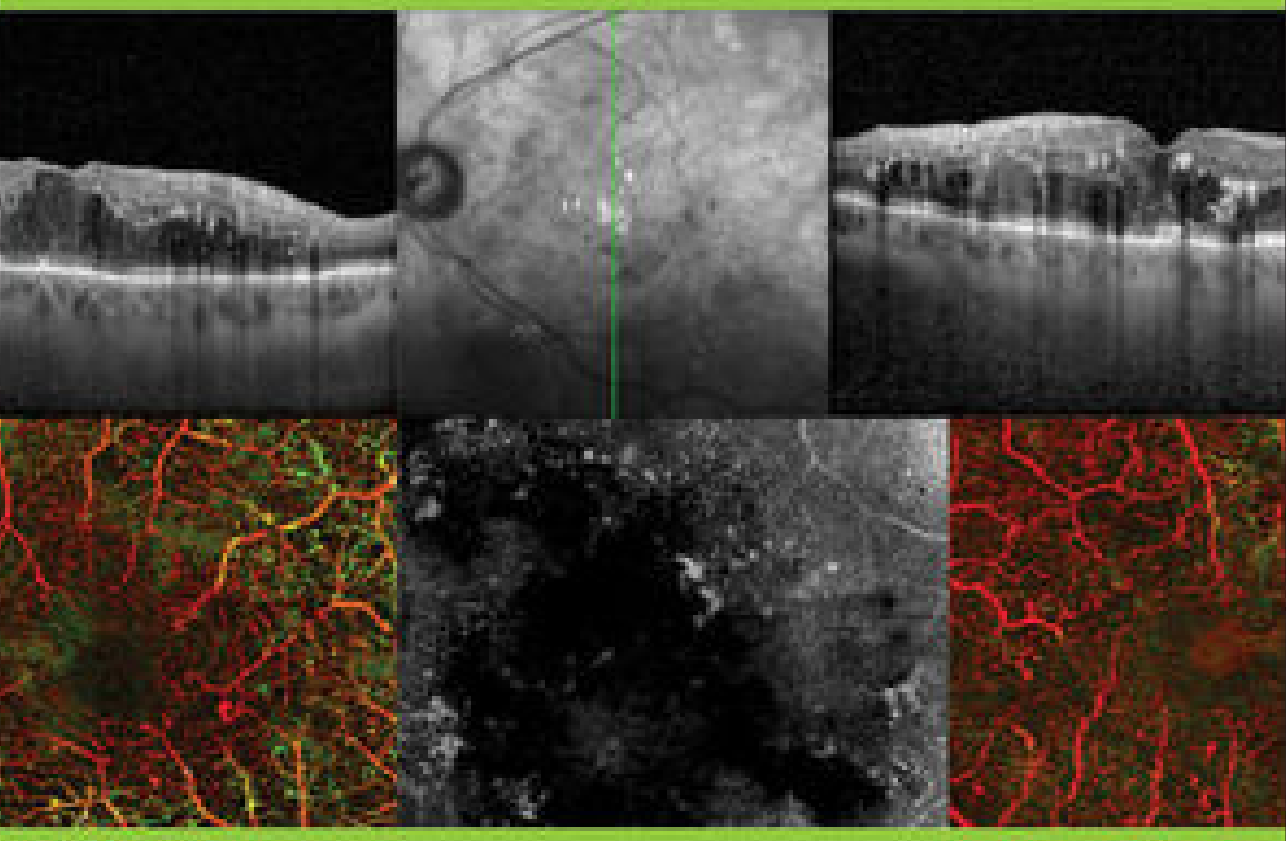
EDITED BY
FRANCESCO TECILAZICH

# MICROVASCULAR DISEASE IN DIABETES



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# Microvascular Disease in Diabetes

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*Edited by Francesco Tecilazich, MD, PhD*

*Endocrinology and Metabolic Diseases,*
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*To my Mother and Father,*
*To Francesca and our children*

# Contents

# Foreword to: *Microvascular Disease in Diabetes*

*Edited by Francesco Tecilazich, MD, PhD*

*Division of Endocrinology and Metabolic Disease, San Raffaele Scientific Institute, Milan, Italy*

Boston, Massachusetts is a big city with dozens of hospitals and research centers, but is a small town when it comes to the diabetes care and research community. When Francesco Tecilazich arrived in Boston in 2009 to work as a fellow with Aristidis Veves at the Joslin Diabetes Center, I was glad to welcome him to our community. I had known Francesco Tecilazich for many years, since 2003, when he was an endocrinology fellow and I was a visiting Professor for a year at the Divisione di Endocronologia, Universita di Verona, Verona, Italy. In Verona, we spent time between tasks to talk about the ins and outs of diabetes biomedicine and Italian cuisine. Likewise, in Boston, we met frequently to discuss these most essential and absorbing topics.

Over the years in Boston, understanding of the molecular, physiological, and pathological basis of microvascular disease in diabetes continued to grow. After working with Aristidis Veves, Francesco Tecilazich expanded his vision and moved across town to work with Maura Lorenzi at the Schepens Eye Research Institute. Thusly well-trained and becoming a well-renowned expert in diabetic microvascular disease, he returned to Italy to continue his work at the prestigious San Raffaele Scientific Institute, Milan, Italy. He has been busy. In this book, *Microvascular Disease in Diabetes,* Francesco Tecilazich has assembled an impressive team of expert authors, who together establish the current edge of biomedical scientific knowledge of microvascular disease in diabetes.

Diabetes mellitus continues to arise unabated worldwide. Much of this is type 2 diabetes, where macrovascular cardiovascular disease dominates morbidity and mortality, but microvascular complications also contribute to suffering. Type 1 diabetes also continues to arise alarmingly and unabated worldwide according to multiple surveillance reports. Unlike type 2 diabetes, the best type 1 diabetes prevention strategies are not well understood, despite ongoing major efforts. In type 1 diabetes, microvascular complications dominate morbidity and mortality, with macrovascular cardiovascular disease contributing to suffering in later life. Furthermore, strategies to reduce cardiovascular disease morbidity and mortality are becoming well developed, with clear roles for blood pressure and LDL cholesterol reduction, and evolving roles for older therapies like aspirin and newer therapies like GLP1 mimetics and SGLT2 inhibitors. Strategies to reduce microvascular disease morbidity and mortality include scrupulous glycemic and blood pressure control but are otherwise less well developed and need the kind of knowledge contained here.

With diabetes increasing globally, the urgency to better understand and control the microvascular complications of diabetes increases apace. *Microvascular Disease in Diabetes* squarely addresses this need. An impressive collection of chapters by international experts provides in-depth reviews of the state of the science. First, the basics: the pathophysiology, genetics, and epigenetics of microvascular disease in diabetes. Then, the major disease areas: diabetic retinopathy, nephropathy, and neuropathy undergo detailed scrutiny. These major areas are helpfully divided into separate clinical and research chapters. Then, the defiantly stubborn, multi-pathogenic problem of the diabetic foot gets its own clinical and research chapters. Finally, new insights into coronary and cerebral microvascular dysfunction in diabetes round out the last two chapters.

At the end of *Microvascular Disease in Diabetes,* the reader will be thoroughly up to date on current knowledge in this field. However, the reader will also be well-warned that every area of investigation covered in this book is "hot" and moving fast, with new knowledge arising at a regular pace. And so, at the end of this book the reader will be also prepared for the future. I can't really say it better than Andrea Giustina and Stefano Frara, writing in the book's Introduction: "This thorough and comprehensive book integrates new and accessible material on diabetic microvascular comorbidities. It will help investigators, clinicians, and students to improve their understanding, providing additional knowledge, assembled in an easily consultable manner, on pathogenesis, diagnosis, research, and cure of microvascular complications."

Well said! With that, reader, enjoy and learn.

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## Editor Biography

**Dr. Francesco Tecilazich** received his MD and PhD at the University of Trieste Medical School in Italy. Once he completed his residency and clinical fellowship in endocrinology and metabolic disease at the University of Verona Medical School in Italy, his training and career goal has been to become an expert in the study and management of diabetes and its complications and contribute to lessening their impact. He first worked on diabetic neuropathy and impaired wound healing under the mentorship of Dr. Aristides Veves at the Joslin-Beth Israel Deaconess Medical Center, Harvard Medical School, in Boston (USA). He then sought training in diabetic retinopathy, this time wishing to master bench research while continuing clinical and translational investigation. This combination of interests brought Dr. Tecilazich to join Dr. Mara Lorenzi's laboratory at the Schepens Eye Research Institute, Harvard Medical School in Boston (USA). After completing a senior post-doctoral fellowship, he was promoted to the faculty position of Investigator at the Schepens Eye Research Institute-Massachusetts Eye and Ear Infirmary, and Instructor in the Department of Ophthalmology, Harvard Medical School. He next returned to Italy at the IRCCS San Raffaele Hospital in Milan where he resumed clinical responsibilities as an endocrinologist in the Division of Endocrinology and Metabolic Diseases, and continues his research activities on the mechanisms of protection of microvessels against diabetes, and on the search for predictors of diabetic microvascular complications.

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# 1

# Introduction

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The American Diabetes Association (ADA) stated in 2017 that diabetes is a complex, chronic illness requiring continuous medical care with multifactorial risk-reduction strategies beyond glycemic control, including continuous patient self-management education and support as critical issues to prevent acute complications and reduce the risk of long-term complications [1]. At the same time, the International Diabetes Federation (IDF) defined diabetes as a pandemic disease and the major cause of cardiovascular (CV) disease (CVD), chronic renal disease, blindness, and amputation. In 2017, 425 million people were affected by diabetes worldwide [2].

One of the main priorities agreed on at the 2013 United Nations high-level meeting on non-communicable diseases (NCDs) was to halt the number of people living with diabetes as in 2010 [3]. Despite these efforts, the number of diabetic patients is expected to continue growing, reaching 629 million in 2045, regardless of country of residence, sex, race, social, or income levels [2]. Data from the NCD Risk Factor Collaboration (NCD-RisC) showed that the global age-standardized prevalence of diabetes between 1980 and 2014, rose from 4% to 9% in men, and from 5% to 8% in women. [4].

Despite a multitude of investigators around the world working extensively on a cure for diabetes, using different approaches, from islet transplantation to stem-cell therapies, progress is slower than anticipated, and a definitive cure is currently not available and actually still far in the future. Indeed, diabetes is a plague due to the increased risk of multiple micro- and macrovascular conditions, dementia, cancers, and infectious diseases. Notably, people with diabetes are supposed to have double the risk of CVD as compared with sex-, age-, and body mass index (BMI)-matched people with no glycemic derangements [5].

Even if women have historically poorer risk factor profiles, they usually receive lesser CV care compared with men, despite no differences in the safety and effectiveness of medication between women and men [6]. It is noteworthy that women with diabetes have a 44% greater risk of coronary artery disease as defined by the presence of angina, heart failure, and/or myocardial infarction [7], and a 27% greater risk of stroke than men [8], independent of sex differences and other major risk factors. Interestingly, the increased risk of microvascular chronic complications in diabetes has been shown to be a "phenomenon

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with a memory". The first evidence was reported in a study from Dr. Lorenzi's group in the 1980s in which the authors elegantly showed that the microvascular changes induced by hyperglycemia persisted after restoration of normoglycemia [9]. Indeed, on the one hand, these observations have been replicated in humans within the Diabetes Control and Complications Trial (DCCT) 30-years follow-up study [10], and on the other hand, these observations represent the foundation of the study of the alterations induced by diabetes to the epigenome [11]. Therefore, in this complex clinical setting, the prevention of the chronic complications of diabetes is one of the main therapeutic goals. Currently, the only available approach to achieve this goal is an adequate management of blood glucose levels, and good control of blood pressure, cholesterol, triglycerides, and body weight through balanced diet and lifestyle changes [12]. Noteworthily, therapeutic patient education is now considered a crucial element in the treatment and prevention of diabetes: several trials have shown that education is able to improve clinical, lifestyle, and psycho-social outcomes, but so far they have not clarified the ideal characteristics of a comprehensive patient education program in clinical practice [13, 14].

In the past, microvascular disease was thought to affect the smallest blood vessels after a long history of diabetes, while stroke and heart attacks were considered classical manifestations of macrovascular disease [15]. In 2000, the first edition of the *Diabetes Atlas* well described microvascular complications as abnormally thick but weak walls of the vessels, leading to bleeding, leaked proteins, and the slowing of the flow of blood through the body. Diabetic retinopathy (RD), nephropathy, neuropathy, and food lesions (up to amputations) were considered the peculiar manifestations of this condition [15]. However, in the past decade, increasing evidence has been published indicating that functional and structural abnormalities of the coronary microvascular district cause myocardial perfusion impairment and, finally, ischemia [16]. Hyperglycemia causes microvascular dysfunction, modifying several physiological pathways, such as NO and arachidonic acid metabolism, and, consequently, generating increased oxidative stress [17]. At early stages, patients with subclinical levels of diabetes-induced myocardial changes (atherosclerotic changes of coronary arteries and microvascular endothelial dysfunction) are usually asymptomatic. Therefore, if not precociously detected, the disease may advance rapidly, leading to heart failure and death [18].

Intriguingly, hallmark studies such as the DCCT/EDIC (Epidemiology of Diabetes Interventions and Complications) and the ACCORD-MIND (Action to Control Cardiovascular Risk in Diabetes – Memory in Diabetes), have demonstrated a link between diabetes and cognitive dysfunction [19, 20]. In addition, other more recent cohort studies highlighted a strong correlation between both type 1 and type 2 diabetes and the development of dementia, especially of vascular origin [21, 22]. The hypothesis of an association between cognitive impairment and microvascular derangement has been finally confirmed by the observations of a solid correlation between RD, the most frequent microvascular complication, and poor neurocognitive performance in patients with diabetes [23–26], with alterations of both gray and white matter structure [27, 28]. It has been proposed that inflammation may also play a key pathophysiological role in this clinical context. Indeed, it is well known that diabetes is associated with high levels of pro-inflammatory cytokines; accordingly, high levels of inflammatory markers in the cerebrospinal fluid and in the circulation have been related to both RD and cognitive impairment [29, 30].

This thorough and comprehensive book integrates new and accessible material on diabetic microvascular comorbidities. It will helps investigators, clinicians, and students to improve their understanding, providing additional knowledge, assembled in an easily consultable manner, on pathogenesis, diagnosis, research, and cure of microvascular complications.

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# 2

# Pathophysiology

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## Introduction

A conspicuous portion of the pathologies of aging and many manifestations of the complications of diabetes are consequences of microvascular dysfunction or damage. Diabetes induces functional and structural changes in the microvessels; these changes occur through multiple mechanisms and at multiple levels, and have damaging consequences for the tissues.

In human diabetes a long latency period (12–15 years) precedes the appearance of microvascular disease; symmetrically, in rodents, microvascular disease does not become detectable until at least 6 months after the induction of diabetes [1], despite sustained hyperglycemia. This does not necessarily imply, but is consistent with, the presence of systems for protection or repair that are activated and efficient at early stages of diabetes and may lose efficacy at later times. Therefore, the natural history of diabetic microvascular disease could incorporate an early phase of active vascular repair; to date however, there is no systematic evidence for this.

## Anatomo-Physiology of Microvessels

Capillaries consist of endothelium surrounded by basement membrane, with the adjunction of ascent capillaries. Arterioles consist of, from the lumen outwards: endothelium (intima), internal elastic lamina (IEL), smooth muscle (media), and finally loose connective tissue (adventitia). Venules consist of endothelium surrounded by the basement membrane and pericytes. Finally, lymphatic vessels consist of endothelium surrounded by elastic fibers and a lax basement membrane.

The endothelium is a diaphanous monolayer of cells. It constitutes the inner layer lining of the blood vessels, is in direct contact with the circulating blood, and is an important autocrine and paracrine regulator of vascular function. Therefore, the endothelium represents a critical interface between blood and peripheral tissues. Endothelial cells (ECs) synthesize

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and release on the one hand vasodilating factors such as nitric oxide (NO), the prostacyclin PGI2 – a cyclooxygenase-dependent metabolite of arachidonic acid, member of the prostanoid group of eicosanoids [2] –, and endothelium-derived hyperpolarizing factor (EDHF), which induces smooth muscle cell relaxation via hyperpolarization [3]. On the other hand, ECs synthesize and release vasoconstricting factors such as endothelin-1 (ET-1), prostaglandins, and angiotensin II (ANG-II).

Smooth muscle surrounds the endothelium, and represents "the muscle behind vascular biology" as it is the effector of vascular constriction and dilation. The smooth muscle is formed by layers of small, spindle-shaped mononucleated cells, known as vascular smooth muscle cells (VSMCs). The number of VSMC layers varies according to the location and size of the vessel: many in elastic arteries, as few as one in resistance arteries [4]. The layers of VSMCs are separated by sheets of elastic laminae, and the IEL separates the endothelium from the smooth muscle [5]. Gap junctions are intercellular connectors that allow various molecules, ions, and electrical impulses to directly pass between neighboring cells in the vessels, as throughout the whole body. "Homo" gap junctions connect same cell types (EC–EC and VSMC–VSMC); "Hetero" gap junctions – also known as myoendothelial gap junctions (MEGJs) – connect different cell types (EC–VSMC). The diffusion of vasodilation is facilitated via the transmission of membrane hyperpolarization occurring via gap junctions; this phenomenon is known as "spreading vasodilation."

Pericytes are a heterogeneous, tissue-specific, and multipotent population of mural cells present in all vascular beds. Pericytes play a critical role in supporting and stabilizing the microvasculature, and regulate multiple aspects of vascular homeostasis, such as regulation of blood flow, angiogenesis, and vascular permeability [6].

## Regulation of Microvascular Tone

### Endothelial-Dependent Vasodilation

A seminal discovery in vascular physiology was the finding by Furchgott and Zawadzki that arterial vasodilation was dependent on an intact endothelium and on the release of endothelium-derived relaxing factor (EDRF) [7] – this molecule was later identified as NO. In ECs, NO synthase – in the presence of oxygen, NADPH, and other co-factors – catalyzes the oxidation of the amino acid L-arginine to form L-citrulline and NO. NO is a gas that easily diffuses across the cell membrane to the adjacent VSMCs where it leads to a cascade of events (see below), resulting in VSMC relaxation and dilation of the vessel. As described above, ECs secrete other vasodilatatory mediators; however, NO is the main agent.

### Endothelial-Independent Vasodilation

VSMCs represent the final effectors of the vasodilating process, determined by relaxation of VSMCs, which is mainly achieved through three mechanisms: (i) the NO activation of soluble guanylate cyclase with subsequent formation of cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G, causing phosphorylation of myosin light-chain phosphatase and therefore inactivation of myosin light-chain kinase, which ultimately leads to the dephosphorylation of the myosin light chain; (ii) the PGI2 activation

of the prostacyclin (IP) receptor leads to cyclic adenosine monophosphate (cAMP)-mediated activation of protein kinase A (PKA), which in turn determines VSMC relaxation by reducing on the one hand intracellular $Ca^{2+}$, via its extrusion through pumps on the cell surface and on the sarcoplasmic reticulum, and on the other hand by inducing VSMC hyperpolarization, via activation of $K^+$ channels; and (iii) the EDHF-mediated transmission of hyperpolarization from ECs to VSMCs through gap junctions and/or the release of diffusible factors.

### Nerve Axon Reflex (NARV)

Under normal conditions, the ability to increase blood flow to the skin depends on the existence of an intact neurogenic vascular response; typically, this response is equal to one-third of the maximal vasodilatory capacity. This protective hyperemic response, also known as Lewis's triple flare response or the NARV begins with stimulation of C-nociceptive nerve fibers, leading to antidromic stimulation of the adjacent C fibers. The activated C fibers then secrete neuropeptides such as substance P, calcitonin gene-related peptide, and histamine, causing vasodilation and increased blood flow to the injured tissues.

### Myogenic Response

Bayliss in 1902 was the first to describe the myogenic response reporting that "the muscular coat of the arteries reacts, like smooth muscle in other situations, to a stretching force by contraction" and "these reactions are independent of the central nervous system, and are of myogenic nature" [8]. This intrinsic behavior of smooth muscle is independent of neural, metabolic, and hormonal influences [9]. The myogenic response is a complex event, highly regulated by an interplay of signaling mechanisms, and subtle effects of diabetes can interfere at more than one level. The myogenic response to pressure of resistance arteries entails that the VSMCs sense the pressure change, depolarize the membrane potential, and activate voltage-dependent calcium channels in the plasma membrane, so that an influx of extracellular calcium can activate the machinery responsible for actin–myosin cross-bridge cycling and VSMC contraction. While the myogenic response is an intrinsic property of the VSMCs, the events leading to constriction and its final intensity can further be modulated by a number of influences, some enhancing vasoconstriction (e.g. the activity of Rho-kinase pathway), some antagonizing it (e.g. endothelial NO) [10, 11].

### Functional Hyperemia

There is another mechanism of vessel reactivity elicited in the retina: the reduced dilation of retinal vessels to a light stimulus. A flickering source of illumination generates an increased metabolic demand of the neural retina, and the neural–glial elements of the retina trigger vasodilation to increase the blood flow and the provision of oxygen and nutrients to the retina. Of note, the retina does not present sympathetic innervation; therefore, this phenomenon cannot be attributed to Lewis's triple flare response as in the NARV (see above).

### Venoarteriolar Response (VAR)

The VAR is the constriction of small arteries attributed to myogenic mechanisms associated with the changes in pressure. The VAR owes its name to the fact that stretch receptors in small veins cause signals that constrict the "upstream" arterioles [12]. The response does not occur through adrenergic mechanisms; rather, it is likely due to myogenic mechanisms associated with the changes in pressure [12].

## Structural Changes in the Microcirculation

Structurally, the most notable changes induced by diabetes in the microvessels involve the decreased number and size of capillaries [13], and the increased thickness of the basement membrane [14, 15]. The extent of these alterations is related to glycemic control [16]. Thickening of the basement membrane has detrimental consequences on numerous cellular functions, such as vascular permeability; cellular adhesion, proliferation, differentiation, and gene expression; impaired exchange of nutrients; migration of activated leukocytes in the interstitium; and altered elastic properties of the vessel walls [17]. All these events eventually lead to vascular dysfunction.

## Functional Changes in the Microcirculation

In the simplest terms, functional changes are the inability of the microvessels to modify their caliber in response to local stimuli; they can be the result of different conditions, such as: endothelial dysfunction, smooth muscle cell dysfunction, impairment of the nerve axon reflex, defective myogenic reflex, and reduced functional hyperemia.

Under normal physiological circumstances, the mechanisms leading to vasodilatation and vasoconstriction are balanced, so vascular tone and permeability, and the balance between coagulation and fibrinolysis, are finely regulated. Meanwhile, in the case of endothelial dysfunction, this balance is altered predisposing the onset and progression of atherosclerosis. Endothelial dysfunction is associated with decreased NO availability, either through loss of NO production or through loss of NO biological activity [18]. The significance of endothelial dysfunction for the micro- and macrocirculation and the variety of proposed mechanisms affecting normal function will be discussed in further detail.

### Endothelial Dysfunction

Endothelium-dependent vasodilation is impaired in diabetes, irrespective of the presence or absence of long-term complications [19–23]. The endothelium has been shown to be dysfunctional in adolescents with type 1 diabetes, a population that is generally spared from the vascular complications of diabetes [24]. This finding suggests that endothelial dysfunction is present before the development of vascular complications and may play an important role in their development. Endothelial function in diabetes has been shown to be associated with total cholesterol, red cell folate, blood glucose, and duration of diabetes [25–27].

Extensive research effort has focused on the relationship of diabetes and vascular disease; it is currently well established that changes in the endothelial function precede the development of diabetes, and are already present in the prediabetic stage. It is also of interest that endothelial dysfunction is associated with insulin resistance in non-diabetic subjects, suggesting a cause–effect relationship of these two conditions [23].

A study conducted in Dr. Veves' unit found that the vasodilatatory response to ACh was reduced in patients with diabetes complicated by neuropathy alone, neuropathy and vascular disease, and patients with Charcot neuroarthropathy; meanwhile, no difference was found between patients with diabetes not complicated by neuropathy and the healthy controls. In addition, Dr. Veves' group also found that the vasodilatory response was not diminished in subjects with neuropathy and vascular disease compared with subjects with neuropathy alone. Altogether, these data suggested for the first time the fundamental role played by the peripheral nervous system in regulating microcirculation [28].

Impairment in the microcirculation was also found to be present in the absence of large vessel disease. These findings implied that the main reason for reduced microvascular reactivity was the presence of neuropathy, as indicated by the fact that no other abnormalities were found in the non-neuropathic diabetic patients. Further support for this claim is provided by the findings that the coexistence of neuropathy and vascular disease did not result in a greater decrease in endothelium-dependent vasodilation than that due to neuropathy alone.

### VSMC Dysfunction

Data on endothelium-independent vasodilation function in complicated and non-complicated diabetes is controversial [29–33]. Studies by Dr. Veves' group suggested that endothelium-independent vasodilation is decreased in patients with diabetes [28]. Using laser Doppler imaging, measurements of vasodilatory response to iontophoresis of sodium nitroprusside – a NO donor – on VSMC function has been shown to be significantly reduced in diabetic patients with vascular disease, suggesting that the endothelium-independent response may be spared. Since ACh stimulates the production of NO, it was surmised that an impaired NO production was responsible for the impaired vasodilatory response observed.

### NARV Impairment

Nerve dysfunction contributes to the diminished vasodilatatory response observed in diabetes. Measurements in patients with a diabetic neuropathic foot have shown that this neurovascular response is impaired, leading to a significant reduction in blood flow under conditions of stress. It has been postulated that the observed reduction in the NARV in diabetic neuropathy is related to both impaired C-nociceptive fiber function and impaired ability of the microvasculature to respond to vasomodulators secreted by these fibers [34]. Evidence for this vasodilatatory impairment related to the presence of diabetic neuropathy is provided by studies in Dr. Veves' lab. In diabetic patients with neuropathy, neuropathy, and peripheral vascular disease, and with Charcot arthropathy, the iontophoretic response to ACh in skin areas adjacent to this substance but not in direct contact with it was significantly reduced compared with patients with uncomplicated diabetes and to healthy subjects (a phenomenon called indirect response) [35].

The impairment in axon-related vascular reactivity is believed to further aggravate the diabetic microcirculatory abnormalities, leading to a vicious cycle [28].

### Defective Myogenic Response

According to Poiseuille's law, blood flow is related to the fourth power of the vessel radius. Thus, arteries that do not constrict normally when perfusion pressure increases allow a larger fraction of pressure and flow to reach the capillaries. The pathogenic consequences intrinsic to a defective myogenic response to pressure can also explain why systemic hypertension is the primary co-pathogenic factor for both diabetic retinopathy and nephropathy. As demonstrated by Rassam, Patel, and Kohne [36], while control subjects constricted their retinal arteries in response to all increments in systemic blood pressure induced experimentally, T1D patients failed to constrict their arteries even at the smallest increment in blood pressure, and manifested large increases in retinal blood flow, especially when very hyperglycemic [36]. For nephropathy, the defective myogenic response of the glomerular afferent arteriole is known to contribute to hyperfiltration and to further worsen hyperfiltration when systemic hypertension develops [37, 38]. In animal models of diabetes an impaired myogenic response to pressure is reported in several vessels, from the ophthalmic arteries [39] to the arterioles of the cremaster muscle [40, 41], to the cerebral [42, 43] and coronary [43] arteries.

Although a defective myogenic response in T1D patients and animal models has been known for many years as a feature of the loss of vascular autoregulation, the appreciation that it can represent an early event is very recent. The reason is that only with the removal of the great equalizer (severe hyperglycemia), has it become possible to capture early individual differences in the path to complications, retinopathy in particular. In the early 1990s most if not all T1D patients had increased retinal blood flow at steady state [44]. In contrast, contemporary T1D patients with or without early retinopathy have absolutely normal retinal hemodynamics at steady state [45–47]. It was only upon the application of a challenge that Dr. Lorenzi identified patients with an abnormal myogenic response. The challenge was a simple change from the sitting to the supine position, which causes an increase in retinal perfusion pressure. In response to the postural challenge approximately 50% of T1D patients without clinical retinopathy manifested lack of arterial constriction in response to the increased perfusion pressure, i.e., a defective myogenic response [46]. When considering that a simple change in position from sitting to reclining, or physical exercise [48, 49], augments perfusion pressure in the eye, it is evident that an insufficient myogenic response will place the retinal capillaries at risk many times a day, every day.

Our group has recently defined more precisely the potential role of the defective myogenic response, as an accelerator of microvascular disease in the retina. We observed that the abnormality (i) tended to predict the appearance of retinopathy, (ii) was not required for the development of retinopathy, and (iii) was associated with accelerated onset of retinopathy [45].

### Reduced Functional Hyperemia

Some T1D patients present reduced dilation of retinal vessels to a light stimulus. The reduced dilation tends to be more pronounced in TD1 patients with retinopathy, but also

occurs in TD1 patients without clinical retinopathy, thus representing an early abnormality [50, 51]. Studies by Dr. Schmetterer's group indicate that the reduced arterial dilation in T1D is not due to decreased reactivity of the retinal arterial smooth muscle cells (SMCs), as the response to a direct vasodilator was normal [52]. Nor is it due to neural retina dysfunction, as the abnormal dilation occurs in the presence of normal pattern electroretinogram [51]. This abnormality has never been investigated with prospective follow-up studies, and the presentations of results have not included individual data to learn whether the abnormality is present in all patients or is more pronounced in some patients than in others.

### Endothelial Dysfunction

Endothelial dysfunction is expressed as increased smooth muscle growth, vasoconstriction, impaired coagulation, thrombosis, and atherosclerosis. The main causes of endothelial dysfunction in diabetes have been postulated to include hyperglycemia, insulin resistance, and inflammation as possible mediators of abnormal endothelium-dependent responses.

### Role of the Immune System and Leukostasis

The dysregulation of the immune system in diabetes is responsible for the activation of the inflammatory response, which in turn generates oxidative stress and increases insulin resistance, thereby favoring the development of microvascular complications. For example, the expression of ICAM-1, a molecule that mediates the adhesion of leukocytes to the endothelium via binding to LFA-1, is increased in diabetes and has been proposed to be a key factor in the onset and progression of diabetic nephropathy, to the extent that inhibition of ICAM-1 has been reported to slow the progression of nephropathy in diabetic animals [53, 54].

Interestingly, in animal models of diabetes, one of the earliest initial findings in microvascular disease is represented by an increased number of leukocytes firmly adherent to the microvessels. This phenomenon, known as diabetic leukostasis and interpreted as a detrimental event for a long time, [55] is constituted by two principal cellular components: monocytes and granulocytes [56]. Our group has recently demonstrated that a discrete subpopulation of monocytes, called patrolling monocytes (PMo), coincide substantially with diabetic leukostasis (Tecilazich and Lorenzi, unpublished). PMo are characterized by a distinct phenotype as they crawl on the endothelium at a speed that is up to 1000 times slower than the typical leukocyte rolling; [57] present a peculiar anti-inflammatory biosynthetic profile [58]; and exert protective and healing actions in different tissues and contexts [59–61]. Studies in our unit clearly indicate that the reduction of leukostasis by removal of PMo in transgenic mice profoundly exacerbates the microvascular pathology seen in diabetes. Moreover, our studies on the transcriptome of these cells show that PMo respond to diabetes by activating an anti-inflammatory, anti-apoptotic, and vasculo-protective program (Tecilazich and Lorenzi, unpublished). This finding is striking as other immune cells respond to diabetes in the opposite fashion [62–65]. Altogether, our data support the hypothesis that PMo dampen the stress induced by diabetes on the microvessels, and that ultimately leads to diabetic microvascular disease. Quite interestingly, the role of monocytes in the progression of diabetic

microvascular disease has been suggested based on the findings that inhibition of CCL-2 improved diabetic nephropathy [66, 67]; however, CCL-2 is expressed on inflammatory monocytes, but not on PMo.

Further investigation is needed to validate this protective system, and hopefully to ascertain from this – and/or from other endogenous systems – the types of molecular actions that protect the vessels in early diabetes; and thus be enabled to mimic, complement, or leverage such actions via exogenous interventions over the duration of diabetes.

## Role of Hyperglycemia

Hyperglycemia causes endothelial dysfunction primarily through the induction of oxidative stress. The molecular mechanisms involved in this process include the enhanced activation of protein kinase C (PKC); synthesis of vasoconstrictor prostanoids; activation of poly (ADP-ribose) polymerase (PARP); generation of oxygen-derived free radicals; synthesis of endothelin-1 (ET-1); induction of the polyol pathway; and generation of advanced glycosylated end products (AGEs). An additional mechanism is the reduction of the $Na^+$–$K^+$ ATPase activity.

### AGEs

When proteins are exposed to hyperglycemic environments, a non-enzymatic reaction (Maillard) determines the formation of Shiff bases that can be rearranged through non-enzymatic glycosylation to form Amadori products, and eventually AGEs. AGEs contribute substantially to the increased vascular permeability of diabetes by altering protein structure and function, by modifying the extracellular matrix's structure, and by triggering inflammation. The blockade of a specific receptor for AGE (RAGE) reverses diabetes-mediated vascular hyperpermeability [68], and limits the generation of reactive oxygen species (ROS). Interestingly, the relationship between ROS and AGEs is bidirectional as the inhibition of ROS prevents the generation of AGEs, suggesting that the autoxidative process plays an important role in the complex reaction cascade leading to AGE.

### Polyol Pathway

Aldose-reductase (AR) – a key factor in the polyol pathway – normally inactivates aldehydes by reducing them to alcohols. In the presence of intracellular hyperglycemia, glucose enters the polyol pathway: AR reduces glucose to sorbitol, an organic osmolyte, utilizing the cofactor NADPH; subsequently, sorbitol dehydrogenase oxidizes sorbitol to fructose. The result of excess polyol pathway activation is: increased sorbitol (which causes osmotic stress), increased fructose (which is a very potent glycation agent), increased ROS (see below), and decreased intracellular antioxidants NO and glutathione – this is the result of the consumption of NADPH, cofactor in the synthesis of sorbitol, NO, and glutathione [69]. The accumulation of sorbitol also causes the depletion of other osmolytes, like myo-inositol and taurine [70], leading to vascular dysfunction and to nerve conduction defects [71]. In fact, the depletion of myo-inositol impairs the phosphoinositide metabolism and decreases phosphoinositide-derived diacylglycerols, which impairs neural PKC activation, with subsequent reduction of Na+–K + -ATPase activity [72]. Finally, the increased activity

of AR plays a critical role also in the downstream activation of MAPK (mitogen-activated protein kinase) [73], PARP [74], and NF-κB [73].

More than 32 randomized controlled trials have tested the efficacy of AR inhibitors (ARIs), such as ranirestat and epalrestat, in the treatment of diabetic neuropathy. A meta-analysis involving 879 ARI-treated and 909 control (placebo or no treatment) participants, showed no overall significant difference in the treatment of diabetic polyneuropathy between the groups [75].

#### Oxidative Stress

Oxygen-derived free radicals – such as superoxide and other ROS – are physiologically produced in the vascular cells by NADPH oxidase, xanthine oxidase, the mitochondrial electron transport chain, uncoupled endothelial NOS, and arachidonic acid metabolism pathways. Oxidative stress is generated when the production of oxygen-derived free radicals exceeds the buffering capacity of available antioxidant defense systems. Diabetes promotes oxidative stress on the one hand by increasing lipid peroxidation products and protein carbonylation, and on the other hand by decreasing antioxidant efficiency through decreased activity of superoxide dismutases, catalases, glutathione peroxidase, and thioredoxin. Oxidative stress in turn induces cell dysfunction or death through the degeneration of biological macromolecules: nucleic acids, lipids, and proteins. Vitamin E is a potent free radical scavenger, and its effect on vessels has been tested at low and high doses: at low doses (400 IU/day) it has no effect on cardiovascular outcomes in patients with diabetes [76]. An initial promising study using high doses (1800 IU/day) showed normalized hemodynamic abnormalities – suggesting that administration of antioxidants could potentially reduce diabetic vascular complications [77] –; subsequent studies unfortunately did not replicate this finding [78], and some studies actually suggested an association of high-dose Vitamin E with worsening of vascular reactivity

#### PKC

The PKCs are a superfamily of cytoplasmic serine/threonine kinases, ubiquitously expressed, involved in a wide range of intracellular signaling, such as: oxidant, inflammatory, mitogenic, and angiogenic. There are 10 PKC isoforms, grouped as classic, novel, and atypical: the most consistently associated with diabetic microvascular disease are PKC α, β, and δ; whereas PKC ε has been suggested to play a protective role in the kidney [79], and has been recently associated with hepatic insulin resistance [80]. In vessels, PKC regulates proliferation, neovascularization, permeability, and contractility; the production of extracellular matrix, the synthesis of cytokines, the activation of cytosolic phospholipase A2, and the inhibition of the $Na^+$–$K^+$-ATPase pump. The effects of pharmacological inhibition of PKC-β overactivation with the bisindolylmaleimide ruboxistaurin (RBX) were tested in diabetic animals, in whom they improved renal, retinal, and neural function [81–84]. The effects of RBX have been tested in patients with proliferative retinopathy, leading to decreased incidence of vision loss [85]; in patients with late-stage nephropathy, leading to decreased incidence of end-stage renal disease [86]; and in patients with severe peripheral neuropathy, in whom it led to significantly decreased symptoms of neuropathy, even though it failed to achieve the primary end point [87].

### Transforming Growth Factor (TGF)-β

TGF-β is a cytokine that plays important roles in the maintenance of vascular homeostasis by regulating the production of extracellular matrix, the replication and survival of ECs, the interactions of ECs and pericytes, and the remodeling of vessels. In addition TGF-β has been shown to be involved in the pathogenesis of disease, such as Marfan and other vasculopathies. In experimentally diabetic animals, Tgf-β synthesis is increased in the kidney [88] and the retinal vessels [89]; furthermore, treatment with anti-Tgf-β antibodies prevents nephropathy [88]. Altogether, these observations have proposed TGF-β is a contributor to, if not a master-mediator of, diabetic microvascular disease. However, data on the role of TGF in vascular disease are conflicting. Recent work from our unit indicates that, in diabetes, the retinal vessels become dependent on an increase in TGF-β signaling to maintain their integrity [90]. Our results are consistent with findings of other groups that used different approaches in different contexts [91–93]. It is noteworthy that TGF is known to be a negative regulator of lymphocytes, and to exert anti-inflammatory actions on mono-macrophagic immune cells.

### Hexosamine Pathway

The first step of the glycolytic pathway is the conversion of glucose-6 phosphate to fructose-6 phosphate. In the presence of intracellular hyperglycemia, not all the fructose-6 phosphate enters the glycolytic pathway; some in fact is diverted to the hexosamine pathway, where it is first converted to glucosamine-6 phosphate, with the concomitant conversion of glutamine to glutamate (a reaction catalyzed by glutamine:fructose-6 phosphate amidotransferase; GFAT), subsequently glucosamine-6 phosphate is converted to uridine diphosphate N-acetyl glucosamine (UDP-GlcNAc). UDP-GlcNAc – a high-energy sugar nucleotide donor – can glycosylate lipids and proteins, can induce the generation of other UDP sugars, or can induce O-GlcNAc modifications of nucleocytoplasmic proteins (e.g. transcription factors, signaling components, and metabolic enzymes) [94], thereby altering gene expression profiles of cells exposed to high glucose.

### PARP

PARP is a nuclear enzyme that responds to oxidative DNA damage by activating an inefficient cellular metabolic cycle, often leading to cell necrosis. Activation of PARP is associated with changes in microvascular reactivity; in addition, PARP's activation – besides being related to endothelial dysfunction in patients with diabetes – has been observed also in healthy patients at risk of developing diabetes [95]. These findings overall suggest that changes in the microcirculation due to PARP activation may begin in the prediabetic state.

### ET-1

ET-1 is a potent vasoconstrictor. Hyperglycemia, ROS, and AGEs induce the synthesis of ET-1 through the activation of NF-κB [96]. The exact mechanism by which hyperglycemia induces its increase is not fully understood; however, it seems that its upregulation is a consequence of NO-Ang-II imbalance. Another possible pathway responsible for the increase of ET-1 is the PKC-mediated induction of the endothelin-converting enzyme (ECE)-1, which activates ET-1 [97].

### Vasoconstrictor Prostanoid Synthesis

Studies in diabetic animals have indicated that an abnormal endothelial production of vasoconstrictor prostanoids – such as thromboxane $A_2$ ($TXA_2$) and prostaglandin $H_2$ ($PGH_2$) – may have a role in endothelial dysfunction. However, studies conducted in humans have failed to confirm the role of vasoconstrictor prostanoids in diabetic microvascular disease. Moreover, endothelium-dependent dilation of the macrovessels, studied by flow-mediated vasodilation in healthy subjects, was unaffected by aspirin, suggesting that the mechanism is independent of vasoconstrictor prostanoids [98].

## Role of Insulin Resistance

Besides its anabolic action, insulin also exerts a hemodynamic action that induces vasodilation and capillary recruitment. The effect on vasodilation is exerted through insulin's modulation of the synthesis and release of NO [34, 99]; in fact, insulin stimulates endothelial NOS by mediating the activation of signaling pathways involving the recruitment of phosphoinositide-3 (PI-3) kinase, which eventually leads to the phosphorylation of endothelial NOS and thereby NO synthesis. It has been proposed that as much as 25% of insulin's stimulatory effect on muscle uptake of glucose is related to its hemodynamic actions [100].

In addition, insulin determines dilation of the capillaries and relaxation of the presphincter, via activities on its receptor on the ECs. In this way insulin's metabolic action is enhanced, both by recruiting new capillary beds and by redirecting the capillary blood flow more toward insulin-sensitive tissues (muscle and adipocytes), and away from insulin-independent tissues (bone and skin) [101].

There is a well-established bidirectional link between hyperinsulinemia and endothelial dysfunction: on the one hand, exposure of vascular endothelium to hypertriglyceridemia and elevated low-density lipoprotein (LDL) cholesterol particles – stereotypical of insulin resistance states – is responsible for reduced NO availability [102]; on the other hand, endothelial dysfunction contributes to impaired insulin action, by altering the transcapillary passage of insulin to target tissues. Although the molecular mechanisms determining the metabolic and vascular abnormalities associated with the insulin resistance state have yet to be entirely elucidated, the impaired NO production clearly appears to play a pivotal role.

## Role of Inflammation

The origins of the increased inflammatory status in type 1 and type 2 diabetes are profoundly different; in both settings however, hypertension and dyslipidemia – in concert with hyperglycemia – play a critical role in the maintenance and exacerbation of inflammation. An early feature of inflammation is the increased levels of cytokines such as: C-reactive protein (CRP), TNF-$\alpha$, interferon $\gamma$, and interleukins-1$\beta$, -2, -4, -6, and -17. Inflammatory cytokines disrupt vascular permeability and impair the vasoregulatory responses. In addition these molecules exert procoagulant activities (i) by inhibiting anticoagulant pathways, (ii) by impairing fibrinolysis (via increased expression of plasminogen activator inhibitor (PAI)-1 and of tissue factor), (iii) by directly activating platelets, and (iv) by increasing the circulating levels of coagulation factors, such as fibrinogen and factor VIII [103].

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# 3

# Genetics of Diabetic Microvascular Disease

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## Introduction

Diabetic microvascular complications are a familiar burden to the diabetic patient. One-third of patients will develop diabetic kidney disease (DKD), which is the leading cause of end-stage renal disease (ESRD) in adult patients and is linked to increased cardiovascular morbidity and premature death. Diabetic retinopathy is the most common cause of blindness in working age people in the world. Diabetic neuropathy takes many forms, the most common ones being peripheral neuropathy and cardiovascular autonomic neuropathy. Most diabetic patients will develop at least milder forms of neuropathy. The microvascular complications are inter-related and it may sometimes be difficult to study one of the complications in particular, completely independent of the two others.

It is evident that the microvascular complications are complex disorders where many genetic factors are involved. In this chapter, we will mainly focus on the genome-wide hypothesis-free approaches to identify common genetic variation related to the diabetic microvascular complications (nephropathy, retinopathy, and neuropathy). Some interesting recent or larger-scale candidate gene efforts are also mentioned. Understanding the genetic basis of the microvascular complications may give us clues to a better understanding of the pathogenesis of diabetic microvascular disease and thus potentially provide us with tools to develop new therapeutic strategies to prevent the complications.

## Genetics of DKD

### Heritability of DKD

Heritability of DKD was first suggested by studies indicating familial clustering of DKD, with the phenotype definitions ranging from microalbuminuria to persistent proteinuria and ESRD [1–4]. Heritability estimates of albuminuria in subjects with type 2 diabetes (T2D)

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range from 30 to 40% [5–7], and up to 75% for estimated glomerular filtration rate (eGFR) [7]. While the sibling risk of DKD was estimated as 2.3-fold in 537 families with at least two siblings with type 1 diabetes (T1D), a higher risk ratio of 2.9 was obtained in a subset of probands with ESRD [4]. A similar increase of heritability for more severe DKD definitions was obtained from a recent study that utilized genome-wide genotyping data of 2843 subjects with T1D to estimate the heritability of DKD with varying phenotype definitions: the narrow-sense heritability of broadly defined DKD (micro- or macroalbuminuria or ESRD) was estimated to be 35%, when it was as high as 47% for ESRD [8].

## Candidate Genes for DKD

Over the decades, genetic variants in a myriad of positional and biological candidate genes have been investigated for DKD, but few candidate genes were robustly replicated. Among the most studied polymorphisms is an insertion/deletion variant rs1799752 (also tagged by another variant, rs4344) that affects the cellular concentration of angiotensin converting enzyme (ACE) [9]. ACE inhibitors and other medical agents inhibiting the renin-angiotensin-aldosterone system (RAAS) are currently the main treatment option to slow down the progression of DKD. Despite a striking 26 000 subjects included in a meta-analysis of 63 candidate gene studies for the *ACE* variant, only modest evidence of association with DKD was found, mainly in the subgroup of Asian subjects with T2D [10]. Characteristic to the candidate gene studies, the vast majority of the 63 included studies only had tens or few hundreds of subjects, which may also partially explain the vague results.

One large-scale candidate gene effort tested 344 single nucleotide polymorphisms (SNPs) and other variants from 127 candidate genes for DKD in a meta-analysis of three studies including 2499 European subjects with T1D. A variant in the *UNC13B* gene was deemed significant after correction for multiple testing, with an odds ratio (OR) of 1.63 and p-value of $2.3 \times 10^{-5}$ for rs13293564 after fine-mapping and combined meta-analysis with the replication study [11].

In order to systematically evaluate the cumulative evidence of the reproduced candidate genes, Mooyaart et al. conducted a literature-based meta-analysis investigating 132 publications on genetic variants for DKD in T1D and/or T2D. The meta-analysis yielded positive results for 24 variants from 17 distinct loci, including the ACE insertion/deletion variant [12]. However, such analysis may suffer from publication bias, and the results may be overly optimistic.

Another effort to assess the role of the previously suggested genetic loci was conducted in the Genetics of Nephropathy, an International Effort (GENIE) consortium by Williams et al., where they re-evaluated a selection of previously supported genetic variants for DKD in up to 6366 subjects with T1D, out of which 2966 were defined as cases with persistent proteinuria or ESRD [13]. A previously reported association with a combined phenotype of ESRD and proliferative diabetic retinopathy (PDR) at rs1617640 in the *EPO* gene (encoding erythropoietin) was not replicated, but the association remained genome-wide significant after a combined meta-analysis though attenuated (p-value $2 \times 10^{-9}$). While nominal evidence (i.e. p-value $<0.05$) was obtained for some of the other loci, most of the previously reported associations were not replicated [13].

## Linkage Studies for DKD

The family-based linkage studies provided a hypothesis-free way to search for novel chromosomal loci linked with a disease, without prior knowledge of the biological mechanisms behind it. The linkage studies on DKD have resulted only in few chromosomal regions reaching a robust logarithm of odds (LOD) score. Even though first reported in a candidate region linkage analysis of chromosomal regions containing genes for the renin–angiotensin system [14], many whole-genome linkage studies on DKD found suggestive evidence of linkage on broadly the same chromosomal region in chromosome 3q21–25 [15–18], well summarized in [15]. However, this region never reached robust statistical significance. To fine-map the linkage region, a positional case–control candidate gene meta-analysis of multiple cohorts identified variants near the *NCK1* gene with relatively high p-values ($7\times10^{-6}$) [19]. A more recent multi-cohort study including 175 families from Finland, Denmark, and France identified a linkage with diabetic nephropathy at chromosome 22q11 with LOD score 3.6, but no fine-mapping efforts were performed to narrow down the source of the linkage [18].

## Genome-Wide Association Studies on DKD

The vague results from the whole-genome linkage studies, and the lack of robust replication of the candidate genes highlighted the need for genome-wide, hypothesis-free searching for genetic variants associated with DKD in a larger number of subjects. Early high-throughput association studies in Japanese subjects with T2D initially evaluated with 50 000 polymorphisms [20], and later with 80 000 polymorphisms, suggested association with DKD at *ACACB* gene encoding acetyl coenzyme A (CoA) carboxylase 2 [21], and with rs741301 in the *ELMO1* gene [22]. Despite multiple subsequent replication attempts, the role of rs741301 and other variants in *ELMO1* remains unclear, with some studies supporting and others refuting the finding [8, 13, 23–27]. A further early implementation of a genome-wide association study (GWAS) utilized pooled DNA of 547 ESRD cases and 549 T1D subjects without DKD, genotyped on a GWAS chip with 555 352 SNPs, but found only suggestive evidence of association with ESRD with $p<0.0001$ for variants in in *ZMIC1* and musculin genes [28].

In a GWAS on DKD including 820 cases and 885 controls of European ancestry from the US Genetics of Kidneys in Diabetes collection, no locus reached the statistical significance required for genome-wide significance, defined as p-value $<5\times10^{-8}$, but suggestive associations were found near *FRMD3* ($p=5.0\times10^{-7}$) and *CARS* genes ($p=3.1\times10^{-6}$), and on chromosomes 7p and 13q. Supportive evidence of association was found for the *FRMD3* and *CARS* loci in the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) Study [29]. In subsequent studies, variants in the *FRMD3* were associated with ESRD in African American subjects with T2D after accounting for a major genetic risk factor for non-diabetic renal disease at *MYH9* locus [30] and in a family study of European American patients with T2D [31], but not in subjects of European ancestry with T1D [13]. The association at the *MYO16/IRS2* locus on chromosome 13q was supported by Japanese [32] and European American patients with T2D [33]. Subsequent analysis of the US GoKinD GWAS data using SNP imputation

revealed four additional loci with a suggestive p-value $<10^{-5}$, e.g. rs7071071 in the *SORBS1* gene [34]. Of note, other variants in the *SORBS1* gene were later reported as potentially associated with DKD in a GWAS of European subjects with T1D; after evaluation in GoKinD US, rs1326934 was strongly associated with DKD with $p = 3.5\times10^{-9}$, but the signal was attenuated to $p = 0.009$ after replication in two additional European cohorts [35].

The first GWAS meta-analysis with genome-wide significant findings was performed by the GENIE consortium. With 6231 subjects with T1D at the discovery stage, and a total of 11 847 patients in a meta-analysis including the replication cohorts, the study identified two loci genome-wide significantly associated with ESRD: rs7583877 in *AFF3* ($p = 1.2\times10^{-8}$) and rs12437854 intergenic between the *RGMA* and *MCTP2* genes ($p = 2.0\times10^{-9}$; Table 3.1). Functional studies on renal epithelial cells suggested that *AFF3* is involved in the renal fibrosis [36]. While little is known of the *RGMA* and *MCTP2* genes, and the affected gene may be located much further away from the main association signal, the same locus was one of the main findings also in a GWAS data mining approach performed in a subset of the subjects [37]. For the DKD phenotype the strongest association in the GENIE GWAS was obtained at the *ERBB4* gene ($p = 2.1\times10^{-7}$ for rs7588550); *ERBB4* gene expression analysis indicated co-expression with collagen genes, also associated with renal fibrosis [36]. Conditional mouse overexpression and knock-out models suggest that *ERBB4* has an important role in the development of kidneys [38], and ErbB4 has been previously suggested as a therapeutic target molecule for the treatment of cardiovascular disorders, schizophrenia, and cancer [39]. Recent work showed protection from albuminuria in STZ-induced hyperglycemic wild-type and miR-146a$^{-/-}$ mice when pan-ErbB inhibitor was administered, suggesting ErbB4/EGFR as a druggable target for DKD [40].

A gender-stratified GWAS analysis in 3652 Finnish subjects with T1D identified rs4972593 between the *SP3* and *CDCA7* genes on chromosome 2q31.1 associated with twofold risk of ESRD in women with p-value $<5\times10^{-8}$, and the finding was replicated in other cohorts in the GENIE consortium [41]. No effect was seen in diabetic men or in subjects with T2D. While the RegulomeDB suggest potential regulatory activity for a SNP in full linkage with rs4972593, no eQTL association was identified to link the SNP to expression of any flanking genes. Nevertheless, the gene expression of *SP3* is among the most gender-specific ones in human glomeruli of diabetic patients [42], and Sp3 transcription factor has been shown to directly bind to the estrogen receptor α [43], providing a potential link with the gender-specific association.

A trans-ethnic GWAS including 13 736 subjects with T2D with European American, African American, Mexican American, or American Indian ancestry from the Family Investigation of Nephropathy and Diabetes (FIND) study found variants between the *SCAF8* and *CNKSR3* genes associated with DKD with p-value $<5\times10^{-8}$, with particularly strong association in the American Indian population (rs12523822, $p = 5.7\times10^{-9}$) [44]. *CNKSR3* is a scaffolding platform that stimulates epithelial sodium channels in response to aldosterone [45]. As inhibition of the RAAS is the main therapy for DKD and other proteinuric kidney diseases, *CNKSR3* is a plausible target gene behind the association. The *MYH9* locus, which is one of the main genetic risk factors for non-DKD in African Americans, was near genome-wide significantly associated ($p = 7.7\times10^{-8}$) with kidney disease in African American subjects with T2D. However, the authors note that this is likely due to a proportion of study subjects with non-DKD [44].

**Table 3.1** Genome-wide association study (GWAS) on diabetic kidney disease (DKD).

| Study | Trait | Discovery population, N (cases/controls) | Replication/meta-analysis, $N_{REP/META}$ (cases/ controls)[a] | Loci identified for diabetic kidney disease (DKD) |
|---|---|---|---|---|
| Tanaka 2003 [20]<br>Shimazaki 2005 [22] | DKD | Japanese type 2 diabetes (T2D), N = 188 (94/94) | $N_{REP}$ = 732 (466/266) | rs11643718 (*SLC12A3*) $p = 8.7\times10^{-5}$, OR = 2.53<br>rs741301 (*ELMO1*) $p = 8\times10^{-6}$, OR = 2.67 |
| Maeda 2010 [21] | DKD | Japanese T2D, N = 188 (94/94) | $N_{REP}$ = 1312 (754 /558) | rs2268388 (*ACACB)* $p = 5.35\times10^{-8}$, OR = 1.61 |
| Pezzolesi 2009 [29]<br>Pezzolesi 2010 [34] | DKD | European American type 1 diabetes (T1D), N = 1705 (820/885) | $N_{REP}$ = 1304 (132/1172) | rs10868025 (*FRMD3*) $p = 5.0\times10^{-7}$, OR = 1.45<br>rs451041 (*CARS*) $p = 3.1\times10^{-6}$, OR = 1.36<br>Imputation suggested four additional loci, e.g.<br>rs7071071 (*SORBS1*) $p = 4.5\times10^{-6}$ |
| McDonough 2011 [51] | end-stage renal disease (ESRD) | African American T2D, N = 1994 (965 T2D-ESRD cases/1029 NDCtrl) | 709 T2D-ESRD/690 NDCtrl; 1246 T2D controls w/o DKD; 1216 non-diabetic ESRD | 19 potential loci for kidney disease in T2D |
| Sandholm 2012 [36] | DKD, ESRD | Caucasian T1D DKD: N = 6231 (2916/3315); ESRD: N = 6652 (1399/5253) | $N_{META}$ = 11 847 | **ESRD: rs7583877 (*AFF3*) $p = 1.2\times10^{-8}$, OR = 1.29**<br>**ESRD: rs12437854 (*RGMA/MCTP2*) $p = 2.0\times10^{-9}$, OR = 1.8**<br>DKD: rs7588550 (*ERBB4*) $p = 2.1\times10^{-7}$, OR = 0.66 |
| Sandholm 2013 [41] | ESRD | Finnish T1D N = 3652; $N_{Females}$ = 1193 (258/935) | $N_{META}$ = 2697 (688/2009) women | **rs4972593 (*SP3/CDCA7*) $p = 3.9\times10^{-8}$, OR = 1.81 in women** |
| Sandholm 2014 [47] | albumin excretion rate (AER)/albumin to creatinine ratio | Finnish T1D, N = 1925 | $N_{REP}$ = 3750 Caucasian T1D | **rs1564939/rs10011025 (*GLRA3*) $p = 1.5\times10^{-9}$ in Finnish discovery.**<br>rs2410601 (*PSD3/SH2D4A*) $p = 3.9\times10^{-6}$ |

(*continued*)

**Table 3.1** Continued

| Study | Trait | Discovery population, N (cases/controls) | Replication/meta-analysis, $N_{REP/META}$ (cases/ controls)[a] | Loci identified for diabetic kidney disease (DKD) |
|---|---|---|---|---|
| Sambo 2014 [37] | DKD, ESRD | Finnish T1D, N = 3464 (multiple phenotypes) | $N_{REP}$ = 4263 European T1D (multiple phenotypes) | Data mining suggested six loci:<br>rs12137135 (*WNT4-ZBTB40*)<br>rs17709344 (*RGMA-MCTP2*)<br>rs1670754 (*MAPRE1P2*)<br>rs12917114 (*SEMA6D-SLC24A5*)<br>rs2838302 (*SIK1*) |
| Germain 2015 [35] | DKD | Caucasian T1D, N = 1462 (683/779) | $N_{META}$ = 7861 (3661/4200) | rs1326934 (*SORBS1*) p = 0.009, OR = 0.83<br>in random-effect meta-analysis |
| Iyengar 2015 [44] | DKD | multi-ethnic T2D, N = 6197 (3223 DKD cases/1686 T2D ctrls/1288 NDCtrl) | $N_{META}$ = 13 736 (including 6229 NDCtrl) | **rs12523822 (*SCAF8/CNKSR3*) $p = 5.7\times10^{-9}$, OR = 0.57 in American Indians** |
| Teumer 2016 [53] | ACR | up to 54 450 Caucasians, including 5825 diabetic (mostly T2D) | $N_{META}$ = 7787 diabetic | rs649529 (*RAB38*) $p = 5.8\times10^{-7}$,<br>rs13427836 (*HS6ST1*) $p = 6.3\times10^{-7}$ |
| Pattaro 2016 [52] | estimated glomerular filtration rate (eGFR), chronic kidney disease (CKD) | up to 133 413 Caucasians, out of which 16 477 diabetic | $N_{META}$ = 16 477 diabetic | **eGFR: rs12917707 (*UMOD*) $p = 2.5\times10^{-8}$**;<br>In diabetic subset $p < 0.05$ for 19/53 loci for eGFR in general population |
| Sandholm 2017 [8] | DKD, CKD, ESRD | European T1D, N = 5156 (multiple phenotypes) | $N_{META}$ = 12 540 Caucasian T1D (multiple phenotypes) | Suggestive associations at rs61277444 (*PTPN13*), rs7562121 (*AFF3*), rs1989248 (*CNTNAP2*), and rs72809865 (*NRG3*) |

[a] Replication/meta-analysis population description (e.g. "European T1D") is the same as the discovery population unless otherwise stated.
$N_{REP}$: N in replication studies, divided to (cases/controls). $N_{META}$: N in combined meta-analysis of discovery and replication studies. $N_{META}$ is given, rather than $N_{REP}$, if joint meta-analysis was performed to obtain the final results. NDCtrl: Non-diabetic control (without kidney disease).
Findings reaching genome-wide statistical significance (p-value $< 5\times10^{-8}$) are highlighted with bold text.

Even though the current view of the clinical course of DKD is more versatile, albuminuria has been considered as the classical hallmark of diabetic nephropathy [46]. A GWAS on albuminuria as a continuous trait in 1925 Finnish patients with T1D identified five SNPs with $p < 5 \times 10^{-8}$ in the *GLRA3* gene. The replication attempt in 3771 additional patients of European ancestry resulted in a nominally significant ($p = 0.04$) association at rs1564939 in the opposite effect direction [47]. The authors hypothesized a population-specific effect and suggested that this warranted further replication in Finnish patients to confirm or refute the finding.

To account for the various phenotypic manifestations of DKD, a recent work from the SUrrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools (SUMMIT) consortium reported GWAS in 12 540 subjects with T1D using seven phenotypic definitions of renal complications of varying severity, based on either albumin excretion rate (AER), eGFR, or both. Suggestive associations were identified in or near *PTPN13*, *AFF3*, *CNTNAP2*, and *NRG3* loci, even though no locus reached genome-wide significance [8]. Evaluation of previous loci supported association with ESRD at rs2838302 at *SIK1*, originally identified in a GWAS data mining approach of 3464 subjects with T1D [37]. Genetic comparison with related traits showed that alleles known to increase body mass index (BMI), and risk of T2D, were associated with DKD traits, suggesting that BMI and metabolic changes leading to T2D are causal risk factors for DKD in subjects with T1D. Analysis of genome-wide correlation also suggested a shared genetic background with DKD and failure at smoking cessation [8].

The GWAS on chronic kidney disease (CKD) and eGFR in the general population has identified multiple loci affecting the kidney function [48, 49]. Previous efforts to assess the effect of the loci affecting general population in diabetic subjects suggested that variants in *GCKR*, *SHROOM3*, and *UMOD* [50], and in the *MYH9/APOL1* locus in African Americans [44, 51], play a role also in subjects with T2D, while no association has been found in subjects with T1D [8, 36]. The observed associations may to some extent arise due to a substantial proportion of subjects with T2D having coincident T2D and non-DKD [44]. The largest GWAS meta-analysis on CKD and eGFR in the general population to date included 133 413 individuals at the discovery stage and up to 42 166 individuals in replication, and resulted in 53 robust loci (p-value $< 5 \times 10^{-8}$) for kidney traits [52]. Evaluation of these 53 loci among the 16 477 included subjects with diabetes (mostly T2D) revealed significant association with eGFR at rs12917707 at *UMOD* (p-value $= 2.5 \times 10^{-8}$) and nominal associations ($p < 0.05$) at 19 loci. Another GWAS studying albuminuria as a continuous trait in up to 54 450 subjects from the general population, with a subset of 7787 subjects with diabetes (mainly T2D), found suggestive evidence of association at *HS6ST1* (rs13427836, $p = 6.3 \times 10^{-7}$) and *RAB38/CTSC* loci (rs649529, $p = 5.8 \times 10^{-7}$) in subjects with, but not without diabetes [53]. Even though the *CUBN* locus, previously associated with albuminuria in the general population [54], did not reach genome-wide significance in diabetic subjects, the effect was larger in subjects with than without diabetes [53].

## Sequencing Efforts for DKD

In addition to the chip-based approaches, next-generation sequencing-based studies are also emerging in order to address the low-frequency and rare variants affecting the disease

risk, with a premise of higher effect sizes because of either capturing the true causal variant rather than a proxy, or capturing underlying direct changes in the protein structure rather than changes in the gene regulation. A sub-study of a larger whole-exome sequencing (WES) study of subjects with and without T2D investigated the role of *RREB1* (ras-responsive element binding protein-1) gene for diabetic ESRD in 529 African American cases with T2D and ESRD and in 535 population-based controls. The findings were followed up by subsequent genotyping in replication and trait segregation studies, suggesting that variants in *RREB1* modulate the risk of T2D, ESRD, and non-diabetic renal disease, but the results should be taken with caution as the locus has been previously associated with T2D [55].

WES of 997 subjects with T1D from the SUMMIT consortium did not identify any variant that would reach the exome-wide significance ($p < 5 \times 10^{-7}$), but among the strongest associations was a common variant in the 3'UTR of *ERBB4* gene [8]; other variants not in linkage disequilibrium were previously suggestively associated with DKD in the GENIE consortium GWAS with partially overlapping subjects [36] (see Section 3.2.4 Genome-wide association studies on DKD).

## Genetics of Diabetic Retinopathy

### Heritability of Diabetic Retinopathy

There is evidence that genetic factors may play an important role in the development of diabetic retinopathy. Familial clustering of various degrees of retinopathy has been reported worldwide [56–59]. In the DCCT, first-degree relatives (with T1D or T2D) of T1D probands were studied and they reported a strong familial clustering with an OR of 3.1 for severe diabetic retinopathy in relatives of probands with and without diabetic retinopathy [56]. Only weak evidence was shown for the phenotype "any retinopathy" [56]. In a study of sib-pairs with T1D, the risk of a T1D proband was 9.9-fold if a T1D sibling had retinopathy, and the risk of the proband was higher if the proband was female [59]. Heritability estimates for retinopathy are on average around 25%, but the estimates vary and may be as high as 52% depending on ethnicity, severity of retinopathy, and type of diabetes [60–62]. As for DKD, it seems that the heritability is higher with a more severe phenotype.

### Candidate Genes for Diabetic Retinopathy

Biologically relevant candidate genes for diabetic retinopathy have been extensively assessed in both T1D and T2D but thus far any positive findings have been difficult to replicate [63, 64]. Abhary and co-workers performed a systematic meta-analysis in September 2008 where they identified 702 publications on candidate genes for diabetic retinopathy. Twenty genes and 34 variants had been studied in multiple cohorts. In a meta-analysis, the aldose reductase gene (*AKR1B1*) was highlighted as an important susceptibility gene for retinopathy ($p = 1 \times 10^{-4}$) together with suggestive evidence of association ($p < 0.05$) for genetic variants in genes *NOS3*, *VEGF*, *ITGA2*, and *ICAM1* [65].

In another meta-analysis, SNPs in 2000 cardiovascular candidate genes were genotyped in 2691 subjects with T2D from the CARe (Candidate gene Association Resource) cohort.

After Bonferroni correction, they found the strongest associations with diabetic retinopathy for rs6128 ($p = 0.0001$) in *SELP* (P-selectin) and rs6856425 tagging α-L-iduronidase (*IDUA*) ($p = 2.1 \times 10^{-5}$). However, the findings could not be replicated in independent cohorts [64].

Few candidate gene studies reach genome-wide significance ($p < 5.0 \times 10^{-8}$). A recent paper studied 134 SNPs in two thiamine transporters and two transcription factors and identified two potentially interesting SNPs in the *SLC19A3* gene that were associated with a lower rate of severe retinopathy. When a combined phenotype of severe retinopathy and ESRD was used, the association became even stronger [66]. The association with the combined phenotype at rs12694743 reached genome-wide significance ($p = 2.30 \times 10^{-8}$ after correction for $HbA_{1c}$ and BMI) in a meta-analysis including the discovery cohort (the FinnDiane Study) and two replication cohorts (Wisconsin Epidemiologic Study of Diabetic Retinopathy [WESDR] and DCCT/EDIC) [66].

Regarding positional candidate genes see also replication/validation studies of GWAS loci in Section 3.4.

### Linkage Studies for Diabetic Retinopathy

Some linkage analyses for diabetic retinopathy have been performed in T2D, but they have so far provided only suggestive linkage to a number of chromosomal regions, with the strongest linkage observed at Chr1p36 [60, 67, 68].

### GWAS on Diabetic Retinopathy

A compilation of current GWAS data on interesting genetic loci for diabetic retinopathy is shown in Figure 3.1. Most of the GWASs so far have been performed in patients with T2D. Only one studied patients with T1D [69]. Half of the studies have been performed in Asian populations (Chinese, Taiwanese, and Japanese) [70–72], one in Mexican Americans [73], and two in Caucasians [69, 74]. The phenotype has varied from any retinopathy to severe sight-threatening retinopathy.

The first GWAS for diabetic retinopathy was performed by Fu et al. in a Mexican American cohort of only 103 cases with PDR (more severe moderate non-proliferative diabetic retinopathy [NPDR]) and 183 controls without retinopathy (normal to early NPDR). Only nominally significant findings ($p < 0.0001$) were observed as two directly genotyped and 32 imputed SNPs were associated with severe retinopathy in 13 different chromosomal regions [73].

Genome-wide significant findings have so far been shown by only two GWASs, as presented in Figure 3.1. In a study of 749 Chinese T2D patients, Huang et al. first identified 12 SNPs with $p < 1 \times 10^{-6}$ for diabetic retinopathy, which after adjustment for $HbA_{1c}$ and duration of diabetes resulted in four genome-wide significant loci (rs17376456 in *KIAA0825* on Chr5q.15, rs2038823 in *HS6ST3* on Chr13q, rs4838605 in *ARHGAP22* on Chr10q, and rs12219125 in *PLXDC2* on Chr10p) [72]. Out of these loci, supporting evidence ($p < 0.05$) has been subsequently reported for *ARHGAP22* and *PLXDC2* [75, 76] (Figure 3.1).

The most recent GWAS was performed in 844 Caucasian T2D patients for sight-threatening diabetic retinopathy. They replicated their top SNPs from the discovery cohort in two

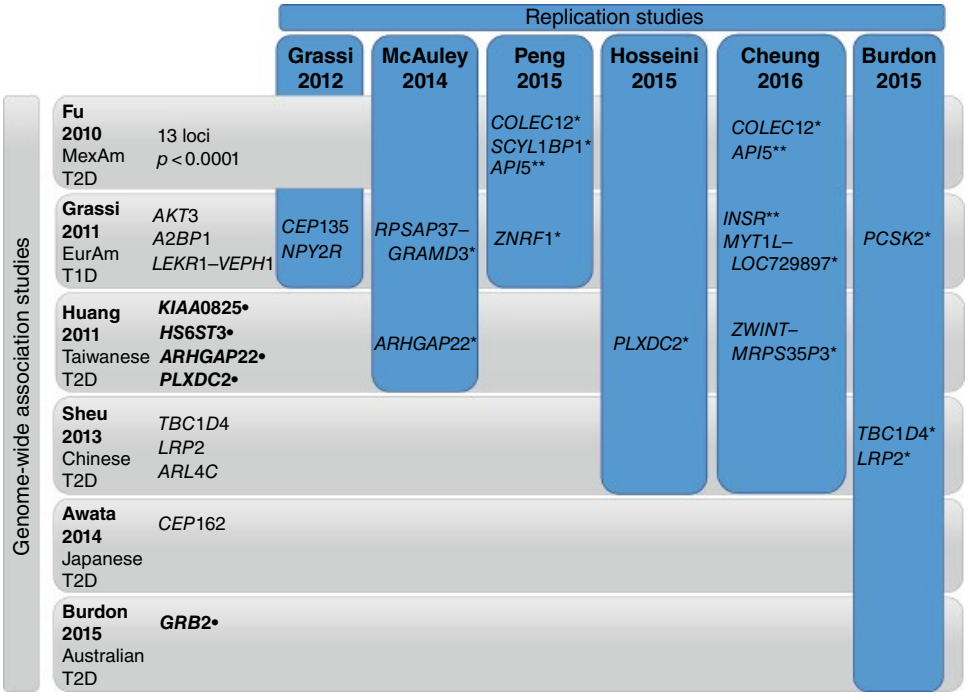


**Figure 3.1** Genome-wide association studies on diabetic retinopathy, and subsequent replication attempts. The horizontal bars indicate Genome-wide association studies (GWASs) and list their key findings. The vertical bars indicate replication studies; loci with evidence of replication ($p < 0.05$) are indicated on the horizontal level corresponding to the GWAS on left. Of note, the loci are named according to one or more nearest or flanking genes, even though no functional link is established between the gene and the association signal. **p-value < 0.05 after correction for multiple testing was calculated based on the number of evaluated single nucleotide polymorphisms. References for the GWAS and replication studies: Fu 2010 [73]; Grassi 2011 [69]; Huang 2011 [72]; Sheu 2013 [70]; Awata 2014 [71]; Burdon 2015 [74]; Grassi 2012 [77]; McAuley 2014 [75]; Peng 2015 [78]; Hosseini 2015 [76]; Cheung 2016 [79].

T2D and one T1D independent cohort and performed a meta-analysis, which ended up with one genome-wide significant SNP rs9896052 ($p = 4.15 \times 10^{-8}$) located upstream from the *GRB2* gene. They further used a mouse model of proliferative retinopathy and showed increased *GRB2* expression in the retina [74].

The fact that several of the early GWASs on diabetic retinopathy never tried to replicate their main findings in independent cohorts highlights the importance of "positional candidate gene" studies that have tried to replicate or validate the earlier identified loci [75–79]. Figure 3.1 summarizes the replicated findings ($p < 0.05$). Of these studies, the ones by Grassi et al. [77] and Hosseini et al. [76] were performed in T1D, the rest in T2D. Of note, only variants in *API5* [78]. Figure 3.1 summarizes the replicated findings ($p < 0.05$). Of these studies, the ones by Grassi [79] remained significant after correction for multiple testing.

In a meta-analysis of 1907 well-characterized subjects with T1D from the DCCT/EDIC Study and the WESDR, none of the tested 34 SNPs from four previous GWASs was replicated

after correction for multiple testing [76]. Nominally significant associations ($p < 0.05$) were observed for severe diabetic retinopathy at the *PLXDC2* locus and for mild diabetic retinopathy in the *PPARG* gene in the same direction as the original findings. Cheung and co-workers studied 2566 Chinese T2D patients with and without retinopathy [79] in an attempt to replicate 38 SNPs with suggestive evidence for association ($p < 5 \times 10^{-4}$) from four previous GWASs [69, 70, 72, 73]. The strongest association was found for an intronic SNP rs2115386 of *INSR* for both sight-threatening DR (STDR) and PDR. Four other SNPs were nominally significantly associated with either STDR or PDR [79].

### Sequencing Efforts for Diabetic Retinopathy

So far, no large attempts have been published. A small WES study with 43 subjects with and 63 without diabetic retinopathy despite 10 years' duration of diabetes observed excess rare variants in three genes (*NME3*, *LOC728699*, and *FASTK*, $p < 5 \times 10^{-8}$) in subjects without diabetic retinopathy [80]. Validation of the findings is still needed.

## Genetics of Diabetic Neuropathy

### Heritability of Diabetic Neuropathy

Diabetic neuropathy is a complex and quite heterogeneous disorder with a plethora of potential phenotypes to study. While there are many established environmental risk factors for neuropathy such as poor glycemic control, overweight, and smoking, the genetic component of diabetic neuropathy is largely unknown. In a family study, the risk for neuropathy in a T1D proband was twofold if a T1D sibling had neuropathy and the risk was twice as high if the proband was female [59]. Based on GWAS data in the Genetics of Diabetes and Audit Research Tayside Study (GoDARTS) cohort, the authors estimated the narrow-sense heritability of painful neuropathy to be 11.0% [81]. In a later sex-specific analysis, they continued by reporting heritability estimates for painful neuropathy of 30.0% for men and 14.7% for women [82].

Heritability results regarding cardiac autonomic neuropathy (CAN) are less consistent. In the Framingham Heart Study, the variance in heart rate variability (HRV) attributable to genetic factors was estimated to be 13–23%, when first-degree relatives and unrelated subjects were studied [83]. On the other hand, in a Hungarian twin cohort, genetic factors did not seem to substantially influence cardiac autonomic function [84].

### Candidate Genes for Diabetic Neuropathy

Far fewer candidate gene studies have been performed for diabetic neuropathy than for nephropathy and retinopathy partly because of the complex phenotype. In addition, only a few findings from the candidate gene studies have been confirmed. A recent meta-analysis of five studies on the *ACE* insertion/deletion polymorphism and four studies on the 677C>T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene suggested that there might be a role for these genes in the development of diabetic neuropathy

[85]. Other potentially interesting candidate genes for diabetic neuropathy that have been suggested include among others *GLO1* [86], *APOE* [87], *VEGF* [88], *eNOS* [89], and *GPX1* [90], and are well reviewed in Politi (2016) [91]. An interesting finding comes from the German Diabetes Study (GDS), where in a population of 538 recent-onset (<1 year) subjects with T1D (n = 163) and T2D (n = 373), Ziegler and co-workers investigated the association of 18 different quantitative measures of neuropathy with nine tagging SNPs in the transketolase gene (*TKT*). The association of the *TKT* SNPs rs7648309 with total symptom score (p = 0.024) and rs62255988 with warm thermal detection threshold in the hand (p = 0.049) remained statistically significant after strict adjustment for multiple testing with the number of phenotypes and SNPs [92].

### GWASs on Diabetic Neuropathy

To our knowledge only two GWASs, both from the GoDARTS cohort, have to date been performed for diabetic neuropathy [81, 82]. Both papers used a robust phenotype of neuropathic pain in patients with T2D. The case definition was based on the use of at least one of five drugs known to be prescribed specifically for neuropathic pain (duloxetine, gabapentin, pregabalin, capsaicin cream/patch, and lidocaine patch) as well as a sign of sensory neuropathy (in the first study only). Controls did not take any of these five drugs or any other more unspecific pain-relieving medication. No minimum duration of diabetes was required for the controls. The main finding of the first study was a cluster of markers with a suggestive association for neuropathic pain next to the *GFRA2* gene [81]. The top SNP rs17428041 in this region had an OR of 0.67 (95% CI 0.57–0.78) and a p-value of $1.77 \times 10^{-7}$. In a second study with a slightly larger cohort, they performed sex-specific analyses for painful neuropathy and found suggestive evidence (top SNP rs71647933, $p = 2.74 \times 10^{-7}$) between the genes *ZSCAN20* and *TLR12P* in women and near *HMGB1P46* (top SNP rs6986153, $p = 8.02 \times 10^{-7}$) in men [82]. No replication of the findings in the two studies was performed.

## Future Directions

During the past 5 years, the genetic research on diabetic complications has moved from candidate gene and linkage studies to GWAS and WES studies, with novel genetic risk factors emerging. However, many of the reported signals still require further confirmation in independent studies, and these can be assumed to explain only a small proportion of the heritability of microvascular complications. Larger studies with careful phenotyping, as well as novel approaches are needed to identify additional genetic risk factors for diabetic complications.

### Larger Studies Are Needed

Increasing the number of samples has markedly increased the number of susceptibility loci for many common traits including T2D [93]. While genetic consortia to perform GWAS meta-analyses already exist for DKD, albeit still moderate in size [8, 36, 44, 53], no GWAS meta-analyses have been performed on diabetic retinopathy or diabetic neuropathy, except

for the GWAS on diabetic retinopathy by Grassi et al. including 2829 subjects from two studies [69]. Furthermore, the number of subjects included in some of the GWASs on diabetic retinopathy is very small, in many cases with limited replication. Therefore, continuous recruitment of subjects and international collaboration to increase the total number of subjects is essential for discovery of further genetic susceptibility loci for diabetic complications. Preliminary reports from larger GWAS meta-analyses are emerging e.g. a GWAS on DKD in up to 8000 subjects with T2D from the Hong Kong Diabetes Registry [94], a GWAS meta-analysis on DKD including both T1D and T2D subjects from the SUMMIT consortium [95], and GWAS meta-analysis on DKD with up to 20 000 subjects with T1D from the Diabetic Nephropathy Collaborative Research Initiative (DNCRI) [96].

While much of the genetic research on common traits has concentrated on subjects of European origin, the GWAS on diabetic microvascular complications, particularly diabetic retinopathy, have been performed on various ethnic groups [69–74]. Furthermore, a large-scale candidate gene study of diabetic retinopathy including over 2000 genes was performed as a multi-ethnic meta-analysis [64]. Recently, a trans-ethnic GWAS analysis identified a novel susceptibility locus for DKD near the *CNKSR3* gene particularly evident in the American Indian population, but directionally consistent also in subjects of European and Mexican ancestry [44]. Both further GWASs for various ethnicities, as well as trans-ethnic GWAS meta-analyses can provide novel cues to genetic factors behind diabetic complications.

## How to Define the Phenotype?

The choice of the best potential phenotype is crucial. Even though the numbers increase statistical power, in some cases it may be fruitful to concentrate on more homogenous sub-groups of subjects, as demonstrated by the *SP3-CDCA7* locus associated with ESRD only in women [41]. In search for susceptibility genes for DKD, multiple phenotypic definitions have been employed with the aim to discover genes affecting various stages of the disease with different pathogenic mechanisms [8, 36]. A similar approach may be particularly useful for diabetic neuropathy with its spectrum of diverse symptoms.

Studies on early phenotypic alterations have been criticized because of the uncertain final outcome of these early traits e.g. microalbuminuria is sometimes classified as case and sometimes as control or early signs of CAN may be even reversible [97]. Theoretically, more severe forms of complications, like ESRD, should be more robust and reduce potential misclassification bias. However, there is high mortality in cardiovascular disease already before the development of ESRD. In a Finnish study of patients with T1D more than 25% of the patients with proteinuria died before developing ESRD [98]. For genetic variants that increase the risk of both ESRD and cardiovascular mortality, the risk allele would be diluted, or even inversed, due to selective mortality.

### Variability in the Phenotypic Definitions

The large variability in the definitions of the microvascular phenotypes creates difficulties for the comparison of data between studies and for fruitful collaboration attempts. The variability and quality of studied phenotypes may explain lack of replication in, for example, diabetic retinopathy. While laser treatment of the retina may be considered a

robust case phenotype, in contrast absence of laser treatment is not a good control definition. However, there is always a delicate balance between achieving the best phenotype quality and reaching large enough patient numbers. One solution has in this case been to use several different case/control cut-offs for the same phenotype [8]. In large consortia, a good harmonization is important to reduce the heterogeneity in phenotype between participating cohorts. While some cohorts may not be able to provide cases and controls fulfilling the primary harmonized phenotype definition, using several phenotype definitions enables the maximal use of all patients in addition to assessing various disease stages.

#### Criteria for Controls

In general, in case–control settings the duration of the controls often seems to be rather short. Since the beginning of candidate gene studies in diabetic complications, especially DKD, durations such as 15 years for T1D and 10 (or even less) years for T2D have been considered sufficient to classify patients as controls if no signs of complication are present. The treatment of diabetes has evolved and postponed the onset of complications. Therefore, much longer duration of diabetes for controls is probably needed. There is a need for studies addressing the potential risk of misclassification with short diabetes duration in controls, and on the other hand, loss of statistical power with too stringent requirements.

#### Do Genetics of Microvascular Complications in T1D and T2D Differ?

Part of the genetic background in the pathogenesis of diabetic complications is most likely the same in both types of diabetes, however, there may also be genetic markers specific for one or the other type. Some papers analyze pooled diabetes cohorts and do not even report specific characteristics on the diabetic population. Especially for DKD, it should be noted that a significant proportion of subjects with T2D may have kidney disease due to non-diabetic causes, while the majority of kidney disease in subjects with T1D is due to diabetic nephropathy [99]. Therefore, the genetic risk factors for non-DKD may also be relevant in the T2D population [52], but less evident in subjects with T1D.

### Low-Frequency and Rare Variants May Affect Diabetic Complications

#### Sequencing of Rare and Low-Frequency Variants

The research focus for the genetics of microvascular complications of diabetes has thus far been mainly on the common variants detectable with GWASs. Preliminary reports of whole-genome sequencing for DKD are emerging [100]. While eventually whole-genome sequencing should cover all coding and non-coding, common and low-frequency or rare variants, and the WES studies targeting the protein coding parts of the human genome are a suitable starting point for the search of rare variants, thus far the WES efforts on DKD have not robustly identified any rare variants, or genes enriched for protein truncating or changing variants [8]. For diabetic retinopathy, three genes were reported enriched for rare variants in subjects without diabetic retinopathy, but replication in other studies is required to confirm these findings as well [80]. Currently, the genotyping chips targeting the exonic content simultaneously with GWAS genotyping provide an interesting and cost-effective approach to detect low-frequency and rare variants. While very rare and de novo mutations cannot be identified with the exome chip approach, it is feasible in large studies of thousands of subjects [96].

### Linkage Analysis with GWAS Data

Most of the current genetic studies, including candidate gene, GWAS, and WES, are based on association tests. Nevertheless, the previously much employed family-based linkage studies are still a valid approach, as when combined with the exome- and genome-wide genotyping and sequencing platforms, they may be particularly efficient in the search for low-frequency and rare variants. Family-based association analysis was used to support the role of *FRMD3* in DKD [31], and preliminary reports for genome-wide linkage studies on DKD based on modern-day, dense SNP genotyping in small pedigrees are emerging [101].

### Genetics May Reveal Biology and Infer Causality

Genetic findings of related traits can be utilized in many ways to improve our understanding of diabetic complications. Analysis of genetic risk scores for related traits suggested that high BMI and metabolic changes leading to T2D are causal risk factors for DKD [8]; furthermore, inverse genome-wide correlation was found with the LD-score regression method between DKD and smoking cessation, supporting the clinical finding that smoking cessation is beneficial for avoiding DKD [8, 102].

In biomarker research, the causality of certain biomarkers for diabetic complications has been evaluated with the Mendelian Randomization method, based on the genetic factors that affect the biomarker levels. For example, urinary kidney injury molecule 1 (KIM1) predicts progression of DKD even though it does not add prognostic benefit on top of AER or eGFR. Nevertheless, Mendelian Randomization suggested that KIM1 is a causal risk factor for reduced eGFR in subjects with T1D [103]. On the contrary, serum uric acid was independently associated with the decline in eGFR, but Mendelian Randomization suggested that it is not a causal risk factor for DKD, but rather a downstream marker of kidney damage [104]. In addition to serum or urine biomarkers, the Mendelian Randomization approach was also applied on BMI suggesting that elevated BMI is a causal risk factor for DKD [105].

## Conclusions

In the future, better phenotyping, more collaboration and larger consortia, and exploration of the low-frequency and rare variations are essential to identify the genetic causes behind diabetic microvascular complications. By guiding us into the complex biology behind the complications, genetics may help us develop new therapeutic tools to improve the prognosis of the diabetic patient.

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# 4

# Epigenetics of Diabetic Microvascular Disease

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## Diabetic Complications Include Microvascular and Macrovascular Injury

In diabetes, hyperglycemia is a causative factor of endothelial dysfunction and, in the long term, of vascular complications [1]. Diabetic microvascular complications, contributing to nephropathy, neuropathy, and retinopathy, substantially contribute to the mortality and morbidity burden of the disease. Poor glycemic control and associated hyperglycemia is not the only risk factor – with genetic predisposition [2], dyslipidemia [3], and hypertension [4] also being substantial contributors – but it is a quantitatively major player in inducing microvascular injury, acting via different metabolic mechanisms.

Different pathways leading to microvascular hyperglycemic damage have been identified, which include (i) the polyol and hexosamine pathways, (ii) hyperglycemia-induced generation of advanced glycation end-products (AGE), and (iii) overproduction of superoxide radicals by the mitochondrial electron-transport chain [5]. These altered pathways lead to amplification of intracellular responses leading to production of endothelial proliferative factors such as vascular endothelial growth factor (VEGF) and tumor growth factor (TGF), causing expansion of the microvasculature and activation of the NF-κB pathway, which initiate persistent sub-chronic inflammatory events [5]. Hyperglycemia also affects the renin–angiotensin systems and endothelial NO production, resulting in vasoconstriction, alteration of the hemodynamic control, and hypertension [6]. To assess the role of ill-controlled glycemia in promoting vascular complications, prospective interventional large-scale studies, in type 1 and 2 diabetic cohorts, were undertaken to investigate the clinical benefits of tight glycemic control. Such studies, and their still ongoing follow-up observational phases, conclusively demonstrated that diabetic complications do become apparent long after the initial exposure to hyperglycemia. This phenomenon, referred to as hyperglycemic/metabolic memory or legacy effect is now explained by the occurrence of long-lasting epigenetic alterations capable of maintaining the negative effects of earlier hyperglycemia even after glycemic levels have been normalized.

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## The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study in Type 1 Diabetic Patients, and the UKPDS Study in Type 2 Diabetic Patients, Support the Existence of the Hyperglycemic Memory Phenomenon

### DCCT/EDIC Study

The DCCT study, started in 1983, compared standard versus intensive glycemic control in a cohort of type 1 diabetic patients over a period of 6.5 years. Within the study group (in which tight glycemic control was obtained via multiple daily insulin injections or insulin pump) glycated hemoglobin and glycemia significantly improved as compared with the control group (one or two insulin injections/day, as for clinical guidelines at the time). Improved glycemic control was associated with a reduced risk of development of retinopathy and neuropathy, as well as with a reduced risk of developing microalbuminuria and albuminuria [7]. At the end of the DCCT, and because of the positive outcome in the study group, participants from both study arms were encouraged to apply the intensive glycemic control regimen. Thereafter, DCCT developed into a longer-term follow-up, observational study, named the EDIC study (Epidemiology of Diabetes Interventions and Complications). The aim of EDIC was to assess the longer-term consequences of tight glycemic control originally applied to the DCCT study arm. At the end of DCCT, glycated hemoglobin was significantly lower in the study group; however, throughout the course of the EDIC study, glycated hemoglobin levels tended to decrease in the conventional group (due to the application of tighter glycemic control) while it increased in the intensive group (perhaps due to lower compliance), reaching similar levels by year five [8]. Longer-term follow-up determined that the group that had received intensive therapy during both DCCT and EDIC had lower incidence of hypertension and better kidney functionality [8], as well as decreased risk of cardiovascular disease [9].

Since during the EDIC study glycemic differences between the two study groups were lost, the poorer clinical outcome experienced by the former (i.e. during DCCT) conventional treatment group over the course of the EDIC follow-up likely derives from the DCCT period of poorer glycemic control. This supports the idea of "metabolic memory" and the occurrence of delayed effects of prior exposure to ill-controlled hyperglycemia.

Recent surveys monitoring DCCT/EDIC participants, >35 years after the start of the study, still show a lower incidence of diabetic complications in the original DCCT interventional tight glycemic control group [10], supporting the hypothesis that past exposure to hyperglycemia can prime the long-term development of diabetic complications (Figure 4.1). Strong evidence of the importance of early and tight glycemic control in type 1 diabetes comes from the recent observation that the overall mortality in the DCCT/EDIC study group is similar to that of the general population, while the DCCT/EDIC control group displayed a higher mortality rate [11].

### The UKPDS Study

Early tight glycaemic control is also a key intervention to reduce the incidence of diabetic complications in type 2 diabetic patients. The UKPDS demonstrated, in newly diagnosed

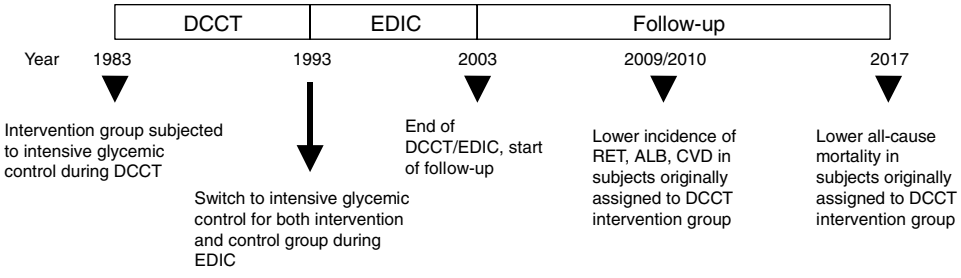


**Figure 4.1** Diabetes Control and Complications Trial-Epidemiology of Diabetes Interventions and Complications (DCCT-EDIC) demonstrates that predisposition to develop diabetic vascular complications is maintained long after switching from a poor glycemic control to a tight glycemic control regimen. Schematic diagram representing the sequence of the DCCT study, EDIC study, and follow-up. Different glycemic control regimens and long-term follow-up clinical results are reported on the timeline. Retinopathy (RET), albuminuria (ALB), cardiovascular disease (CVD). Sustained incidence of diabetic complications, and overall mortality, was observed in the follow-up in the DCCT control group initially assigned to conventional insulin therapy.

type 2 diabetic patients, that a 10-year intensive glycemic control led to a 25% risk reduction in developing microvascular endpoints [12]. Furthermore, the 10-year follow-up study (in which glycemic differences were lost between the two study arms, in the same way as the DCCT/EDIC) demonstrated a persistent reduction of microvascular risk in the intervention group and also demonstrated risk reduction for macrovascular events (myocardial infarction) and all-cause mortality [13].

Collectively, the DCCT/EDIC and UKPDS studies prove that early exposure to hyperglycemia can contribute to later development of diabetic complications, even subsequently to the implementation of tight glycemic control, thus explaining the observed "hyperglycemic memory" phenomenon, and underscore the importance of early glycemic control to avoid the development of diabetic microvascular complications [14].

## Models of Hyperglycemic Memory

The "metabolic memory" hypothesis inferred from the DCCT/EDIC and UKPDS has also been experimentally supported by research in animal models and cell culture models.

### Animal Models

In a "classic" study, diabetic dogs kept under poor glycemic control for 5 years developed diabetic retinopathy. Insulin therapy to restore glycemic control fully prevented diabetic retinopathy if initiated within 2 months from diabetes outset. However, a parallel group kept under poor glycemic control for 2.5 years and then switched to insulin therapy, although not showing early signs of retinopathy at 2.5 years, developed retinopathy at year 5, despite the late initiation of insulin injections, indicating that the progression of the disease was elicited by earlier hyperglycemia [15].

In a similar experimental study, islet transplantation in diabetic rats within 6 weeks of development of overt diabetes alleviated the development of diabetic retinopathy,

while transplantation after 12 weeks from the onset of diabetes was less beneficial, indicating that changes caused by early hyperglycemia are irreversible [16].

### Cell Culture Models

The earlier use of endothelial cell culture models demonstrated that vascular dysfunction engendered by hyperglycemia (or – more appropriately – high concentrations of glucose in the cell culture media) is associated with permanent alterations of gene expression. The persistence of gene expression in response to transient hyperglycemia was first shown in human endothelial cells by M. Lorenzi group, and was indeed identified as a "phenomenon with a memory" [17]. This pioneering work indicated that overexpression of fibronectin and collagen IV mRNAs in cultured human endothelial cells exposed to high glucose (HG) concentrations was maintained even once cell culture conditions were normalized for glucose [17]. Subsequent studies showed that several protein markers of glucotoxic stress, such as PKCβ and the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase complex p47phox subunit, retained overexpression upon return to normoglycemic cell culture medium. Pharmacological normalization of reactive oxygen species (ROS) or overexpression of uncoupling protein-2 (UCP2) blocked hyperglycemia-induced ROS production and reverted hyperglycemia-induced PKCβ and p47phox expression, proving the role of ROS in determining the persistence of cellular stress after glucose normalization [18]. The increase in ROS, however, appears to be a primary, yet reversible response to high concentrations of glucose [19]. A more robust target mediating the establishment of a glycemic memory has been shown to be the increase of the transcription factor NF-κB, which was persistent even upon a return to normoglycemia, and may dictate the long-lasting expression of NF-κB target genes, *MCP-1* and *VCAM-1*. In a series of cell culture studies on endothelial cells, several groups independently demonstrated that transient HG concentrations induce permanent epigenetic changes, more specifically histone methylation, that dictate persistent gene activation despite a return to normal glucose (NG) concentrations [20].

## The "Epigenetic Code"

The term "epigenetics" – where the Greek prefix *epi-* means *over, upon* – is meant to represent any genetic-like phenomenon whose transmission is independent of the information contained in the DNA sequence. Epigenetics refers to stable and to a certain extent heritable patterns of gene expression that do not involve changes in DNA sequence. Critical epigenetic marks related to regulation of gene transcription are histone post-translational modifications and DNA methylation on cytosines. The arrangement of various types of epigenetic marks determines the so-called histone code [21]. Overall, epigenetic regulation is dictated by the interplay between: (i) DNA methylation/demethylation on cytosine, with methylation having a silencing effect; (ii) post-translational modifications on histone proteins, including methylation, acetylation, and phosphorylation; and (iii) micro RNA (miRNA)-mediated processes.

These epigenetic phenomena synergize with the "classical" control of gene expression mediated by transcription factor complexes at genes' promoters, and the concerted action

of chromatin-modifying enzymes is an upstream mechanism of transcriptional control, which dictates chromatin accessibility for the transcription factors. Finally, the action of miRNAs is yet another regulatory layer to dictate the final translational response of a given gene.

## DNA Methylation

In mammals, DNA methylation occurs primarily on cytosines in CpG dinucleotides and is a crucial DNA modification to negatively modulate gene expression, and favoring chromosomal condensation [22]. Methylation of DNA is catalyzed by a family of DNA methyltransferases (DNMTs) encompassing DNMT1, DNMT3a, DNMT3b, and DNMT3L. Cytosine methylation occurs via the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the 5′ position of the cytosine ring, generating 5-methylcytosine. Transcriptional silencing and chromatin compaction initiated by DNA methylation involves a hierarchy of regulatory events including the recruitment on the chromatin template of methyl-binding proteins (MDBs), histone deacetylases (HDACs) and chromatin remodeling factors [23]. HDACs, mainly HADC1 and HDAC2, by generating deacetylated chromatin, provide an additional repressive step. HDAC1 directly binds to DNMT3a and DNMT1 [24], while HDAC2, which contributes to the maintenance of hypoacetylated heterochromatin, is associated with DNMT1 and DNMT-associated protein 1 [25].

Most of the CpG dinucleotides in the genome are unmethylated, but specific and CG-rich stretches of DNA, called CpG islands, which are mostly localized in promoter and/or regulatory regions, are subjected to methyl-modifications during development [26]. Importantly, in diabetes and diabetes-related complications, aberrant DNA methylation patterns have been observed during both the development and the progression of the disease [27]. The analysis of blood samples from diabetic patients suggests that DNA hypomethylation is related to decreased levels of SAM, the physiologic donor of methyl groups, due to decreased activity of methylenetetrahydrofolate reductase (MTHFR) [28]. The epigenetic code hypothesis supports the idea that DNA methylation and histone post-translational modifications are interrelated events in dictating gene regulation [23]. However, which epigenetic modification acts as primary event is still debated. The analysis of the kinetics of silencing of transgenes suggests that an initial event leading to transcriptional silencing is the loss of transcriptionally permissive histone acetylation and histone 3 lysine 4 (H3K4) methylation, followed by transcriptionally repressive H3K9 methylation and, as a last event, promoter DNA methylation, which stabilizes the silent epigenetic state [29]. Whether such a sequence of molecular events takes place to determine epigenetically based transcriptional alterations in microvascular diabetic complications is still an unexplored research field.

## Histone Methylation in Models of Hyperglycemic Variability

Histone methylation can be either transcriptionally activatory or repressive, depending on the targeted lysines and arginines, and plays a central role in the determination of epigenetic persistence. Histone arginine methylation mostly relates to transcriptional activation, while lysine methylation can confer opposite transcriptional regulatory functions. Methylation of H3K4, H3K36, and H3K79 is associated with a transcriptionally active state. In contrast, methylation of H3K9, H3K27, and H4K20 usually correlates with transcriptional

suppression. Lysine residues can be mono-, di-, or trimethylated, and the selective action of methylase/demethylase enzymes in achieving such precise methylation patterns controls chromatin accessibility and transcriptional activity [21].

Histone arginine and lysine methyltransferases belong to three distinct enzymatic classes, namely (i) the protein arginine methyltransferases (PRMTs) family; (ii) SET-domain-containing lysine methyltransferases; and (iii) non-SET-domain proteins DOT1/DOT1L. Lysine methyltransferases exhibit high substrate specificity, usually modifying a specific lysine on a specific histone protein [30].

SET7/9 histone H3K4 methyltransferase activity, in particular, has been demonstrated to contribute to the induction of persistent activating epigenetic changes in endothelial cells exposed transitorily to HG concentrations [31]. Similar hyperglycemic-dependent epigenetic changes have also been observed in monocytes [32] and pancreatic cells [33]. In monocytes, gene knockdown of SET7/9 inhibited the TNFα-induced expression of its target gene, the p65 subunit of the NF-κB complex [32], and, similarly, specific reduction of H3K4 monomethylation and decreased p65 NF-κB subunit was demonstrated in response to HG concentrations in SET7/9 knockdown endothelial cells [31]. Overall, a model can be proposed whereby SET7/9, through persistent induction of H3K4 methylation, contributes to the maintenance of HG-induced transcriptional programs in diabetes (Figure 4.2). In addition, it should be noted that CARM1 (co-activator-associated arginine methyltransferase 1), belonging to the histone arginine methyltransferase family, also enhances NF-κB-mediated gene transcriptional activation via methylation of H3R17 in monocytes [34]. Collectively, these results suggest that histone lysine/arginine methyltransferases SET7/9 and CARM1 cooperate to drive hyperglycemia (or HG in cell culture studies)-induced epigenetic modifications, determining the transcriptional response to hyperglycemia.

## Epigenetic Changes in Microvascular Models of Glycemic Memory

Although DCCT/EDIC and UKPDS clinical trials suggest that early exposure to hyperglycemia contributes to the development of diabetic complications even years after the improvement of glycemic control, the underlying epigenetic mechanisms are still being studied. In diabetes, an early clinical marker is the occurrence of a persistent sub-clinical inflammatory condition. In type 2 diabetes, inflammation is promoted by the secretion of TNFα and other pro-inflammatory cytokines by insulin-resistant adipocytes [35]. In type 1 diabetes, a contribution to the inflammatory state also is also provided by hyperglycemia-induced endothelial damage [36]. As NF-kB is the central regulator of pro-inflammatory genes, several studies have investigated the link between hyperglycemia and NF-κB activation. In monocytes, activation of NF-κB and transcriptional induction of TNFα following exposure to HG concentrations was dependent on histone H3 and H4 hyperacetylation at the TNFα gene promoter [37]. Interestingly, the same study also demonstrated TNFα gene promoter hyperacetylation in monocytes derived from type 1 and type 2 diabetic patients, providing a first illustration of an epigenetic mechanism in the pathophysiology of diabetes [37].

More advanced approaches based on genome-wide analysis of histone H3K4 and H3K9 methylation revealed, in cultured monocytes, HG-dependent histone methylation defects in genes linked to inflammation and oxidative stress pathways [38].

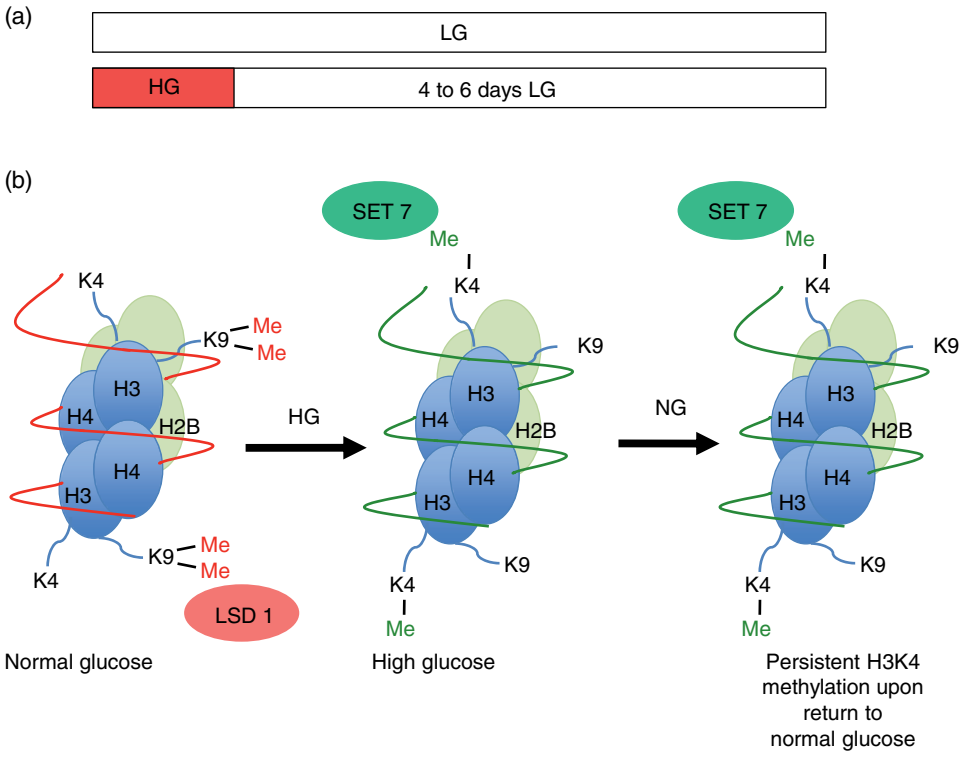


**Figure 4.2** Major histone H3 and H4 post-transcriptional alterations induced by exposure to high glucose (HG) concentrations. Exposure of endothelial cells to HG, leads to the simultaneous recruitment of the histone H3K4 methyltransferase SET7 and histone H3K9 demethylase LSD1 at the p65-NF-κB promoter region, resulting in erasure of the repressive H3K9 methyl marks and methylation of the transcriptionally activatory H3K4 methyl mark. Such epigenetic conformation is retained upon return to normal glucose (NG) concentration in the cell culture medium. (*See color plate section for color representation of this figure.*)

A perhaps more definitive proof of the occurrence of persistent epigenetic alterations associated to hyperglycemia has come from genome-wide profiling of epigenetic changes in lymphocytes and monocytes obtained from a sub-cohort of DCCT/EDIC. In this study, monocytes and lymphocytes from 30 case and 30 control DCCT/EDIC subjects were studied for histone activatory (H3K9 acetylation and H3K4 trimethylation) and repressive (H3K9 dimethylation) marks by chromatin immuno precipitation (ChIP). Immunoprecipitated material was analyzed by promoter tiling arrays analysis (ChIP-chip) covering genomic regions around the transcriptional start site (TSS) of all annotated human genes. H3K9 acetylated loci were increased in monocytes from controls, i.e. study participants doing conventional therapy during the DCCT phase. Such H3K9 acetylation was associated with the glycemic history of the control subjects, and inflammation-related *STAT1*, *TNFa*, and *IL1A* gene loci were among the hyperacetylated genomic regions [39]. This study provided the first clinically relevant description of histone post-translational modifications (PTMs) in type 1 diabetes, allowing associations to be drawn between epigenetic changes and the $HbA_{1c}$ profile of DCCT/EDIC study participants over a 30-year period (Figure 4.1) and

supporting the idea that epigenetically based hyperglycemic memory involves the simultaneous interaction of different chromatin-modifying enzymes.

## miRNAs Contribute to the Control of Glucose Homeostasis and Are Dysregulated in Diabetes

MiRNAs belong to a class of small (21- to 23-nucleotide) non-coding RNAs originally discovered as developmental regulators in *Caenorhabditis elegans* [40]. miRNAs regulate gene expression, in a sequence-specific manner, at the post-transcriptional level by cleavage or transcriptional repression of their target mRNAs. miRNAs are able to control highly complex regulatory networks of gene expression, as each miRNA can target multiple mRNAs [41].

In mammals, miRNAs also play a key role in modulating transcription, and it is increasingly appreciated that miRNA dysregulation is associated with a range of pathological states including diabetes and diabetes-related cardiovascular complications [42].

A number of miRNAs have been shown to be critical in the control of insulin secretion and glucose metabolism. Studies in rodent models have shown the involvement of miRNAs in the insulin production and secretion machinery and pancreatic β-cell function and development (miR375) [43] and glucose homeostasis (miR125a, miR29) [44]. Coincident to their physiological roles, miRNA dysregulation has been linked to the progression towards, and the pathogenesis of, diabetes. Table 4.1 summarizes confirmed roles for miRNAs in metabolic processes subject to alterations in diabetes. Other important miRNAs linked to β-cell dysfunction and type 2 diabetes physiopathology are miR96/miR124, whose overexpression lowers the response of β-cells to secretagogues by affecting the insulin exocytotic machinery [45], and miR34a/miR146, which mediate lipotoxicity-induced β-cell dysfunction [46].

Finally, a most prominent miRNA responsible for the regulation of whole-body glucose homeostasis is miR29, which is highly upregulated in insulin target tissues (skeletal muscle, fat, liver) of diabetic Goto–Kakizaki rats and, when overexpressed in adipocytes, promotes insulin resistance [47].

The increasing evidence that miRNAs regulate glucose homeostasis, and might contribute to the development of diabetes when dysregulated, suggests that miRNAs could be potential targets for therapeutic intervention. Furthermore, the remarkable stability of miRNAs in serum and plasma of human samples provides a new window toward revolutionizing early diagnosis of diabetes. These studies could lead to new strategies and specific targets to develop therapies, with recent studies showing significantly altered expression profile of serum miRNAs in diabetic patients, with 128 different miRNAs being potential clinical markers for early diagnosis of diabetes [48].

## Future Prospects

A substantial amount of literature indicates that epigenetic defects can contribute to the establishment of diabetes and its microvascular complications. Recent progress in understanding the interaction between DNA methylation, histone PTMs, and miRNA dynamics

**Table 4.1** miRNA-mediated epigenetic regulation of metabolism.

| Controlled biological event | miR(s) | Target mRNA | Function | Reference |
|---|---|---|---|---|
| **Glucose metabolism** | miR29a | N.D. | Potentially implicated in inhibition of insulin action, overexpressed in insulin target tissues in type 2 diabetic rats | [44] |
| | miR145 | IRS-1 | Downregulates IRS-1 | [51] |
| **Insulin synthesis** | miR96<br>miR124a | SNAP25<br>Rab3a u<br>**Rab27a**<br>**Noc2** | Regulation of insulin exocytosis. miR96/124a target several components of the exocytotic machinery | [45] |
| | miR30d | N.D. | Regulator of pro-insulin gene expression | [52] |
| | miR34a/<br>miR146 | p53,<br>VAMP-2,<br>TRAF6 | Involved in free fatty acid-induced β-cell dysfunction and apoptosis | [46] |
| | miR375 | Many targets | Pancreatic islet specific miRNA; negatively regulates glucose-stimulated insulin release | [43] |
| **Lipid metabolism** | miR14 | Drice | Regulator of fat metabolism (triacylglycerol levels) in *Drosophila melanogaster*, acts on the apoptotic effector caspase Drice | [53] |
| | miR122 | ? | Liver specific, controls of hepatic lipid metabolism; its inhibition results in reduction of circulatory cholesterol levels | [54] |
| | miR143 | ERK5/<br>BMK1 | Regulates proliferation and differentiation of adipocytes | [55] |

in determining transcriptional outcomes suggests that hyperglycemia can induce persistent epigenetic changes – even once glycemic control is improved – which contributes to the development of diabetic microvascular complications.

From a clinical perspective, the continuing follow-up of the DCCT-EDIC and UKPDS studies now allows us to determine with increased precision the clinical impact of prior exposure to hyperglycemia, and it appears that the mortality for the study groups originally assigned to tight glycemic control is lowered, lending more weight to the "metabolic memory" hypothesis. The development of high throughput sequencing technologies and more sophisticated epigenetic methodologies will allow us to precisely determine the extent of specific epigenetic events driving gene responses in type 1 and 2 diabetes. Defining the epigenetic basis that leads to "metabolic memory" and associated diabetic microvascular complications will provide critical insights into the interpretation of persistent epigenetic gene-activating events associated with DNA methylation and other histone modifications as well as miRNA expression patterns.

The recent identification of epigenomic alterations on biological samples obtained from well characterized study groups of the DCCT/EDIC trial [39] improved our current

understanding of hyperglycemia-induced alterations within a real patho-physiological context [49, 50]. A more precise definition of the molecular events that lead to the phenomenon of "hyperglycemic memory," may lead to new strategies and specific targets to develop therapies to tackle the long-term deleterious effects of previous hyperglycemia.

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# 5

# Diabetic Retinopathy – Clinical

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## Introduction

Diabetic retinopathy (DR) is a chronic sight-threatening microvascular complication that can result in blindness. As with many other retinal disorders, DR is taking advantage of new treatment strategies, which are now changing the course of this disease. For years, the best treatments aimed at disease stabilization. Nowadays, new therapies have become available and improvements in visual outcomes are now possible when appropriate treatments are delivered on time. Intravitreal therapies based on anti-vascular endothelial growth factor (anti-VEGF) and steroids have dramatically changed the management and outcomes of microvascular complication of diabetic eyes. Anatomical recovery is now possible avoiding irreversible loss of retinal tissue. On the other hand, these new treatment modalities have increased the disease burden, requiring regular patients' controls, more frequent treatments, and reorganization of the workload of ophthalmologic departments.

Other important changes in the management of DR are the new imaging techniques lately introduced in the clinical practice. Until recently, the assessment of retinal damage was based on standard fundus photographies, fluorescein angiography (FA) and optical coherence tomography (OCT). Nowadays, retinal vascular networks can be also imaged in a dyeless and non-invasive fashion, with OCT angiography. This novel technique, based on the motion of red blood cells against static tissues as intrinsic contrast, allows visualization of retinal (superficial and deep) and choriocapillary plexuses, providing new insights on DR. However, this recently introduced imaging modality is still under development and more details of the retinal periphery should be obtained to expand its use in DR. Indeed, FA is still essential in the clinical practice of ophthalmologists dealing with DR and improvements in this imaging modality have arrived too. The main drawbacks of FA included the limited field of view, which has been addressed by the introduction of new fundus cameras. Widefield and ultra-widefield fundus cameras with filters for FA can capture in a single frame almost the entire retina and allow simultaneous images of both central and peripheral retina in each phase of the angiogram.

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The management of DR is thus rapidly evolving and we are now in the middle of a revolution for both patients and ophthalmologists. These continuous improvements, however, require constant updates for clinicians treating diabetics with retinopathy, particularly on diagnostic and therapeutic strategies.

## Epidemiology

DR and the other microvascular complications of diabetic eyes are major public health issues. Recently, different principal concerns are emerging in the epidemiology of DR [1]. First, there is clear evidence of a global increase in the numbers of diabetic patients, and estimates of retinopathy are expected to rise as well. In 2000, diabetes mellitus had a worldwide prevalence of over 170 million, which increased to ~415 million in 2015 [2, 3]. Among those with diabetes in 2010, almost one-third was found to have some form of DR [4]. However, the prevalence of DR among patients varies accordingly to the type of diabetes. Patients with type 1 diabetes show a higher prevalence when compared with those with type 2, with a higher risk of visual loss [5]. On the other hand, even if DR still remains a leading cause of visual loss among the working-age population, advancements in diagnosis and treatment have reduced the incidence of blindness for DR, particularly in high-resource countries [1, 6, 7]. Results of these improvements are sometimes observed in the course of the same clinical study. For instance, in the Wisconsin Epidemiologic Study of Diabetic Retinopathy, the progression of DR was slower in the last 13 years of the study when compared with the previous 12 years [7]; those trends were confirmed in many others cohorts, highlighting the achievements in the management of DR.

## Screening

Screening of DR has demonstrated effectiveness in reducing visual loss among diabetic patients in many countries [8, 9]. In recent years, screening programs and telemedicine have gained attention for the early recognition of DR among diabetic patients. Guidelines have been proposed for the screening of DR, even if timing and management may vary. For type 1 diabetes, after 5 years from the diagnosis the American Academy of Ophthalmology (AAO) recommends annual screening for DR with a dilated eye examination [10]. For type 2 diabetes, ophthalmological screening at the diagnosis is suggested, with strong evidence for adults [11]. Thereafter, annual fundus examination is recommended as DR remains asymptomatic until advanced stages. Poor accessibility to ophthalmologists may affect adherence to these guidelines. Indeed, different procedures are emerging to obtain a more systematic screening of DR with the help of telemedicine. These include digital fundus photographs, which can be obtained in different ways, including by fully automated non-mydriatic devices and portable retinal imaging tools [12, 13]. Images can then be reviewed and graded by eye care specialists, to direct the patient toward the most appropriate management. These systems for the screening of DR using tele-ophthalmologic platforms have demonstrated cost-effectiveness and are gaining recognition worldwide.

## Risk Factors

With a worldwide increase of diabetic patients in recent years, DR is emerging as the principal cause of avoidable blindness affecting the working-age population. Among patients with DR, different risk factors may promote the development and progression of retinopathy. As prevention still remains the best treatment, all these factors should be addressed and corrected whenever possible. Poor glycemic control and chronic hyperglycemia have been proven to be the most consistent risk factors for the development and progression of DR and diabetic macular edema (DME), in association with the duration of the disease [7]. The World Health Organization (WHO) and the American Diabetes Association (ADA) set HbA1c level ≥48 mmol/mol (or ≥6.5%) as glycemic threshold for the diagnosis of diabetes, on the basis of the increased risk of DR with higher values [14]; this underlines the strong correlation that exists between DR and hyperglycemia. Blood pressure is another major risk factor for DR [15]. Control of hypertension yielded a 47% reduction of a significant deterioration of visual acuity and a 34% reduction in anatomic worsening of DR among type 2 patients [16]. Furthermore, risk factors have a different impact on different countries. In developing areas, obesity and unhealthy lifestyle produced a major raise in the incidence of diabetes and DR, while in developed countries part of the recent increase in diabetic patients was attributed to higher life expectancy [7]. Health disparities contribute to explaining this phenomenon, where higher medical access may mask the drawbacks of a sedentary lifestyle and overweight in developed countries. Also ethnic differences, genetic predisposition, and socioeconomic status may influence the incidence of DR [17].

Another important risk factor for eye disease is the duration of diabetes, which strongly predicts the onset and the progression of DR. Almost every patient with type 1 diabetes develops signs of DR after 20 years of the disease [18]. In type 2 diabetes, the duration of the disease is not always easy to assess and the presence of age-related comorbidities may affect the onset of DR; however, different studies have found that disease duration is an independent risk factor for DR as well [7, 18].

## Clinical Patterns

Ocular abnormalities in diabetic patients have complex pathogenesis being related to many clinical conditions. Even if diabetes may affect the majority of the eye tissues, the most common causes of visual loss derive from the retina.

DR is a chronic and progressive disorder, usually divided into two different stages with different management and clinical signs: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR), as illustrated in Table 5.1. Both of these stages may be further complicated by the presence of DME, which increases the extent of visual impairment.

### Non-Proliferative Diabetic Retinopathy (NPDR)

Also known as background retinopathy, this condition is the earliest stage of DR (Figure 5.1). If left untreated, NPDR may progress to the proliferative form. In particular, glycemic

**Table 5.1** Classification of the severity of diabetic retinopathy (DR) accordingly to main clinical findings observed on dilated fundus examination (based on the Diabetic Retinopathy Study classification).

| Severity of the disease | Findings on dilated fundus examination |
|---|---|
| No apparent retinopathy | No abnormalities |
| Very mild non-proliferative diabetic retinopathy (NPDR) | Microaneurysms only |
| Mild NPDR | Microaneurysms, hard and soft exudates, mild intraretinal hemorrhages |
| Moderate NPDR | Previous findings associated with mild intraretinal microvascular abnormalities (IRMAs) and/or venous beading and/or severe intraretinal hemorrhages |
| Severe NPDR | Any of the following with no signs of proliferative diabetic retinopathy (PDR):<br>• >20 intraretinal hemorrhages in each retinal quadrant<br>• Definite venous beading in ≥2 retinal quadrants<br>• Prominent IRMA in ≥1 retinal quadrant |
| Very severe NPDR | ≥2 of the characteristics of severe NPDR |
| PDR | ≥1 of the following:<br>• Neovascularization<br>• Vitreous/pre-retinal hemorrhage |
| High-risk PDR | ≥1 of the following:<br>• Neovascularization at disc greater than or equal to about one-quarter to one-third disc area<br>• New vessels elsewhere at least one-half disc area in size<br>• Vitreous hemorrhage |

control and the other contributing factors should be addressed to prevent any further progression in long-lasting diabetics.

### Clinical Findings

- *Retinal microaneurysms* are the clinical hallmark of NPDR and the first sign to appear on ophthalmoscopic examinations. Loss of pericytes around the endothelium of retinal capillaries generates those saccular/fusiform dilatations, which appear as tiny red dots within the retina on fundus examination [19]. However, on funduscopic examination microaneurysms are indistinguishable from dot hemorrhages, which can occur after their rupture and bleeding. Microaneurysms are more frequently found in the posterior pole or temporally, even if their presence is often not limited to these areas. Patients with only microaneurysms are usually asymptomatic.
- *Intraretinal hemorrhages* are another common finding of NPDR, generally not producing any symptom. Depending on their location, they appear with two main different shapes: those located deeper (within the inner/outer plexiform layer or the inner nuclear layer) are known as dot-blot hemorrhages, because they are perpendicular to the retinal surface;

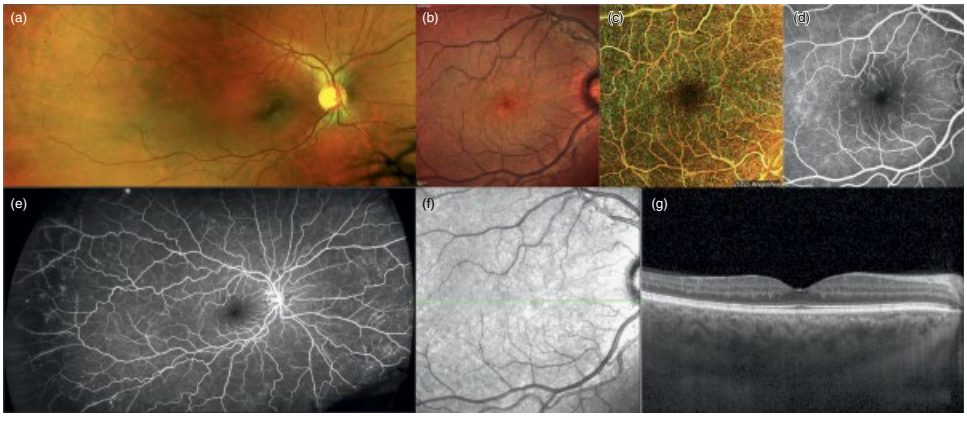


**Figure 5.1** Multimodal imaging of non-proliferative diabetic retinopathy (NPDR). Rare microaneurysms and intraretinal hemorrhages are observed on widefield color imaging (a) and on multicolor imaging of the posterior pole (b). Microaneurysms in optical coherence tomography (OCT) angiography of the posterior pole (c); this color depth map reconstructs vessels from the superficial to the deep retina. Fluorescein angiography shows hyper-fluorescent spots with mild leakage corresponding to few microaneurysms at the posterior pole (d) and in the retinal periphery (e), where initial peripheral capillary non-perfusion is observed. Infrared imaging of the posterior pole (f) and the structural OCT B-scan (g), which rules out the presence of macular edema. (*See color plate section for color representation of this figure.*)

those located within the nerve fiber layer are known as flame-shaped, occurring parallel to the retinal surface.

- *Hard exudates* are yellow lipid exudates within the retina. They occur from leaking microaneurysms or impaired capillaries and are often organized in clusters, which may appear with a circinate configuration. Hard exudates often follow fluid exudation in the retina.
- *Soft exudates*, also known as cotton wool spots, are microinfarctions of the retinal fiber layer, suggesting a more advanced stage of NPDR. Ophthalmoscopically they are whitish lesions, with undefined borders and fluffy appearance.
- *Others signs* include venous beading, intraretinal microvascular abnormalities (IRMA), vascular loops, and the large areas of capillary non-perfusion typical of severe NPDR.

**Diagnostic Tools**

Many exams may help in the diagnosis, staging, and follow-up of DR. Recently, new imaging modalities have been introduced in clinics in addition to traditional devices.

- *Dilated fundus examination* is the standard diagnostic modality in most of the patients with NPDR, and is adequate and sufficient for proper diagnosis and identification of progression to more severe stages [20].
- *Fundus photography* is a valuable technique for monitoring DR, regularly performed in most of the clinical trials. Recently, wide- and ultrawide-field digital fundus cameras have been developed, with the advantage of capturing above 100° of the retina with a single frame (Figures 5.1–5.3). On fundus cameras, additional filters may be applied to obtain further information and details.
- *FA* is a widely used imaging technique for the study of retinal disorders. FA requires an intravenous injection of sodium fluorescein, a dye solution that fluoresces when exposed to continuous light excitation between 465 and 490 nm of wavelength [21]. Sodium fluorescein is generally well tolerated, but side-effects may occur, including nausea and a transient yellowish tinge of the skin (common), vomiting, extravasation under the skin, vasovagal crisis, and allergic reactions as hitching, hives, and, rarely, bronchospasms and anaphylaxis [21]. Even if sodium fluorescein is mainly eliminated within the first 24 h by the kidney and the liver, there are no contraindications in patients with renal failure or heart diseases [21, 22]. Contrarily, FA is usually deferred during pregnancy, at least during the first trimester [21]. FA illustrates the severity of DR by providing information on retinal perfusion, endothelial permeability, and vascular abnormalities. Normally, fluorescein does not diffuse through intact endothelium of retinal vessels and retinal pigment epithelium, which form the inner and the outer blood–retina barriers, respectively. In mild and moderate stages of NPDR, FA is usually unnecessary [23]. However, it can easily differentiate patent microaneurysms (hyper-fluorescent) from retinal hemorrhages (hypo-fluorescent). In severe NPDR, FA may help to detect areas of capillary non-perfusion, IRMA, venous beading, endothelial hyperpermeability, and rule out the presence of new vessels. According to different clinical trials, a careful clinical examination and fundus photographs might be sufficient to follow the progress of severe NPDR, but FA can facilitate the differentiation from PDR [24, 25].

- *OCT* is a non-invasive imaging modality that provides extremely valuable information on each layer of the retina and of the choroid. Structural OCT produces cross-sectional 2D images of the target tissue, typically using an infrared light source. In NPDR, structural OCT can localize retinal exudates and highlights vitreomacular abnormalities. Recently, OCT angiography has been introduced and it is rapidly expanding as a non-invasive and dyeless technique [26]. It allows visualization of the retinal and choroidal plexuses, providing separate information on each of these vascular layers [26]. Vascular abnormalities occurring in NPDR, including microaneurysms and retinal non-perfusion, can be visualized with OCT angiography.

#### Treatment Strategies

In the treatment of NPDR, different emerging concepts illustrate most of the potential strategies available. First, the majority of the therapies for DR at the early non-proliferative stages are still not directly ophthalmological. Second, preventive treatments used to reduce the incidence of DR are also useful to slow its progression; strong evidence sustains many of these primary interventions, which include lifestyle modifications, tightening of glycemic control, diet plans, and correction of hypertension and dyslipidemias [16, 25, 27–31]. Third, a personalized approach is suggested rather than the application of standard fixed protocols, which may result in poor adherence to the treatment plan.

- *Laser photocoagulation*. For diabetic patients at the non-proliferative stage of DR, laser treatment of unperfused peripheral retina is not indicated for mild to moderate forms. The Early Treatment Diabetic Retinopathy Study (ETDRS) demonstrated limited reduction of the progression to severe visual loss; 5 years after laser therapy, the incidence of severe visual loss was 2.6 and 3.7% in treated and untreated patients, respectively [25]. In more severe forms of NPDR at high risk of conversion into proliferative DR, the benefits derived from early scatter photocoagulation are more favorable compared with the side-effects; these forms should then be treated as PDR for the high rate of progression [25].

### Proliferative Diabetic Retinopathy (PDR)

PDR is a sight-threatening complication sustained by chronic retinal ischemia (Figure 5.2). Among diabetic patients, more than 50% of patients with type 1 diabetes and 10% of those with type 2 diabetes develop PDR after two decades of illness [32, 33]. At increased risk of PDR are patients with advanced NPDR; approximately 50% of those patients will convert to PDR yearly [34].

#### Clinical Findings

- *Neovascularizations*. By definition, PDR is characterized by the presence of new vessels, which are promoted by chronic retinal ischemia secondary to capillary non-perfusion. These new vessels grow as fibrovascular structures perpendicularly from the retina toward the vitreous, which acts as a scaffold [25]. New vessels usually arise from the optic disc (neovascularization of the disc) or the inner retinal layers (neovascularization elsewhere). However, if the retinal ischemia is left untreated, new vessels can also develop

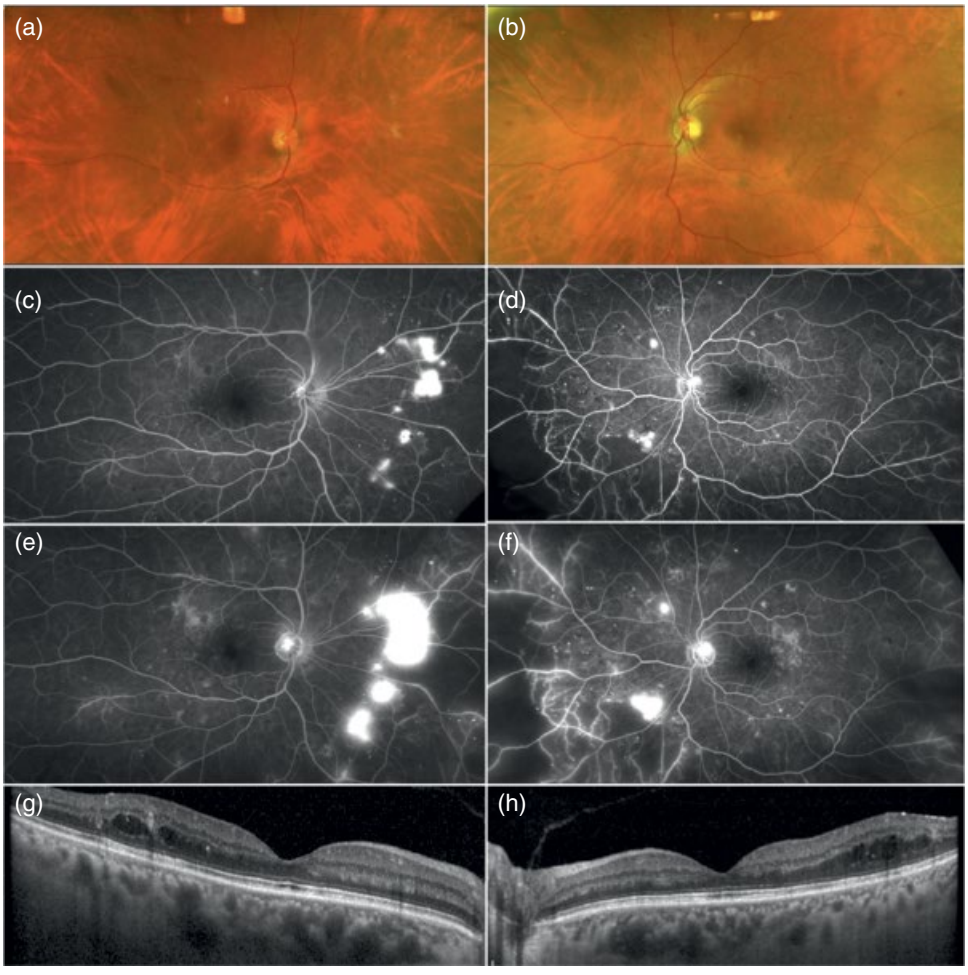


**Figure 5.2** Multimodal imaging of proliferative diabetic retinopathy (PDR). Microaneurysms and intraretinal hemorrhages are observed on widefield color imaging of both eyes (a–b). Fluorescein angiography (FA) (c–f) better highlights new vessels (early intense hyper-fluorescence – c and d – increasing on late phases of the angiogram – e and f –), and detects peripheral capillary non-perfusion and vascular leakage. Structural optical coherence tomography (OCT) (g–h) documents DME not involving the center. (*See color plate section for color representation of this figure.*)

from the iris (*rubeosis iridis*) and the filtration angle, obstructing the aqueous drainage and resulting in neovascular glaucoma. New vessels have the typical shape of a "carriage wheel," and are usually identified on fundus examination. If any doubt exists, FA can differentiate new vessels (which show profuse leakage of the dye during the exam), from abnormal vascular lesions such as IRMAs.

- *Fibrovascular structures*. New vessels may evolve into fibrous structures, which may contract; this may promote scarring, vitreoretinal tractions, retinal breaks, macular holes, and even a tractional retinal detachment, which requires a prompt surgical repair.

- *Vitreous hemorrhages*. New vessels are fragile and could bleed spontaneously into the vitreous. If the hemorrhage is confined to the virtual space between the retina and the vitreous (subhyaloid hemorrhage), erythrocytes may settle inferiorly and be absorbed. Contrarily, when the hemorrhage breaks into the vitreous, the clearing process is usually slower (weeks to months); the blood may remain localized (vitreous hemorrhage) or diffuse into the vitreal chamber (hemovitreous) obscuring the vision of the retina and causing a sudden visual loss. Hemovitreous may require surgical removal (pars plana vitrectomy).

**Diagnostic Tools**

Imaging modalities for the diagnosis and follow-up of PDR are similar to those used for NPDR, with more emphasis on FA to assess ischemia and new vessels, and with a major role of ultrasonography, particularly in advanced PDR, when fundus exploration could be limited by media opacities.

- *FA* allows easy identification of unperfused retina and new vessels. Abundant leakage from the early phases of the angiogram is the hallmark of retinal neovascularizations. This technique highlights also the integrity of the inner and outer blood–retinal barriers, which may affect the management of PDR.
- *Ultrasonography*. Standardized echography (A- and B-scan) is a valuable technique to assess the status of the retina in the presence of media opacities, such as vitreous hemorrhage or complex fibrovascular structures. In particular, ultrasound is routinely part of the preoperative work-up before any surgical intervention to the posterior segment of the diabetic eye when the fundus is not visible. Ultrasonography is able to detect retinal detachments, vitreoretinal tractions, and retinal breaks, and can confirm the presence of vitreous hemorrhage.
- *Structural OCT* provides information on the integrity of each retinal layer, central retinal thickness, and the presence of intraretinal fluid for macular edema, which may be associated with PDR. Is also helpful to recognize vitreomacular tractions, macular holes, preretinal hemorrhage, disorganization of retinal inner and outer layers, and new vessels in the macular area or on the optic disc. Added diagnostic value comes from the possibility to quantify retinal thickness into topographic maps that provide a reference for the follow-up.
- *OCT angiography* provides valuable information on the status of PDR and capillary dropout at the posterior pole. At the moment, this technique is still limited by the relatively small field of view, which reduces the information on the retinal periphery. However, composite images obtained with the most recent prototypes are overcoming this limitation, showing promising results in the study of retinal periphery [35].

**Treatment Strategies**

- *Laser treatment*. In the management of PDR, panretinal laser photocoagulation (PRP) has been the standard treatment modality for decades. Evidence for the use of PRP came from different randomized clinical trials, which include the ETDRS and the Diabetic Retinopathy Study [36–38]. These landmark trials demonstrated the effectiveness of PRP

in reducing by 50% the progression of PDR into severe visual loss (<5/200). PRP consists of scattered photocoagulation of the retina with 1200–1800 laser burns, excluding the macula and the optic disc; it is always performed outside the vascular arcades, extending toward the equator. PRP aims at the regression of new vessels after the scarring of the retinal burns; for these reasons, its therapeutic effects are not immediate but require some weeks to appear. Side-effects of PRP include irreversible loss of retinal tissue, reduction in night vision, and poor dark adaptation. Even if the physiopathology of PRP is still debated, its effects seem to be related to the ablation of the ischemic retina. This downregulates the expression of vasogenic factors driving the neovascularization process, such as the VEGF. In addition, PRP reduces oxygen demands by ablating retinal pigment epithelium (RPE) and retinal cells, which improves the perfusion of the untreated retina and promotes the regression of new vessels.

- *Anti-VEGF agents*. With the advent of anti-angiogenic drugs capable of reducing VEGF levels, a new era for the treatment of PDR has begun. Different molecules of this category have been developed for intravitreal injections and are on-label for the treatment of macular edema associated or not with PDR. The anti-VEGF agent ranibizumab, for instance, has been demonstrated to be effective against DME associated with PDR [39, 40]. In the "Protocol S" of the Diabetic Retinopathy Clinical Research Network (http://DRCR.net), intravitreal ranibizumab was non-inferior to laser photocoagulation in PDR in visual outcome and new vessel regression at 2-year follow-up [41]. The "Protocol S" also showed that intravitreal ranibizumab was associated with less peripheral visual field loss, less need for surgical treatment of PDR, and reduced incidence of DME when compared with PRP [41]. Another anti-VEGF molecule, the VEGF-trap aflibercept, has demonstrated protective effects against the progression to PDR in diabetics with macular edema during the VIVID and VISTA trials [42]. However, the management of PDR with anti-VEGF agents still present issues related to both cost burden and long-term efficacy after discontinuation of intravitreal injections. While the effects of PRP are long-lasting and require few follow-up visits, anti-VEGF agents need to be repeated on a monthly/as needed basis. Furthermore, there are potentials side-effects related to systemic diffusion of intravitreal anti-VEGFs. On the other hand, PDR is often associated with DME and the use of anti-VEGFs addresses both these complications, improving the visual outcome. The use of anti-VEGF agents also offers another treatment option when it is not possible to immediately complete the PRP, helping to control the ischemic sequelae. Furthermore, anti-VEGF agents have been shown to be a viable treatment strategy combined with surgery and laser in advanced PDR complicated by vitreous hemorrhage or by neovascular glaucoma [43].
- *Surgical treatments*. Surgical intervention becomes necessary for non-clearing vitreous hemorrhages, tractional or rhegmatogenous retinal detachments, and pre-retinal fibrous membranes. Pars plana vitrectomy (PPV) with intraoperative endo-laser photocoagulation is the standard surgical treatment option and is usually performed under local anesthesia [44]. PPV requires extensive removal of the vitreous gel, which increases the clearance of VEGF and reduces the duration of future anti-VEGF treatments [45]. Preoperative anti-VEGF agents have been used to reduce intraoperative bleeding from fibrovascular membranes [46, 47], even if concerns still exist regarding the possibility of worsening tractional bands [48]. In order to release these tractional forces, which can contribute to retinal detachments and DME, the use of other intravitreal agents such as ocriplasmin

has been advocated [49]. These agents promote an enzymatic vitreolysis and a release of vitreomacular tractions, which can worsen DME [50]. Furthermore, the posterior vitreous hyaloid acts as a scaffold for growing vessels and an enzymatic vitreolysis of the vitreous can prevent the development of neovascularizations [48].

## Diabetic Macular Edema (DME)

DME is emerging as a major cause of visual loss in diabetic patients, occurring both in NPDR and PDR (Figure 5.3) [3]. DME prevalence among diabetics has been reported to vary from 1.4 to 12.8% [51]. However, these data are affected by many factors, including the definition used for DME, the diagnostic method, the stage of DR, and the type of diabetes.

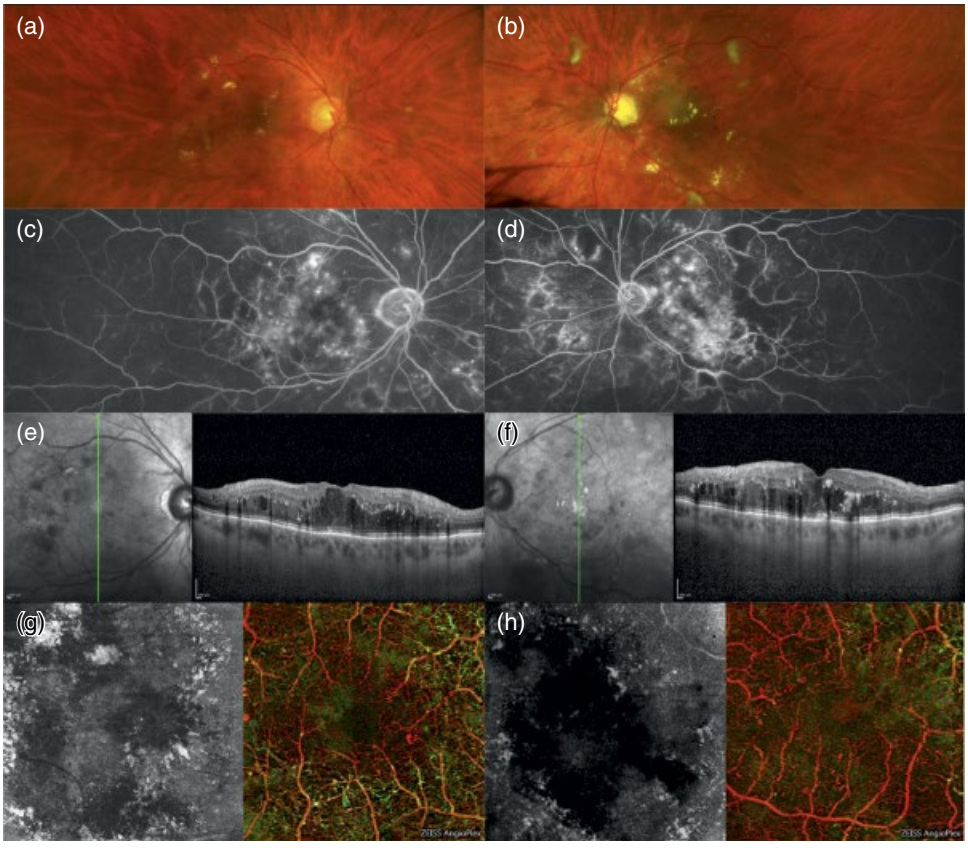


**Figure 5.3** Multimodal imaging of diabetic macular edema (DME). Microaneurysms, intraretinal hemorrhages, hard and soft exudates are observed on bilateral widefield color imaging (a–b). Late phases of fluorescein angiography (FA) show breakdown of the blood–retina barrier, vascular leakage, and areas of capillary non-perfusion (Panels c–d). Structural optical coherence tomography (OCT) highlights DME and retinal exudates on B-scans (e, f) and on en-face imaging (g – left panel, h – left panel). An OCT angiography color depth map, which reconstructs vessels from the superficial to the deep retina, confirms the presence of microaneurysms (g – right panel, h – right panel). (*See color plate section for color representation of this figure.*)

DME is typically defined as a retinal thickening at the posterior pole and it is often associated with hard exudates, even if different subtypes can be identified (see "Classification"). In the pathophysiology of DME, chronic hyperglycemia activates multiple pathways, which can ultimately lead to microvascular impairment, dysfunction of the blood–retinal barrier, and fluid exudation [52]. Furthermore, many investigations have elucidated the important role of the VEGF in vascular permeability and DME [53]. However, the pathophysiology of DME seems to have a more complex origin. Growing evidence suggests that neurodegeneration and inflammation may contribute to and precede the development of clinical signs of DME [54]. As for DR and DME the present treatment strategies have dramatically changed over the past few years. With the introduction of intravitreal therapies that can block inflammation and VEGF release, a new standard of care has become available. As chronic DME permanently damages the retina, a personalized therapeutic plan should be developed to avoid persistent visual deterioration.

## Classification

Different classifications have been proposed for DME. The ETDRS divides DME into two main clinical entities based on fundus biomicroscopy: clinically significant (CSME) and non-clinically significant macular edema [55]. CSME is defined by the presence of at least one of the following characteristics: (i) retinal thickening within 500 μm of the fovea; (ii) hard exudates within 500 μm of the fovea with adjacent retinal thickening; (iii) retinal thickening of ≥1 disc area, within 1 disc diameter of the fovea.

Another classification has been proposed by the AAO, where DME was defined by the presence of any type of retinal thickening or hard exudates at the posterior pole, distant from the macula in mild DME, approaching the center of the macula in moderate DME, and involving the macula in severe DME [56].

Recently, biomicroscopic patterns of DME have been integrated into a novel treatment algorithm that suggests the best treatment strategy for each subtype (Table 5.2) [57]. When the DME is localized and confined around leaking vessels with a cluster of hard exudates, it is classified as vasogenic. Conversely, when there is a diffuse retinal thickening compared with the limited number of microaneurysms and exudates, DME is classified as non-vasogenic. Tractional DME is instead associated with abnormalities of the vitreoretinal interface, such as epiretinal membranes or vitreomacular tractions.

**Table 5.2** Classification of diabetic macular edema (DME) according to the main clinical findings observed on fundus examination.

| Different patterns of diabetic macular edema (DME) | Findings on dilated fundus examination |
|---|---|
| Vasogenic DME | Retinal thickening associated with microaneurysms and cluster/circinate of hard exudates |
| Non-vasogenic DME | Diffuse retinal thickening, with a scant number of hard exudates and microaneurysms |
| Tractional DME | Retinal thickening in the presence of epiretinal membrane or vitreomacular traction |

Another simplified classification divides DME according to whether it involves the macula (center involved) or not.

### Clinical Findings

- *Retinal thickening*. Although the original definition of CSME proposed by the ETDRS in 1985 was based on fundus examination and did not require any further instrumental assessment, now we can detect and measure subtle retinal thickening using retinal imaging (e.g. OCT). Retinal thickening can be localized, often around microaneurysms and traction points, or can diffusely involve the posterior pole.
- *Microaneurysms and hard exudates*. Microaneurysms are often associated with DME and are considered one of the main sources of exudation. Leaking fluid contains water, proteins, and lipids. While the fluid may be more rapidly adsorbed, lipids and proteins could persist more in the retina forming hard exudates, which are signs of current/previous macular edema.
- *Epiretinal membranes and vitreoretinal tractions*. Abnormalities of the vitreoretinal interface could be associated with DME and contribute to its genesis. The term "cellophane" maculopathy refers to the presence of a thin hyper-reflective band upon the retinal surface on fundus examination.
- *Advanced findings associated with chronic DME*. These include macular holes, retinoschisis, and RPE atrophy, which can all complicate DME.

### Diagnostic Tools

- *OCT*. OCT is able to identify retinal thickening for fluid exudation before the development of cystic spaces among retinal layers. Furthermore, it can localize retinal exudates and disorganization of inner and outer retinal layers, which can complicate DME and predict visual outcomes [58]. Structural OCT has become an indispensable tool in the management of DME, also by providing accurate references for its follow-up.
- *FA*. FA is particularly useful to identify microaneurysms and leaking vessels responsible for DME. Once identified, focal laser treatment can be applied, aiming at closing the leaking points. Impaired blood–retinal barriers can be observed in late phases of FA as a diffuse leakage of the dye around leaking points.

### Treatment Strategies

Since the beginning of the 1980s, DME has been treated with focal/grid laser photocoagulation when associated with visual impairment [55]. Although laser has been considered the standard treatment for years, its efficacy remains limited, as it improves vision in a low number of eyes (<5%), while another portion may be unresponsive [59]. Other treatment options include surgery, which may carry significant risks, and intravitreal injections of therapeutic drugs, as illustrated in figure 5.4. This last treatment modality presents a relatively good safety profile and achieves a visual recovery in a significant proportion of patients. For these reasons, intravitreal pharmacotherapies are now considered the new standard of care for most DME.

- *Laser photocoagulation*. Laser treatments of DME have been assessed by the ETDRS, which confirmed the benefits of focal and grid laser photocoagulation [55, 59, 60]. These

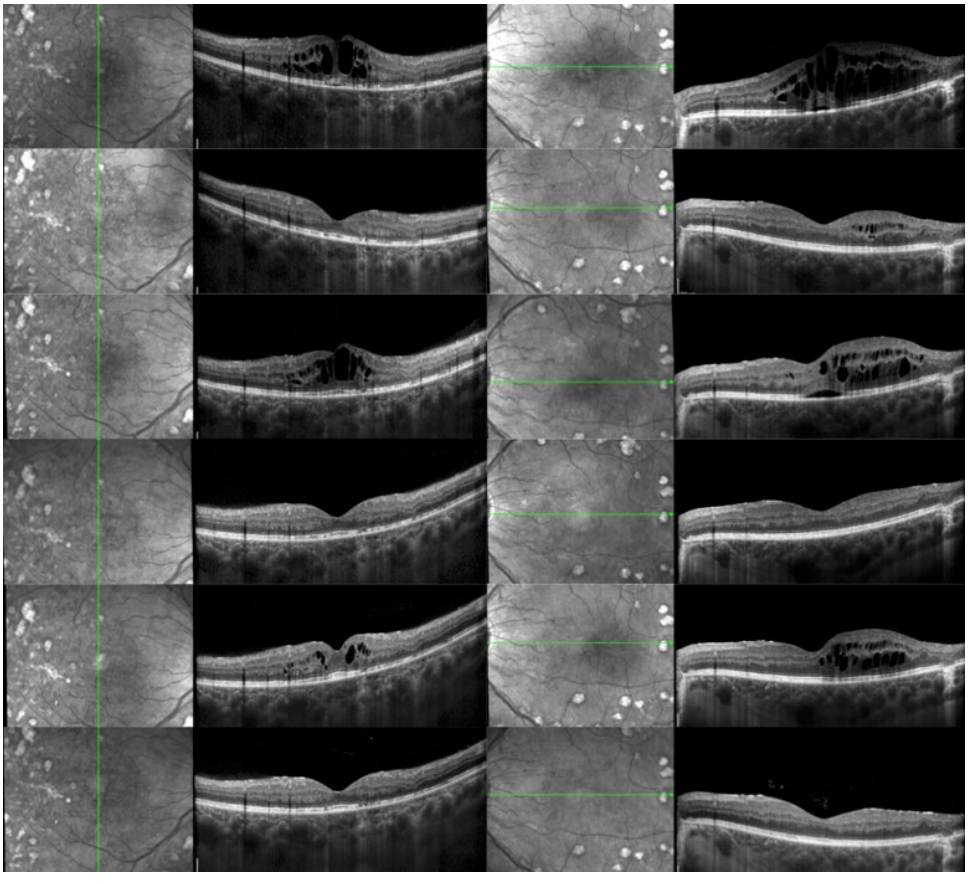

**Figure 5.4** Structural optical coherence tomography (OCT) of diabetic macular edema (DME) before and after therapy. Structural OCT B-scans document reabsorption of DME after each intravitreal injections of anti-VEGF performed in both eyes (right and left panel, respectively). Baseline (top row), 2 weeks after the first injection (second row), 4 weeks after the first injection (third row), 2 weeks after the second injection (fourth row), 4 weeks after the second injection (fifth row), and 2 weeks after the third injection (bottom row). (*See color plate section for color representation of this figure.*)

studies showed a reduction in visual loss in nearly 50% of treated patients when compared with untreated controls. The ETDRS also provided recommendations for DME treatment procedures [59, 60]. "Focal" laser photocoagulation has aimed to treat leaking aneurysms identified with FA, which contribute to localized retinal thickening, while "grid" laser photocoagulation aimed to treat a diffuse CSME applying multiple laser burns distant from the fovea and from the optic disc. The main limitation of these laser treatments includes poor visual improvement and severe side-effects, such as visual field scotomas, hemorrhages, and enlargement and coalescence of multiple laser scars. This has led to the development of new laser treatments, including subthreshold micropulse laser, "light" laser treatments, and semiautomated and automated photocoagulator with eye-trackers [61–63].

- *Intravitreal pharmacotherapies*. The efficacy of intravitreal injections of anti-VEGF agents and steroids has been demonstrated by several DME randomized trials [40, 64–83].

  Anti-VEGF agents available for DME include bevacizumab, ranibizumab, and aflibercept [64–71]. Those agents are more effective than laser treatments on visual outcomes, achieving visual recovery in a significant proportion of patients [67]. For instance, ranibizumab achieved better visual outcomes when compared with focal/grid laser photocoagulation at 3 years [72]. However, the duration of intravitreal anti-VEGFs is limited and DME tends to recur, requiring retreatments. Retreatments can be performed using monthly-fixed protocols, as-needed schedules, and treat-and-extend regimens. In the last, treatment intervals are gradually extended accordingly to the response of each patient [68]. However, all of these regimens often require frequent and sustained injections to maintain a good functional and anatomical outcome [64–71]. When frequent administrations are not possible or medical history includes recent strokes or myocardial infarctions, anti-VEGFs should be avoided.

  Due to these limitations, intravitreal steroids have gained attention in DME. These include triamcinolone acetonide, dexamethasone, and fluocinolone acetonide, which have turned out to be effective also in diabetics unresponsive to anti-VEGFs. Triamcinolone acetonide has achieved suboptimal results in DME when compared with laser or ranibizumab [76, 77]. Sustained delivery systems have therefore been developed in order to improve efficacy and reduce steroid side-effects, such as cataract and glaucoma. These include a dexamethasone intravitreal implant, which is a biodegradable device providing drug delivery for up to 6 months, and a fluocinolone acetonide insert, which is a non-biodegradable implant releasing 0.2 μg per day for up to 36 months [78–83]. Both these sustained delivery systems have proved to have good efficacy and a good safety profile in DME.

  Recently, the effect of pharmacologic vitreolysis by means of intravitreal injections of ocriplasmin has been evaluated in order to reduce vitreous tractions, which can contribute to DME [84].

## Conclusions

DR still represents a major cause of visual impairment worldwide and, if left untreated, has the potential to end in blindness. For decades the main treatment strategies relied on laser photocoagulation and aimed at disease stabilization. Nowadays, new treatments have become available that can lead to visual recovery without irreversible loss of retinal tissue. Development in retinal imaging fosters these advancements and sets novel endpoints for DR treatments. However, in this new era for the management of DR clinicians are faced with many challenges, including decisions on the most appropriate strategy for each case and the economic burden of new therapies. Ongoing and future studies will provide the clinicians with the best evidence for their decisions and better care for their patients.

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# 6

# Diabetic Retinopathy – Research

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Diabetes has deleterious effects on multiple tissues including the visual system. Diabetic retinopathy (DR) is one of the major causes of blindness in the working-age population [1]. The two broadly classified forms of DR are non-proliferative diabetic retinopathy (non-PDR) and proliferative diabetic retinopathy (PDR). Non-PDR is associated with microaneurisms, hemorrhages, and exudates, while PDR results in neovascularization, fibrous membranous growth, leading to vision loss [1–3]. Although the detrimental effects of non-PDR and PDR are known, the mechanistic understanding of the development and pathogenesis of DR is still under investigation. While some *in vivo* models currently in use are able to reproduce aspects of the non-PDR phenotype, there are no *in vivo* model systems that replicate PDR seen in humans. Keeping this in mind, this chapter summarizes the overall research findings that have helped us gain a better understanding of the effect of hyperglycemia on retinal physiology and function.

## Retina and Induction of Hyperglycemia

The retina is an extended part of the central nervous system and the tissue architecture is highly organized. It comprises neuronal cell types such as ganglion, amacrine, horizontal, bipolar, and photoreceptor cells. The nutritional demand for the retina is satisfied through two vascular supplies, namely retinal and choroid vasculature [4]. It also has glial cells such as astrocytes and Müller cells to help maintain the blood–neural barrier [5]. Basally located retinal pigment epithelial (RPE) cells also play a key role in maintaining the blood–retinal barrier [5] as well as playing a vital role in the visual cycle. Although the retina is often described as an immune privileged tissue, microglial cells constantly monitor to help maintain the tissue homeostasis.

In humans, DR has been shown to affect vasculature, cause reactive gliosis, induce inflammation, and affect neurons in the retina [6–8]. The effects of hyperglycemia on retinal cells have been largely characterized in rats and mice. The most commonly used models are chemically induced diabetic models, where streptozotocin (STZ) or alloxan are

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administered in rats or mice to destroy the insulin-producing β-cells in the pancreas [9] and study the effect. Other genetic mouse models like $Ins2^{Akita}$ mice (which carry a spontaneous insulin gene mutation) [10] and AKIMBA (a cross between $Ins2^{Akita}$ mice and Kimba mice that transiently overexpresses human vascular endothelial growth factor (VEGF) in the photoreceptors) [11] are widely used to characterize the relationship between hyperglycemia and retinal cell abnormalities. The topics below cover the scientific advancements made using *in vivo* and *in vitro* models to address each of the diabetes-related retinal complications.

## Hyperglycemia and Retinal Microvasculature

Retinal microvessels consist of endothelial cells and pericytes. Analysis of postmortem human retina with DR revealed both pericyte and endothelial loss, with the presence of acellular capillaries [12]. Similar to human conditions, chemically induced hyperglycemic rats and mice display TUNEL positive pericytes and endothelial cells, with acellular capillaries in the retina [12, 13]. In addition, mice that carry a mutation in the insulin gene ($Ins2^{Akita}$ mice) also display acellular capillaries 36 weeks post-hyperglycemia [10]. These data suggest a role for hyperglycemia in pericyte–endothelial loss.

Several possible mechanisms have been reported to induce pericyte and endothelial cell death due to high glucose. Culturing bovine retinal pericytes and rat retinal endothelial cells under high glucose conditions has been shown to affect mitochondrial morphology and membrane potential leading to apoptosis [14, 15]. One possible mechanism implicated in high glucose-induced mitochondrial damage in retinal capillary cells is mediated through MMP-9 [16]. Using high glucose *in vitro* studies and *in vivo* chemically induced diabetes mice, it has been demonstrated that elevated glucose level increases MMP9 expression and activity [16, 17]. Chemical induction of diabetes in MMP-9 knockout mice preserved mitochondrial morphology and suppressed apoptosis of capillary cells [17].

In addition to causing mitochondrial dysfunction, high glucose is reported to increase Siah1/GAPDH interaction and induce apoptosis of primary human pericytes [18]. By contrast, inhibition of Siah1/GAPDH using siRNA or peptides suppressed high glucose-induced pericyte death [18]. The other pathway shown to be involved in pericyte cell death is angiopoietin 2 (Ang2)-integrin α3β1 signaling [19]. Ang2 has been shown to be elevated in diabetic mouse models and the increase in Ang2 is suggested to cause pericyte loss [19, 20] via integrin α3β1 activation of p53 [19]. Inhibition of integrin α3β1 using neutralizing antibodies blocks pericyte cell death by suppressing p53 activation [19]. These data suggest that Ang2 likely plays a role in diabetic retinal pericyte loss.

## Hyperglycemia and Reactive Gliosis

The retina has two types of glial cells, astrocytes and Müller cells. Among several other functions, these glial cells play a key role in maintaining retinal vascular integrity. Abnormality in Müller cells with increased glial fibrillary acidic protein (GFAP) expression was reported in human DR [6]. Studies conducted in experimental diabetic rats and mice

show that both astrocytes and Müller cells are affected by hyperglycemia [21, 22]. For instance, 6 weeks post-induction diabetic rats decreased astrocyte cell density [22]. Since astrocytes play a vital role in maintaining the blood–retinal barrier, it has been demonstrated that the loss of astrocytes was accompanied by vascular leakage in hyperglycemic mice [23]. Interestingly, it is reported that Ang2, in addition to playing a role in pericyte loss, also induces astrocyte cell death through integrin αvβ5 signaling [23]. While integrin αvβ5 is suggested to act as a receptor for Ang2 [23] treatment of brain astrocytes with Ang2 in the presence of high glucose increased integrin αvβ5 levels and cell death [23]. Blockage of Ang2 and/or integrin avb5 decreased astrocyte cell death *in vitro* and *in vivo* of hyperglycemic mice retina [23].

Besides changes to the astrocytes, Müller cells have shown to undergo changes in hyperglycemic rats. Six weeks of hyperglycemia in rats increased GFAP expression in Müller cells [22], suggestive of reactive gliosis. *In vitro*, maintaining Müller cells under high glucose escalated mitochondrial fragmentation resulting in increased cytochrome C levels and cell death [24]. A study reports that thioredoxin-interacting protein (TXNIP) in response to high glucose triggers Müller cell mitochondria fragmentation leading to cell death [25]. Deletion of TXNIP in rat Müller cells *in vitro* prevented high glucose-induced mitochondrial damage [25]. In addition, it was also reported that TXNIP levels were increased in diabetic rat retina and that knockdown of TXNIP suppressed Müller cell reactive gliosis [25]. These results indicate that diabetes affects both astrocytes and Müller cells survival in the retina, which can affect tissue homeostasis.

## Hyperglycemia and Neurodegeneration

There is a notion that neuronal loss is an early event that occurs in DR which results in vision impairment [7]. Increased TUNEL positive cells have been observed in the outer nuclear layer of diabetic human retinal sections, suggestive of neuronal cell death and poor visual function [7]. In the case of experimental diabetic mice model, a transient loss of neurons is observed a month after hyperglycemia, the neuronal loss then continued after 6 months post induction of diabetes [26].

Interestingly, retinal and nerve fiber layer thinning has been observed in postmortem human eyes with diabetic mellitus even with no or minimal retinopathy complications [27]. Similar results are found in chemically induced and diabetic spontaneous mutant obese mice [27]. These mice show progressive ganglion cell loss associated with nerve fiber layer thinning after the induction of diabetes [27]. These results suggest that irrespective of retinopathy complications, hyperglycemia could damage retinal neurons.

## Hyperglycemia and Inflammation

Inflammation is thought to play a role in DR pathogenesis and has been widely investigated. Inflammatory cytokines such as IL-1β, TNF-α, and IL-6 have been shown to be present in the vitreous and aqueous humor of patients with diabetic macular edema and PDR [28, 29]. In addition, microglial activation is seen in human eyes with DR [30].

When compared to normal healthy eyes, increased association of microglia is observed in the areas of microaneurysms and neovascularization [30]. The trigger for the inflammatory response in DR has been characterized in experimental diabetic rodent models. It is reported that expression of Iba-1 and TNF-α is increased in diabetic rat retina [31]. As diabetes has been shown to affect multiple cell types in the retina, inflammatory cytokine release and the activation of microglia could be mediated by any of the cell types affected by hyperglycemia.

Studies have shown that astrocytes and Müller cells can induce inflammation in response to high glucose [32–34]. Culturing of astrocytes under high glucose conditions has been shown to induce IL-1β and TNF-α mRNA levels [32]. Similarly, Müller cells are also shown to upregulate IL-1β and TNF-α mRNA levels through a CD40-mediated pathway in experimental diabetic mice [33, 34]. Induction of diabetes in mice is shown to increase ICAM-1 and CD40 levels in Müller cells and endothelial cells [33]. Moreover, stimulation of endothelial and Müller cells with CD40 upregulated ICAM-1 expression [33]. On the contrary, induction of diabetes in CD40 knockout mice suppressed ICAM-1 levels [33]. Further investigation revealed that Müller cell-specific expression of CD40 upregulated TNF-α, IL-1β, and ICAM-1 leading to leukostasis [34].

In conclusion, the overall research findings thus far indicate that diabetes-related complications in the retina affect the majority of the cell types. Some of the cell types are affected in the early phase, while others are late responders. The other common theme observed is that most of the cell types undergo apoptosis in response to high glucose through mitochondrial damage. Taken together, these data suggest that a comprehensive therapeutic approach that targets early and late responders is required to tackle DR and to restore vision.

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# 7

# Diabetic Nephropathy – Clinical

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Diabetic nephropathy (DN) is a kidney disease that develops as a consequence of chronic hyperglycemia. From a clinical point of view, it is defined as the presence of persistent proteinuria (>500 mg/24 h) in patients with concomitant retinopathy and elevated blood pressure in the absence of urinary tract infections or other renal or cardiac diseases [1, 2]. This clinical phase has been defined as overt nephropathy, clinical nephropathy, proteinuria, or macroalbuminuria.

In the early 1980s, a number of studies showed that small amounts of albumin in the urine, undetectable by conventional methods, were predictive of the later development of proteinuria in both type 1 and type 2 diabetic patients. This stage of DN was termed microalbuminuria or incipient nephropathy [3].

Diabetic retinopathy is present in almost all type 1 diabetic patients with nephropathy, whereas only 50–60% of proteinuric type 2 diabetic patients have retinopathy [1, 2]. DN is at present the main cause of chronic kidney disease in patients starting renal replacement therapy and is accompanied by an increased cardiovascular mortality [1, 2].

## Epidemiology

DN is prevalent among African Americans, Asians, and Native Americans rather than in Caucasians [3].

The overall prevalence of micro- and macroalbuminuria is around 30–35% in both types of diabetes. According to the European Diabetes Prospective Complications Study Group (EURODIAB) [3] the cumulative incidence of microalbuminuria in type 1 diabetes was 12.6% over 7.3 years and 33% after an 18-year follow-up in Denmark [3]. In patients with type 2 diabetes, the incidence of microalbuminuria was 2.0% per year and the prevalence evaluated 10 years after the diagnosis was 25% in the UK Prospective Diabetes Study (UKPDS) [3]. The prevalence of DN ranges between 5 and 20%, in type 2 diabetic patients mainly because of ethnic differences.

*Microvascular Disease in Diabetes*, First Edition. Edited by Francesco Tecilazich.

Proteinuria develops in 15–40% of patients with type 1 diabetes, with a peak of incidence after 15–20 years of diabetes followed by a progressive decline [3].

Of interest, the prevalence of end-stage renal disease (ESRD) caused by diabetes did not significantly change between 1990 and 2010 [4]. DN rarely develops before 10 years of duration of type 1 diabetes, while approximately 3% of newly diagnosed type 2 diabetic patients are already affected by overt nephropathy [3].

The risk of developing DN in a normoalbuminuric patient with a diabetes duration of longer than 30 years is very low. This indicates that the duration of exposure to hyperglycemia is not sufficient to explain the development of DN and suggests that only a subset of patients is susceptible to the renal complication [5].

The cumulative incidence of ESRD in proteinuric type 1 diabetic patients is 50% 10 years after the onset of proteinuria, compared with 3–11% 10 years after the onset of proteinuria in type 2 diabetic patients [1].

ESRD in type 1 diabetic patients with nephropathy remains the major cause of decease, accounting for 59–66% of deaths [1]. Cardiovascular disease is also a major cause of death (15–25%) in relatively young type 1 diabetic patients with nephropathy [1].

## Natural History

When compared with other renal disorders, the natural history of renal involvement in type 1 diabetes follows a very characteristic pattern.

After a long period (10–15 years or more) characterized by the substantial absence of specific symptoms (with the exception of renal hypertrophy and increased glomerular filtration rate that may develop in some patients [6]), in approximately 30–35% of the patients the renal function starts to deteriorate, a dysfunction first evidenced by the selective excretion of albumin with urine (microalbuminuria) [7]. Even though it is commonly assumed that type 1 and type 2 diabetic patients share most pathogenic and clinical features of renal damage, some relevant differences in the onset and progression of DN between type 1 and type 2 patients should be taken into consideration.

Most type 2 diabetic patients show an early onset of arterial hypertension, that often precedes the renal involvement. In these patients, the presence of microalbuminuria or even proteinuria can sometime be demonstrated in concomitance with the clinical diagnosis of diabetes [8].

## Stages of DN

Seminal studies performed by Mogensen and co-workers allowed the classification of the progression of DN into five stages [7].

*Stage I* is characterized by renal hypertrophy and hyperfiltration with glomerular filtration rate (GFR) usually greater than 120 ml/min [6]. This stage is the earliest clinical form of DN in type 1 diabetes. This stage may be reversible with long-term insulin treatment and tight glucose control [9] and it usually spans for several years. Approximately 10% of type 2 diabetes patients usually present with persistent microalbuminuria at diagnosis, and

50–60% are already hypertensive. Most type 2 diabetic patients do not show increased GFR values at this stage. Increased GFR has been convincingly demonstrated only in type 2 diabetes among Pima Indians and, in some cases, in a subgroup of the general Caucasian population [10].

*Stage II*, or incipient nephropathy, may develop several years after the development of the initial dysfunctions [7]. At this point, renal hypertrophy may still be found [6], and GFR starts returning to apparently normal values. This "normal GFR" may actually reflect some glomerular damage caused by long-term hyperfiltration. Albumin excretion is usually normal but may increase to some extent in cases of stress [7].

*Stage III* represents the first detectable clinical manifestation of diabetic renal disease. Renal size and GFR may still be normal to slightly elevated. Microalbuminuria becomes detectable at this point ranging between 20 and 200 μg/min. In the patients with the highest amount of microalbuminuria, a parallel decrease of GFR may be seen. At this point, slight elevations in blood pressure are noted [7]. The majority of diabetic patients with microalbuminuria will develop overt nephropathy if no specific treatment is undertaken.

*Stage IV* is the stage of overt DN. Patients with stage IV disease have persistent proteinuria, usually greater than 500 mg/day corresponding to a urinary albumin excretion greater than 200 μg/min. GFR is reduced getting close to end-stage levels over time. Hypertension is a classic feature of this stage of the disease.

*Stage V* is characterized by end-stage renal failure with atrophic kidneys and GFR lower than 20 ml/min. At this point, volume-dependent hypertension develops and dialysis is usually required. Once developed, the rate of progression of DN is variable, but the mean interval between the diagnosis of type 1 diabetes and the development of overt proteinuria is 17 years. Mean interval to ESRD is approximately 23 years. While some of these patients will die because of uremic complications, the majority of diabetic patients with DN will die because of cardiovascular diseases. The risk of cardiovascular disease is 30–40 times higher in patients with nephropathy when compared with patients free of renal disease despite a similar duration of diabetes [11].

## Morphologic Changes

The progression of DN is accompanied by renal structural changes [1]. In presence of overt nephropathy all renal compartments, glomeruli, tubuli, extraglomerular blood vessels, and the interstitium, show abnormalities. The sum of these various morphologic changes and the interplay among them determine the clinical course of the complication.

Advanced-stage diabetic glomerulopathy is easily recognized, both in the diffuse or the nodular form. Diffuse diabetic glomerulopathy is characterized by a mesangial expansion, detectable with the periodic acid–Schiff reaction. The characteristic Kimmelstiel–Wilson nodules appear as acellular accumulations that are circular on section. Fibrinoid lesions in the tuft occur in advanced stages. Another peculiarity is represented by capsular drops inside the Bowman's capsule. The glomerulopathy appears grossly similar in type 1 and type 2 patients [12].

Electron microscopy provides a more detailed definition of the structure involved. In case of advanced stages of the complication, the mesangial regions occupy a large proportion of

the glomerular tuft, with an increased content of matrix. Further, the basement membrane of the capillary walls is thicker than normal. The severity of diabetic glomerulopathy is therefore estimated by the change of thickness of the basement membrane, of the mesangium, and of the amount of matrix [13].

## Pathophysiology

The pathogenesis of DN remains largely unknown. There is now convincing evidence that basic pathophysiological mechanisms eventually leading to nephropathy are similar in type 1 or 2 diabetes [14]. However, in type 2 diabetes other harmful factors, related or not related to diabetes, such as hypertension, obesity, dyslipidemia, and ischemic renal disease caused by arteriosclerosis, could also injure the kidney resulting in complex patterns of nephropathy [15]. Although it is still unclear whether it is hemodynamic or structural changes that have the major impact in the development of DN, it is now quite clear that the two processes are interrelated. On a molecular level, hyperglycemia and advanced glycation end-products (AGEs) were shown to be key players in the development of DN [15]. Recent evidence also suggests that an increase in reactive oxygen species (ROS) formation induced by high glucose-mediated activation of the mitochondrial electron transport chain represents an early event in the development of diabetic complications [15]. In parallel, a variety of growth factors and cytokines are induced by the increased ambient glucose through complex signal transduction pathways involving protein kinase C, mitogen-activated protein kinases, and the transcription factor NF-κB [15].

High glucose, AGEs, and ROS act together to induce growth factors and cytokines such as transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF) [16, 17].

TGF-β has been recognized as a modulator of extracellular matrix (ECM) formation and renal hypertrophy. Overexpression of TGF-β in diabetic glomeruli contributes to the matrix accumulation by increasing synthesis and decreasing degradation of extracellular proteins such as fibronectin, type I collagen, type IV collagen, and laminin [18], which are key factors in the pathogenesis of DN. TGF-β also decreases matrix degradation by inhibiting proteases and by activating protease inhibitors (plasminogen activator inhibitor-1). Finally, TGF-β promotes the cell–matrix interaction by upregulating the synthesis of integrins, the cell surface receptors for matrix [19, 20]. TGF-β messenger RNA (mRNA) expression is markedly increased in renal biopsy specimens from patients with proven diabetic kidney disease [21, 22].

VEGF is a major angiogenic factor and is also referred to as the vascular permeability factor, due to its ability to induce vascular permeability and leakage [23, 24]. Increasing evidence suggests that TGF-β and VEGF are key pathogenic factors in early stages of DN. Serum and urinary TGF-β levels correlate with the severity of microalbuminuria [25, 26].

The activation of the renin–angiotensin system by high glucose, mechanical stress, and proteinuria with a subsequent increase in local formation of angiotensin II (ANG II) causes many of the pathophysiological changes associated with DN [15]. Although ANG II was shown to be a central mediator of the glomerular hemodynamic changes associated with the progression of DN, several non-hemodynamic effects of ANG II may also be important

in the progression of the complication. In fact, it has been shown that ANG II is involved in almost every pathophysiological process implicated in the development of DN such as production of ROS, upregulation of cytokines, synthesis of cell adhesion molecules and of profibrotic growth factors, which in turn give rise to mesangial cell proliferation, induction of TGF-β expression, increased synthesis of ECM, increased synthesis of plasminogen activator inhibitor-1 by endothelial and vascular smooth muscle cell, and macrophage activation and infiltration [27]. ANG II also increases the adrenal synthesis of aldosterone, a factor involved in the progression of renal injury [28], and accelerates the glomerular transcapillary passage of plasma proteins, the principal cause of proteinuria [28]. Recent evidence suggest that proteinuria by itself also contributes to the progression of renal injury [29]. Experimental observations indicate that an excess of filtered proteins may promote renal damage. *In vivo*, proteinuria is associated with the renal expression of cell adhesion molecules and chemoattractants, precursors of tubulointerstitial inflammation and fibrosis [30].

## Clinical Course

Keen and colleagues [31] were the first, in 1969, to demonstrate elevated urinary albumin excretion rate in newly diagnosed type 2 diabetic patients. The same phenomenon was also documented in patients with short-term type 1 diabetic patients who had poor glycemic control [32]. This abnormal but subclinical albumin excretion rate was defined as "microalbuminuria." In addition to hyperglycemia, many other factors can induce microalbuminuria in diabetic patients, such as hypertension, massive obesity, heavy exercise, various acute chronic illness, and cardiac failure [33, 34]. The day-to-day variation in urinary albumin excretion rate is however high, ranging between 30 and 50% [35]. Consequently, more than one urine sample is needed to confirm the diagnosis of persistent microalbuminuria. Urinary albumin excretion within the microalbuminuric range (20–200 μg/min) in at least two of three consecutive sterile urine samples is a generally accepted definition of persistent microalbuminuria [1]. Persistent microalbuminuria has not been detected in type 1 diabetic children younger than 12 years [36] and it is exceptional in the first 5 years of type 1 diabetes [1]. Conversely, screening for DN must be initiated at the onset of type 2 diabetes, since 7% of these patients were shown to already have microalbuminuria at that time [3]. For patients with type 1 diabetes, the first screening is recommended 5 years after the onset of the disease [3]. The screening should not be performed in the presence of conditions that increase urinary albumin excretion, such as urinary tract infection, hematuria, acute febrile illness, vigorous exercise, short-term pronounced hyperglycemia, uncontrolled hypertension, and heart failure [3].

Initial retrospective studies in type 1 diabetic patients [37] observed a risk of progression from microalbuminuria to proteinuria of near 80% over the subsequent 6–14 years, while more recent studies [37] have suggested that the percentage of microalbuminuric patients progressing to proteinuria over 10 years is 30–45%. Microalbuminuria in some cases can also spontaneously regress to normoalbuminuria [38]. Krolewski et al. defined regression of microalbuminuria as the 50% reduction in urinary albumin excretion from one 2-year period to the next [38] and demonstrated a regression of microalbuminuria in 58% of

patients with type 1 diabetes after a 6-year follow-up, suggesting that elevated urinary albumin excretion does not always imply progressive nephropathy [38]. Parving et al. confirmed these data showing that, among 277 type 1 diabetic patients with microalbuminuria, 28 regressed to normoalbuminuria either transiently or permanently [39].

Persistent albuminuria (>300 mg/24 h) implies instead a progressive decline of GFR coupled to a raised blood pressure [1]. In the case of DN, albuminuria is the first sign of ESRD and peripheral edema is the first symptom. Fluid retention is frequently observed early in the course of this renal disease [1].

Along with DN, diabetic patients may also present retinopathy and other complications of diabetes. Blindness due to severe proliferative retinopathy or maculopathy is approximately five times more common in type 1 and type 2 diabetic patients with DN compared with normoalbuminuric patients [1]. Macroangiopathy, as evidenced by stroke, carotid artery stenosis, coronary heart disease, and peripheral vascular disease, is two to five times more common in patients with nephropathy [1].

Peripheral neuropathy is present in virtually all patients with advanced nephropathy. Foot ulcers with sepsis leading to amputation occur frequently (>25%), probably as a result of a combination of neural and arterial disease [1]. Autonomic neuropathy may be asymptomatic and may show up as abnormal cardiovascular reflexes, or may result in debilitating symptoms. More than half of the patients with advanced DN have symptoms of autonomic neuropathy: gustatory sweating, impotence, postural hypotension, and diarrhea [1].

## Treatment

It takes several years to move from the onset of diabetes to the development of DN. This evidence suggests that our major efforts should be aimed toward the prevention of the complication. Once it has developed, it is nonetheless quite important to treat DN both in its early and later stages because of the high morbidity and mortality rate associated with the disease [1]. The best possible preventive strategy for DN is based on the treatment of its risk factors: hypertension, hyperglycemia, smoking, and dyslipidemia [3].

## Primary Prevention

Primary prevention in patients with no clinical and biochemical signs of renal damage is achieved by strict glycemic control obtained by insulin or oral antidiabetic agents [40–42], as required, and/or with the maintenance of blood pressure levels <120/80 mmHg [43]. Of interest, early antihypertensive treatment is effective in preventing the subsequent development of microalbuminuria only when hypertension has already developed [43, 44]. Intensive treatment of glycemia aiming at HbA1c < 7% should be pursued as early as possible to prevent the development of microalbuminuria as demonstrated by the Diabetes Control and Complications Trial (DCCT) (39% of reduction of the incidence of microalbuminuria in patients allocated to the intensive insulin treatment) [3].

## Secondary Prevention

Secondary prevention aims to prevent or slow down the progression from micro- to macroalbuminuria. Blood pressure control along with strict glycemic control represent the first-line approach. Recent guidelines suggest the need to maintain a diastolic blood pressure level <80 mmHg and a target level of <125/75 mmHg in young patients and in those with renal impairment and/or with daily proteinuria >1 g. At this stage, the use of other antihypertensive agents in addition to angiotensin converting enzyme (ACE) inhibitors may be necessary to achieve optimal blood pressure levels [1]. The first clinical trial [45] showing the renoprotective effect of the inhibition of the renin–angiotensin system was performed in patients with type 1 diabetes with a daily proteinuria greater than 500 mg. Treatment with captopril slowed the rate of decline of creatinine clearance and was associated with a 50% reduction in the risk of death, dialysis, or renal transplantation. Subsequent studies confirmed that the blockade of the renin–angiotensin system (ACE inhibitors or angiotensin receptor blockers) is renoprotective in patients with type 1 [46] and type 2 [47] diabetes with microalbuminuria. Finally, the protective effect of ACE inhibition was confirmed also in patients with non-diabetic chronic nephropathies [48].

In parallel, the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group demonstrated that tight glycemic control prevents the progression of DN in type 1 diabetic patients [49]. In line with these findings, a significant reduction of the cumulative incidence of nephropathy was demonstrated in type 2 diabetes treated with intensive blood glucose control in the Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) study [50].

## Tertiary Prevention

Tertiary prevention concerns the reduction of the progression rate toward the development of end-stage renal failure by optimal blood pressure control that might also be obtained by the simultaneous inhibition of ACE and the angiotensin receptor (dual blockade) [51, 52]. Hypoproteic diet [53] and the control of dyslipidemia [54] may also interfere with the rate of progression of the complication [55, 56].

## Perspectives

The inhibition of the renin–angiotensin system has become an established treatment for DN since 1993 but, although expected, a subsequent significant reduction of the number of diabetic patients requiring dialysis or renal transplant could not be demonstrated [4, 57]. A recent study actually showed that, unlike other complications of diabetes, the cumulative incidence of patients reaching ESRD has not significantly changed in the past 20 years [4]. Based on this evidence it is becoming evident that at least some key points in the pathogenesis of DN are still missing and only by clarifying these “dark sides” will it be possible to set up a final and successful therapeutic approach for the prevention/treatment of this life-threatening complication of diabetes.

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# 8

# Diabetic Nephropathy – Research

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Diabetic nephropathy (DN) eventually affects about one-third of patients with both type 1 and type 2 diabetes. The consequences of this complication of diabetes are devastating. DN represents a leading cause of end-stage renal failure and a strong predictor of premature death and cardiovascular disease [1]. The risk of developing this complication is not linear with time, reaches a peak between 10 and 20 years of diabetes, and then declines, thus suggesting that only a subgroup of diabetic patients may be susceptible to renal complications [2]. That genetic factors may play a role in the development of DN is suggested by the clustering of this complication in families [3]. Furthermore, arterial blood pressure can be higher in the parents of type 1 diabetic patients with proteinuria than in the parents of age- and duration of diabetes-matched patients with normoalbuminuria, suggesting that an inherited predisposition to essential hypertension may be related to the susceptibility to DN in diabetic patients [4, 5].

## Essential Hypertension and DN

A major breakthrough in the research field of DN has been the discovery of its association with familial predisposition to essential hypertension [4, 6]. That predisposition to hypertension may contribute to DN was initially suggested after the observation that type 1 diabetic patients with microalbuminuria (the early phase of DN) already have a slight, but significant increase in blood pressure [7, 8].

Further support for the hypothesis was provided by the finding that DN is associated with an increased activity of sodium/lithium counter-transport (Na/Li CT) in red blood cells, an established marker of predisposition to essential hypertension [9–11]. Genetics of essential hypertension and DN partially overlap, so any gene candidate to explain the pathogenesis of hypertension can, at least potentially, also be considered as a susceptibility gene for DN [12].

Despite the initial enthusiasm surrounding the potential use of Na/Li CT activity as a marker for the early detection and treatment of individuals predisposed to develop

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hypertension and\or DN, its use has been so far restricted to epidemiologic studies, as specificity and sensitivity of the tests are still too low to justify any clinical use [7, 13, 14].

Among other possible candidate genes, the renin–angiotensin system (RAS) has been suggested to explain, at least in part, the genetic component of essential hypertension and DN. In fact, dysfunctions of renal hemodynamics similar to those caused by RAS activation are characteristic features of the early phases of renal involvement in type 1 diabetes and predict the subsequent development of clinical DN [15]. In parallel, pharmacological blockade of the system by means of angiotensin converting enzyme (ACE) inhibitors has been shown to have a beneficial effect on the progression of clinical proteinuria [16].

Renin was the first RAS gene to be considered and subsequently excluded as a major determinant of essential hypertension [17]. Despite this evidence, renin is still under investigation as a possible candidate gene for DN based on observations not related to its blood pressure-regulating role. A few reports have in fact described an association between plasma levels of prorenin, the inactive precursor of renin, and microvascular complications of diabetes [18, 19].

The gene for ACE is presently the most studied candidate gene for DN. Besides converting angiotensin I to angiotensin II, this enzyme regulates the microcirculation by inactivating bradykinin, a peptide with vasodilating effects [20]. In the general population, the D allele was found to associate with high serum levels of ACE in a dose-dependent manner. DD homozygotes have higher ACE concentrations than II homozygotes, with ID heterozygotes having intermediate values [21]. The D allele is also a risk factor for myocardial infarction among Caucasian individuals [22].

Although hypertension seems not to be involved, the finding of a relation between ACE genotype and cardiac disorders has potential implications for DN if one considers that myocardial infarction occurs with excess frequency not only in patients with DN but also in their non-diabetic relatives [15].

In the kidney, ACE variants might determine increased glomerular perfusion pressure and filtration rate, two dysfunctions found in the subgroup of type 1 diabetic patients who subsequently develop renal complications [23].

Genes regulating insulin sensitivity may also play a role in hypertension and DN [23]. Interestingly, many of the clinical features of the syndrome of insulin resistance (hypertension, dyslipidemia, increased coronary risk) occur with increased frequency among type 1 diabetic patients with microalbuminuria [24] or overt proteinuria [24], suggesting a possible link between insulin resistance and susceptibility to DN.

Another appealing hypothesis to explain different susceptibilities to onset/progression of DN is that there could be a genetic variability in the structure or in the synthesis of the different glomerular components. These genetic factors may be especially important in determining the rate of progression of nephropathy once the first renal lesions have appeared [17].

The thickening of the basement membrane and the expansion of the mesangium observed in type 1 diabetes appear to be mainly related to an excess synthesis (or reduced degradation) of collagen IV [25].

Another component of the glomerular basement of particular interest is heparan sulfate, the most abundant proteoglycan in this filtering apparatus. Besides contributing to the

structural organization of glomerular basement membrane (GBM), heparan sulfate is thought to play a role in determining the charge selectiveness of glomerular filtration by means of its negatively charged sulfate groups [26]. In fact, the experimental removal of this proteoglycan from the GBM results in a loss of anionic sites and albuminuria [27, 28]. Hyperglycemia affects the metabolism of heparan sulfate and this abnormality contributes to the development of DN by promoting an increased filtration of proteins [29].

## Role of Genetics in the Pathogenesis of DN

How genetic predisposition may team up with hyperglycemia to induce the development of DN remains so far unclear. Of interest, in a long-term study of type 1 diabetic patients who received kidney transplants because of end-stage renal disease (ESRD) due to DN, only about 50% of the patients experienced a recurrence of nephropathy during a post-transplant period of 6–14 years. The recurrence did not correlate with any potential transplant risk factor. This finding suggests that there may be intrinsic differences in the resistance of the donor kidneys to the development of DN [30].

Focusing on the kidney as the cause of DN, Brenner and Chertow [31] advanced the concept that oligonephropathy (retardation of renal development as occurs in individuals of low birth weight) increases the risk of systemic and glomerular hypertension in adult life and the risk of developing renal disease following exposure to stressing factors, such as chronic hyperglycemia. Intrauterine growth retardation, defined as birth weight below the 10th centile, gives rise to a significant reduction in nephron number [32].

Birth weight is known to vary directly with height. Adults with short stature (reduced height within the normal range) have an increased risk for development of DN [33] and for development of microalbuminuria in non-diabetic men [34]. Furthermore, there is an increased risk for the development of DN in women with type 1 diabetes who have experienced intrauterine growth retardation [35]. Although intrauterine growth retardation indicates an elevated risk of developing DN, the proportion of cases of nephropathy attributable to low birth weight is small, probably less than 10% [35]. These findings support the hypothesis that genetic predisposition of factors operating *in utero*, or early childhood, or both, contributes to the development of DN. In line with this hypothesis, oligonephropathy acquired later in life also acts as a risk factor for the initiation and progression of DN [36].

In the past few years, based on a genome-wide approach, a large number of linkage and association studies have been performed with the aim to clarify the genetic bases of DN [37, 38]. Several loci have been proposed as candidates for DN, and many more will be suggested as knowledge of the human genome will expand. While some genes have been implicated because they are associated with diabetes type 1 itself, others have been proposed on the basis of their possible function in the chain of events leading to renal complications [37, 38].

The potentiality of these new candidate genes as markers of the complication on one hand and as pharmacologic targets on the other is presently under investigation.

## Polyol Pathway and Non-Enzymatic Glycation

Two alternative mechanisms have been proposed as possible links between the excess of glucose and diabetic complications: activation of the polyol pathway and non-enzymatic glycation of proteins [39, 40].

Genetic differences in the activities of these pathways may explain the inter-individual variability in susceptibility to DN [17].

Most of the target tissues/organs susceptible to diabetic complications, such as nerves, lens, some renal cells, blood cells, and endothelial cells [41] are independent of insulin action.

The excess glucose that enters these cells stimulates the production of sorbitol, a sugar alcohol or polyol and fructose [39].

Accumulation of these metabolites, especially sorbitol, leads to an increase in intracellular fluids followed by complex biochemical changes including a decrease in Na/K ATPase activity and myoinositol content [39].

Two enzymes control the polyol pathway: aldose reductase, catalyzing the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent production of sorbitol from glucose, and sorbitol dehydrogenase, oxidizing sorbitol to fructose [39]

Another mechanism that has been proposed to explain glucose-mediated damage is non-enzymatic glycation of proteins [40].

Glucose interacts with the free amino groups of proteins to originate an aldimine (N-glucosylamine) and then a ketamine (1-amino-1-deoxyketose or Amadori product). These molecules are further modified to form highly reactive compounds that interact with other amino groups and undergo several intramolecular rearrangements to form a variety of advanced glycation end products (AGEs) [40].

The accumulation of AGEs may contribute to the development of diabetic complications by inducing the crosslinking of structural proteins of the extracellular matrix such as collagen and laminin and by impairing their function [40]. Other adverse effects would be produced by the interaction of AGEs with specific membrane receptors, a phenomenon that ends up by triggering the secretion of diverse cytokines and growth factors [40].

Individuals genetically protected from AGE accumulation should, at least in principle, be less susceptible to the development diabetic complications [17].

## Environment

Several studies have clearly demonstrated that both environmental and genetic factors are implicated in the development and progression of DN [12].

The evidence for cigarette smoking as a risk factor for DN is still under debate. Many studies have found cigarette smoking to be a significant risk factor for advanced stages of DN [42–44], but other investigations have not fully confirmed this association [45, 46]. Only a few studies have examined smoking as a risk factor for the onset of microalbuminuria; some have shown a significant association between the development of microalbuminuria and smoking [47, 48], whereas others have not [49, 50].

The impact of glycemic control on the progression of DN has also been considered [14]. Blood glucose levels are clearly a major determinant of HbA1c levels [51] and biological

variations of HbA1c levels are important predictors of the development and progression of diabetes complications.

A close correlation between blood pressure and rate of decline in glomerular filtration rate (GFR) has been documented in type 1 and 2 diabetes [14]. Systemic blood pressure elevation to a hypertensive level is an early and common phenomenon in DN [14]. The risk of developing microalbuminuria and proteinuria is increased in patients with higher levels of blood pressure [14].

Dyslipidemia is one of the major risk factors for the development and progression of DN [52] and lipoprotein abnormalities such as higher plasma levels of very low-density lipoprotein, low-density lipoprotein and triglycerides, and lower levels of high-density lipoprotein tend to be more frequent in patients affected by DN [52]. A number of studies have reported that lipids may induce both glomerular and tubulointerstitial injury through mediators such as cytokines, reactive oxygen species, chemokines, and by inducing hemodynamic changes [52]. Clinical studies in patients with DN showed that lipid control can be associated with an additional reduction in proteinuria [52]. Experimental studies demonstrated that lipid-lowering agents exerted a certain degree of protection from the development of DN [53]. Altogether, there is some evidence that lipid reduction can preserve GFR and decrease proteinuria in both types of diabetes [53].

Finally, short-term studies in normoalbuminuric and microalbuminuric type 1 diabetic patients have shown that a low-protein diet (0.6–0.8 g/kg/day) reduces urinary albumin excretion and hyperfiltration, independently of changes in glucose control and blood pressure [14]. Longer-term trials in type 1 diabetic patients with DN suggest that protein restriction significantly reduces the decline of kidney function [14].

## Perspectives

### Sodium–Glucose Cotransporter-2 (SGLT2) and DN

Early increases of kidney volume and GFR have been shown to predict the subsequent development of DN [15, 54–56]. These renal dysfunctions are, at least in part, explained by diabetes-associated increased filtered glucose followed by augmented sodium and glucose reabsorption by the proximal tubule through the SGLT2 (increased kidney volume). This sequence of events finally ends up by decreasing the distal sodium delivery to the macula densa with activation of the tubuloglomerular feedback and consequent increase of GFR. Selective inhibitors of SGLT2 have been recently introduced in clinical practice to improve the glucometabolic control taking advantage of their glycosuric effect [57–60]. These same drugs, by increasing the sodium delivery to the macula densa, should be able to reduce the tubuloglomerular feedback, counteract the tendency toward the increase of kidney volume and GFR, and, finally, prevent the development of DN [61–63].

### B7-1 and DN

T cell activation is mediated by the interaction of the immune-related protein B7-1 (expressed by antigen-presenting cells, also known as CD80) with CD28 and with T-lymphocyte antigen 4 (CTLA-4, expressed by T cells) [64]. Induction of B7-1 in podocytes has been associated

with the presence of proteinuria in lupus nephritis [65], focal segmental glomerulosclerosis [66], and, more recently, in DN [67]. This finding suggested the possibility to use abatacept (CTLA4-Ig), a B7-1 inhibitor, as a possible treatment for DN. This hypothesis is presently still under debate after the recent demonstration that B7-1 seems not to be induced in podocytes of both human and experimental DN [68].

## Animal Models

Animal models have been quite useful in dissecting the pathogenesis of a number of different diseases and in testing novel therapies. The increasing availability of genetically modified animals actually facilitates mechanistic studies that could not otherwise be performed in humans. For these reasons a reliable and easy-to-use animal model to study the mechanisms involved in the development of a specific disease, as well as to test the efficacy of new drugs is crucial. One of the major problems that can be encountered while studying the pathogenesis of DN is the lack of preclinical models able to recapitulate important functional, structural, and molecular features of advanced human diabetic kidney disease [69–73]. In order to rationalize the development of novel preclinical models of DN, the nephropathy subcommittee of the Animal Models of Diabetic Complications Consortium (AMDCC) has published the following validation [72] criteria for rodent models based on the clinical and pathological features of human DN:

1) more than 50% decline in GFR over the lifetime of the animal;
2) greater than 10-fold increase in albuminuria compared with controls for that strain at the same age and gender;
3) histopathologic findings which include:
   - advanced mesangial matrix expansion (with nodules);
   - any degree of arteriolar hyalinosis;
   - GBM thickening (>25% increase compared with baseline by electron microscopy morphometry);
   - tubulointerstitial fibrosis.

## DN in Induced Model of Type 1 Diabetes Mellitus (T1DM)

Alloxan and streptozotocin are two glucose analogues capable of inducing diabetes secondary to the necrosis of pancreatic β-cells. Both compounds are commonly used for producing artificially induced T1DM, which causes kidney damage similar to that seen in the case of human DN [69–73].

Induced diabetic mice do not develop hypertension and exhibit severe hyperglycemia and significant weight loss, possibly due to the catabolic effects of insulin deficiency, as well as volume depletion associated with osmotic diuresis. For this reason, intermittent treatment with small amounts of insulin to avoid weight loss without reversing hyperglycemia may be required in long-term studies.

Finally, taking into account the evidence that male animals are more susceptible to diabetes induction, females are rarely used in these kinds of studies.

Previous reports have shown that differences in susceptibility to DN can be found in mice based on their genetic background and strain.

## DN in Spontaneous Model of T1DM

### $Ins2^{Akita}$ Mice

These mice carry a spontaneous mutation of the *Ins2* gene resulting in a misfolding of the aminoacidic sequence of insulin, which is toxic to pancreatic β-cell [74]. $Ins2^{Akita}$ mutation is autosomal dominant and heterozygote mice develop significant hyperglycemia at 3–4 weeks of age, while homozygous mice usually die in the perinatal period. Males develop a more aggressive insulin deficiency than females and for this reason are more often used for experimental studies.

The Akita mouse develops moderate levels of albuminuria and relatively slight structural changes, including an increase in mesangial matrix, thickening of GBM, and depletion of podocytes [71].

Recently the backcrossing of the mutation with other strains such as DBA2 [75] and 129SV [76] demonstrated that the genetic background of Akita mice contributes to the severity of both albuminuria and histological changes (see Table 8.1).

**Table 8.1** Renal impact of superimposed diabetes on different animal strains.

| | Albuminuria | Renal pathological changes (6 months of age) | Hypertension |
|---|---|---|---|
| **C57BL/6** | Mild to moderate but less than 10-fold higher compared with control mice at 6 months of age | Glomerular hypertrophy<br>Mild to moderate mesangial matrix expansion<br>Thickening of the GBM<br>No tubulointerstitial fibrosis (but enlargement of the tubule and tubular cell atrophy) | No |
| **DBA/2** | Marked, 10-fold higher at 5 weeks of age | Severe mesangial matrix expansion and nodular glomerulosclerosis<br>Arteriolar hyalinosis<br>Thickening of the GBM<br>No tubulointerstitial fibrosis (but enlargement of the tubule and tubular cell atrophy) | No |
| **CD1** | More than 10-fold higher when compared with control mice | Moderate mesangial matrix expansion (but no nodular lesions)<br>Increased glomerular surface area<br>Tubulointerstitial fibrosis | No |
| **129/Sv** | Mild to moderate but less than 10-fold higher compared with control mice at 6 months of age | Mild to moderate increase in mesangial matrix expansion<br>No tubulointerstitial fibrosis (but tubular damage) | Yes |
| **Rats (Sprague–Dawley)** | 10-fold higher than control rats at 24 weeks of age | Mild increase in mesangial matrix (but no nodular lesions)<br>Accumulation of collagen IV in glomerular basement membrane<br>No tubular damage and no tubulointerstitial fibrosis | No |

The effects of diabetes on blood pressure also varied among the strains: 129SV and C57Bl/6 are more susceptible than DBA2.

## Non-Obese Diabetic (NOD) Mice

This strain is a polygenic model of spontaneous T1DM [69–73]. NOD mice develop spontaneous insulitis at the age of 4–5 weeks, and overt diabetes emerges at the age of 24–30 weeks when most of the pancreatic β-cells are destroyed. Incidence of diabetes is four times higher in female than in male NOD mice.

Even though NOD is the most common model used to study the genetic and immunologic bases of the pathogenesis of T1DM, only a few investigators choose the NOD mouse to study the pathogenesis of DN because of the variable age of onset of diabetes that characterizes this murine strain.

Finally, recent studies have shown that albuminuria in hyperglycemic NOD mice is seven times higher compared with normoglycemic NOD mice and that modest histological alterations can be found in glomeruli and in the proximal tubules.

## OVE26 Friend leukemia virus B (FVB) Mice

OVE26 mice overexpress calmodulin in pancreatic β-cells, which results in the development of type 1 diabetes within hours of birth due to deficient insulin production [77].

Male heterozygous OVE26 diabetic mice manifest a higher albuminuria when compared with other models: significant albuminuria is already detectable at 2 months of age and at 4–5 months of age is almost 100-fold higher than the basal level.

The GFR of OVE26 mice significantly increases from 2–3 months of age and then decreases significantly from 5–9 months. Diastolic blood pressure, measured with a tail cuff, is increased during the first 3 months of age.

Histological changes include: increase in mesangial expansion with formation of Kimmestiel–Wilson nodules, enlarged glomeruli, a thickening of the GBM, podocyte loss, and tubulointerstitial fibrosis. Inflammation and increase of kidney volume have also been described.

According to the literature [77], this model seems to be the best at replicating the human form of disease but a recent study also suggests that its phenotype is probably due to the genetic background (FVB): in fact after a single cross with other strains (C57 or DBa2) a 17-fold reduction of albuminuria, together with a less severe renal histopathology was observed without significant change in glucose metabolism.

This strong genetic susceptibility to DN suggests OVE26 FVB as a good model to study loci containing sequences important to the susceptibility to DN.

Unfortunately another main issue concerning the use of this model is due to the low viability and the poor breeding capability.

## DN in Spontaneous Models of T2DM

### db/db Mice

This model is characterized by a mutation in the leptin receptor (LepRdb/db) that results in abnormal splicing and, as a consequence, in a defective receptor for the adipocyte-derived hormone leptin [69–73]. The defect in leptin signaling produced by the LepRdb/db deletion affects hypothalamic responses, leading to the development of hyperphagia, obesity, hyperlipidemia, hyperinsulinemia, insulin resistance, and diabetes (more severe in males than in females) originally recognized in the C57BLKS/J strain.

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# 9

# Diabetic Neuropathy – Clinical

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## Introduction

One of the most common and most feared complications of diabetes is neuropathy. Neuropathy can result in pain, loss of protective sensation, muscle weakness, loss of autonomic control of end organ function, or some combination of these problems simultaneously [1].

Diabetes has a worldwide impact; we currently expect over 350 million people to have diabetes globally by the year 2030 [2]. The vast majority of these individuals will have type II diabetes, approximately 90–95%, while the remaining will have type I diabetes [2]. In general, those individuals with type II diabetes will develop neuropathic complications far earlier than those with type I diabetes. Many individuals with type II diabetes may have a diagnosis of neuropathy at the time they are diagnosed with diabetes [3, 4]. In contrast, individuals with type I diabetes will not generally present with neuropathy until at least 5 years after development of diabetes, and some individuals may not develop neuropathy after decades of type 1 diabetes [5].

The complications of diabetic neuropathy are restricted to those individuals who meet the diagnostic criteria for diabetes, using fasting blood glucose levels, hemoglobin A1C levels, or the results of an oral glucose tolerance test. However, research suggests that a diagnosis of prediabetes with or without metabolic syndrome may predispose to the microvascular complications of prediabetic neuropathy [3]. This topic is outside the scope of the current chapter and the reader is referred to several excellent publications discussing this topic [3, 4, 6].

## Classification of Diabetic Neuropathies

Diabetes can cause a bewildering array of neuropathies, which can create challenges to the clinical recognition of these problems and result in confusing nomenclature in the literature. Table 9.1 outlines some of the common presentations of diabetic neuropathy.

*Microvascular Disease in Diabetes*, First Edition. Edited by Francesco Tecilazich.

**Table 9.1** Forms of neuropathy.

| Neuropathy type | Comments | Onset | Type of diabetes |
|---|---|---|---|
| Distal symmetric polyneuropathy (DSPN) | Most common presentation for neuropathy | Gradual onset, slowly progressive | Type 1 and 2 |
| Small fiber neuropathy | Often an early presentation of neuropathy | Gradual onset, often slowly progressive (but with exceptions) | Type 1 and type 2 |
| Autonomic neuropathy | Diverse manifestations including cardiac, gastrointestinal, genitourinary, sudomotor, hypoglycemia unawareness | Can occur early in diagnosis, but typically is slowly progressive | Type 1 and type 2 |
| Treatment-induced neuropathy | Associated with rapid improvement in glucose control after prolonged periods of hyperglycemia | Days to weeks | Type 1>>type 2 |
| Radiculoplexus neuropathy | Severe pain and weakness are common findings | Often occurs in older individuals with sudden onset | Type 2 |
| Mononeuropathies | Mononeuropathies at the carpal tunnel, ulnar nerve or common peroneal are common | Sudden | Type 2>type 1 |
| Cranial neuropathies | Third, fourth, and sixth nerve palsies occur. Thought to be ischemic. Gradual improvement over time | Rapid onset. Very gradual resolution of symptoms | Type 2>type 1 |

## Distal Symmetric Polyneuropathy (DSPN)

DSPN is the classical "stocking and glove" distribution of neuropathy that is described in every introductory medical textbook [1, 7, 8]. Nearly 50% of individuals with diabetes will develop this form of neuropathy. It is typically a slowly progressive neuropathy that begins with sensory changes in the toes, and gradually progresses more proximally due to the "dying back" phenomenon of axonal loss in peripheral nerve dysfunction [1, 7].

Despite the frequent description of the stocking and glove distribution of neuropathy in the literature, this is an uncommon and very advanced presentation of DSPN. In a typical presentation, sensory loss or pain occurs in the toes and gradually spreads to the feet over many years. Sensory loss does not extend to the hands until after the neuropathy approaches the knees. Symptoms associated with distal symmetric diabetic polyneuropathy are typically classified as positive (i.e. a spontaneous symptom that is a result of a damaged nerve) or a negative symptom (the loss of function due to a damaged nerve) [9]. Positive symptoms typically include tingling, paresthesias, burning, or shooting pains. Negative symptoms may include loss of sensation or a perception of coldness in the affected region [9]. In some cases, the neuropathy may affect the distal motor system resulting in loss of muscle mass in the feet and signs may include weakness of toe extensors and flexors [1, 4].

There are several terms reported in the literature to further characterize distal symmetric neuropathy in diabetes. These terms include "sensory," "motor," and "poly." A distal, symmetric, sensory neuropathy suggests a typical length-dependent neuropathy that predominantly affects sensory fibers. A distal, symmetric, sensori-"motor" neuropathy involves both sensory and motor fibers [10]. The addition of "poly"- to neuropathy implies the involvement of both large myelinated and small unmyelinated nerve fibers, but does not specify the proportion of nerve fiber involvement (nearly every form of neuropathy in diabetes is considered a "polyneuropathy" because of the extensive involvement of many different nerve fiber types) [10].

Pain, or other uncomfortable sensory perception, is noted in approximately 50% of patients with a DSPN. Pain is reported in a number of different ways, and may include burning, tingling, stabbing, aching, or cold pain. There is a typical diurnal variation to the character of the pain, with a peak of pain at night, which often interferes with sleep. In many cases pain is less severe during the day, with distraction or activity often mitigating the more severe symptoms [1, 4, 10].

Some patients with DSPN have loss of sensation as their only symptom of neuropathy. In a typical clinical scenario, these individuals may not bring their neuropathy to the attention of their physicians because they have no pain, it is only if they develop ulcerations or other non-healing wounds to their feet that they may seek medical attention. Careful screening of at-risk individuals can be valuable in identifying these patients to prevent the development of ulcerations, and amputations [1, 4, 10].

**Diagnosis**

The diagnosis of DSPN is generally made on clinical grounds through a combination of symptoms, signs, and the judicious use of electrophysiologic testing (in select cases) [5]. Examinations that suggest a length-dependent sensory loss (using a 10-g monofilament, vibration, proprioception, pain, or thermal sensation) and reduced or absent distal reflexes will support the diagnosis of DSPN. Symptoms will generally occur within the region of sensory loss, although up to half of patients with DSPN may be asymptomatic [5]. Electrodiagnostic testing is generally reserved for unusual cases, or to answer a specific clinical question. Electrodiagnostic testing is not required in routine, uncomplicated cases [11, 12].

**Management**

The management of DSPN typically follows the management of diabetes in general. A focus on glucose control is of critical importance, particularly in those individuals with type 1 diabetes [5]. Other risk factors for neuropathy prevention are important in type 1 diabetes and should be considered: lipid control, blood pressure management, and smoking cessation. For those individuals with type 2 diabetes, glycemic control is also of value, but the associated risk factors may play a much larger role, so lipids, blood pressure, and tobacco use should all be monitored closely [5].

Many individuals with DSPN will report neuropathic pain, and will require pain management as one of their therapeutic goals. A brief overview of the treatments of neuropathic pain is outlined in Figure 9.1. There are several important guidelines to consider when managing neuropathic pain. The use of a single agent to control pain is preferable to

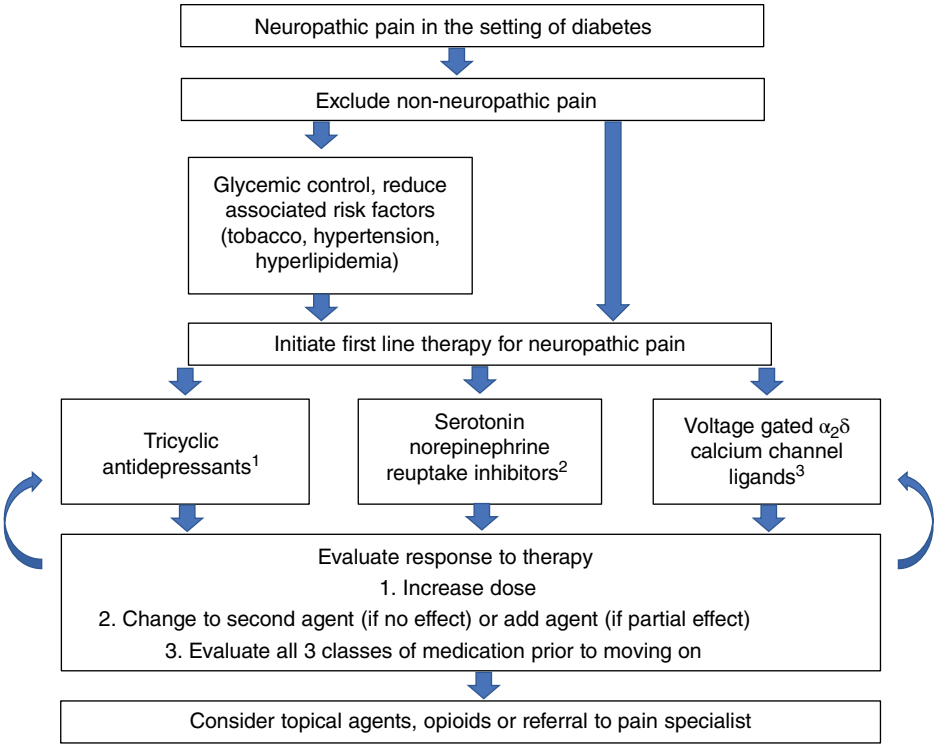


**Figure 9.1** Treatment of neuropathic pain.

polypharmacy, particularly in the setting of expansive medications lists that are common in individuals with diabetes [13]. The goal is to reduce pain to a manageable level, not necessarily to prevent all pain, which may only be achieved with excessive side-effects. It is particularly important to reassess pain severity at every visit. If neuropathic pain has gradually abated, the slow titration down of chronic medications for pain is an important patient care item that is often overlooked.

## Small Fiber Neuropathy

Small fiber neuropathy presents in a similar fashion to DSPN of diabetes, but by definition, predominantly affects small unmyelinated nerve fibers [14]. In diabetes, small unmyelinated nerve fibers are frequently involved earlier than large myelinated nerve fibers, so may be the presenting form of neuropathy for individuals with prediabetic neuropathy or with neuropathy at the time of diagnosis of type 2 diabetes [15].

Small unmyelinated nerve fibers mediate pain, thermal sensation, and autonomic function. The clinical description of a small fiber neuropathy generally refers to the involvement of fibers that involve pain and temperature detection, while autonomic neuropathies are usually classified separately [5, 10]. The exception to the classification of autonomic neuropathies separately is the inclusion of peripheral sudomotor (sympathetic cholinergic) nerve fibers that control sweat function, which are often included as part of the small fiber

neuropathy definition [16]. Pain associated with small fiber neuropathy often presents with burning and stabbing pain in the distribution of nerve damage, sometimes with accompanying contact allodynia or hyperalgesia [10]. In other scenarios patients have painless small fiber neuropathy, and may only notice decreased thermal or pain sensitivity (typically stepping into a bathtub, they won't notice hot water until above the area of involvement). With peripheral sudomotor nerve fiber involvement, there is a gradual loss of distal sweat function [17, 18]. Clinically, the loss of peripheral sweating may result in compensatory proximal hyperhidrosis, which is often the presenting patient complaint. As the area of peripheral sweat dysfunction increases, peripheral thermoregulatory function is impaired and there is central compensation and proximal hyperhidrosis. Physicians should be cautious in treating complaints of proximal hyperhidrosis because this increases the risk of hyperthermia.

**Diagnosis**

A diagnosis of diabetic small fiber neuropathy is most often made through evaluation of clinical symptoms and signs. A history of burning or stabbing pain in a length-dependent fashion, in combination with loss of thermal or pain sensitivity in the same distribution, is generally all that is necessary for the clinical diagnosis of small fiber neuropathy. In some cases, the history or examination findings may not support the clinical diagnosis and therefore additional supportive testing is required.

The current gold standard for the pathologic diagnosis of small fiber neuropathy is the use of a skin biopsy with analysis of intra-epidermal nerve fiber density [19]. Standard guidelines and normative data by age and gender have provided clear recommendations on the use of skin biopsy in patients with suspected small fiber neuropathy [19, 20]. A 3-mm punch skin biopsy, taken 10 cm above the lateral malleolus, provides sufficient tissue to determine the relative proportion of small nerve fibers in the skin, and can aid in diagnosis of small fiber neuropathy [21]. Some cutaneous tissue structures, such as sweat glands and pilomotor fibers, are also present within skin biopsies and may provide additional information about the structural integrity of the peripheral autonomic nervous system in diabetes [22–24]. A skin biopsy is not required for a diagnosis of small fiber neuropathy, but may provide clarity in unclear cases.

Other tests of small nerve fiber function include quantitative sensory testing (QST) and quantitative sudomotor axon reflex testing (QSART). QST tests the capacity of the person to respond to a standardized sensory stimulus that can include pain and thermal detection thresholds in the setting of a small fiber neuropathy. Although relatively sensitive and specific, QST is less useful in the setting of cognitive impairment, fatigue, or feigned sensory loss [25–28]. QST has not generally achieved widespread clinical use because of relatively poor reimbursement. QSART quantifies the peripheral sudomotor (sweat) output from a local area of nerve stimulation [29]. QSART is sensitive and specific for the presence of a distal sudomotor neuropathy, although care must be taken to control for environmental factors, confounding medication effects, age, and gender in the analysis [16, 29].

**Management**

The management guidelines for small fiber neuropathy in diabetes mimic those of DSPN. The goal of treatment is prevention of disease progression. In most cases the diagnosis of

small fiber neuropathy is an early microvascular manifestation of diabetes, but suggests that the individual is clearly susceptible to neuropathy development and additional care must be taken to avoid severe long-term complications. Goals of care include a focus on hemoglobin A1C, lipid control, normal blood pressure, and avoidance of tobacco and excessive alcohol. In many cases neuropathic pain is a confounding variable, and must be considered a goal of care. Treatments for neuropathic pain are highlighted in Figure 9.1.

### Autonomic Neuropathy

Autonomic neuropathy is a common and potentially debilitating complication of diabetes. Frequently abbreviated in the literature as diabetic autonomic neuropathy (DAN), it is challenging to concisely report the epidemiology of the problem because of the diffuse potential organ system involvement. The frequency of neuropathy reported will depend entirely on what organ system is studied, and what tests are used to identify the problem. Symptoms of DAN may involve the cardiovascular, genitourinary, gastrointestinal, pupillary, sudomotor, and neuroendocrine systems [30–34]. The prevalence of autonomic neuropathy is typically reported by organ system, because of the wide variability of the organ specific autonomic neuropathy across individuals with diabetes. The incidence of autonomic neuropathy increases with the duration of diabetes, and with the level of glycemic control [5, 34].

Cardiovascular autonomic neuropathy (CAN) in diabetes carries significant morbidity and mortality. Initially this may manifest as a resting tachycardia, but this may progress to exercise intolerance, orthostatic hypotension, syncope, intra-operative cardiovascular instability (and risk of arrhythmias), and silent myocardial infarction [5, 34]. Several studies have reported an increased risk of mortality in individuals with CAN [32, 35]. In the ACCORD trial, it was reported that there was a two to three times higher risk of mortality in individuals with CAN, compared with those individuals with diabetes that did not have CAN [36]. However, the exact mechanism underlying the relationship between CAN and the risk of mortality is not fully elucidated. Orthostatic hypotension, defined as a fall in systolic blood pressure of $\geq$20 mmHg, or diastolic fall in blood pressure of $\geq$10 mmHg, is a sign of more advanced CAN. The diagnosis of orthostatic hypotension may be complicated because a number of commonly prescribed medications (such as antidepressants, antihypertensives, bladder antispasmodics) can cause orthostatic hypotension and should be considered in the diagnosis because orthostatic hypotension as a side-effect of medication use carries a much lower mortality rate [37].

Gastrointestinal autonomic neuropathies include gastroparesis, gastroesophageal reflux disease, chronic diarrhea, or constipation [38]. Symptoms of gastroparesis may include nausea, vomiting, food intolerance, early satiety, bloating, or abdominal pain [39–41]. The severity of gastroparesis will fluctuate with glycemic control and the presence of gastroparesis will confound glycemic control because of irregular food and medication absorption. This can create a vicious cycle of increasingly difficult glycemic control, particularly for those individuals that take insulin, because the variable absorption of food results in both over- and under-treatment with resultant hypo and hyperglycemia. Other gastrointestinal manifestations of neuropathy can include heartburn, regurgitation, bronchospasm, laryngitis, or chronic cough related to reflux [42]. Finally, diabetic diarrhea can occur in rare cases and can be particularly difficult to manage. The diarrhea is typically nocturnal, profuse,

and can be associated with fecal incontinence [42]. Some individuals with significant gastrointestinal dysfunction may progress to chronic constipation [42].

The most common presentation of gastrointestinal autonomic neuropathy is sexual dysfunction. In men, retrograde ejaculation and erectile dysfunction is very common, and may be seen in up to 50% of men with diabetes [43, 44]. In women, sexual dysfunction may manifest as diminished vaginal lubrication and painful intercourse (dyspareunia) [44]. Bladder dysfunction in diabetes may manifest as urgency, frequency, but may progress to diminished capacity to sense bladder fullness, resulting in urinary retention and overflow incontinence [45].

The peripheral sympathetic cholinergic system is commonly involved in patients with diabetes. The loss of sweat function typically follows along with the stocking and glove distribution seen with the sensory neuropathy [17]. Progressive loss of sudomotor nerves fibers causes dry, cracked skin that may provide a portal to infection [46]. With progressive sympathetic cholinergic neuropathy, thermoregulatory capacity is impaired and proximal hyperhidrosis is a common compensatory mechanism, and also a common patient complaint [47, 48].

Other autonomic manifestations may include pupillary dysfunction. Of particular concern to patients is difficulty with pupillary dark adaptation, which will directly impair nocturnal driving creating a loss of independence [49]. One of the more feared complications of diabetes is hypoglycemia-associated autonomic failure [50, 51]. With recurrent episodes of hypoglycemia, there is a blunted neuroendocrine response to future episodes of hypoglycemia with diminished release of epinephrine and glucagon. Individuals then develop defective glucose counter-regulatory mechanisms and have hypoglycemia unawareness, typically resulting in more profound and more frequent hypoglycemia.

#### Diagnosis

The diagnosis of DAN is based on a combination of symptoms and signs of a particular end organ dysfunction [52, 53]. For example, diabetic gastroparesis will present with nausea and vomiting of food eaten hours earlier. Gastroparesis can be confirmed by a gastric emptying study [41].

One of the earliest clinical manifestations of DAN is a resting tachycardia and changes to peripheral sudomotor function [16, 34]. There may be no associated symptoms at that time. A diagnosis of early DAN can be made through a battery of autonomic function tests. These autonomic tests may include measures of parasympathetic function (the heart rate response to deep breathing, the heart rate response to a Valsalva maneuver, the heart rate response to standing), and sympathetic adrenergic function (the beat-to-beat blood pressure response to a Valsalva maneuver, the blood pressure response to standing or tilt table testing), and the tests of sympathetic cholinergic function (the sudomotor response to QSART, thermoregulatory sweat testing, or other sweat test) [29, 54]. A summary of autonomic function tests is provided in Table 9.2.

#### Management

The management of the myriad manifestations of DAN is particularly challenging. The diffuse innervation of the autonomic nervous system and the widespread impact on end organ function does create many barriers to effective care. A detailed discussion of the

**Table 9.2** Common tests of autonomic function.

| Autonomic function test | Technique |
|---|---|
| Parasympathetic function testing | • Heart rate response to deep breathing (expiratory to inspiratory ratio)<br>• Heart rate response to Valsalva maneuver (Valsalva ratio)<br>• Heart rate response to standing (30:15 ratio) |
| Sympathetic adrenergic function | • Beat-to-beat blood pressure response to the Valsalva maneuver (the phase 2 blood pressure recovery, and phase 4 blood pressure overshoot)<br>• The systolic and diastolic blood pressure response to tilt table testing<br>• The systolic and diastolic blood pressure response to active standing |
| Sympathetic cholinergic function | • Quantitative sudomotor axon reflex testing (QSART)<br>• Sympathetic skin response (SSR)<br>• Thermoregulatory sweat testing (TST) |
| Gastrointestinal autonomic testing | • Gastric emptying scintigraphy<br>• Isotope base breath testing |
| Urologic autonomic testing | • Urodynamic studies<br>• Post-void residual |
| Pupillary autonomic function testing | • Pupillometry |

management of DAN is beyond the scope of this chapter, but readers are referred to several publications on the topic [41, 42, 55–58].

Symptoms of autonomic dysfunction that often require treatment in individuals with diabetes include orthostatic intolerance, gastrointestinal dysfunction, sudomotor abnormalities, and urologic and sexual dysfunction. It is important to establish realistic treatment goals with patients; expectations should target symptom improvement, but the disease is unlikely to be cured and long-term therapy may be required.

Orthostatic intolerance, including symptoms associated with orthostatic hypotension, is usually treated by a combination of pharmacologic and non-pharmacologic therapies. Non-pharmacologic therapies include education, compression stockings (which need to be considered cautiously in the setting of a diabetic peripheral neuropathy – they can cause pressure ulcers), adequate fluid and salt intake, and lifestyle modifications. Pharmacologic therapy may be required and could include a volume expanding agent (such as fludrocortisone) or a sympathomimetic agent (such as midodrine, droxidopa, or ephedrine). The combination of pharmacologic and non-pharmacologic therapy can improve symptoms of orthostatic intolerance in many patients. Unfortunately, other comorbid complications of diabetes such as renal failure, cardiac disease, and retinopathy may complicate treatment paradigms. Treatment of gastrointestinal disorders should address the particular symptom involved: gastroparesis, constipation, or diarrhea. Treatment of sexual and urogenital dysfunction often requires a combination of pharmacologic intervention and lifestyle modifications for peak effectiveness.

Patient education is critical for the management of DAN. Given sufficient education and motivation, many patients will become active partners in their treatment, which

will provide a feeling of empowerment and control over a disease that is often frustratingly resistant to treatment.

## Treatment-Induced Neuropathy of Diabetes (TIND)

TIND, also described as insulin neuritis, has been reported since 1933 [59]. Thought to be a rare disorder, this is a neuropathy precipitated by a rapid improvement in glycemic control in patients with chronic hyperglycemia. It has been described more recently as treatment-induced neuropathy because it is the rapid change in glycemic control that precipitates the problem, not the choice of agents used to control the glucose. It can occur with insulin, oral hypoglycemic medications, or even with diet control [60].

Classically, the neuropathy in TIND develops 2–3 weeks after a significant improvement in glycemic control, generally with a decrease in the hemoglobin A1C by 3% points or more within 3 months [61]. A study of all diabetic neuropathy patients over a 5-year period revealed that >10% of all cases of diabetic neuropathy were precipitated by an abrupt improvement in glycemic control, consistent with TIND [61]. The neuropathy is typically small fiber with burning and shooting paint that occurs in a length-dependent fashion. The greater the change in glycemic control, the larger the distribution of neuropathy, and the more severe the pain. In addition to the small fiber sensory neuropathy, individuals also develop an associated autonomic neuropathy that can cause orthostatic hypotension, gastroparesis, nausea, sexual dysfunction, and sudomotor dysfunction [60, 62].

In addition to the development of neuropathy, other microvascular manifestations are frequently noted. There is an acute worsening in retinopathy severity in the setting of TIND [62]. This was also reported in the Diabetes Control and Complications Trial (DCCT) as early worsening retinopathy [63]. There is also renal involvement that typically manifests as worsening microalbuminuria during the development of TIND [62]. These microvascular complications that develop in parallel in the setting of rapid glycemic control strongly implicate TIND as a diffuse microvascular process.

### Diagnosis

The diagnosis of TIND is often very challenging unless it is considered as part of the differential diagnosis. In individuals with TIND, there is a rapid improvement in glycemic control that occurs 2–8 weeks prior to the onset of symptoms [61]. The decrease in hemoglobin A1C will exceed 3 points in 3 months, but unless regular testing of the glycosylated hemoglobin A1C occurs, this is easily missed. The larger the drop in HbA1C, the more prominent the clinical manifestations of TIND, and the easier it is to identify. However, in cases with more subtle decreases to the hemoglobin A1C, the clinical manifestations may simply be the new onset of burning pain in the feet, which may typically be overlooked and presumed to be just the onset of a length-dependent diabetic neuropathy [61].

In cases of TIND, neurophysiologic testing (including nerve conduction studies) is often not helpful because TIND is frequently an isolated small fiber neuropathy without significant large nerve fiber involvement. Autonomic function testing can aid in defining any autonomic involvement, but is not required for diagnosis. A skin biopsy with analysis of intra-epidermal nerve fiber density can often be helpful in determining the extent and severity of small nerve fiber involvement, but is not necessary for making a clinical diagnosis [60].

### Management

One of the most significant aspects of treatment for individuals with TIND is the avoidance of major fluctuations in glycemic control. Individuals with TIND that relax their glycemic control may have some improvement in neuropathic pain which creates a significant negative feedback loop that results in sustained hyperglycemia [59, 64]. Unfortunately, many individuals are then at risk for future episodes of TIND, but frequently associated with more severe complications in subsequent iterations of TIND [65]. Therefore, individuals with TIND are counseled on maintaining a stable hemoglobin A1C and any future changes should not exceed a 2-point change in the A1C over a 3-month period of time.

Individuals with TIND often present with severe neuropathic pain [60]. The pain typically requires pharmacologic management as suggested in Figure 9.1. In many cases, polypharmacy is required to treat the pain. The symptoms of autonomic dysfunction less frequently require treatment, because they are typically overshadowed by the neuropathic pain. Tricyclic antidepressants, often successfully used for treatment of neuropathic pain, may worsen a resting tachycardia or exacerbate orthostatic hypotension, so should be used with caution.

## Diabetic Radiculoplexopathy

Historically referred to as "diabetic amyotrophy," the acute to subacute onset of lumbosacral or cervical radiculoplexus neuropathies can be a clinically dramatic presentation. Diabetic radiculoplexus neuropathies are typically seen in individuals over 50 years of age, and are more common in men. The radiculoplexus neuropathies are also more common in individuals with type 2 diabetes, and may present prior to a diagnosis of diabetes [66, 67].

The classic presentation for diabetic lumbosacral radiculoplexus neuropathy (abbreviated to the only slightly less cumbersome DLRPN) is severe unilateral pain in the thigh, hip, and back that extends to the entire leg, and may move to the contralateral limb over a period of months. The pain is typically severe, and will often bring the individual to medical attention. The pain is followed by muscle weakness in the distribution of pain. Examination findings will evolve over time from minor sensory change, to diffuse weakness, muscle atrophy, and loss of tendon reflexes. Symptoms may progress for up to 18 months, but will eventually stabilize. Most patients will experience improvement, but it is often incomplete [67–69]. Approximately one-third of cases present as a cervical radiculoplexus neuropathy rather than with lumbosacral involvement [67]. In some situations, the upper limbs may become involved after a lower limb has stabilized or is starting to improve. The most recent evidence from nerve and muscle biopsies suggests that DLRPN is a microvasculitis that causes ischemic injury to local nerves [67, 70].

### Diagnosis

The onset of severe pain followed by motor weakness in a limb of someone with diabetes should always raise a suspicion for DLRPN. Electrodiagnostic studies show multifocal involvement of nerves, roots, and nerve plexus with asymmetries in compound muscle action potential amplitudes. Imaging of the plexus by MRI may reveal nerve root enhancement and lumbar puncture will typically demonstrate an elevated protein with normal cell count [67].

### Management

Care is generally supportive, and consists of pain control and physical rehabilitation. Several studies have investigated the use of immunosuppressive therapies in the role of treating a potential microvasculitis. The use of both methylprednisolone and immunoglobulin have been attempted in clinical trials, and although promising have not achieved sufficient evidence for routine clinical use [71–73].

## Mononeuropathies

Individuals with diabetes have a higher prevalence of focal mononeuropathies than the general population [74, 75]. The most common mononeuropathies include carpal tunnel syndrome, ulnar neuropathy at the elbow in the upper extremity and common peroneal, deep peroneal and tibial neuropathies in the lower extremities.

### Diagnosis

A combination of clinical history, examination findings, and electrodiagnostic studies are used to determine the presence of a mononeuropathy. In some cases, an underlying peripheral neuropathy may complicate electrodiagnostic studies and create diagnostic confusion [75, 76]. Clinical judgment must guide management decisions.

### Management

Conservative management of upper extremity mononeuropathies follows the same standard guidelines as in non-diabetic cases. Splinting and avoidance of nerve injury is the primary focus. Nerve decompression in more advances cases or in patients that do not respond to conservative therapy can be considered [77–80]. For lower extremity nerve compression syndromes, the recommended guidance is less clear. Decompression of lower extremity mononeuropathies is not recommended, and there is no role for splinting [81]. Education about avoidance of pressure on the common peroneal nerve (i.e. avoidance of leg crossing) is appropriate.

## Cranial Neuropathies

Diabetes is one of the more common causes of cranial nerve palsies, and may cause a third, fourth, or sixth nerve palsy. The patient that presents with a third (oculomotor) nerve palsy will have a relatively acute onset of pain behind or around the eye, followed by weakness or paralysis of the oculomotor muscles, with ipsilateral ptosis. Those with fourth or sixth nerve palsies will have more isolated eye movement abnormalities. The pathophysiology for the third, fourth, and sixth diabetic cranial neuropathies is thought to be microvascular ischemia [7, 74, 82]. There are many case reports of facial (seventh) nerve palsy in diabetes, but no clear evidence to date that these are interrelated [83].

### Diagnosis

The diagnosis is made by history and examination.

**Management**

The natural history of the cranial neuropathies is for gradual improvement, in some cases with complete resolution. Care is generally supportive management of the diplopia with a focus on diabetes control.

## Screening for Neuropathy

According to the 2016 American Diabetes Association recommendations, all patients with type II diabetes should be assessed for diabetic peripheral neuropathy starting at the time of diagnosis and at least annually thereafter. Those individuals with type I diabetes they should be assessed 5 years after the time of diagnosis and at least annually thereafter. In general, screening for DSPN should include a careful history with a focus on sensory changes or weakness, a neurologic examination focusing on sensory, strength, and reflex testing. In patients with DSPN or other microvascular complications, symptoms and signs of autonomic neuropathy should be assessed as well. All patients should undergo testing with a 10-g monofilament to identify patients at risk for spontaneous ulceration and amputation.

## Summary of Management of Diabetic Neuropathy

The management of all neuropathic complications of diabetes reflects the general guidelines for diabetes control. A focus on glucose control is of critical importance, particularly in those individuals with type 1 diabetes [5]. Other risk factors for neuropathy prevention are important in type 1 diabetes and should be considered: lipid control, blood pressure management, and smoking cessation [5]. For those individuals with type 2 diabetes, glycemic control is also of value, but the associated risk factors may play a much larger role, so lipids, blood pressure, and tobacco use should all be monitored closely. Regular physical activity is an important, and often overlooked, aspect of diabetes care.

In the setting of neuropathic pain, treatment options should be considered to maximize pain relief and minimize side-effects. In any patient with neuropathy involving the feet and legs it is necessary to have a discussion about foot care safety and fall risks. Patients should examine their feet daily, and contact their physician if there is any sign of complication.

1) Tricyclic antidepressants: Those commonly used for neuropathic pain include amitriptyline, nortriptyline, and desipramine. Doses begin as low as 10 mg, and can be titrated to clinical effect (generally <200 mg). At doses ≥100 mg cardiac side-effects become more prominent. An ECG should be performed in anyone with a history of cardiac disease or over the age of 40 years. Anticholinergic effects are prominent and should be used cautiously in the elderly.
2) Serotonin norepinephrine re-uptake inhibitors: Doses of 20–60 mg/day for duloxetine and 37.5–225 mg/day for venlafaxine. Side-effects include nausea, somnolence, weight gain or loss, sexual side-effects, serotonin syndrome.

3) Alpha2-delta calcium channel ligands: Doses of 300–3600 mg/day for gabapentin and 50–600 mg/day for pregabalin. Side-effects include somnolence, dizziness, peripheral edema, weight gain, and dizziness.

When considering treatment for neuropathic pain, the general guidelines for evaluation are suggested. First, the exclusion of non-neuropathic causes of pain is recommended. Reduction of risk factors that may worsen pain is then considered. Treatments of neuropathic pain are then considered based on age, medical illness, and potential drug–drug interactions. The last few steps require consideration and modification based on the treatment response.

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# 10

# Diabetic Neuropathy – Research

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## Basic and Translational Research in Diabetic Neuropathy

### Animal Models of Diabetic Neuropathy

In mice and rats, guidelines were established by the Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes (EASD) in a conference sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the Juvenile Diabetes Research Foundation (JDRF) [1] in order to formulate a plan to characterize neuropathy in diabetic rodents, to minimize inter-investigator variability, to improve reproducibility both in animals and translation to human studies, and to maximize the impact of the studies. The discussion was divided into five areas: (i) status of commonly used rodent models of diabetes, (ii) nerve structure, (iii) electrophysiological assessments of nerve function, (iv) behavioral assessments of nerve function, and (v) the role of biomarkers in disease phenotyping [1]. A neuropathy phenotype in rodents was defined as the presence of statistically different values between diabetic and control animals in two of three assessments (nocifensive behavior, nerve conduction velocities, or nerve structure). The participants proposed that this framework would allow different research groups to compare and share data, with the emphasis being on data targeted toward the therapeutic efficacy of drug interventions. Guidelines to assess diabetic neuropathy have also been provided by the National Institutes of Health through the Diabetic Complications Consortium (DiaComp; www.diacomp.org), formerly the Animal Models of Diabetic Complications Consortium. DiaComp has helped to identify and characterize novel animal models of diabetic complications and provided a central resource for phenotyping protocols and established criteria to define and characterize diabetic complications as a means to minimize inter-investigator variability and maximize the impact of new studies.

The most useful animal models for diabetic neuropathy should ideally mimic the human condition in terms of etiology, metabolic changes and the specific characteristics of the neuropathy. However, even well-characterized diabetic animals are still only models of

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human diabetic neuropathy and, while they are essential in understanding the pathogenesis and potential treatment of human diabetic neuropathy, they cannot hope to exactly replicate data obtained in well-designed human studies. Once an animal model is selected, a comprehensive characterization of diabetes, the neuropathic phenotype, and the metabolic and physiologic profile are essential to completely understand the implications of the results on diabetic peripheral neuropathy pathogenesis. Here, we discuss important considerations that must be addressed when designing and selecting the appropriate mouse models for type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) diabetic neuropathy studies.

Many experimental models of diabetic neuropathy have been evaluated and it is beyond the scope of this review to evaluate all available animal models that are reviewed elsewhere [2]. In T1DM, the streptozotocin (STZ) diabetic rodent is most frequently used by investigators. Hyperglycemia from STZ administration is induced using one of two paradigms recommended by DiaComp: a single high dose (HD – approximately 150 mg/kg) or consecutive multiple low doses (LD – approximately 50 mg/kg/day for 5 days). The progressive β cell death that occurs with LD STZ is more representative of T1DM. The greatest problem with the STZ rodent is that the diabetes is uncontrolled and therefore the animals generally have a poor condition and high mortality from manipulations required in evaluation that is not seen in most humans with well-managed T1DM. The effect is also highly dependent on using very freshly prepared STZ, the strain and gender of the mouse, and even the mouse diet. Thus, even under ideal conditions, with LD STZ, there is a significant failure rate in inducing diabetes and a high post-injection mortality rate that is not observed with models of T2DM. Spontaneous T1DM mouse models, include the non-obese diabetic (NOD) and $B6Ins2^{Akita}$ (Akita) mice. NOD mice develop diabetes due to an inheritable polygenic immunodeficiency against pancreatic β cells [3]. The problem with the NOD mouse is that development of diabetes and neuropathy is unpredictable and not uniform. Thus, large numbers of mice are required to perform intervention studies. Furthermore, factors such as housing status and diet and gender can affect diabetes development, with female mice more likely to develop early and more severe T1DM symptoms [3]. Akita mice robustly develop diabetes at 7 weeks of age but development of neuropathy is more limited and unreliable [4, 5].

Numerous rodent models of T2DM are available and include the Zucker diabetic rat [6], the low capacity running (LCR) rat [7], the db/db and ob/ob mice (reviewed in [2]). However, the model that most closely resembles human impaired glucose tolerance (IGT) and early T2DM in humans and which is favored by investigators is the high fat diet (HFD) mouse. T2DM is the result of a combination of factors, including genetic background, a high fat, high carbohydrate Western diet, and sedentary lifestyle. These factors culminate in insulin resistance and often present as IGT and prediabetes before the onset of overt hyperglycemia and diabetes. Furthermore, T2DM is a component of the metabolic syndrome. Therefore, features of the metabolic syndrome, including obesity, cholesterol, high blood pressure, and triglycerides, may also contribute to diabetic neuropathy pathogenesis in T2DM. HFD-fed mice have a gradual onset of metabolic imbalances that are more characteristic of the human condition. HFD mice exhibit weight gain, increased adiposity, moderate hyperinsulinemia, and IGT but do not have elevated glucose levels [8, 9]. Diet alone is sufficient to induce neuropathy in C57BL/6 mice [9]. When fed a HFD consisting

of 58% kcal from fat, mice developed hallmarks associated with prediabetes after 16 weeks, including increased weight gain, IGT, and normal levels of fasting glucose. Neuropathy was also present, as evidenced by decreased motor and sensory nerve conduction velocities (NCVs) and impaired behavioral responses to mechanical and thermal stimuli. Since these initial observations, several studies have investigated the effect of a HFD on the development of neuropathy in mice. Collectively, these studies provide irrefutable evidence that increased dietary fat predisposes mice to nerve dysfunction in the absence of T2DM. Regarding sex-based differences, male mice are more predisposed to diet-induced obesity (DIO) and gain weight more consistently than female mice (www.jackson.org). Furthermore, male and female mice develop different levels of pain, making comparisons of mechanical and thermal thresholds difficult between studies [9–11]. The severity of nerve dysfunction may also be dependent on the duration of HFD feeding [8, 10]. Whereas a 54% HFD for 8 weeks promotes significant deficits in motor NCVs and tactile responses but no significant differences in sensory NCVs, thermal responses, or intraepidermal nerve fiber density (IENFD) [8], a 45% HFD for 34 weeks induces deficits in all measures of nerve dysfunction, except for mechanical responses [10]. Increasing the percentage of dietary fat content induces greater levels of weight gain and insulin resistance in mice and may shorten the time to induction of neuropathy. HFD chow typically ranges 32–60% kcal from fat. In humans, 60% fat would be extreme. The type of fat also influences the severity of diabetes [12, 13]. Mice readily develop obesity when placed on a HFD consisting primarily of saturated, obesogenic fat (e.g. lard, coconut oil), whereas mice on a diet of unsaturated fat (fish oil) do not gain as much weight [12, 13] and are more sensitive to insulin [14]. It is also important to keep in mind that diets composed of plant-derived ingredients may exhibit batch-to-batch variations reflective of changes in the growing season; thus, purified ingredients may offer improved consistency between studies for future cross-study data interpretation [15]. Finally, chows may also contain phytoestrogens that reduce the degree of weight gain, so it is important that these components and micronutrients are carefully matched to those in control chow for DIO studies [16]. However, despite these caveats, the HFD rodent provides a representative model of prediabetes and neuropathy.

## Basic Pathophysiology of Diabetic Neuropathy

### Changes in Redox Potential

Oxidative stress is one potential mechanism of injury to the peripheral nervous system (PNS) [6]. In experimental diabetes, levels of oxidative stress and reduced antioxidant defense parallel severity of neuropathy. In support, blocking oxidative stress in the diabetic animal restores normal blood flow and sciatic and saphenous NCVs. Increased metabolic mitochondrial flux due to high glucose results in increased formation of reactive oxygen species (ROS) including peroxinitrite, superoxide, and hydroxyl radicals, and deficits in mitochondrial respiratory function [17]. Generation of ROS increases mitochondrial permeability transition, opening of the adenine nucleotide translocase/voltage-dependent anion channel and resulting in mitochondrial swelling that disrupts the integrity of the outer membrane [18]. In addition, it leads to membrane lipid peroxidation and degradation of DNA, all of which are associated with neuronal or axonal injury [19]. Ultimately this leads to ballooned mitochondria with disrupted cristae [19] and loss of mitochondria [20].

In contrast, upregulation of pathways that block oxidative stress prevents axonal injury in diabetic neuropathy. For example, overexpression of manganese superoxide dismutase (SOD2), the enzyme responsible for mitochondrial detoxification of oxygen radicals, decreases superoxide in cultured dorsal root ganglion neurons and blocks cellular injury. Diabetic neuropathy was observed in the C57BL/6Jdb/db mouse after streptozotocin treatment, and decreased expression of SOD2 in these animals increased diabetic neuropathy [21].

The mitochondrion is a key site for generation of ROS. Mitochondrial stress is particularly important in T2DM but to a lesser extent is important in T1DM [20, 22–24]. The generation of ROS can be decreased by stabilizing the inner mitochondrial membrane potential and reducing generation of superoxide and other radicals or by increasing antioxidant defense. Increasing levels of uncoupling proteins such as UCP1 or UCP3 that prevent hyperpolarization of the inner mitochondrial membrane potential will reduce the formation of ROS and ameliorate neuronal injury [18]. An important marker of oxidative stress is a reduction in the glutathione system that protects diabetic neurons and Schwann cells from injury. Diabetic patients with distal symmetric polyneuropathy showed significantly lower values of reduced glutathione (GSH) and a reduced glutathione/oxidized glutathione (GSH/GSSG) ratio [25]. In patients with diabetic neuropathy, there is an increased consumption of glutathione in mononuclear cells from patients. In contrast, malondialdehyde and oxidized low-density lipoprotein (LDL) levels were unchanged in diabetic neuropathy. Another recent approach has been to manipulate nuclear factor erythroid 2-related factor 2 (Nrf2), which mediates expression of various antioxidant proteins via antioxidant response element (ARE) binding sites. Nrf2 appears to bridge the link between neuroinflammation and apoptotic pathways impacting progression of diabetic neuropathy [26]. Under normal conditions, Nrf2 is inactive and remains in the cytosol. Acute hyperglycemia increases the expression of Nrf2, but persistent hyperglycemia decreases Nrf2 expression. Downregulation of Nrf2 causes various microvascular changes that result in diabetic neuropathy. Targeting Nrf2 activators as potential therapies may provide insight into new therapies for diabetic neuropathy. For example, natural compounds such as curcumin, sulforaphane, resveratrol, and vitamin D can activate Nrf2 and, thus, promote antioxidant pathways to mitigate oxidative stress [27]. Another factor that can activate Nrf2 is taurine, found naturally in the body. Supplementing diabetic rats with taurine did not alter body weight and blood glucose concentration, but reduced the serum malondialdehyde concentration in diabetic rats and alleviated neuroinflammation by suppressing nuclear factor κB (NF-κB) expression and enhancing Nrf2 [28].

Fisetin, a phytoflavonoid that simultaneously targets NF-κB and Nrf2, shows a beneficial effect in experimental diabetic neuropathy [29]. Fisetin was administered at 5 and 10 mg/kg for 2 weeks in streptozotocin diabetic rats. Neuropathy, as determined by nerve conduction studies and sciatic nerve blood flow deficits, was ameliorated in treated rats. Importantly, fisetin reduced levels of interleukin-6 and tumor necrosis factor-α in sciatic nerves of diabetic rats ($p < 0.001$) suggesting that a combined approach to modifying oxidative stress and neuroinflammation may be a more effective approach. The mechanistic target of rapamycin (mTOR) is another promising agent for the development of novel regenerative strategies for the treatment of diabetes mellitus. mTOR and its related signaling pathways impact cellular metabolic homeostasis, insulin resistance, insulin secretion,

and programmed cell death with apoptosis and autophagy [30]. mTOR is the central element for the protein complexes mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) and is a critical component for a number of signaling pathways that involve phosphoinositide 3-kinase (PI 3-K), protein kinase B (Akt), AMP activated protein kinase (AMPK), silent mating type information regulator 2 homolog 1 (SIRT1), Wnt1 inducible signaling pathway protein 1 (WISP1), and insulin/insulin growth factor 1 [31]. mTOR, sirtuins, and insulin/insulin growth factor 1 signaling are longevity pathways involved in an array of different processes, including metabolism, and neuronal plasticity that are critical in preventing neuropathy and in axonal repair. As a result, mTOR represents an exciting target for the treatment of diabetic neuropathy.

Nitrosative stress can increase nerve injury in diabetes [32]. In humans, gastric bypass, in particular Roux-Y gastric bypass (RYGB) can improve neuropathic symptoms, independent from glycemic control. The procedure is associated with improved nitrosative injury [33]. In this study, the decrease in nitrotyrosine correlated with improvement in the neuropathy disability scale up to 12 months. The decrease in methylglyoxal after 6 months correlated with a decrease in the neuropathy disability scale after 12 months ($p = 0.003$). Peroxynitrite resulting from the reaction of superoxide with nitric oxide causes oxidative stress and leads to poly ADP-ribosylation, mitochondrial dysfunction, impaired stress signaling, and protein nitration. In diabetic mice treated with the peroxynitrite decomposition catalyst Fe(III) tetramesitylporphyrin octasulfonate (FeTMPS, 10 mg/kg/day) or protein nitration inhibitor (−)-epicatechin gallate (20 mg/kg/day) for 4 weeks, there was partial correction of neuropathy [34]. This supports the concept that peroxynitrite decomposition catalysts may be used in treatment of diabetic peripheral neuropathy.

#### Role of Mitochondrial Dysfunction in Diabetic Neuropathy

Diabetes-induced oxidative damage in neurons, axons, and Schwann cells has been proposed as a unifying mechanism for diabetic neuropathy. One mechanism for generation of oxidative stress is that an increased metabolic influx into mitochondria increases respiration and results in a high proton gradient, leading to increased production of ROS. The increase in generation of ROS results from mitochondrial dysfunction that is induced by an increase in mitochondrial inner membrane depolarization and degeneration of mitochondria in peripheral nerves [6, 20, 22, 35–37]. These impaired mitochondria may be rescued by regeneration of new mitochondria. Thus, activation of mitochondrial regeneration may be protective under conditions where significant mitochondrial degeneration is present.

Molecular methods allow manipulations of the mouse genome to overexpress or knockout expression of individual genes. The ease of genetic manipulation has provided important tools for study of the pathogenesis, prevention, and treatment of diabetic neuropathy. There are three key genes, which code for proteins that are transcriptional factors or activators of transcriptional factors and promote function and regeneration of mitochondria **(**Figure 10.1). These are: the $NAD^+$-dependent deacetylase SIRT1, peroxisome proliferator-activated receptor-gamma co-activator 1α (PGC-1α), and mitochondrial transcription factor A (TFAM). We tested if overexpression of the gene or knockout of the gene would protect or exacerbate diabetic neuropathy.

PGC-1α is a transcriptional co-activator and a master regulator for mitochondrial biogenesis in many tissues including the nervous system [38–40]. PGC-1α is a promising target for

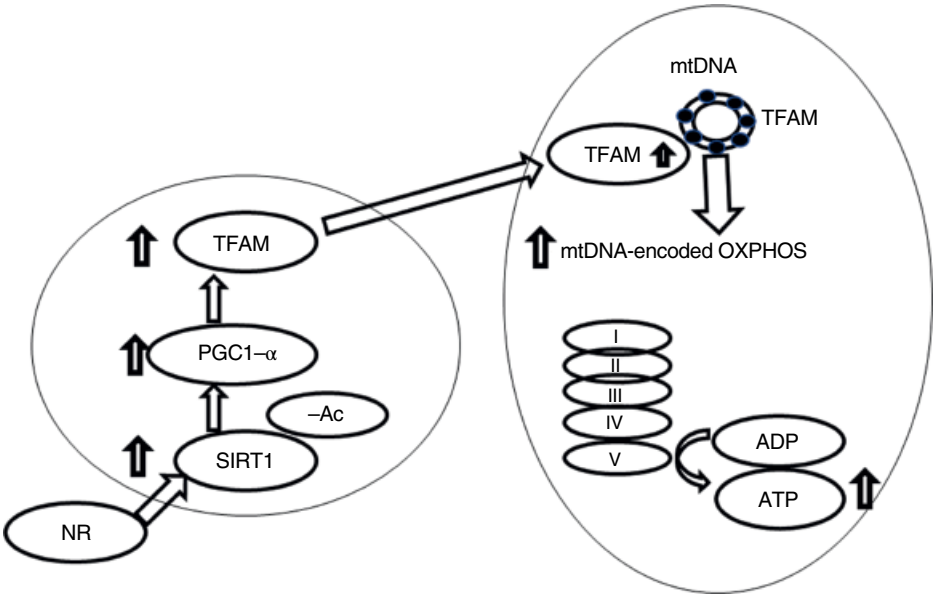


**Figure 10.1** The majority of mitochondrial proteins are synthesized in the nucleus and shuttled to the mitochondria. Key transcription factors such as silent mating type information regulator 2 homolog 1 (SIRT1), peroxisome proliferator-activated receptor-gamma co-activator 1α (PGC-1α), and mitochondrial transcription factor A (TFAM) are important in regulating mitochondrial function and preserving mitochondrial DNA in the peripheral nervous system (PNS) in response to increased metabolic loads provided by hyperglycemia or hyperlipidemia in diabetes. SIRT1 regulates PGC-1α activity via deacetylation, whereas PGC-1α regulates expression of TFAM via nuclear respiratory factor, NRF1 and 2. In this review, the importance of these factors in protecting against diabetic neuropathy is discussed.

therapy for neurological disease. For example, the pan-PPAR agonist, bezafibrate, upregulates PGC-1α and exerts beneficial effects in a transgenic mouse model of Huntington's disease [41]. PGC-1α activates transcriptional factors such as nuclear respiratory factor 1 (NRF1) and TFAM, which in turn induces mitochondrial respiration proteins and protects and replicates the mitochondrial genome [24, 39, 42, 43]. PGC-1α has been mapped to chromosome 4P15.1, a region associated with basal insulin levels in Pima Indians, who are identified as having a high risk of developing diabetes and diabetic-related complications [44]. Common polymorphisms of PGC-1α are associated with conversion from IGT to diabetes [45]. Thus, it is likely that PGC-1α and its downstream signaling intermediates are important in normal mitochondrial regulation in diabetic subjects [46]. We used PGC-1α knockout mice [PGC-1α (−/−)] to study the effect in diabetic neuropathy. Loss of PGC-1α, a protein that is essential for mitochondrial function and energy homeostasis, causes neuropathy that is worsened by diabetes [20]. This observation was supported by evidence from other groups showing that phosphorylation and expression of AMPK/PGC-1α and mitochondrial respiratory chain complex proteins are downregulated in dorsal root ganglia of both diabetic rats and mice [17, 22, 47]. Control of bioenergetics in the diabetic peripheral nerve is in part controlled by the SIRT1-AMPK and PGC-1α signaling axis in

which the upstream members (SIRT1-AMPK) sense the metabolic demands of cells, whereas the downstream member, PGC-1α, regulates mitochondrial biogenesis and function [17, 22, 23, 47, 48]. There was no difference in plasma glucose, hemoglobin A1C, or insulin levels between PGC-1α wild-type diabetic and PGC-1α knockout diabetic mice, indicating that exacerbation of peripheral nerve injury associated with reduced PGC-1α levels is independent of glycemic control. However, there was a significant increase in total cholesterol and triglyceride levels in non-diabetic PGC-1α knockout compared with PGC-1α wild-type mice and PGC-1α knockout diabetic compared with PGC-1α wild-type diabetic mice. These findings are consistent with recent observations that increased lipid levels are associated with neuropathy [49] possibly by ox-LDL interaction with the receptor LOX-1 [10, 50, 51]. Impaired fatty acid oxidation is strongly correlated to impaired activation of PGC-1α [52] and adipose-specific ablation of PGC-1α results in mitochondrial dysfunction [53]. Thus, mitochondrial PGC-1α has a critical role in lipid metabolism. Loss of PGC-1α may also worsen neuropathy due to impaired antioxidant defense related to impaired mitochondrial function. Several lines of evidence support this idea: PGC-1α was markedly reduced in diabetic dorsal root ganglion neurons; the mitochondria in neurons from PGC-1α non-diabetic mice contained abnormal vacuoles consistent with mitochondrial degeneration; there was a decrease in mitochondrial respiratory function; gene expression of glutathione peroxidase and SOD2 were reduced; protein levels of transcription factors that regulate mitochondrial transcription and are important for normal mitochondrial homeostasis (e.g. TFAM and NRF1) were significantly decreased in dorsal root ganglion neurons of both PGC-1α knockout non-diabetic and diabetic mice; levels of oxidized proteins consistent with increased oxidative stress were significantly increased; and overexpression of PGC-1α, using an adenoviral construct, prevented the generation of ROS in adult mouse dorsal root ganglion neuron cultures [20]. The role of PGC-1α in mitochondrial regulation is supported by: (i) data showing an inverse correlation between muscle PGC-1α levels and mitochondrial activity in humans with insulin resistance and diabetes [54, 55] and (ii) previous studies showing PGC-1α knockout mice have degenerating mitochondria in the central nervous system [56].

TFAM lies downstream of PGC-1α and has an important role in protecting the integrity of mitochondria. In the humanized TFAM overexpressing transgenic mouse model, levels of TFAM are increased only twofold in dorsal root ganglion neurons [24]. Overexpression of TFAM reversed experimental diabetic neuropathy. TFAM prevents slowing of nerve conduction velocity, reduces mechanical allodynia, and decreases the loss of intraepidermal nerve fibers. The transgenic mice expressing human TFAM protein increased total TFAM levels and mtDNA copy number to the same extent. This suggests that the mtDNA copy number per se does not affect TFAM gene expression. The proposed explanation is that the interaction between TFAM and mtDNA is dynamic and that the presence of one increases the stability of the other. This interaction is probably beneficial from a regulatory point of view because small changes in TFAM protein levels or mtDNA levels result in rapid adjustment to maintain a constant optimal ratio between TFAM and mtDNA [57]. This is supported by the findings that TFAM interacts with mtDNA to wrap mtDNA to form a nucleoid structure similar to histones in the nucleosome [58–60]. Since mitochondria can generate ROS due to their respiratory activity, perhaps TFAM functions in multiple roles to promote mtDNA transcription and replication, and wraps mtDNA to protect it

from attack by ROS [58–60]. The blood results showed that there was no difference in glucose or insulin levels between wild-type diabetic and TFAM transgenic diabetic mice. Although human TFAM is likely to be expressed in all tissues because the promoter is not tissue- or cell-specific, TFAM overexpression does not reduce the severity of diabetes. Thus, protection against peripheral nerve injury is localized to PNS mitochondria and is independent of glycemic control. The results showed that there is a net loss of both mtDNA and TFAM in chronic experimental diabetes. After acute diabetic exposure, there is an attempt to upregulate both mtDNA and TFAM, albeit the increase is not significant. It could be argued that acute exposure to hyperglycemia increases TFAM levels, mtDNA, and mitochondrial biogenesis to meet the high energy demand in neurons [61]. However, in chronic hyperglycemia with concurrent generation of ROS, the regulation might change from physiological to pathological and could eventually lead to a decline in mtDNA, TFAM, and mitochondrial function. MtDNA is particularly susceptible to oxidative injury due in part to the following factors: first, its location within mitochondria where the respiratory complexes I and III are potential sites for the generation of superoxide radicals and, second, the limited repair activity against DNA damage within mitochondria [62]. Under normal conditions, the toxic effects of ROS are prevented by such scavenging enzymes as superoxide dismutase, glutathione peroxidase, and catalase as well as by other non-enzymatic antioxidants. However, when the production of ROS becomes excessive, or if the levels of antioxidant enzymes decrease, then oxidative stress might have a harmful effect on the functional and structural integrity of biological tissue. Dorsal root ganglion neurons from TFAM transgenic mice are able to scavenge high glucose-induced ROS much more efficiently than those from wild-type mice. However, this scavenging ability did not appear to be due to an increase in the expression of the antioxidant enzymes SOD2 or glutathione peroxidase. Our results with mitochondrial respiration showed no significant increase in ADP-stimulated State 3 and resting State 4 respiration. All these findings suggest that the protective effect of TFAM overexpression is apparent only under chronic conditions of oxidative stress.

SIRT1 acts upstream to coordinate the SIRT1/PGC-1α/TFAM pathway to regulate mitochondrial oxidative energy metabolism and neuronal protection. Using an inducible neuron-specific SIRT1 overexpressing (SIRT1OE) C57BL6 mouse, SIRT1 is able to reverse diabetic neuropathy in HFD diabetic mice [63, 64]. Diabetes reduces $NAD^+$, SIRT1, and PGC-1α levels in dorsal root ganglion neurons. SIRT1 overexpression normalizes these levels. These findings are consistent with the concept that the SIRT1/PGC-1α/TFAM pathway can reverse neuropathy in a model of T2DM. SIRT1 requires $NAD^+$ as a cofactor to deacetylate histone, PGC-1α, and other transcriptional factors. Treatment with a precursor of $NAD^+$, nicotinamide riboside (NR) can reverse neuropathy in the HFD mouse, which is a model of T2DM [65–67]. Adult C57BL6 mice were fed a HFD (60% calories from fat) for 2 months until they developed neuropathy. Then, 150 mg/kg or 300 mg/kg NR was mixed with HFD and fed every day for 2 months [65, 66]. Neuropathy improved with treatment in both groups but more effectively with 300 mg/kg NR. There was no change in control diet animals. NR treatment decreased the HFD-induced increase in triglycerides and non-esterified fatty acids, and normalized the IGT test. In HFD mice, there was a decrease in the $NAD^+$ level, in SIRT1 activity, and in PGC-1α levels in dorsal root ganglion neurons. NR normalized these measurements.

### Protein Kinase C (PKC) and Neuropathy

Increased PKC and corresponding increases in diacylglycerol (DAG) levels are associated with hyperglycemia. Increased aldose reductase pathway activity or glycolytic pathway flux promotes de novo DAG synthesis by glycerol-3-phosphate following increased levels of intracellular glyceraldehyde-3-phosphate. Chronically elevated DAG then increases PKC activity. Activation of PKC isoforms (α, β, δ, ε, ξ) is reported in some tissues prone to diabetic complications. Hyperglycemia-induced oxidative stress may also mediate the adverse effects of PKC-β isoforms by the activation of the DAG-PKC pathway. Treatment with antioxidants may prevent glucose-induced cellular injury and inhibit DAG-PKC activation. Activation of PKC promotes vasoconstriction and ischemia, nitric oxide dysregulation, and increased leukocyte adhesion that further adds to the pathogenesis of diabetic neuropathy. The high-affinity PKC β inhibitor, ruboxistaurin mesylate, has been evaluated for treatment of human diabetic neuropathy in a randomized, Phase II, double-blind, placebo-controlled parallel-group trial. The trial compared 32 mg/day or 64 mg/day robuxistaurin with placebo for 1 year. The primary endpoint of the trial was changes in vibration detection threshold and the secondary endpoints were effects of robuxistaurin versus placebo on the Neuropathy Total Symptom Score-6 (NTSS-6), and other clinical and electrodiagnostic measures of neuropathy. In this study, the primary and secondary efficacy measures did not differ between treatment groups. However, a subgroup of patients with less severe diabetic neuropathy showed a trend toward improvement in the primary and secondary efficacy measures [68].

### Advanced Glycation End Products

Increased tissue glucose induces the generation of advanced glycation end products (AGEs) in peripheral nervous tissue. Tissue glucose is metabolized to 3-deoxyglucosone (3-DG), methylglyoxal, and $N^{\varepsilon}$-(carboxymethyl) lysine. Binding of these AGE ligands to the receptor for advanced glycation end products (RAGE) can result in diabetic complications. AGE and RAGE accumulation in nerve perineurial, epineurial, and endoneurial microvessels of patients with IGT results in increased expression of the transcription factor NF-κB [69, 70]. AGE–RAGE interactions further lead to the generation of ROS and the activation of PKC, transforming growth factor β (TGF-β) [71], mitogen-activated protein kinase (MAPK), and transcription factors such as NF-κB [72].

RAGE is thought to be one of the potential contributors to the neurotoxicity [73]. It has been shown that RAGE activation leads to an increase in proinflammatory molecules, oxidative stressors, and cytokines. The proinflammatory effect of RAGE is mediated by its binding to ligands such as AGE, S100/calgranulin, and amphoterin. Activation of these ligands leads to subsequent activation of downstream pathways such as NF-κB, signal transducer and activator of transcription (STAT), and c-Jun N-terminal kinase (JNK). An important target for treatment of AGE-induced diabetic nerve injury is the glyoxalase system. The glyoxalase system is a highly specific enzyme system existing in all mammalian cells that is responsible for the detoxification of dicarbonyl species, primarily methylglyoxal. It has been implicated as playing an essential role in preventing the increased formation of AGEs. When glyoxalase 1 was knocked out in cells, formation of methylglyoxal and associated protein modifications were blocked [74] suggesting that glyoxalase 1 could be an important target for treatment in diabetic neuropathy [75].

### Modifying Molecular Chaperones

Individual molecular chaperones and chaperone complexes are expressed under normal and disease states. Molecular chaperones such as heat shock protein 90 (Hsp90) and Hsp70 assist in folding nascent polypeptides into their final biologically active conformations and in the refolding of aggregated and denatured proteins. As a result, the chaperones assist in directing proteins toward degradation via the proteasome or by autophagy [76, 77]. Pharmacologic modulation of the molecular chaperones can improve insulin resistance [78] and peripheral neuropathy [79]. Hsp90 modulators such as dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride (DMAG), KU-32, and KU-596 show promise in treating diabetic complications in animal models and may afford a novel and effective disease-modifying approach for humans [80].

### Inflammation and Neuropathy

Inflammation is an emerging pathogenic mechanism of diabetes and its complications. The NF-κB pathway is part of the central machinery initiating and propagating inflammatory responses. The present study envisaged the involvement of the NF-κB inflammatory cascade in the pathophysiology of diabetic neuropathy using BAY 11-7082, an IκB phosphorylation inhibitor [81]. STZ was used to induce diabetes in Sprague–Dawley rats. BAY 11-7082 (1 and 3 mg/kg) was administered to diabetic rats for 14 days starting from the end of 6 weeks post diabetic induction. Diabetic rats developed deficits in nerve functions and altered nociceptive parameters and also showed elevated expression of NF-κB (p65), IκB and p-IκB along with increased levels of IL-6 and TNF-α and inducible enzymes (COX-2 and iNOS). Furthermore, there was an increase in oxidative stress and decrease in Nrf2/HO-1 expression. We observed that BAY 11-7082 alleviated abnormal sensory responses and deficits in nerve functions. BAY 11-7082 also ameliorated the increase in expression of NF-κB, IκB, and p-IκB. BAY 11-7082 curbed the levels of IL-6, TNF-α, COX-2, and iNOS in the sciatic nerve. Lowering of lipid peroxidation and improvement in GSH levels were also seen along with increased expression of Nrf2/HO-1. Thus, it can be concluded that NF-κB expression and downstream expression of proinflammatory mediators are prominent features of nerve damage leading to inflammation and oxidative stress, and BAY 11-7082 was able to ameliorate experimental diabetic neuropathy by modulating neuroinflammation and improving antioxidant defense.

### TGF-β and Neuropathy

Regulation of TGF-β1 has been associated with diabetic nephropathy and retinopathy, predominantly by increasing generation of the extra cellular matrix. However there is evidence that TGF-β is upregulated in STZ diabetic rats with neuropathy. In dorsal root ganglion and sciatic nerve from diabetic animals TGF- β 1 and TGF- β 2 are increased and are associated with increased neuronal death [71]. In diabetic dorsal root ganglion neurons using quantitative real-time PCR (QRT-PCR), TGF-β1, and TGF-β2 mRNA, but not TGF-β3, were increased at the 4- and 12-week time point [71]. In sciatic nerve TGF-β3 mRNA was primarily increased. In diabetic rat sciatic nerve, TGF-β formed homo- and hetero-dimers, of which $\beta_2/\beta_3$, $\beta_1/\beta_1$, and $\beta_1/\beta_3$ were significantly increased, while the TGF-$\beta_2/\beta_2$ homodimer was decreased. In dorsal root ganglion neurons, pretreatment with TGF-β neutralizing antibody prevented the increase in total TGF-β protein observed with high glucose. In dorsal root ganglion neurons, exposed to high glucose, TGF-β2 > β1 increased the

percentage of cleaved caspase-3 compared with high glucose alone and TGF-β neutralizing antibody inhibited the increase. TGF-β isoforms applied directly to dorsal root ganglion neurons reduced neurite outgrowth and this effect was partially reversed by TGF-β neutralizing antibody. These findings implicated upregulation of TGF-β in experimental diabetic peripheral neuropathy and suggested a potential new target for treatment of diabetic peripheral neuropathy.

Patients with T2DM and clinically detectable serum TGF-β1 showed positive correlation with NCVs suggesting that this cytokine might be used as a biomarker for diabetic peripheral neuropathy [82]. TGF-β increases Smad3 signaling thereby affecting metabolism and energy homeostasis and reducing inflammation and ROS production [83].

#### Metabotropic Glutamate Receptors (mGluRs) and Oxidative Injury

mGluRs are a subfamily of glutamate receptors that are G-protein-coupled and linked to second messenger systems [84]. Glutamate is the principal neurotransmitter in the CNS that is transported to the axon terminals [85]. The machinery for production, paracrine release, and recycling of glutamate occurs in sensory ganglia and includes the enzymes amidohydrolase, glutaminase [86, 87], glutamate aspartate transporter (GLAST), glutamate transporter 1 (GLT1) [88], as well as the recycling enzyme glutamine synthetase [86, 89].

The glutamate carboxypeptidase II (GCP II) inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) is protective against glucose-induced programmed cell death and neurite degeneration in dorsal root ganglion neurons in a cell culture model of diabetic neuropathy [84], likely by activating mGluR2/3. Preclinical data indicate that GCPII inhibitors ameliorate diabetic neuropathy in animal models. Direct or indirect activation of mGluR2/3 in animal models protects against development of diabetic neuropathy [90]. mGluR2/3 agonists prevent glucose-induced neuronal injury in dorsal root ganglion neuronal cultures only in the presence of Schwann/satellite glial cells, by increasing glutathione and maintaining mitochondrial function [84, 91, 92]. N-methyl-D-aspartate (NMDA) receptor antagonists are not as protective as mGluR2/3 agonists, suggesting that ionotropic pathways are not involved in this pathway [84, 91, 92]. Together these findings are consistent with the concept that mGluRs may protect against cellular injury by regulating oxidative stress in models of diabetic neuropathy **(**Figure 10.2).

#### Growth Factors and Diabetes

In animal models of diabetes, levels of several growth factors may be reduced, and treatment with growth factors may improve neuropathy. Reduced levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor-I (IGF-I) occur in models of diabetes. Administration of these factors protects against diabetic neuropathy in animals (reviewed in [19]). NGF primarily supports the survival of small-fiber sensory neurons associated with pain and temperature sensation. NGF, substance P, and calcitonin gene-related peptide gene expression are upregulated in the dorsal root ganglia of diabetic mice when they develop mechanical allodynia, which suggests that inhibition of NGF action may be a strategy for treating painful diabetic neuropathy [94].

BDNF may improve glycemic control, and at least in animal models may reduce the severity of diabetic neuropathy [19]. BDNF enhances neurite outgrowth, supports dorsal root ganglion neuronal survival, is upregulated in injured peripheral nerve, and promotes

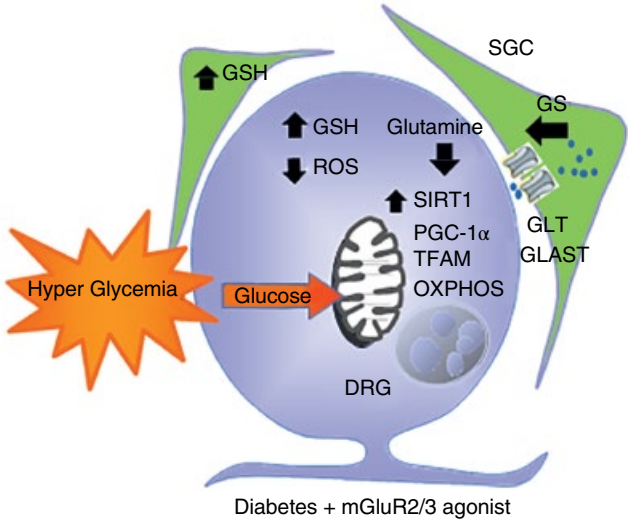


**Figure 10.2** Glutamate transporter 1 (GLT1) and glutamate aspartate transporter (GLAST) are present in satellite glial cells (SGCs) to transport extracellular glutamate into the SGC where glutamine synthetase (GS) converts it to glutamine, which is eventually recycled to the neuron for conversion into glutamate. In diabetes, hyperglycemia-induced oxidative stress affects glutamate transport proteins, increasing extracellular glutamate. LY379268 treatment promotes glutamate uptake and likely decreases extracellular glutamate. In addition, mGluR 2/3 receptors present in dorsal root ganglion neurons decrease extracellular glutamate. Thus, activation of mGluR2/3 receptor with an agonist is likely to sensory transmission and importantly nociceptive transmission [93].

axonal regeneration. Systemic administration of BDNF also decreases non-fasted blood glucose in obese diabetic C57BLKS-Leprdb/leprdb (db/db) mice and this effect can persist for weeks after cessation of BDNF treatment [95].

IGF-I activity is reduced in experimental models of diabetes. Sensory neurons and supporting Schwann cells from diabetic rodents express lowered amounts of IGF-I and IGF-I receptor and are susceptible to glucose-induced injury [96]. In sciatic nerve from diabetic animals serum IGF-I and IGF-I mRNA are decreased. Changes in IGF-I are coupled with evidence that administration of IGF-I reduces dorsal root ganglion neuronal injury, ameliorates neuropathy in diabetic rats, and promotes nerve regeneration (reviewed in [19]). In man, IGF-I and IGF-I receptor levels are decreased in diabetic patients with neuropathy, compared with those diabetics without neuropathy [19]. Taken together these changes indicate that IGF-I plays a critical role in the development of diabetic neuropathy. However, promising results with growth factors in experimental diabetic neuropathy have not translated to human clinical studies [19, 95].

## Clinical Research in Diabetic Neuropathy

There are currently no disease-modifying treatments that have been definitively shown, in randomized clinical trials, to reduce or reverse diabetic sensory polyneuropathy. However, it is important to identify individuals with impaired glucose regulation and neuropathy

because aggressive diabetic control and lifestyle interventions can delay the onset of diabetes and may reverse small-fiber neuropathy associated with early diabetes mellitus. Unfortunately, trials in diabetic neuropathy have been hampered by insensitive and poorly reproducible outcome measures. Although both large- and small-fiber neuropathy occur in T2DM, in subjects with IGT, a small-fiber neuropathy is more common [97–106]. Importantly, developing appropriate endpoints has been a problem in diabetic neuropathy because many of the endpoints have proved too insensitive in clinical trials. The US Food and Drug Administration held a public workshop on "Clinical Development Programs for Disease-Modifying Agents for Peripheral Neuropathy" in February 2013 that included determining outcome measures that could be used to assess the efficacy of disease-modifying agents. There was agreement that the IENFD was a sensitive outcome measure and that there was a need to establish content validity in clinical scales [107]. IENFD shows relatively rapid improvement with exercise interventions in diabetic neuropathy and is a sensitive and reliable measure in measuring change in early diabetic neuropathy [97–106]. Furthermore, IENFD directly correlates with increasing diabetic neuropathy severity, and is safe and easy to perform [108, 109]. The IENFD currently represents the gold standard in measuring change in small-fiber neuropathy. Clinical scales and their relative value in diabetic neuropathy have been assessed and compared [106]. Another endpoint that has been recently evaluated and shows promise is the sweat gland innervation density [110, 111].

#### Treatment of Hypertension

Improvement in hypertension has been frequently observed to parallel improvement in neuropathy, although the exact reason is uncertain. Thiazide diuretics aggravate abnormal glucose metabolism in both diabetic and non-diabetic patients probably because of decreased sensitivity of pancreatic β-cells to glucose [112]. Thus, in patients with hypertension thiazide diuretics should be stopped and an alternative medication considered. Suitable choices include an angiotensin-converting enzyme inhibitor (ACEI) or an angiotensin-receptor blocker that may reduce the risk of diabetes [113] or severity of diabetic neuropathy [114]. In preclinical studies, there is also clear evidence that an ACEI combined with an endopeptidase inhibitor is most effective in reducing the severity of experimental diabetic neuropathy [115]. Recently, the ACEI quinapril has been shown to improve parasympathetic dysfunction in diabetic cardiac autonomic neuropathy [116]. Sixty-three consecutive patients with both T1DM and T2DM were randomized to quinapril 20 mg/day or placebo for 2 years. In the treatment group, the expiration/inspiration ratio and mean circular resultant increased ($p < 0.05$) but other measures of autonomic neuropathy did not change. In the placebo group, all cardiac autonomic indices deteriorated, except the Valsalva ratio, which did not change. Thus improvement of hypertension may parallel improvement in neuropathy. However ACEIs may also affect oxidative and nitrosative stress pathways as well as improving vascular reactivity [117]

#### Improved Glycemic Control

The Diabetes Control and Complications Trial (DCCT) established clear links among impaired glycemic control, neuropathy, and retinopathy. This study prospectively followed 1441 insulin-dependent participants with T1DM for a mean of 6.5 years to assess the effect of intensive insulin therapy on the development of diabetic complications (reviewed in [95]).

Patients were divided into a primary-prevention and a secondary-intervention group, and treated with intensive or conventional insulin therapy. In the secondary-intervention cohort, intensive insulin therapy reduced the appearance of clinical neuropathy by 60% over a 5-year follow-up. The results for patients who had neither retinopathy nor significant albuminuria at the start of the study (primary-prevention cohort) were even more impressive. In this group, intensive therapy reduced the appearance of neuropathy by 69% compared with only 10% with conventional therapy, indicating that early optimal glycemic control can prevent the development of neuropathy prior to developing retinopathy and microvascular injury. Furthermore, it is clear from these data that any increase in glucose above normal is associated with an increased risk of end organ injury, including neuropathy.

Although studies show clear improvement in outcomes for diabetic and somatic neuropathy for T1DM, the data for T2DM are less clear [118]. Despite these reservations, improvement in T2DM was observed in the UK Prospective Diabetes Study (UKPDS) study. In this study 3867 patients with newly diagnosed T2DM were randomly assigned to intensive therapy with a sulfonylurea (chlorpropamide, glibenclamide, or glipizide), with insulin, or conventional diet therapy. After 10 years there was an aggregate 25% risk reduction ($p = 0.0099$) in microvascular endpoints [119]. Thus, intensive blood-glucose control by either sulfonylureas or insulin substantially decreased the risk of microvascular complications in patients with T2DM, however there was a higher rate of hypoglycemic complications, particularly in the insulin treatment group [119]. In the Steno type 2 randomized study, improved glycemic control in T2DM was associated with a lower rate of progression of autonomic neuropathy [120].

Importantly, improved glycemic control has been shown to have a sustained benefit on diabetes and its complications. The NeuroEDIC study found a reduced prevalence of neuropathy in a group of type 1 diabetics that received intensive treatment compared with the standard treatment group during the DCCT study. However, although intensive glycemic control reduced the severity of neuropathy, it was not able to arrest damage to peripheral nerves. Thus, 34% of subjects in the former intensive treatment group and 41% of those in the former conventional treatment group developed clinical neuropathy [121]. The NeuroEDIC (neuropathy assessment during Epidemiology of Diabetes Interventions and Complications) study provided more conclusive data for cardiac autonomic neuropathy than it did for peripheral neuropathy. After adjusting for the effects of age, it was found that the prevalence and incidence of cardiac autonomic neuropathy was significantly lower in the subjects formerly receiving intensive treatment compared with the former conventional treatment group [122]. A possible explanation for the greater effects of glycemic control on cardiac autonomic neuropathy versus somatic diabetic neuropathy may be that there is a difference in the effect of metabolic memory on the small nerve fibers that are measured by cardiac autonomic tests and the large-fiber function that is measured by traditional nerve conduction studies.

#### Diet and Lifestyle Interventions

Glucose modifying drug therapy is typically not appropriate in patients with impaired glucose regulation due to cost and potential for serious side-effects such as hypoglycemia. A more suitable approach for these patients would be a lifestyle intervention that could

arrest the underlying process that leads to neuropathy and its associated functional disability. At present there is no evidence from a randomized study that a lifestyle intervention would reverse somatic neuropathy. However, there is evidence that a lifestyle intervention can be more effective than a drug intervention in preventing conversion from IGT to diabetes. The Diabetes Prevention Program (DPP) study was designed to determine if a more intensive dietary and exercise intervention or metformin treatment was effective in preventing or delaying the onset of T2DM in people with IGT or impaired fasting glycemia. The DPP demonstrated that lifestyle changes reduced the risk of developing T2DM by 58% in adults with impaired fasting glycemia or IGT who were at high risk of developing diabetes [123]. Metformin also reduced the risk of developing T2DM, but was less effective than weight loss and increased physical activity [123]. To prevent one case of diabetes in 3 years, only 6.9 persons would have to participate in the lifestyle-intervention program, whereas 13.9 would have to receive metformin [123]. This clearly demonstrated that the lifestyle intervention was nearly twice as effective as drug therapy.

The long-term safety and tolerability of metformin has been examined during the open-label extension of the DPP [124]. No significant safety issues were identified and metformin was well tolerated with gastrointestinal symptoms, which were initially more common in the metformin group compared with placebo, declining over time. Throughout the unblinded follow-up, weight loss remained significantly greater in the metformin group compared with placebo (2.0 vs 0.2%, $p = 0.001$) and this was related to the degree of adherence [124]. Furthermore, adherence to placebo did not affect weight loss and participants in the metformin group with low adherence lost weight. This suggests that metformin, rather than adherence to positive health behaviors, was the relevant factor. Examining the 10-year cost effectiveness of the interventions used in the DPP, lifestyle was found to be cost-effective and metformin was marginally cost-saving compared with placebo [125].

Lifestyle changes in patients with IGT, who are at the earliest definable stages of hyperglycemia, may also be effective in preventing diabetes-associated complications such as peripheral neuropathy. The Impaired Glucose Tolerance Causes Neuropathy (IGTN) study was a natural history study that enrolled participants with IGT or impaired fasting glycemia and mild neuropathy and gave them general dietary and physical activity advice with goals that were similar to those used in the DPP "lifestyle intervention" group. In the IGTN study, participants who lost weight and/or increased their physical activity with a concomitant improvement in metabolic control demonstrated a reduced progression of neuropathy based on the IENFD obtained from a 3-mm skin punch biopsy and were able to actually regrow their epidermal nerve fibers [126, 127]. More recent studies have underscored the importance of intervention during the earliest stages of impaired glucose regulation and that a lifestyle intervention can be more effective than a drug intervention in preventing conversion from IGT to diabetes [123]. Furthermore, studies have shown that an exercise intervention can prevent the development of neuropathy in subjects who have T2DM but do not have neuropathy [97, 101, 128], may affect pain and symptom outcomes in subjects with neuropathy, and is safe [99, 100]. However, randomized controlled studies to test if a lifestyle intervention can reverse or slow the progression of diabetic neuropathy are required.

**Alpha-Lipoic Acid**

Alpha-lipoic acid has a disease-modifying effect and in several studies has been shown to improve symptoms in diabetic neuropathy. However, the evidence to support its use is not strong because clinical trials with alpha-lipoic acid have been completed using a variety of study designs, routes of administration, and sample sizes [129–132]. In the multicenter randomized double-blind placebo-controlled ALADIN III (Alpha-Lipoic Acid in Diabetic Neuropathy III) trial, there was a small but significant improvement in the Neuropathy Impairment Score (NIS) in alpha-lipoic acid-treated patients, but no significant improvement in the Total Symptom Score (TSS) [130]. In the Deutsche Kardiale Autonome Neuropathie (DEKAN) Study, there were small improvements in the cardiac autonomic spectral analysis in alpha-lipoic acid-treated patients [133]. In the SYDNEY2 trial, 181 diabetic patients received once-daily oral doses of 600 mg (n = 45) (ALA600), 1200 mg (n = 47) (ALA1200), and 1800 mg (ALA1800) of ALA (n = 46) or placebo (n = 43) for 5 weeks after a 1-week placebo run-in period [131]. The primary outcome measure was the change from baseline of the TSS. Secondary endpoints included the Neuropathy Symptoms and Change (NSC) score and the NIS. The mean TSS decreased by 51% in ALA600, 48% in ALA1200, and 52% in ALA1800 compared with 32% in the placebo group ($p < 0.05$ vs placebo). Significant improvements favoring all three alpha-lipoic acid groups were also noted in the NSC score, and the NIS was numerically reduced. Thus, oral treatment with alpha-lipoic acid for 5 weeks improved neuropathic symptoms and deficits in patients with diabetic sensorimotor polyneuropathy. Based on this study, 600 mg alpha-lipoic acid once daily appears to provide the optimum risk-to-benefit ratio [131]. Further support for alpha-lipoic acid therapy is provided by a meta-analysis of four trials (ALADIN I, ALADIN III, SYDNEY, NATHAN II) comprising n = 1258 patients (alpha-lipoic acid n = 716; placebo n = 542) with the foot TSS as the primary outcome measure and the NIS for the lower extremity (NIS-LL) as a secondary outcome measure [134]. The NIS for the lower extremity (NIS-LL) was a secondary outcome measure. Finally, in the NATHAN I study [132], 460 diabetic patients with mild-to-moderate diabetic sensorimotor polyneuropathy were randomly assigned to oral treatment with 600 mg alpha-lipoic acid once daily (n = 233) or placebo (n = 227) for 4 years. The primary endpoint was a composite score of the NIS-LL and seven neurophysiologic tests. Secondary outcome measures included NIS, NIS-LL, nerve conduction, and quantitative sensory tests (QSTs). Change in primary endpoint from baseline to 4 years showed no significant difference between treatment groups ($p = 0.105$). However, when measured independently, change from baseline was significantly better with alpha-lipoic acid than placebo for NIS ($p = 0.028$), NIS-LL ($p = 0.05$), and the NIS-LL muscular weakness subscore ($p = 0.045$). More patients showed a clinically meaningful improvement and fewer showed progression of NIS ($p = 0.013$) and NIS-LL ($p = 0.025$) with alpha-lipoic acid than with placebo. Part of the reason that there was failure of the primary study endpoint to detect a difference was that nerve conduction and QST results did not significantly worsen in the placebo group. Global assessment of treatment tolerability did not differ between the groups.

The overall results of the alpha-lipoic acid studies indicate that chronic treatment with alpha-lipoic acid at an optimal dose of at least 600 mg/day is safe and improves some neuropathic deficits in patients with diabetic polyneuropathy. However, limitations in

endpoint measures have precluded a definitive conclusion as to whether alpha-lipoic acid reverses or prevents diabetic polyneuropathy. In a post-hoc analysis of the NATHAN 1 trial, improvement and prevention of progression of NIS-LL with alpha-lipoic acid vs. placebo after 4 years was predicted by higher age, lower BMI, male sex, normal blood pressure, history of cardiovascular disease (CVD), insulin treatment, longer duration of diabetes and neuropathy, and higher neuropathy stage. Participants treated with alpha-lipoic acid who received ACEI showed a better outcome in heart rate during deep breathing (HRDB) after 4 years [135]. Thus, better outcome in neuropathic impairments with alpha-lipoic acid was predicted by normal BMI and blood pressure and more severe diabetes and neuropathy. Improvement in cardiac autonomic function was predicted by ACEI treatment.

#### Benfotiamine and Neuropathy

Thiamine plays an essential role in energy metabolism. Benfotiamine (S-benzoylthiamine O-monophoshate) is a synthetic S-acyl derivative of thiamine. Once absorbed, benfotiamine is dephosphorylated to lipid-soluble S-benzoylthiamine. Benfotiamine administration increases the levels of intracellular thiamine diphosphate, a cofactor necessary for the activation of, resulting in the reduction of tissue level of AGEs. The anti-AGE effect of benfotiamine makes it effective for the treatment of diabetic neuropathy [136].

Another effect of benfotiamine is to reduce accumulation of triosephosphates in diabetes. Excess triosephosphates can be removed via the reductive pentosephosphate pathway. However, this pathway is impaired in diabetes by mild thiamine deficiency. The expression and activity of the thiamine-dependent enzyme transketolase in the pentosephosphate pathway is consequently decreased. Correction of thiamine deficiency in experimental diabetes by high-dose therapy with thiamine and the thiamine monophosphate prodrug, benfotiamine, restores disposal of triosephosphates by the reductive pentosephosphate pathway in hyperglycemia. This prevents activation of multiple biochemical pathways that cause injury in diabetes: PKC, hexosamine, glycation, and oxidative stress pathways [137]. High-dose thiamine also corrects dyslipidemia in experimental diabetes – normalizing cholesterol and triglycerides. IGT is observed with thiamine deficiency and thus dietary thiamine may prevent T2DM.

Benfotiamine has been assessed as a clinical therapy in a double-blind, placebo-controlled, phase-III study [138]. A total of 165 patients with diabetic polyneuropathy were randomized to one of three treatment groups: benfotiamine 600 mg per day, benfotiamine 300 mg per day, or placebo. After 6 weeks of treatment, the primary outcome parameter, the Neuropathy Symptom Score differed significantly between the treatment groups in the per-protocol but not in the intention to treat population ($p = 0.055$). The TSS showed no significant differences after 6 weeks of treatment. The improvement was more pronounced at the higher benfotiamine dose and increased with treatment duration. In the TSS, "pain" showed the best response to treatment. Treatment was well tolerated in all groups.

#### Treatment of Neuropathic Pain

Almost one-third of patients being managed for painful diabetic neuropathy in a tertiary care setting achieve meaningful improvements in pain and function in the long term [139]. Strong clinical research evidence supports the use of pregabalin, duloxetine, amitriptyline,

gabapentin, venlafaxine, opioids, and topical capsaicin for the treatment of diabetic neuropathic pain [140]. Pregabalin significantly improves pain irrespective of the length of time since onset of neuropathic pain [141]. Sodium valproate may show a weak neuropathic pain effect but is not recommended for use in women of childbearing potential, and patients need to be monitored for hepatotoxicity and thrombocytopenia [142]. Topical capsaicin acts on the transient receptor potential channel 1 (TRPV1) receptor and lacks the systemic side-effects of other neuropathic pain medications. In high doses, it may help some patients [143] but it may cause loss of intraepidermal nerve fibers. In general, there is considerable interest in this class of receptors as therapeutic targets. The TRP on nociceptive neurons are sensitive to temperature: heat-sensitive TRPV1, warm-activated TRPV3, cold-responsive TRPA1, and cool-activated TRPM8 channels [144]. Site-specific resiniferatoxin (an ultra-potent capsaicin analogue) is being evaluated as a "molecular scalpel" to achieve permanent pain relief. In the past few years a number of potent, small-molecule TRPV1, TRPV3, and TRPA1 antagonists have been advanced into clinical trials for the treatment of neuropathic pain. Early TRPV1 antagonists caused hyperthermia and impaired noxious heat sensation, placing patients at risk for scalding injuries. Another local agent is lidocaine. However, there is only class III evidence supporting the use of lidocaine in diabetic neuropathic pain [140]. For many other diabetic neuropathic pain medications there is insufficient evidence of efficacy. These include: carbamazepine, oxycodone, oxcarbazepine, carisbamate, topical clonidine, zonisamide, lacosamide, and lamotrigine [140, 145–152].

Ongoing research in the field includes studies with PL37. PL37 is the first orally administered dual inhibitor of enkephalinases (DENKI). It is active at the level of peripheral nociceptors, increasing the concentrations of enkephalins at the site of the painful stimulus, thus reducing degradation by the peptidases neutral endopeptidase and aminopeptidase N. The effect may be as potent as those of morphine, but without the side-effects [153]. Other medications such as botulinum neurotoxins (BOTOX) have been tested in diabetic neuropathy. BOTOX (most of the literature is with onabotulinumtoxin A) is probably effective (level B) in painful diabetic neuropathy [154]. Recent studies with tanezumab, a humanized monoclonal antibody against NGF, have shown some effect at high dosage in neuropathic pain [155]. Patients received subcutaneous tanezumab 20 mg or placebo on day 1 and week 8. The primary endpoint was change from baseline in average diabetic polyneuropathy pain. The mean diabetic polyneuropathy pain reduction from baseline to week 8 was greater with tanezumab vs placebo ($p = 0.009$). Neuropathy assessments showed no meaningful changes.

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# 11

# Diabetic Foot – Clinical

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## Introduction and Size of the Problem

Diabetic foot is the most frequently recognized complication of diabetes, with a prevalence of active foot ulcers of 1.7 identified at screening among persons with diabetes [1], and an annual incidence rate in the global population of persons with diabetes of 6.3% [2].

Recent data from the International Diabetes Federation estimate that, annually, foot ulcers develop in 9.1–26.1 million people with diabetes worldwide. The lifetime incidence of foot ulcers, previously estimated to be 15–25% among persons with diabetes [3], is now considered to be between 19 and 34% of the diabetic population [4]. The risk of death at 5 years for a patient with a diabetic foot ulcer is 2.5 times higher than the risk for a patient with diabetes without a foot ulcer [5]; at 10 years it is two times higher [6].

More than half of diabetic foot ulcers become infected [7], and about 20% of moderate or severe foot infections lead to some level of amputation [8]; mortality after diabetes-related amputation exceeds 70% at 5 years [9].

Furthermore, diabetic foot ulcers and associated infections are a powerful risk factor for visits to the emergency department, and admissions to the hospital [10]; these rates exceed the rates for congestive heart hospital admissions among diabetic patients [11].

Similarly, the direct costs of treating diabetic foot complications exceed the treatment costs for many common cancers [12], justifying the definition of "diabetic cancer" used when referring to the diabetic foot. In the United States a total of US$176 billion is spent annually on direct costs for diabetes care; as much as one-third of this expenditure is lower extremity-related [13].

These data are sobering, and they underline from many points of view how the diabetic foot is the most serious complication of diabetes.

*Microvascular Disease in Diabetes*, First Edition. Edited by Francesco Tecilazich.

## Pathogenesis of Diabetic Foot Complications

Diabetic foot ulcer is an outcome of a complicated amalgam of various risk factors such as peripheral neuropathy, peripheral vascular disease (PVD), foot deformities, arterial insufficiency, trauma, and impaired resistance to infection [14]. Diabetic foot lesions frequently result from a patient having simultaneously two or more risk factors, with diabetic neuropathy playing a central role.

### Neuropathy

Peripheral neuropathy in diabetes is one of the major causes of foot ulcers [15]. Neuropathy is a disease affecting the nerves causing impairment in sensations, movement, and other health-related aspects depending upon the nerve affected. Up to 66% of patients with diabetes present with peripheral neuropathy in the lower extremity [16].

Neuropathy in diabetic patients is manifested in motor, autonomic, and sensory divisions of the nervous system.

In motor neuropathy damage to the motor nerves alters the ability of the body to coordinate movements, and induces the formation of foot deformities, hammerhead toes, and claw foot. Motor neuropathy triggers atrophy in foot muscles leading to alterations in the foot anatomy and causing osteomyelitis.

Sensory neuropathy causes wreckage of the sensory nerves present in the extremities. Recurrent foot injuries are outcomes of sensory neuropathy causing disruption in skin integrity and providing a viable route for microbial invasion leading to unhealed wounds, which in severe stages form chronic ulcers. Loss of protective sensation leads to ulcers caused by ill-fitting shoes, exposure to heat, and hurt due to foreign agents [17].

Autonomic neuropathy leads to deportation in functions of sweat and sebaceous glands in the foot, which in turn leads to dry skin and predisposition to fissures. As a result, the natural moisturizing ability of the foot is lost, and the overlying skin becomes more vulnerable to breaks and development of infection [18].

Loss of sensation, foot deformities, and limited joint mobility can result in biomechanical loading of the foot itself, producing high pressure in some areas, to which the body responds with thickened skin (calluses). This leads to a further increase of the abnormal loading, often with subcutaneous hemorrhage and, eventually, ulceration (Figure 11.1).

### Peripheral Vascular Disease

PVD is an atherosclerotic occlusive disease of the lower extremities, and being diabetic is an important risk factor for PVD [19]. PVD is an important cause leading to the development of foot ulcers in about 50% of cases; it accounts for 70% of deaths in type 2 diabetes [20]. Diabetic patients have a higher incidence of atherosclerosis, thickening of

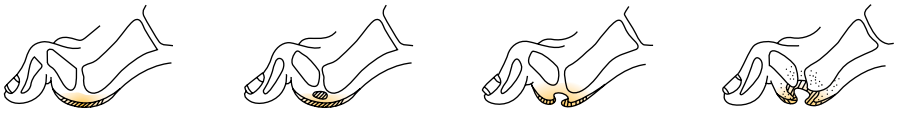

**Figure 11.1** Illustration of ulcer due to repetitive stress.

basement membranes of the capillaries, hardening of the arteriolar walls, and endothelial proliferation. Atherosclerotic blockages of large and medium-sized arteries such as the femur-popliteal and aorta-iliac vessels lead to acute or chronic ischemia. In combination with digital artery disease, ulcers can develop and quickly progress to gangrene due to inadequate blood flow.

Diabetics have scanty arterial blood supply and therefore peripheral ischemia is a cause of ulceration in about 35% of cases. Improper supply of blood to the peripheries leads to poor wound healing, which worsens the situation [21].

Decreased arterial perfusion causes abatement of peripheral pulses and the patient risks ulceration and infection with impaired healing rates, finally leading to a chronic state involving gangrene and amputation. PVD is not considered as an independent risk factor – it combines with neuropathy to become a leading cause of non-traumatic amputations [22].

The majority of foot ulcers are neuroischemic – caused by combined neuropathy and ischemia. In these patients symptoms may be absent because of the co-presence of neuropathy, despite severe pedal ischemia, leading to a late diagnosis and to a late referral to doctors, increasing the probability of an amputation.

### Other Risk Factors

Several contributory factors are associated with diabetic foot ulcers. Studies have testified that a history of ulceration or amputation, foot pressure, peripheral edema, poor socioeconomic backgrounds, plantar callus formation, ischemia, nephropathy, retinopathy, poor glucose control, old age, and prolonged diabetes are important predisposing factors causing diabetic foot ulcers. Insufficient health care and education are also reported to be an important risk factor for foot ulcers [23].

## Prevention

To prevent foot ulcerations among patients with diabetes, identification of those at risk of a foot ulcer is essential. Foot examination is an easy method to achieve this goal, and should specifically include screening for peripheral neuropathy and research for signs and symptoms of peripheral artery disease (PAD) [24], such as:

- absence of foot pulses (tibial posterior artery and/or dorsal pedal artery);
- ankle-brachial index <0.9;
- foot deformity or bony prominences;
- limitation of joint mobility;
- presence of callus or other signs of abnormal pressure;
- poor foot hygiene;
- inappropriate footwear or barefoot walking;
- previous ulcer and/or amputation;
- loss of protective sensation (impaired pressure perception according to the Semmes–Weinstein monofilament test and/or impaired vibration perception according to the 128 Hz tuning fork test).

**Table 11.1** Risk classification system.

| Category | Characteristics | Screening frequency |
|---|---|---|
| 0 | No peripheral neuropathy | Once a year |
| 1 | Peripheral neuropathy | Once every 6 months |
| 2 | Peripheral neuropathy with peripheral artery disease and/or a foot deformity | Once every 3–6 months |
| 3 | Peripheral neuropathy and a history of foot ulcer or lower extremity amputation | Once every 1–3 months |

The International Working Group on Diabetic Foot (IWGDF) has recently implemented a risk classification system that should guide subsequent preventive management. In detail, four categories have been identified, as shown in Table 11.1.

According to this table, at-risk patients require more frequent foot screening than patients who are not at risk. The aim of more frequent screening is early identification of factors that can increase the chances of developing a foot ulcer (as reported above), followed by providing appropriate preventive foot care.

Pre-ulcerative signs on the foot, such as callus, blisters, or hemorrhage, appear to be strong predictors of future ulceration [25], and require immediate treatment by a foot care professional.

The effectiveness of treating these pre-ulcerative signs on the prevention of a foot ulcer has been indirectly investigated, focusing on the importance of callus removal to reduce plantar pressure, an important risk factor for ulceration [26].

At-risk patients should also be instructed not to walk barefoot, in socks, or in thin-soled standard slippers [27], to daily inspect their feet and the inside of their shoes, to daily wash their feet, to wear properly fitting footwear, and, when a foot deformity or a pre-ulcerative sign is present, to wear therapeutic shoes, custom-made insoles, or toe orthosis [28–30].

## Treatment

When prevention strategies fail, foot ulcers occur. This is always a dramatic event, as the majority of non-traumatic amputations on diabetic patients are preceded by the development of an ulcer (over 85%). The cornerstones of diabetic foot therapy are illustrated in later chapters, but as a very brief summary we might say that neuropathic ulcers need offloading while vascular ulcers need revascularization. Many studies around the world have shown that setting up a multidisciplinary foot care team is associated with a drop in the number of diabetes-related lower extremity amputations (up to 85% according to various data).

## Treatment of Neuropathic Diabetic Foot Ulcers: Offloading

As there are no current treatments available to completely ameliorate the effects of neuropathy, the present central tenet in treatment and prevention of plantar diabetic foot ulcers focuses on the redistribution of pressure [31], commonly referred to as “offloading.”

There is a plethora of offloading modalities available (total contact casts, removable or non-removable knee-high devices, forefoot offloading shoes, therapeutic footwear and so on), but recently the IWGDF clarified the ones supported by significant evidence.

To heal a neuropathic plantar forefoot ulcer without uncontrolled infection and ischemia in a patient with diabetes, it is strongly recommended to use a non-removable knee-high device with an appropriate foot–device interface; this in fact leads to a significantly higher proportion of healed ulcers when compared with removable offloading devices (including footwear and walkers) [32, 33]. Notably, there is evidence to suggest that a removable walker rendered irremovable is as effective as a total contact cast in healing these kinds of ulcers [34].

When a non-removable knee-high device is contraindicated or not tolerated by the patient, a removable knee-high walker with an appropriate foot-device interface should be considered, but only when the patient can be expected to be adherent to wearing the device [35, 36].

If even a knee-high device is contraindicated or cannot be tolerated by the patient, the use of a forefoot offloading shoe, cast shoe, or custom-made temporary shoe should be considered, but also in this case only when we expect the patient to be adherent [37].

There is no evidence available on how to treat non-plantar neuropathic foot ulcers, even though such lesions also require relief from mechanical stress. Depending on the type and location of the ulcer, the use of shoe modification, temporary footwear, toe spacers, or orthoses should be considered.

When conservative treatment fails, and the patient is at very high risk for ulcer progression and amputation, a surgical offloading intervention should be considered.

Achilles tendon lengthening, joint arthroplasty, single or pan-metatarsal head resection or osteotomy, or digital flexor tenotomy are the choices, but the quality of evidence in the literature is low, as these surgical procedures can be applied only to selected patients and have possible serious complications such as infections of surgical sites, gait problems, occurrence of acute Charcot neuroarthropathy, and transfer ulcers [38, 39], so it is not clear if the benefits outweigh the potential harm.

Finally, crutches, wheelchairs, and bedrest are also very effective in promoting healing of neuropathic diabetic foot ulcers, even if the vast majority of patients for whom these devices are prescribed do not have the upper-body strength, endurance, or willpower to use these devices.

## Treatment of PAD: Revascularization

PAD, defined as any atherosclerotic arterial occlusive below the level of the inguinal ligament resulting in a reduction in blood flow to the lower extremity, is the major cause of lower leg amputation in persons with diabetes [40].

Diabetes is a risk factor for PAD, and a prevalence rate of 10–40% in the general population of patients with diabetes has been reported; around 50% of patients with a diabetic foot ulcer have co-existing PAD [7].

Identifying PAD among patients with foot ulcerations is important, because its presence is associated with worse outcomes, such as slower (or lack of) healing of foot ulcers, lower

extremity amputations, consequent cardiovascular events, and premature mortality [7, 41]. However, the identification of PAD may not be easy, as diabetic foot ulcer patients frequently lack typical symptoms of disease, such as claudication or rest pain even in the presence of severe tissue loss [42, 43], and arterial calcification, foot infection, edema, and peripheral neuropathy may adversely affect the performance of diagnostic tests for PAD.

Although a properly performed medical history and clinical examination can suggest the presence of PAD in a patient with a foot ulcer, their sensitivity is too low to rule out PAD, as palpable pulses may be present despite the presence of significant ischemia. Furthermore, an Ankle Brachial Index (ABI) $< 0.9$, usually a useful test to detect PAD in non-diabetic patients, has a low diagnostic utility in diabetic patients with medial wall calcification (Monckeberg sclerosis) of the arteries in the lower leg, which affects this test by inducing false negative results (ABI $> 1.3$). In contrast, detection of a triphasic pedal arterial waveform with a handheld Doppler appears to provide stronger evidence for the absence of PAD. The most useful test to predict wound healing in diabetic patients with PAD is TcpO2 $> 25$ mmHg.

The aim of revascularization is to restore direct flow to at least one of the arteries of the foot, preferably the artery that supplies the anatomical region of the wound; this procedure leads to a limb salvage rate of 80–85% (versus 50% in non-revascularized diabetic patients) and to an ulcer healing rate at 1 year of >60% [44, 45].

PAD in patients with diabetes has a number of characteristics that render it more difficult to treat. The atherosclerotic lesions are multilevel and particularly severe in tibial arteries, with a high prevalence of long occlusions [46]. The predilection for multiple crural vessel involvement combined with extensive arterial calcification increases the technical challenges associated with revascularization using either an open bypass or endovascular techniques.

Currently, there is poor evidence to establish which revascularization technique is superior, since the major outcomes – healing, amputation, and complications – are similar between the two techniques [44]. The results of both open and endovascular procedures greatly depend upon the local availability and expertise in a given center, as well as the morphological distribution of PAD [47]. Open and endovascular revascularizations are increasingly combined, and an individual choice should be made for each patient; however, these patients should be managed in centers where both approaches are available.

The perioperative mortality rates of revascularization procedures in patients with diabetes and an ischemic foot ulcer are <5%, but major systemic in-hospital complications (cardiovascular and renal disease, especially) have been observed in about 10% of patients in both open and endovascular series, probably reflecting the poor general health of these patients. The outcomes in patients with diabetes and end-stage renal disease are worse, with a 5% preoperative mortality and 1-year mortality of about 40% [44].

As a consequence, revascularization procedures should be avoided in those patients with an unfavorable risk–benefit ratio, such as patients who are severely frail, have a short life expectancy, have poor functional status, are bedbound, or those with a large-sized tissue necrosis that renders the foot functionally unsalvageable.

## Diabetic Foot Infections (DFIs)

### Introduction

Foot infection is the most frequent diabetic complication requiring hospitalization and the most common precipitating event leading to lower extremity amputation [48, 49]. Furthermore, the development of a foot infection in a diabetic patient is associated with substantial morbidity, reduced physical and mental quality of life [50], need for health care provider visits, wound care, antimicrobial therapy, and frequently surgical procedures.

DFI can involve soft tissues (skin, fascia, tendons, muscle), joints, and bones, and usually begins with a break in the skin leading to an open wound that becomes colonized and then infected. While diabetic neuropathy is the primary cause of foot wounds, the presence of PAD increases the risk of a wound becoming infected [51].

### Diagnosis

DFI must be diagnosed clinically, based on the presence of local and systemic signs and symptoms of inflammation. This represents a challenge for clinicians, as the presence of biohumoral and clinical markers of severity of infection (such as feverishness, chills, leukocytosis, raised erythrocyte sedimentation rate (ESR), and raised C-reactive protein (CRP) levels) may not be present in a diabetic patient due to a deficient immune system response.

Wound cultures should be used to determine the causative organisms and their antibiotic sensitivities, but not for diagnosis, because all skin wounds harbor microorganisms, and their mere presence (even if they are virulent species) cannot be taken as evidence of infection.

To assess the severity of any DFI, the Infectious Disease Society of America classification scheme, reported in Table 11.2, is strongly recommended.

Diabetic foot osteomyelitis (DFO) is the most frequent DFI, being found in up to 60% of patients hospitalized for a foot infection [52], and develops by contiguous spread from overlying soft tissues, penetration through the cortical bone, and into the medullary cavity.

A definite diagnosis of DFO is a challenge for clinicians too, requiring the presence of both histological findings and isolation of bacteria from an aseptically obtained bone sample [53].

A probable diagnosis of bone infection is reasonable if there are positive results on a combination of diagnostic tests such as probing to bone [54], serum inflammatory markers (ESR and CRP) [55], plain X-ray (cortical bone interruption or fracture), magnetic resonance imaging (MRI) [56], or radionuclide scanning [57].

Any bacteria can be potentially involved in DFI [58, 59]. An acute infection of a superficial wound in a previously untreated patient is usually caused by aerobic gram-positive cocci, while deep or chronic wounds often harbor polymicrobial flora, including aerobic gram-negative and obligate anaerobic bacteria. Recent and prolonged antibiotic therapy can predispose to unusual or antibiotic-resistant pathogens. Infections requiring hospitalization are often polymicrobial and may include various types of aerobes and anaerobes [60].

**Table 11.2** The diabetic foot infection (DFI) classification by the Infectious Disease Society of America.

| | |
|---|---|
| No systemic or local symptoms or signs of infection | **Category 1**<br>**Absence of infection** |
| Presence of two or more of the following signs/symptoms (after exclusion of other causes of inflammatory response of the skin):<br>• Local swelling<br>• Erythema between 0.5 and 2 cm around the wound<br>• Local pain or tenderness<br>• Local warmth<br>• Presence of pus | **Category 2**<br>**Mild infection** |
| Infection involving structures deeper than skin and subcutaneous tissues (bone, joint, muscle) or erythema extending more than 2 cm from the wound margin | **Category 3**<br>**Moderate infection** |
| Presence of SIRS (systemic inflammatory response syndrome) | **Category 4**<br>**Severe infection** |

Bacteria most often reported as causing DFI include most aerobic gram-positive cocci (*Staphylococcus aureus* and other staphylococci, streptococci) and gram-negative rods (Enterobacteriaceae, *Pseudomonas aeruginosa*) and common obligate anaerobes (Bacteroides).

## Treatment

Surgery is the cornerstone of DFI treatment. It should be a priority in acute life-threatening infections such as necrotizing fasciitis, deep abscess, gas or wet gangrene, and compartment syndrome, as in these cases early intervention may be associated with better outcomes [61].

The aim of urgent surgical treatment is to drain any deep pus and to minimize tissue necrosis by decompressing foot compartments and to removing dead and infected tissue, according to the anatomical structure of the foot (Figure 11.2).

Operative treatment of a DFI should be carried out by surgeons with thorough knowledge of the anatomy of the foot and the ways in which infection spreads through its fascial planes [63].

Bone resection and amputation is often necessary when there is extensive soft tissue necrosis or to provide a more functional foot for those patients who have undergone previous surgery or minor amputations with residual biomechanical consequences that can potentially result in an unstable foot.

Antimicrobial therapy is another cornerstone of DFI treatment, as required by virtually all clinically infected diabetic foot wounds. The choice of the specific antibiotic agent has to be based on the isolated causative bacteria, their susceptibilities, the clinical severity of the infection (according to the classification shown above), and, last but not least, the financial cost. The selection of the initial antibiotic regimen is usually empirical; the antibiotic should be selected to cover the most likely infecting organisms, and subsequently modified according to the severity of infection, and the clinical or microbiological information.

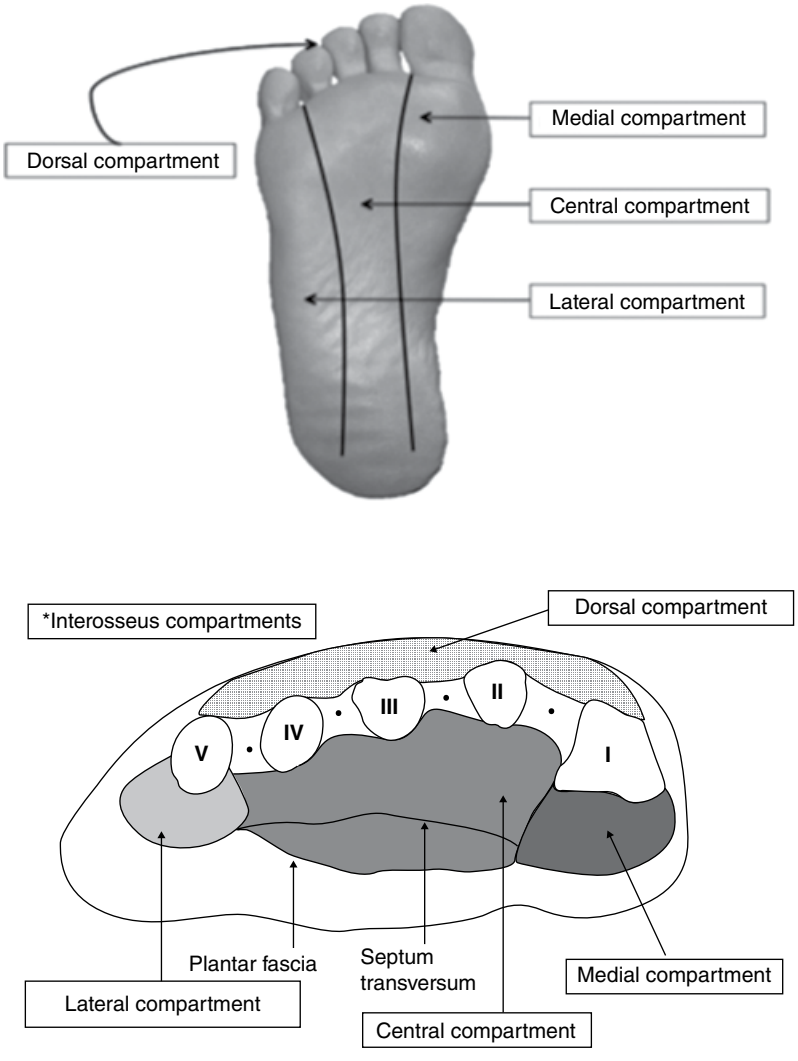


**Figure 11.2** Foot compartments [62].

Antimicrobial agents that have demonstrated clinical effectiveness in published prospective studies are:

- *cephalosporins* (cephalexin orally; cefoxitin, ceftizoxime, ceftibiprole, ceftaroline parenterally);
- *penicillin/beta-lactamase inhibitor combinations* (amoxicillin/clavulanate orally; ampicillin/sulbactam, piperacillin/tazobactam, and ticarcillin/clavulanate parenterally);
- *carbapenems* (imipenem/cilastatin and ertapenem, parenterally);
- *fluoroquinolones* (ciprofloxacin, levofloxacin, and moxifloxacin, orally or parenterally);
- *other agents*: clindamycin (orally and parenterally); linezolid (orally and parenterally); daptomycin (parenterally); tigecycline (parenterally); vancomycin (parenterally).

DFO can be successfully treated both with infected bone resection and/or with medical therapy alone. There are no current published data that provide information on which is the best choice; a medical treatment should be preferred in those patients who are unstable for surgery, in whom the infection is confined to small forefoot lesions; conversely, surgical treatment should be reserved for infections associated with bone necrosis or exposed joint, in patients at high risk for antibiotic-related problems, or when the infecting pathogen is resistant to commercially available antibiotics; however, there is still no current consensus [64].

## Wound Healing

Wound healing is a complex process involving highly regulated responses and specified cell types [65], and can be enhanced by the appropriate choice of a topical regime. Foot ulcers are more frequently destined to heal if clinicians base their treatment on the following principles. However, even optimum wound care cannot be effective if the cornerstones of ulcer treatment are missing.

As outlined before:

1) a neuropathic ulcer needs relief of pressure and protection (offloading);
2) an ischemic ulcer needs restoration of skin perfusion (revascularization);
3) an infected ulcer requires antibiotics or surgical treatments.

If these statements are satisfied, a proper local wound treatment forms an important component of the management of the diabetic foot.

### Debridement

Wound debridement is a central tenet for healing any ulcer; when systematically performed, it transforms a chronic into an acute ulcer [66], which represents the first healing step of an ulcer. Debridement consists of the removal of dead, damaged, or infected tissue, which improves the healing potential of the remaining healthy tissues. Different techniques are recognized: [67]

- *Surgical or sharp debridement*: recommended for necrotic and infected wounds. This is the most effective and fastest debridement technique.
- *Autolytic debridement*: a selective process in which the necrotic tissue is liquefied and digested.
- *Mechanical debridement*: involves the removal of unhealthy tissue using a dressing that when changed leads to scrubbing of the wound, to the removal of exudates and of dead tissues, and to bleeding.
- *Enzymatic debridement*: a technique that ensures the removal of dead tissue by topical enzymes; it is recommended for sloughy, infected, necrotic wounds where surgical debridement is contraindicated.
- *Maggot debridement:* a technique in which maggots or fly larva, raised in a sterile environment, are used; the larvae feed on the necrotic tissue and on the bacteria present at the wound site, and secrete antimicrobial enzymes, which stimulate the progression of wound healing.

### Dressings

Wound care plays a pivotal role in the management of diabetic foot ulcers, promoting a moist wound healing environment [68]. The ideal wound dressing should be sterile, easy to use, cost effective, maintain a moist wound healing environment, absorb excess exudate, not contaminate the wound with foreign particles, protect the wound from microorganisms, allow gaseous exchange, control wound odor, and provide thermal insulation and mechanical protection [69].

There are numerous topical regimens and devices available, and the choice of the best one should be based on the type of wound tissue, its complexity, and properties [70].

Currently there is either insufficient or no evidence to justify the use of any of the preparations, and to prefer it to any of the others. In the absence of any specific indication, clinicians should use the dressing with the lowest financial cost, but which supports moist wound healing while controlling any exudate.

### Topical Negative Pressure Wound Therapy (NPWT)

This technique has an important role in modulating the wound healing environment, and its use in diabetic foot wounds is encouraging. NPWT applies continuous or intermittent negative pressure to the wound via a substance that fills the wound; therewith, NPWT has been shown to be effective in extracting wound exudate, in reducing the frequency of dressing changes, in stimulating granulation tissue formation [67], and in reducing the ulcer's dimensions. Moreover, this technique may increase tissue perfusion by inducing neoangiogenesis of small vessels, and may also encourage offloading by rendering deambulation difficult. The main clinical application of NPWT is on post-surgical wounds, where there is a documented benefit in both the time to healing, and in the proportion of ulcers healed [71].

### Other Topical Treatments

Currently, there is no evidence for the use of growth factors, bioengineered skin, electricity, magnetism, ultrasound and shockwave devices, or herbal therapies to improve wound healing in diabetic foot ulcers, while the use of hyperbaric oxygen therapy (HBO) should be considered as a complement in reducing major leg amputation numbers, even though further evidence is required, above all to identify the population most likely to benefit from its use.

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# 12

# Diabetic Foot – Research

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## Introduction

The "small vessel disease" concept previously associated with peripheral artery disease in diabetes has been disproved with the publication of studies clearly showing the presence of arteriolar occlusive disease in the limbs of both non-diabetic and diabetic individuals [1, 2]. A number of studies published in the following years have also dispelled the concept of "small vessel disease" and additionally demonstrated the diabetic peripheral artery disease can be treated with surgical or endovascular revascularization techniques [1–4]. Moreover, in physiologic studies where the vasodilator drug papaverine was administrated into femoropopliteal bypass grafts, the vessels of diabetic patients exhibited similar vascular reactivity with the vessels of non-diabetic patients [3]. These findings, together with more than 40 years of clinical experience in successful arterial reconstruction in diabetic patients, have not only refuted the "small vessel disease" notion, but also led investigators to further research and establish the underlying changes in diabetic microcirculation [4].

According to recent work, even though occlusive disease of the microcirculation is not present in diabetic patients, their microcirculation and especially the arterioles and capillaries are compromised. Impairment in diabetic microvascular function is characterized by elevated vascular permeability and dysfunctional autoregulation of blood flow and vascular tone. Hyperglycemia and insulin resistance are believed to cause metabolic dysregulation and lay the foundation for microvascular dysfunction. These metabolic changes generate structural and functional remodeling at the capillary and arteriolar level.

*Microvascular Disease in Diabetes*, First Edition. Edited by Francesco Tecilazich.

## Anatomy of the Skin and Microcirculation

The skin consists of two main layers: a superficial layer of stratified epithelium, the epidermis, and the dermis, an underlying layer of mostly connective tissue that makes up the bulk of the skin. The epidermis is rich in keratin and has no blood supply, with nutrition being provided by the papillary portion of the dermis. The dermis is composed of an upper papillary layer and a lower reticular layer characterized by the abundance of extracellular matrix proteins, namely collagens, fibronectin, and elastin. It accommodates an extensive microvascular network that supplies tissues with nutrients and removes waste products.

Skin microcirculation comprises the thermoregulatory arteriovenous shunt flow and the nutritive capillary blood flow and is arranged in two horizontal plexuses, an upper plexus located immediately below the papillary dermis, and the lower cutaneous plexus deeper in the dermis. The entire skin vasculature is variable and dependent on the anatomical site. In a healthy foot, approximately 80% of the total blood flow circulates through the arteriovenous shunts while the remaining 20% passes through the nutritive capillary bed [5]. These nutritive capillaries are arranged into functional units, the capillary loops, with every dermal papilla being supplied by up to three capillary loops [6]. Because the transport of metabolites and nutrients from the blood flow to the tissues occurs at the capillary level, the integrity of the microcirculation correlates with whole-skin homeostasis (Figure 12.1).

## Structural Changes in the Microcirculation

With regard to structure, the most significant changes that impact the microcirculation in diabetes are a decrease in the capillary size and the thickening of the capillary basement membrane [7, 8]. There is, however, no difference in skin capillary density between healthy

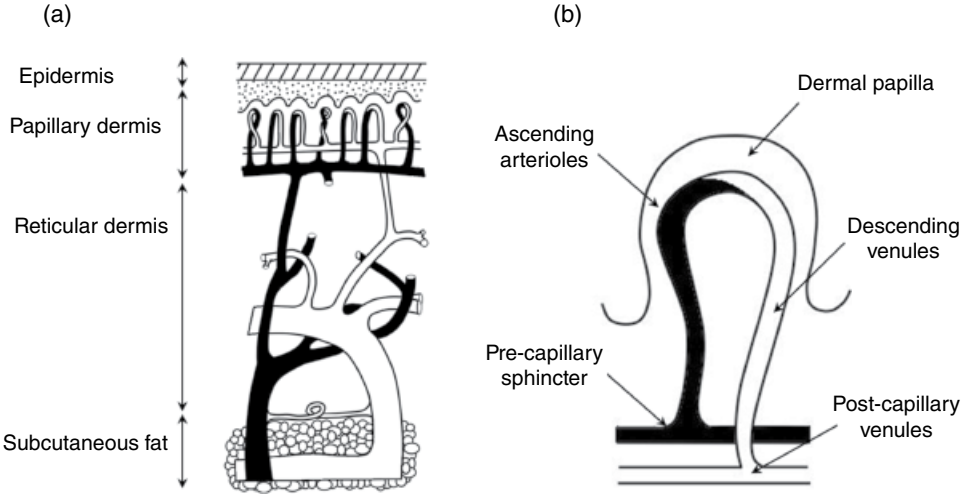


**Figure 12.1** Schematic diagram of the microcirculation in human skin. (a) The distinct layers of the skin are epidermis, papillary dermis, reticular dermis, and subcutaneous fat. The upper nutritive capillary loops are located in the papillary dermis, while the thermoregulatory shunt circulation is found in the lower dermis. (b) A single capillary loop within a dermal papilla.

and diabetic individuals [9]. The aforementioned structural changes appear more pronounced in the lower limbs, possibly due to the presence of elevated hydrostatic pressure [10]. The degree of the basement membrane thickening has been reported to correlate with glycemic control, with poorly controlled diabetic patients exhibiting thicker basement membrane [11].

In the diabetic foot, muscle capillaries have been shown to have thickened basement membrane [12]. The successive order of events that culminates in basement membrane thickening begins with the augmented shear force and hydrostatic pressure present in the microcirculation. These are believed to induce an injury response to the microvascular endothelium, leading to the secretion of various extravascular matrix proteins. Consequently, thickening of the basement membrane and arteriolar hyalinosis arise [13].

Changes in the basement membrane can have an impact on various cellular functions, including gene expression, adhesion, proliferation, and differentiation, and could therefore affect vascular permeability and lead to vascular dysfunctions. Basement membrane thickening results in impairment of nutrient exchange and in migration of activated leukocytes between the interstitium and the capillary. Moreover, the mechanical properties of the capillary are affected, causing a dysfunction in vasodilation capability [14]. As a result, the physiological hyperemic response to injury is compromised, diminishing the compensatory arteriolar dilatation following local injury, and therefore the hyperemic response is decreased [15]. It is noteworthy that basement membrane thickening apparently does not cause narrowing of the capillary lumen and arteriolar blood flow measurements are within the normal range or even elevated in spite of these alterations [16].

## Functional Changes in the Microcirculation

The reported inability of the microcirculation to undergo proper vasodilation in response to injury has been characterized as a functional ischemia and has been shown to arise spurred by a number of factors present in a diabetic individual's microcirculation. The changes in the lower extremity microcirculation have been suggested to play a critical role in the impaired wound healing associated with chronic non-healing diabetic foot ulcerations. Many studies have examined these modifications, with particular focus placed on the changes in the microcirculation, muscle metabolism, and nerve function of the diabetic foot.

The functional changes in the microcirculation involve diminished elasticity of the capillaries – and as a result vasodilating capacity – and dysregulated nutrient exchange and cellular migration. These abnormalities are believed to be a consequence of smooth muscle cell (SMC) dysfunction, endothelial cell (EC) dysfunction, and defective nerve axon reflex. Even though the exact molecular mechanisms of SMC and EC dysfunction have yet to be elucidated, measurements of diabetic neuropathic foot have demonstrated that there is an abnormal neurovascular response, causing a considerable decrease in the blood flow under stress conditions.

The reduced expression of poly(ADP-ribose) polymerase and endothelial nitric oxide synthase have been associated with the functional impairment of the diabetic foot microcirculation [17, 18]. In addition, endothelial nitric oxide synthase expression is decreased in peripheral neuropathy, pointing to a relationship between endothelial

dysfunction and neuropathy. Under conditions of stress including trauma and pain, the C fibers generate and release peptides such as neuropeptide P, substance P, neurotensin, and others that amplify vessel permeability and induce vasodilation. This process is a protective mechanism in response to stressors and it has been demonstrated to be dysregulated in diabetic patients regardless of the presence of neuropathy with the most significant dysregulation reported in the neuropathic foot [19–21]. Finally, this impairment at the foot level can be regarded as functional ischemia and could possibly be another contributing factor for the poor wound healing in diabetic foot ulcers.

## Functional Changes in the Diabetic Foot

In the absence of peripheral neuropathy, the resting total microcirculation at the skin level of the diabetic foot is similar to that of the non-diabetic foot. However, in the presence of neuropathy, the capillary blood flow has been found to be diminished [18, 22]. This could denote a cutaneous blood flow maldistribution with a concomitant functional ischemia. The hyperemic response is, as previously highlighted, impaired in the diabetic foot microcirculation, hence failing to reach maximum blood flow in response to stress or injury.

The functional changes in diabetic microcirculation seem to affect the vasodilation ability of precapillary arterioles and capillaries during events of stress or injury. Clinical examination of the diabetic foot with neuropathy and ulceration could present a warm limb with distended veins and palpable pulses; however, a contradictory finding is that this foot could also be functionally ischemic. Diabetic autonomic neuropathy with sympathetic denervation may result in the opening of the subpapillary arteriovenous shunts causing an elevation of the blood flow maldistribution between the subpapillary vessels and the nutritive capillaries [9, 23]. Thus, despite the fact that there appears to be no diminution in the vascularization of the foot, the skin microcirculation will be sizably diminished [24–26]. Eventually, arteriovenous shunting additionally exacerbates the functional ischemia, as reported by studies utilizing Doppler sonography, venous occlusion plethysmography, and venous oxygen tension measurements [27].

### Vasodilation

The endothelium is a monolayer of cells that constitutes the inner lining of blood vessels. By being in constant direct contact with circulating blood, ECs function as essential paracrine and autocrine regulators of vascular function, and provide a critical interface between the tissues and blood components. SMCs are small mononucleated cells with a spindle-like morphology that encircle the endothelium and are composed of a variable number of layers according to vessel size and location. Larger arteries (elastic arteries) are formed of many layers of SMCs alternating with layers of elastic laminae, while precapillary arteries (resistance arteries) may only have one SMC layer [28]. The internal elastic lamina (IEL) lies between the endothelium and SMC layer [29]. Gap junctions allow direct communication between the cells of the artery wall. These junctions enable the passage of small molecules and electrical current and exist both between same cell types (EC–EC and SMC–SMC)

known as homo gap junctions, and between distinct cell types (SMC–EC), called myoendothelial (MEGJs) or hetero gap junctions. MEGJs consist of an EC projection that extends to a SMC via an IEL perforation [29]. The transmission of membrane hyperpolarization through the gap junctions is an essential part of the process known as "spreading vasodilation," which enables the diffusion of vasodilation from stimulated to unstimulated nearby vessel portions.

Arterial vasodilation is dependent on intact endothelium and the release of a compound named endothelium-derived relaxing factor (EDRF) that causes arterial SMC relaxation following the release of ACh and other vasodilators [30]. This substance was subsequently identified as endothelial-derived nitric oxide (NO), a gas synthesized from precursor L-arginine during a reaction with nitric oxide synthase as a catalyst. Following its release from the ECs, NO diffuses to the neighboring SMCs and incites SMC relaxation via activation of cyclic guanosine monophosphate (GMP) and hyperpolarization of SMC membrane. The activation of cyclic GMP induces SMC guanylatecyclase causing increase of cGMP levels, decrease in intracellular $Ca^{2+}$ concentration, and finally resulting in SMC relaxation and therefore vasodilation.

ECs produce and secrete both relaxing factors such as NO and prostacyclin (PGI2) and contracting factors such as prostaglandins, angiotensin II (ANG II), and endothelin-1 (ET-1). NO is the principal vasodilatory regulator, however additional vasodilating mediators exist, such as PGI1, a cyclooxygenase-dependent metabolite of arachidonic acid [31]. Another noteworthy agent is endothelium-derived hyperpolarizing factor (EDHF), whose name derives from the fact that its vasodilating mechanism is abrogated by depolarizing potassium ($K^{2+}$) concentration or by $K^{2+}$ channel blockers. Direct hyperpolarization of the SMC membrane is rather induced through nitrosylation of the K-ATP channel and subsequently a rise in the activity of the pump. This mechanism is of great importance as it counteracts the effects of vasoconstrictive agents [32].

Under physiological conditions, there is a balance in the mechanisms inducing vasoconstriction and vasodilation and therefore vascular tone and permeability, and balance between fibrinolysis and coagulation are all precisely regulated. However, in the event of endothelial dysfunction, the balance is perturbed, prompting the onset and advancement of atherosclerosis. Endothelial dysfunction correlates with reduced availability of NO, either through the loss of NO production or through the loss of NO biological activity [33]. The importance of endothelial dysfunction for the microcirculation and the different mechanisms influencing normal function will be further examined.

#### Endothelium-Dependent Vasodilation

Most of the studies are in agreement that endothelium-dependent vasodilation is impaired in diabetes, regardless of the presence of long-term complications [34–38]. The first research studies on endothelium-dependent vasodilation employed venous occlusion plethysmograpgy, while later studies utilized a non-invasive method, flow-mediated vasodilation. The results from all these studies have demonstrated that endothelium-dependent vasodilation is dysfunctional in adolescent patients with type 1 diabetes, a population that is usually not affected by the macro- and microvascular diabetic complications [39]. This points to the presence of endothelial impairment before the development of vascular complications and may in fact be a critical factor toward their subsequent development.

Moreover, endothelial function in type 1 diabetes has also been reported to have an association with blood glucose levels, total cholesterol, and diabetes duration [40–42].

With regard to type 2 diabetes and its association with vascular disease, there have been numerous studies examining this relationship. It is now well established that there is an impairment of endothelium-dependent vasodilation both in macro- and microcirculation in type 2 diabetes. In addition, these changes are known to be already present at the prediabetic stage and thus precede the development of the disease. Finally, it has been shown that endothelial dysfunction is associated with insulin resistance in healthy individuals, alluding to a cause–effect relationship between these two states [38].

According to a study completed in our unit, the vasodilatory response to ACh was decreased in patients with diabetes and neuropathy, neuropathy and vascular disease as well as subjects with Charcot neuroarthropathy, while there was no difference between individuals with diabetes without neuropathy and the non-diabetic controls. We also discovered that the vasodilatory response was not attenuated in patients with neuropathy and vascular disease in comparison to patients with only neuropathy. Taken together these findings underscored for the first time the pivotal role that is played by the peripheral nervous system in the pathogenesis of diabetic foot ulceration and in mediating microcirculation [18]. Moreover, impairment in the microcirculation was also observed in the absence of large vessel disease. These results suggested that the primary reason for diminished microvascular reactivity was the presence of neuropathy, due to the fact that there were no additional defects detected in the non-neuropathic diabetic subjects. In support of this argument, neuropathy and vascular disease together did not lead to a further reduction in endothelium-dependent vasodilation compared with neuropathy alone.

#### Endothelium-Independent Vasodilation

The vascular SMCs being the final effectors of the vasodilation process are generally regarded as "the muscle of vascular biology." The vasodilating process is regulated by the relaxation of vascular SMCs, primarily completed through NO activation of gauntlet cyclase with subsequent generation of cyclic GMP, which in turn triggers protein kinase G, actuating phosphorylation of myosin light chain phosphatase and thereby inactivating myosin light chain kinase, which finally causes dephosphorylation of myosin light chain. EDHF controls vasodilation via transmission of hyperpolarization from ECs to vascular SMCs through gap junctions and/or the secretion of diffusible factors. Endothelium hyperpolarization is dependent on the activation of the intermediate conductance calcium-activated potassium channels (IKCa) and the small conductance calcium-activated potassium channels (SKCa) on the surface of ECs.

The reports on endothelium-independent vasodilation in complicated and non-complicated diabetes are controversial [43–47]. Newer data have indicated that endothelium-independent vasodilation is diminished in diabetic patients [18]. By employing laser Doppler imaging, measurements of the vasodilatory response to iontophoresis of sodium nitroprusside on vascular SMC function have been found to be significantly decreased in patients with diabetes and vascular disease, pointing to the possibility that endothelium-independent response is spared. Due to the fact that ACh elicits the generation of NO, it was hypothesized that an impaired NO production was the causative factor for the abnormal vasodilation detected.

### Nerve Axon Reflex

Nerve dysfunction contributes to the reduced vasodilatory response reported in diabetes. Under physiological conditions, the elevation of blood flow to the skin relies on the presence of an intact neurogenic vascular response. This protective function, known as Lewis' triple-flare response or nerve axon reflex vasodilation (NARV) starts with the stimulation of C-nociceptive nerve fibers, causing antidromic activation of the neighboring fibers. The stimulated C fibers subsequently release neuropeptides such as substance P, histamine, and calcitonin gene-related peptide (CGRP), leading to vasodilatation and augmented blood flow to the injured tissues (Figure 12.2). Generally, this response makes use of one-third of the maximum vasodilation capacity and is dependent on the presence of an intact vascular response.

Research on the diabetic foot with neuropathy has revealed that this neuromuscular response is dysregulated, resulting in a significant decrease in blood flow under stress. The reported reduction in NARV in diabetic neuropathic patients has been associated with both defective C-nociceptive fiber function and inability of microvasculature to effectively respond to vasomodulators released by these fibers [48]. There is strong evidence provided from studies in our unit that support the relationship between this vasodilatory impairment and diabetic neuropathy. In more detail, in diabetic subjects with neuropathy, neuropathy and peripheral vascular disease, and with Charcot arthropathy, the iontophoretic response to ACh in the skin for areas close to this substance but not directly in contact with it was significantly decreased as opposed to individuals with non-complicated diabetes and with healthy controls – a phenomenon known as indirect response [49].

The impairment in axon-related vascular reactivity is thought to additionally exacerbate the diabetic microcirculation defects and thus creates a vicious circle [18]. Therefore, in diabetic foot with neuropathy, the implication of the C-nociceptive fibers not only causes the well-established changes in pain perception, but also leads to impaired vasodilation under stresses such as injury and infection.

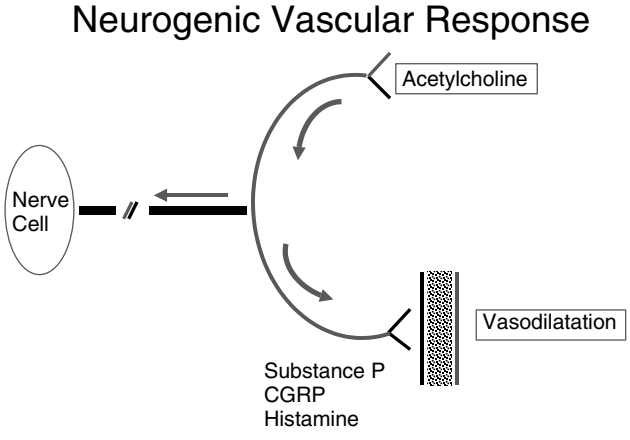


**Figure 12.2** Stimulation of the C-nociceptive nerve fibers results in antidromic stimulation of the adjacent C fibers, which release substance P, calcitonin gene-related peptide (CGRP), and histamine that cause vasodilatation and increased blood flow.

## Mechanisms of Endothelial Dysfunction

Endothelial dysfunction is manifested with elevated leukocyte interactions, vasoconstriction, SMC growth, vascular inflammation, defective coagulation, thrombosis, and atherosclerosis. There are several lines of evidence that indicate impaired endothelial function in individuals with both type 1 and 2 diabetes mellitus [36, 37]. The causes of endothelial dysfunction have been suggested to include hyperglycemia, insulin resistance, and inflammation as potential regulators of impaired endothelium-dependent response.

### Endothelial Dysfunction and Hyperglycemia

A number of mechanisms have been suggested for impaired endothelial function in hyperglycemia, primarily via triggering of oxidative stress. The principal mechanisms implicated in this process include the activation of protein kinase C (PKC), the elevated production of vasoconstrictor prostanoids, the attenuation of $Na^+/K^+$-ATPase activity, poly (ADP-ribose) polymerase (PARP) activation, generation of oxygen-derived free radicals, the elevated synthesis of endotelin-1 (ET-1), the activation of the polyol pathway and the formation of advanced glycosylated end products (AGEs).

#### *Protein Kinase C (PKC)*

PKC is a superfamily of cytoplasmic serine/threonine kinase isoenzymes. Increased activation of PKC, a major contributor in intercellular signal transduction for hormone and cytokines, could be caused by hyperglycemia and increased fatty acids existing in type 2 diabetes [50]. PKCs take part in vascular cell signal transduction and regulate various signaling, including oxidant, inflammatory, mitogenic, and angiogenic effects, in diabetic vascular tissues that may promote atherosclerotic cardiovascular disease [51, 52]. PKC activation causes elevated production of extracellular matrix proteins and cytokines, it increases contractility, permeability, and vascular cell proliferation, activates cytosolic phospholipase A2, blocks the activity of $Na^+/K^+$-ATPase, and mediates angiogenesis through the expression of growth factors, such as vascular permeability factor (VPF) [53]. Ruboxistaurin (RBX) mesylate is a PKC inhibitor that specifically prevents PKC-β overactivation. RBX has been shown to ameliorate neural function in animal models of diabetes [54]. However, in a double-masked randomized clinical trial carried out among individuals with diabetic peripheral neuropathy, RBX failed to achieve improvement of quantitative sensory testing for vibration detection threshold among all symptomatic patients. Nevertheless, the study demonstrated that treatment with RBX at the dosage of 64 mg, compared with placebo, conferred a significant improvement in the Neuropathy Total Symptoms Score-6 (NTSS-6) at 6 and at 12 months [55].

#### *Vasoconstrictor Prostanoid Synthesis*

Research in diabetic animals has also suggested that dysregulated endothelial production of vasoconstrictor prostanoids may also cause EC dysfunction. Elevated amounts of thromboxane A2 (TXA2) and prostaglandin H2 (PGH2) have been measured in sections of diabetic vascular tissue. In human studies, on the other hand, the exact role of vasoconstrictor prostanoids is not established. Flow-dependent vasodilation in healthy individuals, which acts as an index of endothelial function, is unaltered by aspirin, hence indicating

that it is entirely modulated by endothelium-derived nitric oxide (EDNO) and is not dependent on vasoactive prostanoids [56].

***Poly (ADP-Ribose) Polymerase (PARP)***

A number of studies have also provided insights into the contribution of PARP in endothelial function [17]. PARP is a nuclear enzyme that in response to oxidative DNA damage activates an inefficient cellular metabolic cycle that often results in cell necrosis. The activation of PARP is not only associated with endothelial dysfunction in diabetic patients, but has also been reported in healthy subjects at risk for developing diabetes [17]. It was observed that activation of PARP correlated with alterations in the vascular reactivity of the skin microcirculation, reinforcing the hypothesis that PARP activation leads to changes in microvascular reactivity. These results overall suggest that disturbances in the microcirculation because of PARP activation could possibly arise in the prediabetic state.

***Oxygen-Derived Free Radicals***

It has been suggested that oxidative stress is also a contributor to the development of diabetic vascular complications, through an augmented generation of oxygen-derived free radicals. This elevated production in diabetes causes the direct inactivation of endothelium-derived NO, therefore decreasing the bioavailability of EDNO [57]. In animal models of diabetes, endothelium-derived free radicals impaired EDNO-mediated vasodilation. In human studies, daily administration of vitamin E, a powerful free radical scavenger, had no apparent impact on cardiovascular outcomes in subjects with diabetes with complications [58]. However, earlier studies demonstrated that high-dose vitamin E (1800 IU/day) administration normalized hemodynamic defects, indicating that antioxidant consumption may attenuate the risks of diabetic vascular complications [59]. Subsequent studies focused on long-term high-dose vitamin E detected no beneficial effects on endothelial function or left ventricular function in type 1 and type 2 diabetic individuals [60]. Moreover, high-dosage vitamin E also correlated with exacerbation in some vascular reactivity measurements in comparison with healthy individuals.

***Endothelin-1 (ET-1)***

Hyperglycemia, oxidative stress, and AGEs incite secretion of ET-1, a potent vasoconstrictor, via activation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) [61]. The upregulation of ET-1 appears to be a result of the NO–Ang II imbalance, but the precise mechanism by which hyperglycemia induces this rise is as yet not fully understood. Another potential pathway causing the upregulation of ET-1 could be the PKC-mediated activation of the endothelin-converting enzyme (ECE)-1, which catalyzes the conversion of the inactive form of ET-1 to its active form [62].

***Polyol Pathway***

In hyperglycemic conditions, glucose enters the polyol pathway. In this pathway aldose reductase (AR), using NADPH as a cofactor, catalyzes the reduction of glucose to sorbitol, an organic osmolyte. Next, sorbitol dehydrogenase oxidizes sorbitol to fructose, with production of NADH from NAD+. Because NADPH is pivotal for synthesizing NO and glutathione, the polyol pathway activation leads to elevated amounts of sorbitol,

fructose – a 10-times more potent glycation agent than glucose – and ROS, and to reduced concentrations of NO and glutathione [63]. Several studies have demonstrated the increased activity of AR during hyperglycemia- and diabetes-induced oxidative-nitrosative stress [63] and downstream activation of MAPK (mitogen-activated protein kinase) [64], PARP [65], and NF-κB [64].

The rise in sorbitol levels leads to the depletion of other osmolytes, like myo-inositol and taurine [66]. The depletion of myo-inositol impairs phosphoinositide metabolism and this is partly responsible for the reduction in $Na^+/K^+$-ATPase activity. The proposed mechanism by which myo-inositol impacts $Na^+/K^+$-ATPase function, is through the inhibited activation of neural PKC caused by reduced phosphoinositide-derived diacylglycerols [67]. The $Na^+/K^+$-ATPase is implicated in cellular homeostasis and the functions of contractility, proliferation, and differentiation. Thus, impairment of this mechanism can result in vascular dysfunction and the early onset reversible nerve conduction defect in experimental diabetes [68].

In the past few decades over 30 randomized controlled trials have examined the efficacy of AR inhibitors (ARIs), such as epalrestat and ranirestat, in alleviating diabetic neuropathy. A meta-analysis of 879 ARI-treated and 909 control (placebo or no treatment) participants, revealed no significant difference in the treatment of diabetic polyneuropathy between the groups [69].

#### *Advanced Glycosylated End Products (AGEs)*

AGEs are the products of a non-enzymatic reaction when proteins are subjected to hyperglycemic conditions. The resultant Schiff bases can be reorganized to form Amadori products, AGEs, and reactive oxygen species. Elevated AGE levels have been observed in patients with diabetes and may enhance vascular permeability in diabetes, as the blocking of a receptor for AGE rescues diabetes-mediated vascular hyperpermeability [70]. Moreover, the produced reactive oxygen species have been reported to result in severe dysregulation of coronary blood flow and cellular hemostasis, causing the serious macrovascular lesions typically present in diabetic subjects after more than a decade with the disease [71]. It is also noteworthy that inhibition of reactive oxygen species also impedes the production of AGE products, indicating that the autoxidation mechanism is a key player in the complex reaction cascade resulting in AGE generation.

### Endothelial Dysfunction and Insulin Resistance

In addition to its anabolic effect, insulin also has a hemodynamic effect that leads to peripheral vasodilation and vascular recruitment. Insulin regulates vasodilation through mediating the production and release of NO [49, 72]. The stimulation of NO by insulin is regulated by the activation of signaling pathways, which involves the recruitment of phosphoinositide-3 (PI-3) kinase that ultimately results in the phosphorylation of endothelial nitric oxide synthase. It has been suggested that as much as a quarter of insulin's stimulatory effect on muscle glucose uptake is associated with its hemodynamic functions [73]. Through direct contact with the endothelial barrier, insulin controls dilation of the capillaries and relaxation of the precapillary sphincter. Through this mechanism the metabolic action of insulin is augmented, both by recruiting new capillary beds and by redirecting the capillary

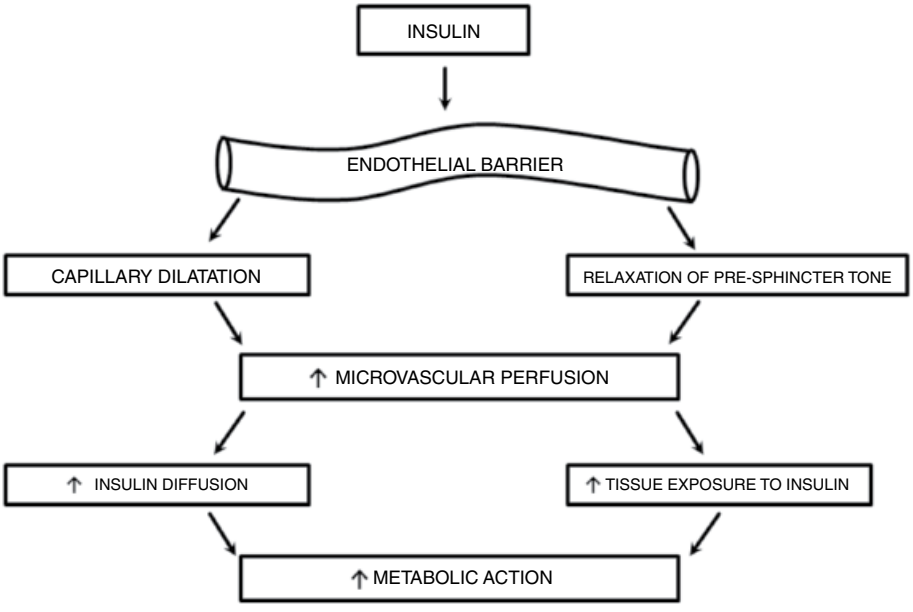


**Figure 12.3** Hemodynamic and metabolic actions of insulin. Insulin promotes an increase in recruitment of microvessels, expansion of the capillary network, and perfusion of the microcirculation, through relaxation of the precapillary sphincter tone and dilatation of the capillaries. Insulin consequently diffuses into the interstitium more readily and the target tissues are exposed to high insulin concentrations. This culminates in an enhanced insulin-mediated glucose metabolism.

blood flow more toward insulin-sensitive tissues such as muscle and adipocytes, and at the same time away from insulin-independent tissues such as bone and skin [74] (Figure 12.3).

The bidirectional link between hyperinsulinemia and endothelial dysfunction is well established. The exposure of the vascular endothelium to hypertriglyceridemia and to increased small dense low-density lipoprotein (LDL) cholesterol particles, typically present in insulin resistance states, causes diminished NO availability [75]. Furthermore, endothelial dysfunction contributes to inhibited insulin action, by modifying the transcapillary passage of insulin to its target tissues. Even though the exact molecular mechanisms governing the metabolic and vascular dysfunction accompanying the insulin resistance state have yet to be entirely delineated, the abnormal generation of NO clearly seems to be of great significance.

**Endothelial Dysfunction and Inflammation**

The increased levels of inflammation present in type 1 and type 2 diabetes have distinct origins. In type 1 diabetes, although inflammation plays an important part in the long-term development of disease, the onset is a result of islet inflammation that is believed to be a localized phenomenon induced by local autoimmune activation. In type 2 diabetes the inflammatory process is a consequence of systemic etiologic factors including insulin

resistance and central obesity. In both types of diabetes, hypertension and dyslipidemia in conjunction with hyperglycemia are primarily responsible for the maintenance and aggravation of inflammation.

Early on the activation of the inflammatory process is characterized by the secretion of chemokines including fibrinogen, interleukins 1 and 6, and monocyte chemoattractant protein 1. These substances stimulate the release of interstitial and vascular cellular adhesion molecules like VCAM-1, ICAM-1, and E-selectin, and recruit monocytes and other immune cells [76]. The secretion of inflammatory cytokines augments vascular permeability, alters the vasoregulatory responses, and enhances the adhesion of leukocytes to the endothelium. In addition, these cytokines promote thrombus formation by facilitating pro-coagulant activity, through inhibition of anticoagulant pathways and impairment of fibrinolysis by raising the expression of plasminogen activator inhibitor 1 and of tissue factor, through activation of platelets and acute phase reactions [76].

## Pathophysiology of Diabetic Wound Healing

Acute wound healing is a dynamic, highly orchestrated, and complex process that begins as soon as tissue integrity is disrupted. It involves a multitude of cell types, molecules, and biological cascades, and has been classically divided into four partly overlapping phases: hemostasis, inflammation, proliferation, and remodeling phase. All different stages have extensively been reviewed elsewhere [77–79].

Non-healing wounds are open wounds that fail to achieve closure in a reasonable amount of time and typically wounds that fail to heal after a 12-week period are characterized as chronic [80]. Chronic diabetic wounds start as acute wounds, but the healing process is inhibited and stalled in different phases. Thus, normal acute ulcer repair fails to occur. Several growth factors, cytokines, proteases, as well as cellular and extracellular components are key players in the wound healing process. In diabetic individuals, this wound healing process is affected by hyperglycemia, chronic inflammation, micro- and macrocirculatory dysfunction, hypoxia, and neuropathy (Figure 12.4).

### Hyperglycemia

Hyperglycemia facilitates non-enzymatic glycation of collagen and other proteins and contributes to the generation of AGEs. These end products decrease the solubility of the extracellular matrix and perpetuate the inflammatory response present in diabetes [81, 82].

### Chronic Inflammation

One of the hallmarks of diabetes is the presence of a chronic low-grade inflammatory state. Moreover, in diabetic individuals there is also a prolonged inflammatory response after injury that has deleterious effects on wound closure. Individuals with diabetes, regardless of their risk for developing foot ulcers, have been observed to have elevated amounts of inflammatory cells in the dermis and especially surrounding the vasculature. Similar results were reported in the skin of diabetic mice, rats, and rabbits [83, 84]. In addition, the

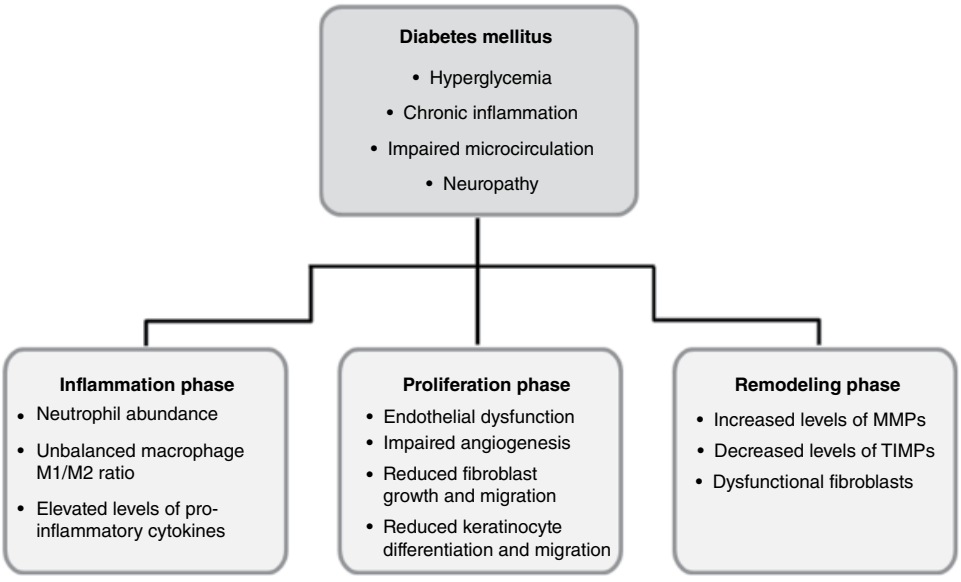


**Figure 12.4** The complications of diabetes mellitus and how they result in impairment of a number of different processes in the distinct stages of wound repair.

pro-inflammatory (M1) to anti-inflammatory (M2) macrophage ratio, an indicator of chronic inflammation, is found to be higher in the dermis of diabetic rabbits both pre- and post-injury in comparison with healthy controls [84]. Other studies have demonstrated [85] that this prolonged inflammatory response involves the continuous secretion and increased levels of pro-inflammatory cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α [86, 87].

Studies in diabetic mice have revealed that there is an abundance of neutrophils and macrophages within the chronic wounds, while neutrophil ablation results in expedited wound closure. These findings, in conjunction with the ability of neutrophils to generate a large number of reactive oxygen species and proteases that can damage healthy tissue, indicate that neutrophils may also be key players in the impaired wound healing observed in diabetes [88]. Furthermore, fibroblasts isolated from diabetic wounds exhibited poor proliferation, higher apoptosis, and reduced migration capacity [89, 90]. Keratinocytes have also been reported as abnormal, displaying increased proliferation but reduced differentiation and impaired migration capacity, factors that could also contribute to incomplete wound repair [85, 89]. Moreover, growth factor expression and their functional levels have been found to be decreased in diabetic wounds [87, 91]. Higher levels of matrix metalloproteinases (MMPs) also localize in diabetic wounds, while levels of tissue inhibitors of MMPs (TIMPs), the proteins that inhibit MMP activity, are lower [86, 92]. To be more specific, in wound fluid of diabetic foot ulcers, the MMP 1, 2, 8, and 9 levels are increased while the TIMP-1 and TIMP-2 are at the same time diminished [93]. These increased amounts of MMPs are responsible for degrading extracellular matrix components, such as fibronectin and collagens, and severely impair the healing process [94].

### Impaired Angiogenesis and Vasculogenesis

Angiogenesis and vasculogenesis are also characteristically defective in the non-healing diabetic wound. Endothelial progenitor cell (EPC) numbers are decreased in diabetic patients at risk of foot ulceration and in patients with active diabetic foot ulcers [95]. Hyperglycemia and chronic inflammation are considered the main contributing factors for EPC dysfunction and abnormal EPC recruitment from the bone marrow in diabetes [96, 97]. It has also been suggested that the non-enzymatic glycation of the basement membranes in the vasculature leads to hindering of EPC homing capacity and therefore impaired blood vessel regeneration [97].

### Hypoxia

Sufficient oxygen supply is pivotal for cell metabolism and energy production. Impaired tissue perfusion and poor oxygen supply due to microvascular and macrovascular disease promote a hypoxic wound environment and impair the healing process. Hypoxia delays wound repair by raising the levels of free oxygen radicals. Chronic hyperglycemia-induced inflammation even further increases oxidative stress and prolongs wound healing [82].

### Impaired Neuropeptide Signaling

Peripheral nerve fibers located in the skin are activated immediately following injury and produce different neuropeptides into the microenvironment of the wound. Neuropeptides such as substance P, neuropeptide Y (NPY), and CGRP impact mast cells, ECs, fibroblasts, and keratinocytes, and contribute to vasoregulation and angiogenesis [98, 99]. The expression of these neuropeptides has been reported to be diminished in diabetes. Moreover, their reduced expression and altered function could play an essential role in the delayed healing observed in diabetic wounds. In diabetic neuropathy the nerve axon-related vasodilation (Lewis triple-flare response) is decreased or even completely absent [100]. The activation of C-nociceptive fibers should effectuate retrograde activation of adjacent fibers to secrete vasomodulators, such as substance P, NPY, CGRP, catecholamines, and histamine, that induce vasodilatation and hyperemia during tissue injury. The impaired Lewis triple-flare response reported in diabetic patients leads to functionally ischemic tissues, even in the absence of disturbed macrocirculation [101].

## Adjunctive Treatment Options

Adjunctive therapies for diabetic foot ulcer treatment have been employed for many years with novel approaches regularly emerging. Some of the most promising among them include bioengineered skin substitutes, extracellular matrix proteins and growth factors, hyperbaric oxygen, and electrical stimulation. In addition, a number of newly introduced techniques are currently under investigation. These include wearable technologies, such as smart socks that can record applied pressure in order to prevent the development of diabetic foot ulcers and three dimensional printed skin equivalents. Animal experiments

have showed encouraging results but further studies are necessary before definitive conclusions about the effectiveness of these techniques can be drawn [102–104].

## Bioengineered Skin Substitutes

There are two products approved by the US Food and Drug Administration (FDA) and available in the market for diabetic foot ulcer treatment, Apligraf® and Dermagraft®. Apligraf is a graft that consists of a cultured living dermis and a sequentially cultured epidermis, derived from neonatal foreskin discarded samples. It is made up of four components: extracellular matrix, viable allogeneic dermal fibroblasts, epidermal keratinocytes, and a stratum corneum. The artificial extracellular matrix consists of a bovine type I collagen lattice and acts as a scaffold where fibroblasts grow and deposit a multitude of extracellular matrix proteins such as different types of collagen, fibronectin, elastin, and glycosaminoglycans. The dermal fibroblasts also synthesize growth factors that promote wound healing, stimulate the formation of new dermal tissue, and provide factors that facilitate the maintenance and function of the overlying epidermis. The keratinocytes form the epidermis, also secreting growth factors to stimulate wound healing through fibrosis, chemotaxis, angiogenesis, and other cellular activities that are often disarranged in chronic wounds [105]. The stratum corneum is a natural barrier that protects against mechanical damage, infection, or wound desiccation [106]. Dermagraft is an allogeneic, human neonatal foreskin-derived dermal fibroblast culture, grown on a bioabsorbable polyglactin mesh scaffold. The fibroblasts produce a rich network of extracellular matrix components such as collagen and glycosaminoglycans [107]. Dermagraft also includes growth factors such as platelet derived growth factor A (PDGF-A), insulin-like growth factor (IGF), keratinocyte growth factor (KGF), transforming growth factors (TGF-α, TGF-β1, TGF-β3), and vascular endothelial growth factor (VEGF). Importantly, the matrix proteins and the growth factors retain their activity after implantation into the wound bed.

In addition to the above, many other products have been designed and are available: a cellular dermal graft derived from human donor tissue, bilayer dressing mimicking normal skin, medical grade polymer covered with a plasma polymerized functional surface, different forms of hyaluronic acid, three dimensional bioabsorbable collagen based extracellular matrix, dressing composed of collagen, or amniotic membrane tissue graft products to name a few [108, 109]. However, none of them has been FDA approved for diabetic foot ulcer treatment, as there is a paucity of satisfactory clinical data to support their therapeutic efficiency.

## Growth Factors

Growth factors are crucial regulators in all the cellular processes implicated in wound healing, such as cell proliferation, migration, angiogenesis, leukocyte extravasation, and extracellular matrix deposition. Therefore, over the past 20 years administration of topical formulations of growth factors has been employed for diabetic foot ulcer treatment [110]. PDGF in a gel form or becaplermin is the only growth factor that has been FDA approved for the treatment of diabetic foot ulcer and is marketed as Regranex Gel (Smith & Nephew plc., London, UK), being commercially available since 1998 [111, 112]. PDGF is a protein

typically associated with platelets, ECs, and macrophages. It causes inflammatory cell activation, promotes cell growth and migration, as well as production of extracellular matrix components. PDGF treatment is widely used in diabetic foot ulcers and a 34% improvement in 12-week healing rates has been demonstrated [113]. However, the FDA has included a warning on the package of this product, since patients who have used three or more tubes of becaplermin present increased risk for cancer mortality [95]. Moreover, other growth factors that have been shown to stimulate the wound healing process in different ways such as modulation of cell proliferation, differentiation, or stimulation of neovascularization, include basic fibroblast growth factor, recombinant human epidermal growth factor, recombinant human VEGF, nerve growth factor, and growth factors isolated from platelet-rich plasma [110, 114–116]. Nonetheless, there are no trials in the literature to confirm beneficial outcomes of using these growth factors for diabetic foot ulcer treatment [117].

### Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy has been employed as an adjunctive treatment for diabetic foot ulcers for more than 40 years and has been reported to decrease the incidence of major amputations in diabetic subjects with ischemic foot ulcers. This treatment may exert a beneficial influence on microbial balance, soft tissue infection, and angiogenesis. In diabetic microvascular disease, the distances between capillaries are increased and their function is impaired. In the presence of these pathological conditions the oxygen is forced to diffuse for longer distances, therefore increased PO2 levels are necessary at the edge of the capillaries [118]. However, the benefit of hyperbaric oxygen therapy remains controversial because of conflicting data in the literature [119, 120]. Recently, the findings from a study investigating hyperbaric oxygen therapy were published. The authors studied and analyzed 6259 individuals with diabetes, sufficient lower limb arterial perfusion, and foot ulceration extending through the dermis. Based on their results, hyperbaric oxygen therapy was not found to enhance wound repair or to reduce the likelihood of amputation [121].

### Negative Pressure Wound Therapy

Negative pressure wound therapy (NPWT) entails locally applying sub-atmospheric pressure to a wound [122]. The observed effectiveness of NPWT could be attributed to several mechanisms of action, including the extraction of the excess third-space fluid from the area [123], the decrease of bacterial load, a mechanical force applied to the wound bed, or the stimulation for forming dense granulation tissue with abundant small blood vessels via EPC recruitment [124]. Nevertheless, there are studies with conflicting findings, such as a retrospective review of 25 patients that revealed a growth in the bacterial load of 43% in wounds treated with NPWT [122]. The recommendations of an international consensus for the use of negative pressure therapy diabetic foot ulcer treatment were positive for diabetic foot ulcers without ischemia [125]. However, more studies are required to substantiate the utilization of NPWT in routine clinical practice.

### Electrical Stimulation and Shockwave Therapy

Both electrical stimulation and shockwave therapy could also potentially be options for adjunctive treatment for diabetic foot ulcers. Pulsed electromagnetic field stimulation appears to reduce the doubling time of fibroblasts and ECs in culture and to also have bacteriostatic and bactericidal effects by increasing the migration of neutrophils and macrophages [126].With regard to shockwave therapy, this therapeutic approach seems to promote the early expression of angiogenesis-related growth factors. Hence, it facilitates enhanced vessel ingrowth that ameliorates blood supply, stimulates cell proliferation, and accelerates tissue regeneration and repair [127]. Nevertheless, there is a lack of clinical trials in the literature, which demonstrate any benefits of these two methods for diabetic foot ulcer healing [117].

## Future Therapeutic Perspectives

Despite current treatments, a quarter of individuals with diabetic ulcers will still undergo foot amputation. Novel treatment strategies for successful healing of diabetic foot ulcers could be focused on correcting the factors that lead to impaired wound repair or alleviate their deleterious effects.

### Gene Therapies

Delivery of genes that encode growth factors such as PDGF and VEGF, which promote wound healing, has been proposed as a future treatment option for ulcers that persist and gain no benefit from the current standard of care [128]. Viral vectors are the most established technique for gene delivery, and replication-defective adenovirus encoding PDGF or VEGF has been used in both human and animal studies with beneficial effects on wound repair. More specifically, topical application of a bovine collagen gel enriched with a replication-defective adenovirus encoding PDGF resulted in rapid reduction of wound size and complete ulcer healing in individuals with diabetes and neuropathic ulcers, with no manifestations of serious safety concerns [129]. Local delivery of VEGF using adenovirus vector has also been shown to accelerate wound healing in animal studies [130].

### Stem Cell Therapies

Stem cell-based therapies consist of adult stem cell direct administration onto the wound to promote repair, and have shown promising results both in clinical trials and in experimental animal studies. Furthermore, EPCs are recognized as an essential target of therapeutic angiogenesis for treatment of diabetic microvascular and macrovascular complications. The use of stem cells in diabetic wounds has been investigated in a number of clinical studies with encouraging outcomes. Intramuscular injections of autologous peripheral blood mononuclear cells (PBMCs), bone marrow mesenchymal stem cells (BM-MSCs), or bone marrow-derived mononuclear cells into the ischemic lower limb of patients with diabetes mellitus and critical limb ischemia significantly enhanced blood flow and

resulted in full wound closure, while importantly no major adverse effects were reported [131, 132]. In addition, local application of autologous bone marrow cells has also been used in a diabetic subject with chronic venous and neuroischemic wounds and has been shown to enhance tissue vascularization and accelerate wound healing with no systemic adverse events [133], while local application of BM-MSCs encapsulated within a fibrin polymer system in individuals with chronic ulcers led to improved wound healing [134]. Local application of allogeneic MSCs has so far been tested only in animal studies with favorable results in wound closure [135]. A recently conducted systematic review and meta-analysis of seven total studies involving 224 diabetic patients, confirmed the benefits of utilizing stem cell-based therapy for the treatment of diabetic foot ulcers. Still, well-designed randomized controlled trials are needed in the future in order to further validate and update these findings [136].

### Norleu3-Angiotensin-(1–7)

Recent studies have shown that angiotensin receptors are abundantly expressed in human skin and that the tissue renin–angiotensin system plays an essential part in the wound repair process [137]. A number of studies have demonstrated that angiotensin II and angiotensin (1–7) heptapeptide promote wound healing in various animal models [138]. Enhancement of wound repair is postulated to be a result of augmented progenitor cell proliferation, blood vessel formation, and elevated extracellular matrix proteins production and deposition [138, 139]. NorLeu3-angiotensin (1–7) is an angiotensin (1–7) analog and it has been shown to have more potency than its parent protein in stimulating wound healing [140]. Topical application of NorLeu3-angiotensin (1–7) cellulose gel has been reported to be successful in accelerating wound healing in individuals with diabetes and foot ulcers with no apparent significant adverse events [141]. A phase III clinical trial is currently being conducted examining the efficacy and safety of NorLeu3-angiotensin (1–7).

### Neuropeptides

Substance P has been administered topically on open excision-type wounds of non-diabetic rats with favorable outcomes. Rats treated with substance P exhibited rapid decrease of wound area and enhanced wound contraction compared with the control group. Substance P administration resulted in elevated levels of VEGF and TGF-β1, as well as increased TNF-α and reduced IL-10 expression on the first days post-wounding. Furthermore, local application of substance P accelerated all phases of the healing process based on the histopathological results of the wound skin specimens [142, 143].

### Cytokine Inhibition

Another therapeutic approach that could enhance wound healing is the polarization or recruitment of anti-inflammatory macrophages (so called M2 macrophages) to the wound area that would subsequently promote cell proliferation and angiogenesis. Classically activated macrophages (M1 macrophages) secrete a variety of pro-inflammatory cytokines, such as IL-1 and IL-6, that appear to be key moderators in sustaining the pro-inflammatory

macrophage phenotype characteristic of diabetic wounds. Inhibition of the IL-1b pathway in diabetic mice has been demonstrated to facilitate the switch from a pro-inflammatory to an anti-inflammatory or regenerative macrophage phenotype that stimulates the production of growth factors, and therefore wound healing [144]. Additional large-scale clinical studies are required to adequately assess the potency and safety of these new treatments and to elucidate whether they can be recommended or preferred from current treatment options to improve wound repair and decrease lower limb amputation incidence in diabetic patients.

## Conclusion

To conclude, even though an occlusive disease of the microcirculation does not really exist, functional impairment of the microcirculation in the diabetic foot may be a causal factor for manifestation of secondary complications of the disease, for instance foot ulcerations and infections. The microcirculation at the diabetic foot level exhibits both structural and functional alterations. The nerve axon-related microvascular reactivity is distinctly impaired in diabetic subjects and there is an increasing amount of evidence and a growing belief that both the inability of proper vasodilatation and the dysregulation in the nerve axon reflex are essential contributors to the impairment of wound repair in diabetic individuals. Additional experimental studies are required to dissect the exact molecular mechanisms governing the observed endothelial dysfunction in neuropathic and diabetic patients.

With a rapidly rising incidence of diabetes worldwide, there is a parallel increase in the frequency of diabetic foot ulcers and therefore the need for more efficient treatment options is stronger than ever. Many promising alternatives to the current standard of care that could prevent, revert, or retard the progression of diabetic foot ulcers are emerging. However, extensive clinical trials and experimental data are necessary to establish the beneficial effects and safety of these treatments.

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# 13

# Coronary Microvascular Dysfunction in Diabetes – Clinical and Research

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## Introduction

Coronary microcirculation plays a fundamental role in the regulation of myocardial perfusion. In the past three decades, a whole body of evidence has demonstrated that functional and structural abnormalities of the coronary microvascular district may result in myocardial perfusion impairment and ischemia [1–3]. This condition, generally defined as coronary microvascular dysfunction (CMD), underpins a wide spectrum of pathophysiological mechanisms and may translate into (or contribute to) several clinical entities, including stable angina, acute coronary syndromes, and cardiomyopathies [4, 5]. In particular, CMD has a high prevalence (up to 63.9%) in patients with suspected ischemic heart disease [6–9], and it is independently associated with adverse outcome [9, 10].

In patients with diabetes mellitus, coronary artery disease (CAD) is the leading cause of mortality [11]. Although much of the increase in CAD risk can be accounted for by the presence of diabetes-associated coronary risk factors such as hypertension, dyslipidemia, and obesity, a significant proportion of it remains unexplained [12]. Among the proposed mechanisms to elucidate this finding, the deleterious effects of diabetes on endothelial function seem to play a central role.

Indeed, among diabetic patients without known CAD, those with impaired coronary flow reserve (CFR) have event rates comparable with those of patients with a prior CAD, whereas those with preserved CFR have event rates comparable with those of non-diabetics [13].

CMD in diabetes may have specific pathophysiological features, partly distinguishing it both from CMD of non-diabetic patients and from diabetic microangiopathy affecting the retina, renal glomerulus, and peripheral nerve. Furthermore, it may become clinically apparent encompassing a wide range of manifestations related to atherosclerosis such as chronic angina and acute chest pain or related to diabetic cardiomyopathies, such as heart failure-related signs and symptoms.

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## Clinical Classification and Mechanisms of CMD

On the basis of the clinical settings in which it occurs, CMD can be classified into four types: dysfunction occurring in the absence of CAD and myocardial diseases (Type 1), dysfunction in the presence of myocardial diseases (Type 2), dysfunction in the presence of obstructive epicardial CAD (Type 3), and iatrogenic dysfunction (Type 4) after percutaneous coronary intervention and/or coronary artery grafting [4].

Though the diabetic patient may experience CMD in each of these settings, mechanisms specifically pertaining to diabetes pathophysiology drive Type 1 and Type 3 CMD in particular, with Type 1 being the first to develop in diabetic CMD natural history. This is due to the timing of microvascular dysfunction, which develops well before the macrovascular disease [14].

Several pathogenetic mechanisms may promote CMD. The relative contribution of each to the clinical framework may vary as a function of the individual risk factors and genetic background. Specifically, conventional cardiovascular (CV) risk factors (i.e. smoking, arterial hypertension, diabetes, dyslipidemia) all have been demonstrated to be involved in microvascular dysfunction [15]. The mechanisms through which the causative factors translate in CMD may be categorized as structural, functional, or extravascular.

Structural alterations include vascular remodeling, vascular rarefaction, and perivascular fibrosis. These features have been documented particularly in hypertensive patients and in those with aortic stenosis [16–19]. The unfavorable hemodynamic changes pertinent to both conditions cause low coronary perfusion pressure due to increased extravascular compressive forces with elevated systolic and diastolic wall stress and impaired relaxation, triggering hypertrophy of smooth muscle cells, increased collagen deposition in the tunica media, intimal thickening, and endothelial degeneration, ultimately resulting in the structural alterations outlined above. Recently, also diabetes has been implied in the pathogenesis of microvascular rarefaction and pericyte loss in both human hearts and animal models [20].

Functional alterations include reduced coronary microvascular dilatation and increased coronary microvascular constriction. These components may variably associate, resulting from the combination of multiple pathways' impairment, triggered by several factors (Table 13.1).

The physiology of microvascular dilatation includes both endothelium-dependent and endothelium-independent mechanisms [15]. Endothelial cells play a significant role in the regulation of blood flow at the level of pre-arteriolar vessels, with nitric oxide (NO) production and release being the most important mechanisms of endothelium-mediated vasodilatation. These are also the first mechanisms to be lost in the case of endothelial dysfunction. Specifically, excess generation of reactive oxygen species (ROS), leading to increased superoxide anion production (which is triggered by several conditions including diabetes, obesity, smoking, and hyperlipidemia), is the most frequent pathway involved in impaired NO synthase (NOS) activity and increased NO degradation.

Notwithstanding the established role of endothelium-independent vasodilation [21–23], the involved cellular pathways associated with CMD remain incompletely characterized. Overall, they are related to impaired endothelium-independent smooth muscle cell relaxation, in response to substances mediating metabolic, flow-mediated, and reactive regulation of coronary blood flow (CBF) (i.e. adenosine, $H^+$, $CO_2$, $H_2O_2$, $\beta_2$ agonists,

**Table 13.1** Coronary microvascular dysfunction develops from a combination of vasodilatation impairment and increased vasoconstriction caused by various stimuli.

| Pathophysiological mechanisms | | Stimuli |
|---|---|---|
| **1) Impaired vasodilation** | Endothelium-independent | Adenosine<br>Catecholamines |
| | Endothelium-dependent | Flow-mediated<br>Acetylcholine<br>Bradykinin<br>Histamine<br>Serotonin |
| **2) Increased vasoconstriction** | | Acetylcholine<br>Catecholamines<br>Endothelin-1<br>Serotonin |

prostacyclin, and NO) [24–28]. While most traditional cardiovascular risk factors may be associated with impaired endothelium-independent relaxation, its precise contribution to CMD pathogenesis remains unclear. Furthermore, aberrant vasoconstrictive stimuli (i.e. endothelin-1) or vasodilatative stimuli in the setting of a diseased endothelium (acetylcholine, serotonin, catecholamines) may further participate in CMD pathogenesis [29–31].

To conclude, elevated left ventricular filling pressures may further reduce CBF, through increased microvascular compression at the level of the subendocardial layers of the myocardium [32]. This mechanism may be particularly relevant in diabetic cardiomyopathy, whose main hallmarks are left ventricular hypertrophy and decreased left ventricular compliance leading to diastolic dysfunction [33, 34].

## CMD in Diabetic Patients

In patients with diabetes different pathogenic mechanisms may lead to the development of CMD. Although the diabetic patients often have a high burden of cardiovascular risk factors, coronary vascular dysfunction may occur even in non-smoking patients or those without arterial hypertension and dyslipidemia [35, 36], and even when CV risk factors are present, they do not completely account for the increased risk observed [12].

Indeed, several features involved in the pathogenesis of the diabetic microangiopathy may play a role in CMD.

- *Hyperglycemia*. Microvascular complications (different from macrovascular ones), also in the coronary district, are linearly associated with the degree of hyperglycemia, may be apparent even before the clinical onset of diabetes [37–40], and may be positively influenced by glycemic control [41]. Different mechanisms may explain these findings:
  a) *Oxidative stress*. Intracellular hyperglycemia in endothelial cells causes mitochondrial ROS overproduction, which leads to the activation of five major mechanisms (i.e. increased polyol pathway flux, increased formation of advanced glycation end

products (AGEs), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C isoforms, and overactivity of the hexosamine pathway) finally resulting in tissue damage and endothelial dysfunction [42, 43]. Furthermore, both glucose and ROS upregulate endothelial arginase I and II, which compete for the substrate L-arginine with the endothelial form of NOS (eNOS). Arginase additionally increases ROS production, further impairing NO bioavailability [44, 45]. Recently, arginase I has been demonstrated to be also upregulated in the red blood cells (RBCs) of diabetic patients as an effect of increased vascular NOS1-derived $H_2O_2$, and to account for decreased NO bioavailability and resulting endothelial dysfunction. In the coronary microvasculature (CMV), the endothelial dysfunction also appears to be related to the increased oxidation of fatty acids, linked to the insulin-resistant-triggered pathways, thus accounting for specific pathobiological features that distinguish diabetic CMD from systemic microangiopathy [46]. Moreover, ROS production directly uncouples endothelial NOS and lower NO production, thereby interfering with endothelium-dependent vasodilation.

b) *Inflammation*. Chronic hyperglycemia increases levels of inflammatory markers, including C-reactive protein (CRP) [47]. There is evidence that myocardial CRP, rather than being a simple marker, may participate in endothelial toxicity and microvascular rarefaction [48, 49].

c) *Depletion of vascular growth factors*. Hyperglycemia may lead to a reduction in the levels of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and NO, potentially affecting microvessel density and integrity [50].

- *Cardiac autonomic dysfunction*. The balance impairment of the cardiac autonomic nervous system contributes to functional CMD in diabetic patients, resulting in impaired CBF during increased sympathetic stimulation [36, 51]. Notably, this mechanism occurs also in a significant number of diabetic patients without apparent systemic autonomic neuropathy [36, 52, 53].
- *Genetic variants*. Approximately 25% of diabetic individuals do not develop microvascular complications, irrespective of glycemic control [54], and glycemic status and traditional CV risk factors only partially account for microvascular complications [55]. These findings point toward a role of genetic variants in the pathogenesis of microvascular complications [56]. In this context, genetic polymorphisms of NOS3 affect endothelial cell survival and responses to stress [57], as well as the risk of CAD and myocardial infarction [58], and VEGF isoforms derived by alternative splicing of the VEGF gene may influence neoangiogenesis and coronary microvessel density [50, 59].
- *Insulin resistance*. The relation between insulin resistance and CMD is not fully established. Insulin has dose-dependent coronary vasodilatory properties through the L-arginine-NO pathway and sympathetic activation [60–62], and it may normalize coronary arterial sensitivity to adenosine [63]. Insulin resistance may thus directly impair vasodilator reserve. Moreover, the chronic hyperinsulinemic state may lead to an increase in plasma triglyceride, a decrease in high-density lipoprotein cholesterol, and an increase in arterial blood pressure [64], further contributing to the development of the CMD related to CV risk factors. Another proposed mechanism linking insulin resistance to CMD concerns the mismatch between perfusion and metabolic demand of the heart potentially induced by regional patterns of insulin sensitivity/resistance [15, 65].

The main feature of diabetic CMD, in which the aforementioned pathogenetic components translate, is the endothelial dysfunction resulting in impaired endothelium-dependent vasodilation. However, the endothelium-independent vasodilation may also be abnormal [39], and following long-lasting diabetes, structural alterations may also occur. Regarding structural abnormalities of the coronary bed, both the inflammatory pathway [48] and the depletion of pro-angiogenic factors [50] due to hyperglycemia may lead to selective coronary microvessel rarefaction [20, 56]. Also, the formation of AGEs may modify microvessel structure through glycation, severely impairing their function [66], leading to cardiac fibrosis and diastolic dysfunction [67, 68], potentially resulting in heart failure with preserved ejection fraction (HFpEF), the main clinical manifestation of diabetic cardiomyopathy [33].

Even if the contribution of CMD to the clinical phenotype of diabetic cardiomyopathy has not been currently quantified relative to the other implied pathogenic mechanism, recent evidence has been provided regarding the relevant role of CMD in HFpEF. Indeed, among 202 unselected HFpEF patients (55 with diabetes), CMD was present in 75% and was associated with markers of heart failure severity (pro-brain natriuretic peptide and right ventricular dysfunction) [69].

## Clinical Assessment of CMD

The assessment of CMV is unique in the field of CAD in the sense that coronary microvessels are not amenable to anatomic imaging. In contrast, CMV state can only be assessed indirectly, through the measurement of CBF and its response to the administration of appropriate vasodilators or vasoconstrictors. In particular, several vasodilators are currently employed in clinical practice, and their use is the mainstay in the non-invasive assessment of CMV, including the endothelium-independent vasodilators adenosine and dipyridamole, the most widely used drugs. On the other hand, the use of a vasoconstricting stimulus (i.e. acetylcholine) generally requires invasive assessment of the coronary circulation, since epicardial or microvascular coronary vasospasm must be adequately excluded, which can currently only be determined through the use of invasive coronary arteriography (ICA).

### Non-Invasive Imaging Technique for the Assessment of CMD

#### Transthoracic Doppler Evaluation

Coronary CBF velocity can be measured using pulse wave Doppler in the coronary arteries using transthoracic echocardiographic color flow-guided imaging. In particular, the left anterior descending (LAD) coronary artery can be generally identified in its middle-to-distal tract in a large proportion of patients, namely 78–91%, using a left parasternal view with the patient laying on the left side [70]. CBF in the right coronary artery can be identified in the posterior descending artery (PDA) using a long axis view. Adequate imaging of the PDA artery can be obtained in approximately 70–80% of subjects. The distal part of the circumflex artery can be visualized in an apical four-chamber view, and adequate images

can be obtained in approximately 40–70% of the subjects [71, 72]. Due to convenience and higher rate of adequate imaging, isolated measures in the LAD are most commonly performed. CBF is measured in the resting condition by measuring the diastolic peak velocity at the Doppler spectral signal of the CBF. Maximal CBF is subsequently measured in the same way after obtaining maximal coronary hyperemia through the use of vasodilating drugs, most commonly intravenous adenosine 140 mcg/kg/min or intravenous dipyridamole (generally 0.56 mg/kg over 4 min or 0.84 mg/kg injected over 6 min) [71–73]. Since Doppler measurements are angle dependent, care should be taken in maintaining the same relative position of the incident ultrasound and the patient throughout the exam. The ratio of maximal CBF over resting state CBF provides a measure of CFR. The values of CFR are clearly normal when above 2.5, borderline between 2.0 and 2.5, and a CFR value below 2.0 is considered abnormal [74].

#### Contrast Echocardiography

Ultrasound contrast agents consist of microbubbles approximately the size of a RBC of high molecular weight inert gases, generally fluorocarbon, stabilized by a lipid coating [75]. These microbubbles resonate in response to ultrasound, returning a signal of low intensity and high frequency that can be detected by the ultrasound probe, and therefore visualized as a hyperechoic signal on echographic images. Since microbubbles are strictly intravascular, myocardial contrast enhancement during echocardiography reflects myocardial blood pool [76]. In particular, when the myocardium is fully saturated during continuous infusion of contrast, signal intensity is proportional to capillary density. Following microbubble destruction by a brief burst of high mechanical index ultrasound, the dynamics of myocardial contrast replenishment can be analyzed to evaluate myocardial blood flow (MBF) velocity. In particular, it takes approximately 5 s to fully replenish the myocardium, corresponding to a capillary MBF velocity of approximately 1 mm/s, with any decrease in MBF proportionally slowing down the replenishment proportionally to the blood flow impairment [77]. MBF can be estimated quantitatively as the product of maximum myocardial enhancement in dB and myocardial MBF velocity in dB/s [78]. The ratio of maximal hyperemia MBF, obtained during adenosine infusion, to resting MBF provides an estimate of CFR. CFR calculation can be performed in each of the 17 myocardial segments on apical transthoracic echocardiographic views, and the values may eventually be grouped in coronary territories.

#### Cardiovascular Magnetic Resonance (CMR) Imaging

CMR can be used to assess MBF. In particular, myocardial perfusion imaging on CMR is based on the changes of myocardial signal intensity during first-pass CMR images after injection of gadolinium-based paramagnetic contrast agents (Figure 13.1) [79]. In particular, 40–60 images are acquired per slice using the electrocardiogram R-wave as a trigger for the acquisition [80]. The acquisition protocol generally lasts approximately 60 s and requires breath holding to minimize motion artifacts. Signal intensity curves can then be calculated for each pixel over a set of images. From the analysis of signal intensity curves, one can estimate MBF [80]. The ratio of maximal hyperemia MBF to resting state MBF provides an estimate of CBF reserve [81].

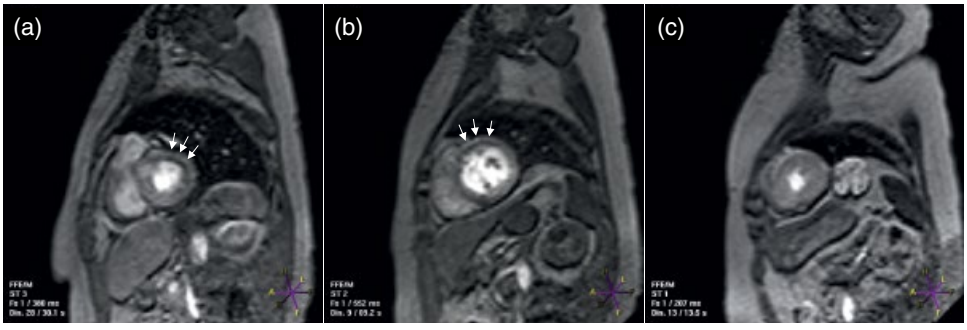


**Figure 13.1** Example of patient with microvascular angina detected by cardiac MRI. Short-axis first-pass dynamic gadolinium cardiac magnetic resonance images from base to apex (A through C, respectively) during dipyridamole infusion stress (0.56 mg/kg over 4 min). A hypoperfused subendocardial region, depicted as a region of lower enhancement, is detectable in the anteroseptal, anterior, and anterolateral walls of the left ventricle (white arrows). (*Source:* From Magnoni M. et al. [79]. Permission obtained from Wolters Kluwer Health, Inc.)

#### Positron Emission Tomography (PET)

PET is a nuclear imaging technique that employs positron-emitting tracers to investigate biological processes. These tracers emit a positron during their nuclear decay, which subsequently interacts with an electron resulting in the annihilation of both particles to produce two photons with opposite directions, which are then detected by the imaging apparatus. PET provides an accurate quantitative measure of MBF per unit of myocardial mass when appropriate tracers and mathematical models are used [82]. Currently, the most widely employed and validated tracers are $^{15}$O-labeled water ($H_2{}^{15}O$) and $^{13}NH_3$. These tracers have a very short half-life with the advantage of allowing the use of adequate tracer concentration without exposing the patient to a high radiation dose. On the other hand, they necessitate in situ production by a cyclotron, which significantly increases the cost of the exam, and therefore its widespread availability. The quantification of MBF in the resting and hyperemic states allows the ready quantification of CFR and allows the distinction of reduced CFR due to reduced maximal MBF, or due to increased resting MBF, as in the case of hyperadrenergic states [82].

### Invasive Techniques for the Evaluation of CBF

Invasive evaluation of CBF is currently regarded as the gold standard evaluation of the coronary microvascular function. Indeed invasive techniques allow the direct and accurate measurement of CBF. On the other hand, the widespread use of these methods is hampered by high costs and, most importantly, by the risk of serious complications that pertain to invasive strategies.

#### Indicator Dilution-Based Methods

According to Fick's principle, the blood flow into an organ can be measured using an indicator as long as the amount of indicator taken up by the organ per unit of time is

known and the arteriovenous difference in terms of indicator concentration is known. Based on this assumption, several invasive methods for the quantification of CBF have been developed.

#### Thermodilution

In its original description, a catheter was inserted into the coronary sinus. Subsequently, a known volume of room-temperature saline was infused at a fixed rate, and blood temperature was measured downstream. The reduction in temperature measured is proportional to CBF. No distinction between coronary perfusion territories can be made in this way, and the unlikely stability of the catheter within the coronary sinus makes this method unsuitable for detecting small changes (i.e. less than 30%) in CBF [83]. Subsequently, the introduction of miniaturized thermal sensors incorporated into coronary wires has allowed intracoronary thermodilution. In this case, a 3-ml bolus of room-temperature saline is injected into a coronary artery, and the distal sensor captures the resulting temperature shift. CBF is inversely proportional to temperature transit time. CFR can be calculated as the ratio of hyperemic on resting CBF [84].

#### Gas Wash Out Method

In this case, the indicator is an inert gas, generally argon or xenon. The differential concentration between the site of injection and downstream in the coronary sinus allows the calculation of total CBF. The gas can be used as a radiotracer and eventually inhaled for 5 min at a known concentration. Using gas chromatography, one can then measure the concentration of gas both in the coronary artery and in the coronary sinus. Knowing the tissue concentration of the gas and the arteriovenous difference, CBF can be calculated according to Fick's law. Alternatively, gas can be injected directly into the coronary artery, and regional CBF can then be calculated based on the scintigraphic detection of the radiolabeled gas [85].

#### Intracoronary Doppler Flow Wire

Intracoronary Doppler wires allow the evaluation of CBF velocity directly in the coronary arteries. According to the continuity equation, CBF velocity multiplied by the coronary artery cross-sectional area yields CBF. Currently, the main limitation of this method is the quantification of the coronary artery cross-sectional area, even when quantitative coronary angiography is used [86]. At the same time, coronary microvascular resistance can be calculated to obtain a quantitation of coronary microvascular function. In particular, coronary microvascular resistance equals the difference between mean aortic pressure and right atrial pressure divided by CBF.

Currently, the diagnosis of CMD as well as its clinical manifestation, microvascular angina, remains a complex clinical challenge. The diagnosis may usually be made in the presence of normal or non-obstructed coronary arteries documented by ICA or non-invasive coronary computed tomography angiography (CCTA), objective indirect signs of inducible ischemia (chest pain and/or ECG ischemic changes, such as ST depression during exercise or pharmacological stress test), and the evidence of reduced CFR by non-invasive or invasive techniques (Figure 13.2).

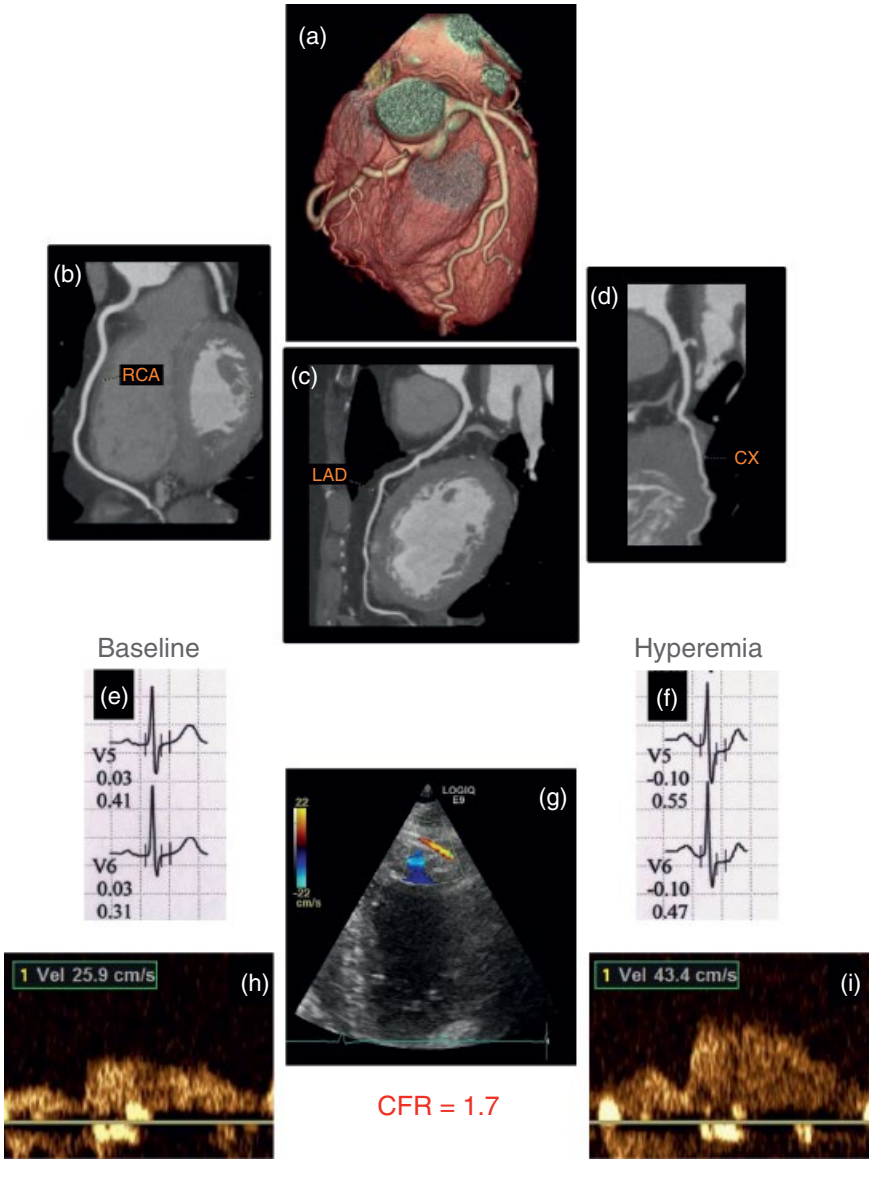


**Figure 13.2** Illustrative example of the comprehensive non-invasive diagnostic workflow in a patient with coronary microvascular dysfunction (CMD). Panels A to D: anatomical imaging by coronary computed tomography angiography (CCTA) shows absence of obstructive and non-obstructive atherosclerotic plaques in epicardial coronary arteries. Panels E to I: functional imaging test by transthoracic Doppler echocardiography and ECG monitoring at rest and during hyperemic stimuli (intravenous dipyridamole). Panels E and F: slight ST depression at the peak of dipyridamole infusion with respect to baseline. Panel G: color Doppler flow imaging of the middle-to-distal portion of left anterior descending artery (LAD). Panels H and I: Doppler-derived coronary flow velocity of LAD at rest (H) and during hyperemia (I), used to coronary flow reserve (CFR) (coronary flow velocity during maximal vasodilation/coronary flow velocity at rest). In this patient CFR was 1.7, consistent with the presence of CMD. (*See color plate section for color representation of this figure.*)

## Treatment for CMD

Current evidence relating to the efficacy of pharmacological treatments for CMD is still weak and sparse, with most available studies being small and not randomized, further varying in enrolment criteria and study end points [87].

Regardless of the clinical phenotype in which CMD translates, or to which CMD contributes, the individual background of CMD risk factors should be corrected, through evidence-based treatment of arterial hypertension, lipid-lowering therapies, smoking cessation protocols, weight, and glycemic control [88]. Of note, while poor glycemic control correlates with increased cardiovascular risk, whether glucose lowering improves cardiovascular outcomes is less well established [89, 90]. Further, it is unclear if glycemic control can contribute to the prevention or reversal of diabetic CMD. In this regard, no correlation between optimal glycemic control and CMD was demonstrated among patients with and without type 2 diabetes [91].

In the setting of microvascular angina, the latest European Society of Cardiology guidelines for the management of stable CAD recommend the use of secondary prevention treatments such as aspirin and statins with class of recommendation I and level of evidence B [88]. Despite their founded prognostic role in epicardial CAD, no definite parallel evidence is available for CMD populations. Nevertheless, statins improve CFR, ischemia, and exercise capacity in patients with CMD, possibly through pleiotropic mechanisms related to NO elevation and endothelin-1 reduction [92–94].

CMD patients may have heterogeneous responses to anti-ischemic drugs with established efficacy in epicardial CAD, thus often requiring experimentation with different drug combinations before satisfactory symptom control. Evidence regarding the efficacy of short-acting nitrates on chest pain patients with CMD is inconclusive [85]. However, their use may be considered especially with a background of beta blocker or calcium channel blocker therapy. Long-acting nitrates have not shown positive effects in this population [95, 96].

Beta blockers, in particular, nebivolol and atenolol, are the anti-ischemic first-line treatment in CMD, especially when high resting heart rate or increased sympathetic activity is present [88, 96–98]. Their use should be avoided if concomitant vasospastic angina is present as they may exacerbate symptoms in this setting [99].

Calcium channel blockers should be used as first-line therapy when beta blockers are contraindicated [88], or a significant variable threshold of effort angina is present [100]. Only dihydropyridine calcium channel blockers should be used in combination with beta blockers when symptom control is not achieved.

Angiotensin-converting enzyme (ACE) inhibitors improve CFR and exercise capacity in CMD, possibly by improving endothelial dysfunction as well as counteracting the vasoconstrictor effects of angiotensin II. The use of ACE inhibitors should be especially considered in patients with diabetes and/or arterial hypertension [88, 101, 102].

Further anti-ischemic drugs (including alpha blockers, ivabradine, nicorandil, ranolazine), drugs inhibiting nociception (xanthine derivatives), and drugs acting on myocyte metabolism (trimetazidine) may be of benefit on an individual basis in CMD patients unresponsive to first-line treatments [87, 88].

Patients with refractory angina to optimal or maximally tolerated medical therapy may benefit from additional non-pharmacological interventions such as spinal cord stimulation and enhanced external counterpulsation [88].

### The Effects of Glucose-Lowering Drugs on CMD

Beyond glucose control, glucose-lowering drugs exhibit favorable effects on different aspects of cardiovascular pathophysiology and subsequent clinical outcomes [103], thus potentially orientating therapeutic choice in the diabetic patient with CMD.

- *Biguanides*. Metformin may enhance NO production by activation of the AMP-activated protein kinase and, thus, potentially improving CMD [104]. In a small double-blind, randomized placebo-controlled study of non-diabetic women with microvascular angina, metformin 500 mg twice a day was associated with significant reduction in chest pain incidence, microvascular response to acetylcholine in the forearm, and maximal ST segment depression [105].
- *Thiazolidinediones*. Activators of PPAR-γ may protect vascular function and improve endothelial dysfunction in diabetes mellitus [89]. In type 2 diabetic mice models, rosiglitazone was associated with increased NO-mediated dilation of coronary arterioles, enhanced vascular catalase activity, and reduced vascular NADPH oxidase activity [106], and pioglitazone has preserved the microvascular structure [107]. In humans, rosiglitazone increases the endothelial number and migratory activity of cultured progenitor cells of diabetic patients [108]. Furthermore, patients with metabolic syndrome who were treated with rosiglitazone showed both a reduction in circulating inflammatory markers and improvement in endothelial function [109].
- *Sodium-glucose cotransporter 2 (*SGLT2*) inhibitors*. Empagliflozin exhibits a potential cardioprotective effect beyond its glucose-lowering effects, which may result, among different mechanisms, from the improved coronary microvascular function. Indeed, empagliflozin may increase barrier function, microvessel density, eNOS phosphorylation, endothelial-dependent relaxation, and endothelial cell survival, through inhibition of diabetes-induced mitochondrial fission by an AMP-activated protein kinase, as assessed in diabetic mice [110].

### Future Perspectives for the Treatment of CMD in Diabetes

Different therapeutic strategies, targeting pathways that may specifically contribute to CMD in diabetic patients, are under current clinical or preclinical evaluation.

- *Mineralocorticoid receptor (*MR*) inhibitors*. Excess MR activation contributes to vascular injury in diabetes [111, 112]. In this context, MR blockade may reduce CMD, as assessed in a rodent model of angiotensin II-dependent cardiovascular injury [113] and in type 2 diabetes patients without clinical ischemic heart disease, where the addition of spironolactone to ACE inhibitor therapy was associated with improved CFR in quantitative PET [114].
- *Rho-kinase inhibitors*. Rho-kinase-mediated calcium sensitization of the myosin light chain in smooth muscle cells leading to vasoconstriction may contribute to diabetic cardiomyopathy, retinopathy, cerebrovascular disease, and nephropathy [115]. Fasudil, a Rho-kinase inhibitor, was demonstrated to improve CMD and early-stage diabetic retinopathy, thus representing a potential valid therapeutic option in these settings [116, 117].
- *Arginase inhibition*. Arginase inhibition through the intra-arterial infusion of N(ω)-hydroxy-nor-l-arginine markedly improved endothelial function in diabetic patients

with and without CAD disease [118, 119]. Whether chronic oral therapy may be effective remains to be tested; nonetheless, this finding suggests that arginase may serve as a potential therapeutic target for improvement of microvascular function.

- *PolyADP-ribose polymerase-1 (*PARP-1*) inhibitors*. Increased PARP-1 activity contributes to microvascular dysfunction in type 2 diabetes. PARP-1 inhibitors, currently used in the oncologic field, may improve microvascular function in type 2 diabetic mice through the restoration of the eNOS-cGMP pathway, thus representing an appealing target for future clinical evaluation [120]. As detailed above, changes in the function of RBCs induced by ROS stimulating arginase I cause endothelial dysfunction in patients with diabetes [121]. This novel finding suggests that RBCs, specifically RBC arginase I, are potential therapeutic targets for the treatment of endothelial dysfunction in this population. Additional studies are needed to develop therapeutic interventions that address this mechanism of endothelial injury.
- *Neoangiogenesis stimulation*. Intramyocardial injection of human CD34+ cells may improve symptomatic and prognostic outcomes through neoangiogenesis stimulation in patients with chronic refractory angina [122]. Given this role in capillary rarefaction of diabetes, the promotion of neoangiogenesis may also be an appealing strategy in patients with diabetic cardiomyopathy or microvascular angina. In this regard, pro-angiogenic gene therapy via thymosin beta 4 (Tb4) or vascular endothelial growth factor-A (VEGF-A) was found to induce capillary growth in the hibernating myocardium of a transgenic diabetic pig model (InsC94Y). Importantly, only Tb4 yielded sufficient microvessel maturation and macrovascular growth to improve perfusion and myocardial function [20].

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# 14

# Cerebral Microvascular Disease in Diabetes – Clinical and Research

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## Introduction

Both type 1 and type 2 diabetes mellitus are related to microvascular complications, such as retinopathy, neuropathy, and nephropathy. Over the past few decades, interest in the brain as a target organ for diabetes-related microvascular complications has accumulated. This interest was primarily driven by the recognition that diabetes was also related to neurocognitive problems, such as problems with memory, attention, or doing multiple things at the same time. It was almost a century ago that Miles and Root published the first ever article discussing cognitive problems in a group of diabetic patients in 1922 [1]. It was the first paper that demonstrated that diabetes also negatively affects the brain. Although the underlying mechanisms were clearly unknown, the clinical problem was known. Further evidence that diabetes is also related to damage in the brain comes from early postmortem studies. For example, in the late 1960s Reske-Nielsen and her colleagues performed a series of postmortem studies in young adults with type 1 diabetes with a long disease duration. They identified a series of changes in the brain that could be related to damage to small cerebral vessels, demyelination, ischemic damage, and atrophy [2, 3]. They concluded that this pattern was sufficiently different from other clinical conditions to justify using the term diabetic encephalopathy. It wasn't however until the expansion of neuropsychology into the field of somatic diseases and the introduction of widely available magnetic resonance imaging (MRI) in later years that research into the effect of diabetes on the brain accelerated.

In this chapter, we will focus on expressions of cerebral microvascular vessel disease in patients with type 1 and type 2 diabetes. Neuroradiologically, cerebral small vessel disease is defined as ischemic white matter hyperintensities, cerebral lacunar infarcts, and cerebral microbleeds [4, 5]. The first focus will be on postmortem studies in both patient groups. Thereafter, we shift toward the classical neuroradiological expressions of cerebral small

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vessel disease in humans with diabetes. We then start focusing on changes in gray and white matter integrity, and how brain regions communicate with one another, and how microvascular disease, peripheral and central, influence these cerebral parameters. After this we will detail how cerebral microvascular disease is associated with clinical parameters, including cognitive functioning and dementia. We will end with how these findings can be translated to personalized diabetes care and suggest future areas for study.

## Postmortem Studies of the Brain in Diabetes

Before the wide availability of MRI scanners, postmortem studies were the only available option to study the changes in the brain due to diabetes. In 1966 Reske-Nielsen and colleagues from Aarhus University in Denmark published the results of postmortem study in young type 1 diabetes patients [3]. They included 16 patients between the ages of 21 and 43 years (mean of 31 years), of whom 10 were male. The mean disease duration was 24 years (range between 16 and 36 years) with a mean onset age of 7 (range between 3 and 18 years). Despite the relatively young age of these patients, all had retinopathy and nine were blind or nearly blind. Many had nephropathy, heart disease, mental disturbances, and hypertension, and all patients had signs of neuropathy. They all died due to the effects of diabetic angiopathy. A consistent pattern of atrophy of the dentate nucleus, demyelination of the cranial nerves, fibrosis of the leptomeninges, softening of brain areas, and angiopathy was observed in these young patients. This pattern seemed to have a dual pathogenesis: on the one hand ischemia, which is caused by the angiopathy; and on the other hand, a more diabetes-specific brain abnormality pattern of demyelination and atrophy. From this the authors concluded that the term diabetic encephalopathy was justified, although this term never caught on.

In 1973 an autopsy study was published by Aronson including 5479 autopsies of which 677 individuals had diabetes, and most had type 2 diabetes [6]. In these patients with type 2 diabetes, infratentorial encephalomalacia, or softening of brain tissue, was more prevalent than in the brains of non-diabetic subjects. Aronson also showed that infarctions within the brain were more prevalent in the diabetes group than in the non-diabetes group, suggesting a relationship between type 2 diabetes and cerebral small vessel disease. The presence of cerebral small vessel disease in type 2 diabetes was later replicated by Kameyamai and colleagues [7]. They also observed severe cerebral atherosclerosis in these patients. None of their patients, however, died from their cerebral small vessel disease. In the recent Finish Vantaa 85+ study 553 residents of the city of Vantaa were included and examined several times [8]. In 291 subjects on whom autopsy was performed 70 had diabetes. Of those diabetic patients 59.7% had cerebral infarctions, one of the expressions of cerebral small vessel disease. This was significantly more than the 44.6% in the group of non-diabetic subjects, with an odds ratio of 1.84, indicating an almost doubled risk of cerebral infarctions in diabetes patients [8]. Interestingly, the risk of presence of beta-amyloid and neurofibrillary tangles – the pathological hallmarks of Alzheimer's disease – was lower in patients with diabetes (odds ratios: 0.45 for beta-amyloid and 0.70 for neurofibrillary tangles). The clinical impact of cerebral small vessel disease is further discussed in the section below.

To summarize, early and also more recent postmortem studies show that cerebrovascular lesions are prevalent in both types of diabetes. Earlier studies also report atrophy, fibrosis, and other pathological changes. These changes may have become less pronounced over the years with the introduction of intensive glucose lowering therapies for type 1 diabetes and better oral hypoglycemic medication for patients with type 2 diabetes, whereas the risk factors for the development of vascular damage, such as hypertension and dyslipidemia, remain, especially during middle age before the development of diabetes [9].

## Cerebral Small Vessel Disease on MRI

Cerebral small vessel disease refers to the progressive damage of small arteries, capillaries, venules, and arterioles in the brain. This process occurs naturally with aging, i.e. older people will have more expressions of cerebral small vessel disease than younger people do. There are also several risk factors that lead to an earlier development of cerebral small vessel disease. Examples are diabetes, hypertension, dyslipidemia, but also other somatic systemic disease such as HIV/AIDS, and even radiation therapy can cause cerebral small vessel disease.

Despite the high cost of MRI scanners they are now widely available and they are a powerful tool to study cerebral small vessel disease in diabetes patients in vivo. With the increasing field strength of the magnet it also becomes easier to detect cerebral small vessel disease. Classically, cerebral small vessel disease is characterized as lacunar infarcts (Figure 14.1 black arrows) and periventricular and deep white matter hyperintensities (Figure 14.1 white (periventricular) and black (deep) squares). Lacunar infarcts, or lacunes appear on MRI sequences as round or ovoid cavities between 3 and 15 mm in diameter. They can be identified on MRI scans with a T1-weighted contrast. They represent occlusion

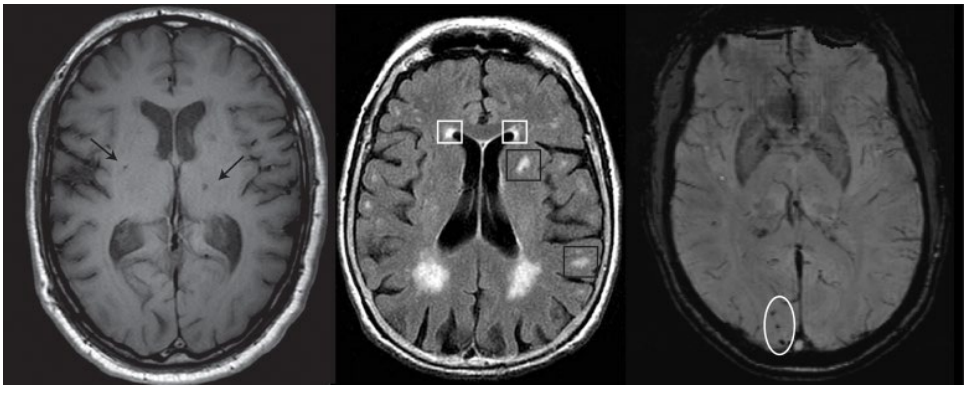

**Figure 14.1** Markers of cerebral small vessel disease on magnetic resonance imaging (MRI) images. On the left side a T1-weighted image showing lacunar infarcts below the black arrows. On the middle image a T2/FLAIR image showing periventricular lesions in the white squares and deep white matter hyperintensities in the black squares. The T2*/SWI image on the right is showing cerebral microbleeds within the white ellipse. FLAIR = fluid-attenuated inversion recovery. SWI = susceptibility weighted image. (*See color plate section for color representation of this figure.*)

of single perforating arterioles and occur mainly in the subcortical regions, such as the thalamus, putamen, and caudate nucleus, but sometimes also in the white matter [4, 10]. Lacunar infarcts are present in up to 28% of the general population but diabetes, mainly type 2, is a strong risk factor [11]. Although they are thought to be clinically silent, studies have shown relationships between the presence of lacunes and cognition, stroke, motor symptoms, and premature death [11]. Presence and location are usually identified by visual rating.

White matter hyperintensities appear as a hyperintense signal on specific MRI sequences in the white matter. The most specific sequences are the fluid-attenuated inversion recovery and T2/proton density weighted images. They are irregular in shape and are mainly found in the deep white matter (black squares in Figure 14.1) or surrounding the ventricles (white squares in Figure 14.1) [4, 10]. White matter hyperintensities on MRI represent ischemia due to a variety of causal mechanisms and are present in 39–96% of the population, depending on the study sample chosen [12]. Various visual rating scales exist to assess the severity of white matter hyperintensities, of which the Fazekas scale is frequently used. It uses a scale ranging from 0 (no lesions), 1 (small punctate lesions), 2 (beginning confluent lesions), to 3 (confluent lesions) [13]. Diabetes, smoking, and hypertension are common risk factors [12], and the presence and severity of these white matter lesions also seem to relate to poorer cognitive functioning [14], and thus are clinically significant.

Cerebral microbleeds represent the third form of cerebral small vessel disease that is visible on MRI (white ellipse in Figure 14.1) [4, 5, 10]. The most useful MRI sequences for the detection of microbleeds are T2*-weighted ones, such as susceptibility weighted images. They show as small dot-like hypointense lesions of about 2–5 mm in size. The presence and the amount of these microbleeds are rated visually and usually divided into infratentorial/deep and supratentorial microbleeds [15]. This distinction is important because of the differential pathogenesis of these microbleeds. Infratentorial and deep cerebral microbleeds are usually related to hypertension and other vasculopathy, whereas supratentorial microbleeds are more related to cerebral amyloid angiopathy and the ε4 allele of the apolipoprotein E protein [15]. The prevalence seems to vary widely, from 5% in the general population to up to 85% in patients with vascular dementia. The Rotterdam Scan Study showed that the presence of supratentorial microbleeds was related to poorer cognitive functioning in the general elderly population [16], whereas the Age, Gene/Environment Susceptibility-Reykjavik (AGES-Reykjavik) study showed a relationship between poorer cognition and mainly infratentorial/deep cerebral microbleeds [17]. In any case, it is indicative that cerebral microbleeds are not clinically silent.

## MRI-Measured Cerebral Small Vessel Disease in Type 1 Diabetic Patients

### White Matter Hyperintensities

The prevalence of cerebral small vessel disease is relatively understudied, with only a few papers reporting on these measures. An early study from 1991 used MRI and identified subcortical or brain stem lesions with hyperintense signal in 11 of the 16 included type 1

diabetes patients compared with 5 of the 40 controls, which was significantly different [18]. More information on complication status, hypertension, lipid control, and other factors related to type 1 diabetes or white matter hyperintensities were not given.

In a general group of 114 young adult type 1 diabetes patients, Weinger and colleagues rated the presence and severity of white matter hyperintensities according to the Fazekas scale [19]. Deep white matter lesions were found in 31% of the type 1 diabetes patients and in 40% of the control group participants. However, this difference of 9% was not statistically significant. Conversely, periventricular white matter lesions were present in 30% of the diabetic patients and in 16% of the non-diabetic controls. This was borderline statistically significant, but lost statistical significance after correction for confounding factors [19]. Within the type 1 diabetes group there were no effects of hypertension, cholesterol levels, levels of lifetime glycated hemoglobin ($HbA_{1c}$), depression, or severe hypoglycemic events. Interestingly, the presence of either background or severe retinopathy was also unrelated to the presence of white matter hyperintensities in this group of young patients [19]. Besides prevalence, the severity of the lesions found did not differ between the groups; only eight patients had grade 2 deep white matter lesions and four had grade 2 periventricular white matter hyperintensities. This study replicated findings from a study in 40 patients with type 1 diabetes who were aged 50 years and above at inclusion [20]. They rated deep and periventricular lesions according to the Scheltens scale [21]. This scale provides a more elaborate rating of white matter hyperintensities than the Fazekas scale, also including the size of lesions. Both deep and periventricular white matter lesions were not more prevalent or more severe in the diabetic group compared with 40 non-diabetic controls. In both groups the prevalence of silent infarcts was 8% [20]. These results are despite the long diabetes duration within the group, and the presence of severe microvascular complications in the majority, of which proliferative retinopathy was the most common, suggesting poorer diabetes control over time. However, given their age, which was on average 60 years, these patients can be considered survivors and may be less representative of the entire type 1 diabetes population. The conclusion that there is no interaction between older age and the prevalence of white matter hyperintensities in type 1 diabetes needs to be drawn with care.

That these last two studies did not find a relationship of white matter hyperintensities with retinopathy is interesting as retinopathy is usually considered to be a proxy for cerebral microvascular disease [22, 23], and it may suggest that there is no direct relationship between peripheral microangiopathy and white matter lesions. This hypothesis seems to be supported by a study performed by Yousem and colleagues, which was published in 1991. They included 40 young type 1 diabetes patients with retinopathy who were compared with 10 age-matched controls [24]. No difference in prevalence or severity of white matter hyperintensities was found. In one of our studies comparing middle-aged patients with type 1 diabetes with proliferative retinopathy or without microvascular complications with matched controls, we also failed to find a difference in white matter hyperintensity prevalence, although no distinction between deep and periventricular lesions was made [25]. This is in apparent contrast with a study by Ferguson and colleagues, who compared 25 type 1 diabetes patients with background retinopathy and a mean age of 32 years with 46 patients without retinopathy and a mean age of 27 years [26]. Both had a mean onset age of 10 years, with a duration of the disease of 22 versus 17 years of age. There were no differences in brain volumes between the groups in the basal ganglia, which are part of the

subcortical structures, but the prevalence of white matter lesions was 33.3% in the group with retinopathy and 4.7% in the group without retinopathy [26].

A recent study decided to take a slightly different approach. Besides using the Fazekas scale to rate prevalence and severity of white matter hyperintensities, Nunley and colleagues also determined volume of the lesions by automated lesion classification [27]. They included 97 middle-aged patients with longstanding type 1 diabetes who all had a childhood onset age and compared them with 81 non-diabetic controls. Here, 32 patients had Fazekas score 2 or 3 relative to six non-diabetic controls. High lesion volume in the type 1 diabetes patients was related to older age, longer disease duration, presence of cardiac autonomic and peripheral neuropathy, higher levels of advanced glycation end products in the skin, and smoking [27]. Interestingly, high lesion volume was not related to glucose control, retinopathy, or blood pressure factors. In this study lacunar infarcts were also assessed, but the prevalence was low. They were found in five patients with type 1 diabetes and were absent in the controls.

## Cerebral Microbleeds

Studies that report on cerebral microbleeds in type 1 diabetes are rare. One recent study by Woerdeman et al. compared the prevalence and severity of white matter hyperintensities, and the prevalence of lacunar infarcts and cerebral microbleeds in 33 patients with proliferative retinopathy and 34 patients without microvascular complications with that of 33 non-diabetic controls [25]. The prevalence of white matter lesions in percentages was higher in patients with proliferative retinopathy, although it was not statistically significantly higher in this group compared with patients without microangiopathy and controls (24.2% versus 14.7% and 15.2%, respectively). Lacunar infarcts were not present in these groups [25]. Seven patients with proliferative retinopathy (21%) had at least one microbleed. This was significantly higher than the 3% (one patient) in the group without microangiopathy and the 9% (three controls) in the controls [25]. The absolute number of microbleeds was low, with six participants having one microbleed, four having two, and one having eight cerebral microbleeds. Not only was the prevalence of microbleeds clearly related to the presence of proliferative retinopathy, it was also related to poorer microvascular functioning in the finger, indicating that microbleeds in type 1 diabetes may be part of a generalized microvascular dysfunction [25].

## Summary

To summarize, there is little known about the prevalence of cerebral small vessel disease detected via MRI in patients with type 1 diabetes. It seems that, in the general type 1 diabetes population, white matter hyperintensities are not more prevalent or severe. However, patients with retinopathy and especially those with end-stage proliferative retinopathy seem to be more susceptible to white matter hyperintensities. There is very little known about lacunar infarcts, but their presence seems limited. This also holds for cerebral microbleeds, although their presence, similar to the prevalence of white matter lesions, seems related to prevalent retinopathy. A limitation of these studies is that, except for one study, they were all performed in young to middle-aged type 1 diabetes patients. We know that

cerebral small vessel disease is more prevalent in the elderly population and that the life expectancy is rapidly rising due to the improvements in diabetes treatment. This means that more type 1 diabetes patients, and not solely survivors, will reach older age and may develop more pronounced cerebral small vessel disease. Identifying the determinants of cerebral small vessel disease may help develop strategies to prevent, stop, or slow down these cerebral changes in an aging population.

## Type 2 Diabetes and Cerebral Small Vessel Disease

### Lacunar Infarcts

More studies have been performed in an effort to identify the relationship between type 2 diabetes and cerebral small vessel disease, as this disease is more prevalent than type 1 diabetes and many epidemiological studies have been performed in elderly populations, which naturally have an overrepresentation of type 2 diabetic patients. For this group, some meta-analyses or systematic reviews are available. A very complete meta-analysis was published in 2006 by van Harten and her colleagues [28]. They included studies that detailed the presence of lacunar infarcts and white matter lesions. Most of these studies covered patients with type 2 diabetes and used MRI, although some also included type 1 diabetes patients or used computerized tomography (CT). Of the 19 studies, 3 included general cohorts, 12 included vascular cohorts and 4 included cohorts of outpatients. Five studies, all including vascular cohorts, used CT to identify lacunar infarcts. In total, 14,953 controls and 2225 mostly type 2 diabetes patients were represented in these studies. For the studies including general cohorts, the odds ratio for having lacunar infarcts in type 2 diabetes was 1.3 (95% confidence interval: 1.1–1.6). For the vascular cohorts, the odds ratio was 2.2 (95% confidence interval: 1.9–2.5) and for the outpatient studies the odds ratio was 1.4 (95% confidence interval: 1.1–1.8). This indicated that patients with type 2 diabetes have a significantly increased risk of having lacunar infarcts compared with the general population, independent of cohort type, although the risk is clearly the highest when patients with vascular disease are included.

This strong and consistent association between type 2 diabetes and the presence of lacunar infarct was replicated by a more recent systematic review summarizing over 25 papers studying this association [29]. A case–control study including 350 type 2 diabetes patients and 363 controls showed that lacunar infarcts were present in 75 (21%) patients and in 58 (16%) of the controls, a significantly higher rate [30]. There was no analysis performed identifying the underlying mechanisms of lacunar infarct presence in this study. However, of the patients included, 72% had hypertension, 23.4% ischemic heart disease, 10.6% had had **a transient ischemic attack** or stroke, and 47.7% had hyperlipidemia [30]. All these are risk factors for lacunar infarct presence and may explain the higher prevalence, as the percentages of these prevalent risk factors are higher than in the control group.

It is clear that there is a strong cross-sectional relationship between type 2 diabetes and lacunar infarcts, but the data on longitudinal changes are less clear-cut. Both the Rotterdam Scan Study and the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) study did not observe a change in lacunar infarct presence over a time period of 3 years in

patients with diabetes [14, 31]. In type 2 diabetes patients, longer disease duration, insulin resistance, nephropathy, and higher blood pressure were found to be related to the incidence of lacunar infarcts in various studies [29]. In acute stroke patients with type 2 diabetes, higher levels of serum triglycerides were also related to an increased prevalence of lacunar infarcts [32].

Given the relationship between type 2 diabetes and lacunar infarcts, it may be hypothesized that people with prediabetes and the metabolic syndrome are also prone to development of infarcts, as these syndromes are related to the same cardiovascular risk factors as type 2 diabetes is. The metabolic syndrome is defined as central obesity, and the presence of two of the following factors: raised triglycerides, reduced high density lipoprotein (HDL) cholesterol, raised blood pressure, and raised fasting plasma glucose [33]. Indeed, in a large study by Kwon et al. it was shown that the presence of the metabolic syndrome in otherwise healthy participants was related to an increased risk of lacunar infarcts (odds ratio 2.18, 95% confidence interval: 1.38–3.44) [34]. Of the separate components, elevated blood pressure (odds ratio 3.75, 95% confidence interval: 2.05–6.85) and impaired fasting glucose (odds ratio 1.74, 95% confidence interval: 1.08–2.80) were related to this increased prevalence of lacunar infarcts on MRI. This was later replicated in a Japanese study showing an odds ratio of 2.43 (95% confidence interval: 1.53–3.87) for having lacunar infarcts in subjects with the metabolic syndrome, with increased BMI, elevated blood pressure, and impaired fasting blood glucose being the components that are independently related to an increased prevalence of infarct [35].

### White Matter Hyperintensities

The relationship between type 2 diabetes and white matter lesions is subject to more controversy [36]. The above-mentioned meta-analysis by van Harten et al. included nine studies with in total 7224 controls and 936 type 2 diabetes patients [28]. For the five studies including vascular cohorts there was a non-significant odds ratio of 1.1 (95% confidence interval: 0.9–1.4) for white matter lesions in the diabetic patients. In the outpatient cohort studies the odds ratio for any white matter lesion was statistically significantly higher for type 2 diabetes patients (2.4, 95% confidence interval: 1.7–3.4), but not for periventricular white matter hyperintensities (odds ratio: 1.8, 95% confidence interval: 0.9–3.6) or for deep white matter lesions (odds ratio: 1.7, 95% confidence interval: 0.9–3.5). Much of this controversy may be due to the various ways of rating white matter hyperintensities on MRI. This can be done by rating scales, such as the Fazekas and Scheltens scales (the latter being more elaborate), which given an idea of severity of the lesions present, by simple yes/no present, or by (semi)automated or manual segmentation of lesions and determine volume.

One such case–control study used an adaptation of the Scheltens scale to determine deep and periventricular white matter hyperintensities in 113 type 2 diabetes patients and 51 controls [37]. The severity score of the deep white matter lesions was significantly higher in the diabetes group relative to the control group, with age and present macrovascular events being risk factors for this higher score [37]. This result was replicated when assessing volume of white matter lesions in 99 type 2 diabetes patients in relation to the volume

of 49 controls. The type 2 diabetes patients had a 56.5% higher lesion volume than the controls [38]. In patients with symptomatic atherosclerotic disease and type 2 diabetes white matter lesion volume was also found to be higher compared with non-diabetic patients [39]. The difference between the groups was 0.31% (95% confidence interval: 0.09–0.53%), which was a lot smaller than previously reported [37, 38]. This may be due to the fact that both patient groups share the same cardiovascular risk factors, whereas in other studies cardiovascular risk factors were not present or were less prevalent in the control groups. In 1366 women studied as part of the Women's Health Initiative MRI study, those with type 2 diabetes showed at baseline a larger white matter lesion volume load diffusely spread throughout the brain. Overall the lesion volume was 21.8% higher in those with diabetes compared with women without diabetes [40]. This finding was also observed in the AGES-Reykjavik study, in which the 462 patients with diabetes (11% of the total) had a higher white matter hyperintensity volume than the non-diabetic group [41].

On the other hand, there have been multiple case–control and population-based studies that did not observe an association of white matter lesions with type 2 diabetes [10, 29]. The case–control study by Moran and colleagues found the white matter lesion volume to be $6.04 \pm 6.99$ ml in the 350 type 2 diabetes patients and $7.10 \pm 8.0$ ml in the 363 controls participating in the study [30]. Although this was not statistically significant, it suggests that type 2 diabetes patients are less likely to develop white matter lesions despite the many cardiovascular risk factors. One reason for this seemingly counterintuitive finding could be that the type 2 diabetes patients are better controlled with respect to risk factors for white matter hyperintensities. For example, the control group in this study used significantly less blood pressure and lipid lowering medication, which was mirrored by more favorable blood pressure and lipid profiles in the patients [30]. The PROSPER study also did not find a difference in white matter lesion volume in the 89 type 2 diabetes patients compared with the 438 control participants [31]. Here the total white matter hyperintensity volume was $4.19 \pm 0.82$ ml in the patients versus $5.34 \pm 0.48$ ml in the controls. The investigators also assessed periventricular (patients: $3.29 \pm 0.69$ versus controls: $4.19 \pm 0.42$ ml) and subcortical (patients: $0.90 \pm 0.16$ versus controls: $1.15 \pm 0.08$) white matter lesion volume, but there were no statistically significant differences [31]. Again it seems that lesion load is higher in the controls than in the patient group. Although this could be due to the sample size difference between the groups, the lipid profile is again more favorable in the patient group, although the blood pressure is lower in the control group. The results of longitudinal changes in white matter hyperintensities in type 2 diabetes are just as heterogeneous as the cross-sectional data are [29, 36].

The PROSPER study also failed to find such an accelerated increase in white matter lesion volume in the type 2 diabetes patients [31]. This is similar to the findings of the study comparing diabetes and non-diabetes patients with clinically manifest atherosclerotic disease [39], and a case–control study of outpatient type 2 diabetes patients after a 4-year follow-up period [42]. Several other population-based studies, however, did observe an association between type 2 diabetes and an accelerated increase in white matter hyperintensities. In the Women's Health Initiative MRI study, 698 of the original 1366 women underwent a second MRI session after a mean follow-up period of almost 5 years. The mean white matter lesion load for diabetic women was, adjusted for baseline, $8.04 \pm 0.36$

and $7.33 \pm 0.10$ ml for non-diabetic women [40]. This difference of 0.71 ml or 9.7% reached statistical significance. Progression of white matter hyperintensities related to type 2 diabetes was also found in two other population-based studies [43, 44]. Because of these contradictory results, it remains unclear what factors relate white matter hyperintensity presence and severity in type 2 diabetes. Some studies have shown poorer glycemic control (i.e. higher $HbA_{1c}$), longer disease duration, and insulin resistance to be possible risk factors [29, 36]. It should be noted that the studies that have reported differences between type 2 diabetes patients and controls show an increase of about 20% in lesion volume. Such differences are, in light of the relatively large inter-individual variation in white matter lesions, relatively small. Taken together, it can be concluded that type 2 diabetes does not have a major effect on the presence and severity of white matter lesions on MRI.

## Cerebral Microbleeds

Compared to lacunar infarcts and white matter hyperintensities, relatively little attention has been awarded to the effect of type 2 diabetes on cerebral microbleeds [10, 29]. As with white matter hyperintensities, the literature for cerebral microbleeds is somewhat contradictory. Two case–control studies did not observe a higher prevalence of cerebral microbleeds in type 2 diabetes patients. The first study, by Moran and colleagues, identified 14 patients (4% of the total) and 22 controls (6% of the total) that had at least one microbleed in the brain [30]. The second study, by Brundel et al., identified 16 of the 48 (33%) type 2 diabetes patients and 20 of the 49 (41%) controls as having at least one microbleed on MRI scans made using a 7 T machine [45]. In both groups the median microbleed amount was 0 but the range was somewhat larger in the type 2 diabetes group (0–13) than in the control group (0–5).

An early systematic review identified that diabetes was a significant risk factor for the presence of cerebral microbleeds with an odds ratio of 2.2 (95% confidence interval: 1.2–4.2) [46]. Hypertension, however, was the strongest risk factor with an odds ratio of 3.9 (95% confidence interval: 2.4–6.4). The AGES-Reykjavik study showed that diabetes patients had a statistically significantly higher prevalence of two or more microbleeds compared with controls (6.9 versus 4.2%), whereas no microbleeds (patients: 86.4% versus controls: 88.7%) and prevalence of a single microbleed (patients: 6.7% versus controls: 7.1%) was not different between the groups [41]. An additional analysis in this study showed that diabetes patients who had either retinal arteriovenous nicking (odds ratio: 2.47, 95% confidence interval: 1.42–4.31) or retinal microhemorrhages/microaneurysms (odds ratio: 2.28, 95% confidence interval: 1.24–4.18) were especially susceptible to having two or more microbleeds in the brain [47]. There are no studies available that have assessed how these microbleeds develop over time, nor are there studies that have systematically assessed the risk factors of microbleed presence in type 2 diabetes.

## Synthesis

There is a clear association between type 2 diabetes and the prevalence of lacunar infarcts. Risk factors are not all that well established yet, but studies suggest that elevated blood pressure, triglycerides, higher fasting plasma glucose levels, and insulin resistance

play a key role. The relationship between type 2 diabetes and white matter hyperintensities is far less clear-cut. There are some studies, cross-sectional and longitudinal, case–control, and population-based, that have shown that patients with type 2 diabetes are more at risk of white matter hyperintensities and higher lesion volume than subjects without type 2 diabetes. There are however at least equal amounts of studies that did not observe any relationship between worse indices of white matter hyperintensities and type 2 diabetes. Moreover, the studies that have found worse results in the patient group compared with the control group show differences of around 20%. As presence and severity of white matter hyperintensities differ significantly between people, such differences are not very pronounced. Therefore, it can be concluded that based on the current state of the literature there is no evidence that type 2 diabetes significantly affects the presence or severity of white matter lesions. Lastly, cerebral microbleeds are poorly studied and current research is both in favor of a difference and no difference in the presence of microbleeds. It seems however that type 2 diabetes patients with retinal abnormalities are more susceptible. A relationship between peripheral microvascular disease, and in particular retinopathy, deserves more attention as it is a promising link that can help predict cerebral small vessel disease in diabetes [48].

## Clinical Impact of Cerebral Small Vessel Disease in Diabetes

The clinical relevance of cerebral small vessel disease in diabetes remains understudied. Clinically, cerebral small vessel disease can have an impact on cognitive functioning, it can contribute to the occurrence of stroke, and it can be a major factor in the development of dementia, and in particular vascular dementia. In the following section, we will discuss the impact of small vessel disease in the brain on these three clinical parameters.

### Cognitive Functioning

It is well known that both type 1 and type 2 diabetes are related to decrements in cognitive performance. In type 1 diabetes, patients commonly show loss of mental flexibility, attention, psychomotor efficiency, and a loss of processing speed [49, 50]. The effect size in the general type 1 diabetes population is about Cohen's $\delta = 0.3$. Cohen's $\delta$ is a measure of the size of an effect between two groups taking into account the means, standard deviations, and group sizes of the groups. It is commonly used in psychological and social sciences to give an interpretation of how large a (statistically significant) effect is. By convention, a $\delta$ of 0.2 is considered small, a $\delta$ of 0.5 medium, and a $\delta$ of 0.8 large [51]. In patients with established proliferative retinopathy the effect sizes can get as high as Cohen's $\delta = 1.0$ [52]. In type 2 diabetes patients, albeit usually older than type 1 diabetes patients, a similar pattern of cognitive dysfunction is seen with similar effect sizes [53]. Specific to type 2 diabetes, though, memory performance can also be affected [53]. A detailed discussion of cognitive decrements is beyond the scope of the chapter. For more information please see the recent review by Ryan et al. [53]. In light of the discussion of the cognitive effects of cerebral small vessel disease in diabetes it is important to state that the cognitive

decrements are modest in terms of effect size, do not allow for the identification of diabetes patient or control based on individual neuropsychological test scores, and are in no way comparable with the deficits seen in patients who develop mild cognitive impairment or dementia.

As cerebral small vessel disease is poorly studied in type 1 diabetes patients it is also relatively unknown what its effect is on cognitive functioning in this group of patients. In the study by Weinger and colleagues, participants with periventricular white matter hyperintensities (independent of group allocation) were at a slightly higher risk of performing more poorly on a delayed memory task (odds ratio: 1.2, 95% confidence interval: 1.06–1.38) [19]. This effect was, however, not specific for the diabetes group, and memory functioning was also not different between patients and controls. The study by Nunley and colleagues that assessed white matter lesion volume in middle-aged patients with long-standing type 1 diabetes correlated lesion load to cognitive functioning in only the type 1 diabetes group [27]. They found that a poorer performance on tests of processing speed was for 13% explained by white matter lesion volume in the patient group. The lower performance on a test of psychomotor efficiency was for 11% explained by white matter hyperintensity load [27]. No studies have assessed the effects of lacunar infarcts or cerebral microbleeds on cognitive functioning in type 1 diabetes. Despite the lack of literature, markers of cerebral small vessel disease on MRI do not seem to be a strong determinant of type 1 diabetes-related cognitive decrements.

In type 2 diabetes more studies have assessed the relationship between MRI-measured cerebral small vessel disease and cognition. The case–control study by Manschot et al. described an association between poorer attention and executive function performance and periventricular white matter lesions on MRI ($z = -0.08$; 95% confidence interval: $-0.15$ to $-0.02$). A similar association was found for processing speed and periventricular white matter hyperintensities ($z = -0.13$, 95% confidence interval: $-0.24$ to $-0.03$) and lacunar infarcts ($z = -0.77$, 95% confidence interval: $-1.14$ to $-0.39$) [37]. Although statistically significant, a 0.08 standard deviation lower performance on attention and executive function or a 0.13 standard deviation lower performance on processing speed in patients with periventricular white matter lesions is a small effect and will likely not be clinically noticeable. A 0.77 standard deviation lower performance on processing speed when having lacunar infarcts is a substantial effect, but it is not comparable with the effects of dementia, in which a 2 or more standard deviation lower performance is expected. A study by Imamine et al. published in 2011 showed that having lacunar infarcts and deep and periventricular white matter hyperintensities both at baseline and after 3 years were moderately related to processing speed at those time-points, but that a change in cerebral small vessel disease severity did not correlate with cognitive decline over time [54]. Cross-sectionally, the case–control study by Moran and colleagues did not find a substantial influence of white matter hyperintensities, lacunar infarcts, or cerebral microbleeds on cognitive functioning in their type 2 diabetes group [30]. This is in apparent contrast with the results from the AGES-Reykjavik study and Women's Health Initiative study in which markers of cerebral small vessel disease were moderately related to poorer functioning on tests measuring memory, processing speed, and executive functioning [40, 41]. Longitudinal analysis of the Women's Health Initiative data also

objectified that an increase in white matter lesion volume over time was related to lower global cognition scores [40]. The longitudinal analysis of the PROSPER study data did not show a relationship between worsening of cerebral small vessel disease markers on MRI and changes in cognitive functioning [31]. Taken together, most studies show an effect of incident cerebral small vessel disease found on MRI on cognitive functioning in type 2 diabetes patients. The effects, however, were mild to moderate, similar to the effects of cerebral small vessel disease on cognition in the general population and are therefore not specific to type 2 diabetes.

## Stroke

The prevalence of stroke in the United States is about 3%, affecting about 7 million individuals [55]. Ischemic strokes make up about 87% of all strokes, with 10% being hemorrhagic stroke, and 3% subarachnoid strokes [55]. Although stroke is a major cause of death, many people survive a stroke and are left with debilitating effects on daily life. Depending on the location of the stroke, tissue damage caused by the stroke itself and by reperfusion after the stroke can lead to cognitive effects ranging from anterograde memory loss, speech production, or comprehension problems, attentional deficits, to executive function problems. Cerebral small vessel disease is a major risk factor for stroke, hypothesized to be involved in up to 20% of all strokes [56]. Other risk factors include older age, hypertension, dyslipidemia, and higher plasma glucose levels [55–57].

Patients with diabetes are at an increased risk of cardio- and cerebrovascular disease. As we have presented in this chapter, type 1 diabetes seems to be related to increased levels of white matter hyperintensity volume and cerebral microbleeds, although a limitation is that the elderly population is not systematically studied. There is also a clear association between type 2 diabetes and markers of cerebral small vessel disease on MRI, especially lacunar infarcts. Combined with vascular risk factors such as hypertension, dyslipidemia, and high fasting plasma glucose levels, it is likely that diabetes is also related to an increased risk of stroke. As stroke however occurs more rarely in younger and middle-aged subjects, most of the literature will be on type 2 and not type 1 diabetes. A meta-analysis summarizing 102 studies showed that diabetes patients have a hazard ratio of 1.56 (95% confidence interval: 1.19–2.05) of having hemorrhagic stroke [57]. This was 1.84 (95% confidence interval: 1.59–2.13) for unclassified stroke, but the highest hazard ratio was for ischemic stroke, which was 2.27 (95% confidence interval: 1.95–2.65) [57]. Risk factors were higher BMI, higher fasting plasma glucose levels, and higher blood pressure and cholesterol levels [57]. How cerebral small vessel disease interacts with the risk of stroke in diabetes patients is not known and should be further studied.

One interesting link that is relevant in diabetes is the link between stroke and retinal changes. A link between cerebral small vessel disease and retinal alterations has been established in several studies, mainly assessing type 2 diabetes [47, 48, 58]. Independent of diabetes, there is also a growing interest in how retinal changes are related to stroke risk and incidence [56]. Future studies will need to identify how peripheral microvascular disease is related to stroke risk and what the potential pathways are.

## Dementia

It is well known that diabetes, again especially type 2 diabetes, is related to an increased risk of dementia [53]. A recent meta-analysis summarizing 19 population-based studies showed that diabetes patients had a relative risk of 1.46 (95% confidence interval: 1.20–1.77) of developing Alzheimer's disease [59]. However, many of the summarized studies did not observe a relationship between diabetes and Alzheimer's disease. The controversy around a potential relationship between Alzheimer's disease and diabetes has also been noted in other reviews and meta-analyses [60, 61]. A retrospective national record study of both type 1 and type 2 diabetes patients who were hospitalized assessed the risk of Alzheimer's disease across different age categories [62]. For type 1 diabetes patients, the odds ratio of having Alzheimer's disease was 1.10 (95% confidence interval: 1.05–1.15) across the age range (30–80+ years). For type 2 diabetes patients this was 0.99 (95% confidence interval: 0.97–1.01). Interestingly, for the category of 80+ years the odds ratio in type 1 diabetes was 0.84 (95% confidence interval: 0.76–0.92), and in type 2 diabetes it was 0.87 (95% confidence interval: 0.84–0.91), suggesting the risk of having Alzheimer's disease is actually lower in diabetes patients at this age than in non-diabetic controls [62]. This weak link between Alzheimer's disease and diabetes is further supported by findings from postmortem studies in diabetes patients [63]. Most postmortem studies have found signs of vascular damage in the brain, but failed to find classical Alzheimer's disease-related pathology, such as amyloid-beta and neurofibrillary tangles [8, 64–66]. These results were further supported by findings that cerebrospinal fluid amyloid-beta$_{42}$ levels in a group of type 1 diabetes patients was increased, instead of decreased, compared with controls [67]. Levels of cerebrospinal fluid tau and phosphorylated tau were increased, which is similar to Alzheimer's disease. However, in Alzheimer's disease higher tau levels have been found to be related to hypertension [68], which was common in this particular group of type 1 diabetes patients [67]. The role of cerebral small vessel disease in Alzheimer's disease is not uncontroversial, but it might be a mechanism through which diabetes is mildly related to the development of Alzheimer's disease.

The meta-analysis by Cheng and colleagues did observe a strong relative risk in diabetes patients to developing vascular dementia (relative risk: 2.49, 95% confidence interval: 2.08–2.97) [59]. The retrospective national record study published by Smolina and colleagues showed similar odds ratios for patients with either type 1 or type 2 diabetes [62]. Across the age groups, the odds ratio of developing vascular dementia in type 1 diabetes was 2.21 (95% confidence interval: 2.13–2.28). For type 2 diabetic patients the odds ratio was slightly lower, but still significantly higher when compared with the control group. The odds ratio was 1.80 (95% confidence interval: 1.77–1.83). This is an intuitive link as diabetes is related to many vascular risk factors, in the case of type 2 diabetes even before the development of diabetes, through obesity and the metabolic syndrome. How cerebral small vessel disease in diabetes is related to the development of vascular dementia is poorly studied, and little is known about the individual contributions of lacunar infarcts, white matter hyperintensities, and cerebral microbleeds to this risk. However, as MRI and postmortem studies have demonstrated a higher prevalence and severity of small vessel disease in the brain it can definitely be hypothesized that cerebral small vessel disease is related to the increased risk of vascular dementia in diabetes and therewith is not clinically silent in nature. More studies on this matter are however highly needed.

## The Influence of Microvascular Disease on the Structure and Functioning of the Diabetic Brain

In diabetes, changes in brain structure and brain functioning are often found to be related to peripheral microvascular disease [22], and are themselves thought to be expressions of microvascular damage in the brain. Therefore, in this last section of the chapter, we will briefly discuss changes in brain structure and functioning in diabetes and how this relates to cognitive functioning. There have been many studies published on various different parameters of brain structure and functioning, including gray matter volume, functional connectivity (i.e. how well brain regions are connected with one another in a functional way), white matter integrity, and even graph theoretical approaches have been used to identify alterations in the brain in relation to diabetes. It is beyond the scope of this chapter to extensively discuss this literature. We will however highlight the most important studies and focus on meta-analyses where possible. As this entire chapter is only on adults with diabetes, we will not summarize the growing literature on brain changes in childhood and adolescent type 1 and type 2 diabetes.

Gray matter structure is commonly analyzed by T1-weighted MRI sequences, which provide a good contrast between gray and white matter and cerebrospinal fluid. There are various techniques available, but three approaches are most commonly used. The first is voxel-based morphometry, in which the MRI scan is divided into gray and white matter, and cerebrospinal fluid and voxels are "counted." Group comparisons can than identify clusters of voxels that have different volumetric numbers, either being gray or white matter or cerebrospinal fluid, between groups. Other methods include measuring cortical thickness, in which the thickness of the gray matter mantel is identified, and brain regions are segmented, based on pre-made cortical atlases.

White matter integrity assesses the microstructural integrity of the white matter. For such an analysis, a diffusion tensor imaging (T2*) MRI scan needs to be carried out. These scans are based on the principle that diffusion of water molecules is highly regular in tissue with natural boundaries, such as white matter fiber bundles, which are highly regular and covered in myelin. Diffusion of water is more random in less regular tissue, such as the gray matter. Although it is tempting to state that alterations in microstructural indices reflect a breakdown of the highly regular structure of fiber bundles or damage to the myelin sheaths, this has yet to be proven in humans, although there is evidence that this is the case in animals.

Functional connectivity refers to the concept that two or more brain regions that are active at the same time are involved in the same process and are functionally connected forming neuronal networks [69]. Such neuronal networks change over time as different processes require different functions and thus different brain regions. Identifying such neuronal networks and functional connectivity can be done by electro- and magnetoencephalography, but also by using functional MRI (fMRI). If a task is used during fMRI acquisition, neuronal networks will show up that are linked to that particular test, whether this is finger tapping or a complex working memory task. Using fMRI during rest offers the unique opportunity to study neuronal networks and functional connectivity independent of tasks. A limited set of between 10 and 20 networks, such as the default mode network, attention, visual, motor, and executive networks, are consistently found in studies and have

been linked to various different functions [70–72]. Differences in the strength of the functional connectivity within these networks can be compared between groups, ultimately identifying areas in which a disease may affect functional connectivity.

This is a very short introduction to the three most important topics of brain changes due to microvascular disease in diabetes. It is by no means exhaustive as many books have been written about each technique. If interested, we suggest consulting the vast literature on these topics.

## Type 1 Diabetes

The effect of type 1 diabetes on gray matter structure has been relatively well studied, but the results have been mixed. Some studies have found lower gray matter volume in the general type 1 diabetes population diffusely distributes throughout the brain [73], only in the frontal [74], or subcortical regions [75], and others have found focal alterations only in patients with established proliferative retinopathy [76]. A meta-analysis of 10 studies assessing gray matter volume including 613 type 1 diabetes patients showed that loss of thalamus volume is the most characteristic alteration (Figure 14.2, panel A) [77]. Lower

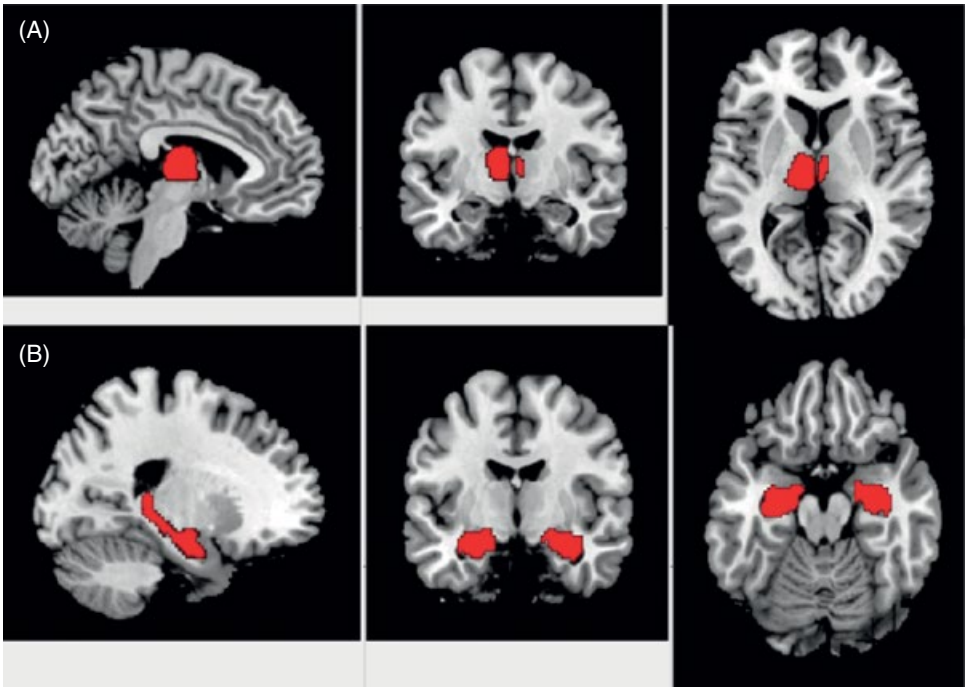


**Figure 14.2** Panel A shows the results of a meta-analysis of 10 studies in which VBM has been used to identify altered gray matter volume. Panel B is the summary of studies using VBM in type 2 diabetes patients. In type 1 diabetes patients the only difference was found in the bilateral thalamus. For type 2 diabetes this was the medial temporal lobe and hippocampus. VBM = voxel based morphometry. *Source:* figure adapted from Moulton et al. [77]. (*See color plate section for color representation of this figure.*)

volume has been found to be a poor predictor of cognitive decrements found in this group of patients, with mild negative correlations [75], if correlations are found.

There is limited literature available on alterations in integrity of the white matter, but the studies that have assessed white matter microstructure show decreased indices in patients relative to controls [78, 79]. The latter study found that having proliferative retinopathy was a strong risk factor for deteriorating white matter microstructure, although focal alterations were also found in patients without any microvascular complications [79]. Both studies found that indices of poorer white matter microstructure were related to poorer overall cognitive functioning, attentional functioning, and processing speed [78, 79].

Functional connectivity, sometimes also called neuronal communication, is a measure of how well different brain regions are connected, either during rest or when performing a task. Only a few studies have assessed this type of brain function. One study, including both patients with and without proliferative retinopathy, showed that during a working memory task during hypoglycemia the brains of patients with proliferative retinopathy activated more brain regions than the brains of patients without retinopathy [80]. As task performance was similar between the two groups, this shows that the brains in patients with proliferative retinopathy have become less efficient as they need to activate more brain regions for similar task performance. When looking to explain cognitive functioning, it is more of interest to study functional connectivity during rest, as no cognitive tasks are involved. A previous study showed there is a distinctly different pattern of functional connectivity changes in type 1 diabetes patients with proliferative retinopathy and without microvascular complications. In this study, functional connectivity alterations were most pronounced in neuronal networks that involve visual and sensorimotor functions, although differences were also found in networks involved in language, attention, and working memory [52]. Independent of which network was studied, patients with proliferative retinopathy showed lower functional connectivity, which is indicative of a less efficient brain network at rest. Patients without any microvascular complications showed increased functional connectivity in networks involved in visual and sensorimotor processes [52]. The higher the level of functional connectivity, the better the cognitive performance in these groups, demonstrating that efficiently and well-organized brain networks have clinical implications [52, 81].

To summarize, just as with cerebral small vessel disease, the literature on brain changes in type 1 diabetes is limited. The currently available studies show that the cortical mantle is relatively spared, but that white matter microstructure and functional connectivity are affected by the disease, especially in the presence of peripheral microangiopathy. Clinically, they are both important for cognitive functioning.

### Type 2 Diabetes

In the meta-analysis by Moulton and colleagues 23 volumetric studies were included containing 1364 patients with type 2 diabetes with a mean age of 63.2 years. Additionally, these articles included 3433 non-diabetic controls with a mean age of 60.5 years [77]. The average disease duration was 10.3 years and patients had moderate glycemic control (7.6% or 60 mmol/mol $HbA_{1c}$). The regional volumetric meta-analysis showed that overall brain volume as well as gray matter volume was lower in the type 2 diabetes patients relative to

the controls. This effect was most pronounced in the orbitofrontal cortex, hippocampus, and basal ganglia. Looking on a voxel-wise level, the bilateral hippocampus showed the most consistent loss of volume in patients (Figure 14.2, panel B) [77]. The meta-analysis consisted of two studies that assessed brain volume longitudinally [40, 42], and found some evidence of progression of brain volume loss over a period of 4 and 5 years, respectively [77]. The volume reductions range between 0.5 and 2%, which corresponds to roughly 2–5 years of normal aging, suggesting a somewhat advanced brain age in type 2 diabetes [29]. The best predictors for loss of brain volume across studies were higher $HbA_{1c}$, longer disease duration, and higher blood pressure [29]. As in type 1 diabetes, the correlations between gray matter volume and cognition are at best weak, if studies found such correlations at all. One study that did find significant associations between cognitive functioning and gray matter volume was the study by Moran et al. in 350 patients. They found that adding gray matter volume to a statistical model attenuated the relationship between type 2 diabetes and performance on memory, visuoconstruction, executive function, and processing speed tasks with 36 to up to 72%, indicating that volume may play a role in type 2 diabetes-related cognitive decrements [30]. Developing microvascular complications is considered to be a main factor in the loss of brain volume in type 2 diabetes [53], but this is not supported by all studies [82].

A growing body of literature involves the assessment of white matter microstructure in patients with type 2 diabetes [10]. In 85 community-dwelling elderly people with diabetes with a mean age of 83.3 years, white matter microstructure was decreased compared with controls[83]. The association with diabetes was particularly significant for microstructure in the hippocampus, posterior cingulate, and prefrontal cortex, and borderline significant for the putamen. Unfortunately, no correlations with cognition were calculated [83]. Another case–control study in younger patients found lower white matter microstructure in the superior and inferior longitudinal, uncinate fasciculi, and in the corpus callosum independent of cerebral small vessel disease, which related to poorer processing speed and memory performance [84]. Other studies have found decreased white matter microstructure in the frontal regions related to longer disease duration [85], in the corticospinal, longitudinal, fronto-occipital fasciculi, and corpus callosum related to higher BMI [86], and in frontal and temporal regions related to memory impairments [87]. A very innovative study in older type 2 diabetes patients (mean age of 71 years) showed a reduction in the extent to which neighboring regions within the white matter are connected with each other, and that this lower connectedness correlated strongly with poorer cognitive functioning in this group [88].

A recent meta-analysis of nine studies that have assessed functional connectivity in type 2 diabetes patients showed that connectivity was lower in patients relative to controls in the lingual, postcentral, and inferior temporal gyri, as well as in the cerebellum, insula, and posterior cingulate cortex [89]. In the precuneus and superior frontal gyrus increased functional connectivity was found in type 2 diabetes compared with controls (Figure 14.3) [89]. Many of the regions observed in the meta-analysis are part of the so-called default mode network. This is active during rest and deactivated during task performance, is thought to be involved in monitoring the inside and outside environment, and is important for general cognitive functioning [10, 90]. A case–control study specifically assessing default mode network connectivity also showed disrupted functional connectivity in type 2 diabetes patients [91]. Interestingly, higher insulin resistance, as measured by the

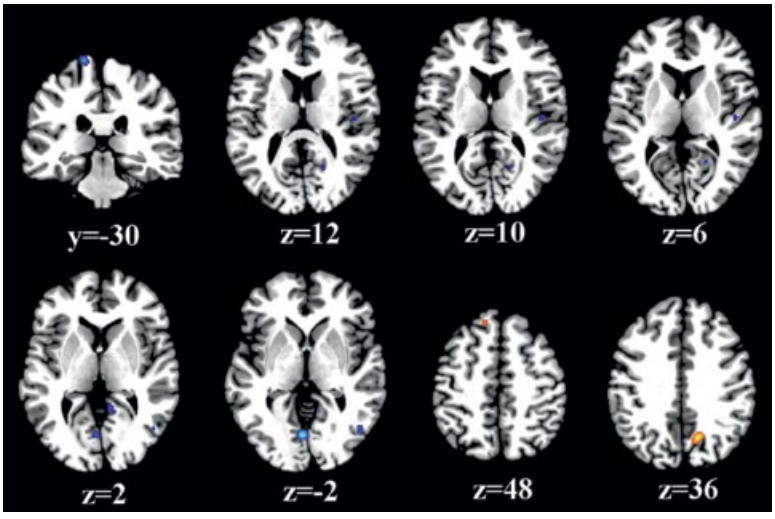


**Figure 14.3** In this figure the results are demonstrated of a meta-analysis of nine functional connectivity studies in which type 2 diabetes patients were compared with non-diabetic controls. The blue clusters signify lower functional connectivity in the patients compared with the controls, and the red/yellow clusters indicate increased connectivity in patients relative to controls. Y and Z give the spatial location of the image on the y- or z-axis. As can be seen the figure shows mainly lower functional connectivity in patients, in the lingual, postcentral, interior temporal, insula, and posterior cingulate regions, and in the cerebellum. Increased functional connectivity in patients relative to controls was found in the precuneus and superior frontal regions. *Source:* the figure was adapted from Xia et al. [89]. (*See color plate section for color representation of this figure.*)

Homeostatic Assessment for Insulin Resistance (HOMA-IR) score, was related to lower connectivity scores in this network. The meta-analysis by Xia and colleagues did not analyze the relationship between cognition and functional connectivity. Other studies have assessed the relationship between functional connectivity and cognition and have found that especially the default mode network is important in proper cognitive functioning in type 2 diabetes [92].

Taken together, gray matter volume, but especially white matter microstructure and functional connectivity, are related to cognitive impairments commonly seen in patients with type 2 diabetes. These changes seem, as far as studied, to be independent of cerebral small vessel disease. However, as many conditions may underlie cerebral atrophy and disruption of white matter microstructure and functional connectivity, cerebral small vessel disease may be involved in these processes in type 2 diabetes [53, 93].

## Conclusions and Future Directions

To conclude, postmortem studies, both older and more recent, have shown that the main damage in the brain of diabetes patients is vascular in nature. With the nowadays wide availability of MRI it has also become possible to study those vascular changes in vivo. In type 1 diabetes patients all the literature, except for one study, has been performed in young or middle-aged adults, an age range in which cerebral small vessel disease is rare.

The little literature that exists is at best contradictory for white matter hyperintensities. Some studies did show an increased prevalence, severity, or lesion volume, whereas others did not find a relationship between type 1 diabetes and white matter lesions. Lacunar infarcts and cerebral microbleeds are even less well studied. It seems that cerebral microbleeds are more prevalent in type 1 diabetes patients, at least in patients with proliferative retinopathy. This was related to altered peripheral microvascular functioning, which suggests that this marker of cerebral small vessel disease may be part of a generalized microvascular dysfunction in patients with longstanding type 1 diabetes. The clinical significance of cerebral small vessel disease remains unclear in this group. In type 2 diabetes there is a clear relationship with an increased prevalence of lacunar infarcts. An association with white matter hyperintensities is controversial and cerebral microbleeds are again little studied. There is some evidence that at least white matter hyperintensity volume increases in patients over time, although studies again show conflicting results. Conflicting results are also found when assessing the association between cerebral small vessel disease and cognition in type 2 diabetes. It also remains unclear what role markers of cerebral small vessel disease play in the association between diabetes, stroke, and (vascular) dementia. It is evident that both types of diabetes are related to structural changes in the cortex, in the white matter microstructure, and in the way brain regions communicate with one another (functional connectivity). These alterations, especially alterations in white matter microstructure and functional connectivity, are strongly related to diabetes-related cognitive deficits.

For future research, there are some important aspects of cerebral microvascular disease that we feel deserve more attention. In type 1 diabetes the focus should shift toward longitudinal studies in older patients. This is important not only because cerebral small vessel disease is more common in the elderly, but also because the life expectancy of type 1 diabetes patients is rapidly increasing, and because the brains of older people are more vulnerable than the brains of young or middle-aged adults. In type 2 diabetes there is still a lack of longitudinal studies and in general the clinical relevance of cerebral small vessel disease is poorly studied. Specifically in the field of type 2 diabetes it will be interesting to include participants in the prediabetic stages. Subjects with prediabetes and the metabolic syndrome share many of the risk factors for cardiovascular and cerebrovascular disease with type 2 diabetes, but in the absence of clinically manifest micro- and macroangiopathy, it will be easier to disentangle the relative contribution of each factor to the risk of developing small vessel disease. Lastly, the field of diabetes needs to take better advantage of newer MRI sequences, something in which this research field is behind. Promising techniques such as arterial spin labeling (ASL) have the capacity to measure perfusion and identify areas of altered perfusion even in the absence of cerebral small vessel disease. Such techniques may in the future help predicting microvascular alterations in patients, on the basis of which interventions could be developed.

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