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NOVEL GENETIC VARIANTS ASSOCIATED WITH DIABETIC NEUROPATHY RISK IN TYPE 2 DIABETES: A WHOLE-EXOME SEQUENCING APPROACH

PhD thesis

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List of Abbreviations

ACCORD - Action to Control Cardiovascular Disease in Diabetes

ACE - Angiotensin-converting enzyme

ADA - American Diabetes Association

ADDITION - Anglo-Danish-Dutch Study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care

ADRA2B - Adrenoceptor alpha-2B

AGEs - Advanced glycation end-products

ALFA - Allele Frequency Aggregator

ALR - Aldose reductase

APOE - Apolipoprotein E

BAM - Binary Alignment Map

Bcl-2 - B-cell leukemia/lymphoma 2

Bcl-XL - B-cell lymphoma-extra large

BMI - Body mass index

BTG2 - B-cell translocation gene 2

CAN - Cardiovascular autonomic neuropathy

CCNI - Cyclin I

CDC34 - Cell division cycle 34

CDK5 - Cyclin-dependent kinase 5

CN - Charcot's neuroarthropathy

CPT - Current perception threshold

CTS - Carpal tunnel syndrome

DAG – Diacylglycerol

DCCT/EDIC - Diabetes Interventions and Complications Study

DFU - Diabetic foot ulcers

DPN - Diabetic peripheral neuropathy

DSPN - Distal symmetric polyneuropathy

E2 - Ubiquitin-conjugating enzyme

EASD - European Association for the Study of Diabetes

ECG- Electrocardiogram

eNOS - Endothelial Nitric Oxide Synthase

ESRD - End-stage renal disease

FHL1, FHL2 - Four-and-a-half LIM domains 1 and 2

GATK - Genome Analysis Toolkit

GDNF - Glial cell line–derived neurotrophic factor

GFRA2 - Glial cell line derived neurotrophic factor family receptor alpha 2

GLO - Glyoxalase

GPx1 - Glutathione peroxidase 1

GRM - Genetic relatedness matrix

GST - Glutathione S-transferase

GSTM1 - Glutathione S-transferase-mu

GSTT1 - Glutathione S-transferase- theta

HWE - Hardy–Weinberg equilibrium

ID3 - Inhibitor of DNA binding 3

IDF - International Diabetes Federation

IGV - Integrative Genomics Viewer

IL-1β - Interleukin-1 beta

IL-4 - Interleukin-4

IL-6 - Interleukin 6

IP3 - Inositol-1,4,5-trisphosphate

IQR - Interquartile range

IWGDF - International Working Group on the Diabetic Foot

LDL - Low-density lipoprotein

LOX-1- Lectin-like oxidized LDL receptor-1

MAF - Minor allele frequency

MAPK - Mitogen-activated protein kinase

MARP - Muscle ankyrin repeat protein

MIR - MicroRNA

MODY - Maturity-onset diabetes of the young

MTHFR - Methylenetetrahydrofolate reductase

mTOR - Mammalian target of rapamycin

MVB12B - Multivesicular body subunit 12B

MYBPHL - Myosin-binding protein H-like

NADPH - Nicotinamide adenine dinucleotide phosphate

NCBI - National Center for Biotechnology Information

NEURODIAB - Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF - Nerve growth factor

NGS - Next-generation sequencing

NTSS-6 - Neuropathy Total Symptom Score-6

OPG - Osteoprotegerin

OR - Odds ratio

PI3K - Phosphoinositide 3-kinase

PIP2 - Phosphatidylinositol-4,5-bisphosphate

PKB - Protein kinase B

PKC - Protein kinase C

PLCB1 - Phospholipase C-beta 1

PRMT1 - Protein arginine methyltransferase 1

QQ - Quantile–Quantile

QST - Quantitative Sensory Testing

RAGE - Receptor for advanced glycation end-products

RANK - Receptor activator of nuclear factor κ B

RANKL - Receptor activator of nuclear factor kappa-B ligand

RBM20 - RNA-binding motif protein 20

RET - Rearranged during transfection

RMI2 - RecQ-mediated genome instability protein 2

ROS - Reactive oxygen species

RXRA - Retinoic acid X receptor alpha

sAnk1 - Small ankyrin 1

SCF - SKP1-Cullin-F-box protein complex

SD – Standard deviation

SLC19A2 - Solute carrier family 19 member 2

SNP - Single nucleotide polymorphism

SNV - Single-nucleotide variant

STZ – Streptozotocin

T2DM - Type 2 diabetes mellitus

TCF7L2 - Transcription factor 7-like 2 gene

TGF-β - Transforming growth factor beta

THTR - Thiamine Transporters

TKT - Transketolase

TNF-α - Tumor necrosis factor alpha

TRPA1 - Transient receptor potential cation channel, subfamily A, member 1

TSA - Thermal Sensory Analyzer

TTN - Titin

UKPDS - United Kingdom Prospective Diabetes Study

UPS - Ubiquitin–proteasome system

VCF - Variant Call Format

VEGF - Vascular eEndothelial gGrowth Ffactor

VGSCs - Voltage-gated sodium channel

VNTR - Variable number of tandem repeat

VSA - Vibratory Sensory Analyzer

WES - Whole-Exome Sequencing

WESDR - Wisconsin Epidemiologic Study of Diabetic Retinopathy

1. Introduction

1.1 Epidemiology of diabetes mellitus and diabetic neuropathy

Type 2 diabetes mellitus (T2DM) is one of the most prevalent chronic diseases worldwide, with rapidly increasing incidence in both developed and developing countries. According to the International Diabetes Federation (IDF), in 2024 approximately 589 million adults (20-79 years) were living with diabetes, 65.6 million of these patients lived in Europe. That number is projected to rise 853 million by 2050. More than four out of five adults with diabetes (81%) reside in low- and middle-income countries. In 2024, the global prevalence of type 1 diabetes was estimated at 9.2 million individuals across all age groups. Among them, approximately 1.8 million were adults aged over 20 years. Type 2 diabetes, which constitutes more than 90% of all diabetes cases worldwide, is presently the eighth leading contributor to global disease burden. Projections indicate that by 2050 it will become the second leading cause. Although type 2 diabetes can present with symptoms similar to those of type 1 diabetes, its onset is usually far less pronounced and frequently asymptomatic. This absence of clear clinical manifestations makes it difficult, and often impossible, to determine the precise time of disease onset. Consequently, diagnosis is frequently delayed, and at any given point an estimated one-third to one-half of individuals with type 2 diabetes remain undiagnosed. According to the IDF, the proportion of people living with undiagnosed diabetes is 16,7% in Hungary. Prolonged delays in diagnosis increase the likelihood of developing chronic complications (1). In line with international trends, the number of individuals with diabetes has been gradually increasing in Hungary as well. According to a survey conducted by the Hungarian Diabetes Association, in Hungary, the prevalence of type 2 diabetes increased markedly between 2001 and 2014 (from 423,000 to 727,000 cases), although the rate of growth has slowed in recent years. Women represented the majority of patients, and the proportion of older individuals (over 60 years) steadily increased. By 2011, every fifth person over the age of 60 was affected by diabetes. During this period, the standardized prevalence rose from 4.2% to 6.4%. In 2014, 94% of all registered diabetes cases were type 2 diabetes, while 6% were type 1 diabetes (2).

Type 2 diabetes mellitus is associated with substantial morbidity, mortality, and socioeconomic burden, largely driven by its chronic complications (3). These complications are traditionally divided into macrovascular (coronary artery disease,

cerebrovascular disease, peripheral artery disease) and microvascular manifestations, which include diabetic retinopathy, nephropathy, and neuropathy (4).

Diabetic neuropathy is a common and serious microvascular complication, affecting up to 50% of individuals with long-standing diabetes (5). According to a multi-ethnic cohort study of adults with type 2 diabetes, the overall prevalence of diabetic peripheral neuropathy (DPN) was 26.7%. Independent and significant risk factors included longer duration of diabetes, inadequate glycemic control, and a history of hypertension, cardiovascular disease, and depressive symptoms (6). 10–15% of individuals with newly diagnosed type 2 diabetes already have distal symmetric polyneuropathy (DSPN), and prevalence can exceed 50% after a decade of disease (7). Painful DPN affects roughly 15–25% of people with diabetes, while cardiovascular autonomic neuropathy occurs in approximately one quarter of patients (8, 9). Globally, approximately 252 million adults with diabetes are undiagnosed, meaning many first present already with complications, including neuropathy (1). According to the IDF, the proportion of people living with undiagnosed diabetes in 2024 is 16,7% in Hungary (1).

1.2. Classification of diabetic neuropathy

Diabetic neuropathy is defined by progressive dysfunction in peripheral nerve fibers, including sensory, motor, and autonomic nerves, in individuals with diabetes, following exclusion of alternative causes (10). DPN encompasses a heterogeneous group of neurological disorders that affect different parts of the peripheral and autonomic nervous systems. The Toronto Consensus Panel on Diabetic Neuropathy classifies these disorders into the following categories, including distal symmetric polyneuropathy, autonomic neuropathies, and focal and multifocal neuropathies (11).

1.2.1. Distal Symmetric Polyneuropathy (DSPN)

The most common clinical form is distal symmetric polyneuropathy, which typically presents with symmetrical sensory symptoms in a stocking-and-glove distribution. Clinical manifestations include paresthesia, dysesthesia, burning pain, and numbness, often accompanied by loss of vibration, temperature, and pain sensation. Advanced disease may lead to muscle weakness and atrophy. DSPN is strongly associated with the development of diabetic foot ulcers, which are a major cause of non-traumatic lower-limb

amputations worldwide. Therefore, DSPN significantly impairs quality of life (12). Vági et al. reported in their meta-analysis that diabetic patients with distal symmetric polyneuropathy have almost double the mortality risk compared to those without. This meta-analysis provides strong evidence that mortality risk is significantly higher in type 1 diabetes compared to type 2 diabetes, with more than a twofold increased risk in type 1. The reason behind the difference between the two groups could be that many younger type 1 patients without DSPN are generally healthy and free of complications, so those who do have DSPN are more likely to have multiple complications, sharply increasing their mortality risk. Compared to this, older type 2 patients often already have several vascular risk factors, for example obesity, hypertension, hyperlipidemia, leading to higher baseline mortality independent of DSPN. This results in a smaller relative increase in mortality associated with DSPN. Moreover, DSPN in type 1 diabetes is mostly related to hyperglycemia, whereas in type 2 diabetes it has a multifactorial origin, including non-glycemic factors, potentially affecting mortality differently. Also, adults with type 1 diabetes often have more severe disease due to longer duration and worse glycemic control, which may further contribute to higher mortality (13).

1.2.2. Diabetic foot ulcers (DFU)

DSPN leads to loss of protective sensation in the feet, increasing the risk of diabetic foot ulcers. Each year, an estimated 18.6 million people worldwide develop a diabetic foot ulcer, with a prevalence of approximately 6.3% (14, 15). These ulcers often become chronic, infected, and difficult to heal, frequently resulting in hospitalization, amputation, and systemic complications (15). Diabetic foot ulcers are responsible for approximately 80% of lower extremity amputations in people with diabetes and are linked to a significantly higher risk of mortality (14). Around half to 60% of diabetic foot ulcers become infected, and approximately one in five cases of moderate to severe infection leads to amputation of the lower limb. Five years after developing a diabetic foot ulcer, roughly 30% of patients die, with mortality exceeding 70% among those who have undergone major amputations. Compared with diabetic patients without foot ulcers, who experience 182 deaths per 1,000 person-years, individuals with foot ulcers face a higher mortality rate of 231 deaths per 1,000 person-years (14).

Diabetic foot ulcers arise from a combination of sensory, motor, and autonomic neuropathies associated with diabetes. Sensory nerve damage reduces protective sensation, making the foot vulnerable to unnoticed injuries. Motor neuropathy contributes to structural deformities and altered biomechanics, while autonomic neuropathy affects skin properties, often leading to dryness and reduced elasticity. These changes commonly result in callus formation. When repetitive stress during walking or standing causes bleeding beneath a callus, removal may reveal a full-thickness ulcer that extends into the subcutaneous tissue. Additional pathways for ulcer development include continuous low-grade pressure, such as that from ill-fitting footwear, which can lead to tissue breakdown, or acute high-pressure injuries from sharp objects causing direct trauma (16).

To evaluate ulcer risk in individuals with diabetes, an annual foot examination is recommended. This assessment should include testing for loss of protective sensation due to neuropathy, checking for peripheral arterial disease, and inspecting the skin for early signs of breakdown (17).

Comprehensive, multidisciplinary management, which often involve podiatrists, infectious disease experts, and vascular surgeons working alongside primary care providers, has been shown to reduce the rate of major amputations compared with standard care. Despite treatment, only about 30–40% of diabetic foot ulcers achieve healing within 12 weeks, and recurrence remains common, occurring in roughly 42% of patients within 1 year and up to 65% within 5 years (14).

1.2.3. Charcot's neuroarthropathy (CN)

Charcot neuroarthropathy is a chronic complication that develops in patients with diabetic neuropathy. It involves progressive destruction and deformity of bones and joints, typically in the foot and ankle, and may present with either painful or painless changes. The condition occurs in limbs that have lost protective sensory innervation, making early detection and management essential to prevent severe disability (18, 19). The reported incidence and prevalence of Charcot neuroarthropathy among individuals with diabetes ranges between 0.1% and 0.4%. In patients with peripheral neuropathy, the prevalence of CN may increase to as high as 35% (20, 21).

The pathogenic mechanisms of CN are not completely understood. According to the neurovascular theory, autonomic neuropathy disrupts normal vascular control, causing increased blood flow (hyperaemia) in the bones. The resulting osteopenia, due to the heightened osteoclastic activity, weakens structural integrity and predisposes to fractures and deformity (22). However, it is also suspected, that sensory neuropathy impairs protective sensation, repetitive minor injuries go unnoticed, eventually leading to joint destruction and deformity. Experimental and clinical observations support this, implicating ongoing trauma in the pathogenesis of CN. This is called the neuro-traumatic theory (19). The third theory, called the neuro-bone-inflammatory (RANKL/OPG) theory, emphasizes the role of inflammation and dysregulated bone remodeling. In CN, there is an imbalance in the receptor activator of nuclear factor κ B (RANK)– receptor activator of nuclear factor kappa-B ligand (RANKL)– osteoprotegerin (OPG) pathway, with elevated RANKL leading to excessive osteoclast activity. Pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), and interleukin 6 (IL-6) also contribute to this destructive cycle (23). Furthermore, increased systemic levels of RANKL, OPG, and inflammatory cytokines have been observed in CN patients compared to diabetic or healthy controls (24).

Taken together, these theories highlight the multifactorial nature of Charcot neuroarthropathy, in which vascular, sensory, and inflammatory mechanisms converge to promote progressive bone and joint destruction in the neuropathic limb.

1.2.4. Cardiovascular Autonomic Neuropathy (CAN)

Cardiovascular autonomic neuropathy is a common microvascular complication of diabetes, characterized by impaired function of the autonomic nervous system that regulates cardiovascular activity. In its initial stages, CAN typically progresses without noticeable symptoms. When clinical signs do emerge, they are usually vague and appear only once the disease has advanced (25). The prevalence of cardiovascular autonomic neuropathy is projected to grow alongside the global increase in both type 1 and type 2 diabetes. Advancing age, prolonged duration of diabetes, and inadequate glycaemic or metabolic regulation are key factors that elevate the risk of CAN (25, 26). Reported prevalence ranges from 29–54% in type 1 diabetes and 12–73% in type 2 diabetes, while

individuals with prediabetes show higher rates (9–38%) compared with those with normal glucose tolerance (0–18%) (25, 27, 28).

The vagus nerve, being the longest autonomic nerve, is usually affected first, leading to parasympathetic dysfunction and sympathetic predominance, clinically manifesting as resting tachycardia (29). With disease progression, patients may develop a fixed heart rate, reduced exercise tolerance, and in advanced stages, orthostatic hypotension, which can cause dizziness, syncope (30). CAN is also strongly linked to silent myocardial ischaemia, with studies showing nearly a twofold increased risk, and has been independently associated with renal complications such as albuminuria, chronic kidney disease, and accelerated decline in kidney function (31, 32).

Evidence indicates that CAN is linked to increased mortality risk, particularly when diagnosed based on multiple autonomic abnormalities, which may reflect more advanced dysfunction or coexisting complications (33). According to Vági et al., CAN independently predicts all-cause mortality, beyond established cardiovascular and other risk factors. The presence of any neuropathy identifies individuals at higher risk, highlighting the need for stricter management of conventional risk factors, including smoking, lipid levels, and blood pressure (34).

1.2.5. Other forms of neuropathy

Focal and multifocal neuropathies, including cranial neuropathies, truncal neuropathies, and radiculoplexus neuropathies, can occur in patients with diabetes. These conditions may present acutely and often resolve spontaneously, but they can significantly impact quality of life during the acute phase. Diabetic lumbosacral radiculoplexus neuropathy, also known as Bruns-Garland syndrome, is characterized by severe, burning pain in the lower back, buttocks, or anterior thighs, often worse at night (35). The prevalence of carpal tunnel syndrome (CTS), the most common entrapment neuropathy, is higher in individuals with diabetes due to glycation and thickening of connective tissues (36). CTS symptoms include pain and paresthesia in the medial portion of the palm, aggravated by hand use, and are often worse at night. The dominant hand is more commonly affected, and there is a higher prevalence of CTS in females. The prevalence of CTS increases with age and obesity (37).

1.3. Pathogenesis of diabetic neuropathy

The pathogenesis of diabetic neuropathy is complex and multifactorial, driven primarily by chronic hyperglycaemia and associated metabolic dysregulations that disrupt both metabolic and vascular homeostasis (Figure 1). Over the past decades, multiple interconnected biochemical pathways have been identified, which together contribute to neuronal damage, impaired nerve repair, and progressive functional decline (38).

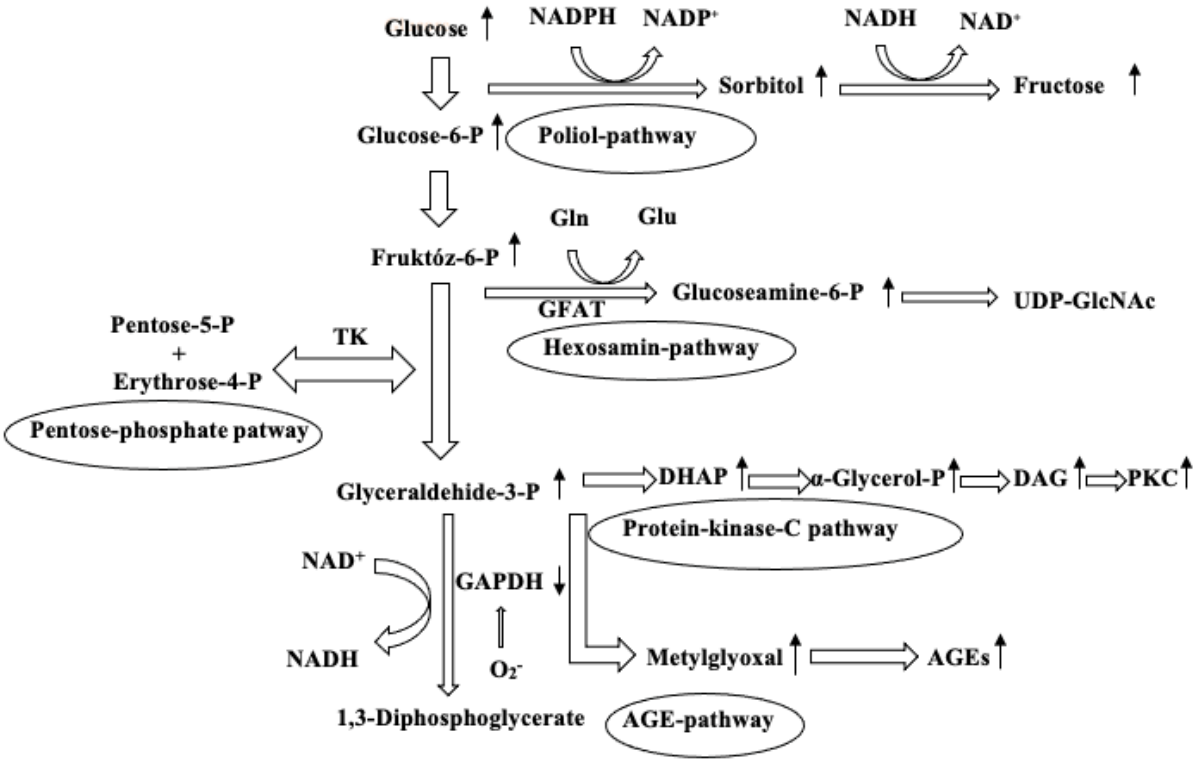

Figure 1. Alternative metabolic pathways activated under hyperglycaemic conditions (39, 40).

1.3.1. Hyperglycaemia-induced metabolic pathways

1.3.1.1. Polyol pathway

One of the most prominent biochemical mechanisms implicated in the pathogenesis of diabetic neuropathy is the activation of the polyol pathway. Under hyperglycaemic conditions, excess intracellular glucose is diverted into this pathway and reduced to

sorbitol by the enzyme aldose reductase. Sorbitol is subsequently converted to fructose by sorbitol dehydrogenase. The accumulation of these sugar alcohols within cells induces osmotic stress, disrupting cellular homeostasis. Moreover, the conversion of glucose to sorbitol consumes nicotinamide adenine dinucleotide phosphate (NADPH), an essential cofactor required for regenerating reduced glutathione, one of the body's most important antioxidant defenses. As a result, glutathione depletion reduces the cell's capacity to neutralize reactive oxygen species, thereby exacerbating oxidative stress. Elevated oxidative stress, in turn, promotes lipid peroxidation, protein modification, and DNA damage, processes that contribute to axonal degeneration and impaired neuronal repair. Beyond its direct neurotoxic effects, polyol pathway activation has also been associated with microvascular dysfunction, basement membrane thickening, and impaired endoneurial blood flow, all of which further compromise nerve integrity and exacerbate diabetic neuropathy (41).

1.3.1.2. Advanced glycation end-products formation and protein kinase C activation

Another important mechanism in the development of diabetic neuropathy is the formation and accumulation of advanced glycation end-products (AGEs). These irreversible modifications of proteins and lipids alter cellular structure and function, impairing axonal transport and flexibility of connective tissue. AGEs also interact with their receptor (Receptor for advanced glycation end-products, RAGE), which activates intracellular signaling cascades such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) amplifying oxidative stress and promoting inflammation in neuronal and vascular tissues (42). The accumulation of AGEs in peripheral nerves and their microvasculature further accelerates structural damage and ischemia (43). Protein kinase C (PKC) activation, driven by elevated diacylglycerol levels, contributes to endothelial dysfunction by reducing nitric oxide availability, enhancing vasoconstriction, and increasing vascular permeability (44).

1.3.1.3. Hexosamine pathway

Hyperglycaemia activates the hexosamine pathway, which leads to aberrant glycosylation of transcription factors, altering gene expression involved in vascular homeostasis and inflammation (38). Fructose-6-phosphate is transformed into glucosamine-6-phosphate

by the enzyme glutamine–fructose-6-phosphate amidotransferase, ultimately generating uridine diphosphate N-acetylglucosamine. The accumulation of this metabolite promotes transforming growth factor beta (TGF-β) production and alters the composition of signaling proteins, which disrupts normal insulin receptor signaling. This interference diminishes insulin's effectiveness, contributing to heightened insulin resistance in tissues (45).

1.3.2. Oxidative Stress and Mitochondrial Dysfunction

Oxidative and nitrosative stress represent an important mechanism underlying the pathogenesis of diabetic neuropathy. Hyperglycaemia induces mitochondrial overproduction of reactive oxygen species (ROS), which overwhelms the cellular antioxidant capacity, leading to damage of DNA, proteins, and lipids (46). Mitochondrial dysfunction not only impairs energy production but also triggers the activation of pro-apoptotic pathways, resulting in neuronal and Schwann cell death. The imbalance between ROS and nitric oxide also promotes peroxynitrite formation, a highly reactive species that exacerbates oxidative injury (47).

1.3.3. Microvascular Dysfunction

The peripheral nervous system is highly dependent on adequate microvascular supply, and endoneurial ischemia is a critical factor in diabetic neuropathy. Chronic hyperglycemia leads to thickening of capillary basement membranes, endothelial dysfunction, and reduced nitric oxide bioavailability. These changes impair vasodilation and reduce nutrient and oxygen delivery to nerve fibers (48). In diabetes, heightened platelet aggregation contributes to the formation of microemboli and the exacerbation of capillary lesions, compromising microcirculatory perfusion and exacerbating tissue ischemia (49). Hypoxia within the nerve is associated with axonal degeneration and demyelination (38). Consequently, microvascular damage acts synergistically with metabolic stress to accelerate the progression of neuropathy.

1.3.4. Inflammatory and Immune Mechanisms

Elevated circulating levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta, and interleukin 6 were reported in patients with diabetic neuropathy (11). These cytokines activate NF-κB signaling and induce the

expression of adhesion molecules, fostering local inflammation and endothelial injury. Inflammatory pathways further disrupt mitochondrial function, enhance oxidative stress, and impair axonal transport.

Moreover, systemic low-grade inflammation associated with insulin resistance and obesity may predispose individuals with diabetes to more severe neuropathic complications (50). Inflammation is therefore not only a consequence but also a driver of neuronal injury.

1.3.5. Dyslipidaemia and lipotoxicity

In addition to chronic hyperglycaemia, dyslipidaemia has emerged as an independent contributor to the pathogenesis of diabetic neuropathy. Abnormal lipid profiles, particularly elevated triglycerides and modified low-density lipoprotein (LDL), promote oxidative stress, mitochondrial dysfunction, and apoptosis in peripheral neurons. Oxidized LDL exerts cytotoxic effects through activation of the lectin-like oxidized LDL receptor-1 (LOX-1), which enhances NADPH oxidase activity, increases superoxide production, and triggers inflammatory signaling. Hyperglycaemia further amplifies this effect by upregulating LOX-1 expression, thereby sensitizing neurons to lipid-induced injury. Taken together, these observations point to dyslipidaemia as both a driver of neuropathic progression and a promising avenue for therapeutic intervention (51).

1.3.6. Growth factor deficiency

Sympathetic and neural-crest–derived sensory neurons, which rely on nerve growth factor (NGF) for survival and function, are affected early in diabetes mellitus. In a study using streptozotocin-induced diabetic rats, Hellweg and Hartung (52) demonstrated significant alterations in endogenous NGF levels. NGF content was reduced in several sympathetically innervated tissues, the sciatic nerve, and the superior cervical ganglion, while paradoxical increases were observed in other peripheral organs, likely due to impaired NGF uptake. Because NGF exerts its trophic effects through retrograde transport, these findings suggest that diabetes disrupts NGF production and/or transport. Such deprivation of NGF may contribute to functional deficits in diabetic neuropathy, including impaired catecholaminergic neurotransmitter synthesis.

1.3.7. Ion channel dysfunction

Another important pathogenic mechanism involves alterations in ion channel function. Dysregulation of sodium, potassium, and calcium channels affects neuronal excitability and contributes to the development of neuropathic pain. Hyperglycaemia-induced changes in channel expression and function can result in spontaneous ectopic discharges and heightened pain perception, further diminishing patient quality of life (53).

The pathogenesis of diabetic neuropathy is multifactorial, involving the convergence of metabolic, vascular, and inflammatory processes. Chronic hyperglycaemia initiates a cascade of biochemical disturbances that promote oxidative stress and mitochondrial dysfunction, ultimately resulting in neuronal injury. In addition, microvascular ischaemia, impaired neurotrophic support, and systemic inflammation compromise the ability of nerves to repair and regenerate. Dyslipidaemia and insulin resistance further exacerbate these mechanisms, creating a vicious cycle of progressive nerve damage and functional decline.

1.4. Genetic Factors Potentially Involved in the Development of Diabetic Neuropathy

In recent years, substantial progress has been made in elucidating the pathogenesis of neuropathy. Earlier epidemiological studies identified several risk factors, including age, female sex, physical labor, lower educational attainment, and disadvantaged socioeconomic conditions (54, 55). Additional factors such as smoking, hypertension, obesity, hypercholesterolaemia, and longer diabetes duration have also been recognized as contributors (56, 57).

Early identification and comprehensive management are essential in the care of individuals with diabetes, underscoring the need for continuous patient education regarding the risks of long-term complications and the importance of regular screening. Achieving and maintaining optimal metabolic control plays a pivotal role in preventing diabetic neuropathies (58). Evidence from the United Kingdom Prospective Diabetes Study (UKPDS) in newly diagnosed type 2 diabetes demonstrated a strong link between glycemic control and the development of chronic complications. Specifically, each 1% reduction in HbA1c was associated with a significant decrease in the risk of both microvascular and macrovascular complications, as well as lower rates of cardiovascular disease and all-cause mortality (59). The Action to Control Cardiovascular Disease in

Diabetes (ACCORD) study, however, demonstrated that intensive glycemic control exerts only a limited influence on the prevention of microvascular complications (60).

Current evidence clearly indicates that the genetic background plays a crucial role not only in modulating the risk factors associated with diabetic neuropathy but also in regulating the underlying molecular mechanisms and pathways.

To date, relatively few human studies have investigated the genetic background of neuropathy, and most of these have focused on the analysis of individual single nucleotide polymorphisms (SNPs). Collectively, these studies have identified approximately 30 genes (61-63) that may play a role in the development of neuropathy (Table 1.).

Table 1. Major genes potentially involved in the development of diabetic neuropathy.

Gene	Variant Type	Role	Publication
Angiotensin-converting enzyme (ACE)	homozygous DD genotype of the I/D polymorphism	Determines ACE activity and serum levels of ACE	(64)
MTHFR gene	C677T polymorphism	Elevates homocysteine levels	(65)
GSTM1 and *GSTT1* genes	homozygous deletion (null genotype)	Reduces enzyme activity (GST protects against endogenous oxidative stress and exogenous potential toxins) and leads to cytogenetic damage	(66)
GLO1 gene	CC genotype	Glo-11 reduces the formation of advanced glycemic end-products (AGEs)	(67)
APOE gene	Ɛ4 allele	Plays a role in the cholesterol and triglyceride metabolism	(68)
TCF7L2 gene	rs7903146, rs7901695, rs12255372	Affects the lipid metabolism and glucose homeostasis	(69)
VEGF gene	C and T alleles	Determines the level of VEGF, which facilitates the proliferation of vascular endothelial cells	(70)
IL-4 gene	VNTR	IL-4 is a cytokine that impacts immune cell chemotaxis and anti-inflammation	(71)

GPX1	rs1050450, C > T	Reduced antioxidant activity	(72)
eNOS gene	rs2070744 (786 T/C) rs1799983 (894 G/T)	Leads to endothelial dysfunction through the change in the synthesis of nitric oxide	(73)
ADRA2B gene	I/D polymorphism	Associated with autonomic dysfunction and increased sympathetic nervous system activity	(74)
MIR146A, *MIR128A* *MIR499A*	rs2910164 (G> C) rs11888095 (C > T) rs3746444 (GG genotype)	Associated with the level of mitochondrial DNA	(75)
SLC19A2, SLC19A3 encoding THTR1 and THTR2		Intracellular transport of thiamine	(76)
Transketolase gene	rs7648309 rs63355988	Loss of protective action in the prevention of diabetic neuropathy	(77)
Glo1 gene	rs1130534 rs1049346	Loss of defense against AGE formation	(78)
Voltage-dependent Na channel beta-2 subunit of Nav1.7	aspartic acid–aspartic acid mutation (D109N)	Hyperexcitability of posterior ganglion neurons	(79)
ANO3 gene	mis-sense heterozygous variants	Increased pain sensitivity	(80)
HCN1 gene	mis-sense heterozygous variant	Increased pain sensitivity	(80)
TRPA1	loss-of-function mutation	Increased pain sensitivity	(80)
TRPV1 and *TRPV4* genes		Painless diabetic neuropathy	(80)
SCN9A, SCN10A, and *SCN11A*	gain-of-function mutations	Neuron hyperexcitability	(81)
Polymorphisms in the *GFRA2* gene	rs4872521 rs4872522 rs10098807 rs11774105 rs17428041 rs17615364 rs11776842 rs12545534 rs11780601	Role in the differentiation and survival of neurons	(82)
ALR2 gene	106C/T polymorphism in the promoter region	Role in nerve conduction velocities	(83)
ALR2 gene	50-(CA)n microsatellite	Susceptibility or defense against diabetic neuropathy	(84)

	polymorphism (Z + 2, Z − 2)		
GPx-1	(rs1050450) 599C/T	Susceptibility to diabetic neuropathy	(85)
CAT	262C/T	Susceptibility to diabetic neuropathy	(85)
Chromosomal loci 1p35.1 and 8p21.3.		Neuropathic pain	(86)
Gene polymorphisms of *ACE, MTHFR, APOE, ALR2, GPx-1, NOS3, CAT,* and *VEGF*		Susceptibility to diabetic neuropathy	(87)
GLP-1, PTEN, insulin, RAGE, HSP27, CW22, and *DUSP1* in the phosphatidylinositol 3-kinase/phosphorylated protein kinase B [PI3/pAkt] signaling pathway		Possible therapeutic targets	(88)
RMI2 gene *MYBPHL* gene *MVB12B* gene *RXRA* gene	rs2032930, rs2032931 rs604349 rs917778 rs2234753	Alters the risk of developing diabetic neuropathy	(89)

1.4.1. Angiotensin-converting enzyme (ACE)

Angiotensin-converting enzyme is a component of the renin–angiotensin system, responsible for converting angiotensin I to angiotensin II, thereby eliciting a potent vasoconstrictive effect. ACE inhibitors have previously been used in diabetic neuropathy to treat microvascular damage, as some studies have demonstrated a protective effect against neuronal dysfunction (90). The ACE gene carries an inversion/deletion (I/D) polymorphism characterized by the presence (I allele) or absence (D allele) of a 287-bp Alu repeat sequence within intron 16. This variation results in three possible genotypes—II, ID, and DD—and is associated with differences in both ACE enzymatic activity and circulating ACE levels (64).

1.4.2. Methylenetetrahydrofolate reductase (MTHFR)

MTHFR catalyzes the conversion of homocysteine to methionine. Variants of the MTHFR gene can lead to reduced enzymatic activity. Among these, the C677T polymorphism of the MTHFR gene is the most common cause of elevated homocysteine levels (91). Hyperhomocysteinaemia exerts detrimental effects on vascular endothelium and smooth muscle cells, leading to structural and functional alterations of the arteries. Previous meta-analyses have demonstrated a clear association between the C677T polymorphism of the MTHFR gene and an increased risk of developing diabetic neuropathy (65).

1.4.3. Glutathione S-transferase (GST)

Glutathione S-transferase provides protection against endogenous oxidative stress as well as exogenous potential toxins. GSTs can protect cells from oxidative damage, a hallmark of various pathological conditions, including neurodegenerative disorders and diabetic neuropathy. The most extensively studied genes are GST mu (GSTM1) and GST theta (GSTT1), along with their polymorphisms. The most common variants of the GSTM1 and GSTT1 genes are homozygous deletions (null genotypes), which are associated with reduced enzymatic activity and cytogenetic damage (92). A previous study demonstrated that the combination of GSTM1 and GSTT1 genotypes significantly increases the risk of developing CAN in patients with type 1 diabetes (66). However, this association has not been confirmed in individuals with type 2 diabetes (93).

1.4.4. Methylglyoxal

Plasma levels of methylglyoxal have shown differing correlations in cross-sectional studies with painful diabetic polyneuropathy and with DPN occurring in type 2 diabetes (94-96). Nevertheless, in the Anglo-Danish-Dutch Study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care (ADDITION)-Denmark cohort, plasma methylglyoxal levels proved to be an independent predictor of DPN occurrence, with a risk ratio of 1.46 (95% CI: 1.12–1.89) (97). Methylglyoxal modifies the nociceptor-specific sodium channel (Nav1.8), enhancing the excitability of sensory neurons and thereby leading to hyperalgesia (94).

Similarly, methylglyoxal has been shown to stimulate the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) (98). TRPA1 is a receptor channel

located in sensory neurons and has been implicated in both inflammatory and neuropathic pain (98). Alterations induced by methylglyoxal in sodium and transient receptor potential channels may potentially contribute to the development of neuropathic pain.

1.4.5. Glyoxalase (GLO)

The glyoxalase system also plays a crucial role in the pathogenesis of diabetic complications. It consists of glyoxalase-1 (Glo-1), glyoxalase-2 (Glo-2), and glutathione, functioning as a defense mechanism against AGE formation. In particular, genetic variants of the glyoxalase 1 (GLO1) gene may induce structural alterations in the glyoxalase binding site (78).

In relation to DPN, of particular interest is a single nucleotide polymorphism in the glyoxalase 1 gene, involving an adenine-to-cytosine substitution at nucleotide position 332 (rs2736654 or rs4746). This specific SNP results in the substitution of alanine with glutamic acid (C332→Ala replaced by A332→Glu), which may potentially affect the morphology of the glyoxalase binding site. The three identified phenotypes of the Glo-1 enzyme are GLO1-Ala/Ala (C332C), GLO1-Ala/Glu (C332A), and GLO1-Glu/Glu (A332A). A reduction in Glo-1 activity has been observed in lymphoblastoid cell cultures homozygous for the A allele, accompanied by increased intracellular methylglyoxal levels and elevated expression of the AGE receptor (99).

Groener et al. investigated the association of the rs2736654 of glyoxalase 1 with diabetes-related complications in a study including 209 patients with type 1 diabetes and 524 patients with type 2 diabetes. The study demonstrated a significantly higher prevalence of the A332A genotype in patients with type 1 diabetes compared to those with type 2 diabetes (35.9% vs. 27.3%; $p = 0.03$). In patients with type 1 diabetes mellitus, no association was found between any of the genotypes and diabetic neuropathy, nephropathy, or retinopathy. In contrast, the C332C genotype was found to be correlated with DPN in patients with type 2 diabetes; 53.7% of individuals carrying the C332C genotype had diabetic neuropathy, compared to 44% of those with the A332A or C332A genotypes ($p = 0.03$; odds ratio: 1.49; 95% confidence interval: 1.04–2.11). This study represents the first comprehensive cross-sectional investigation to suggest a potential association between GLO1 polymorphism and diabetic neuropathy in patients with type 2 diabetes, highlighting the important role of methylglyoxal in the pathogenesis of

diabetic neuropathy (67). Previously, the A332A genotype—rather than the C332C genotype—had been associated with reduced glyoxalase activity (99).

In another study involving 326 participants (101 with type 1 diabetes, 100 with type 2 diabetes, and 125 healthy controls), the association between Glo-1 activity and the GLO1 rs2736654 SNP, as well as two other common SNPs—rs1130534 (G124G) and rs1049346 (5′-UTR)—was investigated. The study found that Glo-1 activity was reduced in individuals carrying the rs1130534 AT and TT genotypes, as well as in those with the rs1049346 TT and CT genotypes. The T alleles of rs1130534 and rs1049346 were associated with reductions in blood Glo-1 activity by 3.1 U/g Hb and 2.8 U/g Hb, respectively. The rs2736654 SNP did not show a significant correlation with Glo-1 activity (78). However, individuals with the C332C genotype were found to have lower Glo-1 activity levels. These findings contradicted the results of the study by Barua et al. (99), which employed a different experimental approach and study population. Nevertheless, they may provide some support for the association between the C332C genotype and diabetic peripheral neuropathy reported by Groener et al. (67).

1.4.6. Apolipoprotein E (APOE)

The three isoforms of the apolipoprotein E gene play an important role in cholesterol and triglyceride metabolism. The presence of the APOE ε4 allele appears to increase the risk of developing severe diabetic neuropathy (68). The transcription factor 7-like 2 (TCF7L2) gene influences lipid metabolism and glucose homeostasis. Examination of three TCF7L2 polymorphisms (rs7903146, rs7901695, and rs12255372) revealed a strong correlation between rs7903146 and CAN (69).

1.4.7. Vascular Endothelial Growth Factor (VEGF)

Human vascular endothelial growth factor promotes the proliferation of vascular endothelial cells. In recent years, elevated VEGF levels have been reported in the presence of diabetic neuropathy (70, 100). Moreover, the presence of the 936C/T mutation in the VEGF gene has been shown to further increase the risk of developing diabetic neuropathy, whereas the T allele appears to confer a protective effect (101).

1.4.8. Interleukin-4 (IL-4)

Interleukin-4 is an important cytokine that regulates immune cell chemotaxis and exerts anti-inflammatory effects. The variable number of tandem repeat (VNTR) polymorphism of the IL-4 gene plays a significant role in the development of diabetic neuropathy (71). Glutathione peroxidase 1 (GPX-1) is an antioxidant enzyme. A polymorphism in this gene (rs1050450, C>T) results in an amino acid substitution from proline to leucine at codon 198, leading to reduced enzymatic activity. The rs1050450 T allele has also been identified as a risk factor for diabetic neuropathy (72).

1.4.9. Endothelial Nitric Oxide Synthase (eNOS)

Endothelial dysfunction contributes to the development of microvascular complications. Endothelial nitric oxide synthase is responsible for the synthesis of nitric oxide. Polymorphisms in the eNOS gene that lead to reduced eNOS expression are thought to be associated with the development of diabetic neuropathy (102). The two most extensively studied SNPs—rs2070744 (−786 T/C) and rs1799983 (894 G/T)—are considered genetic susceptibility factors for neuropathy (73).

1.4.10. Adrenoceptor Alpha-2B (ADRA2B)

The adrenoceptor alpha-2B gene carries a common nonsynonymous mutation (12Glu9) that encodes a receptor protein and results in an insertion/deletion polymorphism of three consecutive glutamates at positions 301–303. This mutation has been associated with metabolic and vascular effects, including obesity, reduced insulin secretion, and the development of diabetes (74, 103-108). Evidence that this I/D polymorphism is linked to autonomic dysfunction and increased sympathetic nervous system activity in the nervous system supports its potential role in the pathogenesis of diabetic neuropathy. When examining the possible relationship between the ADRA2B gene I/D polymorphism and diabetic neuropathy, a higher frequency of the D allele was reported in patients with neuropathy, suggesting that the presence of the D allele may contribute to the severity of this condition.

1.4.11. MicroRNAs (MIR146A, MIR128A, MIR499A)

Investigations into polymorphisms within microRNA (MIR) regions have revealed an association between the risk of developing diabetic neuropathy and the rs2910164 (G>C) variant in MIR146A as well as the rs11888095 (C>T) variant in MIR128A (75). The

rs2910164 genetic variant in MIR146A is associated with a lower risk of diabetic neuropathy, whereas the rs11888095 variant in MIR128A is linked to an increased risk. Spallone et al. also reported an association between the rs3746444 SNP (GG genotype) in the MIR499A gene and DPN. This phenotype was accompanied by a reduction in mitochondrial DNA copy number, which in turn diminished the capacity to counteract oxidative stress and hyperglycemic burden (109).

1.4.12. Thiamine Transporters (THTR-1/THTR-2)

Both thiamine deficiency and impaired regulation of thiamine transporters contribute to the exacerbation of the metabolic effects of hyperglycemia and the development of diabetic complications (40). Intracellular thiamine transport is mediated by two transporters, THTR-1 and THTR-2. Genetic studies have identified mutations in the solute carrier family 19 member 2 and 3 (SLC19A2 and SLC19A3) genes, which encode THTR-1 and THTR-2, respectively, and have been implicated in the development of neurological disorders. Defects in THTR-1 can lead to mitochondrial dysfunction and, consequently, impaired defense against oxidative stress, it can cause a severe genetic disorder, thiamine-responsive megaloblastic anemia, or a form of maturity-onset diabetes of the young (MODY) (76). On the other hand, mutations in the SLC19A3 gene are known to underlie neurological disorders such as early infantile Leigh-like syndrome/atypical infantile seizures and adult-onset Wernicke's encephalopathy.

In a study conducted by Porta et al. (110), genetic variants of the genes encoding thiamine transporters and their associated SP1/2 transcription factors were investigated in patients with type 1 diabetes from the Finnish Diabetic Nephropathy (FinnDiane) cohort. The findings were further validated in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study (DCCT/EDIC) and the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) cohorts. Out of the 134 SNPs analyzed, two SNPs within the SLC19A3 locus—rs12694743 and rs6713116—were identified as being significantly associated with a protective effect against severe retinopathy ($p = 3.8 \times 10^{-6}$; odds ratio: 0.51; 95% CI: 0.38–0.68), as well as against the combined occurrence of severe retinopathy and end-stage renal disease (ESRD) ($p = 7.5 \times 10^{-8}$; odds ratio: 0.31; 95% CI: 0.20–0.47). The association with the combined phenotype reached genome-wide significance in a meta-analysis integrating

data from the WESDR cohort ($p = 2.3 \times 10^{-8}$; odds ratio: 0.28; 95% CI: 0.18–0.44). These SNPs may represent novel, independent risk factors in the development of retinopathy and nephropathy.

1.4.13. Transketolase (TKT)

The role of genetic variability in the transketolase gene was investigated in 240 patients with diabetic nephropathy (111). The study confirmed significant thiamine deficiency in diabetic nephropathy but did not reveal any association between transketolase gene polymorphisms and the presence of diabetic nephropathy. In a subsequent study by the same authors, involving 314 patients with diabetic nephropathy, the effects of 19 SNPs located in six genes encoding enzymes that metabolize glycolytic intermediates (such as transketolase, transaldolase, transketolase-like 1, fructosamine-3-kinase, glyoxalase-1, and glucose-6-phosphate dehydrogenase) were examined. Their findings indicated that the transketolase SNP rs11130362 and the fructosamine-3-kinase SNP rs1056534 together had a significant impact on the progression of nephropathy ($p = 0.00645$). Furthermore, the transketolase SNP rs3736156, both individually ($p = 0.00442$) and in combination with the two aforementioned SNPs, was significantly associated with the occurrence of major cardiovascular events ($p = 0.01014$) (112).

Ziegler et al. (77) investigated the role of genetic variability in the transketolase gene in the development of neuropathy. The study included 165 patients with type 1 diabetes and 373 newly diagnosed patients with type 2 diabetes. Thirteen SNPs within the TKT gene were selected, and several associations were identified between these SNPs and peripheral nerve function. However, most of these associations lost significance after Bonferroni correction, except for the correlation between the rs7648309 SNP and symptom score, as well as between the rs63355988 SNP and thermal sensation. The study was the first to demonstrate an association between diabetic neuropathy and certain TKT SNPs. The findings suggest that TKT may play a protective role in the development of diabetic neuropathy.

1.4.14. Ion Channels

In a case study of a male patient with painful diabetic neuropathy, a mutation (D109N) in the β2 subunit of the Nav1.7 voltage-gated sodium channel was reported, resulting in

hypersensitivity of dorsal root ganglion neurons (79). It is well established that neuropathic pain substantially reduces quality of life in patients with diabetes (113). Previous studies have identified several risk factors for neuropathic pain, including female sex, smoking, age, body weight, and longer duration of diabetes (114-116). Genetic variants of voltage-gated sodium channels (VGSCs) have previously been identified through next-generation sequencing (NGS) and may contribute to the development of neuropathic pain (115, 117). These genes play a crucial role in nociceptors and nerve fibers in the initiation and propagation of action potentials (79, 117).

1.4.15. Glial Cell Line-Derived Neurotrophic Factor Family Receptor Alpha-2 (GFRA2)

A multicenter study (82), which investigated approximately one million SNPs across the entire genome, identified a single locus (chromosome 8p21.3) associated with neuropathy. However, in this study, the presence of neuropathy was defined based on whether the patient had been prescribed medication suitable for the treatment of neuropathy or whether the monofilament test yielded an abnormal result. Within the identified genomic locus, nine SNPs showed a significant correlation. These SNPs were intergenic, located in proximity to the glial cell line–derived neurotrophic factor (GDNF) family receptor alpha-2 and the neurturin receptor gene. The GFRA2 protein is a glycosylphosphatidylinositol-anchored cell surface receptor and a member of the GDNF receptor family. GDNF is a factor that plays a fundamental role in neuronal differentiation and survival. The GFRA2 receptor binds to this protein family. Proper receptor function is required for the corresponding effect. The receptor activates the rearranged during transfection (RET) tyrosine kinase receptor pathway (118). Based on these findings, it is conceivable that genetic polymorphisms in the GFRA2 gene may contribute to susceptibility to diabetic neuropathy.

1.4.16. Aldose reductase (ALR)

The expression of the aldose reductase gene can be induced by methylglyoxal, AGEs, and oxidative stress caused by hyperglycemic conditions. This gene plays a complex role in the complications of diabetes. Sivenius et al. (83) found that the 106C/T polymorphism in the promoter region of the ALR2 gene is associated with reduced conduction velocity

of the peroneal nerve in patients with type 2 diabetes mellitus, whereas the 106C/C genotype is associated with lower amplitudes of sensory nerves.

Other authors identified a polymorphism located in the 5′ region of the upstream regulatory sequence of the ALR2 gene, the 50-(CA)n microsatellite polymorphism, which has more than 10 alleles and has also been associated with diabetic neuropathy. Two major alleles exist, Z−2 and Z+2, where Z corresponds to 24 CA repeats. The study found that the Z+2 allele appears to confer protection against diabetic neuropathy, whereas the Z−2 allele was associated with increased susceptibility to complications in both type 1 and type 2 diabetes mellitus (84).

1.4.17. Glutathione peroxidase 1 (GPx1)

The 599C/T polymorphism (rs1050450) in the glutathione peroxidase 1 gene has been associated with diabetic neuropathy. A similar correlation was found for the 262C/T polymorphism in the catalase gene (85). In a genome-wide association study, Meng et al. identified an association between neuropathic pain and the chromosomal loci 1p35.1 and 8p21.3 (86).

1.4.18. Whole-exome sequencing

In a previous investigation, our research group applied whole-exome sequencing to a cohort of patients with type 2 diabetes, comparing those with neuropathy to those without. Through this approach, we were able to identify five genetic variants that appear to influence susceptibility to diabetic neuropathy. Specifically, two variants located in the RecQ-mediated genome instability protein 2 (RMI2) gene (rs2032930 and rs2032931), along with one variant in the myosin-binding protein H-like (MYBPHL) gene (rs604349), were strongly associated with an elevated risk of neuropathy, with odds ratios indicating a 22- to 49-fold increase. Conversely, two additional variants demonstrated a protective association: the rs917778 SNP in the multivesicular body subunit 12B (MVB12B) gene and the rs2234753 SNP in the retinoic acid X receptor alpha (RXRA) gene, both of which were linked to a substantially reduced probability of developing neuropathy, with risk estimates falling to approximately 0.07–0.08 (89). Taken together, these findings suggest that genetic variation in genes related to genome stability, protein binding, vesicular

trafficking, and nuclear receptor signaling may play an important role in modulating individual vulnerability to diabetic neuropathy.

2. Objectives

In our previous study, we performed whole-exome sequencing in patients with type 2 diabetes mellitus, with and without neuropathy (24 and 24 patients, respectively). In the present study, we sought to extend this work by conducting an in silico analysis of the whole-exome data to identify additional genetic variants that may contribute to susceptibility to neuropathy.

1. Search for genetic variants that increased the risk of developing of neuropathy.
2. Search for genetic variants that reduced the risk of developing of neuropathy.

3. Methods

3.1. Patient selection

Patients with type 2 diabetes mellitus, identified in primary care through a screening program, were referred to our department and enrolled in the present study. To ensure genetic homogeneity, as is customary in genetic research, neither healthy controls nor patients with type 1 diabetes mellitus were included. A total of 48 participants with type 2 diabetes mellitus were included in the study (30 men and 18 women), comprising 24 individuals with neuropathy and 24 without. All participants were in sinus rhythm, and resting electrocardiograms were normal in every case. Based on medical history, comprehensive physical examination (including neurological assessment), resting electrocardiography, and chest radiography, individuals with pulmonary disease, hypertension, ischemic heart disease, heart failure, valvular disorders, or any other condition that could influence the results of autonomic testing were excluded.

Inclusion criteria required a history of type 2 diabetes mellitus for more than five years, adequately controlled glycemic status, and an age range of 18 to 69 years at baseline assessment. Exclusion criteria comprised type 1 diabetes mellitus, poorly controlled glycemic status (HbA1c value above 8%), pregnancy, severe comorbid conditions (e.g., malignancy), psychiatric disorders, immunosuppressive therapy, impaired capacity to provide reliable participation, and neuropathy attributable to causes other than diabetes.

During the 48 hours preceding neurological assessment or the cardiotens test, participants were required to discontinue medications that could influence autonomic or sensory function (e.g., digitalis, beta-adrenergic blockers, atropine, non-dihydropyridine calcium channel blockers, or sedatives) (45). In addition, all participants were instructed to abstain from vigorous physical activity, smoking, caffeine, and alcohol consumption for at least 12 hours before the study.

The study protocol was approved by the local ethics committee (approval number: 37596-8/2018/EÜIG), and written informed consent was obtained from all participants.

3.2. Neurological assessment

Patients were classified into the neuropathy group if sensory and/or autonomic neuropathy was confirmed. Cardiovascular autonomic neuropathy was diagnosed using Ewing's standardized cardiovascular reflex tests and was defined by the presence of at least one abnormal result or two borderline results. Sensory neuropathy was diagnosed based on the presence of at least one abnormal result or two borderline results obtained using a Neurometer, Medoc device, tuning fork, or monofilament.

3.2.1. Evaluation of sensory neuropathy

As it is presented in Figure 2., sensory nerve function was evaluated using a calibrated tuning fork, monofilament testing, the Neurometer® device (Neurotron Inc., Baltimore, MD, USA), the Medoc device (Medoc Ltd., Ramat Yishai, Israel), and the Neuropathy Total Symptom Score-6 (NTSS-6).

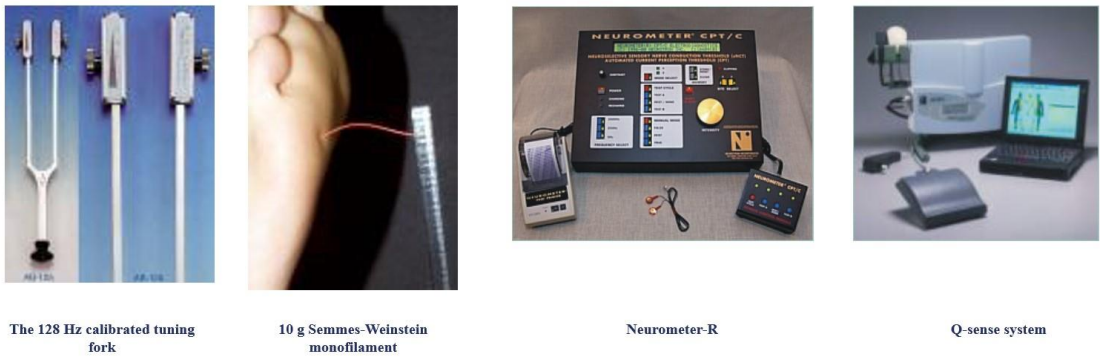

Figure 2. Instruments used for the assessment of sensory neuropathy (119).

3.2.1.1. The 128 Hz calibrated tuning fork

The calibrated tuning fork serves to assess vibration perception (deep sensory function), which reflects the function of large myelinated fibers. The most widely applied instrument in clinical practice is the 128 Hz Rydel-Seiffer calibrated tuning fork (45), and its use is recommended by the European Association for the Study of Diabetes (EASD) and Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes (NEURODIAB) for the evaluation of vibration sensation in outpatients (120).

The device is simple to administer, and the procedure requires approximately one minute. To perform the test, the tuning fork is activated and placed on a bony prominence of both

the upper and lower limbs, and the patient is instructed to indicate the point at which the vibration is no longer perceived. Commonly assessed sites include the tip of the great toe, the medial malleolus, and the dorsal surface of the second metacarpal. The instrument is equipped with a wedge-shaped scale ranging from 1 to 8 on its upper end. When the tuning fork is activated, the scale appears as two diverging lines that gradually converge as the vibration diminishes, allowing a numerical value to be recorded at the moment when the patient reports cessation of vibration perception. The interpretation of the acquired results are presented in Table 2. This method provides a quantitative assessment of vibration threshold and is widely applied in the evaluation of peripheral neuropathy, demonstrating good reproducibility and strong clinical applicability (121).

Table 2. Interpretation of measurements obtained using a calibrated tuning fork (121).

Value obtained from the tuning fork	Evaluation of results
8-7	Normal sensory function
6	Sensory neuropathy is suspected
5-1	Distal sensory neuropathy

3.2.1.2. 10 g Semmes-Weinstein monofilament test

The use of the Semmes–Weinstein monofilament 5.07/10 g is recommended for the assessment of protective sensation. Similar to the calibrated tuning fork, this test is extremely simple and rapid to administer. The evaluation is straightforward, as patients are only required to indicate whether they perceive the pressure exerted by the bent monofilament. The procedure involves applying the 10 g monofilament perpendicularly to the test site at a 90-degree angle for approximately 1.5 seconds (122). Standard test sites include the plantar surface of the hallux and the first, third, and fifth metatarsal heads, although additional points on the foot may be examined to increase sensitivity. Interpretation is straightforward, the test is considered abnormal if the patient does not perceive the pressure exerted by the bent monofilament. Impaired perception of the monofilament is considered a strong predictor of foot ulceration risk, and its application is recommended by the American Diabetes Association (ADA) and the International Working Group on the Diabetic Foot (IWGDF). The method is highly reproducible, cost-effective, and well suited for both clinical and research settings (17, 123).

3.2.1.3. The Neurometer

Current perception threshold (CPT) testing was performed with the Neurometer® (Neurotron Inc., Baltimore, MD, USA) at the median and peroneal nerves using three frequencies (2000 Hz, 250 Hz, and 5 Hz), corresponding to the assessment of large myelinated, small myelinated, and small unmyelinated sensory nerve fibers, respectively (124-126). Normative reference values for these three frequency measurements at the peroneal and median nerves were established by Evans et al. and they are presented in Table 3. (127). CPT testing provides a quantitative and reproducible measure of sensory nerve function, has been widely applied in both clinical and research settings for the evaluation of diabetic neuropathy.

Table 3. Internationally accepted normal values obtained using the Neurometer device (100 = 1 mA) (127).

Nervus medianus		***Nervus peroneus***
Normal range	**Frequences**	**Normal range**
120-398 mA	*2000 Hz*	179-523 mA
22-189 mA	*250 Hz*	44-208 mA
16-101 mA	*5 Hz*	18-170 mA

3.2.1.4. The Medoc device

Similarly to the Neurometer device, this testing method is also based on the Quantitative Sensory Testing (QST) principle and is suitable for assessing all types of sensory nerve fibers. It is non-invasive, reproducible, and provides quantitative results (128). A major advantage of the Medoc system over the Neurometer is that, while the Neurometer applies electrical stimulation, the Medoc system uses physiological stimuli. The Thermal Sensory Analyzer (TSA) II system is designed to assess thermal and pain perception, thereby providing information about the function of small nerve fibers, whereas the Vibratory Sensory Analyzer (VSA) 3000 system measures vibration perception, reflecting the function of large nerve fibers.

Threshold values can be determined using two methods: the Limits method, which is based on reaction time, or the Levels method, which uses constant-intensity stimuli. In our study, we applied the Limits method, in which the stimulus intensity gradually

increases until the patient interrupts the test upon perceiving the sensation. The stimuli follow one another sequentially, and generally four stimuli are sufficient to determine the threshold value. This approach is the most commonly used because it requires only a short testing time.

During the use of the TSA II system, to determine thresholds for cold, warm, and painful cold and heat sensations, a 9 cm^2 ceramic probe is placed on the dorsal surface of the hand or foot. The probe's baseline temperature is 30–32°C, corresponding to the typical temperature of uncovered skin at room temperature. The temperature of the ceramic plate can be adjusted by computer in 0.3°C increments within a range of 10 to 45°C. Under normal sensory conditions, the minimal detectable warm sensation typically appears at 1–2°C above the baseline temperature, while the minimal detectable cold sensation appears at less than 1°C below the baseline. Painful heat perception usually occurs around 45°C, and painful cold perception around 10°C. This method thus provides an assessment of small fiber function. The system automatically compares results to normal reference ranges.

For vibration perception threshold testing, the distal phalanx of the index finger or great toe is placed on the vibration probe, which delivers 125 vibrations per second. The vibration amplitude can be adjusted between 0 and 130 μm. The test result provides information about the condition of large, myelinated Aα fibers. During threshold determination, the patient signals the perception of the vibration by pressing a response button. The system then automatically compares the obtained values with age-matched normative reference ranges.

3.2.1.5. The Neuropathy Total Symptom Score-6 (NTSS-6)

The severity of neuropathic symptoms was evaluated using the Neuropathy Total Symptom Score-6. This questionnaire quantifies both the frequency and intensity of sensory symptoms commonly reported by patients with diabetic peripheral neuropathy, including aching pain or tightness; sharp, shooting, or lancinating pain; allodynia or hyperalgesia; numbness or insensitivity; prickling or tingling; and burning sensations. The NTSS-6 yields a total score ranging from 0 to 21.96 as it is presented in Table 4. A score greater than 0 indicates the presence of at least one sensory symptom, while severe sensory neuropathy is defined by a score exceeding 6 points (129).

Table 4. NTSS-6 score (129).

Symptoms		Severity			
		Absent	**Mild**	**Moderate**	**Severe**
Frequency	**Never**	0,00	0,00	0,00	0,00
	Sometimes	0,00	1,00	2,00	3,00
	Often	0,00	1,33	2,33	3,33
	Continuously	0,00	1,66	2,66	3,66

3.2.2. Evaluation of cardiovascular autonomic neuropathy

In patients with type 2 diabetes mellitus, cardiovascular autonomic function was evaluated using the five standard cardiovascular reflex tests in combination with 24-hour ambulatory blood pressure monitoring.

3.2.2.1. Standardized reflex tests of cardiovascular autonomic function

Cardiovascular autonomic neuropathy was evaluated using five standardized cardiovascular autonomic reflex tests: the heart rate response to deep breathing (beat-to-beat variation), to standing (30/15 ratio), and to the Valsalva maneuver (Valsalva ratio), which assess parasympathetic function, as well as the blood pressure responses to standing and to sustained handgrip, which reflect sympathetic function. According to the Toronto Consensus Panel, the blood pressure response to sustained handgrip is no longer considered an acceptable clinical test but remains applicable for investigational purposes (130). All reflex tests were performed with the Cardiosys H-01 12-lead portable electrocardiogram (ECG) system. Cardiovascular autonomic neuropathy was diagnosed

when at least one abnormal or two borderline cardiovascular reflex test results were present. Abnormal results were scored as 2, borderline values as 1, and normal values as 0, allowing for calculation of the severity of cardiovascular autonomic neuropathy (124, 131).

3.2.2.1.1. Heart rate response to deep breathing (respiratory sinus arrhythmia)

Among the five standard cardiovascular reflex tests, the heart rate response to deep breathing is considered the most sensitive and one of the most rapid to perform. Under normal physiological conditions, heart rate varies continuously, increasing during inspiration and decreasing during expiration, a phenomenon known as respiratory sinus arrhythmia. The response is maximal at a breathing frequency of six breaths per minute. During the test, the patient is instructed to breathe at this rate while electrocardiographic activity is continuously monitored. The test duration is approximately 30 seconds. The difference between the maximum heart rate during inspiration and the minimum heart rate during expiration is then calculated, and a value below 10 beats per minute is suggestive of autonomic neuropathy. This test primarily reflects parasympathetic (vagal) function.

3.2.2.1.2. Heart rate response to standing (30:15 ratio)

The 30:15 ratio test is performed by instructing the patient to lie quietly for three minutes, then to stand up and remain standing for one minute with arms relaxed at the sides while a continuous electrocardiographic recording is obtained. Under normal physiological conditions, heart rate increases transiently upon standing and subsequently decreases. The peak tachycardic response typically occurs around the 15th beat after standing, followed by the peak bradycardic response at approximately the 30th beat. The ratio of the longest to the shortest R–R interval (30:15 ratio) is calculated and serves as an indicator of cardiovascular autonomic integrity. A reduced ratio is suggestive of autonomic dysfunction. This test primarily reflects parasympathetic function.

3.2.2.1.3. Heart rate response to the Valsalva maneuver (Valsalva Ratio)

During the Valsalva maneuver, blood pressure normally decreases with a corresponding increase in heart rate, followed by a sudden rise in blood pressure accompanied by a reduction in heart rate. The test was performed under standardized conditions, with

patients instructed to maintain an airway pressure of 40 mmHg for 15 seconds. The Valsalva ratio is defined as the ratio of the longest to the shortest R–R interval recorded during the maneuver. A reduced ratio is indicative of autonomic dysfunction. This test primarily reflects parasympathetic function.

3.2.2.1.4. Blood Pressure Response to Standing

Under normal physiological conditions, systolic blood pressure increases upon standing, a response that requires intact autonomic nervous system function. For the test, the patient rested in the supine position for 10 minutes, during which baseline blood pressure was recorded. After standing, systolic blood pressure was measured at 1, 3, and 5 minutes. A fall in systolic blood pressure of ≥30 mmHg is considered abnormal and indicative of autonomic dysfunction. This test primarily reflects sympathetic function.

3.2.2.1.5. Blood pressure response to sustained handgrip

The sustained handgrip test, performed by maintaining prolonged contraction of the hand muscles, is normally associated with an increase in both heart rate and blood pressure. An attenuated or absent rise in diastolic blood pressure during this maneuver is indicative of impaired autonomic function.

According to the most recent international recommendations, only four cardiovascular reflex tests are advised for the evaluation of autonomic neuropathy, with the orthostatic hypotension test applied to assess sympathetic innervation and the sustained handgrip test omitted (130). Our research group has provided evidence supporting the omission of the handgrip test. Specifically, we found that its sensitivity for detecting autonomic neuropathy was only 24.6%. More importantly, the sensitivity of the handgrip test for detecting sympathetic neuropathy, as confirmed by the orthostatic hypotension test, was merely 20%, and no correlation was observed between the handgrip test and the other cardiovascular reflex tests. A negative correlation was identified between the handgrip test and the presence of hypertension, indicating that in diabetic patients with hypertension the test rarely yields abnormal values. Taken together, these findings demonstrate that the handgrip test has limited diagnostic value due to its low sensitivity and dependence on hypertension, and therefore it is not recommended for the assessment

of autonomic function (132). Normal reference values for cardiovascular reflex testing are presented in Table 5.

Table 5. Normal reference values for cardiovascular reflex testing (131).

Method	Tested parameter	Normal value	Borderline value	Abnormal value
Tests for the investigation of parasympathetic functions				
Deep breathing test	Beat to beat variation (beats/min)	≥ 15	11-14	≤ 10
Valsalva manoeuvre	Valsalva ratio	≥ 1,21	1,11-1,2	≤ 1,1
Heart rate response to standing	30/15 ratio	≥ 1,04	1,01-1,03	≤ 1,0
Tests for the investigation of sympathetic functions				
Blood pressure (BP) response to standing	Reduction of systolic BP (mmHg)	≤ 10	11-29	≥ 30
Handgrip test	Increase of diastolic BP (mmHg)	≥ 16	11-15	≤ 10

3.3. Genetic analysis

3.3.1. DNA isolation

Genomic DNA was extracted from peripheral blood samples using the HighPure DNA Isolation Kit (Roche, Rotkreuz, Switzerland) according to the manufacturer's instructions. DNA concentration was quantified with the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA)

3.3.2. Whole-Exome Sequencing (WES)

Whole-exome sequencing was performed at the Molecular Biology Laboratory of the Department of Internal Medicine and Oncology, Semmelweis University, which possesses the required expertise and infrastructure to support this procedure.

Whole-exome sequencing was conducted on the Thermo Ion GeneStudio S5 platform, targeting the coding regions of approximately 32,000 genes, corresponding to approximately 50 million base pairs per patient.

Exome library preparation was conducted using the Ion Torrent AmpliSeq RDY Exome Kit (Thermo Fisher Scientific, Waltham, MA, USA) to amplify the target regions of the exome. Genomic DNA concentration was determined with either the Qubit DNA HS or BR Assay Kit (Life Technologies, Carlsbad, CA, USA), and 100 ng of DNA per sample was used for library construction. Ion Xpress barcode adapters were ligated to uniquely label each sample. After amplification, libraries were purified with AMPure XP beads and quantified using a Qubit 2.0 fluorometer. Final libraries were diluted to approximately 100 pM. Three barcoded libraries were pooled per run and loaded onto a single Ion 540 chip. Sequencing was carried out on the Ion GeneStudio S5 platform (Thermo Fisher Scientific).

Following sequencing, AmpliSeq Exome reads were aligned to the human reference genome (hg38). Coverage analysis and initial data processing were conducted using Ion Torrent Suite Software version 5.12. The resulting Binary Alignment Map (BAM) files were subsequently uploaded to the cloud-based Ion Reporter Software version 5.12, where variant calling and raw data analysis were carried out using the Ion Reporter™ AmpliSeq Exome workflow.

Variant annotation was carried out using a series of bioinformatic filters. These included single-nucleotide variant (SNV) filtering; evaluation of synonymous and missense variant effects; application of functional prediction algorithms such as SIFT, PolyPhen, and Grantham; restriction of homopolymer length to ≤6 bases; and assessment of homozygosity. Additional criteria required a minimum allele read count of 100, an allele ratio of 1.0, and a minor allele frequency (MAF) threshold of ≤0.5.

3.4. Bioinformatic and statistical methods

Variant annotation was conducted using ANNOVAR (v03Dec2019), incorporating data from dbSNP, ClinVar, gnomAD, and OMIM. Sequencing reads were visualized with the Integrative Genomics Viewer (IGV), and duplicate reads were marked using Picard tools. Single-nucleotide polymorphism calling was performed on Variant Call Format (VCF) files generated with Genome Analysis Toolkit (GATK). These VCF files were merged using BCFtools, and the resulting variants were further annotated with SnpSift (133).

Variant annotation was performed using the dbSNP reference database (hg38, build 151), obtained from the National Center for Biotechnology Information (NCBI) dbSNP repository.

Quality control of the raw VCF files was carried out using PLINK v1.9 (134). Predicted sex, inferred from SNP data, was compared with the reported phenotypic sex of participants to identify discrepancies. SNP filtering was applied using the following thresholds: missingness rate >0.05, minor allele frequency <0.01, and Hardy–Weinberg equilibrium (HWE) p-value $<1 \times 10^{-10}$. These thresholds were selected in accordance with the recommendations provided in the PLINK v1.9 documentation (135).

Association testing was conducted in the R v4.0.3 environment using the GENESIS Bioconductor package to fit logistic regression models (136, 137). To minimize potential confounding and to account for associations identified during quality control, model estimates were adjusted for age, sex, and relatedness. Genetic relatedness was modeled using a genetic relatedness matrix (GRM) generated with the SNPRelate package (138).

Quantile–Quantile (QQ) plots were generated using the qqman R package, and Manhattan plots were constructed with ggplot2 (139). The most significant SNPs were ranked according to p-values derived from the logistic regression analysis.

All statistical analyses were conducted in R v4.0.3. Continuous variables are presented as means ± standard deviations, and group differences were evaluated using the Mann–Whitney test. Categorical variables are expressed as frequencies, and comparisons between groups were assessed with Fisher's exact test. A p-value <0.05 was considered statistically significant.

4. Results

Data from patients with type 2 diabetes mellitus and neuropathy were compared with those from patients with type 2 diabetes mellitus but without neuropathy. The baseline characteristics of the two study groups are summarized in Table 6. Patients with neuropathy were, on average, older than those without neuropathy. In contrast, no significant differences were observed between the groups in terms of diabetes duration, body mass index (BMI), HbA1c levels, or lipid profile.

Table 6. Demographic and clinical characteristics of the two study groups.

	T2DM with Neuropathy (n = 24)		**T2DM Without Neuropathy (n = 24)**		
	Average	**±SD**	**Average**	**±SD**	***p* Value**
Age (years)	66.5	9.27	56.2	10.8	0.0012
Body mass (kg)	93.8	15.8	86.8	17.4	0.1009
Body height (cm)	172.5	9.9	170.0	10.5	0.4030
BMI (kg/m^2)	31.5	5.00	30.0	5.2	0.1670
Systolic blood pressure (Hgmm)	137.7	15.7	134.0	12.2	0.3842
Diastolic blood pressure (Hgmm)	71.3	7.1	75.0	9.1	0.1718
Duration of diabetes (years)	10.3	6.2	13.2	7.5	0.1322
Sex (male/female)	17/7		13/11		
Fasting blood sugar (mmol/L)	8.92	2.81	8.97	3.18	0.9912
HbA1c (%)	7.49	1.09	7.04	1.00	0.1376
Cholesterol (mmol/L)	4.80	0.88	5.05	1.23	0.5596
LDL cholesterol (mmol/L)	2.91	0.82	3.2	0.94	0.4480
HDL cholesterol (mmol/L)	1.26	0.36	1.17	0.29	0.6441
Triglyceride (mmol/L)	1.85	0.88	2.53	1.73	0.3065

Data are presented as mean ± standard deviation (SD). Differences between groups were analyzed using the χ^2 test.

The results of whole-exome sequencing are presented in Table 7.

Table 7. Summary of whole-exome sequencing results, detailing identified genetic variants with their chromosomal locations, variant positions, reference allele frequencies, and associated odds ratios (ORs) for neuropathy risk. * Minor allele frequencies are based on the European population data from the Allele Frequency Aggregator (ALFA) project (140).

Variant ID	Reference/ Alternative Allele	Position	Gene	Reference Allele Frequency (MAF) of European Population *	Logistic Regression Estimate (β)	Logistic Regression Estimate (β) Standard Error	OR for Reference Allele	*p* Value
rs922984	T/C	chr2:178751160 (GRCh38.p14)	*TTN*	0.070	3.248	0.989	26.69	0.001
rs2291313	T/C	chr2:178767983 (GRCh38.p14)	*TTN*	0.202	2.304	0.738	22.65	0.002
rs4471922	G/T	chr2:178768571 (GRCh38.p14)	*TTN*	0.205	2.304	0.738	22.65	0.002
rs6086563	C/G	chr20:8722498 (GRCh38.p14)	*PLCB1*	0.243	2.787	0.855	25.99	0.001
rs4241602	A/G	chr4:77066198 (GRCh38.p14)	*CCNI*	0.081	4.020	1.264	24.01	0.001
rs2396295	A/G	chr19:536437 (GRCh38.p14)	*CDC34*	0.088	3.213	0.996	25.16	0.001
rs892204	G/A	chr19:536900 (GRCh38.p14)	*CDC34*	0.081	3.213	0.996	25.16	0.001
rs6682221	C/A	chr1:203305408 (GRCh38.p14)	*BTG2*	0.099	−2.761	0.893	0.045	0.002

We identified seven additional genetic variants significantly associated with an increased risk of developing diabetic neuropathy. The rs922984, rs2291313, and rs4471922 variants in the titin gene; the rs6086563 SNP in the phospholipase C-beta 1 gene; the rs4241602 variant in the cyclin I gene; and the rs2396295 and rs892204 variants in the cell division cycle 34 gene were associated with a 22- to 26-fold increased risk. In contrast, the rs6682221 variant of the anti-proliferation factor 2 gene appeared to confer protection, reducing the risk of neuropathy to approximately 0.045. The chromosomal locations and positions of these genetic variants, together with their corresponding odds ratios, are presented in Table 7., which indicates the probability of developing neuropathy. Collectively, these findings highlight potential novel candidate genes that may contribute to the pathogenesis of diabetic neuropathy.

5. Discussion

In a previous study, our group performed whole-exome sequencing in a cohort of individuals with type 2 diabetes, comparing patients with diabetic neuropathy to those without neuropathy. This analysis identified five genetic variants associated with susceptibility to diabetic neuropathy. Two variants in the RecQ-mediated genome instability protein 2 gene (rs2032930 and rs2032931) and one variant in the myosin-binding protein H-like gene (rs604349) were significantly associated with an increased risk of neuropathy. In contrast, two additional variants exhibited protective associations: rs917778 in the multivesicular body subunit 12B gene and rs2234753 in the retinoic acid X receptor alpha gene, both of which were associated with a markedly reduced likelihood of neuropathy.

In this study, we identified several additional genetic variants that may contribute to the pathogenesis of diabetic neuropathy in individuals with type 2 diabetes mellitus. The reanalysis of our previous data acquired from whole-exome sequencing revealed associations with novel loci that expand our current understanding of the molecular underpinnings of this complication.

5.1. Genetic variants that increase the risk of developing neuropathy

The rs922984 variant represents a missense mutation, whereas rs2291313 and rs4471922 are intronic SNPs within the titin gene. Titin is a giant sarcomeric protein that supports myofibrillar assembly during myogenesis, determines the passive elasticity of muscle, and performs diverse signaling functions. It interacts with more than 170 protein ligands, including telethonin, α-actinin, small ankyrin 1 (sAnk1), filamin C, nebulin, tropomyosin, αB-crystallin, four-and-a-half LIM domains 1 and 2 (FHL1, FHL2), calpains 1 and 3, and muscle ankyrin repeat proteins (MARPs). Through these interactions, titin influences key cellular processes such as phosphorylation, calcium binding, and myosin binding (141). It contributes to the maintenance of sarcomere structure both longitudinally and radially and plays a role in active contraction of striated muscle (142). In addition, titin is essential for diastolic function of the heart (143).

Titin is expressed in two principal isoforms, N2B and N2BA. Their relative abundance is predominantly controlled by the alternative splicing factor RNA-binding motif protein 20

(RBM20). RBM20 is a muscle-specific regulator that governs titin splicing within sarcomeric tissues, including both skeletal and cardiac muscle (144).

The isoform composition of titin is a major determinant of passive myocardial stiffness. Upregulation of the N2B isoform has been associated with impaired cardiac contractility and diastolic dysfunction (145). Animal studies demonstrated that RBM20 knockout mice and rats express only the largest titin isoform, N2BA-G. Similarly, loss-of-function mutations in RBM20 result in increased N2BA isoform expression, and affected animals develop idiopathic dilated cardiomyopathy (144, 146). In healthy adult cardiac tissue, the smaller N2B isoform predominates under the regulation of RBM20, whereas absence of this splicing factor leads exclusively to expression of the larger N2BA isoform. These findings highlight RBM20 as a dose-dependent regulator of titin splicing in the heart. Furthermore, Zhu et al. showed that RBM20 expression correlates with insulin levels, thereby influencing titin splicing. Insulin promotes protein synthesis via activation of the mammalian target of rapamycin (mTOR) pathway, which enhances RBM20 expression, whereas inhibition of phosphoinositide 3-kinase (PI3K) or mTOR reduces RBM20 levels (143).

Krüger et al. (147) demonstrated that cardiomyocytes from streptozotocin (STZ)-treated rats exhibited an increased proportion of the stiffer N2B titin isoform in response to insulin. This insulin-driven shift in isoform expression was inhibited by a PI3K blocker, indicating that insulin regulates titin isoform composition in the heart via activation of the PI3K/Protein Kinase B (PKB) signaling pathway. Moreover, insulin-treated cardiomyocytes showed enhanced phosphorylation of titin, further supporting a regulatory role of insulin in titin post-translational modification. These findings link insulin signaling to myocardial structural remodeling through modulation of titin isoforms and phosphorylation status.

The pathogenesis of type 2 diabetes mellitus evolves slowly over several years, typically progressing in a silent and asymptomatic manner. During this preclinical stage, insulin resistance develops and is accompanied by compensatory hyperinsulinaemia, which generally precedes the elevation of fasting blood glucose levels by several years (148, 149). This extended asymptomatic period is nevertheless biologically active, as it is

characterized by metabolic and vascular alterations that can initiate tissue damage and increase susceptibility to chronic complications, including diabetic neuropathy.

The development of diabetic cardiomyopathy has been attributed to multiple mechanisms (150). Cardiovascular autonomic neuropathy has been extensively studied as a contributing factor to its pathophysiology; however, the complexity of this association has been consistently emphasized across publications. Diabetic cardiomyopathy rarely occurs in isolation, as several comorbid conditions frequently coexist with diabetes and contribute to its progression (151). In the early stages of CAN, reduced parasympathetic tone results in tachycardia, which may transiently enhance myocardial contractility and relaxation, but is eventually followed by progressive decline in left ventricular function (152). Echocardiographic studies indicate that the early course of the disease is characterized by impaired left ventricular diastolic function with preserved systolic function (153). Nevertheless, similar to systolic heart failure, this condition is associated with increased mortality (154). Findings from the DCCT/EDIC study demonstrated that in individuals with long-standing type 1 diabetes, the presence of CAN was associated with left ventricular remodeling and hypertrophy (155). Comparable evidence has been reported in type 2 diabetes, where echocardiographic evaluation revealed an increased left ventricular diastolic diameter in patients with CAN (156). Despite these observations, further studies are required to delineate the independent role of cardiovascular autonomic neuropathy in the pathogenesis of diabetic cardiomyopathy.

The two intronic SNPs in the titin gene (rs2291313 and rs4471922) and the rs922984 variant may exert an indirect effect on the diastolic dysfunction observed in diabetic cardiomyopathy.

The rs6086563 variant is an intronic SNP within the phospholipase C-beta 1 (PLCβ1) gene. PLCβ1 encodes a signaling protein that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to generate inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), a pivotal step in intracellular signaling pathways. These pathways regulate processes such as neurotransmitter and hormone signaling, which are critical for the development and function of the central nervous system (157). PLCβ1 shows predominant expression in brain regions involved in higher-order functions, including the frontal cortex, hippocampus, and amygdala, and has been implicated in the regulation of

emotional and cognitive behavior. Dysregulation of phosphoinositide signaling mediated by PLCβ1 may therefore contribute to the pathogenesis of psychiatric disorders (158). Furthermore, homozygous or compound heterozygous loss-of-function mutations in PLCβ1 have been described in children with early-onset epileptic encephalopathy (159).

Alterations in inositol metabolism have been associated with insulin resistance and the microvascular complications of diabetes (160). Clinical studies have demonstrated reduced inositol levels in patients with diabetes (161, 162). Concomitant with myo-inositol depletion, decreases in Na^+/K^+-ATPase activity and nerve conduction velocity have been documented, alongside structural abnormalities such as axonal atrophy, paranodal swelling, and paranodal demyelination (163). These metabolic and morphological alterations are believed to contribute to the pathogenesis of diabetic neuropathy.

The rs6086563 SNP in the phospholipase C-beta 1 gene may contribute to myo-inositol depletion, thereby increasing susceptibility to microvascular complications, including neuropathy.

The rs4241602 variant is an intronic SNP in the cyclin I gene. Cyclin I is an atypical cyclin predominantly expressed in postmitotic cells. By binding to the cyclin-dependent kinase 5 (CDK5), cyclin I forms a critical antiapoptotic complex that modulates the levels of the pro-survival proteins B-cell leukemia/lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-XL) via the mitogen-activated protein kinase (MAPK) signaling pathway (164). CDK5 is a key regulator of neuronal apoptosis and has been implicated in the pathogenesis of several neurological disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, and ischemic stroke (165). Under normal physiological conditions, CDK5 remains inactive, but binding to its activator p35 results in kinase activation and subsequent phosphorylation of multiple substrates involved in neuronal development, axonal growth, and dendritic branching. In response to pathological neuronal stimulation, elevated intracellular calcium triggers the cleavage of p35 to p25. Formation of the p25–CDK5 complex produces a hyperactivated kinase state, leading to widespread substrate hyperphosphorylation and contributing to neurodegenerative processes (166). Furthermore, CDK5 plays an important role in pain signaling within trigeminal ganglion neurons. Enhanced CDK5 activity increases calcium influx and

polymodal nociceptor activity, thereby elevating pain sensitivity and contributing to the development of allodynia, a hallmark of neuropathic pain (167).

In both in vitro and in vivo studies, the use of CDK5 inhibitors has shown neuroprotective effects, indicating that CDK5 may represent a potential therapeutic target for neurological diseases in the future (166).

Our findings suggest that the intronic rs4241602 SNP may be implicated in the development of allodynia, a characteristic feature of diabetic neuropathy.

The rs2396295 and rs892204 variants are intronic SNPs located in the cell division cycle 34 (CDC34) gene. Cell division cycle 34 is a ubiquitin-conjugating enzyme (E2) that plays a pivotal role in regulating cell cycle progression, particularly the transition from the G1 to S phase, through the ubiquitination and subsequent proteasomal degradation of key cell cycle regulators. In endothelial cells, proper control of the cell cycle, mediated by CDC34 and other components of the ubiquitin–proteasome system (UPS), is fundamental to maintaining endothelial homeostasis, vascular integrity, and angiogenic capacity. The UPS, in which CDC34 functions as a core E2 enzyme within the SKP1-Cullin-F-box protein complex (SCF) complex, orchestrates the targeted degradation of cyclins and cyclin-dependent kinase inhibitors. Through this mechanism, it governs endothelial proliferation, quiescence, and cellular responses to stressors such as oxidative damage. Disruption of UPS activity, including dysregulation of CDC34, can result in abnormal endothelial proliferation or premature senescence, ultimately impairing vascular function and contributing to pathologies such as atherosclerosis and defective angiogenesis (168).

Quiescent cells in the G0/G1 phase preserve barrier integrity and vascular tone, whereas controlled proliferation is essential for tissue repair and new vessel formation. The equilibrium between these states, partly maintained through CDC34-dependent proteolysis, is therefore crucial for endothelial health (168, 169).

Recent studies demonstrate that modulation of CDC34 activity, through either pharmacological or genetic approaches, significantly influences endothelial function in vascular pathologies such as atherosclerosis and diabetic vasculopathy (168, 170, 171).

Genetic perturbation studies, including CRISPR-based functional screens, have identified CDC34 and other ubiquitin–proteasome system components as essential modulators of endothelial transcriptional and metabolic programs relevant to coronary artery disease. Dysregulation of CDC34 disturbs the balance between endothelial renewal and senescence, leading to diminished nitric oxide bioavailability, elevated oxidative stress, and activation of pro-inflammatory pathways (170-172).

Pharmacological manipulation of the UPS, including components such as CDC34, remains in the experimental stage. Although proteasome inhibitors and agents targeting cell cycle checkpoints have shown promise in preclinical models by reducing pathological endothelial proliferation and vascular inflammation, their therapeutic translation is limited by off-target cytotoxicity and systemic effects (168, 173).

Taken together, these findings suggest that the rs2396295 and rs892204 SNPs in the CDC34 gene may influence endothelial function and vascular repair in diabetes, thereby contributing to microvascular complications such as neuropathy.

5.2. Genetic variants that reduce the risk of developing neuropathy

The rs6682221 variant is situated within the 2 kb upstream regulatory region of the B-cell translocation gene 2 gene (BTG2). BTG2 is involved in the control of cell proliferation, apoptosis, and growth, and has been characterized as a potential tumor suppressor. In cooperation with protein arginine methyltransferase 1 (PRMT1), BTG2 contributes to neuronal development by regulating arginine methylation (174). Studies in BTG2-deficient mice revealed impaired contextual memory and the accumulation of immature neurons. Furthermore, BTG2 can interact with the promoter of inhibitor of DNA binding 3 (ID3) and suppress its expression (175). Research by Zelin Chen et al. demonstrated that ID3 expression is upregulated during wound healing, which promotes dermal fibroblast activation and enhances neuronal regeneration (176).

Based on our findings, the rs6682221 SNP located within the 2 kb upstream region of the BTG2 gene may be implicated in the regulation of neuronal regeneration, a process that could be relevant to the pathogenesis of diabetic neuropathy.

6. Conclusion

1. Three variants in the titin gene (rs922984, rs2291313, and rs4471922) were found to be of particular interest. Given the central role of titin in sarcomere assembly, myocardial elasticity, and contractile regulation, these variants may indirectly influence cardiac function and contribute to the diastolic abnormalities frequently observed in diabetic cardiomyopathy.
2. Similarly, the rs6086563 intronic variant in the PLCβ1 gene highlights a potential link between altered inositol metabolism, impaired neurotransmitter signaling, and the increased susceptibility to microvascular complications, including neuropathy.
3. Another important finding was the rs4241602 SNP in the cyclin I gene, which may affect antiapoptotic pathways mediated by CDK5 and thereby influence neuronal survival and pain processing, particularly the development of allodynia.
4. In addition, two intronic variants (rs2396295 and rs892204) in the CDC34 gene suggest a connection between impaired endothelial function, vascular repair, and diabetic microvascular disease.
5. Finally, the rs6682221 variant in the BTG2 gene, located within its upstream regulatory region, may play a role in neuronal regeneration through modulation of ID3 expression.
6. These findings emphasize the complex genetic landscape underlying diabetic neuropathy and point toward novel biological mechanisms that may drive its progression. While hyperglycemia and metabolic dysregulation remain fundamental contributors, our results underscore the role of genetic susceptibility in modulating individual risk. Future functional studies are needed to validate these associations and to elucidate their mechanistic impact. Such efforts could ultimately support the development of personalized approaches for risk stratification, early detection, and targeted therapies in diabetic neuropathy.

7. Summary

Diabetic polyneuropathy represents a serious complication that greatly influences both morbidity and mortality in people with diabetes. The condition shows considerable variability among individuals and does not always correspond to metabolic control, implying a potential pathogenic contribution of genetic factors. Despite the limited data currently available on biomarkers of diabetic polyneuropathy, multiple studies indicate the presence of genetic susceptibility.In our study, 24 long-standing type 2 diabetic patients with neuropathy and 24 without neuropathy underwent comprehensive neurological evaluation and whole-exome sequencing. Through this approach, we successfully identified genetic variants that may influence the risk of developing diabetic neuropathy.

We identified three variants in the titin gene out of which two were intronic SNPs (rs2291313 and rs4471922) and one as a missense mutation (rs922984). These variants seemed to have a role in the development of diabetic cardiomyopathy. Another genetic intronic variant that can elevate the risk of diabetic neuropathy is the rs6086563 in the phospholipase C-beta 1 (PLCβ1) gene through the altered inositol metabolism. In our study the rs4241602 SNP in the cyclin I gene seems to have a role in the development of allodynia. Also, rs2396295 and rs892204 which are intronic variants in the CDC34 gene suggest a connection between impaired endothelial progenitor cell function, vascular repair, and diabetic microvascular disease. Finally, the rs6682221 variant in the BTG2 gene, located within its upstream regulatory region, may play a role in neuronal regeneration through modulation of ID3 expression.

Overall, the synthesis of epidemiological, mechanistic, and genetic evidence highlights diabetic neuropathy as a multifactorial disorder, driven by both metabolic insults and genetic predisposition. Improved understanding of these pathways may support the development of targeted prevention strategies and novel therapeutic approaches.

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