
| Glucose | 86.35 | 80.79 | 98.45 | 4.33 | 115.35 | 78.33 | 290.17 | 46.91 | <0.001** |
| ALP | 80.37 | 70.16 | 94.38 | 3.57 | 104.92 | 59.99 | 195.88 | 30.14 | <0.001** |
| CK | 41.01 | 25.35 | 54.36 | 7.13 | 113.33 | 22.91 | 294.78 | 73.81 | <0.005* |
| CRP | 0.38 | 0.15 | 0.96 | 0.14 | 8.01 | 2.90 | 12.91 | 2.81 | <0.001** |

Data are expressed as the mean ± standard deviation.* $p < 0.005$, ** $p < 0.001$, *** $p < 0.0001$, WBCs – white blood cells; RBCs – red blood cells; ALP – alkaline phosphatase; CK – creatine kinase; CRP – C-reactive protein

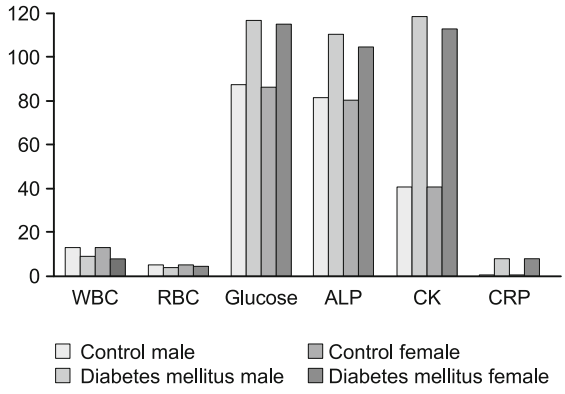


**Fig. 1. Graphical representation of the blood parameter values of control and diabetic males and females.**

antibody (1:100; Invitrogen). Secondary antibody (Cat. no. 85-9043; Invitrogen) was applied for 20 minutes. Then the slides were exposed to streptavidin–peroxidase for 20 minutes. Chromogen diaminobenzidine (DAB, Cat. No. 34002, Invitrogen) was used. Control slides were prepared as described above, omitting the primary antibodies. After counterstaining with haematoxylin, and washing the slide in tap water for 8 minutes and in distilled water for 10 minutes, the slides were mounted with Entellan (Cat. no. 107961; Sigma-Aldrich, St. Louis, MO, USA).

*Statistical analysis*

Statistical analysis was performed with SPSS for Windows software (ver. 15.0; SPSS Inc., Chicago, IL, USA). The Mann–Whitney *U* test was used as appropriate and the results were expressed as means ± SD. $p < 0.05$ were considered to indicate statistical significance.

## Results

*Blood and biochemical findings*

Blood and biochemical parameters of the control group and male and female diabetic groups were compared. The results are shown in Table 1.

*Histological findings*

The structural features and cellular components of the diabetic foot skin lesions of the male and female patients were evaluated histopathologically. In the male patients, leukocytes, lymphocytes, and macrophages were spread diffusely throughout the lesion, with inflammatory cells invading all layers of the blood vessels, especially small blood vessels, and with panarteritis seen in the ligament tissue. Nodular structures were observed in the perivascular area. Degeneration of collagen fibres in the lesion and desquamation of some sweat gland cells were seen. In the dermis, increased inflammation was accompanied by hypertrophic fibroblasts and structural disruption of the extracellular matrix (Fig. 3a). In the female patients, dilation and congestion in the blood vessels were seen, in addition to degenerative changes in the subendothelial layer and perivascular infiltration of lymphocytes. Disorganisation and hyalinisation of collagen fibrils and inflammatory cell accumulation were seen (Fig. 3b).

**Tab. 2. Immunohistochemical expression scores in male and female diabetic foot patients**

| Patient groups | Endothelin-1 | NF-κB | ADAM-15 |
|---|---|---|---|
| Male diabetic foot | 4.0±0.2 | 3,20±0.9 | 3.78±0.2 |
| Female diabetic foot | 3.89±0.8 | 3.68±0.4 | 3.82±0.3 |

0 – none, 1 – weak, 2 – middle, 3 – strong, 4 – strongest. Data are expressed as the mean ± standard deviation (it was not statistically significant protein expression between male and female diabetic food, $p > 0.05$)

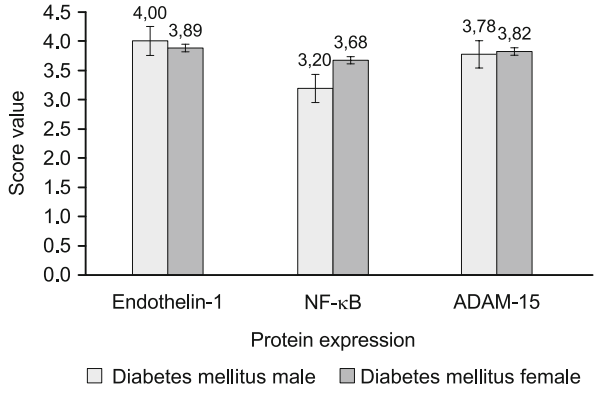


**Fig. 2. Histogram showing histopathological differences and protein expression levels in diabetic groups (Scoring was determined by examining Endothelin-1, NF-κB and ADAM-15 levels in the excised tissues in 10 different regions within the microscope field).**

*Immunohistochemical findings*

In the male patients, endothelial cells of the dilated blood vessels and inflammatory cells around the subendothelial and perivascular areas were positive for ET-1 (Tab. 2). ET-1 expression was increased in fibroblasts and inflammatory cells present among collagen fibrils in the connective tissue (Fig. 3c). In female patients, ET-1 was expressed in the endothelial cells of the blood vessels, in scattered inflammatory cells located in the subendothelial layer, and in inflammatory cells and fibroblasts in the connective tissue in the perivascular area (Fig. 3d). In male patients, NF-κB was expressed in macrophages around blood vessels, and in nodular cells in the perivascular space (Fig. 3e). In female patients, NF-κB was expressed in fibroblasts located near and within the granulation tissue (Fig. 3f). In male patients, ADAM-15 was expressed in endothelial cells, in the basal membranes of blood vessels, and in inflammatory cells and fibroblasts in the lesion (Fig. 3h). In female patients, ADAM-15 expression was increased in the endothelial cells of dilated blood vessels, and especially in the basal membrane (Fig. 3g).

## Discussion

Diabetes mellitus is an increasingly prevalent metabolic disease with systemic and chronic complications. Diabetic foot re-

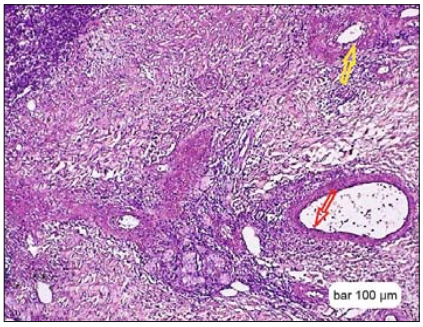


**Fig. 3a. Diabetic group hematoxylin and eosin staining. Diabetic foot of a male patient shows intense inflammatory cell infiltration, with inflammatory cells invading blood vessel layers (red arrow), ligamentous tissue showing panarteritis and degeneration occurring in some sweat gland cells (yellow arrow). Scale bar = 100 µm.**

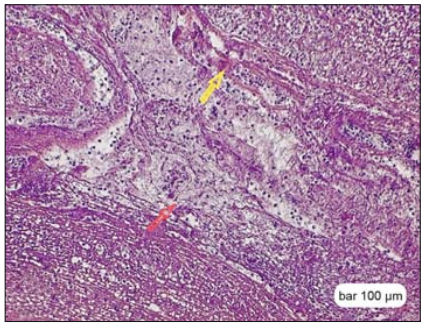


**Fig. 3b. Diabetic group hematoxylin and eosin staining. Diabetic foot of a female patient shows dilation and congestion in the blood vessels, degenerative changes in the subendothelial layer, perivascular infiltration of lymphocytes (yellow arrow), disorganisation and hyalinisation in collagen fibrils and an accumulation in inflammatory cells (red arrow). Scale bar = 100 µm.**

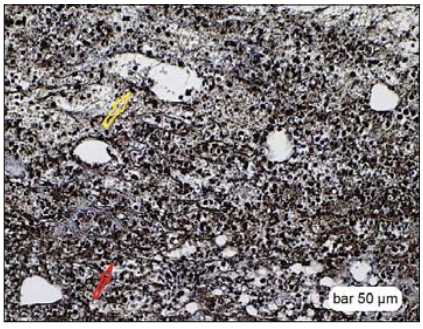


**Fig. 3c. Diabetic group endothelin-1 immunostaining. Foot of a male patient shows endothelin-1 (ET-1) expression in the endothelial (yellow arrow), and inflammatory cells in the subendothelial and perivascular areas (red arrow). Scale bar = 50 µm.**

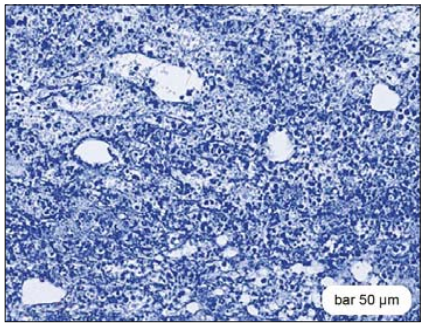


**Fig. 3c*. Diabetic group negative control hematoxylin staining. Scale bar = 50 µm.**

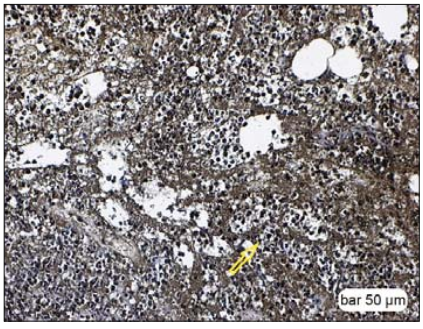


**Fig. 3d. Diabetic group endothelin-1 immunostaining. Foot of a female patient shows ET-1 expression in the endothelial cells, inflammatory cells in the subendothelial layer, and fibroblasts in the connective tissue of the perivascular area. Scale bar = 50 µm.**

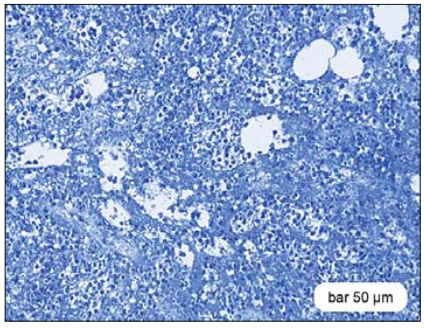


**Fig. 3d*. Diabetic group negative control hematoxylin staining. Scale bar = 50 µm.**

sults from peripheral artery disease and neuropathy, leading to ischemia and infection. Diabetes is accompanied by neuropathic degeneration, vasculopathy, infection, and poor wound healing due to defective collagen production. Diabetics also show decreased fibroblast and endothelial cell proliferation, reduced epithelisation, decreased collagen production, and reduced tensile forces (14). In the diabetic foot tissues of our male patients, leukocytes, lymphocytes, and macrophages were spread diffusely throughout the lesion, with inflammatory cells invading all blood vessel layers, and especially small blood vessels. The diabetic foot of females showed dilation and congestion in the blood vessels, degenerative changes in the subendothelial layer, and perivascular infiltration of lymphocytes. Disorganisation and hyalinisation in collagen fibrils were also seen, in addition to inflammatory cell accumulation (Fig. 3a, b).

Various growth factors play roles in diabetic foot wound healing. The accumulation of collagen IV and macrophages in connective tissue facilitates the healing of diabetic foot ulcers and improves the activity of actin proteins in smooth muscle (3). Histopathologically, diabetic ulcers show neuropathic lesions that are "frozen" in a chronic low-grade inflammatory condition in association with a transient extracellular matrix (15, 16). Persistent hyperglycaemia inhibits wound healing in diabetics, despite fibroblasts, pericytes, keratinocytes, and endothelial cells all being common, because proximal factors impair physiology (17). Endothelial cell dysfunction precedes the appearance of microvascular lesions. Vasoconstriction is associated with marked changes in microvascular blood flow, vascular permeability, and changes in the antithrombotic properties of endothelium (18). In diabetics, endoneurial microangiopathy and basal membrane thickening are important clinical findings of neuropathy. Endoneurial capillary microangiopathy inhibits glucose tolerance and has been reported to be an early and persistent feature of the processes underlying diabetic peripheral neuropathy (19). Venous occlusion together with elevated glucose levels delays wound healing, resulting in nerve entrapment in the ulcer and degenerative changes in peripheral nerves (20, 21). ET-1 levels are increased in diabetic patients when compared with non-diabetic controls, while endothelial receptors are upregulated (22). We found that ET-1 expression was high in vascular endothelial cells, and in inflammatory cells around pre-capillary

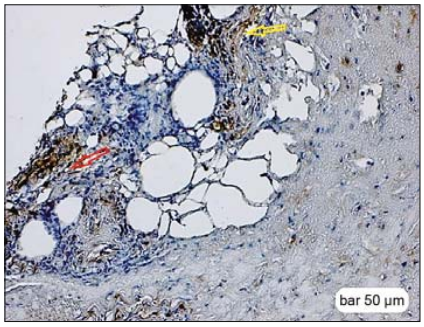


**Fig. 3e. Diabetic group nuclear factor kappa B immunostaining. Nuclear factor kappa B (NF-κB) expression in macrophages around blood vessels (yellow arrow), and in inflammatory cells in the perivascular space, in a male patient (red arrow). Scale bar = 50 µm.**

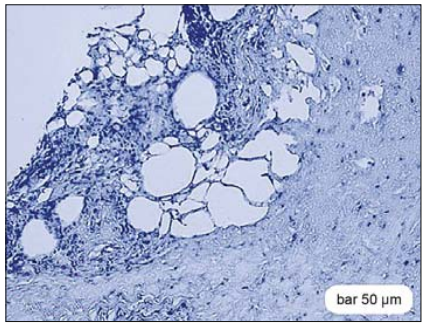


**Fig. 3e*. Diabetic group negative control hematoxylin staining. Scale bar = 50 µm.**

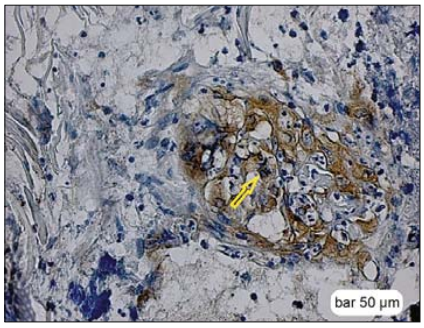


**Fig. 3f. Diabetic group nuclear factor kappa B immunostaining. NF-κB expression in fibroblasts around and within the granulation tissue (yellow arrow). Scale bar = 50 µm.**

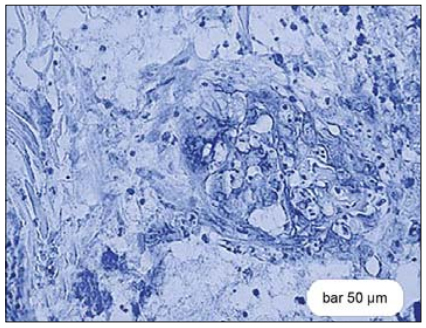


**Fig. 3f*. Diabetic group negative control hematoxylin staining. Scale bar = 50 µm.**

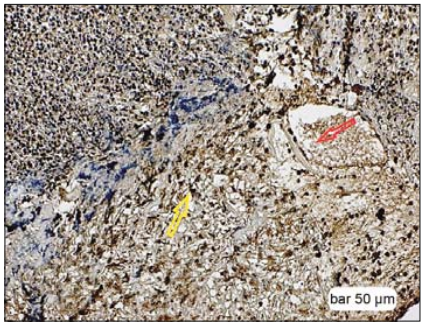


**Fig. 3g. Diabetic group ADAM-15 immunostaining. Disruption of ADAM-15 expression in female patients, increased ADAM-15 expression in connective tissue fibroblasts (yellow arrow), and leukocytes in granulation clusters (red arrow). Scale bar = 50 µm.**

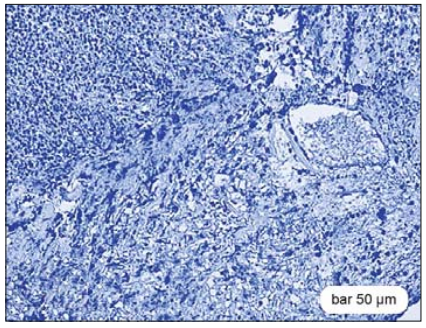


**Fig. 3g*. Diabetic group negative control hematoxylin staining. Scale bar = 50 µm.**

vessels in the stroma. ET-1 expression in pre-capillary vessels in type 2 diabetics results in deterioration of the arterial–venous connection of vasoconstrictor end-feeding capillaries, leading to irregular blood flow in the veins. ET-1 is an important vasoconstrictor that helps to regulate the vasculature via proinflammatory and profibrotic effects. ET-1 release in the endothelial cells of diabetic foot ulcers is an important determinant of the onset of the disease and is thought to have an important role in determining insulin resistance.

NF-κB is one of the most important regulators of proinflammatory gene expression, enhancing the expression of degradative enzymes that rearrange the matrix in cytokine synthesis (23). Activation of the pro-inflammatory pathway also causes insulin resistance and type 2 diabetes. NF-κB activation is correlated with inflammatory disease, but it is difficult to relate NF-κB activity to inflammatory disease because both inflammatory and anti-inflammatory mediators are produced, and disease progression occurs when the balance between these factors is compromised (24). Chronic inflammation inhibits insulin sensitivity via the activation of pathways that are directly associated with the key components of the insulin signalling pathway. This inflammatory response impairs insulin sensitivity by activating the Toll-like receptor (TLR) family, particularly TLR4 (25). TLR activation results in NF-κB activation, and activated NF-κB may affect insulin signalling by stimulating the transcription of various inflammatory genes, such as interleukin (IL)-6, cyclooxygenase (COX)-2 and tumour necrosis factor (TNF)-α (26).

Inflammation in general and pro-inflammatory cytokines in particular play an important role in the function of our immune system. In addition, it has been observed that most tumors' microenvironments are present with immune cells. Inflammation surrounding tumors has been suggested to confer many necessary properties for the growth and the development of those tumors such as proliferation, angiogenesis as well as metastasis. Other important role of inflammation and proinflammatory cytokines involves apoptosis. And, NF-κB is considered a prototypic proinflammatory signalling pathway and plays a role in the expression of proinflammatory genes, including cytokines, chemokines and adhesion molecules. In our study, NF-κB expression was seen in male and female diabetics, in nodular organising cells dispersed in macrophages around blood vessels and in the perivascular space, and in fibroblasts near granulation tissue. NF-κB, an important regulator of inflammation, is induced both in the granulation zone and in macrophages. It is thought to play an important role in wound healing.

ADAM-15 is expressed on the surface of endothelial cells in blood vessels (27). It is capable of digesting gelatine and type IV

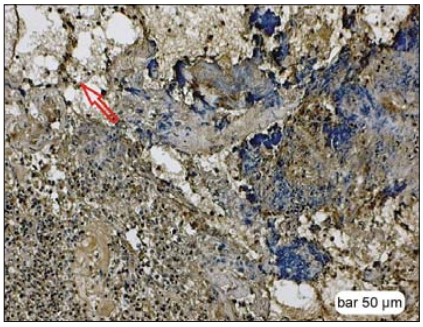


**Fig. 3h. Diabetic group ADAM-15 immunostaining. Disruption of ADAM-15 expression in male patients, ADAM-15 expression in endothelial cells and the basal membrane of blood vessels(red arrow). Scale bar = 50 µm.**

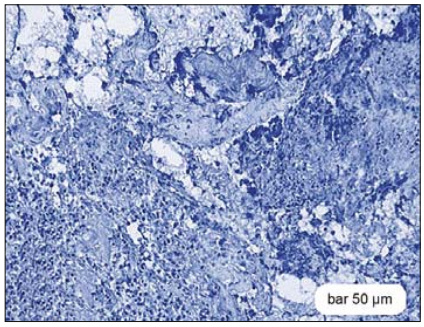


**Fig. 3h*. Diabetic group negative control hematoxylin staining. Scale bar = 50 µm.**

collagen (28), which are necessary for endothelial cell sprouting and migration. The role of ADAM-15 in endothelial cells was first examined in vascular diseases and was subsequently shown to be associated with atherosclerosis (29). ADAM-15 as the most highly over-expressed protease in prostate cancer progression (30), and later studies demonstrated that ADAM-15 was critical for tumor maintenance, cancer cell-endothelial interaction and metastasis (31). We observed ADAM-15 expression in endothelial cells, inflammatory cells, and fibroblasts in the diabetic feet of males and females. ADAM-15 expression was increased in the basal membrane junctional complex between the cell and the membrane, which must be organised so that immune cells can migrate. For migration of inflammatory cells, the extracellular matrix needs to be reshaped if the endothelial cells and endothelial-basement membrane are disrupted. Although ADAM-15 can function as a mediator of diabetic inflammation, it may also induce endothelial cells, contributing to an angiogenic effect. It could also be used therapeutically to prevent inflammation.

In conclusion, ET-1 expression in the endothelial cells of diabetic foot ulcers is an important determinant of insulin resistance at the onset of the disease, and also induces macrophages to express NF-κB, a regulator of inflammation. It is thought that ADAM 15 contributes to angiogenic effects as a means of stimulating endothelial cells.

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