Faculty of Health Sciences
Metabolic and Renal Research Group

Wound healing in diabetes

An intervention study in db/db mice

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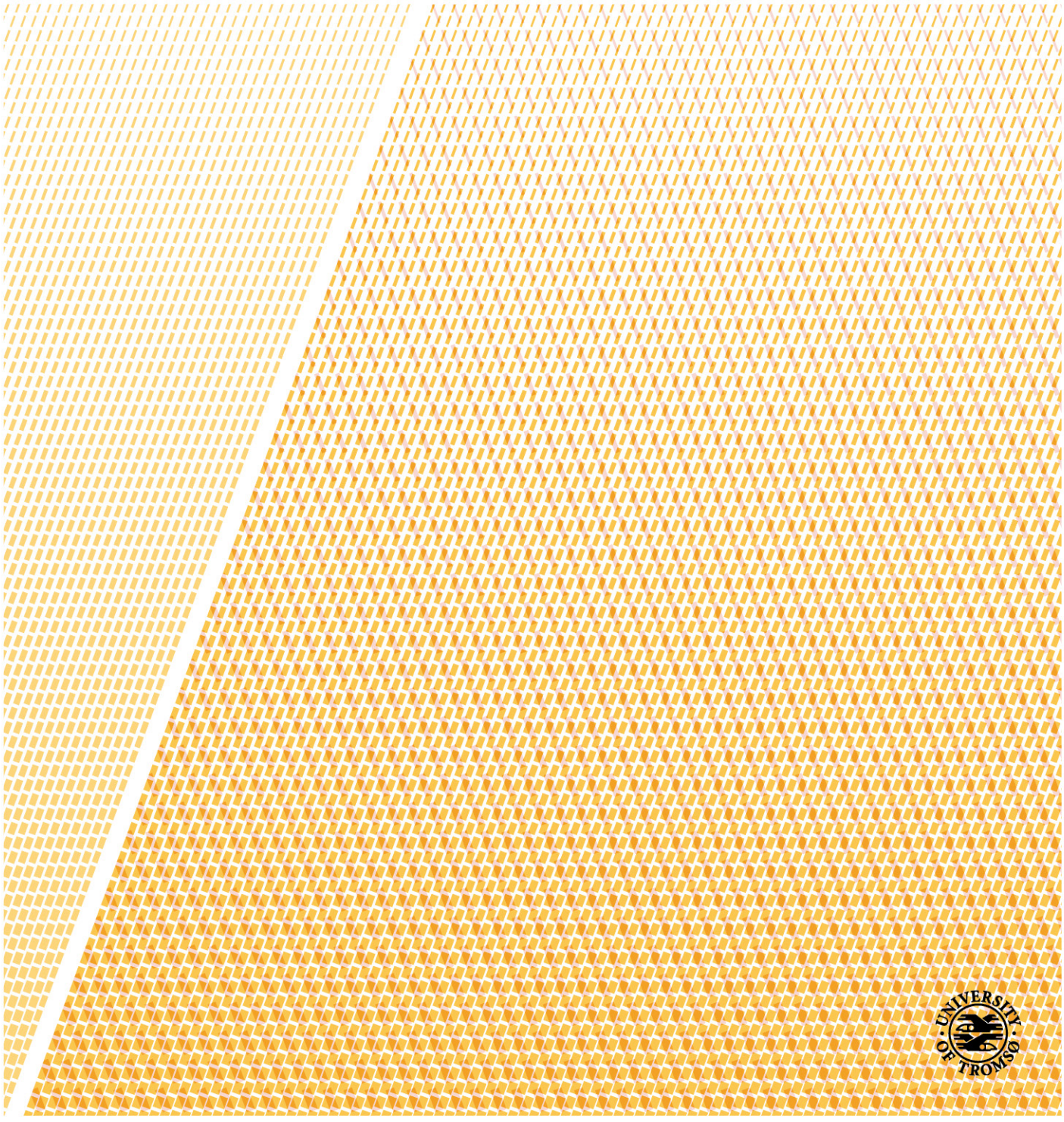
Margrete Berdal

A dissertation for the degree of Philosophiae Doctor – January 2017



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ACKNOWLEDGEMENTS

The work as presented in this thesis was carried out in the Metabolic and Renal Research Group and in Department of Comparative Medicine, UiT The Arctic University of Norway.

I would like to thank all who have been involved in my work, and in particular:

I wish to express my deepest gratitude to my supervisor Trond Jenssen for introducing me into science and for skilful guiding me during these years of work. Your efforts have been crucial. I would also like to heartily thank my co-supervisor Rolf Seljelid for introducing me into science and providing us with aminated-β-1,3-D-glucan for the experiments.

Åse Lund, Hege Iversen Appelbom, and late Jorunn Eikrem have been my extraordinary important colleagues in the technical work of the long-lasting animal experiments. Thank you so much for your excellent work, your positive attitude, and for always creating a positive atmosphere.

The staff at the Department of Medical Biology ("Exp.pat.") should be appreciated for always kindly helping me, in particular Wenche Helen Bakkelund for introducing me into cell biological techniques, Anja Väpsä for preparing the tissue specimens, and Baldur Sveinbjørnsson for assistance in the lab and, together with Robert Hanes, being crucial in the accomplishment of the immunohistochemical wound analysis.

I would like to thank the staff in Department of Comparative Medicine, in particular Nina Løvhaug and Ragnhild Hansen Osnes, for their good animal care and technical assistance.

My nice colleagues at "pauserommet", you know who you are, I would like to heartily thank you for interesting discussions, humoristic stories, chatting, and laughter. You encouraged me to keep on working.

The great office company of Hilde-Merete Storhaug, Manar Kalaaji, Dmitri Svistounov, and Silje Småbrekke is very much appreciated.

My family, my husband Bernt, and our sons Bernhard, Magnus, and Sigbjørn, I would like to thank you so much for your love and joy in my life.

This work was supported by grants from the Norwegian Diabetes Association and the Norwegian Research Council.

LIST OF PAPERS

Paper 1 Berdal M, Appelbom HI, Eikrem JH, Lund Å, Zykova S, Busund L-T, Seljelid R, Jenssen T. Aminated β-1,3-D-glucan improves wound healing in diabetic *db/db* mice. *Wound Rep Reg* 2007; 15: 825-832

Paper 2 Berdal M, Appelbom HI, Eikrem JH, Lund Å, Busund L-T, Hanes R, Seljelid R, Jenssen T. Aminated β-1,3-D-glucan has a dose-dependent effect on wound healing in diabetic *db/db* mice. *Wound Rep Reg* 2011; 19: 579-587

Paper 3 Berdal M, Jenssen T. No association between glycemia and wound healing in an experimental *db/db* mouse model. *ISRN Endocrinology* 2013: 1-6

Paper 4 Berdal M, Jenssen T. Effects of AGE inhibition with aminoguanidine in a diabetic *db/db* mouse wound model. *J Diabetes Mellitus* 2014; 4: 107-114

ABBREVIATIONS

AGE	advanced glycation end product
HbA_{1c}	glycated haemoglobin A_{1c}
DFU	diabetic foot ulcer
IGF-1	insulin-like growth factor-1
MMP	matrix metalloproteinase
NO	nitric oxide
PDGF	platelet-derived growth factor
RAGE	receptor for advanced glycation end products
RCT	randomized controlled trial
ROS	reactive oxygen species
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor

SAMMENDRAG

Betaglukaner kan bedre sårtilheling ved diabetes

Studier som er utført på mus ved UiT Norges arktiske universitet, viser at betaglukaner kan bedre sårtilhelingen ved diabetes.

Diabetes er en sykdom som rammer stadig flere mennesker i Norge. Ved sykdommen er sukkerinnholdet i blodet for høyt på grunn av mangel på eller for dårlig effekt av insulin. Høyt blodsukker er skadelig for kroppen og kan gi langtidskomplikasjoner hvis det ikke behandles. Blant disse komplikasjonene er svekket sårtilheling. Dette kan i verste fall føre til amputasjoner når sår ikke gror.

For at sår skal gro, kreves ulike typer celler, deriblant makrofager. Makrofagene skiller ut vekstfaktorer i såret som fremmer tilhelingsprosessen. Forskning viser at makrofager fungerer dårligere enn normalt ved diabetes. Dessuten skiller de ut mindre mengder vekstfaktorer ved diabetes enn hos mennesker og dyr som ikke har sykdommen.

Tidligere studier har vist at betaglukaner stimulerer makrofager til å fungere bedre. Med dette som utgangspunkt har vi studert effekter av betaglukan og vekstfaktorer på sårtilheling. I eksperimentene ble mus tilført enten betaglukan eller vekstfaktorer i såret. Resultatene viser forbedret sårtilheling ved behandlingen. Resultatene indikerer dessuten at blodsukkernivåene ikke påvirker sårtilhelingen i denne modellen.

Forskning på dyremodeller er viktig for å belyse årsaker til svekket sårtilheling ved diabetes. Musemodellen som vi har anvendt i studiene våre, benyttes av diabetesforskere over hele verden. Våre studier bidrar til ny kunnskap om denne modellen som andre forskere kan få nytte av i fremtiden.

SUMMARY

The prevalence of diabetes is increasing globally. Certain microvascular and macrovascular complications such as cardiovascular disease, retinopathy, nephropathy, neuropathy, and impaired wound healing are associated with the disease. Prolonged exposure to hyperglycaemia plays an important role in their development. Glucose reacts with macromolecules including proteins, lipoproteins, and nucleic acids over time to form irreversible advanced glycation end products (AGEs) that could modify both extracellular matrix and plasma proteins and also alter cellular functions.

In skin of animals and humans with diabetes, increased cross-linking of collagen by glycation and subsequent accumulation of AGEs occur. This is associated with more stiffness and increased insolubility, and could potentially contribute to inhibition of cellular infiltration in the wound, blockage of angiogenesis and attenuation of wound contraction and closure. Furthermore, wound studies in diabetes have demonstrated an aberrant course of inflammation. Macrophages are crucial in inflammation and wound healing, and studies have revealed impaired functioning of these cells in diabetes.

In order to study wound healing, we used leptin receptor deficient *db/db* mice, the most widely used animal model for type 2 diabetes. The aims of our studies were to: 1) examine the effects of a macrophage stimulant, water-soluble aminated β-1,3-D-glucan (AG) with or without additional glucose-lowering treatment with insulin, 2) study potential dose-response effects of AG, 3) investigate if there was an association between wound healing and metabolic variables, and 4) explore potential effects of aminoguanidine, an inhibitor of AGE formation.

In mice that were topically applied with AG, wound closure improved significantly compared to placebo treated controls. The addition of subcutaneous insulin treatment did not further accelerate wound healing. Subcutaneous insulin treatment alone did not promote wound healing. It lowered plasma glucose significantly, but did not normalise it. Experiments on different frequencies of topical AG administration onto the wound revealed dose-dependent effects on wound healing. Wounds applied with multiple AG doses (five or more) closed comparably to growth factor treatment (combined treatment with platelet-derived growth factor and insulin-like growth factor 1) during the early phase (days 0-17) of wound repair. In a separate study, wound closure did not show a significant association with plasma glucose, whereas change in body weight predicted wound healing in our model. Aminoguanidine, either administered topically or systemically, did not improve wound healing. However, the AGE-inhibitor was associated with favourable metabolic changes over time, that is, percentage weight loss and glycated hemoglobin A_{1c} tended to decrease dose-dependently.

1. INTRODUCTION AND BACKGROUND

The presence of foot ulcers and impaired wound healing are among the long-term complications of diabetes (1). Studies have revealed that alterations in the course of inflammation and related growth factors in the wound are involved in these complications (2-4).

Cells from the monocyte-macrophage system play key roles in inflammation and wound healing (5). Diabetes alters the function of these cells (6,7). Furthermore, prolonged exposure to hyperglycaemia is associated with decreased healing rate of foot ulcers (8).

The present work aimed to study the effects of a macrophage stimulant on inflammation and wound healing. The experiments were run with or without additional glucose-lowering treatment with insulin. Comparisons were made to placebo as well as topical applications of platelet-derived growth factor combined with insulin-like growth factor-1. In a separate study we aimed to explore the potential association between glycaemia and wound healing.

1.1 Diabetes mellitus

Diabetes mellitus (from greek *diabainein*: "to pass through" describes the copious urination, and from latin *mellitus*: "sweetened with honey" refers to sugar in the urine) is a complex, chronic illness with increasing global occurrence (9,10). The estimated world prevalence of diabetes in 2013 was 8.3% and correspondingly in Europe 6.8% (10). In Norway, the prevalence of diabetes at present is not exactly known, but a recent study from the Norwegian Prescription Database estimated a prevalence of 0.64% for type 1 diabetes, and 2.4% for type 2 diabetes (11). In the county of Nord-Trøndelag, the occurrence of known diabetes has increased: 2.9% of the population aged ≥ 20 years had diabetes in 1984-86 (First Nord-Trøndelag Health Survey, HUNT 1) while the corresponding prevalence was 4.3% in 2006-08 (HUNT 3) (12). However, more recent data from the Norwegian Prescription Database suggest that the annual increase in incidence of type 2 diabetes has levelled off (11).

Diagnosis and classification of diabetes are performed according to certain criteria, and there are two main clinical entities: Type 1 diabetes, which is due to an immune-mediated inflammatory destruction of the pancreatic β-cells, and type 2 diabetes, which results from an insulin secretory defect and a surplus of glucagon secretion with simultaneous insulin resistance (13,14). Type 2 diabetes is most frequent and accounts for about 90-95% of diagnosed adults in the United States of America (U.S.) (15). According to the HUNT studies, the corresponding part in Norway is at least 80% of all cases with diabetes (12). In addition, there are people with undiagnosed diabetes. Studies have suggested that more than 50% of people with diabetes are unaware of their disease according to the World Health Organization criteria (1). In Norway,

the number of individuals with undiagnosed diabetes varies from 22% to 100% of those with known diabetes as the reference (16). Another group that does not fulfil the criteria of diabetes has impaired fasting glucose (fasting plasma glucose is above normal, but not diabetic) and/or impaired glucose tolerance (IGT), where fasting plasma glucose level is normal and 2-hour glucose is above normal, but not diabetic during a standard oral glucose tolerance test (14). These people are at risk for later development of type 2 diabetes (14). In 2012, 5.9% of the adult population in the U.S. had impaired fasting glucose (prediabetes) while the corresponding percentage of diagnosed diabetes was 9% (15).

1.2 Chronic diabetes complications

Diabetes is associated with certain long-term complications (17). These include microvascular complications typical for diabetes: retinopathy, nephropathy, and neuropathy (17). The macrovascular complications, e.g., stroke or heart disease due to enhanced atherosclerosis however, are not specific to diabetes, but the risk of cardiovascular disease increases 3-8-fold in people with diabetes or impaired glucose tolerance (17). Furthermore, hypertension and dyslipidaemia are commonly coexisting with diabetes, and the patients are at risk of developing cardiovascular disease (14).

The presence of diabetic microvascular complications is largely caused by prolonged exposure to hyperglycaemia (17). A large prospective, randomized, controlled trial (RCT) in type 1 diabetes demonstrated that intensive glucose-lowering treatment, with the goal of nearly normalizing blood glucose concentrations, effectively delayed the onset and slowed the progression of retinopathy, nephropathy, and neuropathy (18). In type 2 diabetes, the United Kingdom Prospective Diabetes Study (UKPDS) showed a significant association between glycaemia and incidence of clinical complications (19,20). In that study, the median HbA_{1c} during 10 years of follow-up (HbA_{1c}, a marker of the average plasma glucose level over the last ~120 days), was reduced by 11% in the intensively treated group (7.0%) compared to the control group (7.9%) (19,21). Furthermore, the study showed 25% risk reduction in microvascular endpoints, but no substantial decrease in risk of macrovascular disease (19). Post-trial monitoring in the UKPDS revealed loss of the difference in HbA_{1c} levels between the intensive and control groups after the first year (20). Despite this, the relative risk reduction for microvascular disease persisted over the subsequent 10-year follow-up, and significant risk reduction for myocardial infarction emerged over time (20). Furthermore, the Steno-2 Study in type 2 diabetes patients with microalbuminuria demonstrated that intensified multifactorial intervention (improved glucose regulation, renin-angiotensin system blockers, aspirin, lipid-

lowering agents, and behaviour modification) compared to conventional treatment had sustained beneficial effects with respect to micro and macrovascular complications and on rates of death from any cause and from cardiovascular causes (22,23). At 21.2 years of follow-up of 7.8 years of the intensified treatment, a median of 7.9 years of gain of life was estimated (24). Besides glycaemia, the extent of diabetic tissue damage is also dependent on genetic determinants of individual susceptibility (17).

1.3 Diabetic foot

The diabetic foot is defined as "Infection, ulceration, or destruction of tissues of the foot associated with neuropathy and/or peripheral artery disease in the lower extremity of people with diabetes" according to the International Working Group on the Diabetic Foot (25) (Figure 1). The ulcer can be superficial, i.e., a "full thickness lesion of the skin not penetrating any structure deeper than the dermis", or deep, i.e., "full thickness lesion of the skin penetrating below the dermis to subcutaneous structures involving fascia, muscle, tendon, or bone" (25). It is deemed chronic once healing is delayed beyond 8 weeks (4).

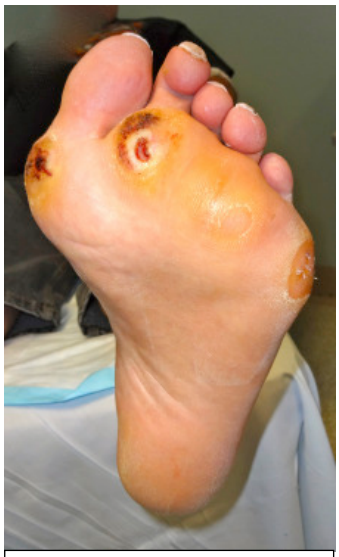

Figure 1.
Diabetic neuropathic foot ulcers overlying the meta-tarsal head. Reproduced with permission from (1), copyright Elsevier.

1.4 Diabetic foot ulcers as a clinical problem

Diabetic foot ulcers (DFU) principally place a major burden on individuals with diabetes, but also on the health care system. In a population-based study from Nord-Trøndelag, 7.4% of persons aged 20 years or older with known diabetes had a history of a DFU not healing within three weeks (26). This proportion is higher than what has previously been reported (27-30).

The lifetime risk of developing a diabetic foot ulcer was estimated to be 15% in the early 1980s (31). Based on more recent studies, the lifetime incidence may be 25% (32). Factors known to be associated with foot ulceration include previous foot ulcers, prior lower extremity amputation, long duration of diabetes (>10 years), poor glycaemic control, and impaired vision (acuity <20/40) (32).

It has been reported that a DFU precedes 84% of non-traumatic lower-extremity amputations (33). Severity of the condition is further emphasized by a meta-analysis of eight reports, including the HUNT 2-study, which concluded that a history of DFU was associated

with an excess risk of all-cause mortality (34,35). This risk was partly attributable to a greater burden of cardiovascular disease (34). Thus, a DFU is a pivotal event in the life of a person with diabetes, and a marker of severe disease. Moreover, the health-related quality of life in DFU patients is poor (36,37).

Without timely and optimal intervention, the wound can rapidly deteriorate and potentially lead to amputation of the affected part of the limb. In Norway, 475 major and minor lower limb amputations due to diabetes were performed in 2015 (38). This corresponds to 2.4 amputations per 1,000 people with the disease (38). A multidisciplinary approach in the treatment of diabetic foot lesions appears to be important in order to avoid lower-limb amputations (39,40).

Diabetic foot disease including ulcerations, infections, and gangrene are the most common causes of hospitalisation among individuals with diabetes. Twenty to 25% of all hospital admission days for patients with diabetes mellitus in the U.S. are related to foot complications (1).

A retrospective cohort study among 8905 diabetes patients in Washington State, U.S., showed that patients with foot ulcers had more inpatient days during the 4-year study period compared to those without an ulcer (30). In the U.S., cost of care for diabetes patients with a foot ulcer was 5.4 times higher in the year after the first ulcer episode compared to diabetes patients without an ulcer (41). Accordingly, a prospective, multicentre European study (821 patients; 14 centres; 10 countries) in consecutive patients with a DFU demonstrated that hospitalisation represented the highest cost, followed by antibiotics, amputations, and other surgery (42). In a smaller Norwegian study of neuropathic DFU patients (n = 12), outpatient visits accounted for 29% of total direct costs, orthopaedic appliances for 28%, and hospitalisation for 20% (43).

DFUs are preventable, and consequently it is important to prioritize and identify individuals at risk (32). Thus, according to practical guidelines, individuals with diabetes should have their feet examined regularly at least once a year, and appropriate measures implemented (44,45).

1.5 Pathogenic mechanisms in the development of diabetic foot ulcers

Pathophysiologic factors being associated with the development of DFUs include peripheral neuropathy, peripheral ischemia, infection, oedema, and callus formation (46).

The greatest single risk factor for the occurrence of foot ulceration is a history of either ulceration or amputation (47). Furthermore, studies showed that the most frequent triad of

component causes was trauma, neuropathy, and deformity, present in more than 63% of the patients (46). Component cause of ulcer occurrence was defined as "Causes of interest that were not sufficient in themselves, but were required components in one or more distinctive causal chains" (46). Trauma alone caused ulceration in 6% of the study patients, and inappropriate footwear was the most common source of trauma (46,48). Furthermore, oedema contributed to the development of 37% of the foot ulcers (46).

1.5.1 Peripheral neuropathy

Peripheral neuropathy is a serious complication of longstanding hyperglycaemia, including damage to autonomic, sensory, and motor nerve fibres. As a contributory factor to the development of DFU it is considered as the most important (49) (Figure 2).

Somatic and autonomic changes seen in patients are broadly duplicated in diabetic rodents, and evidence implicates impaired nerve and ganglion blood flow as a major etiological factor, especially in the early phase of diabetic neuropathy (50). Characteristics of this phase are diminished sensory and motor nerve conduction velocity that are reversible by vasodilator treatment (50). In the longer term, direct effects of hyperglycaemia-dependent oxidative stress

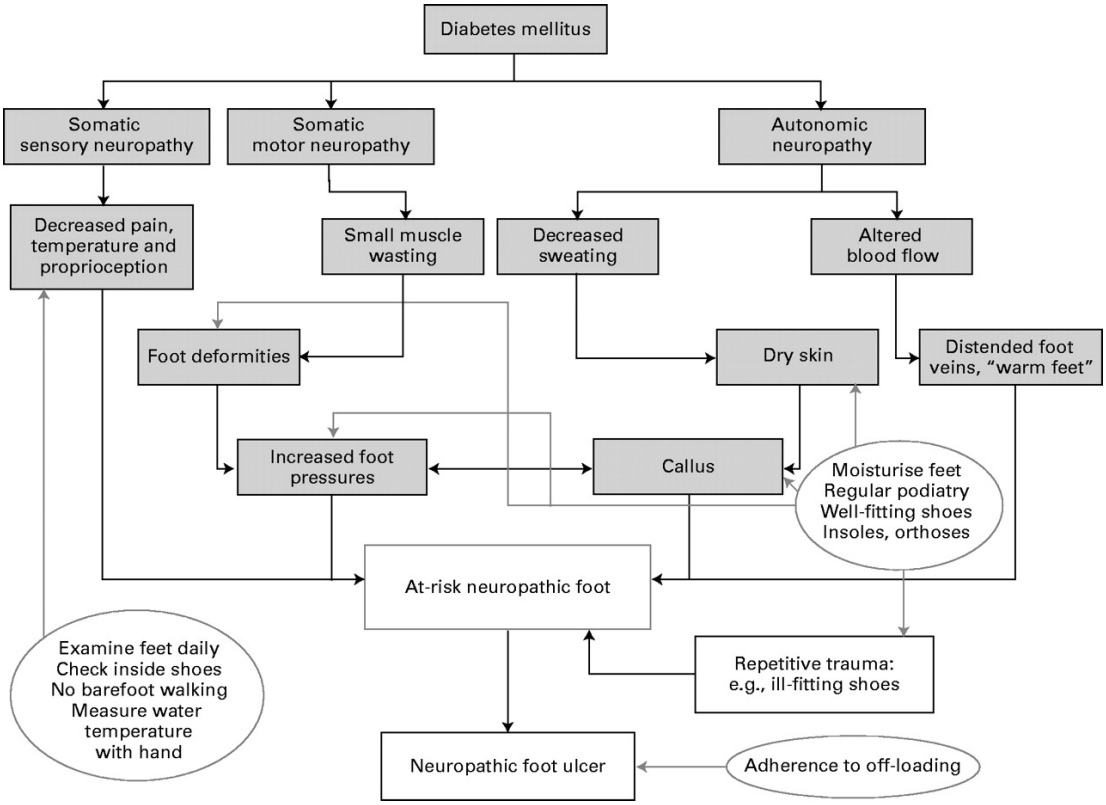

Figure 2.
Causal pathways to foot ulceration emphasizing the key role of the patient in ulcer prevention (light grey spheres and arrows); filled boxes, intrinsic factors; repetitive trauma, extrinsic factors. Reproduced with permission and copyright © of the British Editorial Society of Bone and Joint Surgery (51).

on neurons and Schwann cells are involved in modulating vascular effects (50). Oxidative stress includes disruption of the redox balance and alterations to the level or activity of reducing enzymes that help to maintain the normal redox state (4).

Neuropathy implies damage to the nerve supply of the intrinsic foot muscles. Other components of the causal pathways to neuropathic foot ulceration are summarised in Figure 2. In the absence of peripheral vascular disease, the foot is warm with distended dorsal foot veins on examination (49). With increased pressure combined with undetected repetitive injury (e.g., from inappropriate footwear) inflammation appears, followed by necrosis, and finally ulceration (1).

1.5.2 Peripheral vascular disease

Peripheral ischemia resulting from arterial disease contributed to the development of 35% of the diabetic foot ulcers in a two-centre study (Manchester, UK and Seattle, U.S.) (46). However, ischemia alone was not a sufficient cause of foot ulceration in any patient reported (46).

Atherosclerotic peripheral vascular disease is twice as common in persons with diabetes compared to those without the disease (32). In diabetes, atherosclerotic peripheral vascular disease particularly affects the femoropopliteal and smaller vessels below the knee (tibial and peroneal arteries of the calf) while frequently sparing the pedal vessels (32). Atherosclerosis, defined as plaque-forming degenerative changes of the aorta and of large elastic arteries, is an inflammatory disease of the intima (52). When a large artery occludes because of obstructive atherosclerotic disease, the normal response of collateral formation is impaired in diabetes (53). This makes the downstream tissue more susceptible to severe ischemia (17,53). AGEs appear to play a central role in this deranged pathway (17).

Furthermore, medial sclerosis, which is the calcification of the tunica media without encroachment on the arterial lumen, results in rigid arteries in diabetes (53). Studies have shown an association between increased arterial wall stiffness and reduced arterial flow volume in the lower extremities in diabetes (53,54). By physical examination, the ischemic foot is red, dry and often neuropathic (49).

1.6 Normal cutaneous wound healing

The normal wound healing process after skin injury elapses in a timely manner through three overlapping phases: inflammation, tissue formation and remodelling (55). The first stage – inflammation – starts with haemostasis that includes vasoconstriction. The injury causes damage to the endothelium and exposure of circulating platelets to collagen. This activates the

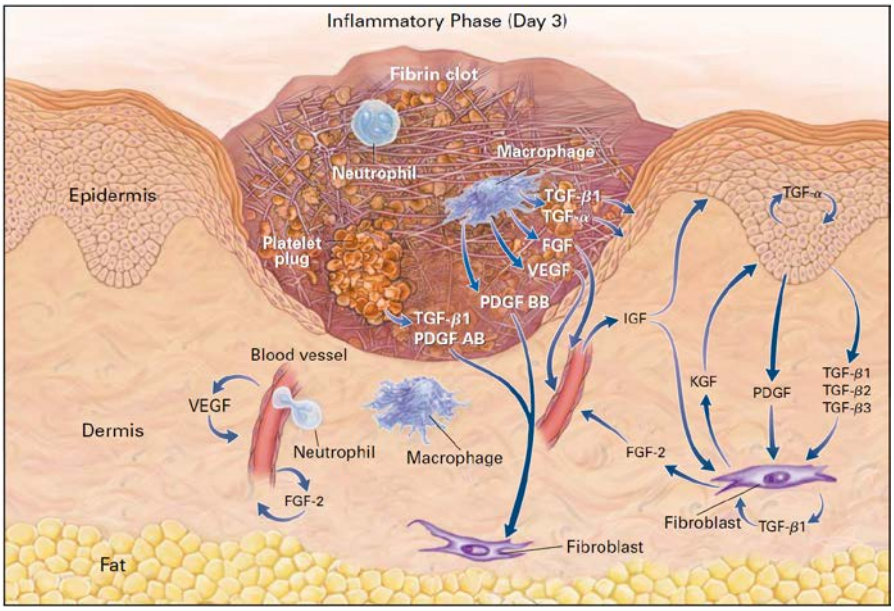

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Figure 3.
A Cutaneous Wound
Three Days after Injury

Growth factors thought to be necessary for the movement of monocytes/ macrophages, fibroblasts, capillary sprouts, and keratinocytes into the wound are shown. TGF-β1, TGF-β2, and TGF-β3 denote transforming growth factor β1, β2, and β3, respectively; TGF-α transforming growth factor α ; FGF fibroblast growth factor; VEGF vascular endothelial growth factor; PDGF, PDGF AB, and PDGF BB platelet-derived growth factor, platelet-derived growth factor AB, and platelet-derived growth factor BB, respectively; IGF insulin-like growth factor; and KGF keratinocyte growth factor.

platelets to secrete growth factors, such as platelet-derived growth factor, that attract and activate macrophages and fibroblasts (56). Furthermore, the platelets become sticky and start forming a platelet plug, a constituent of the macroscopic blood clot (Figure 3). Activation of the coagulation cascade adds fibrin to the clot. The fibrin matrix becomes the scaffold for infiltrating cells to the wound (55). Neutrophils are then recruited to the area of injury in response to activation of complement, degranulation of platelets, and products of bacterial degradation (55). After 2-3 days, monocytes appear in the wound and differentiate into macrophages (Figure 3). Like neutrophils, macrophages remove debris, foreign particles, and bacteria. Furthermore, the macrophages secrete a number of growth factors and cytokines into the wound, such as platelet-derived growth factor (PDGF), and tumour necrosis factor (TNF)-α, a potent inflammatory cytokine (56). Macrophages are thought to be crucial for coordinating later events in the response to injury, but the importance of neutrophils and macrophages in wound repair is incompletely understood (5,55). Data suggest, however, that a deficiency in either cell type can be compensated for by the redundancy in the inflammatory response (55).

The second stage of wound repair – new tissue formation – occurs 2-10 days after injury and is characterized by cellular proliferation and migration of different cell types (55). Initially, keratinocytes migrate over the injured dermis (epithelialisation). New blood vessels then form, and the sprouts of capillaries associated with fibroblasts and macrophages (granulation tissue) replace the fibrin matrix. The angiogenesis is stimulated by several growth factors including

vascular endothelial growth factor and fibroblast growth factor, both secreted by macrophages (56). Fibroblasts are attracted from the wound edge or from the bone marrow. They are stimulated by growth factors released from cells such as macrophages and platelets (platelet-derived growth factor, transforming growth factor β1) (Figure 3). The fibroblasts then proliferate, express integrin receptors, and migrate into the wound space (55,56). Here they are responsible for the synthesis, deposition, and remodelling of the extracellular matrix, which is mainly in the form of collagen (55,56). Some of the fibroblasts differentiate into contractile cells, myofibroblasts, contributing to the wound contraction (55).

The third phase of wound healing – remodelling – starts 2-3 weeks after the injury and lasts for a year or more (55). The fibroblasts continue to synthesise fibrillary collagen, and 3 weeks after injury the wound strength is about 20% of the final one (56). Over time, cells in the wound area undergo apoptosis, and over 6-12 months the acellular matrix is actively remodelled from a mainly type III collagen to one predominately composed of type I collagen (55). During this process, collagen fibrils remodel to form larger bundles with an increase in the number of intermolecular cross-links (56). In parallel with collagen synthesis, the remodelling includes a continuous catabolism of collagen which is controlled by several matrix proteinases (56). The wound gradually gain breaking strength. However, at maximal strength a scar is only 70% as strong as normal skin (56).

1.7 Wound healing in diabetes

The repair process is normally well-regulated with the timely and overlapping phases: inflammation, new tissue formation, and remodelling (55). Diabetes, however, delays this process.

Biopsies from chronic diabetic ulcers have demonstrated increased presence of inflammatory cells (B-cells, plasma cells, and macrophages), decreased numbers of $CD4^+$ T cells, no indication of epidermal growth or migration over the wound surface together with narrowing or occlusion of the blood vessels within the edge of the wound (2,4). Wounds from diabetic mice and rats, however, contained significantly fewer inflammatory cells compared to wounds from nondiabetic animals (3,4,57) (Figure 4).

Healing impairment in diabetes is caused by intrinsic factors (neuropathy, vascular problems, other complicating systemic effects due to diabetes) and extrinsic factors (wound infection, callus formation, and excessive pressure to the site) (58).

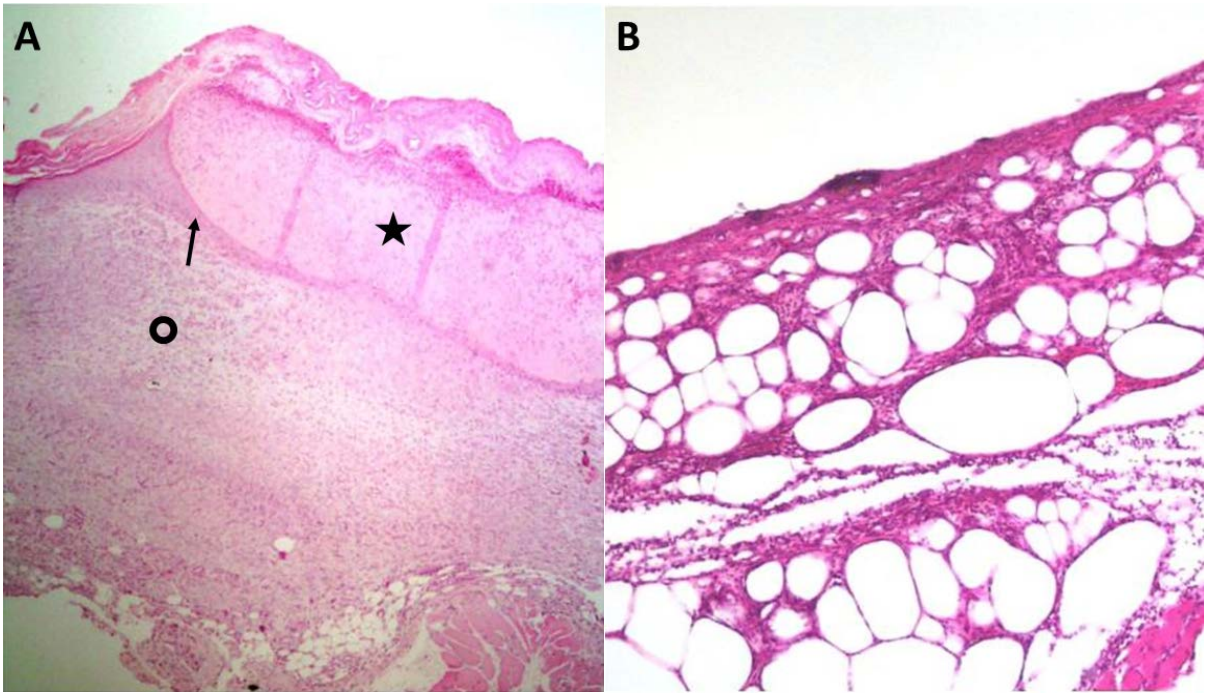

Figure 4.
Normal and Diabetic Murine Skin Wounds 13 Days after Injury
A. Histological photomicrograph of a normal wound bed (*db/+* mouse) showing abundance of cell-rich granulation tissue (⭘) covered by epidermis (⬆) and a scab (★). **B.** Corresponding diabetic wound bed (*db/db* mouse) with sparse granulation tissue and abundant adipose tissue. Haematoxylin-eosin staining (magnification x 100).

1.7.1 Peripheral neuropathy in wound healing

Peripheral neuropathy in murine and human diabetes was associated with fewer dermal nerves compared to controls (59). These nerves release substance P, a neuropeptide that contributes to the cutaneous inflammation during wound healing. In experiments with diabetic *db/db* mice, topical applications of substance P improved wound repair (59).

Moreover, neuropathy in diabetic patients has been shown to reduce the vasodilatory response at the foot level irrespectively of the presence or absence of peripheral vascular disease (60). Accordingly, other studies in humans with diabetes demonstrated a reduced nerve axon-related vasodilation (Lewis triple-flare response), indicating that neuropathy renders the diabetic foot functionally ischemic, as blood flow fails to increase under conditions of stress, e.g., with injury or infection (60).

1.7.2 Vascular problems in wound healing

Peripheral arterial disease (PAD) causing severe perfusion deficit and ischemia affect wound healing in diabetes (53). When inadequate perfusion is identified in a patient with DFU, revascularization should always be considered (53).

Microcirculatory deficiencies in diabetes include a reduction of capillary size, thickening of the basement membrane, and arteriolar hyalinosis (58). The thickening of the basement membrane interferes with physiological exchanges, and leads to altered migration of leukocytes (contributing to infection), decreased maximal hyperaemia, and abnormal auto-regulatory capacity (58).

The formation of new blood vessels from pre-existing capillaries being able to penetrate the wound site is essential to wound healing (4). Patients with diabetes display reduced ability to angiogenesis (17). Limited penetration of new blood vessels into the wound in diabetes restricts entry of inflammatory cells (4). In turn, the total amount of factors released by these cells will decrease. The oxygen supply to the wound will also be poor (4). Topical administration of high glucose to wounds of nondiabetic rats results in inhibition of the normal angiogenic process, suggesting direct roles for glucose toxicity and diminished angiogenesis in diabetes (61,62).

1.7.3 Role of the macrophage in wound healing

Macrophages are crucial cells in wound healing, and diabetes affects their functions (5-7,63,64) (Figure 3). In wound healing, myeloid cells are mobilized from the bone marrow into the circulation, and migrate into the peripheral tissues where they differentiate into macrophages (63). In tissues, these mononuclear phagocytes respond to environmental cues (e.g., microbial products, damaged cells, activated lymphocytes), which implicates that the macrophages undergo a series of modifications in order to maximize their effector functions (65,66). Thus, they become activated. The classical M1 activation results from stimulation by Toll-like receptor ligands and interferon gamma, and the alternative M2 activation follows stimulation by IL-4/IL-13 (66). Consequently, distinct phenotypes occur, M1 and M2 macrophages, respectively, which represent extremes of a continuum in a universe of activation states (66). The M1 phenotype express high levels of pro-inflammatory cytokines, reactive nitrogen and oxygen intermediates, and promote strong microbicidal and tumouricidal activity (63,66). The M2 phenotype, however, is considered to be involved in tissue remodelling and immunoregulatory functions (66).

Under normal wound healing conditions, macrophages initially show the M1 phenotype followed by conversion to the anti-inflammatory M2 phenotype promoting tissue repair (63). During impaired healing associated with diabetes, there is a persistent inflammatory response (6,67). Biopsies from humans with diabetic wounds revealed significantly higher macrophage number compared to nondiabetic controls (2). In diabetic rodents, studies have demonstrated a

later infiltration of macrophages into the wound and decreased macrophage activation (6,7,67). Furthermore, diabetic human and murine wound macrophages exhibited a persistent pro-inflammatory (M1) phenotype expressing high levels of pro-inflammatory molecules (IL-1β, TNF-α, and MMP-9) and low levels of the healing-associated (M2) phenotype markers (CD206, IGF-1, TGF-β1, and IL-10) (6,64,68). This has also been shown in *in vitro* studies of human monocytes and macrophages demonstrating M1-type polarization in response to high glucose levels (69). Topical applications of ex-vivo generated M2 macrophages into skin wounds, however, did not improve wound healing in *db/db* mice (70).

Biopsies from human ulcers and also wounds in *db/db* mice indicated that sustained inflammasome activity in wound macrophages contributes to early healing responses of diabetic wounds (6). A component of the innate immune system, the NOD-like receptor protein (NLRP)-3 inflammasome (NOD = nucleotide-binding oligomerization domain), that is a multiprotein complex including interleukin (IL)-1β-converting enzyme (caspase-1) becomes activated by proinflammatory danger signals (6). Inflammasome activation leads to the formation of active caspase-1, which then activates the proinflammatory cytokines IL-1β and IL-18 (6). Elevated levels of IL-1β have been found in wounds of diabetic humans and mice, which is consistent with increased inflammasome activity (6). By pharmacological inhibition of inflammasome activity in wounds of *db/db* mice, wound healing was improved, macrophages changed from proinflammatory to healing-associated phenotypes, and levels of prohealing growth factors were increased (6).

In diabetic human and murine wounds, the resolution of inflammation was studied (68). Nuclear receptors such as the peroxisome proliferator-activated receptors (PPARs) seem important to promote wound healing (68). The study indicated impaired activity from PPARγ in diabetic wound macrophages (68). Furthermore, experiments with myeloid-specific PPARγ knock-out mice suggested that loss of PPARγ in macrophages was sufficient to prolong wound inflammation and delay healing (68).

Apoptosis, which normally occurs concurrently with re-epithelialisation, was considerably delayed in diabetic *db/db* mice, and was reflected by retarded wound closure (71). Efficient dead cell clearance (efferocytosis) at the wound site is a prerequisite for the timely resolution of inflammation and successful healing (72). Macrophages from wounds in diabetic mice demonstrated impaired efferocytosis (72). This finding was associated with higher burden of apoptotic cells in wound tissue, higher expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and lower expression of anti-inflammatory cytokines (IL-10, TGF-β1) (72).

Observations related to apoptotic cell load in human wound biopsies corresponded to these findings in mice (72).

A mechanism by which macrophages are epigenetically pre-programmed towards a pro-inflammatory phenotype associated with non-healing diabetic wounds has been identified in human tissue and murine models (63).

In diabetic mouse and rabbit wounds, the neuropeptide substance P was deficient, and there was absence of an acute inflammatory response important for wound healing progression (73). Instead, inflammation persisted throughout the healing process. Treatment with substance P induced an acute inflammatory response and modulated macrophage activation toward the M2 phenotype that promotes wound healing (73). Similarly in diabetic mice, the treatment of wounds with transforming growth factor-β1 normalized M1/M2 macrophage polarization in the granulation tissue, restored angiogenesis, and wound healing (74).

In a pilot study of patients with healing (n=5) and nonhealing (n=5) DFUs, the relative expression of proinflammatory and anti-inflammatory genes was assessed from debrided tissue (75). The selected genes were highly indicative of human M1 or M2 macrophage phenotypes *in vitro* (75). The initial value of the M1/M2 score was significantly higher for healing compared to nonhealing wounds (75). These results were in agreement with studies describing the sequential profile of M1 and M2 macrophages in acute wound healing (75). The study suggested that inflammation is beneficial for healing and supports the clinical practice of wound debridement to stimulate inflammation (75).

1.7.4 Complicating systemic effects due to diabetes

High glucose could directly contribute to poor wound healing. In diabetes, glucose reacts non-enzymatically with long-lived tissue proteins, lipoproteins, and nucleic acids to initially form reversible, early **glycation** products (76). The reaction proceeds via more stable Amadori products to irreversible advanced glycation end products (76,77). Studies have demonstrated that high glucose concentrations *in vitro* inhibited proliferation of human fibroblasts, bovine endothelial cells, and murine keratinocytes (4). Human endothelial cells, and monocyte-derived macrophages, together with bovine endothelial cells, displayed increased synthesis and activity of various matrix metalloproteinases (MMPs) with high glucose (4). Furthermore, in endothelial cells hyperglycaemia increased expression of proinflammatory protein ligands such as members of the S100 calgranulin family and high-mobility group box 1 (HMGB1) (17). This hyperglycaemia-induced overexpression was mediated by the ROS-induced reactive metabolite methylglyoxal, which increased binding of the transcription factors nuclear factor kappa B (NF-

κB) and activated protein-1 to the promoters of RAGE (receptor for advanced glycation end products) and RAGE ligands, respectively (17). Consequently, transcription of the corresponding genes could be initiated. Moreover, AGE binding to its receptor in endothelial cells induced the production of ROS, which in turn activated NF-κB, causing multiple pathological changes in gene expression, including thrombomodulin, tissue factor, and vascular cell adhesion molecule 1 (VCAM-1) (17). These effects induced procoagulatory changes on the endothelial cell surface and increased the adhesion of inflammatory cells to the endothelium (17).

Glycated haemoglobin, the important marker of glycaemic control, is an Amadori product (77). In a study on 183 diabetic individuals, HbA_{1c} was a significant predictor of wound-area healing rate (8). Increased cross-linking of collagen by glycation and the subsequent accumulation of AGEs and fluorescent products occurs in the skin of patients and rodents with diabetes, being associated with more stiffness and increased insolubility (4). This could potentially contribute to inhibition of cellular infiltration of the wound, blocking of angiogenesis, and additionally delayed contraction and wound closure (4). Furthermore, an AGE-receptor blocker improved wound healing in rodents with diabetes (78). AGE inhibitors also improved wound healing in human endothelial cell layers *in vitro* and *in vivo* in type 1 diabetic rats (79,80).

Diabetes in humans and animals implicates intracellular hyperglycaemia in a particular subset of cells showing no significant change in glucose transport rate when glucose concentration is elevated (17). Such cells include capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves (81). Intracellular hyperglycaemia generates increased ROS in the mitochondria that disrupts the **redox balance** (82). Free radicals induce DNA strand breaks, and thereby activating poly (ADP-ribose) polymerase (PARP) that synthesises polymers of adenosine diphosphate (ADP)-ribose (17). The key glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) becomes modified with these polymers (17). GAPDH-activity is reduced which leads to shunting of early glycolytic intermediates into pathogenic signalling pathways implicating 1) increased aldose reductase substrate conversion, 2) increased O-GlcNAcylation (modification of proteins by *O*-Linked β-*N*-acetylglucosamine), 3) activation of protein kinase C, and 4) increased AGE formation (17).

Wound healing in diabetes is impaired by mitochondrial superoxide overproduction resulting in defective angiogenesis in response to ischemia (82). In diabetic mouse models of impaired angiogenesis and wound healing, deferoxamine (DFO) improved wound healing by

preventing iron-catalysed reactive oxygen stress and consequently correcting the function of the transcription factor hypoxia inducible factor-1α (82). DFO-treated wounds demonstrated increased collagen density, improved neovascularization, reduced free radical formation, and decreased cell death (82).

An important scavenger of ROS, reduced glutathione (GSH), demonstrated decreased ROS-levels in diabetic foot ulcers and in wounds of diabetic *db/db* mice (4,17). Addition of glutathione monoester to boost the GSH content in the wound of diabetic mice accelerated the healing process (83). GSH content and the activities of various antioxidant enzymes (glutathione reductase, catalase, and superoxide dismutase) were diminished in diabetes in various cells involved in wound healing (platelets, granulocytes, fibroblasts) (4). Consequently, increased oxidative stress could result in damage and strand breakage of cellular DNA in such cells in diabetes, as has been found to be the case (4). This could be disruptive to the normal functioning of these cells and potentially contribute to ulcer formation and poor wound healing (4).

Diabetes is a **chronic**, **low-grade inflammatory condition**, and increased intracellular ROS as resulting from hyperglycaemia activate a number of proinflammatory pathways (17,84). Ligation of proinflammatory ligands (S100 calgranulins, HMGB1) with RAGE causes cooperative interaction with the innate immune system signalling molecule toll-like receptor 4 (TLR4) (17). TLR4 plays an important role in the early stage of cutaneous wound healing (85). In high-glucose cultured human keratinocytes and in skin keratinocytes from diabetic patients, expression of TLR4 and thrombomodulin were both downregulated, possibly through the action of TNF-α (85). This pro-inflammatory cytokine was dose-dependently upregulated by glucose, which is in accordance with enhanced macrophage polarization into the M1 phenotype by high glucose levels or AGEs *in vitro* (69,85,86). Studies have shown that increased TNF-α expression in diabetic wounds impairs wound healing, possibly due to enhanced fibroblast apoptosis and decreased proliferation of these cells (85). Supplementation of soluble thrombomodulin increased TLR4 expression and promoted wound healing *in vitro* in human keratinocytes and *in vivo* in streptozotocin-induced diabetic mice (85).

Studies in skin biopsies from diabetic patients and healthy controls, obtained ahead of ulcer development, revealed increased immune cell infiltration and increased levels of inflammation-associated factors such as MMP-9 and protein tyrosine phosphatase-1B (PTP-1B) in diabetic individuals compared to controls (84). Expression of MMP-9, PTP-1B, and aberrant growth factor levels (platelet-derived growth factor-AA, fibroblast growth factor-2)

were also associated with failure to heal diabetic foot ulcers (84). Furthermore, studies in diabetic *db/db* mice have indicated a detrimental role for MMP-9 in wound healing while MMP-8 was associated with improved wound healing (87). Topical applications of an MMP-9 inhibitor (ND-366) in this rodent model improved wound healing by lowering inflammation and enhancing both angiogenesis and re-epithelialisation (87). The combined topical application of the MMP-9 inhibitor and active recombinant MMP-8 enhanced healing even more (87).

1.7.5 Altered growth factor levels

A large array of growth factors are involved in wound repair. Diabetic wounds exhibit a persistent inflammatory state associated with reduced levels of various growth factors and oxidative stress (6,88). Antioxidant treatment in type 1 diabetic mice improved wound healing, upregulated growth factor expression [vascular endothelial growth factor (VEGF) protein], and reduced oxidative stress in wound tissue (88). Some of the important growth factors are briefly presented here:

Platelet-derived growth factor is a dimeric polypeptide consisting of A and B chains, in homodimer (AA, BB) or heterodimer (AB) combinations (89). Major sources of PDGF are platelets, macrophages, and keratinocytes (56). This growth factor displays a variety of activities including action as a chemoattractant for neutrophils, monocytes, and fibroblasts (3,56). Furthermore, PDGF stimulates cells to secrete growth factors and induces the production of several extracellular matrix molecules (fibronectin, collagen, proteoglycans, and hyaluronic acid) (3,4).

In wound tissue from streptozotocin-induced diabetic rats, PDGF protein was significantly lower, as was messenger ribonucleic acid (mRNA) of PDGF-A and its receptor in genetically diabetic (*db/db*) mice compared with normal controls (89,90). A lack of PDGF protein was also detected in chronic wound fluid from diabetic patients (91). In a placebo-controlled study, PDGF significantly increased the incidence of complete wound closure and also reduced the time to complete closure (92). PDGF-gel was approved as a treatment for chronic neuropathic ulcers resistant to conventional treatment, but is no longer authorized in Europe (see Advanced therapies, page 26) (93).

Insulin-like growth factor (IGF) is another growth factor that is important for wound healing. There are two isoforms of IGF in mammals, IGF-1 and IGF-2 (4). Both are single-chain polypeptides (94). In general, IGF-2 production is highest in foetal life while IGF-1 dominates after birth (95). IGF-1 is mainly synthesised in the liver, but also in platelets,

macrophages, and fibroblasts in healing wounds (94). Production of IGF-1 has been shown to increase in various regenerating tissues, including the brain, peripheral nerves, and muscle after injury and in the kidney after nephrectomy (96). The IGFs circulate in blood and are carried by binding proteins (IGFBPs) to target organs (96). IGF-1 has short-term metabolic effect on glucose transport and long-term growth-promoting effects as a growth hormone-dependent mitogenic agent (94). IGF-1 can induce chemotactic activity in endothelial cell lines, stimulate keratinocyte and fibroblast proliferation and re-epithelialisation, and also increase wound strength (4). In diabetic human and murine wounds, IGF-1 is a marker of healing-associated macrophages (M2) (64). Skin from humans with diabetes demonstrated that IGF-1 was reduced within the basal layer of the epidermis, in fibroblasts, and at the ulcer margins, which could contribute to the slower rate of re-epithelialisation in diabetes (97). IGF-1 was also present in human endothelium, and reduced endothelial insulin/IGF-1 signalling contributed directly to impaired wound healing in mice (97,98).

Levels of IGF-2 mRNA are high in most foetal tissues and decline rapidly after birth (96). Similar to IGF-1, IGF-2 is known as a potential mitogen for normal and neoplastic cells (95). Furthermore, there is strong evidence that activation of IGF-2 (by its E2F transcription factor 3) is present in different types of cancer (95). No IGF-2 mRNA and IGF-1 mRNA, however, were detected in unwounded nondiabetic and diabetic murine skin (96). The appearance of IGF-1 mRNA and IGF-2 mRNA was delayed and their protein content decreased in wounds of the genetically diabetic mice (96). In these diabetic wounds, IGF-1 mRNA peaked at 14 days after wounding while IGF-2 mRNA peaked 10 days after wounding (96). To speculate, this may indicate their importance during different phases of wound repair. As for IGF-1, IGF-2 was present throughout the epidermis in normal human skin, but with elevated levels in the basal layer and in fibroblasts within the ulcers of patients with diabetes (4).

Treatment of wounds with IGF-1, particularly in combination with its binding protein IGFBP-1, in streptozotocin-induced diabetic rats, *db/db* mice, and normal rabbits accelerated healing (94,99). Furthermore, wound treatment with IGF-1 as gene therapy enhanced wound healing in *db/db* mice *in vivo* and in human skin *in vitro* (100).

Transforming growth factor-β1 (TGF-β1) is one of three isoforms of TGF-β secreted by platelets and macrophages of mammals (56). It acts as a potent chemoattractant for monocytes, macrophages, lymphocytes, neutrophils, keratinocytes, and fibroblasts, facilitates cellular movement, and induces such cells to release growth factors, stimulates angiogenesis, and enhances extracellular matrix (ECM) deposition (4). Moreover, TGF-β1 is inhibiting

proteolytic degradation of ECM (101). In wound fluid from diabetic rats, the levels of TGF-β (isoform not reported) were diminished, and in humans with diabetic foot ulcers, the normal elevation of TGF-β1 found in acute wound healing was absent (101,102). Furthermore, AGE inhibition with aminoguanidine was associated with decreased superoxide levels and increased TGF-β1 expression in wound tissue of type 1 diabetic rats (103). Topical applications of TGF-β1 in type 1 diabetic mice have demonstrated improvement in wound healing, probably through mechanisms compensating for the imbalance between macrophage phenotypes (M1, M2) (74). TGF-β1 is a cytokine derived from the healing-associated M2 macrophage phenotype (74).

1.7.6 Cellular profile and properties

Cellular infiltration in diabetic wounds is downplayed by impaired function of the infiltrating cells. The bactericidal activity of neutrophils was found to be reduced in diabetic patients, and their monocytes were less responsive to induction of chemotaxis (4). In endothelial cells, high glucose levels inhibited migration and delayed replication (62). The morphologically altered fibroblasts from chronic diabetic ulcers had significantly diminished proliferative ability and mitogenic response to various growth factors compared with those from normal diabetic skin (104-106). Moreover, dermal fibroblasts from patients with diabetes demonstrated reduced collagen synthesis and reduced activities of several antioxidant enzymes, while levels of MMP-2 and pro-MMP-3 protein were increased (4). Human keratinocytes exhibited reduced migration and decreased proliferation capacities under hyperglycaemic conditions (107).

As previously mentioned (section 1.7.3), apoptosis was considerably delayed in diabetic murine wounds (71). In nondiabetic epithelial cells and fibroblasts, apoptosis can be stimulated by TGF-β1, so lack of this growth factor could contribute to the delay, also in diabetes (108,109). Addition of PDGF in combination with IGF-2 resulted in significantly increased apoptosis in wounds of diabetic *db/db* mice (71).

1.7.7 Extracellular matrix

The content of collagen and glycosaminoglycans (GAGs) in the skin of rodents and patients with diabetes is diminished, which in turn has been associated with decreased wound strength (110-113). Lower levels of collagen and GAGs in diabetic rodents and humans with diabetes were associated with decreased synthesis of these extracellular matrix components (114-117).

Diminished NO synthesis may also contribute to the decreased collagen content and wound-breaking strength as demonstrated in type 1 diabetic rats (4,118). The enzymes

catalysing NO synthesis, inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS), demonstrated lower levels in wound tissue of diabetic *db/db* mice (119,120). Moreover, oral administration of an NO donor or the NOS substrate L-arginine in diabetic rats increased wound collagen deposition (121,122). Experiments in diabetic patients with periwound subcutaneous injections of L-arginine improved healing of ulcers (123).

Proteases, predominantly MMPs and neutrophil-derived elastase, as well as their inhibitors (tissue inhibitors of MMPs, the TIMPs) are highly regulated throughout the process of wound healing (124). The action of proteases allow for the migration of cells into the wound resulting in regenerative tissue and infiltration of blood vessels, and further on to re-epithelialisation and wound contraction (125).

Studies have shown that chronic wounds have elevated MMPs and lower TIMPs compared to healing wounds (124). This can have a detrimental effect on wound healing, and lead to degradation of the extracellular matrix and growth factors (IGF-1, TGF-β1) (4,124). In a randomized controlled trial, treatment with collagen/oxidized regenerated cellulose/silver normalized the wound microenvironment (MMP-9, elastase, TIMP-1), protected against infection, and improved wound healing in patients with diabetic foot ulcers (124).

1.8 "State-of-the-art": treatment and care of people with diabetic foot ulcers

The DFU constitutes a breakdown of the skin barrier that normally protects the underlying tissues against invasion of pathogens. In diabetes, more than 50% of foot ulcers develop infection (126). The diagnosis of a diabetic foot infection is clinical and based on symptoms and signs of inflammation such as redness, increased temperature, oedema, pain/tenderness, and purulence (126). However, the clinical signs of a foot infection could be dampened because of diminished leukocyte function, peripheral arterial disease, and neuropathy in diabetes (126). About 50% of diabetic patients with a deep foot infection lack a systemic inflammatory response that indicates infection (i.e., diminished or lack of elevation of erythrocyte sedimentation rate or C-reactive protein, normal white blood cell count and body temperature), leading to a delayed diagnosis (126).

The diagnosis of infection is not based on culture results and therefore only leads to systemic antibiotic treatment when associated clinical signs are present (e.g., enlarging wound size, satellite areas of breakdown, surrounding cellulitis, and probing to bone) (126).

Treatment and care of individuals with a DFU should be done according to thorough assessment. Guidelines and specialists in developed countries recommend the treatment to be

performed by the specialist health service/multidisciplinary foot care team, which is in line with a number of studies (39,44,127,128).

Principles of ulcer treatment are: 1) relief of pressure and protection of the ulcer, 2) restoration of skin perfusion, 3) treatment of infection, 4) metabolic control and treatment of comorbidity, 5) local wound care, and 6) education of the patient and his/her relatives (45).

1.9 Advanced therapies for diabetic foot ulcers

Studies have suggested that if a DFU is not 50% smaller at week 4 despite optimal care, it is unlikely to heal by week 12 (129). When standard treatments fail to heal chronic ulcers, advanced therapies are considered (130). These options of treatment are not established in routine management (45). However, an early decision to use advanced therapies improves the likelihood that these interventions will be effective (126).

The most readily available advanced therapy is autologous skin transplantation (autograft) (126). Hyperbaric oxygen therapy (HBOT) is another option for adjunctive treatment, and it has been used for about 40 years (131). This treatment could be considered in poorly healing wounds (45). However, larger prospective RCTs are needed to properly evaluate HBOT in people with chronic wounds (131).

Yet other options are bioengineered skin and skin grafts (dermal fibroblast culture and fibroblast-keratinocyte co-culture) that improved healing of clean neuropathic ulcers when compared to placebo (132). Negative pressure wound therapy (NPWT) is another treatment modality that causes a suction effect which deforms the extracellular matrix and promotes cellular proliferation (126). Consideration of NPWT is recommended in postoperative wounds, but further evidence is needed to substantiate the role of NPWT in routine clinical practice, including the treatment of chronic DFUs (45,132).

There are studies on a number of other advanced therapies, including application of cells (platelets, stem cells), and growth factors [for a review, see (132)]. Evidence is limited for these therapies, and a recent systematic review concluded that with the possible exception of NPWT in post-operative wounds, there is little published evidence to justify the use of newer therapies (132,133). Studies on platelet-derived growth factor (becaplermin) led to its approval by the U.S. Food and Drug Administration in 1997 for the treatment of diabetic neuropathic foot ulcers (126). However, further studies revealed increased risk of cancer-related death associated with the use of becaplermin (134). It is therefore no longer authorized, at least in Europe (including Norway) (93).

1.10 Beta-glucans in wound healing

Beta-glucans include glucose polymers found in the cell walls of plants, fungi, and bacteria (135). They exert a variety of effects on the immune system, including antitumor and anti-infective activities (135). Furthermore, they have been studied in wound healing in animals and humans with or without diabetes (136-141). We wanted to study the effects of water-soluble aminated β-1,3-D-glucan on wound healing in diabetic mice.

Neutrophils, macrophages, endothelial cells, fibroblasts, and keratinocytes are important cells in wound repair, and pattern-recognition receptors for β-glucans have been identified on these cells (142-145). When a β-glucan binds to its receptor on macrophages, it is rapidly internalized into the cell and stimulates the transcription of cytokines that have functional roles in fibroblast migration and activation (142). Beta-glucans mediate their effects by activating leukocytes and stimulating their phagocytic activity such as the production of reactive oxygen intermediates, inflammatory mediators, and cytokines (135). Exactly how β-glucans mediate the effects on immune function is still unclear (146). Nevertheless, receptors on leukocytes which have been described to recognise β-glucans and mediate their effects include 1) complement receptor 3, 2) lactosylceramide (CDw17), 3) scavenger receptors, and 4) Dectin-1 (135). Among these receptors, Dectin-1 has been shown to be the major receptor for β-glucans on leukocytes, and it is capable of mediating the biological activities of these carbohydrates *in vitro* (146). The ability of Dectin-1 to recognise β-glucans and induce cellular responses is influenced by the structure of these carbohydrates and the cell type expressing this receptor (146). In nondiabetic murine models, Dectin-1 demonstrates a redundant role in the protection against *Staphylococcus aureus* by a water-soluble β-glucan and in multiple organ dysfunction syndrome induced by a particulate β-glucan (zymosan) (146). A water-insoluble linear 1,3-beta-glucan (Curdlan) shows enhanced migration, proliferation, and wound closure in a dectin-1 dependent manner, both *in vitro* and *ex vivo* in healthy human keratinocytes (145). In nondiabetic murine wound macrophages, a particulate β-glucan induces TNF-α transcription (147). In normal human fibroblasts *in vitro* a water-soluble β-glucan, however, was shown to stimulate mRNA expression of the growth factors PDGF-A, PDGF-B, acidic fibroblast growth factor, basic fibroblast growth factor, TGF-α, TGF-β, and VEGF (144).

In nondiabetic wound models, insoluble β-glucan prepared from the yeast *Saccharomyces cerevisiae* was associated with a higher number of macrophages, and fewer neutrophil granulocytes compared to controls (136). Furthermore, re-epithelialisation and the onset of fibroblast proliferation commenced at an earlier stage in (insoluble) glucan-treated

rodent wounds, and the supernatant from (soluble) glucan-activated macrophages increased early wound breaking strength (136,137). This suggested that the wound healing effect of the (soluble) β-glucan was mediated, in part, by macrophage release of soluble growth factors (137). In yet other experiments, a β-1,3 glucan (solution in water) increased wound breaking strength in nondiabetic mice with suppressed macrophage function (148). A glucan that was isolated from *Saccharomyces cerevisiae* and prepared as water-soluble (1-3)-β-D glucan phosphate was tested in nondiabetic rodents (149). Perioperative administration of this glucan significantly increased tensile strength and hydroxyproline content in nondiabetic rat skin wounds and murine colon anastomoses (149). The findings suggested augmented collagen synthesis (149). Zymosan (a particulate beta-glucan-containing extract from yeast (*Saccharomyces cerevisiae*)) was shown to activate nondiabetic murine macrophages to express regulatory-like (M2) markers *in vitro* (150).

A soluble β-glucan, poly-[1-6]-β-D-glucopyranosyl-[1-3]-β-D-glucopyranose (PGG) glucan, reduced the risk of staphylococcal abscess formation in a nondiabetic wound model in guinea pigs (151). A study in nondiabetic rats using a β-1,3-glucan with or without hyaluronic acid-conjugation (to increase solubility) showed that the treatment of experimental corneal alkali burns suppressed the acute inflammatory reaction, as judged histologically by the presence of fewer polymorphonuclear leukocytes (152). Corneal epithelial wound healing was also promoted *in vitro* and *in vivo* (152). Corticosteroid administration in nondiabetic rats subjected to incisional skin wounds impaired wound healing, and additional systemic (intragastric gavage) or topical microparticulate β-glucan treatment improved wound repair in this model (153).

In partial-thickness burns in children, treatment with a β-glucan collagen matrix demonstrated good results, simplified wound care, and decreased post-injury pain compared to standard treatment (140). Whether the children had diabetes or not, was not reported (140). In a multicentre RCT in 1249 patients scheduled for a gastrointestinal procedure, perioperative parenteral administration of PGG glucan reduced serious postoperative infections or death by 39% after high-risk non-colorectal operations (n=391). Ninety seven (25%) of the patients in this group had diabetes type 1 or type 2 (154).

The effects of glucans on skin wound healing have also been studied in experimental models on type 1 diabetes (139,155). One study with streptozotocin-induced diabetic and nondiabetic control rats treated orally with microparticulate 1.3-1.6 β-D-glucan reported a significant increase in macrophage and fibroblast activity compared to controls, as judged from

haematoxylin-eosin-stained microsections (139). Furthermore, wound bursting pressure and hydroxyproline levels in that study were significantly higher in the diabetic group with glucan treatment (139). In recent experiments, potential wound healing effects of the medicinal mushroom *Sparassis crispa*, which contains more than 40% β-glucan, was tested (142,156). Oral administration of *Sparassis crispa* in type 1 diabetic rats significantly accelerated wound closure (156). This effect was associated with significant increases in macrophage and fibroblast migration compared to controls (156). Furthermore, in type 1 diabetic mice topically applied *Sparassis crispa* improved wound healing, and oral administration of the medicinal mushroom in these mice was associated with dose-dependent wound closure (142).

As for experimental type 2 diabetes, macrophage function at the wound site was improved in a study in *db/db* mice after oral treatment with a fermented papaya preparation (FPP) (157). The preparation improved NO production from these cells, increased recruitment of macrophages to the wound site, and accelerated wound closure (157). Intracellular ROS was also detected in these wound-site macrophages (157). The experiments were based on previous data showing that diabetes compromises respiratory burst in alveolar macrophages, and hyperglycaemia inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase function in neutrophils, which is crucial to the respiratory burst (157). Furthermore, the FPP was known to possess antioxidant properties (157). Wound macrophages from FPP-treated mice, however, showed significantly higher ROS production compared to control cells, suggesting that the preparation preserves NADPH oxidase function (157).

Just a few studies appear to address β-glucan treatment of wounds in humans with diabetes (141). In 54 diabetic patients (type 1: 21%; type 2: 79%) with cutaneous foot and leg ulcers, a macrophage stimulating water-soluble β-1,3/1,6-glucan demonstrated good safety results and promising potential as a treatment in a randomized, double blind placebo-controlled phase II study (141). Another RCT in people with diabetes (type 1 or type 2 was not announced) included a structurally different β-glucan and collagen that was as effective as moistened gauze in promoting wound healing in DFUs, with a benefit of marginal significance to ulcers with a duration of less than six months (158).

Beta-glucans exert more effects than already mentioned. They promoted protection from type 1 diabetes in mice (159). They also demonstrated hypoglycaemic activity in obese/type 2 diabetic mice, possibly associated with altered gut microbiota (160). In nondiabetic mice, however, a particulate β-glucan effectively scavenged free radicals (161). This is in line with the findings in a pressure ulcer model in nondiabetic rats where

microparticulate β-glucan treatment was associated with significantly lower levels of malondialdehyde (MDA), an oxidative stress marker, and higher levels of the antioxidant glutathione in tissue samples including skin (162). Furthermore, anastomotic colonic segments from orally β-glucan pre-treated nondiabetic rats exposed to colon anastomosis and preoperative irradiation demonstrated significantly lower levels of MDA compared to controls without β-glucan pre-treatment (163). Similar experiments in type 1 diabetic rats revealed that oral administration of microparticulate beta-D-glucan significantly improved colon anastomosis healing (138). Levels of MDA, however, were reported significantly higher in anastomoses from these rats compared to diabetic controls without beta-D-glucan treatment (138).

A study in tumour bearing mice showed that oral intake of yeast-derived particulate β-glucan converted polarized M2 bone-marrow-derived macrophages and immune-suppressive tumour-associated macrophages to an M1-like phenotype, leading to reduced tumour progression (164). Furthermore, studies *in vitro* and *in vivo* of water-soluble glucans stimulating nondiabetic human monocytes and murine macrophages demonstrated polarization toward the pro-inflammatory M1 state as indicated by enhanced reactive oxygen species (ROS) level, NO production, and secretion of inflammatory cytokines (IL-1β, IL-6, and TNF-α) (165-167).

2. AIMS OF THE STUDY

We performed studies in an experimental murine model of type 2 diabetes (*db/db* mice) in order to:

1. Test potential effects on healing and wound histology of the macrophage-stimulant, water-soluble aminated β-1,3-D-glucan (AG), as tested with or without insulin support therapy.

2. Assess the dose-response efficiency of AG treatment, as well as growth factor therapy (platelet-derived growth factor/insulin-like growth factor-1 in combination) on healing and wound histology.

3. Assess whether plasma glucose or other metabolic variables were predictors of wound closure.

4. Test potential effects of an inhibitor of advanced glycation end products, amino-guanidine (AGu), on wound healing compared to placebo (NaCl 9 mg/mL).

3. METHODOLOGICAL CONSIDERATIONS

3.1 Animals

In our experiments, we used diabetic *db/db* mice with characteristics resembling type 2 diabetes in man. The diabetic state is caused by a recessive mutation (gene symbol, *db*) in chromosome 4 (168). The mutation encodes a deficient leptin receptor leading to the lack of weight-reducing effects of leptin (169). Moreover, homozygous mutants are infertile, obese, hyperphagic, insulin resistant, and consistently develop severe diabetes with marked hyperglycaemia (3,168). Increased plasma insulin concentration is observed already at 10 days of age, and it peaks at 6-10 times normal levels by 2-3 months of age, then drops precipitously to near normal levels (168). This drop is concomitant with atrophy of the islets of Langerhans and a rise in blood glucose concentrations (168). Abnormal deposition of fat is observed at 3 to 4 weeks of age (170). Body weight increases with age and so does also blood sugar concentrations, which remain above 400 mg/dL (~22 mmol/L) until death at 5-8 months (168,170).

This is the most widely used animal model for type 2 diabetes, and has also been used in studies on wound healing (3,78,171,172). We based our wound model especially on a *db/db* mice model reported by Greenhalgh and co-workers (3).

3.2 Randomization of the experimental animals

The experiments were run on batches from the supplier (M & B A/S, Denmark) including 20-40 animals divided into groups of 3-5 animals per cage by the technicians at the Department of Comparative Medicine, UiT The Arctic University of Norway. The animals were non-blinded randomized (by myself) into the intervention groups as mentioned in the papers 1, 2, and 4. Briefly, since we observed that glycaemia and body weight within the batches were different at the time of intervention assignment, I selected animals so that plasma glucose and body weight between intervention groups were as equal as possible. Furthermore, for every batch of mice, animals assigned to the same intervention group stayed in different cages as far as possible.

Since the animals within the batches differed in age by up to two weeks, any differences regarding glycaemia and body weight could, at least in part, be due to characteristics of the animal model (168,170).

3.3 Wound model

In our studies, we established an excisional wound model based on the one by Greenhalgh and co-workers (3) (Figure 5). Other groups have studied a similar wound model (78,172-174). Rodent models have been criticized because the major mechanism of wound closure is con-

traction, whereas re-epithelialisation and granulation tissue formation are the major forces involved in human wound healing (175). However, wound contraction contributes less to the healing of full-thickness skin excisions in diabetic mice than in their nondiabetic littermates (3). This was also evident on inspection of the wounds in our studies.

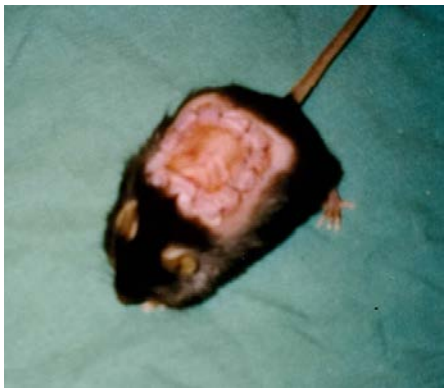

Figure 5.
Wound model in a diabetic *db/db* mouse

3.4 Wound area measurement

Three methods of wound area measurement were used in the study. They are described in detail in the individual papers. First we used a manual method (papers 1, 2, and 3), and in paper 1 we applied a digital method for comparison. Since the digital method became unavailable after a while, we established another digital method for the remaining part of the study (papers 3 and 4). The methods were thoroughly validated (papers 1 and 3), and comparisons of the methods are described in papers 1 and 3 and commented on in the next paragraph.

In more recent studies reported by others, wound areas have been measured by means of a digital camera and image analysis software (6,7).

3.5 Wound area measurement: comparison of the methods

We used Bland-Altman plots for comparison of the methods (see papers 1 and 3), an approach that also has been adapted by others (176-179). In such charts, the average of the observations by the two methods are plotted on the x-axis against the difference between the observations on the y-axis (180). The mean of the differences between the two methods is the estimated bias, i.e., the systematic difference between the methods (180). The standard deviation of the differences measures random fluctuations around this mean (180).

The data in **paper 1** included repeated measurements, meaning several (twelve) observations per mouse (dependent observations). When plotting the average of the areas between the two methods against absolute values of the difference between areas we observed a significant and positive correlation, that is, there was a relationship between difference and magnitude of the areas (paper 1, figure 1A). Consequently, we also plotted the average of the areas measured by the two methods against the percentage (relative) difference between the areas (paper 1, figure 1B).

Twenty-nine of the 600 measurements (4.8%) were outside the ± 2 standard deviations limits of agreement (paper 1, figure 1B). They were measurements of smaller areas (<0.3 cm^2). This may reflect the presence of more frequent measurement error in smaller areas. Related to evaluation of the intervention-effects on wound healing, the larger areas are assumed to be more important, and none of their measurements were outside the limits of agreement of percentage difference between the areas. Thus, the two methods were considered to be in good agreement with each other, and consequently one method could be used in place of the other (176).

In **paper 3**, we also did a method comparison between manual and digital measurements, but with only one wound area measurement per mouse (n=149), i.e., independent observations. One hundred and forty (94%) of these observations were within the limits of agreement. The mean difference (estimated bias) between the two methods was 0.02 cm^2 (180). In summary, the agreement between the methods was quite good. However, the digitized method demonstrated a lower coefficient of variation and was more convenient to perform. The results from this method was therefore preferred for publication.

3.6 Immunohistochemistry

Immunohistochemistry was performed with the aim of detecting wound macrophages (paper 2). As a marker we used the F4/80 extracellular antigen found on murine macrophages (7). This general macrophage marker does not distinguish between different phenotypes of macrophages, such as proinflammatory (M1) and prohealing phenotypes (M2) (64).

However, studies in diabetic *db/db* mice have demonstrated prolonged persistence of macrophages during the late phase of repair (day 13 postwounding), and macrophages isolated from wounds at 10 days after injury exhibited a persistent proinflammatory (M1) phenotype (64,67). Our tissue specimens were taken from wounds at days 13-14 after injury.

3.7 Statistical analysis

Linear mixed model (LMM) analysis was used in our study. LMMs state that observed data consist of two parts, fixed effects and random effects (181). Fixed effects define the expected values of the observations (the regression coefficients), and random effects define the variance and covariance of the observations (181).

Our data were repeated measurements (paper 1, paper 2) or data with heterogeneous variances between batches of mice (paper 3). We had to assume that the repeated observations on the same mouse in our model were correlated (181). Hence, statistical analysis of these data

should address the issue of covariation between the measurements (random effects). This was solved by applying LMM that included the covariance structure into the statistical model (181).

The covariance structures are patterns in covariance matrices (tables). For the repeated measurements, modelling the covariance structure involved modelling variation between subjects (mice) and covariation between measurements at different times on the same animal (181). Since the covariance associated with the repeated observations in our study declined exponentially over time, a first-order autoregressive covariance structure was included in the analyses (paper 1, paper 2).

For the uncorrelated data with heterogeneous variances between batches of animals, a diagonal covariance structure was included in the analyses to estimate if plasma glucose was a predictor of wound healing (paper 3).

If we had ignored the covariance structure in our analyses of data as mentioned, e.g. by applying repeated measurements analysis of variance, it may have resulted in inefficient inference about estimates of the fixed effects (predictor variables) (181).

The remaining statistical analyses are described in detail in the individual papers.

4. MAIN RESULTS

Paper 1: Aminated β-1,3-D-glucan improves wound healing in diabetic *db/db* mice

Macrophages are crucial for normal wound healing, and impaired functioning of these cells has been demonstrated in diabetes. This study was undertaken to see if there was an effect of the macrophage stimulant, water-soluble aminated β-1,3-D-glucan (AG), on wound healing in diabetic (*db/db*) mice. AG (final concentration 11.10 mg/mL in NaCl 9 mg/mL) was topically applied and covered the wound bed (dose 50-100 μL) from day 0 after wounding until complete wound closure, cycling 5 days with and 5 days without treatment to avoid maceration of the skin. The volume of 100 μL (0.1 cc) was based on the method by Greenhalgh and co-workers (3). We observed that AG treatment was associated with significantly higher wound closure rate over 17 days compared to placebo. The addition of subcutaneous insulin treatment significantly improved glycaemic control (plasma glucose, HbA_{1c}). However, wound closure in AG groups with or without insulin treatment was not significantly different.

Histological sections from the wound area at 13 days after injury were scored based on degree of cellular density, granulation tissue formation, vascularity, and re-epithelialisation. Specimens from AG treated wounds demonstrated significantly higher scores compared to controls.

Our study showed that AG treatment improved wound closure, and accordingly histological scores were higher. Better glycaemic control was not associated with further improvement of wound closure.

Paper 2: Aminated β-1,3-D-glucan has a dose-dependent effect on wound healing in diabetic *db/db* mice

This study was conducted in order to see if AG had dose-related effects on wound healing in *db/db* mice with growth factors (platelet-derived growth factor/insulin-like growth factor-1 in combination) as comparators. The macrophage stimulant was topically applied in four dosage regimens from baseline (day 0). The final concentration of AG and the volume applied at each occasion were the same as in paper 1: 1) one dose at day 0; 2) one dose every tenth day; 3) one daily dose for five consecutive days; and finally, 4) cycling five consecutive days with and five days without one daily dose (as in paper 1) until complete wound closure.

There was a significant association between the cumulative number of AG doses and wound closure. Furthermore, the wounds in the two groups with consecutive daily doses closed more completely compared to the two remaining AG groups and the placebo group, but

comparable with the growth factor group. Histological scoring of specimens from wounds at days 12-14 was significantly associated with the number of AG doses. Immunostaining of wound tissue revealed significantly higher numbers of F4/80-positive cells (macrophages) in AG treated compared to placebo treated specimens. In conclusion, this study showed dose-related effects of AG on wound healing in *db/db* mice.

Paper 3: No association between glycaemia and wound healing in an experimental *db/db* mouse model

Hyperglycaemia may be an operative mechanism in diabetes-induced impaired wound healing. However, a link between glycaemic control and wound healing has never been established. This study was performed in order to determine if there was an association between plasma glucose and wound healing in *db/db* mice. Secondarily, potential associations between wound healing and metabolic variables (weight, weight change) were tested.

In 149 animals, we observed no association between fasting plasma glucose and wound healing. Furthermore, change in plasma glucose from start to end of the experiment did not predict wound closure. However, increase in body weight was an independent positive predictor of wound closure. In conclusion, this study suggests that wound healing in *db/db* mice is independent of prevailing glycaemia.

Paper 4: Effects of AGE inhibition with aminoguanidine in a diabetic *db/db* mouse wound model

Advanced glycation end products (AGEs) react non-enzymatically with tissue proteins, lipoproteins, and nucleic acids over time to form irreversible structures involved in diabetic complications, such as impaired wound healing. This study was undertaken to investigate potential effects of the AGE inhibitor aminoguanidine (AGu) on wound healing. Wound closure in AGu-treated groups (topically, systemically, or both) did not improve significantly compared to control animals. Neither did AGu administration prior to baseline (day 0) significantly affect wound healing. However, AGu intake was associated with less body weight loss over time. Furthermore, HbA_{1c} tended to decrease dose-dependently which may be attributed to improved insulin secretion since previous studies on AGu demonstrated beneficial effects on insulin biosynthesis and secretion. Alternatively, AGu might have directly inhibited haemoglobin glycation. In conclusion, the study demonstrated no significant effects of AGE inhibition on wound healing.

5. GENERAL DISCUSSION

5.1 Aminated β-1,3-D-glucan

Wound healing is impaired in diabetes, and studies have demonstrated abnormal functioning of macrophages in the diabetic state (2,3,182). In our studies, we found that application of the macrophage stimulant aminated β-1,3-D-glucan (AG) improved wound healing in an animal model with type 2-like diabetic *db/db* mice (paper 1, paper 2) (Figure 6).

AG is a water-soluble derivative of Curdlan, a linear β-glucan from the bacteria *Alcaligenes faecalis* (183,184). Previous studies have shown that AG protects against life-threatening infections and causes tumour regression in nondiabetic models (166,167,185). Studies on mechanisms behind the effects of AG include experiments in macrophages and monocytes (166,167). AG stimulated monocytes/macrophages with subsequent release of proinflammatory mediators (166,167). Furthermore, it has been suggested that AG binds and internalizes into macrophages via a β-glucan receptor (186).

In our study, AG treated diabetic wounds were histologically more mature, capillarised, and had a thicker granulation tissue compared to controls (paper 1, paper 2) (Figure 6). Histological scoring was dose-related, and AG treatment was associated with significantly higher infiltration of F4/80 positive cells (macrophages) at the wound site (paper 2). These findings appear to be in line with results from the nondiabetic models as previously mentioned (section 1.10) (136,137).

We also found that AG treatment was associated with significantly more collagen deposition (paper 2). In two type 1 diabetic wound models, however, collagen formation was not different between glucan treatment and placebo groups (138,155). This was in contrast to another type 1 diabetic model where a β-glucan, structurally different to AG, increased collagen content (139). Furthermore, a preparation consisting of collagen and oxidized regenerated cellulose (a β-glucan) showed significantly accelerated wound closure in *db/db* mice (187), but collagen formation was not assessed in that study.

The fact that we performed our studies in a type 2 diabetes model may be of importance regarding glucan treatment effects since studies have shown that wound healing impairments in *db/db* mice are more severe than in type 1 diabetes mouse models, such as chemically induced (streptozotocin) or spontaneous genetic (Akita) diabetes (188).

The principle of macrophage stimulation was tested in a study with *db/db* mice and excisional skin wounds (189). In this model, wounds that were topically treated with the macrophage-activating compound, macrophage-activating lipopeptide-2 (MALP-2), closed

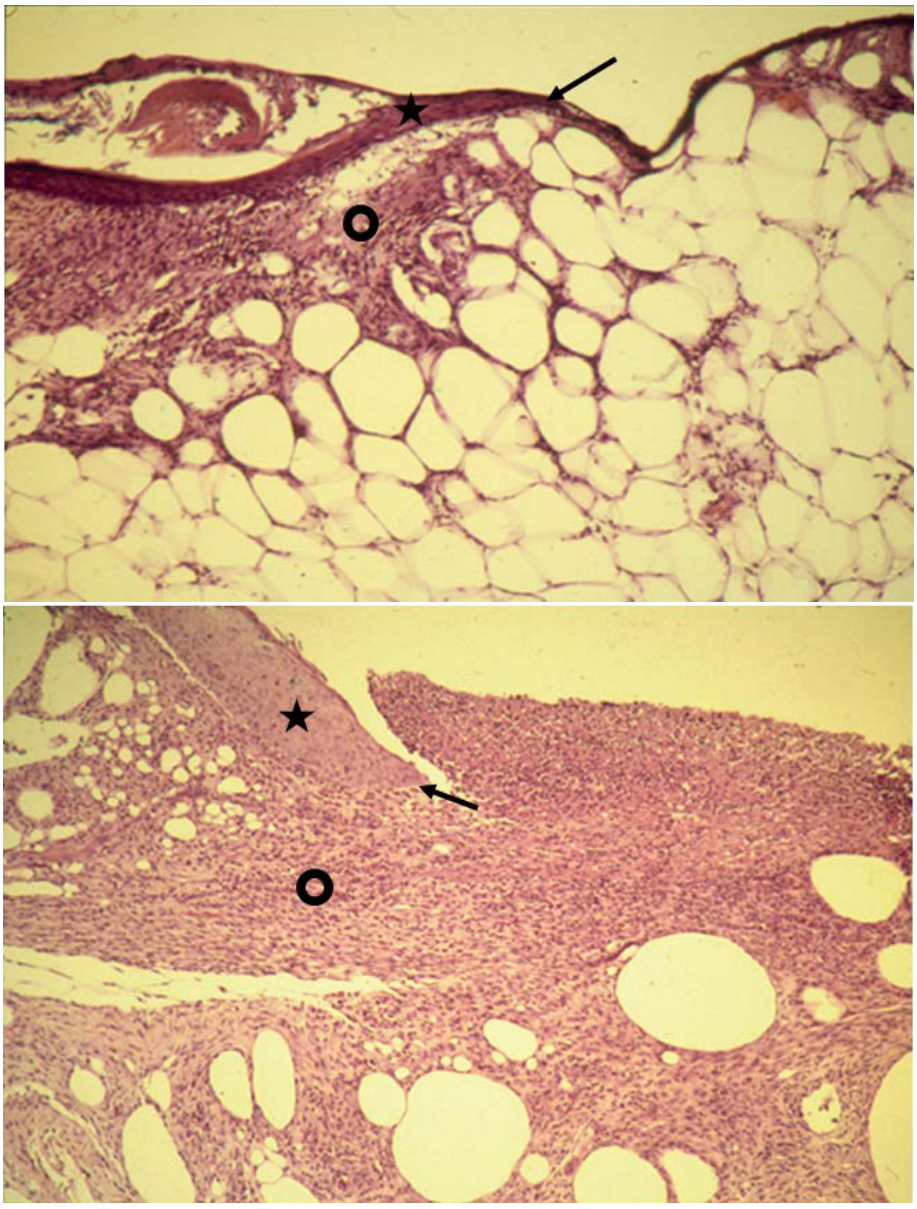

Figure 6.
Diabetic Control and AG Treated Murine Wounds

The photomicrograph in the **upper panel** shows a diabetic (*db/db* mouse) control wound 13 days after wounding. Wound edge (↙) and epidermis (★) cover the sparse granulation tissue (O) and abundant underlying adipose tissue.
The **lower panel** depicts a corresponding photo-micrograph from a diabetic (*db/db* mouse) wound at day 13 treated with AG for 8 days. Wound edge (↖). Thickened epidermis (★) covers the more abundant granulation tissue (O). (Both panels: haematoxylin-eosin staining; magnification x 200).
AG, aminated β-1,3-D-glucan

two weeks earlier than control wounds (189). This appears to differ from data in our model since glucan (AG) treated wounds in our study tended to close significantly more during the early phase (days 0-17) (189), while there was no difference between the groups in time to complete healing (data not shown). Furthermore, MALP-2 was well tolerated in a phase-I clinical trial (190).

Studies on wound healing in type 1 diabetic rats after administration of the β-glucan containing mushroom *Sparassis crispa* have demonstrated increased migration of macrophages and fibroblasts and higher levels of collagen formation (142). These findings are in accordance with our study, including collagen deposition that we semi-quantitatively scored in tissue sections (paper 1, paper 2).

Experiments in a wound model of *db/db* mice have indicated insufficient macrophage recruitment and activation (7). Moreover, applications of IL-1β-treated macrophages to these wounds accelerated granulation tissue formation and wound closure (7). To speculate, since AG causes release of large amounts of IL-1 in nondiabetic models, release of IL-1β in AG treated wounds may have accelerated the healing process as seen in our study (167). In yet another study, inhibition of the IL-1β pathway in wounds of diabetic *db/db* mice using a neutralizing antibody changed macrophages from proinflammatory to healing-associated phenotypes, increased the levels of wound growth factors, and improved wound healing (64). Thus, IL-1β may have a dual and opposing effect in wound healing in *db/db* mice: accelerating granulation tissue formation on one hand with lower release of growth factors (TGF-β1, IGF-1) in the wound on the other (7,64).

Mechanisms of action of AG in our wound model are unknown (paper 1, paper 2). AG was applied topically, and *in vitro* release of proinflammatory cytokines (IL-1β and TNF-α) from peritoneal macrophages did not differ between AG and control groups in our study (paper 1). We would suggest that a topical rather than a systemic effect of AG was operative (paper 1). However, levels of cytokines and growth factors in the wound area, e.g. in wound tissue or fluid, were not measured, and this is a limitation of our study (paper 1, paper 2). Nondiabetic models have suggested that the mechanism of action of AG involves monocyte/macrophage stimulation and both local and systemic release of mediators involved in inflammation, such as prostaglandin E_2, IL-1, and TNF-α (166,167).

5.2 Growth factors

Growth factors are important to wound healing (3,4). In our experiments, we chose to compare the combination of PDGF and IGF-1 with AG alone since Kiritsy and colleagues demonstrated improved wound closure rate with these two growth factors in *db/db* mice (3,172). Our dose of PDGF and duration of the treatment (5 days) were based on the study by Greenhalgh et al. who combined this growth factor with basic fibroblast growth factor (3). In their experiments, application of PDGF or basic FGF for 5 days had the same effect as did application for 10 or 14 days (3). Similarly in our study, the 5 days AG treatment was as effective regarding wound healing as applications within a longer period of time (paper 2).

Wounds treated with growth factors in our model showed 60% closure of wound area by day 17 (paper 2). This was comparable to the AG groups treated with five or more doses,

which suggests that the major effect of AG treatment was mediated by local release of growth factors (paper 2).

5.3 Insulin treatment and glycaemic effects on wound healing

The *db/db* mice that were included in our studies were severely hyperglycaemic, and we tested if hyperglycaemia impairs wound healing (paper 1, paper 2, paper 3). Therefore we aimed at correcting hyperglycaemia with subcutaneous insulin implants and study potential effects on wound repair (paper 1, paper 2). Besides the glucose-lowering effect, insulin also stimulates the growth and development of different cell types, and affects proliferation, migration, and secretion by keratinocytes, endothelial cells, and fibroblasts (191).

Regarding insulin implants as used in our experiments, studies have revealed that the release rate is ~2 U/24 hours/implant of insulin for more than 40 days in rats (192). We chose to use these implants since injections of long-acting insulin (Ultratard) in pilot studies were not effective in controlling blood glucose levels over time (data not shown). The insulin implants were individually dosed (data not shown). Briefly, insulin was initially dosed according to plasma glucose at baseline (paper 1, paper 2). During follow-up, blood glucose was measured (Glucometer Elite™, Bayer Diagnostics) once to twice a week in the insulin group, and if the level was not satisfactory (i.e., stayed >16 mmol/L), we added an extra ½ -1 implant subcutaneously under local anaesthesia (paper 1, paper 2). In these insulin-resistant mice, plasma glucose and HbA_{1c}-levels decreased significantly, by 45% and 25%, respectively, with insulin implants, but not to normal levels (paper 1, paper 2). Wound closure did not improve compared to controls without insulin (paper 1, paper 2).

I am not aware of any other studies that have been published with the use of insulin implants in *db/db* mice. Furthermore, no reports on wound healing in mice of this strain treated with insulin injections have been found. However, Yano and colleagues studied the insulin effects on surgical site infections in *db/db* mice, 16 weeks of age (193). For one week before bacteria inoculation, they injected subcutaneously human intermediate-acting insulin (Humulin N), approximately 10 to 50 IU/animal/day, to control blood glucose levels to below 8 mmol/L (193). Insulin treatment ameliorated the surgical site infection compared to controls as assessed from maximal diameter of the incision (193).

The functional counterpart of the *db/db* mouse, the leptin-deficient *ob/ob* mouse, demonstrated decreased collagen accumulation in a wound model (194). This was not restored by intermediate-acting insulin treatment (4-10 U NPH insulin/animal/24 h, route of administration not reported) in phenotypically obese mice even though blood glucose was

significantly lowered (194). The lack of a positive insulin effect on the wound collagen content in the *ob/ob* mouse is in accordance with our studies (168,194).

In type 1 diabetic mice, blood glucose levels were lowered after intraperitoneal insulin injection, but not to normal levels (on average 250 mg/dL (~14 mmol/L)) (195). Blood glucose levels of the corresponding diabetic and nondiabetic control animals were on average 500 mg/dL (~28 mmol/L) and 150 mg/dL (~8 mmol/L), respectively, which appear similar to our diabetic and nondiabetic control animals (195). Injected insulin doses needed to reduce blood glucose levels appear to be lower in type 1 diabetic mice (Lente insulin, multi-injection regimen, in total approximately 2.56 U/animal/48 h) than in *db/db* mice (subcutaneous Humulin N, approximately 10 to 50 IU/animal/day) (193,195). With this insulin regimen in type 1 diabetic mice, the wound healing improved and was similar to nondiabetic controls (195). This is somewhat contradictory to what we find in *db/db* mice, and severe insulin resistance is implicated in the *db/db* model (196).

In paper 3, we aimed at investigating if there was an association between fasting plasma glucose and wound healing in 149 *db/db* mice. Our data revealed no such association, nor did the change in plasma glucose from the start to the end of the experiment predict wound closure (paper 3). Trousdale and co-workers reported a similar study in 31 *db/db* mice (similar wound model, age, and bodyweight) and detected no significant association between wound closure and fasting blood glucose (173). In yet another study on wound healing, *db/db* mice were joined with wild-type mice through parabiosis, and thus diabetic animals were exposed to factors derived from the wild-type circulation (197). In these *db/db* mice, wound healing was improved compared to corresponding controls, and glycaemia remained unaffected (197). This finding indicates that the primary mechanism of improved wound healing is not through normalisation of blood sugar, but rather related to circulating leukocyte frequencies, improvement in inflammatory markers, or other blood-mediated mechanisms (197). The wounds in these experimental animals had increased presence of macrophages and T-lymphocytes, and 20% of the circulating cells were derived from the nondiabetic partner (197).

The treatment with subcutaneously injected relaxin, a peptide hormone of the insulin superfamily, was associated with significant improvement in wound healing and decreased blood glucose levels in *db/db* mice (198). The effect on wound healing was abrogated by a concomitant treatment with antibodies against vascular endothelial growth factor or CXC chemokine receptor 4, making it less likely that the reduction in blood glucose would contribute to the wound repair (198). This is in line with our studies (paper 1, paper 2).

A recent Cochrane review addressing RCTs on intensive versus conventional glycaemic control for treating diabetic foot ulcers failed to find any completed RCTs with definite results, both for type 1 and type 2 diabetes (199). In another meta-analysis on eight trials including 6960 patients from Europe, U.S., and Japan with type 2 diabetes, intensive glucose control was, however, associated with reduced risk of amputation by 36% (200). Four trials reported amputation without further description (200). The Steno-2 and Veterans Affairs Cooperative Study in type 2 diabetes (VACSDM) specified that the number for amputations was due to ischaemia, and the Veterans Affairs Diabetes Trial specified amputation for ischaemic diabetic gangrene (200). The eighth trial of the meta-analysis mentioned above (200), United Kingdom Prospective Diabetes Study, defined amputation as major limb complications requiring amputation of a digit or any limb for any reason (200).

In patients with type 1 diabetes, the incidence of ischemic foot ulcers related to glycaemic control was studied (201). The individuals were randomly assigned to either intensified or standard insulin treatment (201). During a median of 28 years follow-up, the incidence of ischemic foot ulcers was significantly lower in the intensified insulin-treatment group (3 out of 35) versus the standard treatment group (10 out of 37) (201). Thus, although nobody has found an effect of glucose control on wound healing, there could be a long-term effect to prevent wound formation.

5.4 AGE inhibition

In diabetes, glucose reacts non-enzymatically over time with macromolecules such as long-lived tissue proteins, lipoproteins, and nucleic acids to form reversible, early glycation products, and further on to form more stable Amadori products, and finally irreversible advanced glycation end products (76,77). AGEs are implicated in the long-term complications of diabetes, including impaired wound healing (78,80).

As an inhibitor of AGE formation, aminoguanidine has been studied for many years (202). Regarding wound healing in diabetes and potential effects of aminoguanidine in rodents, there are just a few studies (80,203) and none in *db/db* mice as far as I know.

In our experiments in *db/db* mice, we aimed at inhibiting AGE formation by aminoguanidine administration at an early stage in the development of diabetes in the animals (paper 4). The studies demonstrated no significant improvement of wound healing associated with AGu intervention in any arm (paper 4). Neither was the presumed AGE preventive administration of AGu for up to eleven weeks prior to wounding effective in accelerating wound closure (paper 4). This is in contrast to the findings in other rodent wound models, such as type

1 diabetic rats and nondiabetic (young and old) mice, in which AGu treatment prevented skin flap necrosis or improved wound closure (80,203,204).

The dose and/or the duration of AGu administration may have differed in the experiments. In our model, we used 1 g/L or 5 g/L in drinking water for up to eleven weeks before wounding and four to six weeks after wounding (paper 4). The dose of ~1 g/L is equivalent to ~1 g/kg/day in severely hyperglycaemic rodents (205). Others have administered the same dose of AGu (1 g/L; dose in g/kg/day was not reported) in drinking water for seven days from the day of wounding in type 1 diabetic rats and have seen improved wound repair (203). This dose of AGu in drinking water reduced AGE content in type 1 diabetic rat skin wounds (103).

AGE formation increases with increasing plasma glucose concentration and diabetes duration. In diabetic *db/db* mice, severe hyperglycaemia occurs at 2-3 months of age (168). In our experiments (group 2, n = 21, age 11-13 weeks, AGu 1g/L), average plasma glucose was 27 mmol/L at baseline and 27.6 mmol/L at the end of follow-up (paper 4). This is different from streptozotocin-induced diabetic rats, where acute hyperglycaemia is introduced prior to experiments. Furthermore, the degree of insulin resistance is different in the two models.

Oxidative stress appears to be implicated when AGu protects against skin flap necrosis and also in impaired wound healing in type 1 diabetic rats and *db/db* mice (80,103,206). Levels of the antioxidant glutathione (GSH) in wounds of *db/db* mice were significantly lower compared to nondiabetic controls (4,83).

Nitric oxide is critical for angiogenesis and collagen deposition in wound repair (4). Lower NO levels may be implicated in the absent wound healing effect as seen in our studies since AGu is an inhibitor of iNOS, and NO deficiency at the wound site was reported in diabetes (121).

Aminoguanidine is an AGE inhibitor that traps reactive dicarbonyl intermediates such as methylglyoxal (204,207). *In vitro* studies with pancreatic islets indicated that AGu has beneficial effects on insulin biosynthesis and secretion which is potentially related to inhibition of AGE accumulation (208). Furthermore, *in vivo* studies in type 2 diabetic (*db/db*) mice fed an AGu-containing diet showed reduced decline in serum and pancreatic insulin levels, and the degree of islet morphological degeneration (209). Thus, to speculate on our own studies, the significantly lower level of the Amadori product HbA_{1c} in the long-term and high AGu dose group with a limited number of animals (orally 5 g/L, n=6; group 3) might be related to inhibition of AGE accumulation in the islets, thereby protection of islet function, and consequently improved insulin secretion (paper 4). During the experiment, average fasting

plasma glucose in group 3 declined more compared to the remaining groups, however, the number of animals was too low to reach a statistically significant difference between the groups (paper 4). An alternative explanation could be that, without affecting glycaemia, AGu directly inhibited glycation of haemoglobin as has been demonstrated *in vitro* and *in vivo* in type 1 diabetic rats (210,211).

6. SUMMARY OF STUDY RESULTS

1. Topical applications of the macrophage stimulant, water-soluble aminated β-1,3-D-glucan, significantly improved the early phase of wound healing in *db/db* mice compared to controls. Long-term wound closure was apparently not affected. Additional insulin therapy did not change the rate of wound healing.
   Histological examinations of AG treated wounds revealed a more mature and thicker granulation tissue compared to placebo treated controls.

2. The effect of AG on wound healing was dose-dependent. The effect of growth factors (PDGF and IGF-1 in combination) on wound healing was comparable to the AG treatment (five or more doses).
   Histological examinations of AG treated wounds demonstrated dose-related effects, i.e. higher maturity with more collagen deposition in wounds applied with higher dosage frequency. The number of F4/80 positive cells (macrophages) in the higher dosage frequency AG treated wounds was higher compared to placebo treated control wounds.

3. Wound closure was not significantly associated with plasma glucose in *db/db* mice.
   The change in body weight demonstrated a significant and independent positive association with wound closure, probably reflecting anabolic metabolism.

4. Systemic and/or topical administration of the AGE inhibitor aminoguanidine did not improve wound healing in the *db/db* mouse wound model.

7. FUTURE PERSPECTIVES

- Mechanisms of action of water-soluble aminated β-1,3-D-glucan in our diabetic murine wound model are unknown. We do not know why AG had a favourable effect in the early phase of wound healing. Cytokines, growth factors, and proteases may be implicated (56,125). In future studies, analyses should be performed in wound tissue to reveal potential mechanisms. Suggested methods would be: enzyme-linked immunosorbent assays, immunohistochemistry, and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) gelatine zymography (64,125,157).

- Relevant end points in such studies should be: 1) wound area over time, 2) histological parameters (including granulation tissue formation, macrophage accumulation, angiogenesis, epithelialisation, and collagen deposition), 3) myo-fibroblast accumulation, 4) cytokines and growth factors, e.g. pro-inflammatory cytokines (IL-1β, TNF-α) and VEGF. Markers of the healing-associated (M2) macrophage phenotype could also be relevant (IGF-1, TGF-β1, IL-10).

- Hyperglycaemia-associated AGE formation and oxidative stress are probably of importance in impaired wound healing (17). Effects of the AGE inhibitor aminoguanidine, as used in our study, has been examined for at least thirty years, and more recent compounds that inhibit AGE formation may be more efficient (202,207). Suggested future studies in diabetic patients and rodents include systemic intervention with an antioxidant (e.g., N-acetylcysteine with or without additional L-arginine), or a potent substance that is assumed to decrease the effects of AGEs in wounds (e.g., a thiazolidinedione) (88,207,212). The combined treatment with topical applications of an antioxidant is also worthwhile to consider. In conjunction with clinical end-points, it would be interesting to monitor levels of oxidants, antioxidants, AGEs, proteases, and inhibitors of proteases in wound tissue (64,78,88,125).

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ERRATA

Paper 1:

"Blood glucose" should be corrected to "plasma glucose", and "blood lactate" should be corrected to "plasma lactate".

In the *Materials and Methods* section under the heading *Preparation and application of AG*, 3rd paragraph "followed by 4 days without treatment" should be changed to "followed by 5 days without treatment".

Papers 1, 2, and 4:

In the *Materials and Methods* section under the heading *Animals*, the designation C57Bl/KsBom should be changed to C57BL/KsBom.

Paper 3:

Table 2: In the column *Predictor variables*, "$wt_{day7\text{-}13}$ (%)" should be changed to "$\Delta wt_{day7\text{-}13}$ (%)".

PAPERS 1-4