SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3077.

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Anyagcsere betegségek
című program

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GENETIC FACTORS ASSOCIATED WITH THE DEVELOPMENT OF NEUROPATHY IN TYPE 2 DIABETES

PhD thesis

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Budapest

2024

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List of Abbreviations

ACE- Angiotensin-converting enzyme

ADRA2B-Adrenoceptor Alpha 2B

AGE- Advanced glycation end product

ALR- Aldose reductase

ANO- Anoctamin

APOE- Apolipoprotein E

BAM-Binary Alignment Map

BMI-Body mass index

CAN- Cardiovascular autonomy neuropathy

CAT-Catalase

CPT- Current perception treshold

CWC22- Spliceosome Associated Protein Homolog

DAG- Diacylglycerol

DCCT- Diabetes Control and Complications Trial

DHAP- Dihydroxyacetone Phospate

DIAD- Detection of Ischemia in Asymptomatic Diabetics

DPYSL2- Dihydropyrimidinase-like 2

DSPN- Distal symmetric polyneuropathia

DUSP1-Dual Specificity Phosphatase 1

ECG- Electrocardiogram

EDIC- Epidemiology of Diabetes Interventions and Complications

EGF - Epidermal Growth Factor

eNOS- Endothelial Nitric Oxide Synthase

ESCRT-I -Endosomal Sorting Complex Required for Transport-I

GAPDH- Glyceraldehyde-3-Phosphate

GDNF- Glia Cell line-derived Neurotrophic Factor

GFAT-Glutamine Fructose-6-Phosphate-Amidotransferase

GFRA2- Glia Cell Line-Derived Neurotrophic Factor Family Receptor Alpha-2

Glo1/ Glo2- Glioxalase1/ Glioxalase2

Gln-Glutamine

GLP-1- Glucagon-Like peptide

Glu-Glutamic acid

GPX1- Glutathione peroxidase

GRN- Granulin gene

GST- Glutathione S-transferase

HCN- Cyclic Nucleotide-gated Channel

HSP27- Heat shock protein-27

IL-4- Interleukin 4

Kv -Voltage-gated potassium

LEA- Lower-extremity amputation

MAPK- Mitogen-activated protein kinase

MGO- Methylglyoxal

MIR-MicroRNA

MTHFR- Methylenetetrahydrofolate Reductase

MVB12B-Multivesicular Body subunit 12B

MYBPHL - Myosin Binding Protein H like

NADH-Nicotinamide Adenine Dinucleotide

NADPH- Nicotinamide Adenine Dinucleotide Phosphate

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

NGS- Next-generation sequencing

NINJ2-Ninjurin 2

NP-Neuropathy

NTN4-Netrin 4

NTSS-6- Neuropathy Total Symptom Score-6 (NTSS-6)

OR-odds ratio

PDPN- Painful diabetic polyneuropathy

PI3/pAkt- Phosphatidylinositol 3-kinase/phosphorylated protein kinase B

PKC- Protein Kinase C

PPAR-γ- Peroxisome proliferator-activated receptor gamma

PTEN- Phosphatase and tensin homolog

RAGE-Receptor of Advanced Glycation End product

RET- Rearranged during transfection

RMI2- recQ-mediated genome instability protein 2

RXRA -Retinoic Acid X Receptor Alpha

SCG- Single-Copy Gene

SNP- Single Nucleotide Polymorphism

SNV- Single Nucleotide Variant

TCF7L2 -Transcription Factor 7-like 2

THTR- Thiamine Transporters

TGF-B- Transforming Growth Factor- Beta

TK- Transketolase

TRP- Transient Receptor Potential

UDP-GlcNAc- Uridine 5'-diphospho-N-acetyl-D-glucoseamine

USA-United States

UTR-Untranslated region

VEGF- Vascular Endothelial Growth Factor

VGSCs- Voltage-Gated Sodium ion Channels

VNTR- Variable Number of Tandem Repeat

WES-Whole exome sequencing

WESDR- Wisconsin Epidemiologic Study of Diabetic Retinopathy

1. Introduction

1.1. Neuropathy as an interdisciplinary disease

Today, the number of people with diabetes worldwide is estimated at 425 million, and it is estimated that without appropriate intervention, by 2050, approximately 3.23 billion people (one third of the projected population) will have diabetes, half of whom will suffer from neuropathy and its negative impact on quality of life (1). In the vast majority of cases, neuropathy is not an independent disease, rather it is a manifestation or a related syndrome of other disease. Today, nearly one third of neuropathies are thought to be diabetic, one third alcoholic, and hundreds of rare diseases account for a third (2). The wide range of aetiologies also means that neuropathy is an interdisciplinary subject in the broadest possible sense; some components of this complex clinical syndrome are primarily of borderline neurology and internal medicine, but the surgical, vascular, rheumatological, orthopaedic, paediatric, urological and rehabilitation aspects of the problem are also of great practical importance.

1.2. Pathomechanism of neuropathy

The exact mechanism of the development of diabetic neuropathy is still not fully understood, which has led researchers to explore the biomarkers of this complication. It has long been known that in a group of people with diabetes develop microvascular complications relatively early, while others do not, or if they do, it is much later. This difference is thought to be due to genetic factors.

There are two main mechanisms involved in the development of neuropathy, functional and/or structural damage to the vasa nervorum, and the direct effect of hyperglycemia on neurons. The pathogenetic significance of the metabolic pathway is still under investigation. (3, 4)

The uptake of glucose by nerve tissue occurs along a concentration gradient, and it is not dependent on insulin. As a consequense, hyperglycemia leads to an increase in the amount of glucose entering nerve cells. In the cells hexokinase is a first step of the glycolysis and such is a rate-limiting factor in intracellular glucose metabolism. However, at the limit of maximum capacity, the alternative metabolic pathways, sorbitol and hexosamine

pathways, as well as mechanisms leading to protein kinase C (PKC) activation and enhanced end-glycation product (AGE) formation are amplified (Figure 1.) (5-7).

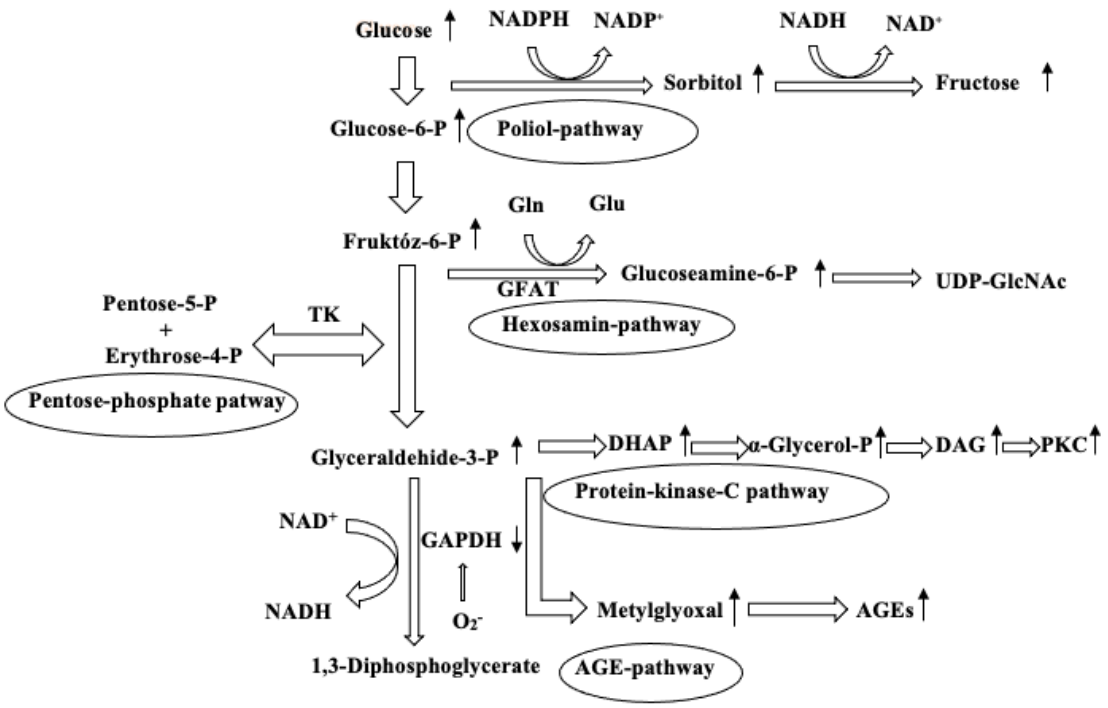

Figure 1. Alternative metabolic pathways activated by hyperglycaemia (5)

The polyol pathway is the longest studied, and as a consequence the most clarified alternative pathway (5, 7). It is related to conversion of glucose through sorbitol as an intermediate to fructose-1-phosphate, which is converted to fructose-1,6-diphosphate. The starter step of this pathway is the conversion of glucose to sorbitol by the enzyme aldose reductase. This enzyme has a lower affinity for glucose than hexokinase. As a result, aldose reductase is activated when the binding capacity of the latter enzyme is fully saturated. The accumulation of sorbitol and fructose injures the nerves in different ways: efflux of diffusible solutes as a result of hyperosmolarity, depletion of NADPH, which causes reduced antioxidant protection, and increased AGE production (8).

The hexosamine pathway involves the conversion of fructose-6-phosphate by the enzyme glutamine-fructose-6-phosphate amidotransferase to glucoseamine-6-phosphate, as well as the formation of UDP-N-acetyl-glucosamine. The end product of this pathway

increases the production of TGF-B and leads to alterations in the formation of signal proteins. As a result, the altered signal transduction of the insulin receptor mitigates the effects of insulin, and increases tissue resistance to insulin (3).

A key enzyme in glycolysis is glycerol aldehyde-3-phosphate dehydrogenase. The activity of this enzyme is known to decrease under conditions of hyperglycaemia and oxidative stress. This results in the amplification of two metabolic pathways: the diacylglycerol protein kinase C activation pathway and the methylglyoxal-induced AGE production. The increase of PKC activity and AGE production cause increased activity of NF-κB, as well as the production of a variety of signalling proteins, vasoactive factors and cytokines (interleukin-1 and -6, TNF-alpha) (9).

The pentose phosphate pathway is another option to reduce intracellular glucose load. Transketolase, which requires vitamin B1 as a cofactor, is the key enzyme in this pathway. Increasing enzyme activity stimulates the conversion of fructose-6-phosphate to pentose-5-phosphate, which may result in a reduction of the adverse effects of the alternative metabolic pathways. Transketolase activity can be stimulated with thiamine or benfotiamine, thereby exerting a protective effect against the four pathogenetic mechanisms of microvascular complications, the glucose-driven hexosamine pathway, the polyol pathway, protein kinase C and the glycation end-product (6, 7).

In recent years, there has been a significant advancement in the understanding of the pathogenesis of neuropathy. Previous epidemiological studies have identified a number of risk factors such as age, female sex, physical work, lower education, and disadvantaged/poor living conditions (10, 11). Other risk factors include smoking, hypertension, obesity, hypercholesterolaemia and duration of diabetes (12, 13). In parallel with the identification of risk factors, research is also being carried out to elucidate the biological mechanisms underlying the risk factors, including the genetic background.

1.3. The prognosis of somatic neuropathy associated with diabetes mellitus

The clinical presentation of somatic neuropathy can be extremely variable. The most common one is the distal type of sensorimotor neuropathy, in which the sensory component is predominant.

In terms of clinical presentation, sensory neuropathy of the distal type is known as a positive sensory syndrome, which includes numbness, tingling, and ants-like sensations.

These predominantly occur at rest, at night, and in a stocking-glove distribution. Stimuli-induced symptoms are characteristic, including the very common phenomenon of allodynia, where a painful response to a painless stimulus is obtained. In clinical practice, patients refer to this as an inability to tolerate contact with the blanket. The negative symptom complex, which is associated with a loss of pain, heat, tactile sensation and sensation of position. Often the patient does not mention any complaints and the damage goes unnoticed. However, they provide a space for the development of lesions, trophic ulcers and infections as consequence (14-16). Distal symmetric polyneuropathia (DSPN) is a leading factor of diabetic foot ulcers and amputations, both associated with increased mortality through infection and chronic inflammation. (17)

A meta-analysis by Vági et al. (18) of 31 cohort studies involving a total of 150,000 diabetic patients revealed a significant increase in all-cause mortality among patients with DSPN compared to those without DSPN.

Lapin et. al found that the risk of mortality was significantly elevated in diabetic patients with painful diabetic peripheral neuropathy (PDPN) compared to those without pain or to diabetic patients without DSPN (19).

One of the most important complications of diabetes mellitus is the diabetic foot syndrome, which has a risk of onset up to 25%, and 2% of thesewill require amputation (20). Diabetes mellitus is the leading cause of lower-extremity amputation (LEA) in Europe and USA, and LEA is associated with significant morbidity and mortality (21). Diabetes mellitus and peripheral vascular disease are associated with increased risk for LEA. Nowadays those who have diabetes have a 10 times higher risk of amputation compared to those without diabetes (22). During a five-year follow-up period, 28- 49% of amputated diabetic patients underwent amputation of the same limb, with subsequent five-year mortality rates ranging from 39-68% (23). Boulton and colleagues observed that reamputation of the ipsilateral limb was necessary in approximately one-third of patients within three years, and in almost 50% of patients after five years (24).

An intact sense of proprioception or joint positioning is crucial for proper walking, particularly on uneven ground (25). DSPN is also associated with balance impairment that could lead to falls and injuries (26).

Type 2 diabetes is recognised as one of the risk factors for fractures. Type 2 diabetic patients have 69% increased fracture risk compared to those without diabetes (27). The

mechanisms underlying the increased fracture risk is not fully understood. It is multifactorial, among which diabetic neuropathy (particularly DSPN) is considered to be one of the most important factors (28). There are lots of mechanism behind that, first of all hyperglycaemia , which may influence bone strength by inducing the accumulation AGEs in bone and increasing the production of nonenzymatic cross-links within collagen fibers (29). Secondly DSPN is related to increased fall risk by impairing the ability of balancing (30). Third, the nervous system has been shown to regulate bone metabolism directly (31) (32).

Diabetic foot-related hospitalisations represent a significant burden on the health system, so early detection and treatment of sensory neuropathy is of paramount importance. It has been demonstrated that proper metabolic control, foot care and self-examination of the foot can significantly improve the quality of life of patients, significantly reduce treatment costs by avoiding amputations, and have a positive impact on prognosis (33).

1.4. The prognosis of cardiovascular autonomic neuropathy in diabetes

Within the symptom complex of autonomic neuropathy, the damage to the cardiovascular system is of particular importance. The prevalence of cardiovascular autonomic neuropathy (CAN) varies from nearly 2% in patients with newly diagnosed or well-controlled diabetes, up to 60% of patients with long-standing type 2 diabetes mellitus (34, 35). Their clinical presentation can be extremely varied, such as resting tachycardia, silent ischaemia, ventricular arrhythmias, QT segment prolongation, orthostatic hypotension, reduced physical performance, intra- and perioperative lability and prothrombotic condition (36, 37).

Autonomic neuropathy also has a significant negative impact on patients' quality of life and increases morbidity and mortality(38) (39) (40) (41, 42). Despite the factors responsible for poor prognosis being brought into the spotlight over the past forty years, their nature still remains poorly understood. In a five-year follow-up study, Ziegler et al. demonstrated that the presence of CAN carries a five-fold risk of mortality to these patients (43).

Resting tachycardia is one of the earliest and most common symptoms of autonomic neuropathy. Large epidemiological studies have shown that it is an independent risk factor for increased cardiovascular and all-cause mortality (44) (45).

The poor prognosis is further reinforced by the higher incidence of asymptomatic ischaemia (46-48) and painless myocardial infarction (49). However the mechanisms of painless myocardial ischemia are complex and not fully understood. Altered pain thresholds, subthreshold by ischemia not sufficient to induce pain and dysfunction of the afferent cardiac autonomic nerve fibers have all been proposed as potential mechanisms (50, 51). Vinik and colleagues, in a meta-analysis of 12 studies, found that diabetic patients with autonomic neuropathy had nearly twice the incidence of silent ischemia compared with individuals without autonomic neuropathy (52). A 4.5-year follow-up study by Valensi et al. (47) found that 50% of diabetic patients with concomitant autonomic nervous system damage and silent ischaemia had a major cardiovascular event. In the Detection of Ischemia in Asymptomatic Diabetics (DIAD) study of 1123 patients with type 2 diabetes, cardiac autonomic dysfunction was a strong predictor of ischemia (48).

Furthermore, damage to the autonomic nervous system increases the risk of ventricular arrhythmias. Prolongation of the QT interval (34) has been attributed to the development of these arrhythmias, as the QT interval is influenced by autonomic nervous tone. Long QT interval can lead to electrical instability and the development of ventricular arrhythmias.

Ziegler et al. in a prospective cohort study found that prolonged corrected QT interval is an independent predictor of mortality in the non-diabetic and diabetic population as well (53).

It is important to know that blood pressure is not a constant parameter; rather, it varies from measurement to measurement, both in healthy individuals and in hypertensive patients. The mean nocturnal blood pressure is typically 15-17 mmHg lower than the daytime mean value (54). It is evident that alterations in the diurnal rhythm of blood pressure and heart rate, as well as the disruption of the sympathovagal balance, the absence of a nocturnal increase in vagal tone and the consequent relative sympathetic predominance, may be identified as pathogenic factors in diabetic patients (54). As a consequence, acute ischaemic events in patients with autonomic neuropathy are most likely to occur in the evening and at night (55). The phenomenon of non-dipping, which is caused by autonomic neuropathy and characterised by a lack of nocturnal blood pressure decline, is also associated with an increased risk of mortality (56).

Investigations into the relationship between autonomic neuropathy and circadian blood pressure changes have demonstrated that alterations in the diurnal rhythm observed in hypertensive diabetic patients are also a consequence of autonomic neuropathy.

In diabetic patients without cardiac diesease, CAN may be a cause of abnormalities in left ventricular systolic and diastolic function (34).

Left ventricular diastolic dysfunction may progress to diastolic heart failure, which is also related to high morbidity and mortality rates (57, 58).

It is known that a number of adverse consequences result from the former, with one of the most important being left ventricular hypertrophy, which is a direct predictor of mortality. However, left ventricular hypertrophy further increases cardiovascular risk by increasing arrhythmia. Parasympathetic neuropathy and consequent left ventricular hypertrophy have also been shown to result in prolongation of the QT interval in non-dipper hypertensives, which is associated with an increased risk of ventricular arrhythmias and consequently sudden cardiac death (59).

Orthostatic hypotension has been identified as a poor prognostic factor for autonomic neuropathy. The main cause of its development is sympathetic autonomic neuropathy, which results, among other things, in a lack of peripheral vasoconstriction, resulting in a lack of compensatory rise in blood pressure on standing (54). Orthostatic hypotension is defined as a fall in blood pressure >30 mmHg systolic or >10 mmHg diastolic blood pressure. Symptoms include dizziness, weakness, visual disturbances, and even syncope, which is essentially related to hypoperfusion of the brain (54). Persistent orthostatic hypotension has been linked to an increase in mortality (60). Furthermore, the inability to elevate systolic blood pressure following a period of standing has been demonstrated to be a predictor of falls in elderly individuals (61).

1.5. Genetic variants potentially playing a role in the development of diabetic neuropathy

Microvascular complications of diabetes, such as neuropathy, appear in some patients after a short duration of disease, but not in some patients after a long duration of diabetes, raising the possibility of genetic susceptibility.

Some studies inquire into genetic basis of diabetic neuropathy, but these studies investigated mostly single nucleotide polymoprhisms (SNPs). In Table 1. (62) we summarize the most relevant studies, and genes that may influence the development of diabetic neuropathy.

Table 1. Major genes that might influences the development of diabetic neuropathy (62)

Gene and Variant Type	Role	Publication
Angiotensin-converting enzyme (ACE) homozygous DD genotype of the I/D polymorphism	Determines ACE activity and serum levels of ACE	(63)
MTHFR gene C677T polymorphism	Elevates homocysteine levels	(64)
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	(65)
GLO1 gene CC genotype	Glo-11 reduces the formation of advanced glycemic end-products (AGEs)	(66)
APOE gene Ɛ4 allele	Plays a role in the cholesterol and triglyceride metabolism	(67)
TCF7L2 gene rs7903146, rs7901695, rs12255372	Affects the lipid metabolism and glucose homeostasis	(68)
VEGF gene C and T alleles	Determines the level of VEGF, which facilitates the proliferation of vascular endothelial cells	(69)
IL-4 gene VNTR	IL-4 is a cytokine that impacts immune cell chemotaxis and anti-inflammation	(70)
GPX1 rs1050450, C > T	Reduced antioxidant activity	(71)
eNOS gene rs2070744 (786 T/C) rs1799983 (894 G/T)	Leads to endothelial dysfunction through the change in the synthesis of nitric oxide	(72)
ADRA2B gene I/D polymorphism	Associated with autonomic dysfunction and increased sympathetic nervous system activity	(73)
MIR146A, MIR128A MIR499A rs2910164 (G> C) rs11888095 (C > T) rs3746444 (GG genotype)	Associated with the level of mitochondrial DNA	(74)
SLC19A2, SLC19A3 encoding THTR1 and THTR2	Intracellular transport of thiamine	(75)

Table 1. Major genes that might influences the development of diabetic neuropathy (62)

Gene and Variant Type	Role	Publication
Transketolase gene rs7648309 rs63355988	Loss of protective action in the prevention of diabetic neuropathy	(76)
Glo1 gene rs1130534 rs1049346	Loss of defense against AGE formation	(77)
Voltage-dependent Na channel beta-2 subunit of Nav1.7 aspartic acid–aspartic acid mutation (D109N)	Hyperexcitability of posterior ganglion neurons	(78)
ANO3 gene missense heterozygous variants	Increased pain sensitivity	(79)
HCN1 gene mis-sense heterozygous variant	Increased pain sensitivity	(79)
TRPA1 loss-of-function mutation	Increased pain sensitivity	(79)
TRPV1 and TRPV4 genes	Painless diabetic neuropathy	(79)
SCN9A, SCN10A, and SCN11A gain-of- function mutations	Neuron hyperexcitability	(80)
Polymorphisms in the GFRA2 gene rs4872521,rs4872522 rs10098807,rs11774105 rs17428041,rs17615364 rs11776842,rs12545534 rs11780601	Role in the differentiation and survival of neurons	(81)
ALR2 gene 106C/T polymorphism in the promoter region	Role in nerve conduction velocities	(82)
ALR2 gene 50-(CA)n microsatellite polymorphism (Z + 2, Z − 2)	Susceptibility or defense against diabetic neuropathy	(83)
GPx-1 (rs1050450) 599C/T	Susceptibility to diabetic neuropathy	(84)
CAT 262C/T	Susceptibility to diabetic neuropathy	(84)
Chromosomal loci 1p35.1 and 8p21.3.	Neuropathic pain	(85)
Gene polymorphisms of ACE, MTHFR, APOE, ALR2, GPx-1, NOS3, CAT, and VEGF	Susceptibility to diabetic neuropathy	(86)
GLP-1, PTEN, insulin, RAGE, HSP27, CW22, and DUSP1 in the PI3/pAkt signaling pathway	Possible therapeutic targets	(87)

Angiotensin-converting enzyme (ACE)

One of the enzymes in the renin-angiotensin-aldosterone system is the angiotensin-converting enzyme, which transforms angitensin I to angiotensin II, evoking a powerful vasoconstrictor effect. In diabetic neruopathy ACE inhibitors have been used to repair microvascular damage, as some studies have revealed a protective effect on neuronal dysfunction (88). The inversion/deletion (I/D) polymorphism of the ACE gene (which means deletion (D allele) or insertion (I allele) of the 287-bp Alu repeat in inton 16) creates three types: II, ID and DD, which has an affect on ACE activity and serum levels of ACE.

Numerus research found significant correlation between the homozygous DD genotype of the I/D polymorphism and increased risk of developing diabetic polyneuropathy (63, 89, 90).

Methylenetetrahydrofolate-Reductase (MTHFR)

Methionine-homocysteine conversion mediated by MTHFR. MTHFR gene variants can cause a decrease in enzyme activity. The most common reason for elevated homocysteine levels is the C677T gene polymorphism in the MTHFR gene (91). Hyperhomocysteinemia has an adverse consequence on vascular endothelium and smooth muscle cells, and cause changes in arterial structure and function. Beetween the C677T polymorphism in the MTHFR gene and risk of developing neuropathy have a clear association in the previous meta-analysis data (64).

Glutathione S-transferase (GST)

From the endogenous oxidative stress and the exogenous potential toxins were protected by glutathione S-transferase. Oxidative stress can contribute to some pathological conditions, including neurodegenerative diseases and diabetic neuropathy. The most widely studied genes and polymorphisms are GST-mu (GSTM1) and GST-theta (GSTT1), and the most common variant is a homozygous deletion (null genotype), which is affiliated to reduced enzyme activity and cytogenetic damage (92). A previous study including type 1 diabetic patients found that the combination of GSTM1 and GSTT1 genotypes significantly increases the risk of developing CAN (65), however, this has not been verified in type 2 diabetes (93).

Methylglyoxal

Methylglyoxal alters the nociceptor-specific Na channel (Nav1.8) and amplifies the excitability of sensory neurons, which leads to hyperalgesia (94). In cross-sectional studies (94-96), type 2 diabetic patients with painful DPN or DPN shows differing correlation in methylglyoxal plasma levels. In the ADDITION-Denmark cohort, the incidence of DPN was independently anticipated by higher methylglyoxal plasma levels with a hazard ratio of 1.46 (95% CI 1.12–1.89) (97)

Transient receptor potential cation channel subfamily A, member 1 (TRPA1) receptor has been associated with inflammation and neuropathic pain in sensory neurons, and the methylglyoxal can stimulate TRPA1 receptor (98, 99).

Glyoxalase

The glyoxalase enzyme system has a crucial role in the pathogenesis of diabetic complications. The glyoxalase pathway consists of glioxalase 1 (Glo1), glioxalase 2 (Glo2) and glutathione, and helps to carry out the detoxification of methylglyoxal, and defends against AGE production. Genetic variants in the glyoxalase 1 can cause modification in the structure of the glyoxalase binding site. In particular, genetic variants in the Glo1 gene may cause changes in the structure of the glyoxalase binding site (77). Groener et al. (66) investigated the possible association of a single nucleotide polymorphism of glyoxalase 1 gene (Glo1 A332C, rs4746 or rs2736654) with the prevalence of microvascular diabetic complications in 209 patients with type 1 and 524 patients with type 2 diabetes. They found that the C332C genotype of the glyoxalase 1 gene has a possible association with diabetic neuropathy in type 2 diabetes, which confirms the important part of methylglyoxal in the development of neuropathy. In a study by Peculis et al (77) including 125 healthy controls, 101 type 1 diabetes, and 100 type 2 diabetes patients, three common single nucleotide polymorphisms (SNPs) in GLO1 were investigated: rs2736654 (A111E), rs1130534 (G124G) and rs1049346 (5'-UTR). It was found that rs rs1130534 and rs1049346 SNPs were associated with reduced GLO1 enzyme activity.

Apolipoprotein E (APOE)

Apolipoprotein E (APOE) has three isoforms and it is a protein involved in cholesterol and triglyceride metabolism. The presence of the Ɛ4 allele of the APOE gene carries a higher risk of developing diabetic neuropathy (67). There is a strong correlation between CAN and the rs7903146 polymorphism of the Transcription Factor 7-like 2 (TCFL7L2) gene.

Vascular endothelial growth factor (VEGF)

Vascular endothelial profileration is assisted by human vascular endothelial growth factor (VEGF)**.** In recent years, VEGF has been linked to the development of diabetic neuropathy (69, 100). The 936C/T mutation in/of the VEGF gene carries an increased risk of developing neuropathy, but the 936T allele may be protective marker against diabetic neuropathy (101).

Interleukin-4

T helper 2 cells, eosinophil, and mast cells produce interleukin-4 which is an important cytokin, as it has an important effect on the formation of endothelial cell adhesion molecules, chemotaxis of immune cells, and antiinflammation. IL-4 gene variable number of tandem repeat (VNTR) polymorphism may be an effective genetic marker in the occurrence of diabetic neuropathy (70).

Glutathione peroxidase (GPx1)

As we all know, oxidative stress has a harmful effect on the vascular system. Glutathione peroxidase (GPx) is an endogenous antioxidant enzyme and assists to detoxifying lipid-peroxides. The rs1050450 C/T polymorphism in the glutathione gene causes an amino acid change from proline to leucine in codon 198 that reduces the enzyme activity. The T allele in the rs1050450 carries a higher genetic risk of developing neuropathy (71).

Endothelial nitric oxide synthase (eNOS)

Endothelial nitric oxide synthase (eNOS) has been shown responsible for the synthesis of nitric oxide. Gene polymorphisms in the eNOS gene can cause decreased eNOS expression and have been implied to be associated with the development of diabetic

neuropathy (102). Shah et al. studied the rs2070744 (786 T/C) and the rs1799983 (894 G/T) gene polymorphisms and it turned out that these SNPs may play an important role in the development of diabetic neuropathy (72).

Adrenoreceptor Alpha 2B (ADRA2B)

Adrenoreceptor Alpha 2B (ADRA2B) gene's non synonymous mutation encodes a receptor protein that puts I/D consecutive glutamates in 301-303 positions. This mutation plays a role in metabolic and vascular effects, like reduced insulin secretion, obesity and the development of neuropathy (73, 103-107). I/D polymorphism is associated with autonomic dysfunction and higher sympathetic nervous system activity, which confirms the role of this polymorphism in the pathogenesis of diabetic neuropathy. The D allele (of the ADRA2B gene I/D polymorphism) has been reveled in higher prevalence in patients with neuropathy, which supports that the presence of the D allele has an impact on the severity of this condition.

MicroRNA (MIR)

When searching for the role of polymorphism in microRNA regions in the development of diabetic neuropathy, it was found that the rs2910164 (G> C) in MIR146A and rs11888095 (C>T) in MIR128A have been associated with the development of this complication (74). The rs2910164 variant in MIR146A decrased, while the presence of the rs11888095 in MIR128A increased the risk of diabetic neuropathy. A study by Spallone et al. (108) found a link between the rs3746444 SNP in the MIR499A gene (GG genotype) and diabetic neuropathy.

Thiamine Transporters (THTR1/THTR2)

It is possible that the thiamine pathway is involved in the development of diabetic neuropathy (7). Thiamin transporters (THTRs), THTR1 and THTR2 are responsible for intracellular thiamine transports. Genetic test shows that mutations of the gene solute carriers (SLCs) SLC19A2 and SLC19A3, encoding THTR1 and THTR2, may play a role in the development of neurological conditions. Malfunctioning of the THTR1 may cause defective mitochondrial function, thus decrease protection against oxidative stress and cell cycle arrest (75). Porta et al. (109) examined two thiamine transporters (SLC19A2/3)

and their transcription factors (SP1/2) testing 134 single nucleotide polymorphisms (SNPs) for a correlation with severe retinopathy or nephropathy or their combination in the FinnDiane cohort.These findings were validated in the DCCT/EDIC and Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) cohorts. In the *SCL19A3* locus, two SNPs (specifically rs12694743 and rs6713116) were found to be significantly associated with a protective effect against severe retinopathy and the combined presence of severe retinopathy and end-stage renal disease.

Transketolase (TK)

In a prospective study involving 314 patients with type 2 diabetes and diabetic nephropathy (including 42 with ESRD) Tanhauserova et al. (110) searched the impact of 19 SNPs in six genes connected to enzymes metabolizing glycolytic intermediates. The researchers found that the rs3736156 SNPs in the transketolase gene on its own and in combination with the two aforementioned SNPs had a noticeable affect on the incidence of major cardiovascular events.

Ziegler et al. (76) were the first to find link between diabetic neuropathy and some TK SNPs. The study involving 165 type 1 and 373 newly diagnosed type 2 diabetic patients, investigated 13 SNPs in TK gene, and found associations between these SNPs and peripheral nerve function, but most of the significance was lost in Bonferroni correlation. Only two correlations were exceptions to this and remained significant: the one between rs7648309 SNP and the symptom score, and the the one between rs63355988 SNP and the warmth perception. These results suggest that TK may have a protective function in the prevention of diabetic neuropathy.

Ion Channels

A case study of a male patient with painful diabetic neuropathy reported an aspartic acid-aspartic acid mutation (D109N) in the voltage-gated Na channel beta-2 subunit of Nav1.7, resulting in hyperexcitability of dorsal root ganglion neurons (78). Neuropathic pain leads to poorer quality of life in people with diabetes (111). Previous studies have identified several risk factors for neuropathic pain, such as female gender, smoking, age, weight and longer duration of diabetes (112-114). Previously, next-generation sequencing (NGS) identified genetic variants of voltage-gated sodium ion channels (VGSCs) that may be

associated with neuropathic pain (113, 115). These genes play an important role in the generation and propagation of action potentials in nociceptors and on the nerve fibre (78, 115-119).

Therefore, Sleczkowska et al. (79) investigated the role of ion channels in painful diabetic neuropathy. They screened voltage-gated potassium (Kv), transient receptor potential (TRP), anoctamin (ANO), and hyperpolarisation-activated and cyclic nucleotide-gated (HCN) ion channel genes which are expressed in peripheral nerves. The researchers used single-molecule inversion probe analysis and NGS. They found that missense heterozygous variants in the ANO3 and HCN1 genes and TRPA1 loss of function were associated with increased pain sensitivity. They also proved that loss-of-function variants in the TRPV1 and TRPV4 genes may be present in painless diabetic neuropathy.

Another study explored the impact of potential pathogenic single-copy gene (SCG) genetic variants in painful and painless diabetic neuropathy and in painful and painless idiopathic neuropathy (80). In this study 1125 patients (237 painful and 309 painless diabetic neuropathy, 547 painful small fibre neuropathy and 32 painless single fibre neuropathy) were proflied by using single molecule inversion probe and NGS. They identified missense heterozygous variants in the ANO3 and HCN1 genes and TRPA1 loss-of-function as being associated with increased pain sensitivity. They also proved that variations in the TRPV1 and TRPV4 genes, which lead to a loss of function, may be associated with painless diabetic neuropathy.

Glia Cell Line-Derived Neurotrophic Factor Family Receptor Alpha-2 (GFRA2)

A single region (chromosome 8 p21.3) was found to be associated with neuropathy in a multicentre study by Meng et al. (81) looking at about a million SNPs across the genome. The definition of neuropathy in this study was that the patient was either taking medication for neuropathy and/or had an abnormal monofilament test. Nine intergenic SNPs showed significant correlations in the genomic locus close to the glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-2 (GFRA2) and the neurturin receptor gene. The GFRA2 protein is a glycosylphosphatidylinositol-coupled cell surface receptor belonging to the GDNF receptor family. GDNF is a factor that has been shown to play an important role in the differentiation and survival of neurons. The GFRA2 receptor binds to these proteins, which activates the RET tyrosine kinase receptor

pathway (120). It is therefore possible that genetic polymorphisms in the GFRA2 gene may contribute to susceptibility to diabetic neuropathy.

Aldose reductase (ALR)

Methylglyoxal (MGO), AGEs and the oxidative stress caused by the hyperglycaemic state can have an effect on the expression of the aldose reductase gene. The study by Sivenius et al. (82) reported that a 106C/T polymorphism in the promoter region of the ALR2 gene is associated with decreased nerve conduction velocities in type 2 diabetes mellitus, whereas the 106C/C genotype was associated with reduced sensory nerve amplitudes.
Another study by Heesom et al. (83) found the 50-(CA)n microsatellite polymorphism, which has more than 10 alleles at 5' in the upstream regulatory region of ALR2, which is also associated with diabetic neuropathy. The Z+2 allele appeared to prevent diabetic neuropathy, while the Z-2 allele was associated with an increased susceptibility to complications in both type 1 and type 2 diabetes mellitus.

Others

The most important gene polymorphisms of diabetic neuropathy (ACE, MTHFR, APOE, ALR2, GPx-1, NOS3, CAT and VEGF genes) were presented in a review article by Jankovic et. al.,(86). This review also assessed epigenomic mechanisms such as DNA methylation. Hyperglycaemia evokes a change in DNA methylation status in white blood cells, which can be a candidate biomarker for PDN. In addition, the protein NINJ2 (ninjurin 2) assists Schwann cells to regenerate after injury. Reduced expression of NINJ2 was observed after increased methylation, which may be involved in the development of neuropathy. Some pathways - nervous system development and/or axonal guidance (netrin-4 (NTN4) and dihydropyrimidinase-like 2 (DPYSL2) genes), glycerophospholipid metabolism (phospholipase and phosphatidylserine decarboxylase), and mitogen-activated protein kinase (MAPK) signalling – showed variation in the progression of PDN by DNA methylation profiles.
Epigenomic mechanisms also include microRNAs and long noncoding RNAs. The following microRNAs have been implicated in the development of diabetic neuropathy: miR9, miR199a3p, miR25, miR146 and miR190a5p. In addition, long non-coding RNAs have been correlated with neuropathy (more than 200 nucleotides in length) via the

MAPK pathway (CCNT2-AS1, RP1-249H1.2 CTD-3239E11.2, RP11-51B23.3, STAM-AS1 and LINC00629). Finally, post-translational histone modifications may be involved in neuropathic pain and peripheral nerve injury-induced neuropathic hypersensitivity.

Miyashita et al. (87) have discussed the possibility of modifying genes such as GLP-1, PTEN, insulin, RAGE, HSP27, CW22 and DUSP1 in the phosphatidylinositol 3-kinase/phosphorylated protein kinase B (PI3/pAkt) signalling pathway by the use of oligonucleotides as a potential treatment for diabetic neuropathy.

However, present data are far from sufficient and are without the extensive, at least whole exome level (WES) approach, which is now essential.

2. Objectives

This study was aimed to perform whole exome sequencing in type 2 diabetic patients with and without neuropathy (24-24 patients respectively).

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

Individuals diagnosed with type 2 diabetes in primary care during a screening program were referred to our department and recruited for the present study. No healthy volunteers or type 1 diabetic patients were involved in order to ensure genetic homogenity as it is usual in genetic studies. There were 48 participants (30 men, 18 women) included who had type 2 diabetes, 24 with neuropathy and 24 without. All the patients were in sinus rhythm, and the resting ECG was normal in all cases. Based on the medical history, detailed physical examination including neurological status, resting ECG and chest X-ray, lung disease, hypertension, ischaemic heart disease, heart failure, vitium and other abnormalities affecting the results of autonomic tests were excluded in all cases.

Inclusion criteria for entry into the study required the presence of type 2 diabetes for more than 5 years, well balanced carbohydrate metabolism and patients had to be between 18–69 years of age at baseline assessment. Exclusion criteria for the present study were type 1 diabetes, unbalanced carbohydrate metabolism, pregnancy, severe diseases (e.g. cancer), psychiatric disorders, immunosuppressive therapy, limited cooperation ability, and neuropathy from causes other than diabetes.

During 48 hours prior to the neurological assessment or cardiotens test, patients were not taking any medication that might have affected autonomic or sensory function (for example digitalis, beta blockers, atropine, non-dihydropyridines calcium channel blockers, and sedatives) (121). All study participants were asked to refrain from vigorous physical exertion, smoking, caffeine, and alcohol consumption for 12 hours prior to the study.

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

3.2. Neurological assessment

3.2.1. Diagnostic testing for cardiovascular autonomic neuropathy

In type 2 diabetic patients, cardiovascular autonomic function is assessed by the five standard cardiovascular reflex tests and the 24-hour ambulatory blood pressure profile.

Cardiovascular reflex tests

Cardiovascular autonomic neuropathy was evaluated by five cardiovascular reflex tests: response of heart rate to deep breathing (beat-to-beat variation), standing (30/15 ratio) and Valsalva manoeuvre (Valsalva ratio), which assess parasympathetic function, as well as blood pressure responses to standing and sustained handgrip estimating sympathetic function. The blood pressure response to sustained handgrip is no longer considered (Toronto Consensus Panel) as an acceptable clinical test but only as an investigational one (122). The Cardiosys H-01 12-lead portable ECG was utilized for all reflex-tests. Cardiovascular autonomic neuropathy was diagnosed if there was at least one abnormal or two borderline cardiovascular reflex tests present. The abnormal value is scored 2, the borderline value 1 and the normal value 0, and the severity of cardiovascular neuropathy can be calculated (123, 124).

Changes of heart rate during deep inspiration (respiratory arrhythmia)

Of the five standard cardiovascular reflex tests, the change in heart rate with deep breathing is the most sensitive and one of the fastest to perform. Under normal physiological conditions, even in healthy people, the heart rate changes constantly, increasing on inhalation and decreasing on exhalation, known as respiratory arrhythmia. It will be maximal at a breathing rate of 6/min. During the test, the patient is asked to breathe in and out at a rate of 6/min while the ECG is continuously monitored. The duration of the test is half a minute. The difference between the maximum heart rate during inspiration and the minimum heart rate during expiration is assessed. If less than 10 per minute, this may indicate the presence of autonomic neuropathy.

Lying-to-standing changes of heart rate (the 30 to 15 ratio)

The 30/15 ratio test involves the patient lying still for 3 minutes, standing up and then standing still for 1 minute with their hands hanging down while a continuous ECG is recorded. Under physiological conditions, the heart rate temporarily increases after standing up and then decreases. Maximum tachycardia may occur on the 15th beat after standing up and maximum bradycardia on the 30th beat. Accordingly, the ratio of the maximum to minimum R-R interval (30/15 ratio) is an appropriate measure to assess cardiovascular autonomic nerve damage.

The changes of heart rate during the Valsalva manoeuvre (Valsalva ratio/quotient)

Normally, when the Valsalva manoeuvre is performed, the blood pressure falls and the heart rate rises, and then suddenly the blood pressure rises and the heart rate falls. The manoeuvre was performed under standardised conditions (40 mmHg airway pressure should be maintained for 15 seconds). The Valsalva quotient is the ratio of the longest and shortest R-R intervals measured during the test.

Lying-to-standing changes of systolic blood pressure

Under physiological conditions, blood pressure rises on standing, which requires an intact autonomic nervous system. During the test, the patient lies supine for 10 minutes and the blood pressure is measured once. The patient then stands up and the blood pressure is measured every 1, 3 and 5 minutes in the standing position.

The changes of diastolic pressure during sustained handgrip

The sustained grip test (prolonged contraction of hand muscles) is usually associated with an increase in heart rate and blood pressure.

The latest international recommendations suggest only four tests instead of the previous five. The orthostatic hypotension test is used to assess sympathetic innervation, and the handgrip test is omitted from the latest recommendations (122). Our working group has demonstrated in recent years that the omission of the handgrip test for the assessment of autonomic neuropathy seems justified. Among the results of our research group, it is worth highlighting that the sensitivity of the handgrip test in detecting autonomic neuropathy was only 24.6%. More importantly, the sensitivity of the handgrip test in

detecting sympathetic neuropathy confirmed by the orthostatic hypotension test was only 20%, and no correlation was found between the handgrip test and other cardiovascular reflex tests. There was a negative correlation between the handgrip test and the presence of hypertension. This means that in diabetic patients with hypertension, the handgrip test rarely shows abnormal values. Therefore, because of its low sensitivity and proven dependence on hypertension, the handgrip test is not recommended for the assessment of autonomic function (125).

The normal, borderline and abnormal values of the five cardiovascular reflex tests are shown in Table 2.

Table 2. Normal values for cardiovascular reflex testing (124)

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

3.2.2. Diagnostic testing for sensory neuropathy

Sensory nerve function was examined by the calibrated tuning fork, monofilament, Neurometer R device (Baltimore, USA) and Medoc Device and Neuropathy Total Symptoms Score-6 (NTSS-6).

The calibrated tuning fork

The calibrated tuning fork provides information about the sensation of vibration (depth perception), i.e. the function of the thick fibres. The most widely used in clinical practice is the 128 Hz Rydel-Seiffer calibrated tuning fork (25), and its use is recommended by the EASD Neuropathy Study Group (NEURODIAB) for the assessment of vibration sensation in outpatients (126).

The test device is very easy to use and the test itself takes approximately 1 minute. To use it, the device is vibrated, then placed on a bony base on both the upper and lower limbs, and the patient is asked to tell us when they no longer feel the vibration. The usual test sites are the tip of the old finger, the medial malleolus and the extensor surface of the second metacarpal. To evaluate the tuning fork, a wedge-shaped scale from 1 to 8 is placed on the top of the instrument. When the instrument is vibrated, the scale splits in two and then gradually merges as the vibration gradually decreases, so that a number can be read when the patient indicates that they no longer feel the vibration.

Meijer et al. (127), in a comparative study of a relatively large number of patients using the full range of neuropathy diagnostic tools, concluded that the calibrated tuning fork is the most appropriate tool for screening and initial testing.

Monofilament

The use of Semmes-Weinstein monofilament 5.07/10 grams is recommended for testing protective sensation. Like the calibrated tuning fork, the test is extremely simple and quick to perform. The evaluation of the test result is also very simple, as the patient only has to say whether or not he/she felt the pressure caused by the bent monofilament. To perform the test, a 10 g instrument is placed on the test site at a 90 degree angle for 1.5 seconds (128).

Neurometer

The Current perception threshold (CPT) was estimated at the median and peroneal nerves with the neurometer at three frequencies (2000 Hz, 250 Hz, 5 Hz), assessing the function of the large and small myelinated and small unmyelinated sensory nerve fibres, respectively (123, 129, 130). For the three frequency measurements of the peroneal and the median nerves, the normal values were established by Evans et al. (131).
The normal ranges of the current sensation thresholds measured with the neurometer on the n. medianus and n. peroneus at different frequencies are shown in Table 3.

Table 3. The internationally accepted normal values with Neurometer devices (100= 1mAmp) (131).

Current perception threshold (frequency)	Nervus medianus (normal range in mm/sec)	Nervus peroneus (normal range in mm/sec)
2000 Hz	120-398	179-523
250 Hz	22-189	44-208
5 Hz	16-101	18-170

Medoc Device

The Q-Sense is the Medoc TSAII-VSA portable, thin-fibre, function focused version of the Q-Sensor that can be used to determine hot thresholds, hot pain thresholds and cold thresholds. As the warm sensation modality is essentially mediated by thin unmyelinated C-type fibres and cold sensation by thin myelinated A-delta fibres, the device is well suited to characterising the functional status of thin fibres. It has the advantage of testing physiological sensation and providing quantitative results, but the patient's cooperation is essential for the test. The test is performed by placing a ceramic plate (Thermode) on the patient's hand or the back of the leg at a basal temperature of 32°C. This is the temperature of the skin at room temperature. The temperature of the ceramic plate can then be varied linearly using a computer and the patient is asked to press a button to indicate a change (limits algorithm).
Measurements are taken four times in each anatomical region and on each side. The cold and warm thresholds (°C) for the right and left lower and upper limbs are determined as the average of at least three successful measurements, and the results obtained for the

right and left limbs are averaged. The normal value of the cold and warm thresholds is influenced by the sex and age of the subject (132, 133). The thermal sensation thresholds were tested by taking the thresholds automatically provided by the Q-Sense software based on the patient's computer recorded data (date of birth [age], sex and anatomical region under investigation) and determining whether the thermal sensation threshold was within the range corresponding to abnormal or intact thin fibre function. A patient with abnormal cold or heat sensation in at least one limb was considered to have thin-fibre neuropathy.

Neuropathy Total Symptom Score (NTSS-6)

The severity of neuropathy symptoms was assessed using the Neuropathy Total Symptom Score (NTSS6). The NTSS-6 questionnaire measures the frequency and intensity of individual neuropathy sensory symptoms commonly reported by patients with DPN (aching pain and/or tightness; sharp, shooting, lancinating pain; and allodynia and/or hyperalgesia, numbness and/or insensitivity, prickling and/or tingling, burning) NTSS-6 scoring ranges from 0-21.96. A score >0 indicates the presence of >1 sensory symptom. Severe sensory neuropathy is defined as a total score of more than 6 points (134). NTSS-6 score system is shown in Table 4.

Table 4. NTSS-6 score(134)

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

3.3. Genetic analysis

DNA Isolation

Genomic DNA was isolated from peripheral blood. This was performed using the Roche HighPure DNA Isolation Kit (Roche, Rotkreutz, Switzerland) according to the manufacturer's instructions. The amount of DNA isolated was measured using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Walsham, MA, USA).

Whole exom sequencing (WES)

Ion Torrent AmpliSeq RDY Exome Kit (Thermo Fisher Scientific, Walzham, MA, USA) was used for the exome library preparation. WES target regions were multiplied by using the AmpliSeq RDY Exome Kit. After that, 100 ng of genomic DNA was quantified using Qubit DNA HS or BR assay (Life Technologies). Samples were bound to specific Ion Xpress Barcode Adapters. Libraries set up this way were amplified and purified by AMPure XP reagent. These libraries were then quantified on a Qubit™ 2.0 fluorometer. The libraries were diluted to ~100 pM final concentration. At a time, three barcoded exome libraries were processed together, and they were combined and placed on an Ion 540 chip. The sequencing was carried out on a Thermo Ion GeneStudio S5 platform (Thermo Fisher Scientific).

After sequencing, the AmpliSeq Exome files were aligned with human reference genome hg38. Ion Torrent software –v5.12- was utilized for coverage analysis. The Binary Alignment Map (BAM) files were uploaded to the cloud-based Ion Reporter software -v5.12-. Ion Reporter™ equipped with AmpliSeq Exome workflow was used for raw data evaluation. The annotation of variants was made by applying several pipelines, such as variant type single nucleotide variant (SNV) filter, synonymous variant effect, missense, functional SIFT scores and/or PolyPhen and/or Grantham, homopolymer length ≤ 6, homozygosity, allele read count ≥ 100, allele ratio = 1.0, and minor allele frequency ≤ 0.5.

3.4. Bioinformatic and Statistical Methods

The variants were annotated using the ANNOVAR software -v03dec2019- (which integrates database information from dbSNP, ClinVar, gnomAD and OMIM. The Integrative Genomics Viewer (IGV) was utilised to visualise the mapped reads. Duplicate reads were flagged using Picard. SNPs were called using GATK Variant Call Format (VCF) files, which were merged using BCFtools and then annotated using SnpSift (135)

The reference database used for the variant annotation was the dbSNP hg38 build 151, downloaded from the NCBI dbSNP database.

PLINK v1.9(136) was used for quality control of the raw VCF files. The phenotypic sex of the individuals was compared with the predicted sex of the samples based on the SNP data. SNPs were filtered based on missingness rate, minor allele frequency, and Hardy-Weinberg exact test p-values with thresholds of 0.05, 0.01, and 1 × 10-10, respectively. Based on the recommendations of and the PLINK 1.9 documentation (137), the quality thresholds were selected as follows.

Association testing was done in R v4.0.3 (138) and logistic regression models were fitted using GENESIS R Bioconductor package (139). In order to eliminate potential confounding effects and because of the possible association detected in our quality control analysis, we controlled the model estimates for age, sex and relatedness. Correction for the latter was made with genetic relationship matrix (GRM) generated using the package SNPRelate (140). A Quantile–Quantile plot was created using the qqman R package (141) and a Manhattan plot was generated with ggplot2 (142). The top 4 SNPs are presented based on logistic regression *p*-values.

All statistical calculations were performed in R v4.0.3 Continuous variables are shown as means and standard deviations. Statistical significance was assessed using Mann-Whitney tests. Categorical variables are presented as frequencies, with differences evaluated using Fisher's exact tests. A test result was defined as significant if the p-value was less than 0.05.

4. **Results**

Characteristics of the two groups studied are shown in Table 5. Type 2 diabetics with neuropathy were significantly older compared to those without neuropaty, but there were no significant differences between the groups in terms of duration of diabetes, BMI, HbA1c and lipid metabolism.

Table 5. Demographic and clinical characteristics of the two study groups. Data are presented as mean±SD or median [IQR]. Between group differences are reported according to $\chi 2$ test.

	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
Trigliceride (mmol/l)	1.85	0.88	2.53	1.73	0.3065

The Manhattan plot showing the logistic regression results of the SNP association analysis can be seen in Figure 2.

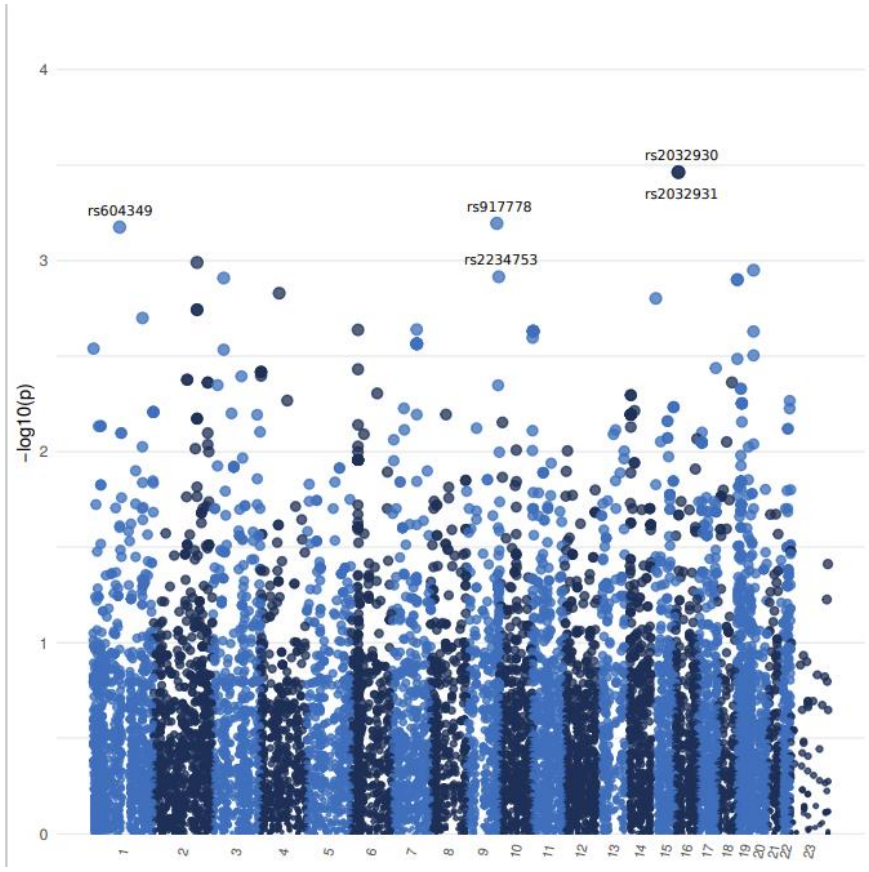

Figure 2. Manhattan plot showing the logistic regression results of the association analysis. SNP positions with the corresponding chromosomes are shown at the x axis. P-value as calculated by the logistic regression analysis are presented at the y axis after -log10 transformation. Those SNPs that were considered in this study are denoted by their rsIDs.

The results of whole exome sequencing are shown in Table 6. (143) and Table 7.

Table 6. The results of the whole exome sequencing. The table shows the location and position of the given genetic variant on the chromosomes and minor allele frequencies, as well as the chance of developing neuropathy which is determined by the OR. *: European population allele frequencies based on ALFA Allele Frequency Aggregator project (144).

variant ID	**alleles minor/ major**	**position**	**gene**	**Minor allele frequency (MAF) of European population***	**Minor allele frequency (MAF) of diabetic patients without NP**	**Minor allele frequency (MAF) of diabetic patients with NP**	**Logistic regression estimate (β)**	**Logistic regression estimate (β) Standad Error**	**OR for minor allele**	**p value without NP vs. with NP**
rs2032930	T/G	chr16:11350573 (GRCh38.p14)	RMI2	0.192	0.021	0.250	3.101	0.866	22.2	0.0003
rs2032931	C/T	chr16:11350612 (GRCh38.p14)	RMI2	0.191	0.021	0.250	3.101	0.866	22.2	0.0003
rs604349	A/G	chr1:109296770 (GRCh38.p14)	MYBPHL	0.095	0.000	0.146	3.885	1.142	48.6	0.0007
rs917778	A/G	chr9:126380963 (GRCh38.p14)	MVB12B	0.206	0.313	0.104	-2.618	0.767	0.07	0.0006
rs2234753	G/A	chr9:134401915 (GRCh38.p14)	RXRA	0.246	0.292	0.167	-2.500	0.770	0.08	0.0012

Table 7. The correlations of genetic variants with neuropathic symptoms and signs as well as neurophysiological parameters in the neuropathy group.Neuropathic symptoms and signs are represented by NTSS6 (see methods sections) (134) Neurophysiological parameters originate from the following measurements: Neurometer R device (Current perception threshold), Thermal sensory analyser, Cardiosys (five cardiovascular reflex tests) (see methods sections) (123, 124, 129, 131, 145).

	SNP	Minor allele present	Minor allele not present	p-value
		mean±SD	mean±SD	
Current perception threshold n. medianus (mm/sec)				
2000 Hz	**rs604349**	448.4±61.5	364.2±91.7	0.0491
	rs2234753	333.2±47.2	376.5±92.8	0.0200
250 Hz	**rs2032930**	175.7±49.8	124.7±47.8	0.0035
	rs2032931	175.7±49.8	124.7±47.8	0.0035
	rs604349	180±14.2	132.8±53.4	0.0180
5 Hz	**rs2032930**	98.1±42.3	74.6±32.5	0.0428
	rs604349	122.4±28.4	78.7±28.5	0.0054
Current perception threshold n. peroneus (mm/sec)				
5 Hz	**rs2032930**	238.4±270.6	169.8±182.2	0.0376
	rs2032931	238.4±270.6	169.8±182.2	0.0376
Beat-to-beat variation (bpm)	**rs2032930**	8.5±5.8	12.2±6.6	0.0226
	rs2032931	8.5±5.8	12.2±6.6	0.0226
	rs2234753	13.4±6.8	10.2±6.3	0.0400
NTSS6 (score)	**rs2032930**	1.3±0.5	1.7±0.5	0.0230
	rs2032931	1.3±0.5	1.7±0.5	0.0230
	rs917778	1.7±0.5	1.4±0.5	NS
	rs2234753	1.8±0.4	1.4±0.5	0.0030
Cold detection threshold (degrees Celsius)	**rs604349**	39.3±4.2	35.8±2.6	0.0419
Heat detection threshold (degrees Celsius)	**rs917778**	30.2±1.2	28.7±3.1	0.0137

4.1. Genetic variants with an increased risk of neuropathy

We were able to identify three genetic variants (rs2032930 and rs2032931 of the RMI2 gene and rs604349 of the MYBPHL gene) that were associated with a 22-49 fold increase in the risk of developing diabetic neuropathy.

The associations of genetic variants with neuropathic symptoms, signs and parameters which showed statistical significance are shown in Table 7.

The rs2032930 showed a significant correlation with current perception thresholds measured at 5 Hz and 250 Hz of the n. medianus (p=0.042 and p=0.003, respectively), at 5 Hz of the n. peroneus (p=0.037), as well as the deep breathing test (p=0.022) and the NTSS (p=0.023). The rs2032931 was associated with the current perception threshold measured at 250 Hz of the n. medianus (p=0.003) and at 5 Hz (p=0.037) of the n. peroneus, the deep breathing test (p=0.022) and the NTTS (p=0.023). There were also correlations between rs604349 genotypes and values measured at 2000 Hz (p=0.049), 250 Hz (p=0.018) and 5 Hz (p=0.005) of n. medianus and Q-Sense warm perception threshold (p=0.042).

Table 7. also shows rs2032930 / rs2032931 is associated with increased risk of developing both DPN and CAN. It also shows that the rs604349 variant is only correlated with sensory neuropathy.

4.2. Genetic variant with reduced risk of neuropathy

Two other variants (rs917778 of MVB12B and rs2234753 of RXRA genes) proved to be protective against neuropathy, reducing the risk to 0.07-0.08. The rs2234753 was significantly associated with current perception threshold measured at 2000 Hz of n. medianus (p=0.020), deep breath test (p=0.04) and NTTS (p=0.003). We also found a correlation between rs91778 and cold perception threshold (p=0.013).

The rs917778 appears to reduce the risk of sensory neuropathy. The rs2234753 variant reduces both DPN and CAN.

5. Discussion

In our study we have successfully identified 5 genetic variants (the rs2032930 and rs2032931 of the recQ-mediated genome instability protein 2 (RMI2 gene), the rs604349 of the myosin binding protein H-like (MYBPHL) gene, the rs917778 of the multivesicular body subunit 12B (MVB12B) and the rs2234753 of the retinoic acid X receptor alpha (RXRA) genes) that may have an impact on the risk of developing neuropathy in type 2 diabetic patients.

5.1. Genetic variants that increase the risk of developing neuropathy

We were able to identify three genetic variants (rs2032930 and rs2032931 of the RMI2 gene and rs604349 of the MYBPHL gene) that were associated with a 22-49 fold increase in the risk of developing diabetic neuropathy.

The rs604349 is an intronic SNP in the MYBPHL gene that appears to increase the risk of neuropathy. This gene has been linked to the circulation of progranulin. Progranulin, a precursor protein, has multiple biological functions and is expressed by many cell types throughout the body, particularly in the skin, gastrointestinal tract and reproductive system (146).

It has different effects in its full length form and after proteolytic cleavage. While in its full form it has an anti-inflammatory role, after cleavage it increases inflammation (147). It is involved in angiogenesis, tumourigenesis, wound repair, cell proliferation, inflammation, and neurodegenerative and metabolic diseases (148). Progranulin has been proposed as a marker of chronic inflammation in obesity and type 2 diabetes through macrophage infiltration of the adipose tissue. Macrophages produce TNF-alpha and IL-6, which induce inflammation (147). Progranulin has been known to alter plasma lipoprotein metabolism, leading to an increased risk of atherosclerosis and chronic inflammatory status (149-151). Progranulin levels in patients with type 2 diabetes who have visceral obesity were shown to be having a 1.4-fold increase in serum progranulin levels (147).

Progranulin levels were positively associated with BMI, fat mass, fasting glucose and insulin levels, and insulin resistance (152). The granulin gene (GRN) is located on chromosome 17q, and GRN gene genetic variants can cause reduced serum progranulin levels (153). However, significant variations in circulating progranulin levels can also be

detected in wild-type GRN carriers. An earlier genome-wide study of 533 subjects revealed a significant association between variations in the CELSR2/PSRC1/MYBPHL/SORT1 loci on chromosome 1p and serum progranulin levels (154). CELSR2/PSRC1/MYBPHL/SORT1 loci with polymorphisms are associated with lower progranulin levels, with rs660240 being the most influential, studied by Tönjes et al (155).

A genetic variant found in our study may affect progranulin levels, leading to elevated blood glucose levels through insulin resistance and a chronic inflammatory state. Hyperglycaemia can lead to the activation of alternative metabolic pathways in neurons as well, and chronic inflammation can lead to neuronal damage.

The rs2032930/rs2032931 are intronic SNPs found in the RMI2 gene and are associated with an increased risk of developing neuropathy. In the literature, they have been attributed to genome stability, tumorgenesis and tumor progression (156). We found no evidence in the literature of an existing relationship between the RMI2 gene and the development of neuropathy.

5.2. Genetic variants that reduce the risk of developing neuropathy

Two other variants (rs917778 of MVB12B and rs2234753 of RXRA genes) proved to be protective against neuropathy, reducing the risk to 0.07-0.08.

The rs917778 is also an intronic SNP in the MVB12B gene. This gene encodes a protein that is part of a heterotetramer (TSG101 (Vps23), Vps28, Vps37 and MVB12A/B) called the endosomal sorting complex required for transport-I (ESCRT-I). The “task” of ESCRT is to help the generation of multivesicular organelles by clustering ubiquitinated proteins The common function of the MVB12A/B proteins is to initiate the downregulation of epidermal growth factor (EGF) receptors (157). EGF is a mitogenic factor that enhances cell differentiation, and it also helps the differentiation, maturation and survival of neurons (158). In animal study by Perez et al. (159) they discovered a protective role for EGF in acrylamide induced neuropathy.

The genetic variant of MVB12B found in our study may alter the lifetime of EGF and thus alter its protective role against the development of neuropathy.

Another genetic variant associated with a reduced risk of diabetic neuropathy is the rs2234753. This is also an intronic SNP in the RXRA gene. RXRA is a member of the

nuclear receptor superfamily and acts as a transcription factor by binding homo- and heterodimers in the promoter of target genes. It is involved in lipid metabolism, cell differentiation and death (160-162).

In obese mouse models, RXR agonists reduce food intake and weight gain, thereby maintaining the balance between blood glucose and insulin sensitivity (163). In an animal study with ducks, it was found that the RXRA gene facilitates the accumulation of fat and the differentiation of pre-adipocytes via the RXRA-C/EBPA signalling pathway. The SNP of RXRA showed an association with average daily feed intake, residual feed intake and feed conversion ratio, suggesting that RXRA is a potent metabolic regulator (164).

A number of metabolic processes involving glucose and lipid metabolism and cell differentiation are regulated by PPARs. The actions of PPARs involve RXR, the vitamin D3 receptor and steroid hormone receptors (165). RXR antagonists have been identified as promising treatment options for T2DM because PPAR-γ with RXRs regulate glucose metabolism (166, 167).

RXRA also activates the PI3K/AKT pathway, which is known to be implicated in the development of obesity and T2DM (168). Insulin is one of the major ligands of the PI3K/AKT pathway.

The PI3K/AKT pathway (reduces gluconeogenesis in liver and muscle, increases body fat accumulation, increases glucose utilisation, increases insulin secretion and reduces appetite) is essential for normal metabolism. Under conditions such as excessive energy intake, it increases the circulation of free fatty acids, which damage beta-cell function and cause insulin resistance. PI3K/AKT signalling pathways are considered promising treatment targets for type 2 diabetes (168).

The rs2234753 SNP of the RXRA gene may have a beneficial effect on glucose metabolism by reducing insulin resistance, thereby limiting the development of microvascular complications such as neuropathy.

6. Conclusion

1. We have successfully identified 5 genetic variants (rs2032930 and rs2032931 of the RMI2 gene, rs604349 of the MYBPHL gene, rs917778 of the MVB12B and rs2234753 of the RXRA genes) that may have an impact on the risk of developing neuropathy in type 2 diabetic patients.
2. We were able to identify three genetic variants (rs2032930 and rs2032931 of the RMI2 gene and rs604349 of the MYBPHL gene) that were associated with a 22-49 fold increase in the risk of developing diabetic neuropathy.
3. We were able to identify two variants (rs917778 of MVB12B and rs2234753 of RXRA genes) that were protective against neuropathy, reducing the risk to 0.07-0.08.
4. The rs2032930/rs2032931 variant is associated with a higher risk of developing both DPN and CAN. It also shows that the rs604349 variant is only correlated with sensory neuropathy.
5. The rs917778 appears to reduce the risk of sensory neuropathy. The rs2234753 variant reduces the risk of both DPN and CAN.
6. After validation on a larger number of cases, we might be able to establish new strategies for early preventive intervention and identify targets for new drug developments in the future.

7. **Summary**

Diabetic polyneuropathy is a complication with a significant impact on morbidity and mortality in diabetic patients. Its occurrence shows significant individual variations, which may not be associated with adequate metabolic control, suggesting a pathogenic role of genetic background. Although the information available on biomarkers of diabetic polyneuropathy is limited, several studies support genetic susceptibility. In our study, 24 long-term type 2 diabetic patients with neuropathy and 24 long-term type 2 diabetic patients without neuropathy underwent detailed neurological assessment and whole exome sequencing. We could successfully identify genetic variants that might alter the risk of developing diabetic neuropathy. The rs604349 is an intronic SNP in MYBPHL (myosin binding protein H like) gene that seems to aggravate the risk for neuropathy. This gene has been linked to circulating progranulin. The rs2032930/rs2032931 are intronic SNPs found in RMI2 (recQ-mediated genome instability protein 2) gene, and appeared to increase the risk of developing neuropathy. In our study, rs917778 and rs2234753 were accompanied with reduced risk for diabetic neuropathy. The rs917778 is also an intronic SNP in MVB12B (multivesicular body subunit 12B) gene. Another genetic variant with reduced risk for diabetic neuropathy is rs2234753. It is also an intronic SNP in the RXRA (retinoic acid X receptor alpha) gene. In summary all 5 SNPs that have been demonstrated to interfere with the risk of diabetic neuropathy in our study can be found in an intronic region of the genes, i.e. they do not get transcribed. Nevertheless, these variants might be parts of the regulating systems at higher levels that indirectly influence pathophysiological processes that may affect the development of neuropathy. In the future, the identification of specific genetic markers associated with diabetic polyneuropathy holds great promise for improving diagnostic accuracy, risk prediction, and the development of targeted therapeutic interventions. By unravelling the genetic background of diabetic polyneuropathy, researchers aim to not only improve our understanding of this complication but also prepare the way for personalized medical approaches that may lead to more effective treatments and management strategies for individuals affected by diabetic neuropathy. In conclusion, investigating the genetic sensitivity to diabetic polyneuropathy is important for advancing our knowledge of this complicated complication, eventually aiming to improve patient outcomes and quality of life through precision treatment approaches.

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10. **Acknowledgements**

I would like to express my gratitude to Professor István Takács, the director of the clinic, for making my scientific work possible.

I am indebted to my supervisor, Zsuzsanna Putz, for her meticulous guidance and comprehensive professional support. I am particularly grateful for the assistance she provided, which extended well beyond the scope of her supervisory duties.

I am indebted to Professor Péter Kempler for allowing me to commence my scientific career in the research group and laboratory he led as an undergraduate student. Furthermore, I am grateful for his original ideas for research, which have shaped my scientific approach throughout my career.

I am grateful to, Professor Péter Lakatos, for his unwavering support of my scientific endeavours.

I would like to express my gratitude to Márton Papp for his invaluable assistance in the statistical processing of the data.

I would like to express my gratitude to my colleagues and all the staff of the Molecular Biology Laboratory. Erika Gulyásné Gáspár was of great assistance in the neuropathic studies, and her work was much appreciated. I would like to express my gratitude to her.

I am indebted to my family for their unwavering support and encouragement throughout my academic career.