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Alterations of endothelial function in patients with diabetes mellitus during the first five years after diagnosis

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Zusammenfassung

Makro- & mikrovaskuläre Komplikationen stellen die häufigsten Ursachen chronischer Gesundheitsprobleme bei Menschen mit Diabetes mellitus dar. Oftmals gehen sie mit atherosklerotischen Veränderungen der Blutgefäße einher und erhöhen nachweislich die Mortalitätsrate. Es wird weithin angenommen, dass eine endotheliale Dysfunktion dem Beginn atherosklerotischer Veränderungen vorausgeht. Diese wird durch eine verringerte flussvermittelte Vasodilatation (FMD) quantifiziert, welche bereits vor dem Auftreten klinischer Symptome detektierbar ist. Innerhalb der prospektiven Deutschen Diabetes Studie wurden 179 Studienteilnehmer mit neu diagnostiziertem Typ 1 Diabetes, 219 Teilnehmer mit neu diagnostiziertem Typ 2 Diabetes, sowie zwei glukosetolerante Kontrollgruppen mit 108 alters- und Body-Mass-Index (BMI)-korrelierten Teilnehmern untersucht. Dabei wurden die FMD, die Stickstoffmonoxid-vermittelte Vasodilatation (NMD) sowie die Intima-Media-Dicke (IMT) der Arteria brachialis ermittelt. Zusätzliche Untersuchungen umfassten anthropometrische Datenerfassung, kardiovaskuläre Parameter, Laboruntersuchungen, Spiroergometrie, Erfassung der Insulinsensitivität mittels euglykämisch-hyperinsulinämischen Clamp-Tests, indirekte Kalorimetrie und bioelektrische Impedanzanalyse. Nach fünf Jahren wurden die endotheliale Funktion und wesentliche Einflussgrößen im frühen Krankheitsverlauf bei 108 Studienteilnehmern erneut untersucht. Während bei Krankheitsbeginn zwischen Diabetes- und Kontrollgruppen kein Unterschied bezüglich FMD und NMD festgestellt wurde, war die IMT jedoch in beiden Diabetesgruppen erhöht und korrelierte mit klassischen kardiovaskulären Risikofaktoren. Innerhalb von fünf Jahren verringerte sich die FMD bei Typ 2 Diabetes um ca. 14% unabhängig von Alter, Geschlecht und BMI. Dies korrelierte mit der IMT, der Insulinresistenz und den Indices der Leberfibrose zu Studienbeginn. In beiden Diabetesgruppen ging ein erhöhter HbA1c, eine niedrige Insulinsensitivität und eine niedrige kardiorespiratorische Fitness (VO_2max) zu Beginn mit einer verringerten FMD nach fünf Jahren einher. Die Mehrheit der Studienteilnehmer mit verringerter FMD konnte die empfohlenen Richtwerte kardiovaskulärer Risikofaktoren nicht einhalten. Die Ergebnisse suggerieren, dass der endothelialen Dysfunktion bei Diabetes morphologische Veränderungen und frühe metabolische Störungen vorausgehen, da zu Krankheitsbeginn die IMT, jedoch nicht die FMD oder NMD eingeschränkt war. Die Verminderung der FMD bei Erstdiagnose eines Typ 2 Diabetes steht im Zusammenhang mit der insuffizienten Kontrolle kardiovaskulärer Risikofaktoren wie BMI, Glykämie, Cholesterinsenkung und VO_2max. Erhöhte Insulinresistenz des Fettgewebes und eine progrediente nicht-alkoholische Fettleber bei Krankheitsbeginn könnten zur Verschlechterung der Endothelfunktion beitragen.

Summary

The most common long-term health problems of diabetes mellitus are micro- and macrovascular diseases, which generally refer to atherosclerotic alterations in blood vessels and subsequently increase mortality rate. It is widely assumed that endothelial dysfunction, represented by decreased flow-mediated vasodilation (FMD), occurs early in the development of cardiovascular disease, even before the onset of clinical symptoms. Within diabetes patients, hyperglycemia and insulin resistance seem to be the most significant factors of impaired endothelial function. The present study includes 179 participants with newly diagnosed type 1 diabetes, 219 participants with newly diagnosed type 2 diabetes and two age- and Body-Mass-Index (BMI)-matched glucose-tolerant control groups with 109 participants, which are all part of the prospective German Diabetes Study. Both groups underwent ultrasound-assisted measurements of FMD, nitroglycerin-mediated dilation (NMD), and intima-media thickness (IMT) of the brachial artery. Additional examinations included anthropometric data, cardiovascular parameters, laboratory analysis, spiroergometry, whole-body insulin sensitivity assessment by euglycemic-hyperinsulinemic clamp tests, indirect calorimetry, and bioelectrical impedance analysis. 108 participants were reevaluated after five years. The present study examined endothelial function and its determinants in type 1 and type 2 diabetes during the early course of the disease. At baseline, no difference was observed in FMD and NMD between diabetic and glucose-tolerant subjects, but both type 1 and type 2 diabetes patients showed higher brachial IMT compared to the respective control groups, which correlated positively with classic cardiac risk factors. During follow-up, FMD declined in persons with type 2 diabetes by about 14% independent of age, sex, and BMI and was associated with baseline brachial IMT, adipose tissue insulin resistance, and liver fibrosis indices. In both type 1 and type 2 diabetes, elevated HbA1c but low M-value and cardio respiratory fitness at baseline were associated with lower FMD. Most subjects with a decline in FMD did not achieve the recommended target values in cardiovascular risk factors. Particularly cholesterol levels were exceeded. The results suggest that morphological changes and early metabolic disturbances may precede endothelial dysfunction in type 1 and type 2 diabetes patients. Accordingly, IMT is increased while FMD and NMD are not impaired at diagnosis of diabetes. In the early course of the disease, the deterioration of FMD in patients with type 2 diabetes is associated with poor glycemic control, low physical activity and off target cholesterol levels. Increased adipose tissue insulin resistance and progressive non-alcoholic liver disease might promote deterioration of endothelial function early after diagnosis of diabetes.

Abbreviations

ABI	Ankle-Brachial-Index
ADA	American Diabetes Association
AGE	Advanced Glycation Endproduct
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AT-II	Angiotensin-II
a.u.	Arbitrary Unit
BMI	Body-Mass-Index
CAC	Coronary Artery Calcification
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CON	Control Group
CRP	C-reactive Protein
CVD	Cardiovascular Disease
DAG	Diacylglycerol
DM	Diabetes Mellitus
eGFR	Estimated Glomerular Filtration Rate
ECG	Electrocardiogram
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-regulated Kinase
ET	Endothelin
FFA	Free Fatty Acid
FFM	Fat-free Mass
FIB	Fibrosis
FMD	Flow-mediated Dilation
FLI	Fatty Liver Index
GDS	German Diabetes Study
GGT	γ-Glutamyl Transpeptidase
GlcNAc	N-Acetylglucosamine
HbA1c	Glycated Hemoglobin
HDL	High-Density Lipoprotein
HLA	Human Leukocyte Antigen
hsCRP	High-sensitivity C-reactive Protein
HSP	Hexosamine Signaling Pathway
ICAM	Intracellular Adhesion Molecule
IKK	IκB-kinase
IL	Interleukin
IMT	Intima-Media-Thickness
IRS	Insulin Receptor Substrate
IVGTT	Intravenous Glucose Tolerance Test
JNK	c-Jun N-terminal Kinase
LADA	Latent Autoimmune Diabetes in Adults
LDL	Low-Density Lipoprotein
MAPK	Mitogen-activated Protein Kinase
MI	Myocardial Infarction
MODY	Maturity Onset Diabetes of the Young
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NF	Nuclear Factor
NMD	Nitroglycerin-mediated Dilation
NO	Nitric Oxide
PAD	Peripheral Artery Occlusive Disease
PAI	Plasminogen Activator Inhibitor
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PKC	Protein Kinase C
PKG	Protein Kinase G
PMV	Pulse Wave Velocity

RAGE	Receptor for Advanced Glycation Endproducts
ROI	Region of Interest
ROS	Reactive Oxygen Species
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
tPA	Tissue-type Plasminogen Activator
UDP	Uridine 5’-diphosphate
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VO_2max	Maximal Oxygen Consumption
vWF	von-Willebrand factor
WHR	Waist-Hip Ratio

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1 Introduction

1.1 Diabetes Mellitus

Diabetes is one of the most massive health issues worldwide, with an estimated 422 million adults suffering from diabetes in 2014 [2]. The global number of persons suffering from diabetes has doubled since 1980, increasing from 4,7% to 8,5%, accompanied by an increase in associated risk factors [2]. Diabetes and higher-than-optimal blood glucose caused 3,7 million deaths, with 34% occurring before the age of 70 [2]. The complications of diabetes mellitus are diverse and include microvascular diseases (diabetic nephropathy and retinopathy), neuropathy and cardiovascular diseases.

Diabetes mellitus is a chronic disease occurring as different subtypes, with type 1 diabetes mellitus (T1D) and subtypes referred to as type 2 diabetes mellitus (T2D) constituting the most frequent types of diabetes Dysfunction in insulin secretion and impaired insulin action at target cells underlie pathological mechanisms in diabetes mellitus. This is reflected by hyperglycemia and a disturbed metabolism of carbohydrates, proteins, and lipids.

1.1.1 Type 1 Diabetes Mellitus

T1D represents a total insulin deficiency due to autoimmune T-cell mediated destruction of pancreatic beta cells, which forces affected people into a lifelong substitution of insulin. It accounts for 5 - 10% of all diabetes patients and primarily appears in childhood and adolescents, but the development of symptoms can occur at any age [3, 4]. T1D occurring after the 30th year of life is described as late-onset autoimmune diabetes in the adult (LADA) if insulin treatment is not required for the first 6-12 months after diagnosis. This form of T1D is characterized by slower disease progression, relates to higher insulin resistance as compared to other T1D patients and higher body mass index, especially when antibody titers are low [5]. It is often mistaken for type 2 diabetes mellitus.

T1D is one of the most common chronic diseases of childhood [6], and the absolute number of incidence is still increasing worldwide since the start of the 20th century [7]. The relative increase is 3% - 4% per calendar year, with a higher increase in children younger than five years of age [8, 9].

Despite this alarming trend, the etiology and mechanisms leading to T1D are not fully discovered yet. A combination of genetic predisposition, environmental factors, and dysregulation of immune response is suspected of setting up the development of T1D. The most critical impact on T1D development with 50% of the genetic susceptibility lies in the human leukocyte antigen (HLA) region on chromosome 6, with HLA class II showing the strongest association [10, 11]. HLA genes induce a preferred presentation of peptides towards autoreactive t-lymphocytes. There is no definitive evidence that certain environmental factors trigger the development of T1D, but due to the early age of onset, it is assumed that nutrition antigens like gluten [12], or virus infections [13] can affect the development of T1D. Insulitis and islet cell antibodies as an established predictive humoral marker represent a pre-stage of T1D long before clinical manifestation [14]. Especially T-lymphocytes are crucial for pancreatic islets infiltration, but B-lymphocytes, macrophages, dendritic cells, and natural killer cells are also involved. Apoptosis of insulin-producing beta cells induced due to inflammation after infiltration of immune cells marking the final step in the development of T1D. Novel prevention strategies in children comprise screening for early stages of T1D according to genetic background and antibody titers, avoiding gluten exposure before the babies reach the fourth month, addresses the gut microbiome and exposure to orally applicated insulin during the first 2 years in life [15].

The risk for complications is significantly increased in T1D with both elevated total mortality and CVD mortality [16]. Especially young age at disease onset is linked to severely increased risk of cardiovascular outcomes and overall reduced lifetime [17].

1.1.2 Type 2 Diabetes Mellitus

T2D is a chronic glucose metabolism disorder representing a relative insulin deficiency, in which reduced insulin effectiveness at target cells and reduced pancreatic beta-cell function lead to a disbalance between insulin offer and insulin need. Due to noticeable heterogeneity in pathogenesis, course of disease and even related comorbidities, there is evidence of subphenotypes within people with T2D, including severe insulin deficient diabetes (SIDD) and severe insulin resistant (SIRD) [18, 19]. These subcohorts show different prevalence of diabetes complications and offer more opportunities for individual and precise prevention and treatment [18].

Affecting 90% of all people with diabetes, it is the most prevalent form of diabetes [20], which generally occurs more often in adult people from the age of 50 [21]. After it was assumed that T2D confines itself in elderly people, an increase in prevalence in children was reported [22], which is associated with the increasing rate of childhood obesity, constituting the primary cause of peripheral insulin resistance [23]. Despite the young age, children with T2D show the same risk profile as adults [24]. Generally, the prevalence is rising in both adolescents and children

[23, 25] in developed countries and developing countries, with an estimated largest increase in regions with low-income levels moving to the middle-income level [20]. This trend is based on changing cultural, social, and lifestyle factors like aging populations, increasing urbanization, reduced physical activity, increased sugar and high-calorie food consumption, as well as less fruit and vegetable intake [20].

The development of T2D is a complex interaction between genetic and environmental factors. Besides genetic components, age, gut metagenome, smoking, alcohol, sedentary behavior, and primarily obesity affect the development of T2D [26, 27].

The World Health Organization defines obesity as excessive fat accumulation in adults with a Body-Mass-Index (BMI) $\geq$ 30 kg/m^2 and overweight as a BMI $\geq$ 25 kg/m^2 [28]. Primarily the visceral fat depots secrete various pro-inflammatory adipokines like tumor necrosis factor-alpha (TNF-α), plasminogen activator inhibitor 1 (PAI-1), resistin, and retinol-binding protein 4 [29]. It was observed that people with high BMI but no diabetes have lower visceral fat amounts [30], whereas people with normal BMI and diabetes suffer from visceral obesity [31]. Obesity is accompanied by increased circulating free fatty acids (FFA), which inhibit insulin secretion in the pancreas's beta cells and glucose absorption in peripheral tissues. Furthermore, T2D is strongly associated with increased insulin resistance.

1.1.3 Other forms of Diabetes Mellitus

Additionally, there are other uncommon manifestations of Diabetes mellitus that lead to diabetic metabolism and associated comorbidities.

Among them are genetic defects in pancreatic beta cells, genetic defects in insulin efficiency, diseases of the exocrine pancreas, endocrinopathies, syndromes, and diabetes induced by infections, drugs, or chemicals. This heterogeneous group of diabetes forms is often summarized as type 3 diabetes mellitus. Among them are several monogenic forms of diabetes, which affect a smaller group of patients. Most of the patients fall ill before the 25th year of life without detecting T1D autoantibodies and a persistent, even though insufficient, insulin secretion, which is known as “maturity-onset diabetes of the young” (MODY).

Another particular form of diabetes is gestational diabetes, often referred to as type 4 diabetes mellitus. It is defined by glucose intolerance that affects women the first time during pregnancy which generally resolves after the end of the pregnancy. It corresponds with T2D in its pathophysiology and predisposes to the development of T2D later in life. The International Diabetes Foundation estimates that around 84% of hyperglycemia during pregnancy in 2019 is due to gestational diabetes [20]. The development of gestational diabetes likely underlies complex mechanisms influenced by the special hormonal status of pregnancy, genetic and epigenetic factors as well as environmental factors and is not fully discovered yet. It is assumed

that pancreatic beta cells can not compensate for the oversupply of glucose, resulting in insulin resistance, hyperglycemia, and an increased supply of glucose to the fetus [32]. Gestational diabetes is associated with an increased risk of several adverse perinatal and long-term health outcomes in both the infant and the mother. Therefore, screening for diabetes is recommended in early pregnancy in women with increased risk, and in all women at 24 weeks of gestation.

1.2 Complications of Diabetes Mellitus

Diabetes mellitus is accompanied by several pathological states, including insulin resistance, hyperinsulinemia, and hyperglycemia, which all contribute to severe long-term consequences. Ultimately, diabetes can result in blindness, kidney failure, diabetic foot syndrome, neural damage, and especially cardiovascular diseases (CVD) (Fig. 1). Even intensive glycemic control provides only a modest cardiovascular benefit, whereas overall and cardiovascular mortality are not affected [33].

The most common cause of death in patients with diabetes mellitus are cardiovascular events. They develop a two-fold higher risk for various vascular diseases than adults without diabetes, independently from other risk factors [34]. Non-enzymatic glycation of proteins of the extracellular matrix, inflammation, and endothelial lesions result in changes in the vessel walls, which are the main reasons for the development of atherosclerosis. Significantly, the periphery and coronary arteries, along with the carotids and brain vessels, are affected, leading to stroke, myocardial infarction (MI), and peripheral artery occlusive disease (PAD).

These changes affect large vessels, resulting in macroangiopathy and terminal vessels involved in microangiopathy. One significant quality-of-life decreasing outcome of microangiopathy is diabetic retinopathy causing 2.6% of blindness in 2010 due to pathological changes of small retinal vessels [35]. Additionally, the prevalence in patients with diabetes to develop any retinopathy is significantly high at 35% [36]. Another dramatic event is the development of a diabetic foot syndrome, generally due to an interaction of diabetic neuropathy and angiopathy leading to chronic wounds with impaired wound healing and a high risk for infection, which repeatedly results in extensive foot ulcers. In severe cases, amputation is often the only therapy available, resulting in 10 to 20 times higher amputation rates towards non-diabetic populations [37]. Furthermore, microangiopathy, especially in synergy with hypertension, causes an advancing restriction of kidney function with the possibility to lead to end-stage renal failure, representing up to 80% of cases of end-stage renal disease [38].

The enormously increased risk in the diabetic population receiving severe complications associated with high morbidity and mortality shows that it is crucial to have reliable diagnostics to prevent a decrease in quality of life.

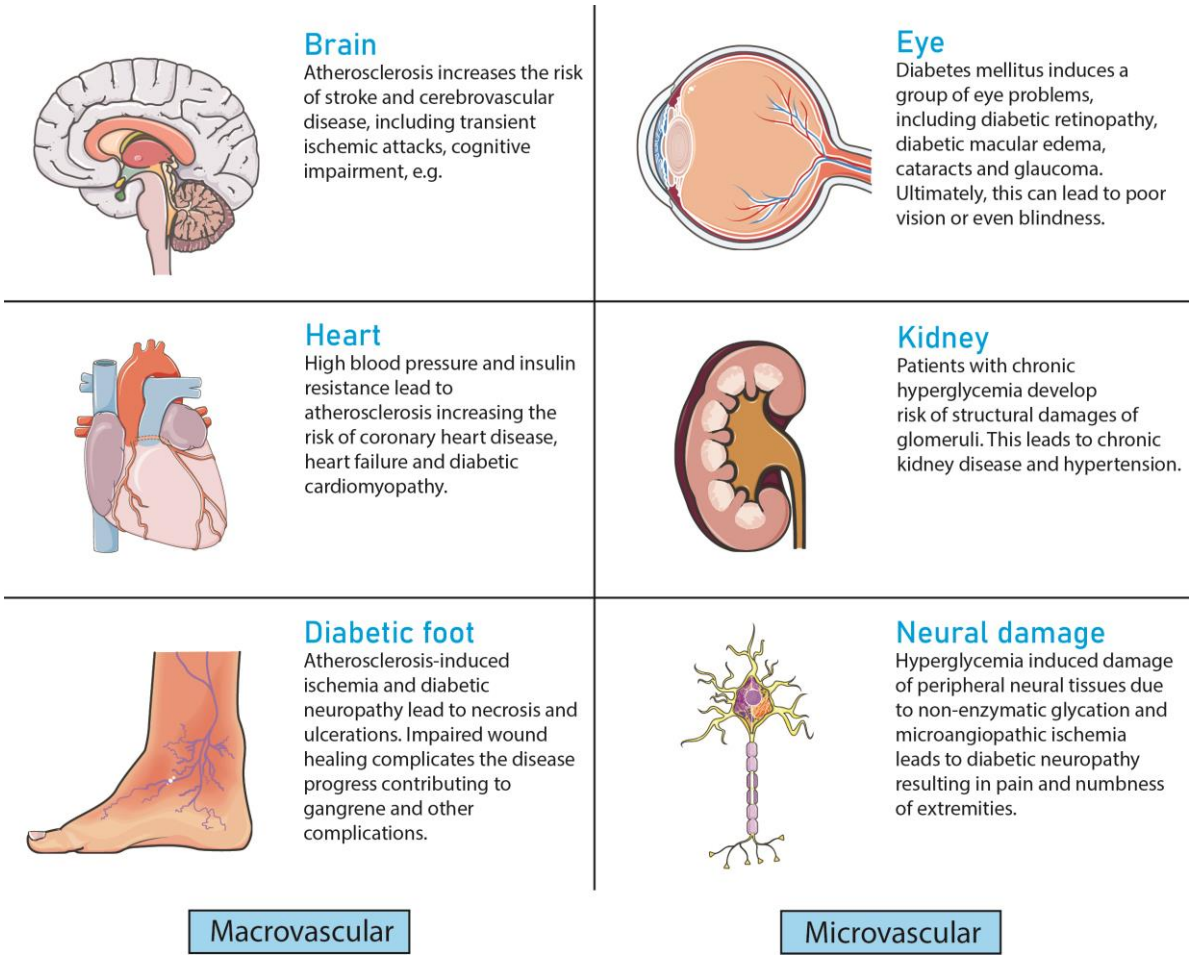

Figure 1. Macro- and microvascular complications in diabetes mellitus.

1.3 Atherogenesis and cardiovascular risk in patients with Diabetes Mellitus

Atherosclerosis-related cardiovascular events are the main reason for increased morbidity and mortality in patients with diabetes mellitus [39]. Even though a high number of older patients with T2D is affected [40, 41], MI is also the main reason for death in people with diabetes under the age of 50 [42]. Contrary to nephropathy or retinopathy, where microvascular complications are more decisive, here macrovascular changes are primarily responsible for disease progression. However, in both forms of angiopathy, disease duration, the quality of metabolic control, and additional risk factors play a crucial role. The process of atherogenesis is depicted in Figure 2.

According to numerous epidemiological and clinical studies, people with DM develop arteriosclerotic vessel alterations more often and earlier than people without DM. Simultaneously it proceeds faster and leads earlier to severe complications like MI and stroke [43]. The high risk most likely arises from the close association between hyperglycemia and other cardiovascular risk factors [34]. As a result of the increased vascular mortality in people with diabetes, a loss of four to eighteen life years appears, dependent on the age of diabetes manifestation [44].

The Whitehall Study showed that the age-adjusted mortality rate (per 100 men and ten years) for coronary heart disease (CHD) is 6,1 in T1D patients and 8,3 in T2D patients compared to 3,9 in controls [45]. The Framingham Study observed people with and without DM for twenty years with a two to three higher incidence in CHD in diabetes [43]. The INTERHEART-study examined the potential risk of various coronary risk factors for diagnosed MI from data of 52 countries with the result that the presence of diabetes mellitus increased the relative risk to 2,37. Interestingly, after adjustment for age and geographical region, the relative risk for women comes to 4,26, whereas men have a relative risk of 2,67 [46]. This finding agrees with a meta-analysis of 37 prospective cohort studies, which shows a sex-specific increased relative risk in women for fatal CHD. It was 50% higher than in men [47].

Even in the early stages of diabetes development, including impaired fasting glucose, the incidence of CHD is increased because of already existing insulin resistance and other classical cardiovascular risk factors [48, 49].

Although patients with T2D are the most affected group, CHD is the most common cause of death in patients with T1D, too. At 55 years, 35% of T1D patients die due to CHD, whereas the numbers in nondiabetic men and women are comparatively low, with 8% in men and 4% in women. The incidence of CHD in young asymptomatic patients with T1D is at approximately 1 to 2% per year [50]. In the older group, including people between 40 to 50 years, over 70% of men and 50% of women with T1D develop coronary artery calcifications (CAC), a marker for arteriosclerotic plaques [51].

Observations of atherogenesis showed different distribution patterns in people with and without DM. While the arterial vessels of nondiabetics are mostly affected segmentally, the atherosclerosis of patients with diabetes extends diffusely over peripheral vessel segments of cerebral-, coronary- and limb arteries. A study using coronary angiography detected a decrease in the average vessel diameter of coronary arteries and a larger extent of lesions in diabetes than in controls [52]. Faster progression of atherosclerosis and excessive intima hyperplasia in diabetes attracted attention after percutaneous transluminal angioplasty, which leads to a higher risk for restenosis and stent obliterations [53].

The comparison of persons with and without DM suffering a MI or cardiovascular death showed that persons with diabetes and no previous MI have the same risk for coronary events as nondiabetic persons with previous MI [39]. A long-term study over eighteen years observing CVD and CHD reveals similar results for the incidence of CHD death. [40] Therefore patients with diabetes have a 2- to 4-fold increase in the development of coronary artery disease (CAD) [54]. Additionally, patients with diabetes carry a higher risk of adverse prognosis after symptoms signifying an acute MI. Thus more than 50% of patients affected by DM with previous MI die within five years, contrary to nondiabetic patients with a rate of under 25% [55].

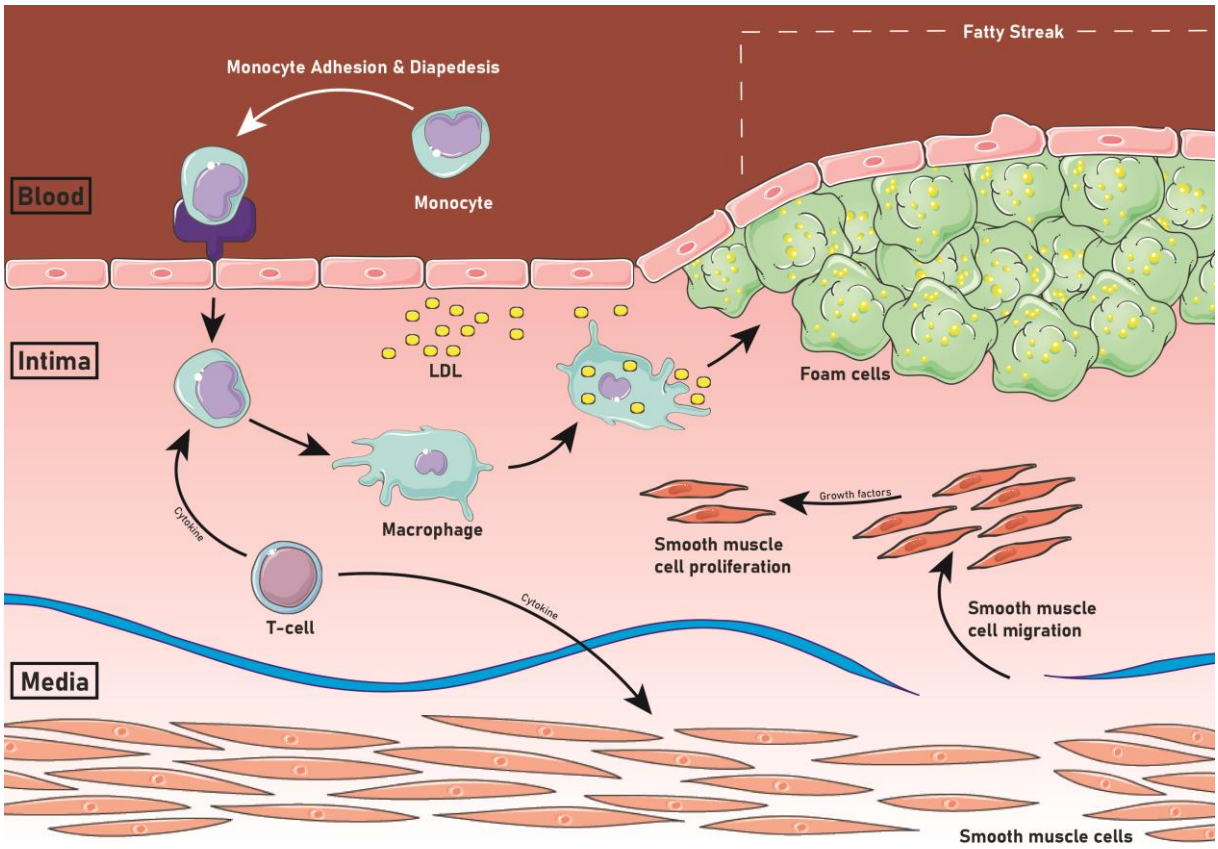

Figure 2. Atherogenesis.

The development of atherosclerotic lesions is based on inflammatory processes within the endothelium. After adhesion to the vessels wall and diapedesis, monocytes differentiate to macrophages and accumulate lipids, transforming into foam cells. Proinflammatory cytokines, secreted by migrated T-cells, enhance this process and simultaneously lead to the migration of smooth muscle cells from the media into the intima. Growth factors induce smooth muscle cell proliferation. Accumulated foam cells and migrated smooth muscle cells lead to fatty streak formation, constricting the vessel and decreasing blood flow, eventually leading to cardiovascular diseases.

Abbreviations: LDL – low-density lipoprotein.

1.4 Endothelial function

Endothelial cells play a crucial role in maintaining physiological metabolism and keeping the human body in a healthy condition. Together they form the endothelium and cover the inner surface of arteries and veins, building a selectively permeable barrier between intravascular and extravascular space. Besides building a physical barrier throughout the cardiovascular system, it also plays a central role in vital functions of the cardiovascular system, including regulation of blood pressure and perfusion, exchange of substances, angiogenesis and vasculogenesis, inflammation, hemostasis, and coagulation [56]. The endothelium provides a non-thrombogenic lining for the cardiovascular system within all vessels [57]. The entirety of all these tasks is summarized under the term endothelial function.

Growing evidence has demonstrated that healthy endothelium is essential to ensure proper maintenance of vascular homeostasis, and therefore, evaluation of endothelial function becomes more and more relevant for predicting cardiovascular risk, even in clinical settings. Over the past decades, it became more apparent that alterations in endothelial function precede the development of CVDs and encourage the development of atherosclerosis [58]. If pathological conditions alter the balanced endothelial regulation, the endothelium's phenotype is modified, and the vascular homeostasis is disturbed, known as "endothelial dysfunction" [59]. Eventually, evaluation of endothelial functions in humans acquired a high significance and served as an excellent surrogate marker of cardiovascular events, which should not be underestimated.

1.4.1 Barrier function

Being a semipermeable membrane is one of the most basic functions of the endothelium. It regulates the transport and transmission of macromolecules between blood and tissues, which cross the endothelial border through transcellular, paracellular or vesicular transport, mainly depending on the type of endothelium. Single endothelial cells are linked by distinct types of adhesive structures or cell-to-cell junctions, including tight junctions, adherens junctions, and gap junctions.

Tight junctions serve the mechanical stabilization of the endothelial cell complex by linking cytoskeletons of participating cells. Adherens junctions are formed by cadherins, building a cell connection, which links the actin filaments of two cells, enhancing their biomechanical stability. Gap junctions are pore-forming protein complexes mainly composed of transmembrane protein connexin, which build a canal tightly connecting the cytoplasm of two cells. They serve the direct signal transmission and the transmission of substances between neighboring cells. Adhesive structures play a crucial role in the regulation of vascular permeability to circulating cells.

1.4.2 Vasoregulation

The endothelium plays a vital role in regulating perfusion and blood pressure. Therefore, it has the ability to produce vasoactive mediators, which influence the vascular tone (Fig. 3). A fundamental trigger is embodied by shear stress applied to the vessel wall. The endothelial cells detect this physical force and transduce it into a cascade of signaling pathways, resulting in the synthesis and release of endothelium-derived relaxing factors, which cause relaxation of underlying vascular smooth muscle cells. In this context, it is of interest which type of shear stress is applied to the vessel's wall. There are two significant types of shear stress. While steady laminar or pulsatile flow provides atheroprotective effects on the vascular wall by enhancing endothelial production of vasodilating mediators, disturbed or oscillatory flow, as observed at atheroprone sites in vivo, stimulates proinflammatory signaling with beginning endothelial dysfunction and subsequent development of atherosclerotic lesions [60, 61].

One of the essential vasodilators in endothelial function is nitric oxide (NO). It is released after detecting increased shear stress on the vessel's wall or through binding substances like bradykinin or prostaglandins on endothelial cell receptors. Both ways increase the activity of Ca^{2+}-canals, which leads to an activation of endothelial nitric oxide synthase (eNOS). eNOS synthesizes NO-radicals from the amino acid arginine. NO diffuses into the surrounding smooth muscle fibers and activates the guanylate cyclase with the following increase of cGMP, which results in activation of protein kinase G (PKG). PKG activates the myosin-light-chain-phosphatase, which dephosphorylates the myosin-light-chain with the following relaxation of the smooth muscle fibers and vasodilation of the vessel wall. In addition, NO leads to hyperpolarization in smooth muscle fibers due to increased potassium conductance, which supports vasodilation.

Depending on the receptor, endothelin-1 (ET-1) is a significant antagonist of NO produced by the endothelium. Binding on ET_A-receptors has a dilating effect on the vessel wall, whereas ET_B-receptors act constringent. ET_A-receptors are mainly located on arteries, while ET_B-receptors are more present in the low-pressure system. Further vasodilating mediators produced by the endothelium are endothelium-derived hyperpolarizing factor and prostacyclin (PGI_2).

There are a few other vasoactive mediators not produced by the endothelium, like thromboxane, prostaglandins, catecholamines (e.g., adrenaline, noradrenaline), or angiotensin II (AT-II). Their effect is partly dependent on the receptor binding.

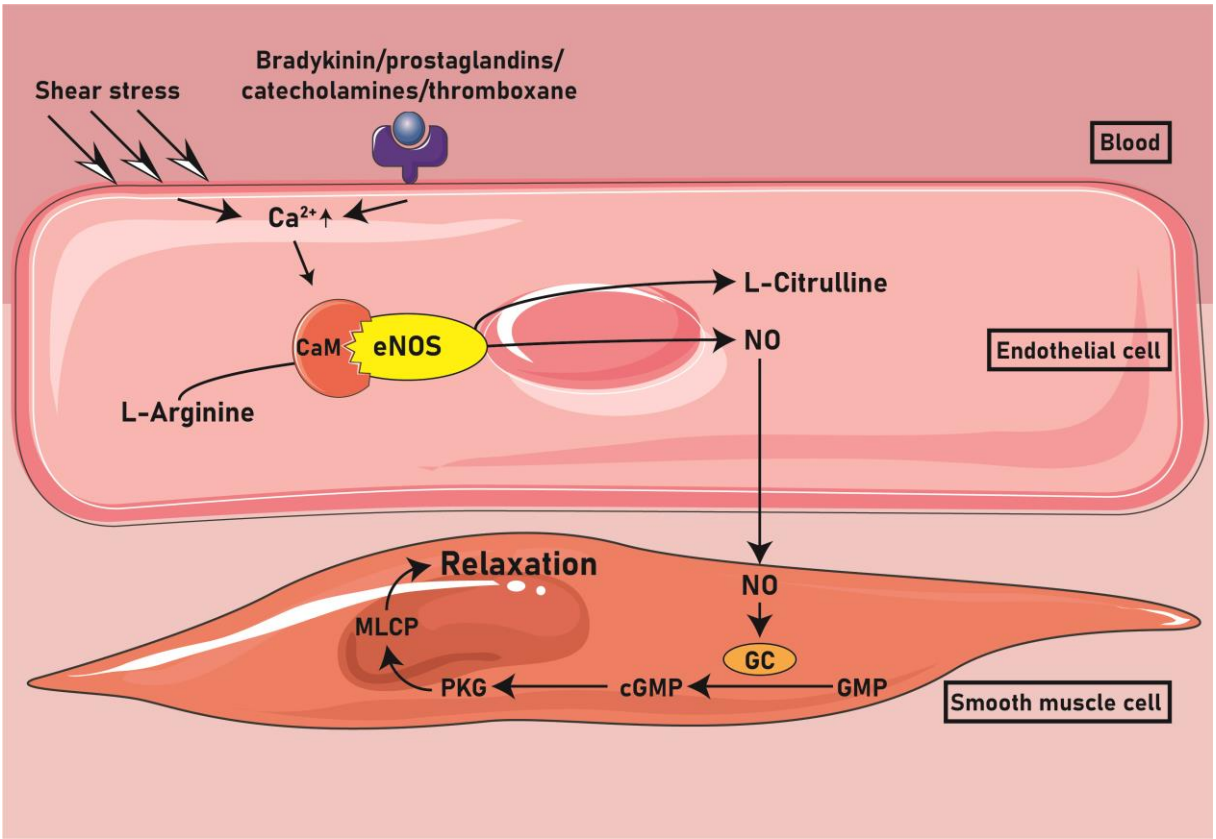

Figure 3. Vasorelaxation as part of endothelial function.

Increased shear stress and various binding substances lead to elevated Ca^{2+} levels within the endothelial cell. CaM now binds to eNOS and enhances eNOS activity, transforming amino acid L-arginine into L-citrulline and vasoactive NO. NO now diffuses into the near smooth muscle cells, stimulating GC, which converts GMP to cGMP. The rising cGMP level activates PKG, which enhances MLCP activity. MLCP dephosphorylates the regulatory light chain of myosin, which ultimately leads to muscle relaxation.

Abbreviations: Ca^{2+} - Calcium, CaM – calmodulin, cGMP – cyclic guanosine monophosphate, eNOS – endothelial nitric oxide synthase, GC – guanylate cyclase, GMP – guanosine monophosphate, MLCP – myosin light-chain phosphatase, NO – nitric oxide, PKG – protein kinase G.

1.4.3 Hemostasis and fibrinolysis

Endothelial cells have numerous tasks in maintaining a non-thrombogenic condition within blood vessels. They regulate thrombosis, thrombolysis, platelet adherence, vascular tone, and blood flow [62]. They are able to produce various vasoactive mediators, including NO and PGI_2, which inhibit platelet aggregation and induce vasodilation.

If the vessel wall is injured, the endothelium takes care of hemostasis and coagulation by switching to an activated state, which is pro-thrombotic, proliferative, and vasoconstricting. Once activated, endothelial cells enhance the adhesion of platelets and neutrophils to the endothelium. The injured endothelial cells secrete most of von-Willebrand-Factor (vWF), which connects exposed sub-endothelial collagen and the thrombocyte surface, supporting the thrombocytes to attach to the vessel wall. The attachment activates the thrombocytes and the coagulation cascade, which results in the creation of a thrombus. Thromboxane A_2 released by activated thrombocytes has a constrictive effect on the vessel wall. NO and PGI_2 continuously synthesized by the endothelium act antagonistic to attachment and aggregation of thrombocytes.

Also participating in fibrinolysis, the endothelium produces tissue-type plasminogen activator (tPA), which activates plasminogen to plasmin [63]. Plasmin splits fibrin and fibrinogen so that the dissolution of the thrombus is ensured.

1.4.4 Inflammation

Regulating diapedesis and migration of immune cells into affected tissues due to immune and inflammatory reactions is another crucial task of the endothelium. Lymphocytes are able to interact with endothelial cells through the constitutively expressed L-selectin receptor, which activates them. The lymphocytes adhere to endothelial cells by the expression of integrins that interact with adhesion molecules, intracellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), and vascular cell adhesion molecule (VCAM) [62]. While ICAM-2 is constitutively expressed on resting endothelial cells, ICAM-1 and VCAM are less expressed, but their expression can be heavenly increased through cytokines and lipopolysaccharides [62]. Binding with the adhesion molecules represents an important step for diapedesis.

50% of intravascular neutrophil granulocytes are bound on endothelial cells, which quickly increases the number of leucocytes in the blood at the beginning of an acute infection. Local leucocytes release cytokines (e.g., bradykinin, histamine, prostaglandins), which lead to vasodilation at the spot of inflammation, slowing down the blood flow. Inflammatory cytokines, like TNF-α and Interleukin-1 (IL-1) by macrophages, activate the endothelium and increase the expression of adhesion molecules on endothelial cells. Together with the hemodynamic changes at the injury site, the chance of adhesion of leucocytes is increased. Vasoactive mediators secreted by leucocytes then lead to endothelial cell contraction as well as leakage of serum and interstitial fluid into the lesion [62]. This process causes maximal leukocyte adhesion to thrombin and histamine-activated endothelial cells. Due to the activation, the endothelium synthesizes chemokines (TNF-α and IL-8) itself, which activate neutrophil chemotaxis and support the diapedesis and migration.

1.5 Evaluation of endothelial function

The endothelial function is characterized by a balanced regulation of vascular tonus, inflammation, and hemostasis. Therefore, several surrogate parameters have been developed to define and measure endothelial function, like flow-mediated vasodilation (FMD), intima-media thickness (IMT), and various blood markers, supported by various imaging techniques such as coronary angiography or high-resolution ultrasound examination.

There are different invasive and non-invasive options to assess endothelial function in humans *in vivo*. Invasive techniques, like intracoronary or intrabrachial infusion of vasoactive agents, are still considered the gold standard for early detection of endothelial dysfunction. However, non-invasive techniques also play an increasingly important role due to their excellent reproducibility, manageable costs, widespread availability in clinical settings, and comparable results [64].

1.5.1 Flow-mediated vasodilation

The most established method to determine endothelial function is the assessment of FMD. FMD can be used as a predictor of CVDs in non-symptomatic patients [65]. Celermajer et al. considered FMD a reliable tool to gather information about vessel conditions and function for the first time [66].

An impaired FMD can be rated as the first sign of the development of atherosclerosis many years before clinical manifestation. The biochemical foundation of FMD measurements is the endothelial NO release after detecting shear stress on the vessel wall. After applying a blood pressure cuff to the forearm, transient hyperemia is induced with increased shear stress. Subsequently, the endothelial cells release NO, which leads to vasodilation. A clinical study in patients with newly diagnosed T2D could detect an endothelial dysfunction embodied by decreased FMD, and even in prediabetic stadiums, an impaired endothelial function was shown [67, 68]. There is a link between increased blood glucose and FMD [69, 70].

Recent literature has no reference values for proving endothelial dysfunction due to dependence on distinct factors, which aggravate establishing a suitable threshold value. However, it can be assumed that the higher the FMD, the more unlikely endothelial dysfunction occurs. FMD-measurement is dependent on various factors, which have to be considered within measurement, evaluation, and interpretation. Many of the influencing factors are linked to endothelial dysfunction. Naidu et al. could establish a link between FMD, sex, BMI, and blood pressure. Regarding sex, men showed a lower FMD than women. Higher BMI and higher blood pressure, regardless of diastolic or systolic higher blood pressure, are accompanied by lower FMD. In contrast, the FMD increases with the reduction of weight [71]. This is supported by a study by Mavri et al., who observed an improvement of FMD in women shortly during a diet [72]. Visceral adiposity, assessed as a waist-hip ratio (WHR) > 0,85, is negatively correlated with FMD [73]. Another study showed that age plays a considerable role in FMD measurement. A study on healthy women showed that FMD of brachial and popliteal artery correlated negatively with higher age [74].

Besides FMD, nitroglycerin-mediated vasodilation (NMD) is measured by applying nitroglycerin sublingually and serves to detect the general responsiveness of endothelium to NO and its ability to dilate. Some literature assumes that NMD could be a more potent marker for predicting future cardiovascular events in patients at risk [75].

1.5.2 Circulating Markers

C-reactive protein (CRP) is an acute-phase protein with proinflammatory effects and can be used as a circulating marker for CVDs. It is assumed that it releases superoxide radicals and is responsible for increased iNOS-activity [76]. While superoxide radicals act harmful on deoxyribonucleic acids and ribonucleic acids, vast amounts of NO synthesized by iNOS are cytotoxic.

Furthermore, different lipid parameters are associated with endothelial function. In particular, oxidated low-density lipoprotein (LDL) and FFAs promote oxidative stress [77, 78]. Visfatin and distinct adipokines synthesized by adipocytes can negatively influence endothelial function with increased oxidative stress [79]. 8-isoprostaglandine and malondialdehyde are metabolic products of lipid metabolism and are considered a reliable marker of oxidative stress [80, 81]. In patients with T2D, increased malondialdehyde concentrations were detected compared to healthy subjects [68].

There are various markers, which partly originate from endothelial cells. NO is an endothelial-dependent vasoactive metabolite, which is decreased in patients with T2D. Another important molecular marker for endothelial dysfunction is E-selectin, which is released after endothelial damage, as well as ICAM-1 and VCAM-1. ICAM-1 positively correlates with T2D and is an indicator of increased cardiovascular risk [82].

1.6 Endothelial Dysfunction in Patients with Diabetes Mellitus

Disturbances in endothelial metabolism and limitations of endothelial functions are known as endothelial dysfunction, characterized by increased endothelial permeability and reduced ability for vasodilation. An essential cause is the reduced release and effect of NO, which acts vasodilatory, inhibits proliferation and migration of smooth muscle cells, prevents activation of thrombocytes, and has an anti-adhesive impact on leukocytes. It occurs that endothelial dysfunction is the first preclinical sign of atherogenesis without showing any clinical symptoms.

Cardiovascular risk factors encourage atherosclerotic changes of vessel walls, and especially a diabetic metabolism combines several pathological mechanisms which can lead to endothelial dysfunction. However, all these mechanisms induce endothelial dysfunction separately and independently of diabetes, which indicates a multifactorial etiology [83]. Some of the exact mechanisms underlying acquired insulin resistance also contribute to endothelial dysfunction, but conversely, insulin resistance itself reinforces the mechanism leading to an insulin-resistant state, revealing mutual relationships within the mechanisms (Fig. 4) [84].

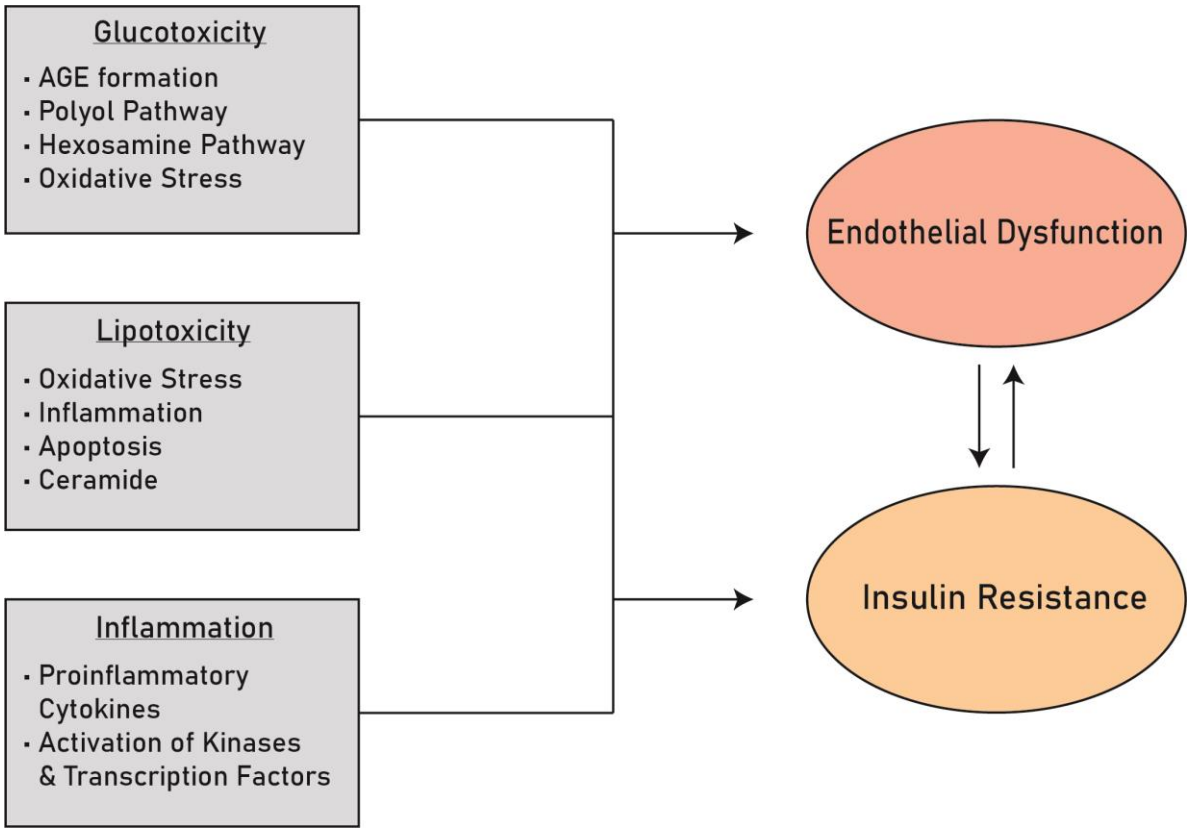

Figure 4. Reciprocal relations in endothelial dysfunction [adapted from Kim et al., 2006 [84]].

Mechanisms of glucotoxicity, lipotoxicity, and inflammation independently lead to endothelial dysfunction and insulin resistance, underlying reciprocal relationships. These associations create a vicious cycle linking cardiovascular and metabolic disorders.

Abbreviations: AGE – advanced glycation end-product.

1.6.1 Insulin resistance and Endothelial Dysfunction

Insulin has essential metabolic tasks and necessary vascular actions, including stimulating NO production in endothelial cells, resulting in vasodilation, increased blood flow, and eventually increased glucose uptake in muscle and fat cells [85].

The effects of insulin are initiated by binding to a ligand-activated tyrosine kinase, expressed by various human cells [86]. Due to the activation by insulin, the receptors can phosphorylate intracellular substrates, including the insulin receptor substrate (IRS) group and Shc. Both operate as docking proteins for subsequent signaling molecules. While phosphorylated IRSs bind to SH2 domains in regulatory subunits of phosphatidylinositol (PI) 3-kinase, which in turn activates the catalytic subunit in Phosphoinositide 3-kinase (PI3K), phosphorylated Shc binds to the SH2 domain of Grb-2, which leads to the activation of guanosine triphosphate (GTP) exchange factor Sos [84]. The resulting cascades ultimately implement the pleiotropic actions of insulin (Fig. 5) [84].

Among the many effects activated by insulin in the endothelium, the distinct pathways also regulate vasomotor control. Insulin activates the predominant vasoprotective PI3K/Akt pathway in healthy individuals, mediating enhanced eNOS expression and activation with subsequent NO production [87].

A prohypertensive pathway originates from Sos, inducing the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) pathway, antagonizing vasomotor control. Sos activates the small GTP binding protein Ras, which then inducts a phosphorylation cascade involving Raf and MAPK/ERK, eventually resulting in increased vasoconstrictor actions of ET-1. MAPK mediates the secretion of ET-1 in endothelial cells induced by insulin-dependent signaling pathways independent of PI 3-kinase–dependent signaling [84].

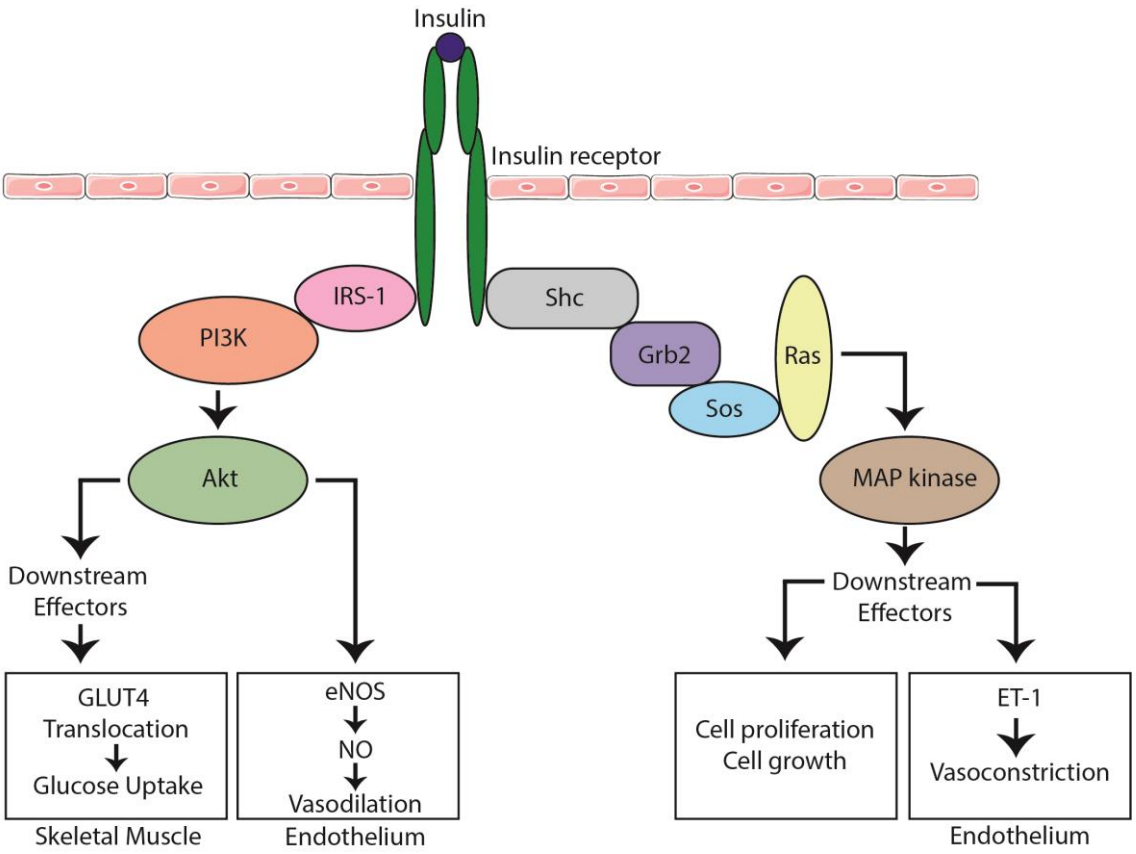

Figure 5. Insulin signaling [adapted from Kim et al., 2006 [84]].

Stimulated by IRS-1 phosphorylation after insulin binding, the PI3K signaling branch eventually leads to GLUT4 translocation, increased glucose uptake in skeletal muscle, and vasodilation in endothelium after stimulating NO production by eNOS. Phosphorylation of Shc enhances the MAP kinase branch, regulating cell proliferation and growth and leading to vasoconstriction due to ET-1 secretion in endothelial cells.

Abbreviations: eNOS – endothelial nitric oxide synthase, ET-1 – endothelin 1, GLUT4 – glucose transporter type 4, IRS-1 – insulin receptor substrate 1, MAP – mitogen-activated protein, NO – nitric oxide, PI3K – phosphoinositide 3-kinase.

Insulin resistance is defined as the impaired impact of insulin on peripheral tissues, including adipose tissue, skeletal muscle, liver, and brain, which may occur years before increased glucose levels. It does not only lay the foundation of developing T2D, it is also prominent in other metabolic disorders like obesity and dyslipidemia, as well as CVDs like hypertension, CAD, and atherosclerosis, which in turn are characterized by endothelial dysfunction. Insulin signaling pathways, which regulate endothelial production of NO, show many similarities with insulin signaling pathways in skeletal muscle and adipose tissue [88]. Therefore, it is assumable that mechanisms, which contribute to insulin resistance and endothelial dysfunction, underlying reciprocal relationships, in which insulin resistance is often linked to the progression of endothelial dysfunction and vice versa [84]. These harmful mechanisms, including glucotoxicity, lipotoxicity, and inflammation, are part of diabetes mellitus and result in a vicious circle, reinforcing the link between metabolic and cardiovascular disorders [84].

Insulin resistance is induced by several mechanisms and molecules, including activation of protein kinase C (PKC), polymorphisms in IRS-1, AT-II, or O-linked glycosylation [89]. In endothelium, insulin resistance predominantly affects the PI3K-dependent signaling by inactivating the IRS but not the insulin receptor itself, so that, under these circumstances, the unaffected MAPK-dependent pathway overdrives [90]. Studies show that patients with IRS-1 polymorphism are both insulin-resistant and develop an endothelial dysfunction due to specific inactivation of the PI3K-dependent signaling pathway resulting in both reduced eNOS gene expression and decreased post-translational NO-producing mechanisms [91, 92]. Another study showed that mice, which are homozygous-null for the IRS-1 gene, are predictably insulin resistant and develop hypertension signs with impaired endothelium-dependent vasodilation [93]. Additionally, patients with a specific polymorphism in IRS-1 that has been involved in direct impairment of eNOS activation may also develop genetically based endothelial dysfunction [92]. Due to compensatory hyperinsulinemia to maintain euglycemia, the MAPK-dependent pathway is enhanced, while the PI3K-dependent pathway is still impaired, resulting in an imbalance between NO and ET-1 production in favor of vasoconstricting ET-1 [94]. This observation is supported by a study that showed that pharmacological blockade of ET-1 receptors positively affects endothelial function in obese and patients with DM but has no effect in lean insulin-sensitive subjects [95]. The MAPK-dependent pathway upregulates the expression of VCAM-1 and E-selectin molecules together with PAI-1, favoring endothelial dysfunction and a prothrombogenic state [96]. Therefore, increased adhesion of monocytes with endothelial cells is observed. Altogether, the antihypertensive effects of insulin are reduced under conditions of insulin resistance due to missing stimulation of NO production.

1.6.2 Hyperglycemia/Glucotoxicity and Endothelial Dysfunction

Hyperglycemia, the most typical indicator of diabetes mellitus, acts harmfully on the endothelium in many different ways. Even healthy test persons show a restricted endothelial function if hyperglycemia is induced [97]. Expression of extracellular matrix and procoagulant proteins, increased apoptosis of endothelial cells, decreased endothelial cell proliferation, and inhibition of fibrinolysis are promoted by a hyperglycemic state, eventually resulting in endothelial dysfunction [98].

The molecular mechanisms underlying hyperglycemia-induced insulin resistance also apply to endothelial dysfunction. That again illustrates the reciprocal connections between insulin resistance, hyperglycemia, and endothelial dysfunction. The hyperglycemic state damages the endothelium, mainly due to the creation of advanced glycoxidation end products (AGE) and increased oxidative stress [99]. A hyperglycemic state is accompanied by reduced NO production, enhanced expression of adhesion molecules, inflammatory gene expression, and leukocyte recruitment [100].

Hyperglycemia features four crucial mechanisms, which play a key role in developing endothelial dysfunction by mainly increasing oxidative stress [101].

1.6.2.1 Advanced glycation end products

Due to hyperglycemia and oxidative stress, AGEs are synthesized after exposure of proteins and lipids to sugars [102]. They inhibit insulin-stimulated phosphorylation of IRS-1 and IRS-2, preventing activation of PI3K/Akt, which leads to the development of insulin resistance. The endothelial proteins cross-link with each other due to AGEs impairing their functions [103]. AGEs also influence vessel elasticity and fluid filtration by modifying extracellular matrix proteins [104]. Regarding atherosclerosis, AGEs play an important role in interactions with macrophages infiltrating endothelial cells. The modification of proteins by AGEs makes it easier for macrophages to infiltrate the endothelial cell, in which they transform into foam cells inducing vascular inflammation and atherogenesis.

Receptors for advanced glycation end products (RAGE) are expressed in endothelial cells, where they promote inflammation by activation of the nuclear factor (NF)-κB pathway and by direct interaction with infiltrated macrophages [105]. Additionally, binding on RAGE activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in reactive oxygen species (ROS) production [106]. In turn, ROS and therefore increased oxidative enhance AGE formation [102].

1.6.2.2 Polyol Pathway

When intracellular glucose levels are elevated due to hyperglycemia, glucose is metabolized via the polyol pathway more frequently [107]. Here, glucose is converted to sorbitol by aldolase

reductase using $NADPH/H^+$ as a cofactor and is then metabolized to fructose by sorbitol dehydrogenase under the consumption of NAD^+ [108]. The increased polyol pathway activity in hyperglycemic patients leads to increased $NADPH/H^+$ consumption, which is not available for the regeneration of glutathione disulfide then. Due to being a significant antioxidant, the neutralization of ROS is impaired. Synthesized sorbitol accumulates within the cells and is considered to be a reason for diabetic neuropathy [109]. Due to the use of $NADPH/H^+$ by eNOS, the endothelial function is affected when the availability of $NADPH/H^+$ is lacking. This leads to reduced NO synthesis and eventually to impaired vasodilation.

1.6.2.3 Hexosamine Signaling Pathway

Physiologically about 1 - 3 % of glucose is metabolized via hexosamine signaling pathway (HSP), resulting in the production of uridine 5'-diphosphate (UDP)-N-acetylglucosamine (GlcNAc) by Glutamine:fructose-6-phosphate amidotransferase, which is the pacemaker enzyme in this pathway [110]. Due to hyperglycemia, physiological glucose metabolism is not entirely sustainable, so increased flux through the HSP occurs. This is another considered mechanism by which hyperglycemia causes insulin resistance and endothelial dysfunction [98, 111]. UDP-GlcNAc is used as a substrate for O-GlcNAc transferase, which causes glycation of eNOS at the Akt phosphorylation site resulting in decreased eNOS activity. O-GlcNAc modifications also occur on proteins involved in insulin signaling, including IRS-1, leading to insulin resistance and its complications [112]. Thereby increased expression of transforming growth factor (TGF)-β and PAI-1 is mediated, which is relevant to the development of vascular complications [113].

1.6.2.4 Activation of Protein Kinase C

There are multiple protein kinase C (PKC) isoforms in the human body, activated by different substances and harm physiological endothelial function. The mitochondrion overproduces superoxide in its respiratory chain if the body is exposed to hyperglycemia. This leads to an inactivation of Glyceraldehyde 3-phosphate dehydrogenase, which correlates with a higher level of glyceraldehyde-3 phosphate, which in turn can be converted to diacylglycerol (DAG) [100, 114], a central activator of PKC. It can also be activated by TNF-α [115] or by the RAGE receptor due to ligation by AGEs [116]. PKC activation leads to reduced eNOS activity with a decreased production of NO [117]. Furthermore, PKC can modulate the endothelial monolayer permeability in various ways. It phosphorylates junctional proteins [118] or enhances the expression of vascular endothelial growth factor (VEGF), ET-1, and thrombin [100], which have permeability-inducing characteristics.

1.6.3 Dyslipidemia/Lipotoxicity and Endothelial Dysfunction

A diabetic state leads to increased lipids in plasma, cells, ectopic and visceral tissues. The effects of these lipid depots and especially those of plasma FFA, reach from oxidative stress via proinflammatory signaling right up to increased apoptosis, harming endothelial structures and functions [119, 120]. In particular, FFA plays a considerable part in inflammation processes and insulin resistance. In a trial, both insulin-mediated glucose uptake and NO-dependent limb blood flow decreased after infusion of FFA [121], implying that insulin resistance and endothelial dysfunction are also linked through elevated FFA levels [84]. FFA metabolites like coenzyme A and DAG activate PKC-θ, which phosphorylates IRS-1 leading to dysfunctional IRS-1 [122]. In addition, increased lipid levels lead to mitochondrial dysfunction uncoupling oxidative phosphorylation, which ends in an additional ROS production [98].

The reciprocal relationship between the contributing factors to endothelial dysfunction is again shown by Den Hartigh et al., who demonstrated that adipocyte NADPH oxidase 4–derived ROS causes adipocyte inflammation and supports the development of insulin resistance [123]. A novel microdialysis technique developed by La Favor et al. can simultaneously measure ROS levels and microvascular endothelial functions in vivo. They observed that NADPH oxidase–derived ROS levels were elevated in obese subjects and correlated with microvascular endothelial dysfunction, which was detected by impaired acetylcholine-induced blood flow increases [124, 125].

Another ROS-producing enzyme, the NADPH oxidase, is also affected by increased lipid levels. FFA stimulates NADPH oxidase by enhancing PKC, and the increased expression of NADPH oxidase contributes to impaired secretion of adipokines, supporting the development of insulin resistance [84, 126]. ROS induced by FFAs unleashes a cascade of proinflammatory cytokines like TNF-α and IL-6 by activating the NF-κB pathway [127]. Primarily TNF-α activates kinases IκB-kinase β (IKKβ) and c-Jun N-terminal kinase (JNK), which also phosphorylate IRS-1/2 [128]. These mechanisms lead to impaired insulin signaling with attenuated activation of eNOS and NO production [129].

Ceramide, a product derived from long-chain saturated fatty acids, has a dual effect on endothelial cells. On one side, it can activate eNOS and therefore increase NO-availability, but otherwise, it is able to inhibit insulin-stimulated activation of Akt [130] and predominantly creates ROS, which generates harmful peroxynitrite by merging with NO, resulting in endothelial dysfunction [131].

1.6.4 Inflammation and Endothelial Dysfunction

Increased circulating inflammatory markers are generally found in cardiovascular disorders like dyslipidemias, CHD, and atherosclerosis, which all go along with endothelial dysfunction [132]. Inflammation also contributes to insulin resistance through several potential biochemical mechanisms [133].

TNF-α is one of the most implicated proinflammatory cytokines in insulin resistance that directly or indirectly induces serine phosphorylation of IRS-1/2, leading to decreased PI-3 kinase and Akt activity. It creates an insulin-resistant state by activating several kinases, including JNK, IKKβ, and IL-1 receptor-associated kinase [134, 135]. These kinases attenuate the effects of PI3K/Akt, leading to reduced NO production and expression of eNOS. Furthermore, the NF-κB pathway induced by previous kinases promotes the expression of adhesion molecules, including ICAM, VCAM, and E-selectin [136]. Aside from that, the NF-κB pathway is, in turn, able to stimulate the expression of proinflammatory substances, like TNF- α [89]. A vicious circle is becoming evident if considering the significant anti-inflammatory effects of NO, which can inhibit NF-κB activity and reduce the expression of leukocyte adhesion molecules VCAM, ICAM, and E-selectin [137]. This provides an even more substantial impact of NF-κB activity, causing extensive damage to endothelial function [138].

Other inflammatory proteins, including CRP and IL-6, were expressed much more highly under the influence of TNF-α [84]. CRP can modulate the expression of proinflammatory cytokines in endothelium and downregulate eNOS expression [139, 140]. Like TNF-α, CRP also increases the expression of leukocyte adhesion molecules and supports the secretion of vasoconstrictor ET-1 [141]. Hence, CRP may also directly contribute to the pathogenesis of atherosclerosis and endothelial dysfunction.

1.6.5 Oxidative Stress and Endothelial Dysfunction

ROS, especially superoxide and hydroxyl radical, have been considered detrimental because of their highly damaging impact on cells and tissues and their pathological implications in several CVDs. They are also considered a significant reason for endothelial dysfunction (Fig. 6) [142].

Increased superoxide has a direct impact on endothelial function due to interacting with NO directly to peroxynitrite ($ONOO^-$), reducing the bioavailability of NO and resulting in other radical or non-radical reactive nitrogen species [143], which down-regulate the PI3-K/Akt pathway leading to a less vasoprotective state [144]. Indirectly, ROS activates PKC-α, PKC-β, and PKC-δ, leading to divergent gene expression for eNOS, ET-1, VEGF, TGF-β, and PAI-1 together with the ROS-dependent activation of NF-κB, which increases pro-inflammatory and prothrombogenic gene expression [145]. Activation of PKC and ROS produced by hyperglycemia contributes to the apoptosis of endothelial cells, enhanced expression of ICAM, VCAM, and E-selectin, as well as pro-inflammatory IL-6 [146, 147]. The predominant effect of PKC-α in modulating the activity of eNOS is yet to be determined. On the one hand, PKC-α takes part in the activation of eNOS in response to fibroblast growth factor [148] and VEGF [149] stimulation. On the other hand, it also directly phosphorylates eNOS at an inhibitory phosphorylation site [84]. In addition, ROS also reduce barrier function [150] and affects NO metabolism by uncoupling eNOS [151]. Uncoupling of eNOS leads to the creation of superoxide instead of NO [152]. This leads to endothelial cell activation and loss of protective mechanisms due to decreased NO availability.

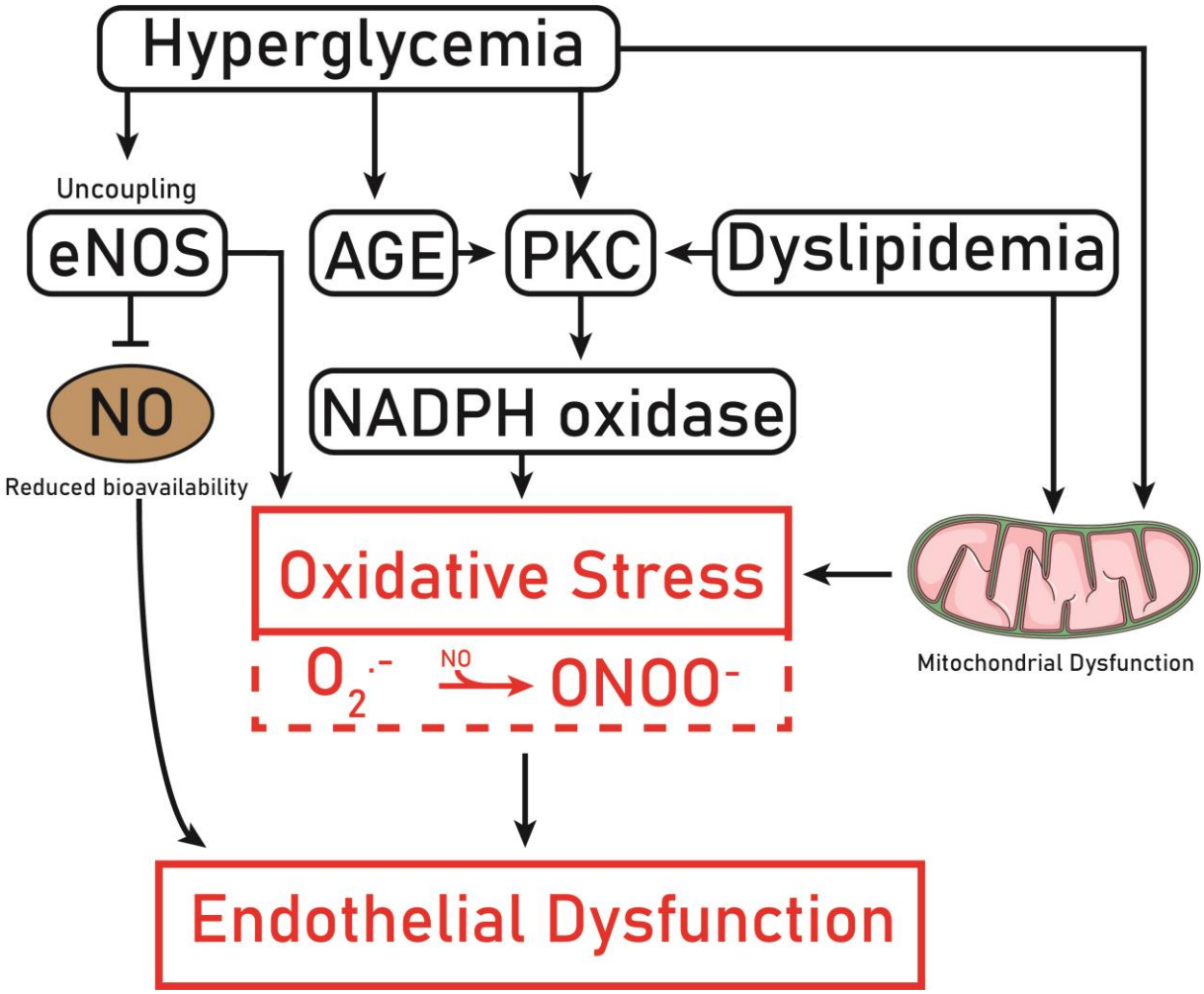

Figure 6. ROS in endothelial dysfunction [adapted from Burgos-Morón et al., 2019 [153]].

Hyperglycemia and dyslipidemia have a substantial impact on the development of endothelial dysfunction by increasing oxidative stress. Hyperglycemia uncouples eNOS, leading to reduced bioavailability of NO and increased production of superoxide. It also increases AGE levels, stimulating PKC activity. Due to the activation of NADPH oxidase by PKC, the production of ROS is increased. Both hyperglycemia and dyslipidemia induce PKC activity directly, and both induce mitochondrial dysfunction resulting in increased oxidative stress. Superoxide reacts directly with NO to harmful peroxynitrite, reducing NO bioavailability and downregulating vasoprotective pathways of insulin signaling.

Abbreviations: AGE – advanced glycation end-product eNOS – endothelial nitric oxide synthase, NADPH - nicotinamide adenine dinucleotide phosphate, NO – nitric oxide, $O_2^{\cdot -}$ - superoxide, $ONOO^-$ - peroxynitrite, PKC – protein kinase C, ROS – reactive oxygen species.

1.7 Intima-media-thickness and cardiovascular risk

The IMT is assessed by measuring the tunica intima and tunica media of an artery. It is mainly performed in a non-invasive ultrasound-supported procedure or, more rarely, with internal invasive ultrasound catheters. Due to its non-invasive technique, reproducibility, manageable costs, and widespread availability in clinical and outpatient settings, the Brightness or 'B-mode' ultrasonography established itself in measuring IMT. Here, the carotid IMT is representative for assessing atherosclerotic progression and regression, revealing a subclinical form of arteriosclerosis.

Many different studies show significant associations between carotid IMT and cardiovascular risk. The Rotterdam Study has demonstrated evidence that IMT measurements are connected with the level of generalized atherosclerosis and cardiovascular events like stroke, essential hypertension, MI, and angina pectoris [154, 155]. The Atherosclerosis Risk in Communities Study displays that a slight increase in IMT is accompanied by a significantly increased relative risk in stroke and MI [156, 157]. Lorenz et al. emphasized in a meta-analysis that IMT is an eminent risk predictor for MI, despite being more reliable in stroke [158]. According to the Heinz Nixdorf Recall Study, primarily the traditional cardiovascular risk factors, particularly diabetes mellitus, are associated with IMT [159]. In epidemiological observations, people with diabetes show a higher IMT accompanied by insulin resistance and central adiposity [160]. The associated increase in morbidity and mortality has been shown in high-risk groups with cardiovascular risk factors and the general population [161]. Another study revealed that fast carotid IMT progression results in a significantly higher rate of cardiovascular events than patients with slower Carotid IMT progression, especially those who had a high initial carotid IMT [162]. Nevertheless, IMT can be decreased by reduction of risk factors and intensive control of blood glucose [163]

IMT of the brachial artery is not used as a standardized marker of cardiovascular risk yet. However, several studies identified the predictive value of brachial IMT in cardiovascular risk assessment, especially in combination with FMD assessment. According to them, brachial IMT correlates positively with the extent of CAD and also the number of involved vessels [164, 165]. In patients with diabetes mellitus, brachial IMT is connected with the CAC score, a marker of coronary atherosclerosis [166]. Koyoshi et al. revealed that brachial IMT was significantly thicker in patients with CAD than without CAD, independent of other risk factors [167]. These results indicate that the assessment of brachial IMT in addition to carotid IMT could help prevent the development of CAD and could gain more clinical significance.

2 Hypothesis

Diabetes and its complications are a widespread disease and pose a massive burden on global health, while the number of affected people continuously increases worldwide. The most common cause of death in diabetes mellitus due to macro-and microangiopathies are cardiovascular events, predominantly stroke and MI. Mainly microangiopathies cause various greatly feared and restrictive complications in diabetes, including retinopathy, nephropathy, and polyneuropathy.

Often patients with diabetes, notably with T2D, carry even more risk factors than only hyperglycemia and insulin resistance, which interact among each other and are closely connected to endothelial dysfunction and a proatherogenic state with the development of severe and mortal outcomes. Generally, endothelial dysfunction, visualized as decreased FMD of the brachial artery, is associated with an increased risk of cardiovascular events. Studies have shown that in preclinical stages of atherosclerosis, endothelial dysfunction precedes the proof of structural alterations, and therefore, endothelial function is compromised even before the onset of clinical symptoms. The prevalence of endothelial dysfunction in patients with diabetes is increased. However, it has not yet been investigated sufficiently how high the prevalence in patients with newly diagnosed diabetes type 1 and type 2 is and how endothelial function develops in the course of the disease. Another important independent risk factor for atherosclerosis is the carotid IMT, representing overall vessel condition. The role of brachial IMT in the development of atherosclerosis is not yet finally analyzed.

This study aims to demonstrate that endothelial dysfunction already occurs in newly diagnosed diabetes type 1 and type 2 and that it deteriorates progressively in the course of the disease. Simultaneously, it wants to show that individuals, which do not hit the target values for classic parameters associated with cardiovascular risk develop an impairment in future endothelial function. In contrast, individuals within the target values would maintain a sufficient endothelial function. Additionally, the relationship between FMD and other surrogate parameters of vascular health will be pursued, especially with brachial and carotid IMT. While a low carotid and brachial IMT would suggest a physiological brachial FMD, a higher carotid and brachial IMT would implicate a decrease in brachial FMD.

3 Material and Methods

3.1 German Diabetes Study

The examinations and results described below are part of the German Diabetes Study (GDS), an ongoing prospective observational cohort study with the primary objective of identifying prognostics factors and mechanisms of related comorbidities in patients with diabetes [19]. The examinations in this particular part of the GDS describe the impact of diabetic subphenotypes on the development of the endothelial function, measured as FMD, in the course of the disease and the relation of endothelial function to other clinical and metabolic variables. The participants undergo measurement at baseline, at five years, and ten years after diagnosis. At the time of analysis, no participant reached the ten-year milestone.

The study is performed according to the Declaration of Helsinki, approved by the ethics committee of the University of Düsseldorf (previous reference number 2478, current reference number 4508), and was registered at Clinicaltrials.gov (Identifier number: NCT01055093).

There are several publications on the results of the GDS, including average values of FMD and NMD in type 1 and type 2 diabetes at baseline representing endothelial function [19]. Additionally, parts of this study referring to changes of endothelial function in the course of disease as well as comparisons to controls are published simultaneously [1].

3.1.1 Study Design

The test persons are recruited from the overall collective of the GDS based on several inclusion and exclusion criteria, as summarized in table 1. The key inclusion criterion is the diagnosis of T1D or T2D within the last 12 months in individuals between 18 and 69 years, including participants with MODY and LADA. Individuals suffering from type 3 or type 4 diabetes mellitus are omitted. Classification and diagnosis of included diabetes types are according to current American Diabetes Association (ADA) recommendations [3].

Key inclusion criteria	Key exclusion criteria	Exclusion criteria for specific examinations
• Diagnosis of T1D and T2D, including MODY and LADA, based on current ADA recommendations [3] • Onset of DM within the last 12 months • Diagnosis of T1D based on diabetes manifestation with ketoacidosis or immediate insulin requirement along with the presence of at least one islet cell-directed autoantibody or C-peptide levels below detection limit • Age of 18 - 69 years	• Secondary DM according to ADA criteria (Type 3 B-H, pancreoprive DM) [3] • Type 4 (gestational) DM, pregnancy • Poor glycemic control (HbA1c > 9.0%) • Hyperlipidemia (triglycerides and low-density lipoproteins > double upper reference limit) • Heart failure (NYHA class ≥ II) • Renal disease (serum creatinine ≥ 1.6 mg/dL) • Liver disease (AST and/or ALT and/or GGT ≥ double upper reference limit) • PAD IV • Venous thromboembolic events • Anaemia, blood donation, or participation in a clinical study within the past three months • Acute infection, leukocytes, immunosuppressive therapy, autoimmune diseases, infection with human immunodeficiency virus, other severe diseases (e.g., active cancer disease) • Psychiatric disorders, limited cooperation ability	• Neurologic examination: corneal disorders and neuropathy from causes other than diabetes • Spiroergometry: ECG abnormalities (alterations of the ST segment, higher grade arrhythmia), unstable angina pectoris, uncontrolled hypertonia • Magnetic resonance spectroscopy/imaging: metallic implants (cardiac pacemaker or defibrillator, cochlear implants, implanted catheters, clips, prosthetic valves), metallic fragments (metal removed from the eye, ever worked as a metal worker), larger tattoos, waist circumference > 135 cm, claustrophobia • Tissue biopsies: effective anticoagulation therapy, platelet aggregation inhibitors > 100 mg acetylsalicylate

Table 1. Key inclusion and exclusion criteria of the German Diabetes Study [19].

Abbreviations: ADA – American Diabetes Association, ALT – alanine aminotransferase, AST – aspartate aminotransferase, DM – diabetes mellitus, ECG- electrocardiogram, GGT – γ-glutamyl transpeptidase, HbA1c – glycated hemoglobin, LADA – latent autoimmune diabetes in adults, MODY – maturity-onset diabetes of the young, NYHA – New York Heart Association, PAD – peripheral artery disease, T1D – type 1 diabetes, T2D – type 2 diabetes.

3.1.2 Subcohort of GDS

For the current study, a total of 398 patients with T1D (179 subjects) and T2D (219 subjects) within the first 12 months of diabetes diagnosis was included, for whom data of endothelial function, M-value, spiroergometry, and bioelectrical impedance analysis were available. Additionally, the data of 109 glucose-tolerant controls were included, for whom the same data was available. The participants of the glucose-tolerant control groups underwent a 75g oral glucose tolerance test to exclude dysglycemia [3] and had no first-degree relatives with known diabetes. Follow-up data were available for 52 patients with T1D and 56 patients with T2D. Only data from operators who had performed over 50 FMD examinations were included to minimize intra- and inter-operator bias [1].

Specific exclusion criteria were applied for particular examinations, including tissue biopsies and spiroergometry, as shown in table 1.

3.1.3 Methods employed in the GDS cohort

Since all present study participants are also part of the GDS cohort, the same methods were used as described [19]. These methods include assessment of anthropometric data, laboratory measurements, spiroergometry, hyperinsulinemic-euglycemic clamp tests to measure peripheral insulin sensitivity together with indirect calorimetry and bioelectrical impedance analysis.

3.1.3.1 Anthropometric measurements and laboratory analysis

All measurements were performed on the first study day, and the subjects had to be fasting for 10 to 12 hours [19]. A calibrated scale with a stadiometer (SECA674, Hamburg, Germany) is used to measure body weight and height for anthropometric data. Measurement of waist and hip circumference is done horizontally with non-elastic tape.

Blood samples for routine laboratory parameters were taken from an antecubital vein and processed and stored under standardized conditions. Standard parameters of clinical chemistry, including plasma glucose, total cholesterol, high-density lipoproteins (HDL), LDL, serum triglycerides, high-sensitivity c-reactive protein (hsCRP), γ-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were measured on a Hitachi 912 analyzer (Roche Diagnostics, Mannheim, Germany). HbA1c is measured on a Variant-II (Bio-Rad, Munich, Germany). Serum concentrations of insulin and C-peptide were measured chemoluminimetrically and FFA microfluorimetrically on a Cobas c311 (Roche Diagnostics, Mannheim, Germany).

Fasting concentrations of insulin and FFA were used to calculate the adipose-tissue insulin resistance index [168]. To define NAFLD, liver steatosis and fibrosis estimates, embodied by fatty liver index (FLI) and FIB-4 index, were computed from routine laboratory parameters [169].

3.1.3.2 Cardiovascular parameters and cardiorespiratory fitness

Assisted by an electrocardiogram (ECG), heart rate was recorded at rest. Blood pressure was measured in a supine position on both arms and both legs. The ratio of the systolic blood pressure measured at the ankle and measured at the brachial artery resulted in the ankle-brachial index (ABI) [170].

On the first examination, an incremental exhaustive exercise test on the cycle ergometer (Ergometrics 900; Ergoline, Bitz, Germany) assessed physical fitness [171]. Arm blood pressure, heart rate, and 12-lead ECG were recorded every 2 minutes during the test. Each participant started the test at 60 revolutions/min [172]. Open-air spirometry measured the respiratory gas exchange (Masterscreen CPX; Jäger/Viasys, Hoechberg, Germany). During exercise, the workload was increased in 16 W/min increments, and the maximal exhaustion (VO_2max) was reached on average after 12 – 15 minutes of exercise.

3.1.3.3 Bioelectrical impedance analysis

Bioelectrical impedance analysis was used to estimate fat mass (FM) and fat-free mass (FFM), both in kg, and to calculate the deriving percent fat mass (BioElectrical Impedance Analyzer System, RJL Systems, Detroit, MI, USA) [19]. Following the attachment of two electrodes on the patient’s right hand and right foot, the resistance of different tissues to the current flow was determined.

3.1.3.4 Modified Botnia clamp test

All participants underwent a modified Botnia clamp test, consisting of an intravenous glucose tolerance test (IVGTT) followed by a hyperinsulinemic-euglycemic clamp test, as previously described and validated [19, 173]. All participants were asked to avoid intense physical activity and stop their oral glucose-lowering medication three days before the clamp. If applicable, the last insulin dose should be injected on the evening before the examination. For blood sampling and infusion of glucose and insulin, two venous catheters were inserted in the antecubital veins of both arms. The IVGTT was started by a bolus of 30% glucose of 1ml/kg (body weight) within 30 seconds into one forearm vein, followed by timed blood sampling for 60 minutes. After 60 minutes, a priming dose of short-acting human insulin (Insuman Rapid; Sanofi, Frankfurt, Germany) was applied [10 mU*kg (body weight)$^{-1}$* min^{-1} for 10 minutes] and continued by constant infusion of 1.5 mU*kg (body weight)$^{-1}$* min^{-1} for 180 minutes until the end of the clamp. A variable 20% glucose infusion was periodically adjusted to maintain euglycemia at 90mg/dl (5 mmol/l).

Whole-body insulin sensitivity was measured from whole-body mean glucose infusion rates during the last 30 minutes of the clamp with glucose space correction (GSC) during steady-state. GSC was calculated as (G180-G150)/30, with G180 and G150 representing glucose values at the timepoints 180 and 150 of the clamp (in mg/dl). 30 represented the time interval between these two measurements (in minutes) [19].

3.2 Assessment of endothelial function

3.2.1 Preparations

Before starting the examination, the test person must meet a couple of requirements. It is required that the test person is fasting for at least eight hours, including high-fat food, Vitamin C-compounds, and all kinds of beverages except for water. In addition, the patient should neither smoke nor be physically active for at least six hours. There should not be an intake of any medication for at least six hours before the examination. Each medication intake should be documented for the last seven days, including antihypertensive medication if there are normotensive blood pressure values (< 140/90 mmHg) under regular medication. An exception is made for test persons who show hypertensive blood pressure values ≥ 140 mmHg systolic or ≥ 90 mmHg diastolic under regular medication. Then it is necessary to apply and document antihypertensive medication in the morning before the examination. However, if possible, the antihypertensive medication should be applied after the assessment of endothelial function. In the case of women, the day of the menstrual cycle has to be documented.

The patient is to be informed about the examination and the risks of the administration of glycerol trinitrate. Table 2 lists both the contraindications measurement of endothelial dysfunction and the application of glycerol trinitrate.

Contradictions of FMD measurement	Contradictions of NMD measurement
• Stage 2 Hypertension (blood pressure ≥ 160/100 mmHg) • congestion syndrome in the arm (e.g., chronic venous insufficiency or Paget-Schroetter disease; diseases of the lymphatic system [e.g., after axillary lymph node dissection]) • Raynaud syndrome	• Hypersensitivity to nitrate compounds • Hypotension (RR systolic < 90 mmHg) or propensity to an orthostatic circulatory disorder • Patients with low filling pressure (e.g., after acute cardiac infarction or left ventricular insufficiency) • Aortic- or mitral stenosis • Diseases with increased intracranial pressure • Simultaneous use of phosphodiesterase-5-inhibitors

Table 2. Contradictions of FMD and NMD measurement.

Abbreviations: FMD – flow-mediated dilation, NMD – nitroglycerin-mediated dilation.

The examination is ECG-assisted, so the patient should undress his torso for applying the ECG-electrodes on his chest and the blood pressure cuff on his right arm. The ECG-electrodes are applied as follows: First rib parasternal left and right, 8. ICS anterior axillary line left.

The blood pressure cuff is placed on the right forearm at the spot with the largest circumference. The upper edge of the cuff should end a maximum of 2 centimeters underneath the tip of the olecranon with the arm in full extension. The stretched-out arm of the test person should be angled 45° in the shoulder joint and placed in a comfortable position with optimal access for the transducer to the measuring point.

Before starting the examination, the test person must lay at least 15 minutes supine in a slightly darkened and temperate room (22°C). Variances of the room temperature > 2°C or shorter laytime have to be documented. During the whole examination, the test person must not speak, move or stand up.

3.2.2 Measurement of FMD

A linear transducer is required for the whole measurement.

The assisting ECG should be placed in a suitable place on the ultrasound screen without disturbing the measurement. QRS complex, ST segment, and T wave should be differentiable.

The transducer should be positioned on the inside of the upper arm maximum of 2 - 3 centimeters above the elbow and depict the brachial artery in longitudinal view.

The focal zone should be on the same level as the vessel, ideally on the vessel wall distant from the transducer. There should be a clear differentiation of the Intima-Media-Border of both vascular walls at the measuring point at least 1 centimeter without artifacts in the vascular lumen, diverting vessels, or significant vessel curvatures. The transducer should be moved as little as possible between the various parts of the examination. The pressure applied on the transducer should be uniform and soft but sufficiently enough to depict the vascular section correctly during the whole measurement.

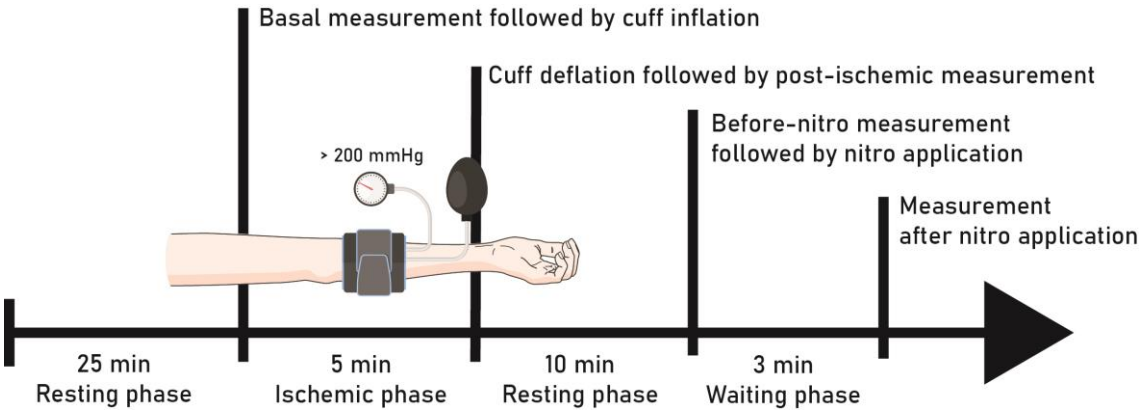

Figure 7. Schedule of FMD measurement.

A schematic representation of FMD and NMD measurement is shown in Figure 7. The first measurement starts with a 10-second loop while the patient is at rest. After that, the blood pressure cuff is to be inflated up to 200 mmHg or 50 mmHg above systolic value (if systolic blood pressure is ≥ 150 mmHg), as far as the patient tolerates. Control of the pressure every 30 - 60 seconds, inflating if necessary. The cuff's pressure is deflated quickly after 5 minutes (tolerance of +/- 10 seconds). Six loops under stress follow precisely 30 seconds after deflating. The first two loops have a length of 20 seconds; the next four loops were 10 seconds long. The transducer must not be moved or taken off during the measurement. Periods >2 seconds between loops have to be documented.

3.2.3 Measurement of NMD

If no contraindications are present, the NMD measurement follows after the FMD measurement. It is necessary to keep a break of at least 10 minutes and adjust the image section used in the FMD-measurement.

Recording a 10-second loop with the patient at rest, then applying one sublingual puff of Nitrolingual spray and letting the test person swallow; precisely 3 minutes after application recording of 6 loops. The first two loops have a length of 20 seconds; the next four loops were 10 seconds long. Here too, the transducer must not be moved or taken off during the measurement. The applied pressure should be sufficient but not too heavy to compress the vessel, and periods > 2 seconds between loops must be documented.

3.2.4 Analysis

After finishing the measurements (including the NMD), there is one loop in rest and six loops under stress for both FMD and NMD, which are analyzed with Brachial Analyzer from Vascular Research Tools 5 of the company Medical Imaging Tools (Coralville, Iowa, USA). If all loops are recorded correctly, the loop at rest and the fifth under stress will be analyzed in FMD and NMD.

3.2.4.1 Analysis of FMD and NMD

After loading the relevant loop, the calibration in horizontal and vertical orientation was set up to 10 units along the scale of the ultrasound screen.

The Region of interest (ROI) was set in at least a 1-centimeter-long vessel segment with a clear differentiation of both vascular walls and without diverting vessels or significant curvatures (Fig. 8). The centerline of the ROI should lay central and parallel to the course of the vessel. The ROI should include both vascular walls but as little surrounding tissue as possible. The vessel diameter is defined as the way between the proximal and distal Intima-line (I-line).

After defining the ROI, the program starts with an automatic analysis of all frames of the loop. If the automatically detected vessel border moves during the analysis, there is the chance to edit single frames afterward. Every relevant loop is analyzed similarly. The same segment of the vessel must be chosen in every loop.

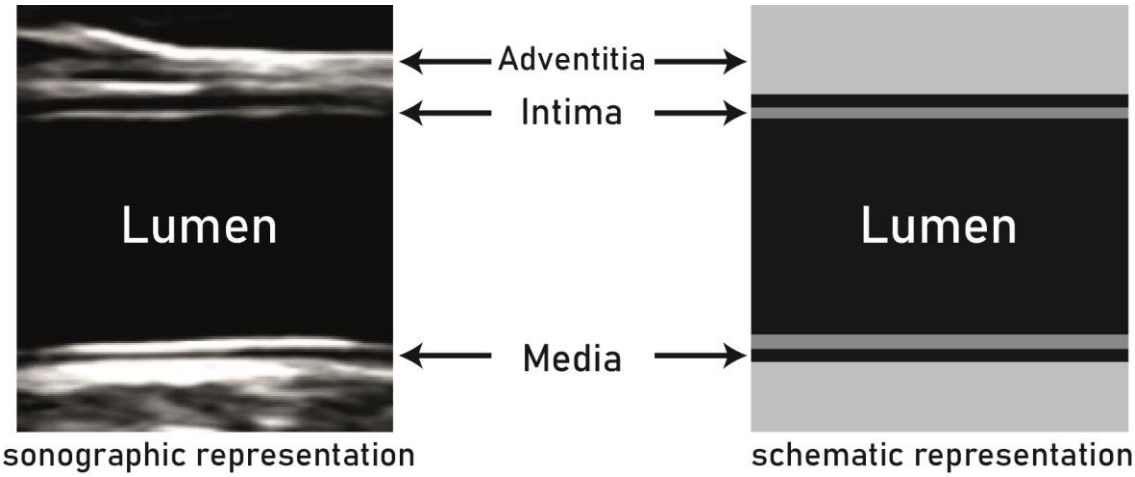

Figure 8. Ultrasonic imaging of the vascular wall.

Additionally, the image quality of the loops at rest before hyperemia and before nitroglycerin-application is evaluated according to 4 different items (Table 3). The points of the individual items are then added up to a final score.

Item	Points		
Is the vessel lumen anechoic and without artifacts?	1	=	very good
	1,5	=	very good to good
	2	=	good
	2,5	=	acceptable
	3	=	bad
Is the Intima-Media-Complex visible as a continuous double line for at least 1 centimeter?	1	=	very good
	1,5	=	very good to good
	2	=	good
	2,5	=	acceptable
	3	=	bad
How many frames (in percentage) were automatically analyzed by Brachial Analyzer?	1	=	> 90%
	1,5	=	80 - 89%
	2	=	70 - 79%
	2,5	=	50 - 69%
	3	=	< 50%
How many diameters were chosen to calculate the mean value?	1	=	4 diameter
	1,5	=	3 diameter
	2	=	2 diameter
	2,5	=	1 diameter
	3	=	0 diameter

Table 3. Evaluation of ultrasonic image quality.

The calculation of FMD and NMD is based on four frames of the specific loop. During the diastole, the vessel diameter is at its smallest state. Therefore, after the automatic analysis of the program, four frames of the loop during the diastole are chosen to determine the arithmetic mean of the vessel diameter calculated with the formula (1) correct to 2 decimal places.

(1) $$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

The calculation of the arithmetic mean of the vessel diameter is performed for all four loops. The standard deviation is calculated for every loop with the formula (2) correct to 2 decimal places.

(2) $$s = \sqrt{\frac{\sum(\mathrm{x}-\mathrm{x_i})^2}{\mathrm{n}-1}}$$

To calculate the FMD and NMD with the previously determined average vessel diameters following formulas are used:

(3) $$\text{FMD [\%]} = \left[\frac{(Vessel\ diameter\ "Stress"\ -\ Vessel\ diameter\ "Rest"}{Vessel\ diameter\ "Rest"}\right] \times 100$$

(4) $$\text{NMD [\%]} = \left[\frac{(Vessel\ diameter\ "Nitro"\ -\ Vessel\ diameter\ "Nitro\text{-}Rest"}{Vessel\ diameter\ "Nitro-Rest"}\right] \times 100$$

Both FMD and NMD are given as a percentage and are calculated correctly to 2 decimal places.

Only data from operators who had performed over 50 FMD examinations were included to minimize intra- and inter-operator bias.

3.2.4.2 Analysis of brachial IMT

The brachial IMT is analyzed with Carotid Analyzer from Vascular Research Tools 5 of Medical Imaging Tools (Coralville, Iowa, USA).

The loop used for brachial IMT is the first recorded loop with the patient at rest before the stress measurement. For calibration, the horizontal and vertical orientation was set up to 10 units along the scale of the ultrasound screen.

Similar to the analysis of FMD and NMD, the ROI is placed in a 1-centimeter-long vessel segment, depicting a clear differentiation of both vascular walls. The centerline of the ROI should lay in the center of the vessel, and the frame defining the ROI should include both vascular walls but as little surrounding tissue as possible. The program should detect the intima border facing the lumen and the inner media border of both vessel walls, followed by an automatic analysis of both IMTs. If the automatically detected vessel border moves during the analysis, there is the chance to edit single frames afterward. Four frames of the loop are chosen to determine the arithmetic mean of the IMT calculated with the formula [1] correct to 2 decimal places. Preferably the four loops selected for FMD can be used for IMT as well. Due to the physical properties of ultrasound, the IMT of the vessel wall distant from the probe must be documented. The image quality of brachial IMT must be evaluated according to the same criteria used for FMD, as shown in table 3.

3.2.5 Comparison measurements

Due to known inter-observer variability in FMD measurements, we performed our own reproducibility study to avoid a possible misinterpretation of our results. 29 consecutive participants of the GDS were examined by two experienced operators, who had performed over 50 FMD examinations. FMD, IMT, and the diameter of the brachial artery were measured as previously described [1].

3.3 Statistical Analysis

Data are presented as means and standard deviation (±SD) for continuous variables and percentages (%) for categorical variables. Skewed data (M-value, triglycerides) were log-transformed before analysis. Matching was performed by propensity score for age, sex, and BMI. Analyses adjusted for age, sex, and BMI were performed to exclude these as confounding factors where necessary. Associations between parameters have been evaluated using linear regression models and corresponding P values. Regression models were used to assess the best predictors of variable changes over time. P values <5% were considered to indicate significant differences or correlations. Statistical analyses were performed using SAS (version 9.4; SAS Institute, Cary, NC, USA).

4 Results

4.1 Study population

This research work is part of the ongoing prospective GDS; therefore, the gathered data originate from participants representing a subcohort of the study [19]. Only data from operators who had performed over 50 FMD examinations were included to minimize intra- and inter-operator bias.

The results comprise the data of 179 T1D patients, 218 T2D patients, and their respective age-, sex- and BMI-matched groups consisting of 109 glucose-tolerant controls (CON1, n = [58] and CON2, n = [51]).

Data of baseline brachial IMT was available from 51 patients with T1D and 74 with T2D. Within the glucose-tolerant controls, we obtained brachial artery IMT data from 88 participants (CON1, n = [43] and CON2, n = [45]).

Baseline data of carotid IMT was available from 14 patients with T1D and 16 with T2D. Within the glucose-tolerant controls, we obtained carotid IMT data from 29 participants (CON1, n = [18] and CON2, n = [11]).

Further, due to the ongoing study design of the GDS, a subset of patients was reevaluated during the 5-year follow-up, including data sets of 52 patients with T1D and 56 patients with type 2 diabetes. Within these data sets, both baseline and follow-up data of brachial IMT were available from 21 patients with T1D and 24 with T2D [1].

The participants of the control group were examined only at baseline.

4.2 Baseline Results

4.2.1 Anthropometric and clinical characteristics

Anthropometric and clinical data are shown in Table 4. As expected, patients with type 1 diabetes were slightly younger than participants of the other groups, with T2D patients representing the oldest group. Independently of age and sex, patients with T2D had higher BMI and WHR than T1D patients and higher WHR than their respective controls [1]. Fasting blood glucose and HbA1c were distinctly higher in both diabetes groups than in the respective controls ($p<0.05$) and higher in T1D patients than in T2D ($p<0.05$) [1]. According to current guidelines [174], the majority of patients with diabetes (76%) had excellent glycemic control at baseline, with the average HbA1c below 7% (53 mmol/mol) [1]. Additionally, hsCRP levels in participants of both groups were higher than in their

respective glucose-tolerant control group ($p<0.05$). No difference was observed in the estimated glomerular filtration rate (eGFR) between all groups.

	CON1	T1D	CON2	T2D
N [m/f]	58 (32/26)	179 (103/76)	51 (36/15)	219 (153/66)
Known diabetes duration [months]	-	6.1±2.6	-	5.9±3.1
Age [years]	37.0±12.5	36.5±11.1	50.8±10.8	51.2±10.3#
BMI [kg/m^2]	25.1±3.9	24.9±4.1	28.7±3.7	30.1±4.9#
WHR	0.86±0.08	0.88±0.09	0.92±0.07	0.96±0.07*#
Fat mass [%]	20.1±8.8	19.6±8.6	28.1±8.1	30.2±10.3
hsCRP [mg/dl]	0.16±0.32	0.22±0.37*	0.18±0.27	0.30±0.29*
Fasting blood glucose [mg/dl]	88±16	133±41*	89±7	127±32*#
HbA1c [% (mmol/mol)]	5.1±0.2	6.6±1.2*	5.3±0.3	6.4±0.9*#
eGFR [ml/min/1.73 m²]	97.6±13.5	101.8±14.6	89.7±11.9	90.0±15.8
Total cholesterol [mg/dl]	186±40	186±42	205±37	199±44
LDL-cholesterol [mg/dl]	114±38	111±34	132±34	128±36
HDL-cholesterol [mg/dl]	65±19	62±18	59±18	45±13*#
Triglycerides [mg/dl]	99±58	90±62	142±192	175±197*
ALT [U/l]	21.8±9.5	23.7±19.0	24.8±10.4	33.7±21.8*
AST [U/l]	23.8±6.6	21.7±7.9*	23.3±6.6	25.7±12.7
GGT [U/l]	20.8±15.9	20.6±16.8	28.6±22.2	42.5±65.72*#
FFA [µmol/l]	476±190	656±290*	526±190	638±256*#

Table 4. Baseline characteristics of the study population [1]

Data are shown as absolute numbers or mean ± standard deviation, as applicable.
Abbreviations: ALT - alanine aminotransferase, AST - aspartate aminotransferase, BMI – body-mass-index, CON1 – age, sex, BMI matched controls for the type 1 diabetes group, CON2 – age, sex, BMI matched controls for the type 2 diabetes group, eGFR – estimated glomerular filtration rate, FFA - free fatty acids, GGT - γ-glutamyl transferase, HbA1c - glycated hemoglobin, HDL – high-density lipoprotein, hsCRP – high sensitivity C-reactive protein, LDL – low-density lipoprotein, T1D – type 1 diabetes, T2D – type 2 diabetes, WHR – waist-to-hip ratio.
**, p≤0.05 T1D or T2D vs. CON1 or CON2, respectively.*
#, p≤0.05 T1D vs. T2D adjusted for age, sex, BMI.

4.2.2 Endothelial function and cardiovascular risk factors

After adjustment for age, sex, and BMI at baseline, no difference was observed in endothelial function assessed by FMD across all groups (Fig. 9A, all $p>0.05$). T1D patients showed higher NMD than T2D patients only if unadjusted, but there was no difference between individuals with diabetes and their control groups (Fig. 9B) [1]. IMT of the brachial artery, representing the local vessel condition, was significantly thicker in both T1D and T2D than in their respective controls, even after adjustment for age, sex, and BMI (Fig. 9C, all $p<0.05$). Differences between T1D and T2D in brachial IMT were only significant if unadjusted. IMT of the carotid, representing overall vessel condition, was only higher in T2D than in the control group (Fig. 9D), but it became insignificant after adjusting for age, sex, and BMI.

Both diabetes groups had lower physical fitness (VO_2max) than their respective controls, but patients with T1D, however, showed significantly higher VO_2max than patients with T2D (Fig. 10A). Participants with T2D also showed higher resting heart rates than their controls and the T1D group (Fig. 10B) and higher systolic blood pressure than in T1D patients (Fig. 10C). There were no baseline differences in diastolic blood pressure or ABI between diabetes and control groups [1].

Regarding lipid metabolism, FFA was higher in both groups than their respective controls ($p<0.05$) and higher in T2D than in T1D. As presented in Tab. 4, the lipid profile showed similarities in total-, HDL- and LDL-cholesterol between patients with T1D and the glucose-tolerant control group. T2D patients showed lower HDL-cholesterol than T1D patients and metabolically healthy subjects. Additionally, the triglycerides were significantly higher than in CON2. Of those affected by T1D, 176 (89.8%) had fasting triglyceride levels <150 mg/dl and 85 (43.8%) achieved LDL levels <100 mg/dl as recommended in current guidelines (22), while of T2D patients only 157 (57.5%) and 55 (20.2%), respectively, achieved the target values [1].

4.2.3 Insulin sensitivity and estimates of liver steatosis and fibrosis

Whole-body insulin sensitivity was reduced in T1D compared to CON1 (12.0±3.5 vs. 9.0±3.2 $mg*kg^{-1}*min^{-1}$, $p<0.001$) as well as in T2D compared to CON2 (10.2±3.1 vs. 6.7±2.8 $mg*kg^{-1}*min^{-1}$, $p<0.001$) (Fig. 10D) [1]. After adjusting for age, sex, and BMI, the adipose tissue insulin resistance index was higher in both diabetes groups than in the respective controls (Fig. 10E). As expected, as assessed by C-peptide levels, the beta-cell function was considerably higher in metabolically healthy controls than in both diabetes groups and higher in T2D than T1D individuals (Fig. 10F).

Surrogate estimates of hepatic steatosis (FLI) were higher in T2D compared to T1D and the control group (Fig. 10G). Surrogate estimates of liver fibrosis (FIB-4) yielded the highest scores for patients with T2D, but there was no significant difference between all the groups after adjustment for age, sex, and BMI (Fig. 10H). Regarding liver enzymes, patients with T1D had lower AST than their control group ($p<0.05$), whereas in T2D patients, GGT was distinctly higher than in T1D and CON2 ($p<0.05$). Additionally, ALT levels were higher in T2D than in their healthy control group at baseline ($p<0.05$) (Tab. 4).

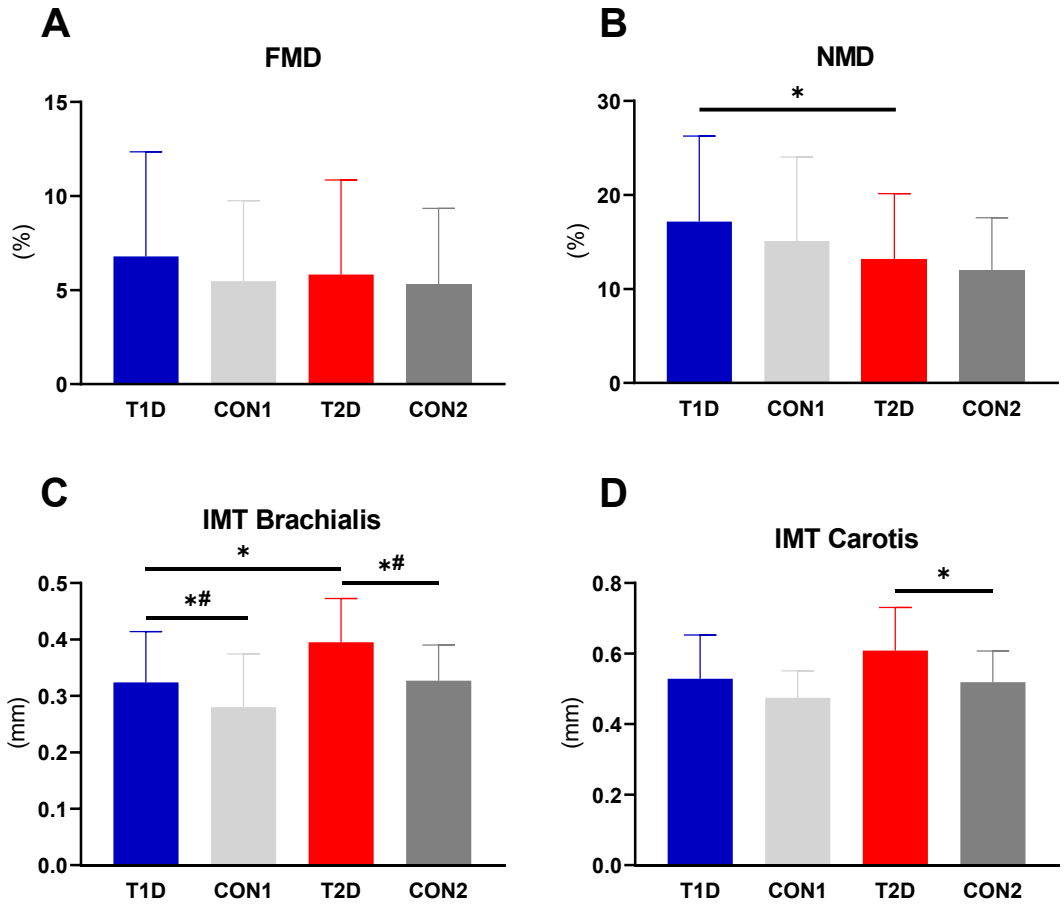

Figure 9. Endothelial function at baseline [adapted from Zaharia et al., 2022 [1]].

Parameters of endothelial function in patients with newly diagnosed T1D, T2D, and matched glucose tolerant humans (CON) showing FMD (A), NMD (B), brachial IMT (C), and carotid IMT (D) at baseline.

Abbreviations: CON1 – age, sex, BMI matched controls for the type 1 diabetes group, CON2 – age, sex, BMI matched controls for the type 2 diabetes group, FMD – flow-mediated dilation, IMT – intima-media-thickness, NMD – nitroglycerin-mediated dilation, T1D – type 1 diabetes, T2D – type 2 diabetes.

*Bar graph with whiskers for standard deviation. *, unadjusted $p < 0.05$. #, $p < 0.05$ adjusted for age, sex, and BMI.*

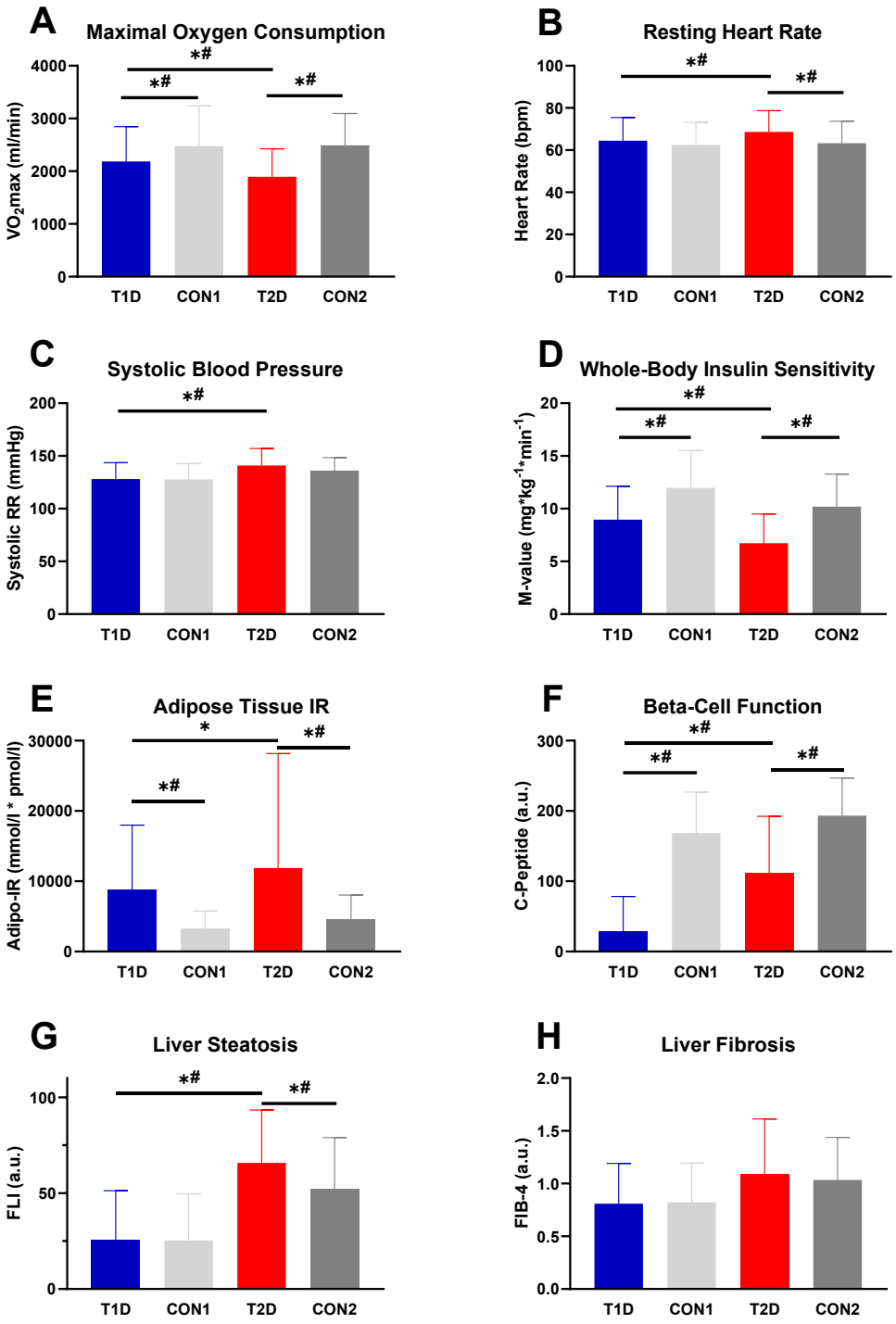

Figure 10. Baseline characteristics [adapted from Zaharia et al., 2022 [1]]

Physical fitness (A), resting heart rate (B), systolic blood pressure (C), insulin sensitivity (D), Adipo-IR (E), beta-cell function (F), and indices of liver steatosis (G) and liver fibrosis (H) in patients with newly diagnosed T1D, T2D, CON1 and CON2 at baseline.

Abbreviations: Adipo-IR – adipose insulin resistance index, CON1 – age, sex, BMI matched controls for the type 1 diabetes group, CON2 – age, sex, BMI matched controls for the type 2 diabetes group, FIB-4 – fibrosis-4, FLI – fatty liver index, FMD – flow-mediated dilation, IMT – intima-media-thickness, NMD – nitroglycerin-mediated dilation, T1D – type 1 diabetes, T2D – type 2 diabetes, VO_2max – maximal oxygen consumption.

*Bar graph with whiskers for standard deviation. *, unadjusted $p < 0.05$. #, $p < 0.05$ adjusted for age, sex, and BMI.*

4.2.4 Associations between endothelial function and metabolic and cardiovascular parameters at baseline

In patients with T1D, FMD correlated positively with NMD at baseline (Fig. 11A). NMD, in turn, correlated negatively with GPT and AP. Both brachial and carotid IMT correlated negatively with eGFR. Furthermore, brachial IMT correlated positively with age, WHR, and both systolic and diastolic blood pressure. In addition, carotid IMT of T1D patients correlated positively with age, GPT, GOT, and surrogate estimates of liver fibrosis, represented by FIB-4.

In T2D patients, there were no significant correlations between FMD at baseline and any other cardiovascular parameter. Decreased NMD at baseline, however, was associated with higher FIB-4. In both brachial and carotid IMT, several correlations with classic cardiovascular risk factors were observed. Thicker brachial IMT was accompanied by higher age, BMI, WHR, HbA1c, and systolic blood pressure. Additionally, FIB-4 was associated with thicker brachial IMT. In T2D thicker brachial IMT went hand in hand with thicker carotid IMT (Fig. 11B). Carotid correlated positively with age, BMI, WHR, ABI, HF, Total- and LDL-cholesterol, HbA1c, AP, as well as diastolic and systolic blood pressure.

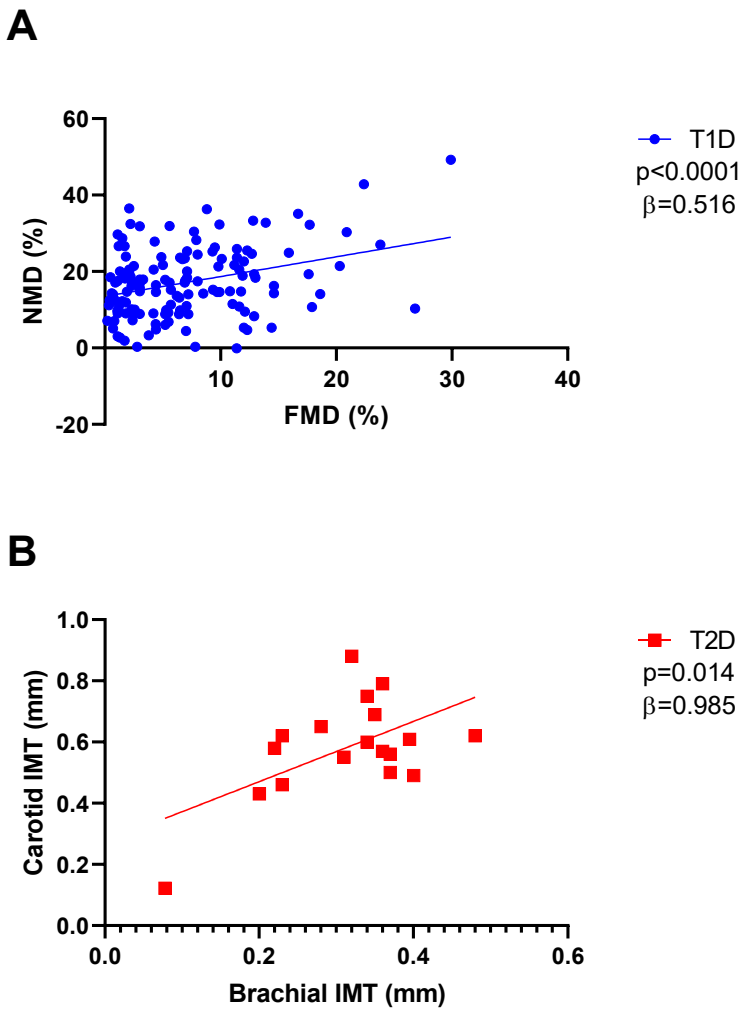

Figure 11. Baseline associations between parameters of endothelial function.

Linear regression models showing associations between baseline FMD and baseline NMD in T1D (A) as well as carotid IMT and brachial IMT in T2D (B).

Abbreviations: FMD – flow-mediated dilation, IMT – intima-media-thickness, NMD – nitroglycerin-mediated dilation, T1D – type 1 diabetes, T2D – type 2 diabetes.

ß, linear regression coefficients, p < 0.05 adjusted for age, sex and BMI

4.3 Follow-up Results

4.3.1 Anthropometric and clinical characteristics

Anthropometric and clinical data of a subset of individuals with T1D and T2D at baseline and 5-year follow-up are shown in Table 5. Over 5 years, the fat mass increased in both T1D and T2D. BMI only increased in T1D. Glycemic control, represented by fasting blood glucose and HbA1c, was worse in both groups, independent of age, sex, BMI, and baseline value [1]. There was no change in WHR, hsCRP, and eGFR.

	Type 1 diabetes		**Type 2 diabetes**	
	Baseline	**Follow-up**	**Baseline**	**Follow-up**
N [m/f]	52 (28/24)		56 (43/13)	
Age [years]	34.7±11.9	39.8±11.9	49.8±10.8	54.9±10.7
BMI [kg/m^2]	24.7±4.1	26.7±4.8*	32.0±6.8	32.5±6.7
WHR	0.9±0.1	0.9±0.1	1.0±0.1	1.0±0.1
Fat mass [%]	18.8±7.9	23.5±9.2*	33.6±14.0	35.1±13.0*
hsCRP [mg/dl]	0.29±0.52	0.39±0.30	0.26±0.37	0.29±0.22
Fasting blood glucose [mg/dl]	137±44	163±47*	118±22	152±46*
HbA1c [%]	6.8±1.2	7.3±1.1*	6.1±0.6	6.9±1.0*
eGFR [ml/min/1.73 m^2]	103±16	100±15	90±15	89±16
Total cholesterol [mg/dl]	190±39	183±38	194±45	196±38
LDL-cholesterol [mg/dl]	112±32	111±32	119±30	127±36
HDL-cholesterol [mg/dl]	64±18	65±21	42±11	43±14
Triglycerides [mg/dl]	87±49	97±67	209±332	211±150
ALT [U/l]	25.5±29.8	21.4±11.7	37.1±24.6	38.0±27.4
AST [U/l]	22.4±11.1	22.3±9.2	24.9±12.1	26.3±15.1
GGT [U/l]	19.2±10.5	21.8±20.1	39.5±24.9	44.4±30.1*
FFA [µmol/l]	683±269	599±353	642±216	713±281

Table 5. Patients' characteristics at baseline and 5-year follow-up.

Data are shown as absolute numbers or mean ± standard deviation, as applicable.
Abbreviations: ALT - alanine aminotransferase, AST - aspartate aminotransferase, BMI – body-mass-index, CON1 – age, sex, BMI matched controls for the type 1 diabetes group, CON2 – age, sex, BMI matched controls for the type 2 diabetes group, eGFR – estimated glomerular filtration rate, FFA - free fatty acids, GGT - γ-glutamyl transferase, HbA1c - glycated hemoglobin, HDL – high-density lipoprotein, hsCRP – high sensitivity C-reactive protein, LDL – low-density lipoprotein, T1D – type 1 diabetes, T2D – type 2 diabetes, WHR – waist-to-hip ratio.
** for $p \leq 0.05$. P values refer to differences between baseline and follow-up adjusted for age, sex, BMI, and baseline value.*

4.3.2 Endothelial function and cardiovascular risk factors

After 5-years, FMD decreased in both T1D and T2D patients (Fig. 12A). There was no difference in NMD in T1D but a decrease in T2D (Fig. 12B). Only the decline in FMD in individuals with T2D remained significant after adjustment to age, sex, BMI, and the respective baseline (-13.9%, p=0.013) [1]. The morphological condition of the vessel, represented by brachial IMT, decreased significantly in patients with T1D (Fig. 12C). However, there were no significant differences between changes in FMD, NMD, and IMT between both diabetes groups (Fig. 12A-C).

Regarding the lipid metabolism parameters (Total-, LDL- & HDL-cholesterol, triglycerides, FFA) (Tab. 5), resting heart rate, systolic or diastolic blood pressure, and VO_2max, we did not detect any significant changes occurring independently of age, sex, and BMI (Fig. 13A-C) [1].

4.3.3 Insulin sensitivity and estimates of liver steatosis and fibrosis

Follow-up data did not show any significant changes in adipose tissue insulin sensitivity within the first five years of diagnosis (Fig. 13E), despite a reduction in whole-body insulin sensitivity in T1D patients by 24% and by 15% in T2D, respectively ($p<0.05$) (Fig. 13D) [1]. Beta-cell function (C-peptide concentrations) decreased only in T1D patients (Fig. 13F) after adjustment for age, sex, and BMI. Patients with T2D showed higher GGT levels at follow-up, but there were no changes in AST nor ALT in any of the groups (Table 5) [1]. Both T1D and T2D patients showed significant progress in NAFLD over 5 years of diabetes duration, as represented by increasing indices of FLI and FIB-4 (both $p<0.001$; Fig. 13G-H) [1].

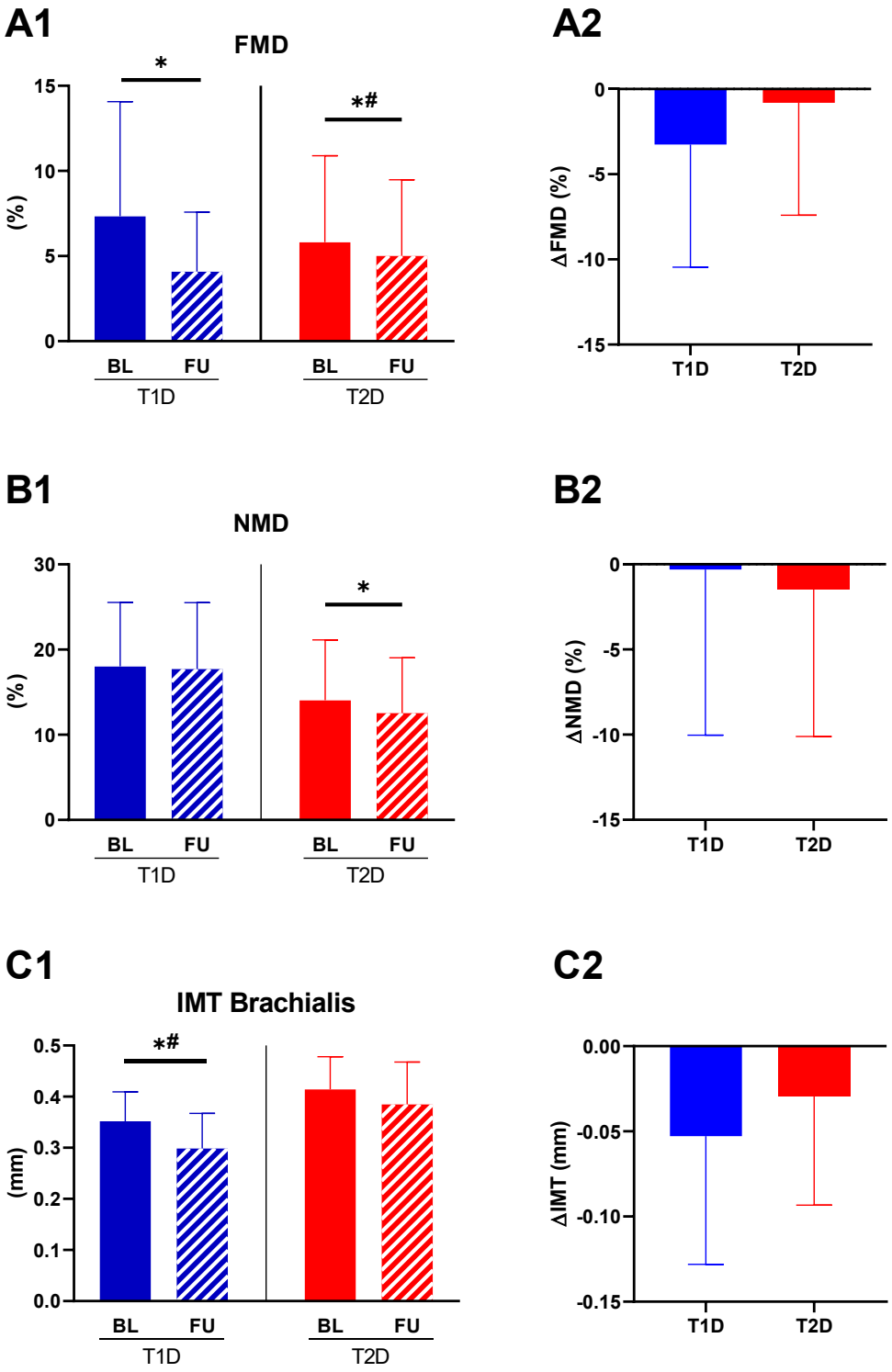

Figure 12. Endothelial function at follow-up [adapted from Zaharia et al., 2022 [1]].

Comparison of parameters of endothelial function at the 5-year follow-up in a subgroup of patients with T1D and T2D, showing both mean values and difference Δ for FMD (A1, A2), NMD (B1, B2), and brachial IMT (C1, C2).

Abbreviations: BL – baseline, FMD – flow-mediated dilation, FU – follow-up, IMT – intima-media-thickness, NMD – nitroglycerin-mediated dilation, T1D – type 1 diabetes, T2D – type 2 diabetes.

*Bar graph with whiskers for standard deviation. *, $p < 0.05$ adjusted for age, sex, BMI, and baseline value.*

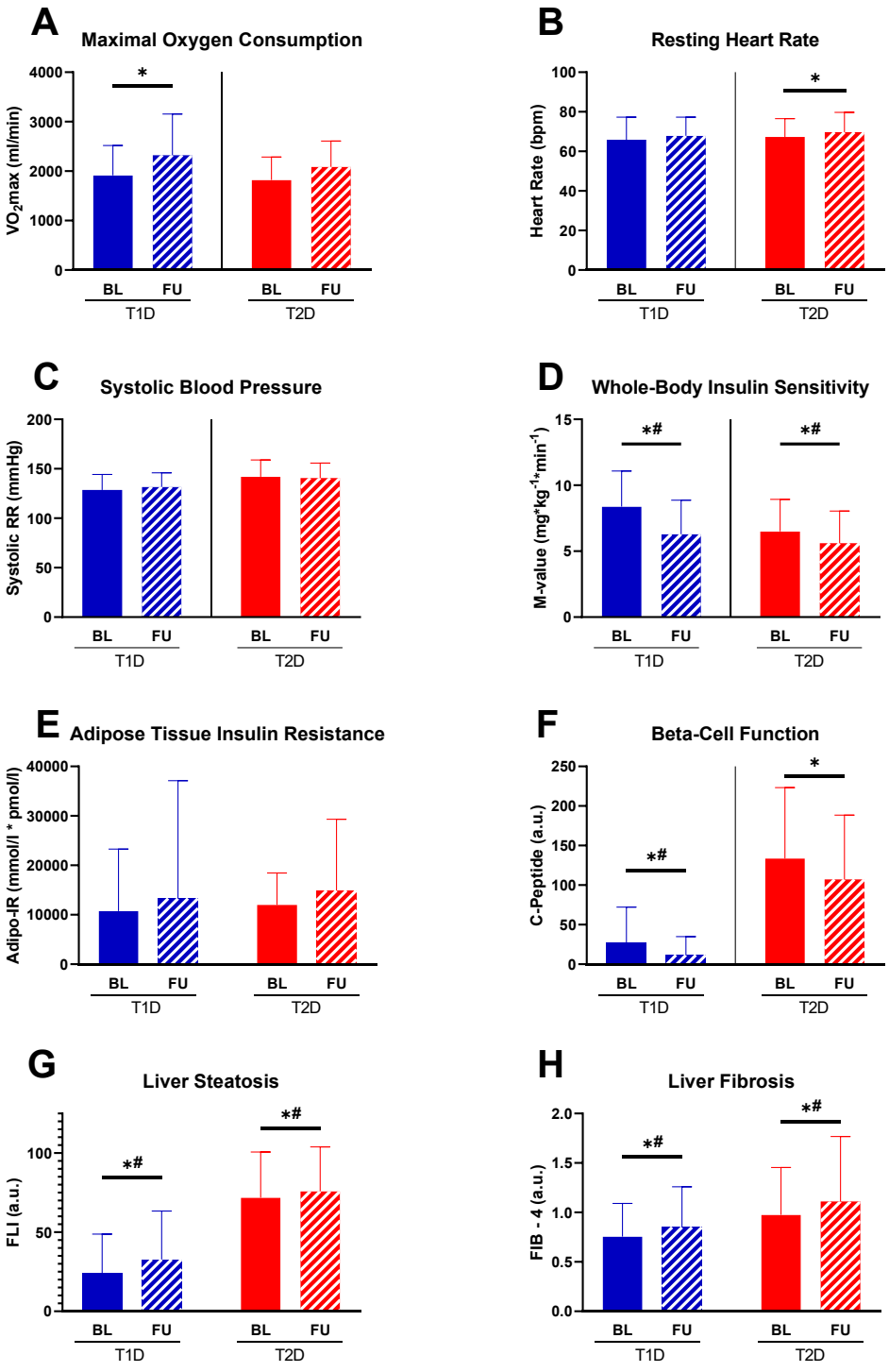

Figure 13. Follow-up characteristics [adapted from Zaharia et al., 2022. [1]]

Comparison of physical fitness (A), resting heart rate (B), systolic blood pressure (C), insulin sensitivity (D), Adipo-IR (E), beta-cell function (F), and indices of liver steatosis (G) and liver fibrosis (H) in patients with T1D and T2D at BL and FU.

Abbreviations: Adipo-IR – adipose insulin resistance index, BL – baseline, FIB-4 – fibrosis-4, FLI – fatty liver index, FMD – flow-mediated dilation, FU – follow-up, IMT – intima-media-thickness, NMD – nitroglycerin-mediated dilation, T1D – type 1 diabetes, T2D – type 2 diabetes, VO_2max – maximal oxygen consumption.

*Bar graph with whiskers for standard deviation. *, unadjusted $p < 0.05$. #, $p < 0.05$ adjusted for age, sex, and BMI.*

4.3.4 Associations between baseline metabolic and cardiovascular parameters and future endothelial function

Changes in FMD in T1D over 5 years positively correlated with changes in eGFR (p=0.008), heart rate (p=0.032) as well as VO_2max (p=0.038). Fat mass, high total- and LDL-cholesterol, as well as high GGT and hsCRP at baseline, predicted a decline of FMD at follow-up after 5 years. Regression models revealed that baseline HbA1c correlated negatively with both changes in FMD and FMD at follow-up in T1D (Fig. 14A). M-value predicted lower FMD in both T1D and T2D. This finding was supported by comparing individuals with the highest and lowest insulin sensitivity (highest/lowest quartile) in each group. Persons with T1D and high M-value at baseline developed no changes in FMD over 5 years, while FMD decreased in individuals with the lowest M-value at baseline (p=0.002) [1]. Future brachial IMT in T1D correlated positively with hsCRP, FLI, and WHR at baseline. Additionally, older people are accompanied by thicker IMT. No convincing predictors for future NMD were observed in T1D.

In T2D, higher VO_2max at baseline was associated with a rise in FMD (p=0.044), even after adjustment for age, sex, and BMI. Whereas elevated baseline FLI, triglycerides, and GGT predicted a more significant reduction of FMD [1]. Persons with T2D showed an inverse correlation between FMD and AST at baseline (p=0.045). Additionally, baseline adipose tissue insulin resistance (p=0.045) and FIB-4 (p=0.029) were associated with a decline in endothelial function in T2D. Interestingly, a thicker brachial IMT at baseline resulted in lower FMD at follow-up in T2D (Fig. 14B). Changes in FMD and changes ABI correlated negatively (p=0.013) as well as changes in NMD and changes in systolic blood pressure (p=0.036). Elevated NAFLD indices FIB-4 and FLI at baseline are also associated with a lower future NMD. Future NMD correlated positively with both M-value and HDL-cholesterol levels at baseline. After five years, negative correlations of NMD in T2D were observed in connection with baseline age, WHR, and GPT. Similar to the preceding parameters of endothelial function in T2D, higher age also results in thicker IMT.

Regression models revealed that age and eGFR best predict the changes in FMD within the T1D group (R^2=0.76, all p<0.05), while age, BMI, and M-value best predict the changes in FMD within the T2D group (R^2=0.66, all p<0.05) [1].

63% of the individuals observed over 5 years show a deterioration in FMD. Looking upon classic cardiovascular risk factors, the majority of subjects do not reach recommended target values [174]. Especially the LDL value is increased. 96% of the subjects with a decline in endothelial function show LDL >70 mg/dL (> 1.8 mmol/l) at follow-up, which is the recommended threshold value for high-risk patients, and still 74% exceed the threshold value of 100 mg/dL (>2.5 mmol/l) for intermediate-risk. Additionally, 62% do not reach a recommended blood pressure below 130/80 mmHg. 53% of individuals with FMD deterioration were current or ex-smokers. At follow-up, the majority (74%) showed high fasting blood glucose levels > 120 mg/dl. However, only 44% missed recommended glycemic control with an HbA1c > 7%.

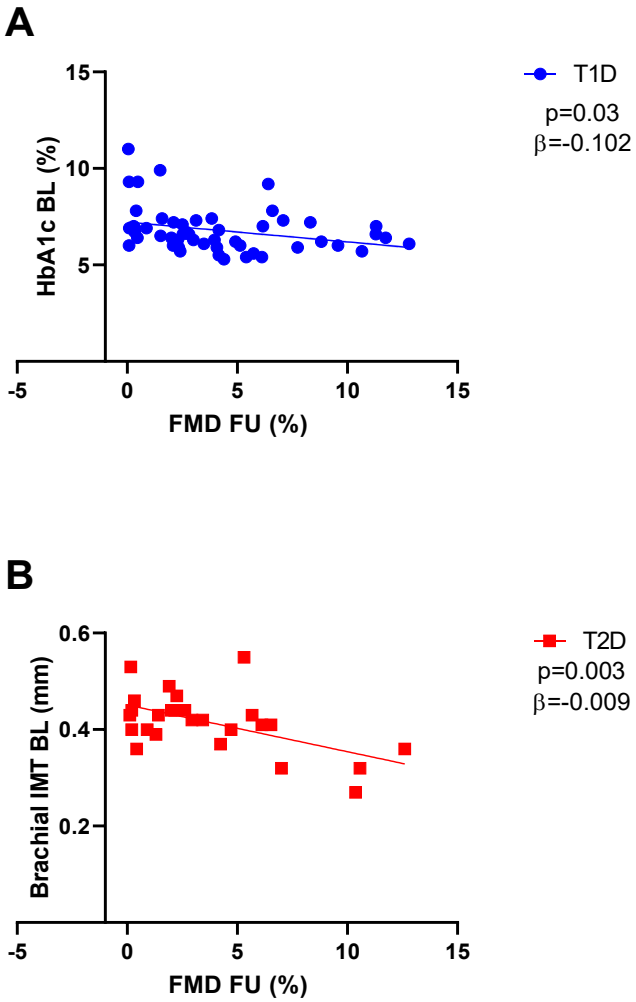

Figure 14. Associations between baseline metabolic parameters and endothelial function at follow-up.

Linear regression models showing associations between baseline HbA1c and future FMD in T1D (A) as well as baseline brachial IMT and future FMD in T2D (B).

Abbreviations: BL – baseline, FMD – flow-mediated dilation, FU – follow-up, HbA1c – glycated hemoglobin, IMT – intima-media-thickness, T1D – type 1 diabetes, T2D – type 2 diabetes.

ß, linear regression coefficients, p < 0.05 adjusted for age, sex and BMI

4.4 Evaluation of comparison measurements

The validation of the FMD method was executed on 29 consecutive participants of GDS using independent measurements of FMD, IMT, and diameter of the brachial artery performed by two experienced examiners. Inter-rater variability coefficients were computed and compared to current literature [175, 176]. The inter-rater agreement of arterial diameter, FMD, and IMT measurements are shown in Figure 15, rendering a coefficient of variation of 3% for the arterial diameter, 25% for FMD, and 9% for IMT.

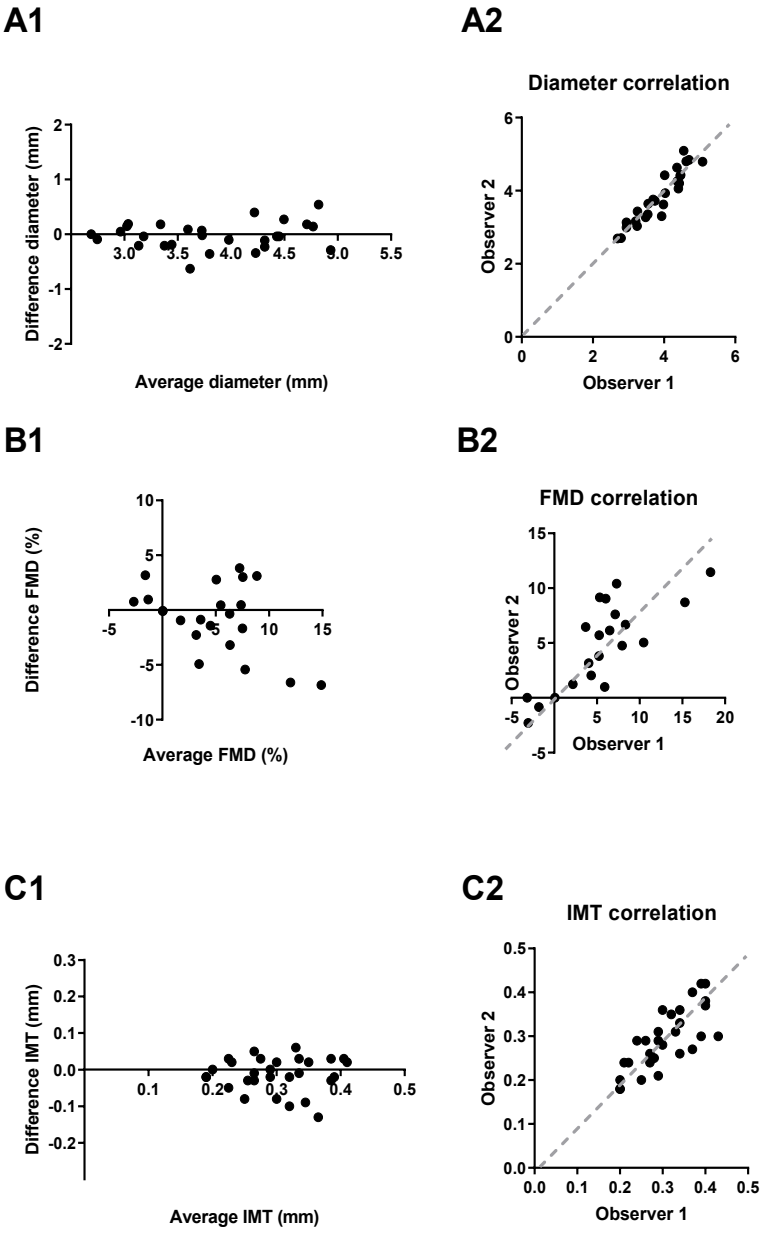

Figure 15. Inter-rater variability.

Bland-Altman plots show the agreement between measurements of the diameter of the brachial artery (A1, A2), brachial FMD (B1, B2), and brachial IMT (C1, C2) from measurements performed by two independent assessors.

Abbreviations: FMD – flow-mediated dilation, IMT – intima-media-thickness.

5 Discussion

The GDS aims to identify prognostic factors and mechanisms underlying the development of related comorbidities and describe the impact of subphenotypes on the course of disease [19]. As part of this prospective longitudinal cohort study, this research work had the specific goal to emphasize the alterations in endothelial function at the beginning of the disease compared to glucose-tolerant controls. It was of particular interest whether the prevalence of endothelial dysfunction, embodied by decreased FMD and NMD, was higher in patients with diabetes mellitus. Additionally, brachial IMT was used to visualize morphological alterations to the endothelium. A further objective was to track the development of endothelial function within the first five years after diagnosis while identifying associations with other cardiovascular risk factors.

In conclusion, there were no differences in early endothelial function in the diabetes cohort used in this study compared to glucose-tolerant controls after adjustment for age, sex, and BMI. However, in both groups with diabetes, increased IMT suggested morphological alterations without functional impairment. Additionally, we observed a decline of 14% in endothelial function in patients with T2D during the first five years after diagnosis, which is associated with estimates of adipose tissue insulin resistance and liver fibrosis at diagnosis. Furthermore, the study could extract predictive value with glycemic control, whole-body insulin sensitivity, and physical fitness representing critical determinants of the development of endothelial dysfunction in diabetes.

Ultimately, within the scope of the evaluation, not all predictions provided beforehand could be confirmed. Hereinafter possible reasons and limitations regarding study design and methods need to be discussed.

5.1 Endothelial dysfunction at diagnosis of diabetes mellitus

In recent literature and meta-analyses, it is widely assumed that an impairment of endothelial function accompanies patients with both T1D [177] and T2D compared to healthy controls [178]. Nonetheless, there are partly contrary results within previous studies. In a population-based study, no associations between diabetes and endothelial dysfunction assessed by FMD were shown [179]. Henry et al. reported reduced FMD only for overt T2D but not for participants with impaired glucose tolerance within the Hoorn Study [70]. However, most of the published works addressing this issue concentrate on endothelial function independent of the stadium of disease, whereas data

availability of endothelial dysfunction at the beginning of diabetes mellitus is lacking. While most studies only used FMD to depict endothelial function, our study includes NMD as an additional functional and brachial IMT as an additional morphological parameter of possible endothelial alterations.

Nevertheless, despite including NMD in our analysis, we could not show a higher prevalence of functional limitations in endothelium in patients with diabetes mellitus at diagnosis than their respective control groups after adjustment for age, sex, and BMI. Once again, previous studies examining NMD revealed conflicting results. While the outcome of the Hoorn Study is consistent with our study, the results of Maftei et al., who detected lower NMD in T1D patients compared to healthy control subjects, contradict our findings [180]. However, the study does not focus on participants at an early stage of the disease. Comparable to a previous study examining participants with T1D [181], direct correlations between FMD and NMD were detected, suggesting that in patients without an untreated prediabetic state, endothelial function and smooth muscle cell function are not yet uncoupled.

Interestingly, while endothelial functionality is not impaired, brachial IMT is significantly thicker in T1D and T2D than in healthy controls. That could support the consideration that structural alterations in endothelium could occur before the impairment of functional aspects and that measurement of brachial IMT could be helpful in the risk assessment of patients with diabetes mellitus since Ono et al. could show an association between thicker IMT of the brachial artery and CAC [166]. That matches the correlation of carotid and brachial IMT in T2D patients observed in the present study, representing overall vessel status. In a recent study, de la Cruz et al. compared brachial FMD and carotid IMT as subclinical atherosclerotic markers in patients with established and newly diagnosed T2D. While carotid IMT was higher in patients with established T2D vs. newly diagnosed T2D, FMD did not differ between the two groups. Furthermore, they revealed that the presence of severe endothelial dysfunction (i.e., FMD <2%) was associated with increased carotid IMT [182]. Our results regarding carotid IMT are consistent with results of other studies like Bonora et al., who figured out that carotid IMT is thicker in patients with non-insulin-dependent diabetes mellitus than in patients without DM [160]. Further, it is widely assumed that diabetes and hypertension increase both brachial and carotid IMT independently [165, 183, 184]. This is in accordance with our study, in which brachial IMT of both diabetes groups and carotid IMT of T2D patients correlated positively with systolic blood pressure. That could warrant intensified antihypertensive treatment even in the early stages of the disease.

The present study adds to these data that functional impairment, embodied by reduced FMD and NMD, may not be necessarily present at diagnosis, but morphological alterations, embodied by thicker IMT, can precede endothelial dysfunction. Still, more evidence at the early stages of diabetes mellitus is required, considering that most published comparisons include participants independent of the time of diagnosis.

5.2 Alterations in endothelial function during the early curse of disease

The present study displayed a decrease in FMD in both T1D and T2D and a decrease in NMD in T2D only. However, after adjustment to age, sex, and BMI, only the decline in FMD in T2D remained significant. During the early course of the disease, patients with T2D decrease endothelial function by about -14%. We identified glycemic control, whole-body insulin sensitivity, and physical fitness as essential determinants of the development of endothelial dysfunction in patients with diabetes. Endothelial function significantly worsened in T2D after five years of disease duration, but not in T1D. Although the change in FMD in T1D is not significant after adjustment, the decrease of about 44% could be ascribed to the insidious development and the prediabetic state in T2D years before clinical diagnosis. In T1D, complications occur earlier, leading to earlier diagnosis with more preserved endothelium and, therefore, a faster decrease of endothelial function.

Vascular function is impaired by chronic hyperglycemia and could contribute to the increased cardiovascular risk in diabetes [185]. It is assumed that endothelial dysfunction predates the onset of hyperglycemia in T2D, so it is likely that other impairments in diabetes, including insulin resistance, altered secretion of adipokines, or abnormal concentrations of metabolites other than glucose, contribute to endothelial dysfunction [89]. Similarly, we could show that besides hyperglycemia, other metabolic alterations at baseline, such as insulin resistance and physical fitness, are relevant predictive markers for endothelial dysfunction in patients with diabetes, independent of age, sex, and BMI. This assumption is supported by a previous analysis including participants of the GDS, which demonstrated that both insulin resistance and hyperglycemia are associated with moderate alterations in cardiac autonomic function, specifically baroreflex dysfunction, in controlled recent-onset T2D individuals during the first five years of disease [186]. This could be linked to increased concentration of the membrane-bound PKC and total DAG levels [187]. PKC plays a vital role in modulating the endothelial monolayer and is induced by insulin resistance, resulting in modulated vascular tone, which could be a primary event that leads to endothelial dysfunction in insulin-resistant states [87].

Together with insulin resistance, impaired mitochondrial function is assumed to contribute to endothelial dysfunction by increasing oxidative stress [188] and is observed in patients with T2D [189] as well as T1D [190]. That leads to the assumption that interventions directed toward restoring mitochondrial function in patients with diabetes might have therapeutic benefits on endothelial dysfunction as well as cardioprotection [191].

Dyslipidemia is a significant risk factor in the development of atherosclerosis, which contributes to an increased risk for cardiovascular events and cardiac vulnerability, especially with other risk factors like insulin sensitivity and hypertension [192, 193]. Therefore, our results warrant further research into the factors influencing the adherence to lipid-lowering therapy and therapeutic success of patients with diabetes, as we showed that most patients do not meet the guideline-recommended targets within the first years after diagnosis. Dyslipidemia, in turn, together with insulin resistance, is related to NAFLD, another risk factor for CVD in both T1D and T2D [194, 195]. In T2D particularly, increased parameters of liver health, including transaminases and surrogate markers for liver steatosis and fibrosis, were associated with decreasing FMD and NMD, but also with increased IMT in T1D, indicating a connection between hepatic alterations and cardiovascular risk, even in the early years of the disease.

Due to the pathomechanisms underlying a diabetic state and the associated comorbidities, antidiabetic drugs focus more and more on cardiovascular protection [196]. These mechanisms include renal protection, lowering chronic inflammation, and reducing ectopic fat deposition [197]. The present study fortifies those observations by showing that eGFR is tightly associated with endothelial dysfunction, particularly in patients with T1D. In clinical practice, however, these drugs are rarely used as first-line therapy. In light of our results, intensified cardiovascular protection instituted from diabetes diagnoses is warranted to reduce their cardiovascular risk [39]. Risk stratification and subsequent targeted management of cardiovascular complications are highly recommended in patients with diabetes [198]. Strict monitoring of blood pressure and specifically the monitoring of the ABI are of interest, as our study shows an association between changes in ABI and FMD over the first five years of disease duration in patients with T2D.

Recent studies proposed that NMD has more potential for predicting future cardiovascular events in patients at risk because of differences in downstream smooth muscle reactivity [75, 199]. The presumable advantage of NMD compared to FMD in detecting early endothelial changes might make it more suitable for monitoring disease progression and assessing cardiovascular risk in patients with diabetes.

However, the present study revealed contrary results, showing that NMD remained unchanged after adjustments for age, sex and BMI, and baseline NMD, while FMD decreased in T2D patients. This is supported by the Hoorn Study, where no difference in NMD between metabolically healthy individuals at the early stage of diabetes was reported [70]. This suggests that early alterations in vasoregulation rely rather on the impairment of endothelial function than on impaired smooth muscle cell function and that FMD is more sensitive to modest changes in endothelial function.

In synopsis with the results at baseline, morphological alterations in endothelium could precede functional impairments in diabetes mellitus. Nevertheless, thicker IMT at baseline can accelerate the development of endothelial dysfunction as observed in T2D patients, who showed increased brachial IMT compared to T1D patients at baseline. That, once more, would draw attention to preventing hypertensive, hyperglycemic, and adipose states, which turned out to be major risk factors of increased carotid and brachial IMT.

5.3 Limitations

5.3.1 Study design

The study benefits from gold-standard methods and the recruitment of well-matched control groups. On the other hand, the participants of the GDS represent a cohort with well-controlled type 1 and type 2 diabetes, whose results cannot be transferred to the entirety of diabetes patients [1]. The follow-up represents a strength of the current study, but the lack of follow-up data for the control group disallows an appropriate comparison of developments. A disadvantage of the GDS being an ongoing study is the loss of follow-up in the diabetes groups. Not all participants reached the follow-up examinations at the time of analysis due to health issues, unwillingness to continue the study, and other various reasons.

5.3.2 FMD as a valid measuring instrument

Despite not being the current gold standard, FMD is considered the most established non-invasive technique for assessing endothelial function and early atherosclerotic alterations. However, the results in our study do not show a clear tendency between FMD at baseline and follow-up. Also, there is wide variance within the values, depicting an inevitable variability within the measurements, which could not be eliminated despite working with large data sets and performing our own reproducibility studies. There are several limitations within both measurement and evaluation, which could lead to mentioned variances.

The assessment method used in this study was carried out according to the International Brachial Artery Reactivity Task Force guidelines for ultrasound assessment of endothelial-dependent FMD of the brachial artery [200]. Even within these guidelines, there is mention of technical and interpretive limitations. It is challenging to relocate the same brachial segment of the baseline measurement after five years at the follow-up visit, even if the same observer performs it. There are several publications relating to intra- and interobserver variability with different results, especially in interobserver variability. While intraobserver variability is mostly negligible due to no statistically significant differences when assessed by a standardized protocol [201], the interobserver variability differs more. Due to the different intra- and inter-observer variability results, Hardie et al. recommended that investigators conduct their own reproducibility studies of sonographic FMD-measurement to avoid misinterpretation [202], as happened in our study. The assessment of observer accuracy further contributes to the methodological strength of our study.

Furthermore, Simova et al. suggested assessing variability examinations on test subjects within or similar to the aimed patient cohort to observe a homogenous group with greater convergence of risk factors [203]. In addition, environmental influences and confounding factors affecting the participants are likely not identical after an interval of five years and can also influence the reproducibility of measurements. For instance, it is unlikely that a female test person is in the same menstrual cycle phase as the baseline visit on the day of follow-up examination, which may affect FMD [204]. Therefore it is recommended that in longitudinal studies, reproducibility measurements should be performed at longer intervals [200]. That underlines the conclusion of Sejda et al. that FMD is difficult to evaluate based on a single measurement [201]. Another aspect is the baseline artery diameter, which affects the percent change in different ways. First, larger baseline diameters generate minor percent change after occlusion [200] and, second, small arteries react relatively more than larger arteries with a higher percent change in vasodilatation [66]. Thus, the best compromise is to report percent and absolute change in diameter [200].

A considerable disadvantage of assessing FMD is the absence of a uniform cut-off value, below which a patient is likely to have endothelial dysfunction. Within several studies, FMD of different study groups were assessed and, if compared, visualize a broad range of possible cut-off points with partly contradictory conclusions. Maruhashi et al. propose a cut-off value for normal endothelial function in brachial artery assessed by FMD of 7.1%, after investigating the FMD of 7277 Japanese subjects with and without risk factors [205]. These results conflict with Lund et al., who measured a mean value in FMD of 2.8 ± 1.6% in 100 young and healthy males with low to moderate cardiovascular risk according to the Framingham Risk Score [206].

The subsequent analysis of FMD is highly dependent on the quality of the ultrasound images. It is often complicated to analyze the same vessel segment in rest and postischemic due to sonographic artifacts or unsteady transducer positioning, despite having visible segments nearby.

Taken together, although the principle of FMD assessment in the brachial artery seems simple, due to its technically challenging application, this method requires extensive training and standardization with the goal of improving the reproducibility of FMD measurements [207].

5.3.3 Alternative diagnostics in endothelial dysfunction

Due to an unavoidable inaccuracy and the lack of standardization in sonographic FMD assessment, other non-invasive tools could be considered more precise in detecting and predicting CVDs.

IMT, pulse wave velocity (PMV), and the augmentation index are non-invasive methods of measurement besides FMD. PMV and augmentation index serve as markers in artery elasticity, whereas IMT gives information about the condition of the vessel wall [208]. IMT in clinical practice is assessed in coronary arteries or carotids. Within our study, we assessed it in both carotid and brachial artery. Decreased FMD correlates with increased PMV and IMT [69, 209]. The European Society of Cardiology considers sonography of carotids and ABI assessment valuable for the overall condition in asymptomatic patients with moderate cardiovascular risk profiles [210]. Assessment of ABI shows a sensitivity of 77% and a specificity of 99%, representing a reliable marker of PAD, which is commonly used in clinical practice and is cited in current guidelines [211]. A threshold value gives ABI an advantage over FMD, which has no importance in everyday clinical practice. Furthermore, the non-uniform standardization and various influencing factors exacerbate the establishment of brachial FMD assessment.

Another study demonstrated that measurements of brachial artery diameter throughout the cardiac cycle reduced technical complexity and analysis time and yielded calculated FMD and NMD values [212].

Besides imaging techniques, there are different circulating biomarkers, which can be used as indicators for endothelial dysfunction, oxidative stress, or inflammation. Parameters including vWF, ICAM-1, VCAM-1, E-Selectin, IL-1, TNF-α, tPA, PAI-1, or thrombomodulin could indicate endothelial dysfunction. hsCRP or homocysteine are associated with inflammation [210]. Measurement of asymmetric dimethylarginine concentration is independent and, therefore, a more predictive value [213]. Only a few circulating markers are eligible for clinical practice due to being non-specific or poor cost-benefit ratio.

Invasive assessment of endothelial function is still considered to be the gold standard. The assessment happens directly in the coronary vascular bed, but numerous disadvantages accompany it. Besides its invasive technique, it is expensive, time-intensive, and limited to those undergoing coronary angiography. Additionally, it is difficult to use it for serial measurements [214].

5.4 Conclusion

Ultimately, the study has not shown a higher prevalence of endothelial dysfunction in individuals with newly diagnosed type 1 or type 2 diabetes in comparison to glucose-tolerant controls after adjustments for age, sex, and BMI in this cohort. Our results suggest that morphological changes and early metabolic disturbances may precede endothelial dysfunction in type 1 and type 2 diabetes patients. Classic cardiovascular risk factors including age, BMI, WHR, systolic and diastolic blood pressure seem to be most influential on the morphological condition of endothelium represented by IMT. Glycemic control, insulin sensitivity, and physical fitness seem to be important determinants of the development of endothelial dysfunction over the early course of diabetes [1]. However, in T1D, changes in FMD and NMD are driven by the age and BMI at diabetes onset, while in T2D, the reduction is independent of age, sex, and BMI. Besides age and BMI, insulin sensitivity at diagnosis best predicted progressive endothelial dysfunction in this group. Therefore, monitoring the progression of endothelial function would also be beneficial in newly diagnosed patients with T2D, and measurement of FMD could be further warranted. The distinct cardiovascular risk patterns of patients with diabetes warrant intensified therapeutic strategies aiming at early prevention of insulin-resistant hyperglycemic and hypertensive states, which can mutually accelerate the progression of endothelial dysfunction. While it is conceivable that favorable modulation of glycemic control, insulin sensitivity, blood pressure, and physical fitness can be translated into a reduction of cardiovascular endpoints, such as endothelial function, this remains demonstrated in large-scale controlled clinical trials.

6 References

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