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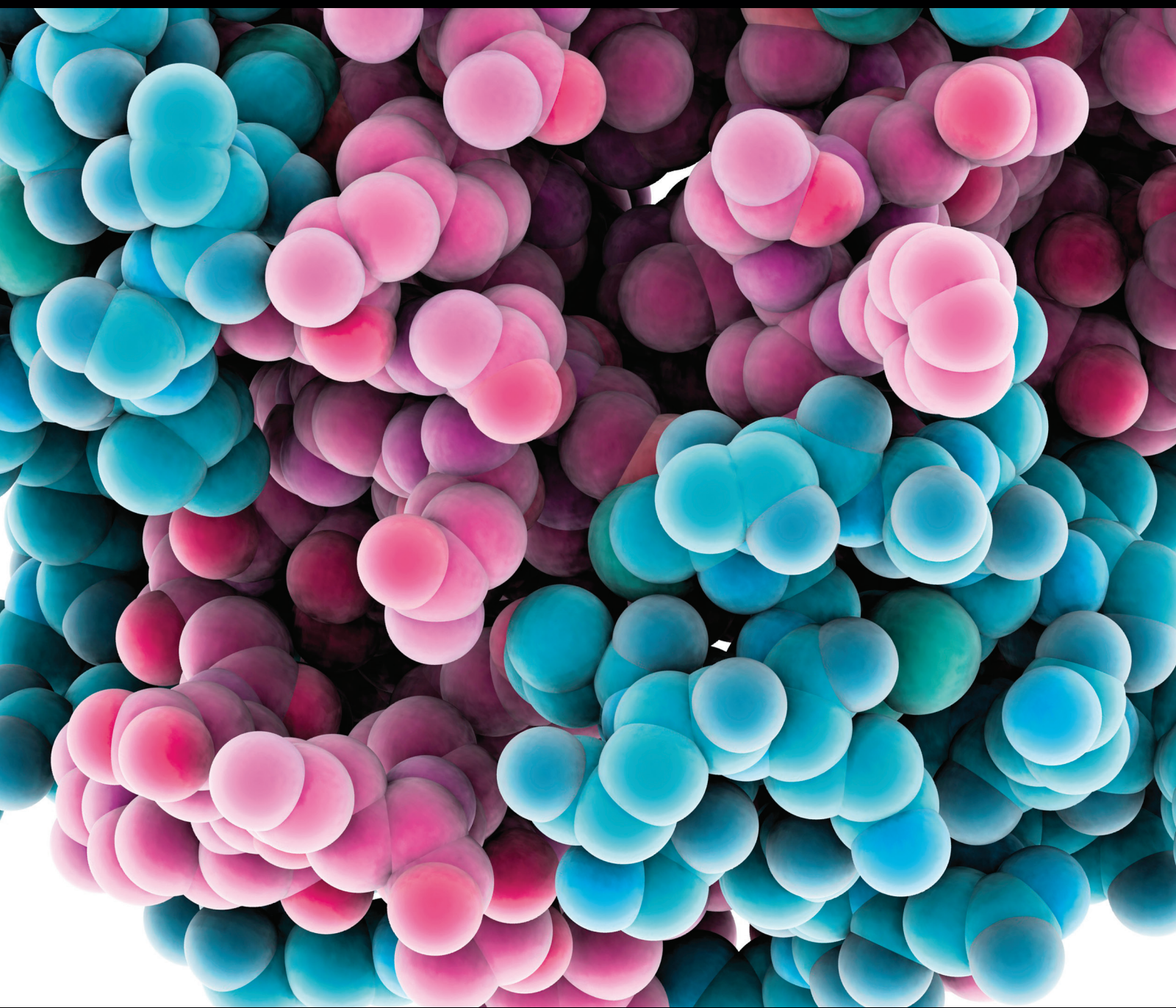
Diabetic Neuropathy: New Insights to Early Diagnosis and Treatments

Special Issue Editor in Chief: Mark Yorek
Guest Editors: Rayaz Malik, Nigel Calcutt, Aaron Vinik, and Soroku Yagihashi



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Editorial Board

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Editorial

Diabetic Neuropathy: New Insights to Early Diagnosis and Treatments

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Diabetic peripheral neuropathy (DPN) is a complex disorder with multiple etiologies that affects about 50% of those with diabetes, and about 30% have painful diabetic neuropathy. Neuropathy can also develop in subjects and animal models with prediabetes/insulin resistance, but without overt hyperglycemia. At this time, there is no known treatment and good glycemic control slows progression of DPN in type 1 diabetes, but not type 2 diabetes [1]. The annual cost for healthcare in patients with DPN in the United States is staggering with estimates exceeding $15.0 billion [2]. Given the increasing incidence of both type 1 and type 2 diabetes and obesity and the impact DPN has on the quality of life of patients and their families and overall healthcare costs, a strategy for the early diagnosis and discovery of an effective treatment is needed.

The early diagnosis and staging of the severity of diabetic neuropathy using patient questionnaires, determining thermal and mechanical sensitivities, and examining nerve conduction velocities require standardization. Whilst recent studies have established good diagnostic utility and reproducibility for sensory nerve evaluation in the skin and cornea, there has been limited application of these techniques in the clinic [3]. For patients with painful diabetic neuropathy, underdiagnosis and adequate control of symptoms remain challenging.

Animal studies have provided considerable insight to the etiology and treatment of diabetic neuropathy. However, there has been a uniform failure to translate these findings to an FDA-approved therapy. Many factors may account for these poor outcomes and need to be addressed at multiple levels. Preclinical studies need to use more predictive/translational endpoints and combination therapies, to identify treatment that at a minimum will slow progression or even repair nerve damage caused by prediabetes/diabetes.

These challenges in DPN lead us to pursue a special issue incorporating the latest clinical and preclinical studies on the diagnosis and treatment of diabetic neuropathy.

F. Picconi et al. assessed whether retinal neurodegeneration has a predictive value in the development of peripheral motor as opposed to sensory unit loss and complimented the work assessing the utility of corneal nerve loss acting as a surrogate marker for DPN [4]. They show that motor unit loss and neuroretinal dysfunction already exist in patients with type 1 diabetes without classical DPN. They concluded that the neuroretina is a potential "window" onto the early neurogenic process in diabetes. These and other studies (see below) suggest that cornea and retinal neurodegeneration may allow early diagnosis of DPN.

X. Jia et al. investigated whether in vivo corneal confocal microscopy could detect improvement of corneal nerve

parameters following improved glycemic control in patients with type 2 diabetes. 32 patients with DPN and 12 age-matched control subjects underwent nerve conduction studies and assessment of corneal nerve morphometry at baseline and after approximately one year. At follow-up, 1/2 of the diabetic subjects had improved HbA1c whilst the other 1/2 continued to have elevated HbA1c. In the subjects with improved glycemic control, corneal nerve fiber density and corneal nerve fiber length increased significantly compared to baseline, whilst those with poor HbA1c showed a significant reduction in sural sensory nerve conduction velocity, corneal nerve fiber density, and corneal nerve fiber length. The authors concluded that corneal nerve fiber repair can be detected when glycemic control improves and that in vivo corneal confocal microscopy could be a sensitive method to assess nerve repair in future longitudinal or interventional studies of DPN.

Sensory nerves in the skin can also be examined as a potential marker for nerve repair in DPN [5]. Saudek et al. examined whether there was a change in the expression of mRNA levels of neurotrophic growth factors and nerve regeneration in the skin 28 months after renal and pancreas transplantation and normalization of glycemia. Transplantation upregulated mRNA levels of multiple neurotrophic growth factors in the skin but was not accompanied by an improvement in skin nerve fiber density or electrophysiological indices of DPN.

R. B. Jansen et al. assessed whether markers of inflammation could be detected in acute Charcot foot compared to the healthy foot. Five subjects with acute Charcot foot were found to have an increased venous-arterial flux of interleukin-6 (IL-6) in the acute diabetic Charcot foot compared to the healthy foot. They also found an increased level of IL-6 and advanced glycation end products in the acute Charcot foot after externally cooling the feet, with no difference in the flux for other markers of inflammation.

B. L. M. Van Eetvelde et al. assessed the influence of clinically diagnosed DPN on respiratory muscle strength in patients with type 2 diabetes. They found that DPN affects respiratory muscle strength in type 2 diabetes patients and concluded that the assessment of respiratory muscle weakness could be of added value in the early screening for DPN in patients with type 2 diabetes.

T. Meldgaard et al. reviewed the diabetes-induced structural remodeling, biochemical dysfunction, immune-mediated alterations, and inflammatory properties of the enteric nervous system and associated structures. They discussed the various patient-reported outcome measures and objective modalities for evaluating dysmotility and outlined the clinical management of diabetic enteropathy. They advocated prompt investigation and intervention, to improve the quality of life for patients with this condition.

M. Bulc et al. explored novel underlying mechanisms of diabetic enteropathy by using double immunofluorescent labelling of substance P, calcitonin gene-related peptide, and leu5-enkephalin on enteric stomach neurons of a porcine model of chemically induced diabetes. The percentage of neurons immunoreactive to substance P and calcitonin gene-related peptide in the myenteric plexus of the antrum, corpus, and pylorus as well as in the submucosal plexus of the corpus was increased. Whilst the number of leu5-enkephalin-like immunoreactivity neurons was increased in the myenteric plexus of the antrum and the submucosal plexus of the corpus, there was a decrease in the myenteric plexus of the corpus and pylorus. The authors concluded that these substances may participate in the development of gastric complications and that future research on the role of these peptides may result in the development of effective pharmacotherapy of gastrointestinal disorders.

In an animal model of type 2 diabetes that combined high-fat feeding and low-dose streptozotocin treatment of Sprague-Dawley rats, E. P. Davidson et al. reported that dietary salsalate in combination with lower amounts of menhaden oil provided greater benefit in diabetes-induced vascular and neural impairment than menhaden oil alone, stressing the importance of combination therapy for the treatment of DPN.

Overall, many of the articles appearing in this special issue stress the importance of early detection for DPN and the need for a successful treatment.

Conflicts of Interest

The author and the guest editors do not have any conflict of interest regarding the publication of this special issue.

Acknowledgments

We express our gratitude to all authors for their excellent contributions and to the reviewers for their dedication. We would also like to express our appreciation to the Editorial Board of the Journal of Diabetes Research for their guidance and commitment to the successful publication of this special issue. The lead guest editor would like to thank the four guest editors for their hard work and cooperation. We hope that this special issue will provide insight to the challenges that remain to find a successful treatment for DPN.

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Rayaz A. Malik
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Research Article

Preserved Expression of Skin Neurotrophic Factors in Advanced Diabetic Neuropathy Does Not Lead to Neural Regeneration despite Pancreas and Kidney Transplantation

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Guest Editor: Rayaz Malik

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes with potential severe consequences. Its pathogenesis involves hyperglycemia-linked mechanisms, which may include changes in the expression of neurotrophic growth factors. We analyzed the expression of 29 factors potentially related to nerve degeneration and regeneration in skin biopsies from 13 type 1 diabetic pancreas and kidney recipients with severe DPN including severe depletion of intraepidermal nerve fibers (IENF) in lower limb skin biopsies (group Tx1 1st examination). The investigation was repeated after a median 28-month period of normoglycemia achieved by pancreas transplantation (group Tx1 2nd examination). The same tests were performed in 13 stable normoglycemic pancreas and kidney recipients 6–12 years posttransplantation (group Tx2), in 12 matched healthy controls (group HC), and in 12 type 1 diabetic subjects without severe DPN (group DM). Compared to DM and HC groups, we found a significantly higher ($p < 0.05$–0.001) expression of NGF (nerve growth factor), NGFR (NGF receptor), NTRK1 (neurotrophic receptor tyrosine kinase 1), GDNF (glial cell-derived neurotrophic factor), GFRA1 (GDNF family receptor alpha 1), and GFAP (glial fibrillary acidic protein) in both transplant groups (Tx1 and Tx2). Enhanced expression of these factors was not normalized following the median 28-month period of normoglycemia (Tx1 2nd examination) and negatively correlated with IENF density and with electrophysiological indices of DPN (vibration perception threshold, electromyography, and autonomic tests). In contrast to our expectation, the expression of most of 29 selected factors related to neural regeneration was comparable in subjects with severe peripheral nerve fiber depletion and healthy controls and the expression of six factors was significantly upregulated. These findings may be important for better understanding the pathophysiology of nerve regeneration and for the development of intervention strategies.

1. Introduction

Diabetic peripheral neuropathy (DPN) is a chronic complication of diabetes mellitus with potential grave clinical consequences such as pain, loss of sensation, foot ulcers, and gangrene, which may result in amputations. During its natural course, DPN progresses from initial functional to late structural changes with nerve fiber loss at its final stage [1].

Several interrelated pathways linked to chronic hyperglycemia are implicated in the pathogenesis of DPN with oxidative stress playing a major role [2]. There are also experimental and clinical data supporting a contributory role of changes in the expression of vascular and neural growth factors, e.g., vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and other neurotrophins in the pathogenesis of DPN [3].

The principal strategy for the prevention of DPN consists of maintaining long-term optimal glycemic control. However, in spite of major advances in the field, this is still not achievable in a substantial proportion of diabetic patients. Interventions targeting the major putative pathogenic pathways of DPN (antioxidants, aldose-reductase inhibitors, inhibitors of glycation, etc.) have been tested in several randomized controlled trials but had been abandoned due to either toxicity or lack of convincing benefit [4, 5]. Moreover, while being effective as preventive measure, good metabolic control has not been shown so far to reverse already established advanced structural nerve damage and fiber loss in DPN.

Patients with long-standing type 1 diabetes mellitus who undergo pancreas/kidney or pancreas transplantation alone represent a population with very advanced diabetic complications including forms of DPN [6]. Long-term normoglycemia can be reestablished after successful pancreas transplantation without further need of insulin injections. Longitudinal follow-up of type 1 diabetic patients undergoing a pancreas (and kidney) transplantation may thus provide novel evidence on the effect of long-term normoglycemia in advanced forms of DPN.

Past trials of preventive and therapeutic interventions in DPN have mostly used clinical symptoms and signs and results of quantitative sensory testing or electrophysiological studies as outcome measures. The introduction of the minimally invasive technique of skin biopsy with determination of intraepidermal nerve fiber (IENF) morphology provides an opportunity for the quantitative assessment of the treatment [7]. In addition, skin biopsy samples can be analyzed for changes in neurotrophic factors in relation to the pathophysiology of neuropathies including diabetic neuropathy [8, 9]. A decreased nerve regenerative capacity has been associated with impaired neurotrophic tone which might reflect to diminished synthesis, secretion, or action of neurotrophic factors in sensory and autonomic nerve fibers [9]. Besides well-established trophic factors, such as the members of the neurotrophin family like NGF or VEGF, novel neurotrophic factors are being discovered which may be deficient in patients with diabetes. Furthermore, the signal transduction pathways of neurotrophic factors may be changed in diabetes and may contribute to either disrupting neuronal survival or nerve regeneration [10].

In our prospective studies which included type 1 diabetic subjects with advanced peripheral neuropathy, we showed that IENF density did not improve even after 2.5 and 8 years of normoglycemia after successful pancreas and kidney transplantation [11, 12]. It is still unknown whether long-term improvement of glycemic control may alter skin neurotrophin levels in advanced neuropathy. We hypothesized that expression of selected neurotrophins and growth factors in the skin might correlate with clinical signs of diabetic peripheral neuropathy and, in contrast to unchanged epidermal nerve fiber density, might be altered following the reestablishment of normoglycemia after pancreas transplantation as a tissue stimulus for neural regeneration. This could provide novel evidence in relation to nerve regeneration following the reestablishment of normal glucose levels.

2. Methods

2.1. Study Design. A comprehensive neurological evaluation comprising clinical assessment, electromyography, autonomic tests, and skin biopsy for IENF quantification and measurement of mRNA of selected neurotrophic factors and receptors was performed in three groups of type 1 diabetic subjects (groups DM, Tx1, and Tx2) and in nondiabetic age- and sex-matched healthy controls (HC). The study was performed at the Institute for Clinical and Experimental Medicine (IKEM) in Prague in 2012–2015 and was approved by the Ethics Committee of IKEM and Thomayer's Teaching Hospital with registration no. G-12-06-43. After the explanation of the aims and study procedures, all participants signed an informed consent to participate in the study.

The DM group included 12 subjects with type 1 diabetes without clinical symptoms of diabetic neuropathy according to the diabetic neuropathy symptom score [13] and a VPT below 15 V. Group Tx1 included 13 subjects who were examined within 2 and 20 weeks (median 8 weeks) following successful pancreas and kidney transplantation (Tx1 1st examination) and reevaluated again within 15 to 30 months (median 28 months; Tx2 2nd examination) of normal function of both grafts. Group Tx2 was comprised of 13 type 1 diabetic subjects who had been normoglycemic for at least eight years after successful pancreas and kidney transplantation. Demographic data for individual groups are shown in Table 1.

2.1.1. Transplantation. Groups Tx1 and Tx2 comprised patients with advanced complications of diabetes including severe diabetic neuropathy and end-stage diabetic nephropathy. Severe diabetic neuropathy was defined by an experienced neurologist by evaluating the neuropathy symptom score, VPT, and EMG. All patients underwent successful pancreas and kidney transplantation and were off exogenous insulin and normoglycemic and had good kidney graft function at the time of study evaluation. The kidney graft was placed into the left iliac fossa. The pancreatic graft was placed intraperitoneally either with systemic or portal venous drainage and anastomosis of the donor duodenum segment to the intestine. Immunosuppressive therapy is comprised of 4-dose induction with anti-T-lymphocyte globulin (Fresenius Biotech GmbH, Gräfelfing, Germany) and prophylactic combination of tacrolimus (through levels 8–15 ng/ml) with either mycophenolate mofetil (1–2 g per day) or sirolimus (trough levels 5–10 ng/ml). Corticosteroids were started at a dose of 20 mg per day and gradually decreased and discontinued 6 weeks after transplantation.

Table 1: Background demographic data and clinical variables.

		Group HC: healthy control subjects	Group DM: type 1 diabetic subjects	Group Tx1: SPK recipients reexamined at 14–30 months	Tx2: SPK recipients at 8 years following transplantation
n		12	12	13	13
Age (years)		43 (28–56)	37 (32–55)	39 (30–57)	44 (33–59)
BMI (kg/m^2)		26.5 (22–37)	26 (22–36)	22.8 (19–28)	24 (20–29)
Diabetes duration (years)		—	18 (4–36)	22 (16–39)	23 (19–50)
Insulin dose (IU/day)		—	48.5 (40–71)	—	—
Pretransplant insulin dose		—	—	32 (20–61)	35 (16–54)
HbA1c	mmol/mol	36 (25–37)	57.5 (47–79)	40 (34–52)/40 (35–54)	37 (32–43)
	%	5.4 (4.4-5.5)	7.4 (6.4–9.4)	5.8 (5.3–6.9)/5.8 (5.3–7.1)	5.5 (5.1–6.1)
Pretransplant HbA1c	mmol/mol	—	—	74 (45–99)	63 (44–84)
	%			8.9 (6.3–11.2)	7.9 (6.2–9.8)
Serum creatinine (μmol/l)		81.3 (59–112)	86.1 (56–101)	103 (62–186)/103.7 (81–154)	100 (75–121)

Data are given as medians and range (in parentheses). If not stated otherwise, the values were determined at the time of investigation.

Subjects in both the Tx1 and the Tx2 groups were enrolled into the study if they fulfilled the following criteria: severe neuropathy before transplantation, no major complication and a rejection-free course after transplantation with normoglycemia, good function of the kidney graft, and informed consent to participate in the study.

2.1.2. Skin Biopsies and IENF Density. Skin biopsies were taken under local anesthesia from the thigh (10–15 cm above the knee) using a 3 mm punch (Stiefel Laboratories, Sligo, Ireland). No sutures were needed due to the biopsy size. Three samples (1 for ENF counts and 2 for mRNA analyses) were taken from each subject.

Samples for IENF density determination were fixed in 4% paraformaldehyde and then processed as previously described [11, 12]. For fluorescence imaging of the panaxonal marker protein gene product 9.5 (DakoCytomation, Glostrup, Denmark), 3 sections per patient were examined. The mean number of IENF per millimeter of epidermis was derived using the software Olympus DP-SOFT (Software Imaging Systems, Münster, Germany).

For RT-qPCR analysis, the skin samples were immediately immersed into RNAlater (Sigma), left for 2 h at 4°C, and then stored at −80°C until analysis.

2.1.3. Electromyography. Nerve conduction studies were carried out using standard techniques on the 3-channel Keypoint Dantec Focus device. Motor nerve conduction velocity (NCV) and a compound muscle action potential of the ulnar and tibial nerve were measured orthodromically. Sensory NCV and amplitude of sensory nerve action potential were measured antidromically in the ulnar and sural nerve. Skin temperature was approximately constant (32°C) during the examinations in both upper and lower extremities.

2.1.4. Vibration Perception Threshold (VPT). The VPT was examined using the biothesiometer probe (Bio-Thesiometer; Bio-Medical Instruments, Newbury, OH) placed over the dorsum of the hallux.

2.1.5. Autonomic Tests. Heart rate responses to deep breathing and the Valsalva maneuver and heart rate and systolic blood pressure responses to standing up, belonging to a battery of standard autonomic function tests (AFTs), were performed with Varia Pulse TF3, a telemetric online computer-aided system (Sima Media Ltd., Olomouc, Czech Republic). Blood pressure was measured with an automated oscillometric device (Omron M4; Matsusaka, Japan). The main outcome parameters were the I-E difference (mean difference of maximal inspiratory and minimal expiratory heart rate), Valsalva ratio (VR; maximal to minimal heart rate during and following the Valsalva maneuver), 30 : 15 ratio (ratio of heart rates around the 15th and 30th heart beats following standing up), and Δ sBP (difference in systolic blood pressure while supine and at 1 min after standing up).

2.1.6. RNA Extraction, Reverse Transcription PCR, and Gene Expression Analysis. After thawing, skin samples were immersed in 1 ml TRIzol reagent (Sigma), dispersed by knife homogenizer (IKA Ultra Turrax, Germany), and extracted into chloroform. RNA isolation from the chloroform extract was performed using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. All PCR reagents and cyclers were purchased from Thermo Fisher Scientific (Foster City, CA). Extracted mRNA (300 ng) was reverse transcribed using SuperScript VILO (Invitrogen). The expression of selected genes was determined using custom-designed TaqMan Gene Expression Array Cards with the following gene-specific TaqMan Assays: CNTF (Hs04194755_s1), CNTFR (Hs00181798_m1), GALR1 (Hs00175668_m1), GALR2 (Hs00605839_m1), GFRA1 (Hs00237133_m1), GFRA2 (Hs00176393_m1), GFRA3 (Hs00181751_m1), NGF (Hs00171458_m1), NGFR (Hs00609977_m1), NGFRAP1 (Hs00918411_s1), NRG1 (Hs00247620_m1), NRG2 (Hs00171706_m1), NRG4 (Hs00945535_m1), NTF3 (Hs00267375_s1), NTF4 (Hs01596132_m1), NTRK1 (Hs01021011_m1), NTRK2 (Hs00178811_m1), TGFA (Hs00608187_m1), NPY (Hs00173470_m1), NPYR1 (Hs00702150_s1), BDNF (Hs03805848_m1), MPZ

(Hs00559329_m1), GFAP (Hs00909233_m1), FGF2 (Hs00266645_m1), FGFR1 (Hs00915142_m1), GDNF (Hs01931883_s1), ERBB2 (Hs01001580_m1), ERBB3 (Hs00176538_m1), and ERBB4 (Hs00955525_m1) (Thermo Fisher Scientific). The suitable endogenous controls, HPRT 1 (Hs99999909_m1) and B2M (Hs99999907_m1), were selected by TaqMan Array Human Endogenous Control Panel (cat. no.: 4366071) as the genes with most stable expression. The analyses were performed on ABI 7900HT Sequence Detection System. Samples were measured as triplicates. All plates were analyzed applying identical conditions. We used the comparative ΔCt method (i.e., relating target gene expression with individual HPRT and B2M expression) for individual assessment. In addition, we compared gene expression in the patient and control groups using the ΔΔCt method when the average normalized expression of each target gene in the skin samples from the healthy control group was used as calibrator. The list of examined neurotrophic factors and their receptors is given in Table 2.

2.2. Statistical Evaluation. All data are expressed as median and range (tables) or median and 1st/3rd quartile plus outliers (graphs). The differences among the groups were first compared using the Kruskal-Wallis analysis of variance, and if p values < 0.05 were found, the differences between individual groups were compared using the Wilcoxon test with Bonferroni correction. Paired data (group Tx2, the 1st and the 2nd examination) were tested by the Wilcoxon signed-rank test. The data obtained by RTq-PCR arrays were analyzed using RT^2 Profiler™ PCR Array Data Analysis software provided by the company together with the PCR array. The relationship between individual parameters was assessed using the Spearman's rank correlations test. Correlations with a p value < 0.05 were considered as significant.

3. Results

3.1. Demography and Metabolism. There were no differences among the 4 groups of subjects in terms of age and BMI ($p > 0.05$, Table 1). Both transplant groups before transplantation and diabetic control subjects (group DM) had elevated values of HbA1c. At the time of examination, however, the HbA1c values in both transplant groups did not differ from healthy subjects, although in the Tx1 group some values at the 1st examination were still abnormal, as the time from transplantation was too short for normalization. High serum creatinine levels in both transplant groups rapidly dropped following the combined pancreas and kidney transplantation and remained stable during the whole follow-up period with the highest registered level at the time of study examination being 186 μmol/l (see Table 1). None of the transplant recipients needed insulin or oral antidiabetic therapy during the study follow-up.

3.2. Vibration Perception Threshold (VPT). Median VPT values in groups HC, DM, Tx1, and Tx2 were 10, 13, 34, and 30, respectively, and were significantly higher in both transplant groups than in the HC and DM groups ($p < 0.001$ and <0.05, respectively). The difference between the diabetic and healthy control groups was not significant. In the Tx1 group, the VPT did not improve during the study follow-up period with the median values remaining almost the same.

3.3. Electromyography. Electrophysiological data are presented in Table 3.

After Bonferroni correction, most nerve conduction velocities and amplitudes were significantly more pathological in the Tx1 and Tx2 groups compared to healthy controls ($p < 0.05$, $p < 0.001$). In the DM group, only ulnar sensory NCV was significantly lower than in healthy controls ($p = 0.034$). Tibial NCV ($p = 0.0625$) showed borderline significance for improvement between the 1st and 2nd examinations in group Tx1.

3.4. Autonomic Tests. The results are summarized in Table 4. Parameters I-E difference and Δ sBP in both transplant groups were significantly more pathological than in both control groups ($p < 0.05$ and $p < 0.01$, respectively). Valsalva ratio was lower in the Tx1 and Tx2 groups than in the HC group ($p < 0.05$ and $p < 0.01$, respectively). Both transplant groups vs. HC group and Tx1 vs. DM groups significantly differed in 30 : 15 ratio ($p < 0.05$).

3.5. Intraepidermal Nerve Fiber Density. In both transplant groups, no or very few IENF were found in skin biopsy samples. Median and range values in the Tx1 and Tx2 groups were 0.4 (0–3) and 0 (0–4.41), respectively, and were significantly lower than in the HC (8.3 (5.9–11.1); $p < 0.01$) or DM (2.1 (0.3–11.5); $p < 0.05$) controls. There was no improvement in group Tx1 after the median 28-month period of normoglycemia ($p = 0.17$). Graphical comparison of the IENF densities is shown in Figure 1.

3.6. Expression of Neurotrophic Factor mRNA in Skin Biopsies. The expression of 29 neurotrophic and related factors in skin samples was compared among the four study groups using the Wilcoxon/Kruskal-Wallis tests. We found significant differences in six: NGF ($p < 0.001$), NGFR ($p < 0.05$), NTRK1 ($p < 0.01$), GFRA1 ($p < 0.01$), GFAP ($p < 0.001$), and GDNF ($p < 0.05$) (see Figure 2). In addition, we found marginal differences ($p < 0.08$) in the expression of NRG2, TGFA, and FGF2. Interestingly, the expression of all these nine factors was higher in both transplant groups (Tx1 and Tx2) than in both HC and DM controls (Supplemental Table 1).

For the six parameters that had a higher expression in groups Tx1 and Tx2 compared to HC and DM, we examined their correlation with the IENF density assessed by fluorescence microscopy (Figure 3). This correlation was statistically significant in all cases with the p value being <0.001 with GFR, <0.01 with NTRK1 and GFAP, and <0.05 with NGFR, GALR1, and TGFA.

We also found a significant correlation between IENF density and clinical indicators of neuropathy severity. As an example, the correlation between IENF density and vibration perception threshold is shown in Figure 4 ($r = -0.63$, $p < 0.001$).

Table 2: List of examined neurotrophic factors and their receptors.

Abbrev.	Name	NCBI reference sequence	Potential role in nerve regeneration	Reference
BDNF	Brain-derived neurotrophic factor	NM_001709	Promotion of nerve cell growth and maturation	[14]
CNTF	Ciliary neurotrophic factor	NG_008776.1	Expressed in myelinating Schwann cells, nerve regeneration following injury	[10]
CNTFR	Ciliary neurotrophic factor receptor	NG_047044.1		[10, 15]
GALR1	Galanin receptor 1	NG_009223.1	Neuroprotection, neuroregeneration	
GALR2	Galanin receptor 2		Neuroprotection, neuroregeneration	
GFRA1	GDNF family receptor alpha 1	NG_050620.1	Expressed in Schwann cells, ensures the response to GDNF	[15, 16] (2002)
GFRA2	GDNF family receptor alpha 2	NG_029215.1	Expressed in Schwann cells, ensures the response to GDNF	[16–18, 19]
GFRA3	GDNF family receptor alpha 3	NG_046894.1		
NGF	Nerve growth factor	NG_007944.1	Essential role in neuronal regeneration	
NGFR	Nerve growth factor receptor	NC_000017.11	Essential role in neuronal regeneration	[19, 20]
NGFRAP	Nerve growth factor receptor associated protein 1	NM_0012826741	Essential role in neuronal regeneration	[19]
NRG1	Neuregulin 1	NG_012005.1	Family of gliotrophic factors that transduce signal through ErbB receptors and are necessary for Schwann cells growth, survival, and differentiation	[21]
NRG2	Neuregulin 2	NC_000005.10	Family of gliotrophic factors that transduce signal through ErbB receptors and are necessary for Schwann cells growth, survival, and differentiation	[10, 21]
NRG4	Neuregulin 4	NC_000015.10		
NTF3	Neurotrophin 3	NG_050629.1	Increase of NT-3 appears to reflect the degree of skin denervation in diabetic neuropathy and may represent a compensatory mechanism	
NTF4	Neurotrophin 4	NG_016289.1	Increase of NT-3 appears to reflect the degree of skin denervation in diabetic neuropathy and may represent a compensatory mechanism	[10, 21, 22]
NTRK1	Neurotrophic receptor tyrosine kinase 1	NG_007493.1	Essential role in neuronal regeneration	
NTRK2	Neurotrophic receptor tyrosine kinase 2	NG_012201.2	Essential role in neuronal regeneration	[21, 22]
TGFA	Transforming growth factor alpha	NG_029975.1	Trophic factor in both central and peripheral neural tissues	[23]
NPY	Neuropeptide Y	NG_016148.1	Increased expression in patients with moderate sensory neuropathy	[24]
NPY1R	Neuropeptide Y receptor Y1	NC_000004.12	Potentially involved in neuronal precursor proliferation	
MPZ	Myelin protein zero	NG_008055.1	Myelin-specific protein, Schwann cells can modulate this gene expression in response to diabetic-induced metabolic derangement	[25]
GFAP	Glial fibrillary acidic protein	NG_008401.1	Glial cell marker	[26]
FGF2	Fibroblast growth factor 2	NG_029067.1	Expressed by the Schwann cells of the peripheral nerves	[27]
FGFR1	Fibroblast growth factor receptor 1	NG_007729.1	Secreted by Schwann cells, essential for neuroregeneration	[16, 27, 28]
GDNF	Glial cell-derived neurotrophic factor	NG_011675.2	Rapidly responses to denervation produced by basal keratinocytes and Schwann cells	
ERBB2	erb-b2 receptor tyrosine kinase 2	NG_007503.1	Neuregulin receptors, response to chronic denervation, expressed in Schwann cells (26 hoke)	[21, 29]
ERBB3	erb-b2 receptor tyrosine kinase 3	NG_011529.1	Neuregulin receptors, response to chronic denervation, expressed in Schwann cells	[21, 29]
ERBB4	erb-b2 receptor tyrosine kinase 4	NG_011805.1		

Significantly negative correlations were found between the parameters of autonomic neuropathy, nerve conduction velocities/amplitudes, and expression of selected factors. In the case of vibration perception threshold and amplitudes, the correlations were positive. These significant correlation were mainly the same neurotrophic factors as per the IENF

Table 3: Electrophysiological data.

	Group	HC	DM	Tx1		Tx2
				1st exam.	2nd exam.	
Ulnar nerve sensory	NCV (m/s)	55.9 (50.6–31.3)	50.8 (42–56.6)*	37.1 (20.3–62.2)*	32.1 (20.4–58.4)	47 (37.1–52.4)
	Amp (μV)	20.7 (12.8–41.7)	15.4 (0.9–31.5)	5.8 (−0.3–18.5) **	10.7 (4.1–35.5)	5.8 (2.9–12.1)***
Sural nerve sensory	NCV (m/s)	54.9 (23.8–64.2)	42.7 (21.3–60.1)	38.5 (22.6–66.4)	48.9 (37.5–64.8)	33.8 (27.5–41.1)
	Amp (μV)	10.7 (5.3–18.2)	8.3 (3.5–17.9)	6 (2–18)	5.9 (3.5–7)	6 (5.2–6.2)
Ulnar nerve motor	NCV (m/s)	61 (51–72)	54.8 (48–62.7)	50.4 (46.8–58.9)**	52.2 (42.7–56.4)	55.2 (51.4–57.2)
	Amp (mV)	7.5 (5.7–10.8)	7.5 (5.4–9.9)	4 (1.4–7.8)**/xx	4.4 (2.4–7.1)	6 (36.4–43.4)
Tibial nerve motor	NCV (m/s)	49.5 (43.3–53.2)	43.6 (37.4–50.6)	37.4 (33.5–44.7)**	43.8 (36–47.5)	41.5 (36.4–43.4)**
	Amp (mV)	9.4 (5–18.6)	7.9 (2.5–17.1)	3 (0.2–0.5)*	5.5 (1–11.4)	3.1 (0.8–3.8)**/x

Data are given as medians and range (in parentheses). * versus HC $p < 0.05$; ** versus HC $p < 0.01$; *** versus HC $p < 0.001$; x versus DM $p < 0.05$; xx versus DM $p < 0.01$.

Table 4: Results of autonomic tests.

	Group	HC	DM	Tx1		Tx2
				1st exam.	2nd exam.	
I-E difference	(beats/min)	18.8 (8.1–33.9)	20.4 (6.4–29.3)	3.1 (0.9–9.9)***/xxx	4.4 (0.8–14.9)	4.9 (2.9–7.8)**/x
Valsalva ratio		1.7 (1.3–2.1)	1.7 (1.1–2.6)	1.2 (1.1–1.6)*	1.3 (1.1–1.6)	1.2 (1.1–1.2)**
30 : 15 ratio		1.13 (0.9–1.14)	1.12 (0.9–1.4)	0.98 (0.9–1.1)*/x	0.99 (0.9–1.1)	0.97 (0.96–1)*
Δ sBP	(mmHg)	1 (−14–12)	3 (−10–17)	37 (2–69)**/xx	18 (4–66)	40 (26–59)**/xx

Data are given as medians and range (in parentheses). * versus HC $p < 0.05$; ** versus HC $p < 0.01$; *** versus HC $p < 0.001$; x versus DM $p < 0.05$; xx versus DM $p < 0.01$.

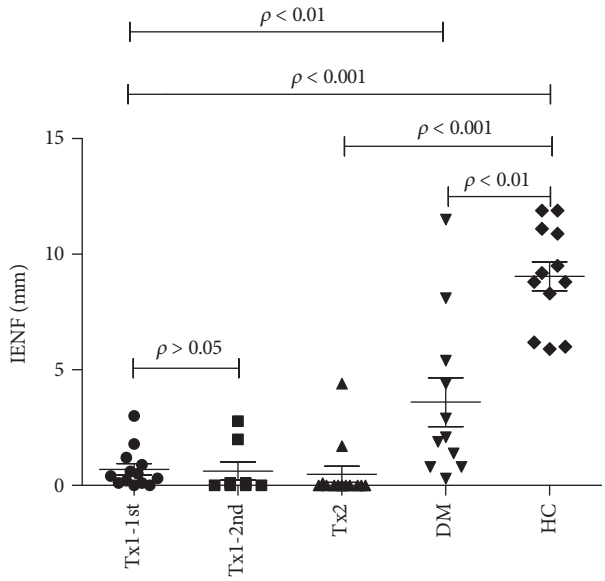

Figure 1: Scatterplot, IENF-density.

density, i.e., GALR1, NGF, NGFR, NTRK1, TGFA, and GFAP. This finding supports the hypotheses that enhanced expression of neurotropic factors is associated with the severity of the neuropathy.

Using stepwise multiple linear regression comprising these 6 factors, we found that IENF density was mainly dependent on GFAP ($p = 0.014$) and NGF ($p = 0.035$). These two parameters accounted for 29% of the IENF density variance.

The neurotropic expression profiles in group Tx1 assessed early after transplantation and after the median 28-month period of normal glucose induced by pancreas transplantation did not change significantly (Figure 5).

4. Discussion

We have investigated skin expression of selected neurotrophic factors and their receptors in subjects with advanced peripheral diabetic neuropathy. In agreement with the experimental data and prevailing clinical views [9, 18, 19, 30], we expected lower expression that would correspond to tissue injury caused by longstanding hyperglycemia and multifaceted metabolic disorders. This assumption, however, was not confirmed. In contrast, with one exception (see Supplemental Table), the expression of all factors was either equal or increased in the Tx1 and Tx2 groups compared with both healthy subjects and diabetic patients without clinically evident neuropathy. Although the upregulated gene expression may not necessarily imply an escalation of protein transcription, we consider this finding novel and important in the search for new targets to stimulate neural regeneration in advanced diabetic neuropathy. Of note, some of these factors had been assessed previously as they could possibly ameliorate advanced nerve damage or they could be of interest for possible interventions [18, 31].

We also conclude that the expression of neurotrophic factors did not change after restoration of endogenous insulin secretion and normalization of glucose homeostasis through pancreas transplantation after 28 months or 8 years

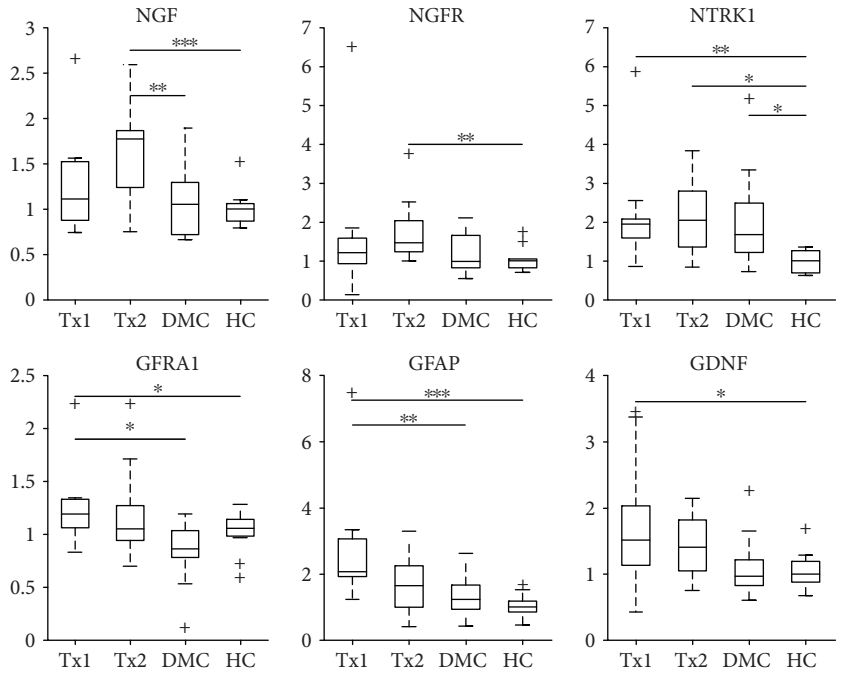

Figure 2: Relative expression of six differently expressed factors. Data are shown as median and 1st and 3rd quartile. Differences between individual groups were assessed by Wilcoxon test with Bonferroni correction: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NGF: nerve growth factor, NGFR: NGF receptor, NTRK1: neurotrophic receptor tyrosine kinase 1, GDNF: glial cell-derived neurotrophic factor, GFRA1: GDNF family receptor alpha 1, GFAP: glial fibrillary acidic protein. +: outlying values.

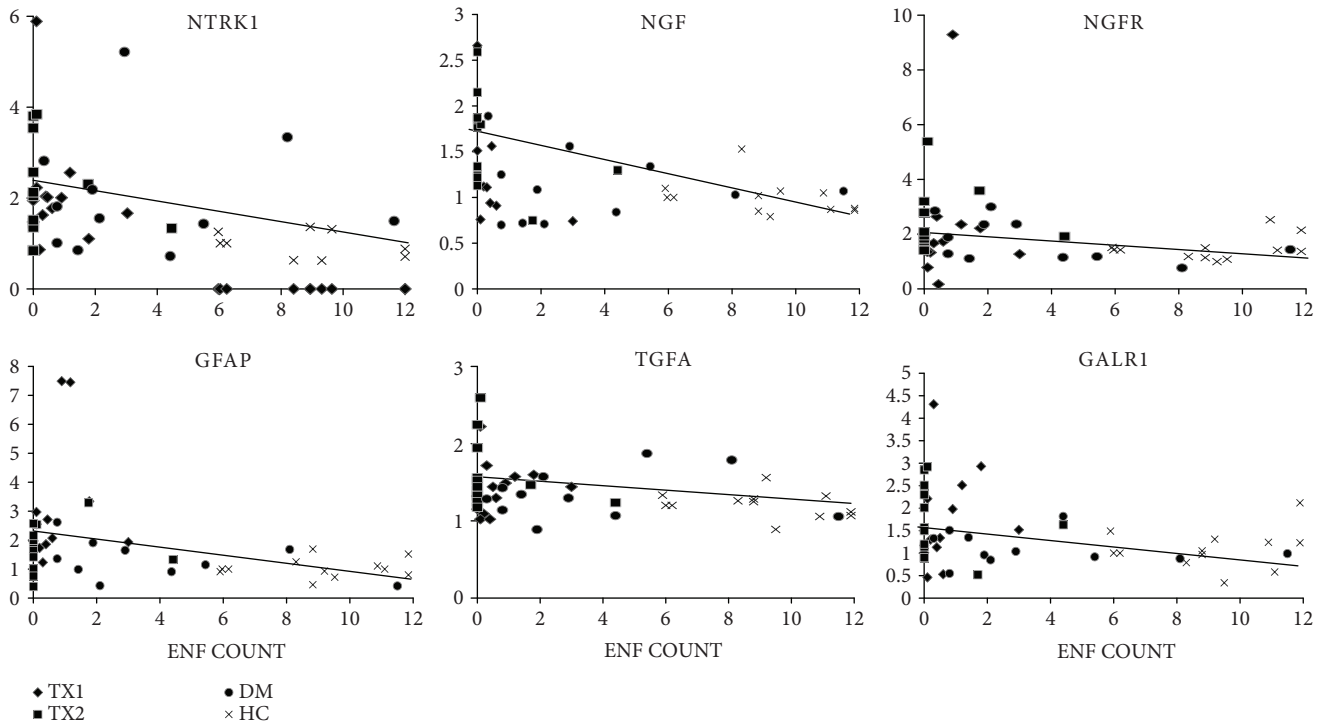

Figure 3: Correlation of intraepidermal nerve fiber density and expression of selected neurotropic factors. IENF count (x-axis) is expressed as number of fibers/mm; relative expression of selected neurotrophins (y-axis) is calculated by ΔΔCt method using normalized expression of each target gene in the HC group as a calibrator.

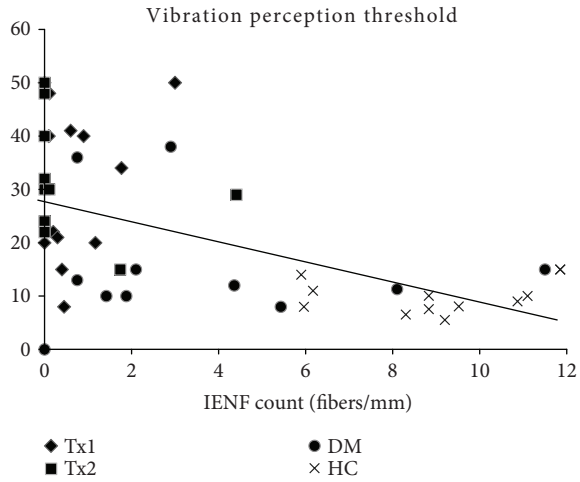

Figure 4: Correlation of intraepidermal nerve fiber density (IENFD) and vibration perception threshold.

of long-lasting normoglycemia when compared with healthy subjects and diabetic subjects without clinically evident neuropathy. At the same time, the enhanced expression profile did not result in any demonstrable regeneration of peripheral sensory nerve fibers. Skin levels of neurotrophins have been assessed in very few studies with somewhat contradictory results, with depletion of skin NGF in patients with diabetic neuropathy [9], and also increased NGF mRNA [17], neurotrophin-3 (NTF3) concentration [32], and expression of the trkA and trkC receptors for NGF and NTF3, respectively [33]. The situation is further complicated by the effect of the duration of diabetes. In an experimental study, Terada et al. [34] demonstrated that motor nerve regeneration after partial denervation was decreased in long-term diabetic rats, whereas it was enhanced in short-term diabetic animals. In a recent work, Uceyler et al. demonstrated decreased skin expression of NGF, NT3, and erythropoietin genes in chronic neuropathies, but the study included only 4 diabetic subjects [8].

A complex analysis of the expression profiles in sural nerve biopsies was performed in 36 subjects with mild to moderate neuropathy in predominantly type 2 diabetic subjects as a part of a larger intervention study [35]. The bioinformatics analysis identified a group of genes which were differentially expressed in samples from patients who showed neuropathy progression over a 52-week period or not. Subsequent examination revealed their relation mainly to inflammatory and lipoprotein subnetworks rather than neurotrophic factors and their receptors. However, this study did not include a nondiabetic control group and assessed progression rather than severity of neuropathy. Further expression studies in skin samples (which are much easier to perform than sural nerve biopsies) should include the candidate genes which have been identified in this work.

Despite the potential effects of the concomitant immunosuppressive therapy, pancreas transplantation represents an optimal model to study the effects of reestablishment of stable normoglycemia in advanced diabetic neuropathy and provides an opportunity to examine whether correction of the principal metabolic noxa, hyperglycemia, may lead to any functional and morphological improvement. In a retrospective analysis of changes in myelinated fiber density in sural nerve biopsies over a 52-week period in mostly type 2 diabetic subjects, HbA1c levels significantly correlated with density improvement but patients with only mild to moderate neuropathy were included [36].

In pancreas and kidney recipients, mostly with advanced neuropathy, several previous studies demonstrated a significant regression of neuropathic symptoms and improvement of electrophysiological indices [37–39]. However, the regenerative capacity probably depends on the stage of the disease [40]. In subjects with impaired glucose tolerance and mild neurological disease, a diet and exercise program was associated with significant increases in intraepidermal nerve fiber density related to improvements in pain and sural nerve action-potential amplitude [41]. In a small study in DPN with the anticonvulsive topiramate, Boyd et al. reported improvements in epidermal nerve fiber density and length [42]. In our recent 8-year prospective work, with the exception of a single patient, we did not show regeneration of epidermal nerve fibers in a group of 12 normoglycemic pancreas and kidney recipients with advanced neuropathy [12]. More optimistic results have been reported with the use of corneal confocal microscopy showing improved density and length of corneal nerve fibers 12 months after SPK [43]. However, the effect in corneal nerves may not predict meaningful neural regeneration elsewhere in the body [44].

We consider it important to know if there is any local attempt to repair severe neural damage. A deficient local response could reflect the consequence of lengthy metabolic disorder. Despite this, we showed that the expression especially of NGF, NGFR, NTRK1, GFRA1, GFAP, and GDNF was upregulated in patients with severe neuropathy as compared to healthy subjects and diabetic patients with subclinical neuropathy. This upregulation of neurotropic factors, however, did not lead to an improvement of electrophysiological parameters or ENF density despite long-term blood glucose normalization and persistence of the enhanced expression profile at 2 and 8 years.

An important shortcoming of our study is that we could not detect the abundance of the specific proteins as we did not have sufficient material for either immunofluorescent or Western blot analysis. We also did not measure the expression of VEGF, which may contribute to the development of neuropathy through its effect on nerve microcirculation or through a direct effect on neurons and other cellular peripheral nerve components. While a decreased production of NGF in various tissues has been implicated in the pathogenesis of experimental diabetic polyneuropathy [45–47], in animal studies, impaired retrograde axonal transport of NGF is also partly responsible for the reduced nerve regenerative capacity [48]. Its increased expression in skin tissue is thus at odds with these findings. Another possibility is that these protein factors need not be transcribed in fully functional forms [19] or, for their full engagement, additional changes are required. We also were not able to determine the specific origin of the upregulated mRNAs which might come from skin fibroblasts, endothelial, or Schwann cells.

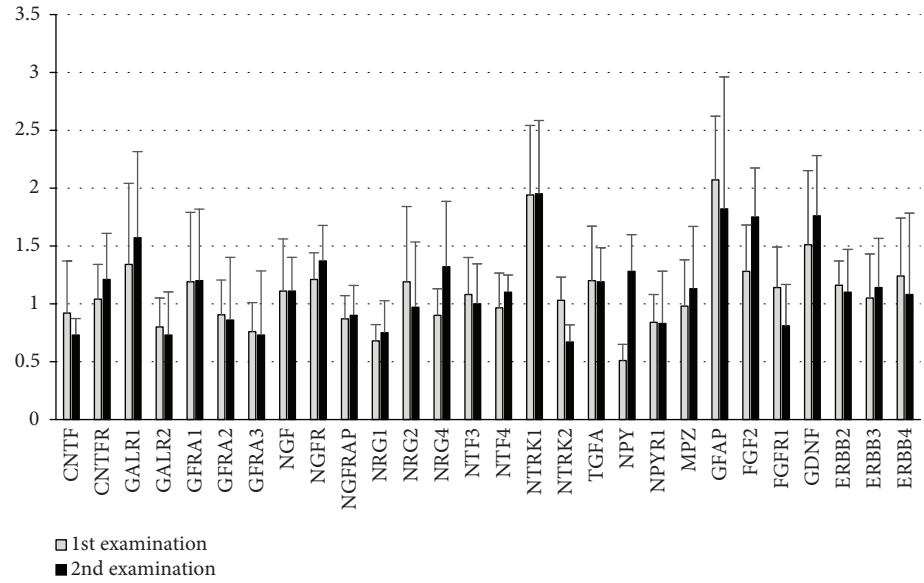

Figure 5: Comparison of the neurotropic factor expression profile in skin biopsies in Tx1 1st and 2nd examinations. The 1st examination was performed early after transplantation and the 2nd examination after the median 28-month period of normoglycemia. Data shown as median and 3rd quartile values. Wilcoxon signed-rank test: $p > 0.05$ for all parameters. The y-axis shows a relative expression of selected neurotrophins calculated by ΔΔCt method using a normalized expression of each target gene in HC group as a calibrator.

The most important conclusion of our study is that local reparatory stimuli in severely nerve-depleted tissue are present but are not efficient.

We acknowledge the lack of a group of patients with advanced diabetic peripheral neuropathy represents a considerable limitation of our study. The local transcription profile of neurotrophic factors at 2–20 (median 8) weeks following successful pancreas and kidney transplantation may not sufficiently reflect the situation before transplantation. Nevertheless, routine performance of skin biopsies in transplant candidates with end-stage kidney failure would have been problematic not only due to a higher risk of local complications. Additionally, waiting times differ among subjects and not all of them reach normal blood glucose levels and good kidney function without episodes of rejection.

This study provides further support to the idea that any therapeutic intervention for diabetic neuropathy should be initiated in the earlier stages before severe neural depletion. Improved metabolic control may be achieved using qualified patient education and emerging technological strategies such as continuous measurement of interstitial glucose level and semiautomatic use of insulin pumps. But if these therapeutic measures fail, then whole organ pancreas or isolated islet transplantation should be considered in subjects with poor metabolic control who do not suffer from advanced diabetic neuropathy.

It remains questionable, whether nerve fiber regeneration in such advanced stages is possible. Stimulating factors may arise from the proximal axons and are transported distally. However, in the case of severe axon damage or death of the whole nerve, regeneration may not by possible unless new neural cells can be transplanted or developed from other cell types.

Abbreviations

AFT:	Autonomic function test
Amp:	Amplitude
DPN:	Diabetic peripheral neuropathy
HPRT:	Hypoxanthine phosphoribosyl transferase
IENF:	Intraepidermal nerve fibers
I-E difference:	Mean difference of maximal inspiratory and minimal expiratory heart rate
NCV:	Nerve conduction velocity
SPK:	Simultaneous pancreas and kidney transplantation
VEGF:	Vascular endothelial growth factor
VPT:	Vibration perception threshold
VR (Valsalva ratio):	Maximal to minimal heart rate during and following the Valsalva maneuver
30 : 15 ratio:	Ratio of heart rates around the 15th and 30th heart beats following standing up
Δ Ct:	Difference in Ct_q values
Δ sBP:	Difference in systolic blood pressure while supine and at 1 min after standing up.

Data Availability

All data used to support the findings of this study are available from the corresponding author upon request. Specifically, primary data concerning all clinical assessments are stored by FS and TH, histological data by LV, and expression studies data by MC from the authors' team. Additional information concerning the study results is given in Supplementary Tables 1, 2, and 3.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Supplemental Table 1: expression of individual neurotrophic factors mRNA in skin biopsies (median, minimal, and maximal values) in individual groups. Supplemental Table 2: Spearman's rank correlation—intraepidermal nerve fiber (IENF) count versus individual factors. Supplemental Table 3: Spearman's rank correlation—vibration perception threshold assessed by biothesiometer (BTM1) versus individual factors. *(Supplementary Materials)*

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Research Article

The Influence of Clinically Diagnosed Neuropathy on Respiratory Muscle Strength in Type 2 Diabetes Mellitus

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Objectives. This cross-sectional study investigated the influence of clinically diagnosed neuropathy (cdNP) on respiratory muscle strength in patients with type 2 diabetes mellitus (T2DM). *Methods*. 110 T2DM patients and 35 nondiabetic healthy controls (≥60 years) were allocated to one of three groups depending on the presence of cdNP: T2DM without cdNP (D−; $n = 28$), T2DM with cdNP (D+; $n = 82$), and controls without cdNP (C; $n = 35$). Clinical neurological diagnostic examination consisted of Vibration Perception Threshold and Diabetic Neuropathy Symptom score. Respiratory muscle strength was registered by maximal Inspiratory and Expiratory Pressures (PI_{max} and PE_{max}), and respiratory function by Peak Expiratory Flow (PEF). Isometric Handgrip Strength and Short Physical Performance Battery were used to evaluate peripheral skeletal muscle strength and physical performance. Univariate analysis of covariance was used with age, level of physical activity, and body mass index as covariates. *Results*. PI_{max}, PE_{max}, and PEF were higher in C compared to D− and D+. Exploring more in detail, PI_{max}, PE_{max}, and PEF were significantly lower in D+ compared to C. PE_{max} and PEF were also significantly lower in D− versus C. Measures of peripheral muscle strength and physical performance showed less associations with cdNP and T2DM. *Conclusions*. The presence of cdNP affects respiratory muscle strength in T2DM patients compared to healthy controls. Both cdNP and diabetes in themselves showed a distinctive impact on respiratory muscle strength and function; however, an accumulating effect could not be ascertained in this study. As commonly used measures of peripheral muscle strength and physical performance seemed to be less affected at the given time, the integration of PI_{max}, PE_{max}, and PEF measurements in the assessment of respiratory muscle weakness could be of added value in the (early) screening for neuropathy in patients with T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common cause of (sensori) motor and autonomic neuropathy [1, 2]. One of the most important and well-recognized clinical manifestations of diabetes-associated neuropathy (NP) is impairment and debilitation in functioning and locomotion due to the development of lower limb skeletal muscle weakness, which is closely related to the severity of NP [3, 4]. Studies using magnetic resonance imaging (MRI) showed accelerated muscle atrophy in accordance with an increased loss of muscle strength in patients with T2DM suffering from symptomatic NP in comparison to T2DM patients without NP and healthy controls [5, 6].

For that matter and from a clinical point of view, the assessment of the influence of NP on muscle function is highly recommended and mainly achieved by means of standardized clinical examinations such as manual muscle testing, isometric and isokinetic dynamometry, Handgrip Strength (HGS), and by functional performance tools (e.g., timed chair stand test (CST) and indirectly by gait analysis and appraisal) [5, 7–9].

Approximately 10–15% of all people aged >40 suffers from NP in which diabetes remains the most common cause. Asides age, diabetes and a set of other distinctive factors causing NP has to be classified as idiopathic in 20–30% of all patients suffering from NP even after thorough investigation. This idiopathic NP is considered as a major culprit of a

person's disability with important social impact due to pain, gait instability, increased risk of falls, injuries, and poor quality of life [10–12].

The association between reduced respiratory function and T2DM has already been described [13], however, the underlying mechanisms are still undisclosed. Klein et al. reported in a systematic review an inverse association between respiratory function on the one hand and blood glucose levels, the severity and duration of T2DM on the other, independent of smoking status or presence of obesity [14]. van den Borst et al. also reported a decreased lung function in T2DM. Metaregression analysis showed, however, that this relation could not be explained by body mass index (BMI), smoking, diabetes duration, or glycated hemoglobin (HbA1c) [15]. Respiratory muscle strength is strongly associated with pulmonary function and may play important roles in the respiratory network, which on its turn depends on intact neural circuitry that orchestrates the interplay between respiratory muscles and intrinsic pulmonary function to maintain adequate ventilation [16]. Kabitz et al. showed that impaired respiratory neuromuscular function, which is strongly related to diabetic polyneuropathy, occurs in T2DM patients as assessed by nonvolitional gold-standard phrenic nerve stimulation [4]. Also, other studies have reported that in patients with T2DM, respiratory muscle weakness can occur and might be associated with autonomic dysfunction [17, 18]. In contrast to the large number of studies examining peripheral muscle weakness in T2DM patients with NP [3, 5, 8, 19–22], only limited research has been conducted regarding the impact of diabetic—or any kind for that matter—NP on respiratory muscle strength [4].

The aim of the present study is to evaluate respiratory muscle strength and function in T2DM patients with clinically diagnosed neuropathy (cdNP) and to compare this with T2DM patients without cdNP and healthy controls. We hypothesize that, compared to healthy controls, respiratory muscle strength and function are decreased in T2DM patients without cdNP and even more impaired in the presence of cdNP.

With respect to the aforementioned hypothesis, the assessment of maximal static Inspiratory and Expiratory Pressure measurements and Peak Expiratory Flow could be considered regarding its added value in the screening for NP.

2. Materials and Methods

2.1. Study Design and Population. In this cross-sectional case-control study, 110 patients with T2DM and 35 healthy controls were included ($n = 145$).

Participants comprised both community-dwelling elderly and elderly living in a residential care setting. Patients with T2DM were recruited by the Department of Endocrinology (University Hospital Ghent) and their general practitioner, and healthy controls by online advertising and flyer distribution. T2DM was diagnosed on two different occasions based on HbA1c assessments according to the Type 2 Diabetes ADA Diagnosis Criteria [23].

Criteria for inclusion were (i) aged 60 years or more, (ii) living in the community or residential care setting, (iii) able to respond adequately to Dutch instructions, and (iv) able to walk independently with or without walking aids. Subjects suffering from (i) major neurological conditions (e.g., stroke, Parkinson's disease, and dementia), (ii) musculoskeletal disabilities (e.g., foot ulcerations, lower extremity amputations, and arthritis with limited joint mobility precluding ambulation), (iii) severe cardiovascular disorders (e.g., exercise-induced chest pain, congestive heart failure (New York Heart Association class III and IV)), and (iv) respiratory diseases (e.g., exercise-induced asthma and COPD (Global Initiative for Chronic Obstructive Lung Diseases (GOLD)) stages 3 and 4) were excluded.

Based on a clinical neurological diagnostic examination performed by trained physical therapists, the population could be divided into three groups: T2DM without cdNP (D−; $n = 28$), T2DM with cdNP (D+; $n = 82$), and nondiabetic healthy controls without cdNP (C; $n = 35$). Control subjects having NP after the clinical neurological examination were excluded as the differentiating etiology of NP was not further examined.

The Ethical Committee of the Ghent University Hospital gave approval to this study, and all participants signed an informed consent. Consequently, this research is compliant with the World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects.

2.2. Outcome Measurements. All measurements were performed on a single morning in a quiet setting and well-lit room with flat surface. At first, anthropometrical data and HbA1c were obtained followed by NP-examinations. Subsequently, respiratory muscle strength, HGS, and the Short Physical Performance Battery (SPPB) were assessed, and a physical activity questionnaire was completed.

2.3. Patient Characteristics. Height, weight, BMI, and body composition (bioelectrical impedance analysis; BIA, Bodystat® 1500MDD) were measured and calculated.

HbA1c was measured using the A1CNow SELFCHECK® (Bayer), an instrument which is well correlated with standardized laboratory HbA1c tests ($r = 0.758$) [24].

Habitual physical activity levels were measured using the physical activity questionnaire for the elderly [25, 26].

2.4. Measurements of Peripheral Clinically Diagnosed Neuropathy. The clinical neurological diagnostic examination consisted of two parts: measurements of the Vibration Perception Threshold (VPT), an assessment of the peripheral large-fiber sensory nerve function, and the Diabetic Neuropathy Symptom score questionnaire (DNS).

The VPT, a valid and reliable measurement, was determined using a Bio-Thesiometer® (Bio Medical Instrument Co., Ohio, USA) on the left and right medial malleolus and on the distal plantar surface of the big toes [27, 28]. VPT was defined as the lowest recorded voltage when subjects indicated the sense of vibration. Each measurement was repeated three times and the lowest reading was considered [29, 30]. Since the threshold at which vibration becomes perceptible is dependent of age, gender, and location, four

percentile rank charts of VPT variation were used [29]. To decide whether vibration perception was within the normal range, a normality cut-off on the 95th percentile was applied. If one of the readings (big toe and the medial malleolus, both left and right) was above the 95th percentile, this criterion was classified as positive.

The DNS, a validated 4-point yes/no questionnaire, has a high predictive value in the screening for diabetic NP when patients score ≥1/4. Meijer et al. compared the validity, predictive value, and reproducibility of the DNS with the Neuropathy Symptom Score (NSS). They found a high correlation between NSS and DNS score ($r = 0.88$) and concluded that the DNS is a fast, easy, reproducible (Cohen's weighted kappa 0.78–0.95), and valid assessment tool to screen for diabetic polyneuropathy [31].

Patients with T2DM were classified as having peripheral cdNP based on at least one of two positive criteria: a VPT exceeding the normality cut-off of 95% or a DNS score of ≥1/4.

2.5. Measurements of Muscle Strength. The maximal static Inspiratory and Expiratory Pressure measurements (PI_{max} and PE_{max}; cm H_2O) were obtained by a Pocket-Spiro Mouth Pressure Monitor with a differential pressure transducer (MPM100; Medical Electronic Construction®). To measure PI_{max}, subjects were seated and asked to exhale slowly and completely up to residual volume and then to perform a maximum inspiratory maneuver during at least 1.5 seconds against a completely occluded airway. Then, a 1-second average including the peak pressure was calculated, indicating the inspiratory muscle strength. PE_{max} was determined under the same conditions while first inhaling completely up to total lung capacity and then performing a maximum expiratory maneuver. For each index, three tests were recorded, and the highest value was used for data analysis [32–35]. The measurement of the maximum static mouth pressures produced against an occluded airway is the most widely used method of measurement and is an easy way to gauge respiratory muscle strength and to determine the severity of respiratory muscle strength impairments [36]. Additionally, Peak Expiratory Flow (PEF; L/m), a cheap, simple, and widely accessible technique with a prognostic value for morbidity [37, 38], was recorded using a Mini-Wright Peak Flow Meter (Henrotech®). This is internationally recognized as the golden standard for PEF measurements [39]. PEF is used as an indicator for respiratory muscles strength in subjects without lung disorders [37].

Isometric HGS (kg) was measured according to the American Society of Hand Therapists guidelines [40] using the Jamar® dynamometer (Sammons Preston Rolyan Inc., Bolingbrook, IL) at the dominant side [37]. The highest grip score of three consecutive trials was retained.

2.6. Measurements of Physical Performance. The SPPB consists of a timed standing balance test (feet together side-by-side, semitandem, and tandem stance), a walk test (time to walk 2.44 meters at usual pace), and a CST (time to raise from a chair and return to the seated position in five times) [41]. Each of the three component tasks was rated from 0 (unable to complete) to 4 (best), and a compiled score was computed by the sum of scores on component tasks (range 0 = worst to 12 = best) [42, 43]. This composite test is often used and validated as a standard assessment of physical performance in research and clinical practice of the ageing population [37, 44].

2.7. Statistical Analysis. Data were analyzed using IBM Statistical Package for Social Sciences (SPSS version 24 for Windows) and were considered significant at $\alpha < 0.05$. After confirming the approximate normality of data using the Shapiro–Wilk test, descriptive statistics for anthropometric, biochemical, and respiratory muscle parameters are presented by arithmetic mean (standard deviation; SD), median (min-max), and by ratio (% and count). Between-groups analysis was performed using univariate analysis of covariance (ANCOVA) with age, level of physical activity, and BMI as covariates. Post hoc comparisons were corrected with the Bonferroni test. A Pearson chi-square test was used for gender and residential status in order to detect all between-group differences.

Linear regression analysis between VPT, DNS, and HbA1c on the one hand and measures of respiratory muscle strength and function (i.e., PI_{max}, PE_{max}, and PEF) on the other was performed with age as confounder.

3. Results

Subject characteristics are shown in Table 1. The healthy control group (C) was significantly younger ($F = 3.487$; $p = 0.017$), had lower BMI ($F = 3.561$; $p = 0.015$), was more physically active ($F = 5.343$; $p = 0.002$), and proportionally a minority was living in a residential setting ($p = 0.002$) compared to the other groups. There was no significant difference in gender distribution between the 3 groups ($p = 0.587$).

HbA1c levels were significantly higher in the diabetes groups compared to the control group ($F = 24.894$; $p < 0.001$), but showed no significant differences between the diabetes groups (D− vs. D+). Also, no significant between-group differences were found for duration of diabetes.

VPT-toe measures (left versus right) and VPT-ankle measures in the C, D−, and D+ are presented in Table 2.

The actual values of DNS reveal a score of zero on the scale of 0–4 in C in contrast with D+ (Table 3).

Linear regression analysis between VPT, DNS, and HbA1c on the one hand and measures of respiratory muscle strength and function (i.e., PI_{max}, PE_{max}, and PEF) on the other has been performed. Age was considered as a confounder since this is the single covariate strongly related to respiratory function, which is less so for the level of physical activity and BMI. The linear regression analysis on respiratory muscle strength (i.e., PI_{max} and PE_{max}) shows that the VPT scores are the only significant explanatory values for the variances, respectively, in PI_{max} (8.2%) and PE_{max} (10.9%). Analyzing respiratory function (i.e., PEF), VPT (2.8%), Hb1Ac (5.5%), and age (17.5%) significantly explain 25.8% of the variance in PEF. The outcome data are documented in Table 4.

Table 1: Subject characteristics.

	C ($n = 35$)	D− ($n = 28$)	D+ ($n = 82$)
Age (yrs)	73 (6.8)	79 (9.9)	**79 (9.1)[a]**
BMI (kg/m^2)	28 (4.0)	**31 (6.2)[a]**	29 (5.3)
HbA1c (%)	5.5 (0.40)	**6.7 (0.81)[a]**	**6.7 (1.24)[a]**
Diabetes duration (yrs)	/	10.5 (8.34)	10.3 (8.64)
Level of physical activity	8.2 (1.24–32.19)	6.4 (0.00–38.35)	**2.6 (0.00–36.41)[ab]**
Male : female			
(%)	43 : 57	39 : 61	34 : 66
(count)	15 : 20	11 : 17	28 : 54
Community-dwelling : RCS			
(%)	71 : 29	**29 : 71[a]**	**37 : 63[a]**
(count)	25 : 10	**08 : 20[a]**	**30 : 52[a]**

Data were expressed as mean (SD), with exception for the level of physical activity as median (min-max), gender, and residential status as ratio (% and count); C: healthy controls; D−: T2DM without cdNP; D+: T2DM with cdNP; yrs: years; RCS: residential care setting; HbA1c: glycated hemoglobin; [a]$p < 0.05$ compared to C; [b]$p < 0.05$ compared to D−.

Table 2: VPT scores.

VPT: highest voltage of the left versus right toe			
	C ($n = 34$)	**D− ($n = 26$)**	**D+ ($n = 74$)**
Means (SD)	20.2 (5.54)	20.3 (6.04)	37.5 (12.26)
min-max	10–38	10–34	10–50
VPT: highest voltage of the left versus right ankle			
	C ($n = 34$)	**D− ($n = 26$)**	**D+ ($n = 70$)**
Means (SD)	23.6 (6.50)	24.0 (7.36)	40.6 (11.60)
min-max	12–35	10–45	7–50

Data were expressed as mean (SD) and minimum-maximum (min-max); VPT: Vibration Perception Threshold; C: healthy controls; D−: T2DM without cdNP; D+: T2DM with cdNP.

Table 3: DNS scores.

	C ($n = 26$)	D− ($n = 22$)	D+ ($n = 70$)
Means (SD)	0 (0)	0 (0)	1.4 (1.35)
Median (min-max)	0 (0-0)	0 (0-0)	1 (0–4)

Data were expressed as mean (SD) and median (minimum-maximum); DNS: Diabetic Neuropathy Symptom score; C: healthy controls; D−: T2DM without cdNP; D+: T2DM with cdNP.

Table 4: Linear regression analysis between VPT, DNS, HbA1c and age on one hand, and PI_{max}, PE_{max} and PEF on the other.

	PI_{max}	PE_{max}	PEF
Explanatory variables	(i) VPT	(i) VPT	(i) VPT (ii) HbA1c (iii) Age
Adjusted R square	0.082	0.109	0.258
p values	$p = 0.003$	$p = 0.001$	$p < 0.001$

PI_{max}: Maximum Inspiratory Pressure; PE_{max}: Maximum Expiratory Pressure; PEF: Peak Expiratory Flow; VPT: Vibration Perception Threshold; HbA1c: glycated hemoglobin.

Table 5 reports on the assessment of respiratory muscle strength between the three groups, corrected for age, physical activity, and BMI. Significant differences were observed for PI_{max}, PE_{max}, and PEF. Post hoc analyses revealed significant lower values in D+ compared to C for PI_{max} ($p = 0.005$), PE_{max} ($p = 0.001$), and PEF ($p < 0.001$). When comparing D− with C, only PE_{max} ($p = 0.039$) and PEF ($p = 0.026$) were significantly lower.

Functional assessment data (i.e., HGS and SPPB) between the three groups are presented in Table 6, corrected for age, physical activity, and BMI.

HGS revealed no between-groups differences ($F = 2.100$; $p = 0.128$). Statistically significant differences were observed on both the SPPB total ($F = 7.209$; $p = 0.001$) as in its subdomains; CST ($F = 4.533$; $p = 0.013$), balance ($F = 3.835$; $p = 0.025$), and gait ($F = 4.130$; $p = 0.019$) with better performance in favor of C. For SPPB total and SPPB gait, post hoc analysis revealed significant higher values in C compared to D− ($p = 0.008$ and $p = 0.043$, respectively) and D+ ($p = 0.002$ and $p = 0.031$, respectively). CST and balance subdomains showed significant better scores for C compared to D+ ($p = 0.010$ and $p = 0.028$, respectively). Considering SPPB balance, only tandem stance showed significant higher results comparing C to D+ ($p = 0.019$).

4. Discussion

The present study was conducted to investigate respiratory muscle strength and function in T2DM and its relation to NP by comparing PI_{max}, PE_{max}, and PEF between T2DM patients with cdNP, T2DM patients without cdNP, and healthy controls.

The key findings of this study were lower measures of PI_{max}, PE_{max}, and PEF in the D− and D+ groups compared to C.

Looking more in detail to the results, all three respiratory muscle outcomes were significantly lower when comparing D+ to C; PE_{max} and PEF were significantly lower in D− and D+ compared to C. Herewith, it seems that the presence of

Table 5: Univariate analysis of covariance (ANCOVA, corrected for age, body mass index, and physical activity) on respiratory muscle strength and function.

	F value p value	C ($n = 35$)	D− ($n = 28$)	D+ ($n = 82$)
PI_{max} (cm H_2O)	$F = 5.289$ $p = 0.007$	64.5 (28.83)	40.7 (25.22)	**36.6 (23.71)**[a]
PE_{max} (cm H_2O)	$F = 6.785$ $p = 0.002$	100.6 (29.58)	**69.5 (29.97)**[a]	**65.2 (31.20)**[a]
PEF (L/min)	$F = 10.600$ $p = 0.001$	471.2 (132.27)	**330.9 (152.07)**[a]	**314.5 (221.26)**[a]

Data were expressed as mean (SD); C: healthy controls; D−: T2DM without cdNP; D+: T2DM with cdNP; PI_{max}: Maximum Inspiratory Pressure; PE_{max}: Maximum Expiratory Pressure; PEF: Peak Expiratory Flow; [a]$p < 0.05$ compared to C.

Table 6: Univariate analysis of covariance (ANCOVA, corrected for age, body mass index, and physical activity) on peripheral muscle strength, balance, and gait.

	F value p value	C ($n = 35$)	D− ($n = 28$)	D+ ($n = 82$)
HGS (kg)	$F = 2.100$ $p = 0.128$	26.9 (12.36)	20.1 (10.15)	17.6 (9.50)
SPPB: total	$F = 7.209$ $p = 0.001$	11 (4–12)	**7 (1–12)**[a]	**6 (1–12)**[a]
(A) CST	$F = 4.533$ $p = 0.013$	3 (0–4)	1 (0–4)	**0 (0–4)**[a]
(B) Balance total	$F = 3.835$ $p = 0.025$	4 (3–4)	3.5 (0–4)	**3 (0–4)**[a]
Side-by-side	$F = 0.508$ $p = 0.603$	2 (2-2)	2 (0–2)	2 (0–2)
Semitandem	$F = 1.334$ $p = 0.268$	2 (2-2)	2 (0–2)	2 (0–2)
Tandem	$F = 3.966$ $p = 0.022$	2 (1-2)	1.5 (0–2)	**1 (0–2)**[a]
(C) Gait	$F = 4.130$ $P = 0.019$	4 (1–4)	**2 (1–4)**[a]	**3 (1–4)**[a]

Data were expressed as median (min-max), with exception for HGS as mean (SD); C: healthy controls; D−: T2DM without cdNP; D+: T2DM with cdNP; HGS: Handgrip Strength; SPPB: Short Physical Performance Battery; CST: chair stand test; [a]$p < 0.05$ compared to C.

NP as well as T2DM has an impact on respiratory muscle outcome. However, an accumulating effect of cdNP and T2DM could not be ascertained.

A posteriori power calculation on respiratory muscle strength and function (PI_{max}, PE_{max}, and PEF) resulted in a power of 0.826, 0.912, and 0.927, respectively.

To understand our NP-related results, the innervation of the respiratory muscles should be explored in depth. While breathing in, the inspiratory muscles contract by recruiting nonvolitional spinal nerves C3, C4, and C5 (the phrenic nerve) innervating the diaphragm, cranial nerve XI, spinal nerves C1 and C2 innervating the sternocleidomastoid and the scalene muscles, and T1 to T12 for the external intercostal muscles. The two expiratory muscle groups (the internal intercostals and abdominals) are usually not used during quiet breathing but are essential in performing expulsive efforts, including cough, vomiting, and defecation. Due to their character, these expiratory muscles are of utmost importance during forced expiration (such as in PE_{max} and PEF, during static and dynamic trunk control, and Valsalva maneuvers). The internal intercostal muscles are innervated by the spinal nerves T1 to T12 and the abdominals by spinal nerves T7 to L1.

The respiratory muscles are generally controlled by the respiratory centers of the autonomic nervous system in the pons and medulla oblongata and are depending on intact motor nerve supply, comparable to all skeletal muscles [45, 46].

Kabitz et al. used bilateral anterior magnetic phrenic nerve stimulation whereby the cortical motor command was bypassed in order to assess respiratory neuromuscular function related to diabetic poluneuropathy in patients with T2DM [4]. They provided the first data regarding cdNP and concluded that cdNP was associated with substantially

impaired respiratory neuromuscular function in patients with T2DM, when stimulating the nonvolitional phrenic nerve. No alterations in respiratory function could be found when assessing volitional respiratory muscle strength. The volitional respiratory neuromuscular function testing was performed by using PI_{max}, PE_{max}, and maximal sniff pressures. PEF, however, was not assessed in this particular study. The exclusion criterion used by Kabitz et al. consisted in the diabetic group of known primary lung diseases, whereas in the control group healthy male subjects experiencing lung, cardiac, or metabolic diseases were excluded [4]. The different eligibility criteria between the research of Kabitz et al. and our own study could explain the different results. We opted for stricter selection criteria such as exclusion of patients with exercise-induced asthma and COPD GOLD stages 3 and 4. It is also of importance to mention the lower mean age of their controls (60.3 years ± 6.9) and the diabetic patients (63.6 years ± 7.5) compared to the present study, which could explain the differences in outcome of the volitional tests on respiratory neuromuscular function [47].

To understand the impact of T2DM as such, we have to focus on muscle mass, muscle fiber type distribution, and vascularization. Checking on the muscle fiber type distribution of the diaphragm in healthy humans, the mean relative occurrence of slow-twitch fibers (type I) is approximately 50%. The remaining proportion is equally divided into two different fast-twitch fibers (type IIa and IIx). Both the inspiratory and expiratory intercostal muscles have at least 10% more type I fibers than the diaphragm and most other skeletal muscles, whereas the expiratory internal intercostal muscles show an almost complete absence of type IIx fibers [46].

In healthy humans, all muscle fibers are surrounded by a certain number of capillaries, depending on their fiber type. In the diaphragm, type I fibers are surrounded by 4–6 capillaries per fiber, whereas slightly less [3–5] are found around type IIa and IIx fibers. However, the calculated values for the fiber area surrounded by each capillary are smaller in the diaphragm than in lower or upper limb muscles. In the expiratory intercostal muscles, more capillaries are found around both type I and type IIa fibers [5, 6] compared to the inspiratory intercostal muscles [4, 5, 46].

In the elderly in general and/or older T2DM patients, abnormalities in muscle morphology have been observed [48, 49]. Studies examining ageing and "accelerated" ageing in the older T2DM patients showed a reduced muscle mass and a decrease in muscle fiber size and number compared to younger controls [50–52]. Fiber size differences, particularly in the type II muscle fibers, seemed to be evident between healthy young men, healthy older men, and older age-matched T2DM patients, suggesting that type II fibers are more prone to muscle atrophy in the latter groups. When examining muscle capillary density (as a parameter of microvascular function), capillaries tended to be less prevalent in the elderly and/or older T2DM patients, implicating lower muscle capillary density. Dilation of these small capillaries could explain the observed shift in the distribution of vessel size with a relative loss of small vessels [52].

Measures of peripheral muscle strength (HGS) and functional performance (SPPB total with CST, balance, and gait as assessment tools) showed a similar profile as the respiratory muscle strength assessments; i.e., C scored better compared to D− and D+.

Analysis of the peripheral muscle strength showed no significant difference in HGS.

SPPB total, showed a significant difference ($p = 0.001$), mainly allocated to gait (usual gait speed over 2m44; $p = 0.019$) since both post hoc analyses showed significant differences of C versus D+ and D−. Walking is a complex motor skill, involving interactions between sensory and motor attributes, but is essentially supported by appropriate muscle strength and balance. Our findings regarding both strength impairment (CST, $p = 0.013$) and total balance changes ($p = 0.025$) with its subtest "tandem stance" ($p = 0.022$) endorse the earlier research results in T2DM and cdNP on gait [53]. The argument that gait performance could be influenced by T2DM alone (without NP) has to be taken into consideration based on previous findings [2, 9]. van Sloten et al. suggested that walking in subjects with T2DM was strongly associated with peripheral NP and decreased muscle strength. This associative result could not be established in our study when T2DM patients were compared to controls [9]. It could be hypothesized that T2DM as such has the same detrimental effects as the presence of cdNP in T2DM on a functional capacity (SPPB total and gait).

Based on our data, we can but conclude that both T2DM and NP significantly influence respiratory muscle strength and function. It was, however, not possible to distinct the initial cause (i.e., neuropathic respiratory impairments or diabetes-related pathology) of decreased respiratory muscle strength and function in our T2DM population.

The linear regression analysis on respiratory muscle strength (i.e., PI_{max} and PE_{max}) suggests that the VPT scores are the only significant explanatory values (respectively, 8.2% and 10.9% of the variances in PI_{max} and PE_{max}) rather than HbA1c, age, and DNS. These results indicate that VPT scores have a larger impact on respiratory muscle strength, supporting the hypothesis that respiratory muscle weakness is due to NP. Analyzing respiratory function (i.e., PEF), VPT (2.8%), Hb1Ac (5.5%), and age (17.5%) significantly explain 25.8% of the variance in PEF. Skloot provided evidence that the ageing process, in the absence of lung disease, alters the intrinsic structure of the lung (changes in collagen fiber network) as well as the supportive extrapulmonary structures (decreased chest wall compliance, reduced curvature of the diaphragm, and loss of respiratory muscle mass). These age-related changes in respiratory mechanics lead to a reduction in expiratory flow and lung volumes and affect lung function [47]. An explanation of the higher impact of HbA1c compared to the VPT scores can be found in the association between increased chronic glycemic exposure to the lung parenchyma and reduced pulmonary function in patients with T2DM [54].

The fact that all existing screening tools and questionnaires only rely on the measurements of appendicular muscles, and based on our results, it should be taken into consideration to integrate PI_{max}, PE_{max}, and PEF in the

screening for respiratory muscle weakness as an indication for the presence of NP in diabetic patients [33]. These findings are supported by Lecube et al. [54] claiming that specific cost-effective screening programs for lung impairment, performed by health care providers, should be investigated in further research.

In the clinical practice of a general practitioner, measurements of lung function (forced vital capacity, forced expired volume after 1 second, Tiffeneau index, and PEF) are already regularly applied, mainly in order to detect COPD or other related respiratory disorders. Additional evaluations of respiratory muscle outcomes, which are easy to manage and have low cost impact (e.g., a portable Peak Flow Meter), could be of additional value and of importance in the screening for NP in the T2DM population.

Overall, impairments of respiratory muscle strength and function (PI_{max}, PE_{max}, and PEF) were slightly more pronounced compared to those of peripheral muscle strength. Since this study had a cross-sectional design, it was not possible to draw any conclusions concerning the timing of the impact on respiratory or peripheral muscles.

The participants were not questioned concerning smoking condition and alcohol consumption, although this information could have an impact on lung function in general, more specific PEF values, and on peripheral NP. However, due to a large group of respiratory disorders (asthma and COPD GOLD stages 3 and 4) were implemented as exclusion criterion, the impact of lung diseases on PI_{max}, PE_{max}, and PEF was significantly reduced. Regarding alcohol consumption, no data were collected which could affect peripheral NP as well. Besides, interviewing subjects about their smoking and drinking habits often lead to ambiguous answers out of exclusion fear.

The cross-sectional design limits drawing conclusions regarding the timing of the impact of causative variables on outcome parameters. Consequently, future research focus on both longitudinal research and the evolution of physical markers and symptoms such as—in this particular study—onset of diabetes, NP, and respiratory and peripheral muscle weakness.

It stands to reason that 82 out of 110 diabetic patients (74.5%) were allocated to the NP group (D+) known as one of the major comorbidities in T2DM patients [1, 2, 55]. Firstly, it is worth mentioning that according to Andersen et al. the prevalence of cdNP increases from 8% in newly diagnosed patients to >40% after 10 years of diabetes [3]. In the present study, the mean duration of diabetes was above 10 years from onset in both D− and D+ groups.

Secondly, the enrolled patients with T2DM showed higher mean age, higher BMI, and lower levels of physical activity compared to controls. Ageing is a well-known nonmodifiable factor for the development of diabetes and lower muscle strength [50]. BMI has a negative impact on muscle strength in a population with insulin resistance (prediabetic situation) and diabetes type 2, which will manifest itself as a decrease of absolute and relative peak torque [56, 57]. Concerning physical activity, low levels have a negative impact on the development of diabetes and on lower muscle strength [57]. In our ANCOVA we encountered this barrier by adding age, BMI, and physical activity to add them as covariates.

Finally, peripheral cdNP was diagnosed by VPT in combination with DNS with a deficient comprehensive neurological examination. Hence, we strongly recommend the use of the Michigan Neuropathy Screening Instrument in future research in order to allocate T2DM patients with/without NP more accurately to the respective groups [58–60]. In addition to these clinical assessments, MRI can be used in the detection of symptomatic NP, and nerve conduction investigations can be performed by means of electroneuromyography to evaluate sensory action potential amplitude and sensory and motor conduction velocity to confirm the presence and the severity of NP [61]. The main drawbacks to MRI and ENMG techniques are the high costs regarding the number of participants (initially 190) and the subject discomfort.

5. Conclusions

Based on a substantial population, this research, focusing on respiratory muscle strength, could conclude that this strength is negatively influenced in T2DM patients with and without peripheral neuropathy. A summation effect in patients with diabetes and neuropathy could not be ascertained. Screening for this muscle characteristic may add value to daily clinical practice of T2DM patients in assessment and follow-up.

Data Availability

The data used to support the findings of this study (BirgitVanEetvelde_20180723_revision.zsav) are included within the supplementary information file(s) (available here).

Disclosure

The data of this paper have been presented as a poster at the 54th EASD Annual Meeting, Berlin, Germany, 1–5 October.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Supplementary Materials

Below are template examples that authors may use to write a Data Availability statement. It will often be appropriate to combine templates and edit them as appropriate. (1) The data used to support the findings (BirgitVanEetvelde_20180723.sav) of this study are included within the supplementary information file(s). Read less. *(Supplementary Materials)*

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Research Article

Association between Early Neuroretinal Dysfunction and Peripheral Motor Unit Loss in Patients with Type 1 Diabetes Mellitus

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Objectives. It has been already confirmed that retinal neurodegeneration has a predictive value in the development of microvascular alterations in diabetic retinopathy. However, no data are available on the association between neuroretinal dysfunction and peripheral motor unit loss. Our study, therefore, was aimed at investigating the hypothesis that retinal neurodegeneration could be considered an early marker of diabetic peripheral neuropathy (DPN). *Methods.* 20 T1DM patients with no symptoms/signs of peripheral polyneuropathy, without DR or with very mild nonproliferative DR, and 14 healthy controls (C) age- and gender-matched were enrolled. The following electrophysiological tests were performed: standard nerve conduction studies (NCS) and incremental motor unit number estimation (MUNE) from the abductor hallux (AH) and abductor digiti minimi (ADM). Neuroretinal function was studied by multifocal electroretinogram (MfERG) recordings, measuring response amplitude density (RAD) and implicit time (IT) from rings and sectors of superior (S)/inferior (I)/temporal (T)/nasal (N) macular sectors up to 10 degrees of foveal eccentricity. *Results.* MfERG RADs from rings and sectors were significantly reduced in T1DM ($p < 0.05$) vs. C. ADM MUNE and AH MUNE were significantly decreased in T1DM ($p = 0.039$ and $p < 0.0001$, respectively) vs. C. A positive correlation between mean MfERG RADs from the central 5 degrees of the four (S, I, T, and N) macular sectors and lower limb motor unit number ($r = 0.50$, $p = 0.041$; $r = 0.64$, $p = 0.005$; $r = 0.64$, $p = 0.006$; and $r = 0.61$, $p = 0.010$, respectively) was observed in T1DM patients. No abnormalities of NCS were found in any subject. *Conclusions.* The motor unit loss on the one hand and neuroretinal dysfunction on the other hand are already present in T1DM patients without DPN. The relationship between neuroretinal dysfunction and motor unit decline supports the hypothesis that neuroretina may represent a potential "window" to track the early neurogenic damage in diabetes.

1. Introduction

Diabetic peripheral neuropathy (DPN) is one of the most debilitating complications of diabetes mellitus (DM), determining sensory loss and neuropathic pain and late weakness [1]. The diagnosis of diabetic neuropathy is also based on the use of neurophysiologic techniques and the evaluation of advanced signs and symptoms. Moreover, the ability of standard electrophysiological techniques (nerve conduction studies and electromyographic evaluation) to identify

neuronal dysfunction is confined to overt neuropathic damage. On the other hand, the new procedures, such as skin biopsy, showed to be helpful in detecting neuropathy in the early and subclinical stages but they are still demanding because they are invasive, require expensive laboratory equipment and trained personnel, and still have limited reproducibility [2]. A painless, noninvasive, cost-effective, and clinically available tool is therefore necessary, for the early detection, staging, and monitoring of peripheral diabetic neuropathy [3].

Recently, emerging evidence shows that the neuropathic process, contrary to the conventional view, is not only confined to the peripheral sensory nerves but also involved the whole nervous system, thus suggesting the possible identification of new observational "windows" of the neurodegenerative process [4]. Particularly, in few studies on animal models and in humans, an early impairment of the motor nerves has also been demonstrated [5, 6]. These alterations are likely associated to a "dying back" of motor nerve terminals, similar to the death process that occurs in sensory epidermal nerve fibers [6].

On the other hand, the process of retinal neurodegeneration is known to precede vascular damage, representing so far an early marker of diabetic retinopathy (DR) [7–9]. However, despite its prominence at clinical examination, vasculature makes up less than 5% of the retinal mass, so that the retina can be more appropriately considered as a vascularized neuronal tissue [10, 11]. Therefore, since hyperglycemia adversely affects the entire neurosensory retina, by accelerating neuronal apoptosis or altering metabolism of neuroretinal supporting cells [11, 12], some authors suggest to consider diabetic retinopathy, as a neuropathy that affects the retinal parenchyma, similar to peripheral diabetic neuropathy [13]. The aim of this observational study is to explore neuronal damage in type 1 diabetes mellitus through new clinically accessible "windows" as the retinal nerve tissue and peripheral motor units and to analyze if their dysfunctions are potential early markers of neurodegeneration in patients without peripheral diabetic neuropathy.

2. Materials and Methods

We followed the methods of Picconi et al. [14]. From the Unit of Endocrinology, Diabetes and Metabolism, S. Giovanni Calibita Fatebenefratelli Hospital of Rome, we recruited 20 patients with type 1 DM (T1DM). 14 healthy participants, without history of ocular disease and no family history of glaucoma or any relevant systemic disease, were enrolled as control group (C), from the medical staff of the Medical Retina Unit, G.B. Bietti Eye Foundation-IRCCS, Rome. Inclusion criteria for the type 1 DM patients were (1) documented diagnosis of type 1 DM, according to ADA criteria [15]; (2) age between 18 and 75 years; (3) treated with continuous subcutaneous insulin infusion or with multiple daily insulin injections; and (4) no signs of retinal vasculopathy (noDR) or very mild nonproliferative diabetic retinopathy (NPDR). Exclusion criteria were (1) symptomatic diabetic polyneuropathy not even with positive sensory symptoms such as pain, burning, paresthesia, or prickling; (2) history of possible confounding diseases (alcohol abuse, vitamin deficiency, malignancy treated with chemotherapy agents, central nervous system diseases, entrapment mononeuropathies, and cervical or lumbosacral radiculopathies); (3) a Michigan Neuropathy Screening Instrument [16] score equal to or greater than 2 points; (4) microalbuminuria (urinary albumin/creatinine ratio > 30 mg/g); (5) spherical refractive error > ±3 diopters, astigmatism (cyl) > ±2 diopters, active or past retinal pathologies, diagnosis of glaucoma or ocular hypertension, and opacities of optical media that could influence functional and structural retinal testing; and (6) history of ocular surgery. This study complied with the principles of the Declaration of Helsinki. All subjects gave their written informed consent.

All subjects underwent a general medical examination and anthropometric parameters. After an overnight fast, blood and urine samples were obtained for the determination of laboratory measurements. Each person underwent a complete ophthalmological examination, with determination of best-corrected visual acuity, anterior segment examination, fundus photography, and multifocal electroretinogram (MfERG) recordings. Neurological evaluation was performed at Disimmune Neuropathies Unit, Policlinico Tor Vergata of Rome. All patients underwent electrophysiological examination including bilateral standard sensory motor nerve conduction studies (NCS) and motor unit number estimation (MUNE). Extensive clinical neurological evaluation was performed; strength was assessed by means of Medical Research Council (MRC) sum score (with a maximum score of 60/60 indicating full strength) [17]. The MRC score of the muscles from which MUNE was derived (abductor digiti minimi (ADM) and abductor hallux (AH)) was also calculated.

2.1. MUNE Evaluation. MUNE is an electrophysiological method that can be used to determine the approximate functioning number of motor neuron units or axons innervating a single muscle. In addition, MUNE methods provide a means of measuring motor unit size, enabling tracking of both loss of motor units and the compensatory phenomenon of collateral reinnervation, and have the advantage of measuring the severity of nerve injury in neuropathy with retained CMAP amplitude; MUNE is already used in neuromuscular disorders such as amyotrophic lateral sclerosis, spinal muscular atrophy, and neuropathies to monitor neuronal loss [18, 19].

Recordings are performed using Medtronic Keypoint EMG equipment (Skovlunde Denmark). Limb temperature is maintained between 32 and 34°C by a heating lamp. Filter settings are 2 Hz/10 kHz. The maximal compound motor action potential (CMAP) is obtained by supramaximal stimulation of the peroneal nerve at the lower limbs and of the ulnar nerve at the upper limbs, with constant current square waves at the fibular head site and from the wrist site, respectively. Measurement of the CMAP negative peak area (from the onset of the first negative peak to the first crossing of the baseline) is preferred to peak-to-peak amplitude or negative peak amplitude measurements, as it minimizes the cancellation error [20] and better considers collateral reinnervation phenomena. Recordings are made from the abductor digiti minimi (ADM) for the ulnar nerve and from

the abductor hallux (AH) for the tibial posterior nerve, with a single active surface electrode over the belly of the muscle, an inactive electrode over the tendon, and a ground surface electrode positioned between the recording site and stimulating site, similar to the arrangement for routine NCS. In this study, we determined the MUNE of ADM and AH using the manual incremental method [21], in which the assumption is made that each small, stepwise increase in CMAP amplitude with slight increments of stimulus intensity represented the addition of another single motor unit potential (SMUP) to the growing waveform [22]. A series of consecutive stimuli of progressively higher intensity are therefore applied at the stimulation site to obtain 10 distinct SMUP, elicited in an all-or-nothing manner. The area of each increment is measured and averaged to get the average SMUP area for that nerve and muscle. As the maximal CMAP area represents the total motor unit population firing together, dividing the maximal CMAP area by the average SMUP area yields an estimate of the number of motor units within that nerve. MUNE value is therefore expressed as maximal CMAP area/average SMUP area. In addition to an estimate of motor unit number, the average SMUP size obtained with these methods is also calculated, in order to quantify the extent of collateral reinnervation.

2.2. MfERG Recordings. VERIS Clinic™ 4.9 (Electro-Diagnostic Imaging, San Mateo, California, USA) was used for MfERG assessment using our previously published method [23–25]. The multifocal stimulus, consisting of 61 scaled hexagons, was displayed on a high-resolution, black-and-white monitor (size: 30 cm width and 30 cm height) with a frame rate of 75 Hz. The array of hexagons subtended 20 degrees of visual field. Each hexagon was independently alternated between black (1 cd/m^2) and white (200 cd/m^2) according to a binary *m*-sequence. This resulted in a contrast of 99%. In all eyes, MfERGs were binocularly recorded in the presence of pupils that were maximally pharmacologically dilated with 1% tropicamide to a diameter of 7–8 mm. Pupil diameter was measured by an observer (LZ) by means of a ruler and a magnifying lens and stored for each tested eye. The cornea was anesthetized with 1% Dicaine. MfERGs were recorded bipolarly between an active electrode (Dawson Trick Litzkow (DTL) bipolar contact electrode) and a reference electrode (Ag/AgCl electrode placed on the correspondent temporal side of the frontal lobe). A small Ag/AgCl skin ground electrode was placed at the center of the forehead. Interelectrode resistance was less than 3 KOhms. Binocular MfERG recording was preferred for helping subjects to have a stable target fixation. Eyes that did exhaustively meet the inclusion criteria were selected from each patient. The signal was amplified (gain 100.000) and filtered (band pass 1–100 Hz) by BM 6000 (Biomedica Mangoni, Pisa, Italy). After automatic rejection of artifacts (by VERIS Clinic™ 4.9 software), the first-order kernel response, K1, was examined.

2.2.1. Ring Analysis. MfERG ring analysis was selected to differentiate changes of the bioelectrical responses of the central foveal regions with respect to the more eccentric retinal areas in the macular region. We analyzed the averaged response obtained from three concentric annular retinal regions (rings) centered on the fovea: 0 to 2.5° (ring 1, R1), from 2.5 to 5° (ring 2, R2), from 5 to 10° (ring 3, R3). We also analyzed the responses from unified rings enclosing responses derived from the total area from 0–5° (R1 + R2) and from the fovea up to 10°(R1 + R2 + R3). For each obtained averaged response, we evaluated the amplitude densities (RAD, expressed in nanovolt/degree^2) between the first negative peak, N1, and the first positive peak, P1, and the implicit time (IT) of the first positive peak (P1).

2.2.2. Sector Analysis. MfERG sector analysis was selected to differentiate changes of the bioelectrical responses of the central macular region in 4 quadrants: inferior (I), nasal (N), superior (S), and temporal (T). We considered isolated and combined responses from the foveal center to external areas (sector 1, S1: 0–2.5°; sector 2, S2: 0–5°; and sector 3, S3: 0–10°) and S1 + S2 and S1 + S2 + S3, respectively. A similar analysis was recently adopted to study retinal functional changes in a hereditary ocular pathology [26].

2.3. Laboratory Measurements. Plasma glucose concentrations were measured by the hexokinase method (Modular P Analyzer, Roche). The intra-assay coefficient of variation (CV) was 1.1%, and interassay CV was 1.9%. The sensitivity of the method was 2 mg/dl (0.11 mmol/l). HbA1c was analyzed by high-performance liquid chromatography (VARIANT 2; BioRad Laboratories, Munich, Germany), with intra- and interassay CV of 0.46–0.77 and 0.69–0.91%, respectively. Plasma total cholesterol, high-density lipoprotein cholesterol (HDL chol), and low-density lipoprotein cholesterol (LDL chol) were analyzed with a colorimetric enzymatic method (CHOD-PAP, Roche Diagnostics). The intra-assay CV was 1%, and the interassay CV was 2.7%. The sensitivity of the method was 0.08 mmol/l. Plasma triglycerides were analyzed with a colorimetric enzymatic method (GPO-PAP, Roche Diagnostics). The intra-assay CV was 1.5%, and the interassay CV was 2.4%. The sensitivity of the method was 0.05 mmol/l. Urinary albumin was determined by the Tina-quant immunoturbidimetric assay (Cobas, Roche Diagnostic, Indianapolis, IN) and urinary creatinine by an enzymatic colorimetric test (Beckmann Coulter, California, USA). C underwent an oral glucose tolerance test, to exclude diabetes and impaired glucose tolerance.

3. Statistical Analysis

The main statistical analysis aimed at assessing the difference among T1DM and C groups in terms of MfERG RADs and MUNE. Thus, a general linear model was applied, allowing to adjust for eventual demographic, anthropometric, and metabolic differences.

Correlations among interval variables were measured through Pearson's index, after appropriate log-transformation when necessary. In order to verify the robustness of correlations, influence statistics (standardized DfBetas) were

Table 1: Clinical and laboratory characteristics of controls (C) and subjects with diabetes (T1DM), mean (SD).

	C $n = 14$	T1DM $n = 20$	p value
Gender (M/W)	5/9	9/11	$p = 0.588$
Age (yrs)	39.07 (14.4)	42.3 (12.4)	$p = 0.721$
Diabetes duration (yrs)	—	17.9 (9.5)	—
BMI (kg/m^2)	22.5 (±2)	24.9 (± 2.5)	$p < 0.001$
Glycemia (mmol/l)	4.9 (0.6)	8.9 (1.4)	$p < 0.0.001$
HbA1c (%) (mmol/mol)	—	7.5 (0.8) 58 (15)	—
Tot chol (mmol/l)	4.0 (0.6)	4.3 (0.44)	$p = 0.089$
HDL chol (mmol/l)	1.7 (0.2)	1.5 (0.2)	$p = 0.002$
Trigl (mmol/l)	0.8 (0.1)	0.75 (0.1)	$p = 0.08$
Microalb/creat (mg/gr)	—	7.2 (5.2)	—
Sural nerve (lateral malleolus)	—	Distal SNAP amp (μV) 13.4 (±4.3)	—
		SCV (ms) 56.4 (±6.9)	—
Tibial nerve (AH)*	—	Distal CMAP latency (ms) 3.2 (±0.4)	—
		Distal cMAP amplitude (μV) 11.9 (±4.6)	—
		MCV (ms) 47.3 (±4.5)	—
Ulnar nerve (ADM)*	—	Distal CMAP latency (ms) 2.2 (±1.7)	—
		Distal cMAP amplitude (μV) 9.8 (±1.7)	—
		MCV (ms) 59.7 (±5.3)	—

*No patient showed abnormalities (temporal dispersion or conduction block) in intermediate and proximal nerve segments. M: men; W: women; BMI: body mass index; HbA1c: hemoglobin glycated; Tot chol: total cholesterol; HDL chol: high-density lipoprotein cholesterol; Trigl: triglycerides; Microalb/creat: microalbuminuria/creatininuria; AH: abductor hallux; ADM: abductor digiti minimi; SNAP: sensory nerve action potential; MCV: motor conduction velocity; SCV: sensory conduction velocity; t-test. Statistical significance $p < 0.05$.

computed and, in case of outliers, correlations were computed after their elimination.

4. Results and Discussion

Clinical and laboratory characteristics of the diabetic patients and of the C group are reported in Table 1. Seven out of 20 patients had mild NPDR. The T1DM and C groups were not different, except for body mass index (BMI), fasting glucose, and HDL cholesterol levels. All patients had an MRC score of 60/60 at neurological examination indicating full strength. From the MfERG analysis, the mean MfERG RADs of R1 (0–2.5°), R2 (2.5–5°), and R3 (5–10°) differed significantly between the C and diabetic groups ($p < 0.01$). Combined sector analysis of mean MfERG RADs from S1 + S2 (0–5°) and S1 + S2 + S3 (0–10°) in superior, temporal and nasal sectors showed significantly reduced values in T1DM subjects vs. C ($p < 0.05$). There was also a reduced, but not statistically significant, RAD value from the inferior sector (0.055). In addition, significant sector X group interaction was found ($F(3,81) = 6.07$; $p = 0.001$), since the difference between DM and C was larger in the temporal and nasal sectors with respect to the superior and inferior sectors (consistently, $p < 0.005$) (Figure 1). For the IT parameter, no significant differences were found between the C and diabetic groups. No significant differences were found between noDR and NPDR patients for both RADs and ITs.

No abnormalities of conventional NCS were found in any subject. The number of motor units was significantly decreased in both the lower and upper limbs in T1DM vs. C (ADM MUNE: 82.55 ± 54.37 vs. 126.96 ± 65.85, $p = 0.039$; AH MUNE 101.87 ± 41.09 vs. 199.90 ± 69.81, $p < 0.001$), while AH SMUP was significantly increased in T1DM vs. C (0.55 ± 0.17 vs. 0.35 ± 0.13 μV/msec, $p = 0.001$) (Figures 2 and 3).

Since BMI and HDL chol were significantly different among the two groups, we added these variables as covariates in the previous analyses and the patterns remained stable.

A positive correlation between the mean MfERG RADs of S1 + S2 of the four sectors (S, I, T, and N) and AH MUNE ($r = 0.50$, $p = 0.041$; $r = 0.64$, $p = 0.005$; $r = 0.64$, $p = 0.006$; and $r = 0.61$, $p = 0.010$, respectively) was observed in

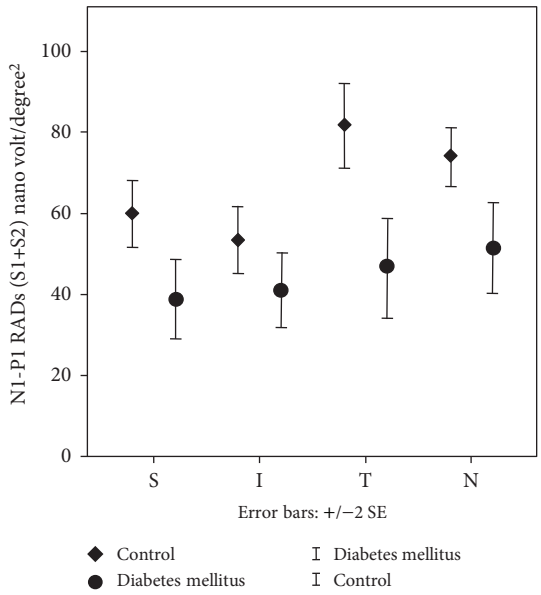

Figure 1: Combined sector analysis of mean MfERG RADs from S1 + S2 (0–5°) in the superior, temporal, and nasal sectors showed significantly reduced values in T1DM subjects vs. C ($p < 0.05$). There was also a reduced but not statistically significant RAD value from the inferior sector (0.055). In addition, significant sector X group interaction was found ($F(3,81) = 6.07$; $p = 0.001$), since the difference between DM and C was larger in the temporal and nasal sectors with respect to the superior and inferior sectors (consistently, $p < 0.005$) N1-P1 RADs are defined as amplitude densities between the first negative peak, N1, and the first positive peak, P1. S: superior; I: inferior; T: temporal; N: nasal macular quadrants.

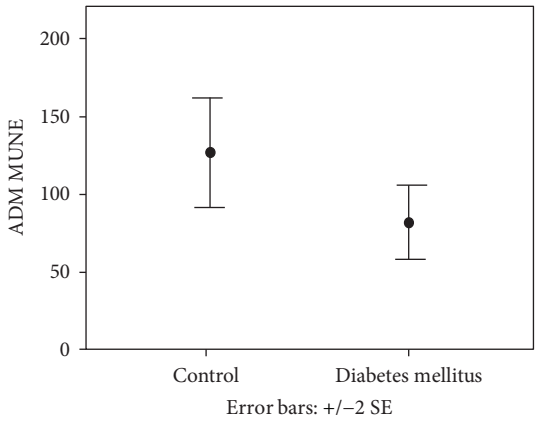

Figure 2: Motor unit number estimation (MUNE) (mean ± SEM) of abductor digiti minimi (ADM) in the diabetic groups and controls. ADM MUNE was significantly decreased in T1DM vs. C ($p < 0.05$).

T1DM patients (Figure 4). Since such correlations could be significantly affected by outliers, influence statistics (standardized DfBetas) were computed. After eliminating cases with values higher than the cutoff (2/sqrt(n)), r correlations resulted similar ($r = 0.49$, $p = 0.065$; $r = 0.64$, $p = 0.015$; $r = 0.64$, $p = 0.013$; and $r = 0.52$, $p = 0.048$, respectively).

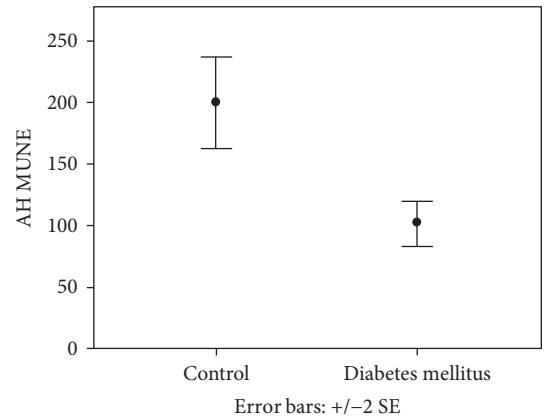

Figure 3: Motor unit number estimation (MUNE) (mean ± SEM) of abductor hallux (AH) in the diabetic groups and controls. AH MUNE was significantly decreased in T1DM vs. C ($p < 0.001$).

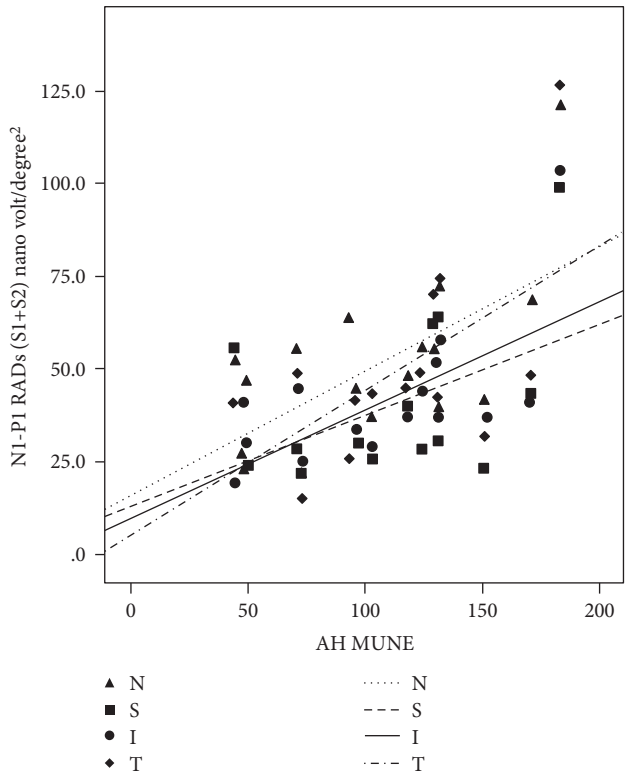

Figure 4: Scatter plot between MfERG RADS of S1 + S2 of the N (nasal), S (superior), I (inferior), and T (temporal) sectors and motor unit number estimation of the abductor hallux (AH MUNE) in type 1 DM patients. A positive correlation between the mean MfERG RADs of the four quadrants (S, I, T, and N) and AH MUNE ($r = 0.50$, $p = 0.041$; $r = 0.64$, $p = 0.005$; $r = 0.64$, $p = 0.006$; and $r = 0.61$, $p = 0.010$, respectively) was observed in T1DM patients. Since such correlations could be significantly affected by outliers, influence statistics (standardized DfBetas) were computed. After eliminating cases with values higher than the cutoff (2/sqrt(n)), r correlations resulted similar ($r = 0.49$, $p = 0.065$; $r = 0.64$, $p = 0.015$; $r = 0.64$, $p = 0.013$; and $r = 0.52$, $p = 0.048$, respectively).

We did not observe a significant correlation between the HbA1c and metabolic parameters and retinal macular function or MUNE (consistently, $p > 0.2$).

In this study, the diabetic subjects were highly selected without DPN, both clinically and after routine electrophysiological examination. Moreover, the good glycemic control and the absence of other microvascular complications and comorbidities allowed to perform these evaluations in the absence of confounding factors. Despite this, we observed a significant reduction in the number of motor units of both the ADM muscle (upper limb) and, more pronounced, the AH muscle (lower limb), consistent with length-dependent neurodegeneration expected in diabetes. The MUNE technique is increasingly used in studies on amyotrophic lateral sclerosis, but it is more rarely used in other peripheral nerve disorders, including diabetic neuropathy [6, 27–30].

Our findings imply that loss of motor units occurs early in the neurodegenerative process in diabetic patients and MUNE can detect motor unit abnormalities, even in the absence of signs of diabetic neuropathy at clinical exam and conventional NCS. Similarly to what is observed for epidermal nerve fiber densities, the dying back of motor nerve terminals occurs early and in the absence of clinical sensory-motor symptoms and signs in DM [31]. We also observed an increase in AH SMUP in the diabetic subjects compared to the control group. In diabetic peripheral neuropathy, a process of chronic denervation, the germination of small intact motor units can lead to larger SMUPs. We know that a motor unit includes a single motor neuron and the group of muscle fibers that it innervates. The increase in the average size of motor units suggests a preferential loss of smaller motor units or the enlargement of these units by compensatory germination [32]. The size of the motor units can also increase with axonal sprouting, which reinnervates the denervated muscle fibers to compensate for the loss of adjacent functional motor units [32–34]. The findings of concurrent AH SMUP enlargement can account for the existence of a similar process already ongoing in these asymptomatic diabetic patients. The only electrophysiological parameter currently used to describe the neuronal loss in neuropathies is the amplitude of distal cMAP, but this parameter remains into normal range until the reinnervation process is effective. Only when a marked loss of the motor units is reached, the force production will decrease in parallel, resulting in the reduction of cMAP amplitude in conventional NSC studies and muscle weakness as described in diabetic patients with more advanced peripheral neuropathy. Then, MUNE may represent an early noninvasive marker of a subclinical DNP. Such a marker has potential therapeutic implications, allowing an early treatment approach for DPN, when the probability of regeneration of sensorimotor fibers is still good [6].

We also showed that multifocal electroretinogram is a valuable tool in order to examine local neuroretinal dysfunction in T1DM with or without diabetic retinopathy. Therefore, it is able to detect functionally the most vulnerable areas, even in the absence of ophthalmoscopic early signs of retinal abnormalities. MfERG allows for the simultaneous recording of the activity of bipolar cells with small contributions from photoreceptors from different areas of the retina [35]. In diabetic population studies, the use of MfERG [36–39] identified significant abnormalities in retinal function, characterized by increased peak latencies and/or reduced amplitudes, suggesting a compromised inner retinal function, secondary to neuronal transmission alterations [40]. The topographic mapping of neuroretinal dysfunction by MfERG has been shown to be predictive of the onset of DR [36] and is able to detect these abnormalities earlier than morphological studies [41, 42]. Recently, in adolescents with T1DM and noDR, an early alteration of the inner retina, confirmed also by a delay of IT in MfERG recordings, has been observed [43]. The nasal retina had abnormal IT compared with the temporal retina, whereas alteration of the amplitude parameter was more evident in the temporal retina in T1DM adolescents [44]. Moreover, Holm and Adrian [45] described that the nasal area of the macula, where there is a higher density of cones and ganglion cells, was more vulnerable to neurodegenerative processes than the temporal region, showing a lower amplitude and longer implicit time in this specific area with the MfERG analysis. The innovative MfERG sector analysis allowed to identify the specific retinal areas of neuroretinal damage that in our group of patients are mainly represented by the nasal and temporal sectors, anatomically crucial regions for the function of collector cells to the small axons of the optic nerve forming the papillomacular bundle. The neurodegenerative theory, for which the photoreceptors are involved early in the course of diabetes in patients, has been also supported by recent *in vivo* studies using adaptive optics ophthalmoscopy [46]. The authors have shown that early pathological disruption of the parafoveal cone mosaic in patients with type 1 diabetes, even before any sign of diabetic retinopathy, was found on fundoscopy. Furthermore, through the use of spectral domain optical coherence tomography (SD-OCT) analysis, we recently observed an increased macular thickness of the inner nuclear layer (INL) and a decrease in the retinal nerve fiber layer (RNFL) thickness in the nasal quadrant of the macular area in T1DM persons with noDR and NPDR, compared to healthy groups. In order to analyze the role of glycemic control on the neuroretina, we also found that only glycemic variability was associated with abnormalities of these specific retinal layers, while no association was observed with HbA1c [14]. Finally, we studied the relationship between functional changes in the neuroretina and early signs of peripheral neuropathic damage. We have found that motor unit loss was associated with the amplitude densities' reduction in all four macular sectors, from the foveal center up to the 5° external areas. At the moment, the available evidence on the link between retinal neurodegeneration and diabetic neuropathy is scanty; it is related exclusively to morphological evidence and identifiable especially in DM1 or DM2 patients already affected by peripheral neuropathic damage diagnosed with standard examinations [47–51]. Recently, in a prospective study, defining longitudinal alterations to the RNFL thickness of the optic nerve head in individuals with DM1, with and without DPN, patients with DPN showed a progressive global RNFL thinning, especially in the superior quadrant. Therefore, it is possible to hypothesize common pathways for retinal and peripheral

neurodegeneration that are independent of DPN risk factors [51]. Polyol pathway activation, hexosamine pathway and protein kinase C (PKC) isoform activation, and accumulation of advanced glycation end products (AGEs) resulting in imbalance of the mitochondrial redox state and in excess formation reactive oxygen species may represent common mechanisms to neuroretinal and peripheral neurodegeneration. Furthermore, a key role of the glial component has been described in the early stages of both neuronal and neuroretinal damages [14, 52, 53]. Despite promising evidence linking morphological alterations of the neuroretina to the overt presence of diabetic neuropathy, a functional analytical approach is required to identify and characterize initial stages of neuropathic damage. Very few studies have investigated the role of early outer retinal deficit at the base of retinal neurodegeneration [46], and, to our knowledge, this is the first work that allowed to observe a relationship between this dysfunction at the early peripheral neuropathic damage. In light of this, our result of an association between neuroretinal (photoreceptors and bipolar cells) dysfunction and subclinical damage of the peripheral nerve in asymptomatic diabetic subjects seems particularly interesting. MfERG recording could represent an accessible, noninvasive, and well-tolerated tool in the detection also of neuronal damage in diabetic patients. However, our study has some limitations: small sample size and the presence in the diabetic population of subjects with diabetic vascular retinopathy, although not proliferating and of a very mild degree, the lack of an association with standardized methods of early DN diagnosis, such as skin biopsy. The inclusion of a cohort of patients with overt DN or a longitudinal observation could allow to understand the associative link between the two neurodegeneration processes. Nevertheless, we believe that the identification of these potential markers of very early neuropathic damage in diabetes (retinal neurodegeneration and morphofunctional alterations of the motor unit) is an important point of strength of our study. Our findings, therefore, strongly support a new vision of the neuropathic damage in diabetes, as an overall neuronal damage.

5. Conclusions

In conclusion, motor unit loss and neuroretinal dysfunction are already present in T1DM patients without DPN. The relationship between neuroretinal dysfunction and early peripheral motor unit decline supports the hypothesis that the neuroretina is a potential "window" onto the early neurogenic process, in diabetes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Parts of this study were presented in abstract form during a poster presentation at the 27th Annual Scientific Meeting of Neurodiab, Coimbra, Portugal, 9–11 September 2017, and as a poster presentation of the 53rd European Association for the Study of Diabetes meeting, 11–15 September 2017, Lisbon, Portugal.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Fabiana Picconi and Giorgia Mataluni have equally contributed.

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Review Article

Diabetic Enteropathy: From Molecule to Mechanism-Based Treatment

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The incidence of the micro- and macrovascular complications of diabetes is rising, mirroring the increase in the worldwide prevalence. Arguably, the most common microvascular complication is neuropathy, leading to deleterious changes in both the structure and function of neurons. Amongst the various neuropathies with the highest symptom burden are those associated with alterations in the enteric nervous system, referred to as diabetic enteropathy. The primary aim of this review is to provide a contemporaneous summary of pathophysiology of diabetic enteropathy thereby allowing a "molecule to mechanism" approach to treatment, which will include 4 distinct aspects. Firstly, the aim is to provide an overview of the diabetes-induced structural remodelling, biochemical dysfunction, immune-mediated alterations, and inflammatory properties of the enteric nervous system and associated structures. Secondly, the aim is to provide a synopsis of the clinical relevance of diabetic enteropathy. Thirdly, the aim is to discuss the various patient-reported outcome measures and the objective modalities for evaluating dysmotility, and finally, the aim is to outline the clinical management and different treatment options that are available. Given the burden of disease that diabetic enteropathy causes, earlier recognition is needed allowing prompt investigation and intervention, which may lead to improvements in quality of life for sufferers.

1. Introduction

The increasing incidence of both type 1 and type 2 diabetes elevates the complications of diabetes as one of the most important current public health issues [1], which causes negative impact on the individual quality of life and increased socioeconomic expenditure. Amongst the diabetic complications with the highest symptom burden, yet frequently underrecognised and suboptimally treated, are those associated with alterations in the enteric nervous system (ENS), hereinafter referred to as diabetic enteropathy. This review will focus on a "molecule to mechanism" approach of diabetic enteropathy and mechanism-based treatments.

2. The Enteric Nervous System

This review will provide a detailed summary of the remodelled and dysfunctional wall of the gastrointestinal (GI) tract and the resulting pathological complications. These include (1) reduced number of intrinsic enteric neurons, (2)

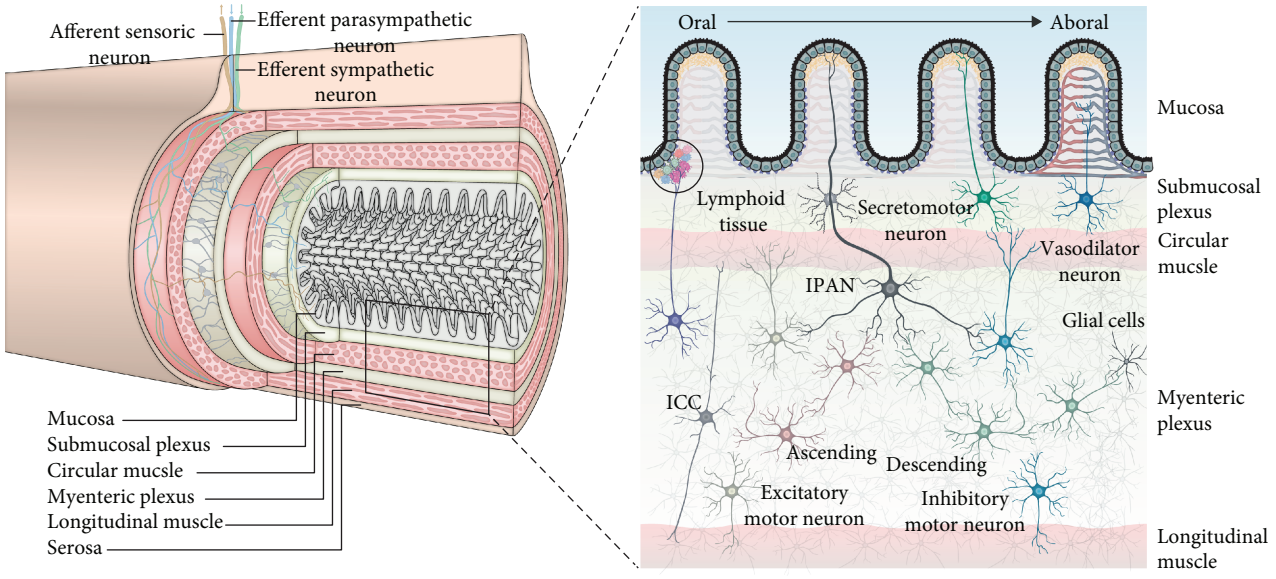

Figure 1: The enteric nervous system. (a) Cross-sectional view. The enteric nervous system (ENS) is embedded in the wall of the GI tract. The neurons are localized in the myenteric and submucosal plexi and are connected by interneurons (depicted in grey). Extrinsic efferent innervation via autonomic sympathetic (green) and parasympathetic (blue) pathways contributes to the regulation and coordination of GI function. Extrinsic afferent sensory nerves (orange) following either vagal or spinal routes provide the central nervous system with information about GI homeostasis. (b) Longitudinal view illustrating a selection of neuronal subtypes. Secretomotor and vasodilator neurons regulate fluid and molecular exchange between gut lumen, tissue, and vasculature. Peristaltic movements (oral contraction and aboral relaxation of intestinal smooth muscle) are facilitated by intrinsic primary afferent neurons (IPANs) activating ascending and descending interneurons, which then activate upstream excitatory and downstream inhibitory motor neurons, respectively. IPANs may initially be activated, e.g., through mechanoreceptors or by acetylcholine secreted by enteric endocrine cells in the luminal epithelial cell layer upon luminal distension. In addition, ENS includes the innervation of gastroenteropancreatic endocrine cells (not shown) and gut-associated lymphoid tissue, responsible for hormone secretion and transmitter release. Although not equally represented, the juxtapositioned networks of enteric glial cells (EGCs) and interstitial cells of Cajal (ICCs) are present in all layers of the GI wall. Note that the thickness of the different tissue layers is not proportionally represented.

structural neuronal changes, (3) intraneuronal biochemical changes, (4) diminished secretion of neurotransmitters, (5) altered immunomodulatory function of the enteric glial cells, (6) neuroinflammation, and (7) altered gut-brain communication through spinal afferents and vagal terminals. These concomitant changes cause altered GI motility and secretory functions and explain—at least partly—the development and maintenance of nausea/vomiting, bloating, early satiety, diarrhoea, constipation, and abdominal pain.

The ENS consists of a complex network of neurons and enteric glial cells (EGCs), which are embedded in the wall of the GI tract. The neurons are localized in the myenteric and submucosal plexi, which are connected by interneurons. The myenteric plexus is situated between the circular and longitudinal muscle layers and influences GI motility. The submucosal plexus is in close proximity to the muscularis mucosae, intrinsic vasculature, and the mucosa [2] (Figure 1(a)) and regulates the secretion of hormones and neurotransmitters. Furthermore, local sensory neurons called intrinsic primary afferent neurons (IPANs) regulate motility and maintain homeostasis. The ENS is supplemented with extrinsic efferent input from the central nervous system via autonomic (both sympathetic and parasympathetic) pathways which also contribute to the regulations and coordination of GI function [3]. Although the majority of enteric afferent axons are confined to the gut wall, a large amount of sensory neurons from the CNS following either vagal or spinal routes have receptive fields in different layers of the GI wall and monitor GI homeostasis [4]. Approximately 80–85% of the nerve fibres in the vagus nerve are afferent and project viscerotopically to the nucleus of the solitary tract [5].

Neurons of the ENS can be categorised according to their connectivity and function (Figure 1(b)). The interstitial cells of Cajal (ICCs), whilst not strictly neuronal, generate and convey electrical impulses to smooth muscle cells facilitating the slow wave peristaltic movement of the stomach and intestines and are referred to as "pacemaker" cells [6].

In summary, the ENS comprises of three panenteric juxtapositioned networks, namely, neurons, EGCs, and ICCs. The detailed role of EGCs is discussed below; however, both enteric neurons and EGCs are particularly vulnerable to hyperglycaemia.

3. Diabetic Enteropathy

Diabetes significantly alters the microenvironment within the ENS due to the effect of, amongst other hyperglycaemia, oxidative stress, neuroinflammation, reduced levels of nerve growth factors, and structural vascular changes [7–9]. In

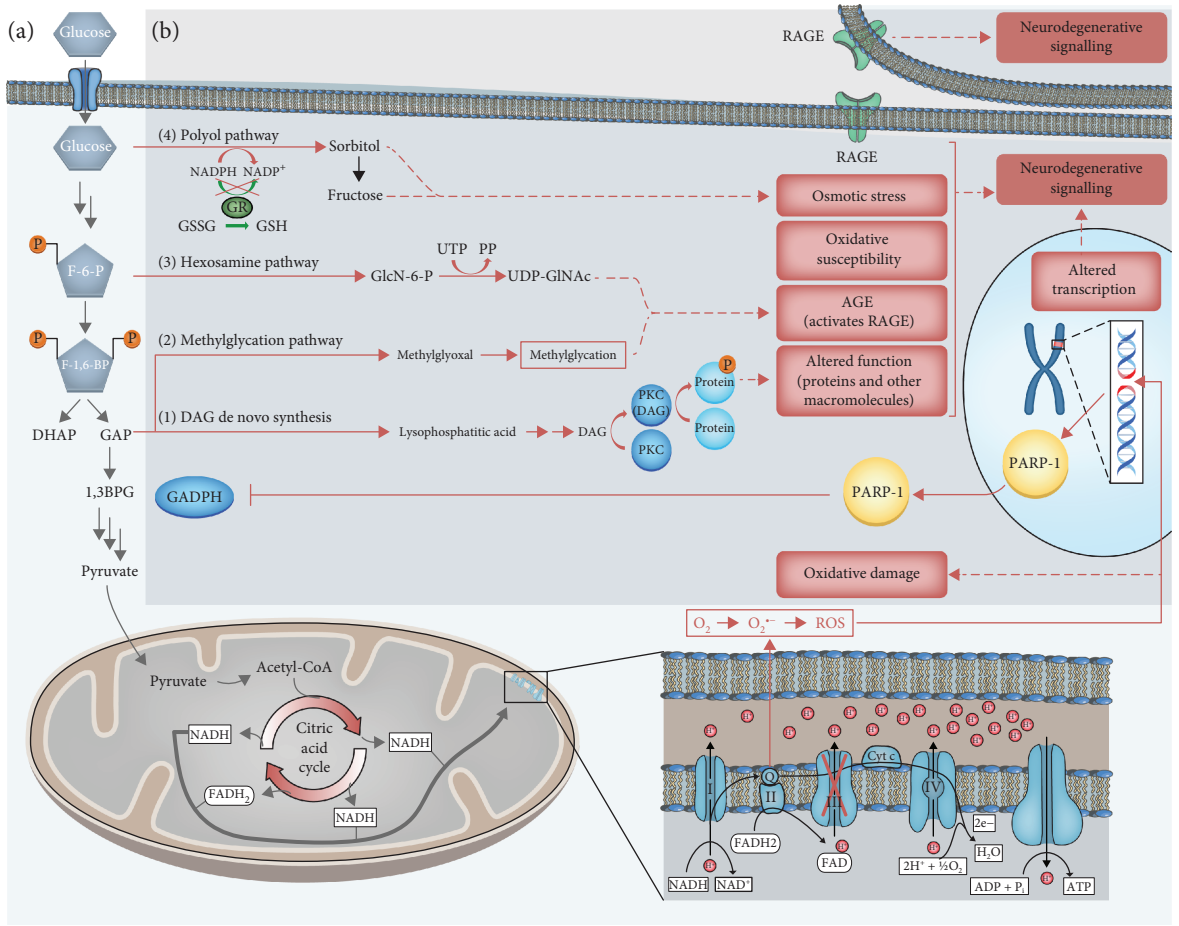

Figure 2: Hyperglycaemia induced intracellular biochemical changes in neurons. (a) Generation of ROS. (b) Consequences of ROS generation. See text and Table 1 for explanation. Abbreviations: 1,3BPG: 1,3-bisphosphoglyceric acid; Acetyl-CoA: acetyl coenzyme A; ADP: adenosine diphosphate; AGE: advanced glycation end products; ATP: adenosine triphosphate; DAG: diacylglycerol; DHAP; dihydroxyacetone phosphate; e^-: electron; F-1,6-BP: fructose-1,6-bisphosphate; F-6-P: fructose-6-phosphate; FAD: flavin adenine dinucleotide (oxidised); $FADH_2$: flavin adenine dinucleotide (reduced); GAP: glyceraldehyde 3-phosphate; GAPH: glyceraldehyde 3-phosphate dehydrogenase; GlcN-6-P: glucosamine 6-phosphate; GR: glutathione reductase; GSH: glutathione; GSSG: glutathione disulphide; H^+: proton; NAD^+: nicotinamide adenine dinucleotide (oxidised); NADH: nicotinamide adenine dinucleotide (reduced); $NADP^+$: nicotinamide adenine dinucleotide phosphate (oxidised); NADPH: nicotinamide adenine dinucleotide phosphate (reduced); O_2: oxygen: $O_2^{\bullet-}$: superoxide; P: phosphor group; PARP-1: poly(ADP-ribose) polymerase 1; PKC: protein kinase C; PP: diphosphate; RAGE: receptor for advanced glycation end products; ROS: reactive oxygen species; UDP-GlcNAc: uridine diphosphate N-acetylglucosamine; UTP: uracil triphosphate.

addition, other aspects such as increased levels of fatty acids, miRNA, endothelia dysfunction or altered enteric microbiota also have been proposed to exert an influence [10] although these are outside the scope of this review.

3.1. Diabetes and Intracellular Biochemical Changes. Neurons have continuously high glucose demand. They cannot allow glycolytic and anaerobic episodes and are further provided with a physiology that fails to regulate episodic glucose uptake under the influence of insulin. Therefore, the neuronal glucose uptake and utilization is highly dependent on the extracellular glucose concentration and facilitated diffusion mediated by primarily glucose transporter 3 (GLUT3); however, other forms including GLUT1, GLUT4, and GLUT8 are also present [11]. Hyperglycaemia in diabetes causes up to fourfold increases in glucose levels, and if this is persistent or repetitive, then intracellular glucose metabolism leads to neuronal damage often referred to as glucose neurotoxicity [12]. These mechanisms are primarily described in the peripheral and central nervous systems, but the same mechanisms are present in the enteric nervous system.

The increased glucose flow through the glycolytic pathway leads to increased levels of pyruvate, which is oxidised in the citric acid cycle. This initiates a continuous elevated flux of electron donors (NADH and $FADH_2$) into the electron transport chain. Subsequently, this leads to an increased voltage gradient across the inner mitochondrial membrane, caused by the efflux of protons from the mitochondrial matrix into the intermembrane lumen by complexes I, III, and IV [13]. At a critical membrane potential threshold, the electron transfer of complex III stalls [13], causing coenzyme Q to donate electrons to molecular oxygen, which generates superoxide, denoted $O_2^{\bullet-}$ (Figure 2(a)). Superoxide is a reactive oxygen species and drives the delirious effects of

Table 1: Accumulation of upstream glycolytic intermediates and their consequences.

Glycolytic intermediate	Alternative pathway	Consequence
Glyceraldehyde-3-phosphate	(1) De novo synthesis of DAG	DAG activates protein kinase C resulting in altered intracellular phosphorylation levels
	(2) Glycosylation pathways	Glycation of various intra- and extracellular proteins and lipids*
Fructose-6-phosphate	(3) Hexosamine pathway	
Glucose	(4) Polyol pathway	Leads to depletion of intracellular NADPH**

* leads to formation of advanced glycation end products (AGE). ** renders the neuron susceptible to oxidation.

increased intracellular glucose concentrations, which ultimately leads to oxidative stress and tissue damage. Interestingly, different populations of neurons have differing degrees of susceptibility to glucose-initiated oxidative stress, which results in pleomorphic neurological sequelae.

Animal models have shown that during hyperglycaemic episodes, the extrinsic sympathetic supply, via coeliac and superior mesenteric ganglia to the ENS, is more sensitive than those deriving from the superior cervical ganglion [14]. Such susceptibility has a number of consequences (Figure 2(b)). Reactive oxygen species cause DNA double-strand breaks, which in return activates DNA repair mechanisms, including the enzyme PARP-1. Activated PARP-1 inhibits the key glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase. This causes the accumulation of upstream glycolytic intermediates [15], which then are diverted into alternative and ultimately pathogenic pathways (Table 1). Glycation of various intra- and extracellular proteins and lipids ultimately results in the formation of advanced glycation end products. Advanced glycation end products activate receptor for advanced glycation end products on the affected and surrounding cells, including myeloid cells, initiating inflammatory and hence neurodegenerative signalling [16]. Depletion of intracellular NADPH renders the neuron susceptible to oxidative damage due to lacking regeneration of the antioxidant glutathione, thereby resulting in a "vicious" cycle.

Over time, the biochemical alterations coalesce with neuronal structural changes and endothelial dysfunction to drive the pathological development of diabetic neuropathy.

3.2. Diabetes and Enteric Neuroinflammation and Oxidative Stress. Inflammation and oxidative stress are two synergistic conditions, which have a significant negative impact on the function of the ENS. *In vitro* studies of human EGCs have demonstrated that inflammation induces proinflammatory pathways leading to alterations in functional signalling pathways linked to GI motility such as mechanical-evoked Ca^{2+} and purinergic signalling [17], indicating that GI dysfunction may indeed be related to inflammation. Whilst such findings have not been comprehensively studied in clinical populations of diabetes, animal models have shown associations between increased oxidative stress and gastroparesis, which could be prevented by treatment with antioxidants [18]. Similar observations have been made in the jejunal tissue of rats with diabetes, where loss of both neurons and EGC was significantly reduced after 120 days of supplement with the antioxidant quercetin [19]. However, trials of antioxidants in adults with diabetes have, to our knowledge, not yet been successful in preventing or improving GI symptoms.

3.3. Diabetes and Structural Neuronal Changes. In animal models of streptozotocin-induced diabetes (streptozotocin is a drug that has preferential toxicity against pancreatic β cells), there is marked degeneration, coupled with a reduction in the density, of neurons in the myenteric plexus [20–24]. In adults with diabetes, there is a reduction in the quantity of colonic ENS, assessed as a total ganglion area by immunohistochemical staining, in comparison to healthy controls [25]. Notably, autonomic neurons, including the ENS, are particularly vulnerable to hyperglycaemia [26]. It has been suggested that diabetes preferentially affects large fibre neurons in the dorsal root ganglion and inhibitory neurons in the gut wall. In particular, selective loss of nitric oxide synthase and neuropeptide Y-expressing inhibitory neurons has been shown in human diabetic colon [25]. However, since the overall motility, coordination, and GI homeostasis are affected in diabetes, it is plausible that IPANs are also vulnerable to chronic hyperglycaemia. Moreover, degeneration and/or loss of ICCs throughout the GI tract has been reported in both animal models and in patients [18, 27], causing reduced frequency of spontaneous muscular contractions. Finally, smooth muscle myopathy [28] and angiopathy [8] are considered a contributing factor in the development of diabetic enteropathy. Taken together, diabetes induces marked structural remodelling of the wall of the GI tract and its neuronal support leading to altered function of the GI tract.

3.4. Diabetes and Immunomodulatory Involvement of Enteric Glial Cells. Besides providing neurotrophic support, EGCs mediate interactions between enteric neurons and other cell types. Through a number of processes, they communicate with immune effector cells, enteroendocrine cells, epithelial cells, and blood vessels, forming a "circuit" that specialises in the control and integration of bidirectional signals from neurons to other cells [29]. Although EGCs exert immunosuppressive and anti-inflammatory effects protecting the ENS against intraluminal foreign antigens, the physiological role of each subtype is still incompletely understood [30]. For example, it has been shown that in diabetes, loss of EGCs throughout the GI tract influences GI function directly. Associated to this, a decreased secretion of neurosupportive factors has been observed [31]. For instance, glial cell line-derived neurotrophic factor mediates differentiation and migration of enteric neurons as well as survival and protection against the adverse effects of hyperglycaemia through

the activation of the neuron-specific Ret tyrosine kinase receptor and coupled PI3K and MAPK pathways in neurons [31, 32]. Taken together, loss of EGC leads to neuronal neglect and apoptosis in the diabetic ENS.

4. Clinical Aspects

As previously described, diabetes results in the loss of neurons causing dysmotility and altered secretion within the entire GI tract and therefore diabetic enteropathy should be considered as a panenteric disorder. For example, oesophageal motor disorders in persons with diabetes has a reported prevalence of up to 63%, which is greater than that of gastroparesis (13%) [33]. A common secondary complication to oesophageal motor disorders is gastroesophageal reflux disease. However, the most thoroughly described GI complication is gastroparesis, defined as delayed gastric emptying in the absence of mechanical gastric outlet obstruction. It is estimated that 5% of adults with type 1 diabetes and 1% of adults with type 2 diabetes develop gastroparesis after 10 years of disease duration [34]. The cardinal symptoms of gastroparesis are early satiety, postprandial fullness, bloating, nausea, pain, vomiting, and weight loss [35]. However, there is considerable interindividual variability in symptoms between patients, with symptom severity being related to the duration of diabetes [35] and poor glycaemic control [35, 36]. From a mechanistic point of view, acute hyperglycaemia reduces the rate of gastric emptying and increases the sense of fullness during gastric distension directly [37, 38]. Changes in gastric emptying lead to unpredictable delivery of nutrition (and thus glucose) and oral pharmacotherapeutic agents into the small bowel [39]. However, it is not known whether chronic poor glycaemic control is the cause or the consequence of gastroparesis, but in reality, it is likely that these factors interact with one another. Notwithstanding the significant symptom burden, gastroparesis is also associated with significant healthcare expenditure. Notably, clinical examinations and hospitalizations due to gastroparesis are increasing as well as the length of stay [40].

The correlation between visceral neuropathy and GI symptoms remains incompletely understood [41]. Lower GI symptoms in adults with diabetes are common, with a twofold increase in the risk of experiencing constipation, diarrhoea, and faecal incontinence [34]. The prevalence increases with poor glycaemic control, and both hard stools and faecal incontinence are reported four times more often in patients with poor diabetes regulation than in those who are with well-regulated diabetes [34]. Several studies have compared the prevalence of GI symptoms amongst adults with diabetes types 1 and 2, but no consistent differences have been found [35].

5. Patient-Reported Outcome Measures

Although patients' experience and perceptions are central to the clinical evaluation, patient-reported outcome measures/questionnaires are helpful in both research and the longitudinal monitoring of response to interventions. The most commonly used are Patient Assessment of GI Disorders-Symptom Severity Index (PAGI-SYM), Gastroparesis Cardinal Symptom Index (GCSI), Gastrointestinal Symptom Rating Scale (GSRS), the Patient Assessment of Constipation Symptoms (PAC-SYM), and Patient Assessment of Constipation-Quality of Life (PAC-QoL).

5.1. PAGI-SYM. PAGI-SYM assesses the severity of common upper GI symptoms. This validated instrument contains 20 items and assesses six subscales: heartburn/regurgitation, postprandial fullness/early satiety, nausea/vomiting, bloating, upper abdominal pain, and lower abdominal pain. This questionnaire allows monitoring of outcomes in clinical practice and trials and is a reliable instrument in subjects with gastroesophageal reflux disease, dyspepsia, or gastroparesis [42].

5.2. GCSI. GCSI consists of three subscales measuring nausea/vomiting, postprandial fullness/early satiety, and bloating derived from the PAGI-SYM. It is based on a 2-week recall, has been validated, and is reliable in assessing symptom severity related to gastroparesis [43]. Conflicting results have been reported concerning the association between upper GI symptoms, as measured by GCSI, in diabetes and objective measures of gastroparesis, e.g., scintigraphic measures of gastric emptying [44–46]. However, the recent study confirmed that the severity of early satiety and postprandial fullness are associated with prolonged gastric emptying [47]. To evaluate the responsiveness to treatment of gastroparesis in clinical trials, the Gastroparesis Cardinal Symptom Index-Daily Diary (GCSIDD) was developed and validated.

5.3. GSRS. GSRS evaluates a wide range of GI symptoms. The questionnaire contains 15 items which are combined into five symptom clusters, namely, reflux, abdominal pain, indigestion, diarrhoea, and constipation [48]. However, the link between GSRS score and objective measures needs a further study. In a clinical setting, the GSRS gives a broader perspective on patients' panenteric GI symptoms in general, in comparison to the GCSI score, which focus on gastroparesis.

5.4. PAC-SYM and PAC-QoL. PAC-SYM and PAC-QoL were developed to evaluate symptom severity and quality of life in patients with constipation. The PAC-SYM is composed of 12 items with three subscales: abdominal symptoms, stool symptoms, and rectal symptoms. It is valid and reliable in the assessment of the presence and severity of constipation symptoms in adults over time as well as the ability to distinguish between responders and nonresponders to treatment [49]. A modified version (M-PAC-SYM) excluding item 7 (rectal bleeding/tearing) has been developed for patients with chronic constipation [49] and may be more relevant for the evaluation of functional constipation in diabetes. The PAC-QoL is a validated and consistent questionnaire [50]. It includes 28 items forming four subscales (worries and concerns, physical discomfort, psychosocial discomfort, and satisfaction) and an overall scale, and thus, it is comprehensive in assessing the burden of constipation on patients' well-being and everyday functioning. In diabetes, there is an existing knowledge gap on the presence of prolonged colonic transit and constipation and the potential implication on the experienced burden. Moreover, bioavailability

of nutrition and on pharmacotherapeutic and glycaemic control warrants further investigation [51].

6. Modalities for Assessing Motility

A number of modalities exist for objectively evaluating diabetic enteropathy. For detailed assessment of GI disorders, objective investigations are a necessary supplement to subjective assessments. Although few has been strictly validated, the most common tests are reviewed here.

6.1. Scintigraphy. Scintigraphic evaluation of gastric emptying is considered to be the gold standard for investigating gastroparesis. This is a quantitative method in which the patient ingests a 99-technicium-radiolabelled standardized meal following which gastric emptying is measured. Although widely available, differences in the delivery of the test and its interpretation have limited the interpretation of results between centres although significant efforts, e.g., from the American Neurogastroenterology and Motility Society and Society of Nuclear Medicine, have aided in the standardization of the performance and interpretation of gastric emptying [52]. Associations between scintigraphic results of gastric emptying and clinical experienced symptoms are poor but have been shown between gastric emptying and fullness/early satiety and nausea/vomiting [53]. Scintigraphy can also be used to measure small bowel transit time and colonic transit time although this requires prolonged sequential scanning and is largely limited to a number of tertiary centres.

6.2. Breath Testing. Nonradioactive ^{13}C isotope bound to a digestible substance, most commonly octanoic acid, can be used as a proxy of gastric emptying. ^{13}C octanoic acid is mixed with a solid meal and ingested, where it is absorbed from the proximal small intestine. Subsequently, it is metabolized in the liver to ^{13}C-CO_2 and can be measured in exhaled breath. Breath testing demonstrates good receiver operator characteristics (sensitivity of 89% and specificity of 80%) in comparison to scintigraphy [54]. In comparison to scintigraphy, ^{13}C octanoic acid does not radiate and sampling can be undertaken in the waiting room. However, concomitant small bowel pathology, such as coeliac disease, can affect breath testing results.

6.3. Manometry. Diabetes affects the oesophageal motility, but studies show contradictive findings covering normal oesophageal motility, delayed oesophageal transit times, and reduced pressure of the lower oesophageal sphincter [55]. However, most of these studies have used conventional manometry catheters, which do not allow for continuous pressure monitoring throughout the oesophagus. In contrast, a newer study in type 2 diabetes used high-resolution oesophageal manometry and found that gastric emptying and oesophageal motility were not generally altered, which possibly suggests that the previous reported extent of gastrointestinal disorders in patients with diabetes may now be reduced due to improved standards of care [56] and better glycaemic control. Furthermore, high-resolution colonic manometry has been used to evaluate physiology and pathophysiology of constipation. Thus, the methodology has been suggested to evaluate the gastrocolonic response, which is potentially mediated by extrinsic neural pathways, and therefore, an absent response could indicate neuropathy in the extrinsic colonic efferents [57, 58].

6.4. Wireless Motility Capsule. This system comprises of an indigestible capsule that continuously measures pressure, temperature, and pH as it traverses the GI tract. Based on stereotypical changes in temperature and pH, segmental and panenteric transit times can be derived [59]. The test involves a standardized meal following which the patient ingests the capsule; data is transmitted wirelessly to a receiver unit worn by the patient until it is expelled. There are a set of robust normal values, and its use has been approved by the Food and Drug Administration [60]. In one study in patients experiencing GI symptoms, 65% had prolonged gastric emptying, 24% had prolonged small intestinal transit time, and 58% had prolonged colon transit time [61]. The findings mirrored another cohort of adults with diabetes and established sensorimotor neuropathy, where 44% had abnormal transit in one or more segments, independent of symptomatology [51]. Therefore, accumulating evidence supports that gastroparesis can coexist with prolonged transit in the small and large bowels as well as low contractility of the colon [61–63]. Beyond pure measurement of transit times, it has recently been proposed that the change in pH across the ileocaecal junction may represent a surrogate marker for caecal fermentation, which itself may influence colonic transit times [51]. Heightened bacterial fermentation in the caecum increases the quantity of short-chain fatty acids, which results in regional acidification. In the future, this may represent a potential therapeutic target in adults with diabetes. In contrast to breath testing, the WMC method provides valuable knowledge such as orocaecal transit [64]; it is, however, more expensive and limited to specialist centres.

6.5. Radiopaque Markers. Radiopaque markers (ROM) are capsules containing plastic beads or rings that are ingested by the patients following which a plain abdominal radiograph is undertaken. Although various protocols exist in terms of the number of capsules to be taken and the number of radiographs undertaken, it is a useful method to delineate whole gut transit time and by proxy colonic transit time as this is the major component of the former [65]. A gastric emptying test with ROM is a widely available screening method to detect delayed gastric emptying in adults with diabetes, where a positive result seems reliable. However, a normal ROM test does not exclude delayed gastric emptying, and if the clinical suspicion of gastroparesis remains, scintigraphy should be performed [53].

6.6. Emerging Techniques. Emerging techniques such as magnetic resonance imaging, the 3D transit system, and the video capsule endoscopy are being developed to assess transit times and motility [66]. Magnetic resonance imaging involves repeated T2-weighted images being recorded by using nonrigid image registration in regional areas of interest, and small and large bowel motor function can be elucidated [67]. The 3D transit system allows continuous tracking of

an electromagnetic capsule ingested by a patient relative to an external plate worn on the abdomen. In addition to transit times, the system allows measurement of the speed, direction, and duration of motility [68]. The video capsule endoscopy is widely used clinically, for instance, to investigate occult gastrointestinal bleeding [69]. Developments in automated software analysis have allowed the systematic quantification of the motion and dynamics of the small bowel [70]. However, whilst these novel techniques offer distinct advantages over and above established methods, further work is needed to define normative values as well as the relation of findings to patient symptoms.

In summary, although most studies have focused on gastroparesis and GI symptoms in diabetes, newer studies show panenteric multisegmental prolongation of transit times prior to the development of clinical symptoms. Taken together, associations between clinical symptoms and sensory abnormalities of the GI tract show conflicting results, which mirrors the neuronal complexity of the ENS, spinal afferents, and central modulation. In the recent study, the evaluation of the brain-gut axis was investigated in adults with diabetes and GI symptoms. The authors provided evidence for the interaction between autonomic neuropathy and peripheral nervous degeneration, as well as changes in the brain processing [71, 72]. Therefore, clinical GI symptoms may not originate from the GI tract but can be developed and maintained through altered central processing. However, in the clinical setting, proactive encouragement of patients to modify lifestyle factors such as improved glycaemic control, daily water intake, dietary aspects, and physical exercise should be emphasized in order to minimise the symptoms of diabetes-induced gut dysmotility.

7. Clinical Management

Diabetic enteropathy has no known cure. The goals of treatment are therefore to slow the progression, relieve symptoms, manage complications, and restore function. The key to preventing or delaying neuropathy is primarily through tight glycaemic control. Such targeted management guided by age, disease duration, and overall health may even improve current symptoms. Dietary and lifestyle advice can give persons with diabetes the tools for better control. Glycaemic control may also improve by the usage of an insulin pump in persons with insulin-dependent diabetes, or sometimes, the preprandial insulin should be given after the meal or in reduced amount when gastroparesis is present. Recently, continuous glucose monitoring devices that allow for glucose readings in real time have become available. Use of continuous glucose monitoring is recommended by national and international medical organisations and expert clinician consensus both in combination with pump and in persons on multiple daily insulin injections [73–76]. Both insulin pump and continuous glucose monitoring reduce the number of hyper- and hypoglycaemic events and thus are believed to be neuroprotective. Beyond optimizing hyperglycaemic control, no available treatments address the underlying polyneuropathy.

The treatment of GI symptoms deriving from diabetic enteropathy is challenging due to the multiple underlying mechanisms. An overview of some of the most frequently applied treatment possibilities is reviewed here.

7.1. Gastroparesis

7.1.1. Nonpharmacological Management. Initial treatment of gastroparesis is based on dietary consulting and improvement of glycaemic control. To enhance emptying of the stomach, low soluble fibre, low fat, and small volume meals are recommended with protein supplementation as needed [77]. If standard dietary modifications are insufficient, small particle size diet as well as liquid and homogenized nutritional supplementations may be initiated with the reservation of postpyloric enteral tube feeding for the most severe cases [78]. Parenteral nutrition should be restricted to cases where all other nutritional treatment modalities have failed.

7.1.2. Prokinetics. Prokinetics (Table 2) have been widely studied in the context of diabetic gastroparesis and generally shown effect in most studies [77, 79, 80]. However, it must be underlined that there is no absolute association between symptom improvement and changes in gastric motility after treatment with prokinetics [81] and most prokinetic drugs are limited to short-term use due to the risk of irreversible tardive dyskinesia (D_2-receptor antagonists) and currently subjected to black box warnings from the FDA and EMA.

Future molecular targets to accelerate GI motility are currently identified, and relamorelin, a synthetic ghrelin analog, has shown promising results, as it increases growth hormone levels and accelerates gastric emptying [82]. Relamorelin has proven to be superior to placebo for symptom relief in phase IIA studies for diabetic gastroparesis, even though vomiting frequency was not reduced. Until today, relamorelin has been well tolerated and is safe in humans without cardiac or neurologic adverse effects, yet it is not approved by the Food and Drug Administration.

7.1.3. Tricyclic Antidepressants. In a retrospective study, low-dose nortriptyline, amitriptyline, and desipramine have shown to reduce symptoms in patients with diabetes, chronic vomiting, and inadequate response to prokinetics [83]. However, in a large multicentre randomized controlled trial in adults with idiopathic gastroparesis, the use of nortriptyline (up to 75 mg per day) compared with placebo for 15 weeks did not improve the overall symptom score [84]. Thus, more evidence is needed to make any conclusive recommendations for diabetic gastroparesis.

7.1.4. Endoscopic Procedures. Most endoscopic procedures evaluated for gastroparesis have been directed towards the pylorus, to investigate whether pylorus spasms may contribute to symptoms and delayed gastric emptying. Intrapyloric injection of botulinum toxin may transiently improve gastric emptying in patients with gastroparesis (idiopathic and diabetic), but after 1 month, the benefit was not superior to placebo [85], and in patients with idiopathic gastroparesis, botox was not superior to placebo in improving either symptoms or the rate of gastric emptying [86]. Other endoscopic procedures include transpyloric stenting and endoscopic

Table 2: Prokinetic for treatment of diabetic gastroparesis.

Drug	Mode of action	Recommended daily dose (formulation)	Comment
Metoclopramide	5-HT_4 receptor agonist D_2-receptor antagonist	10 mg TID (tablet)	Black box warnings for long-term use: (i) FDA < 3 months (ii) EMA ≤ 5 days
Domperidone	D_2-receptor antagonist	10 mg TID (tablet) 30 mg BID (suppository)	Should be avoided in the presence of prolonged QT interval
Erythromycin	Motillin receptor agonist Cholinergic receptor agonist	250 mg TID (tablet)	Clinical efficacy often diminishes after 2–4 weeks due to tachyphylaxia Prokinetic action likely a drug class effect and other macrolides with less toxicity may be used (azithromycin, clarithromycin), but evidence from controlled trials is lacking
Prucalopride	5-HT_4 receptor agonist	2 mg (tablet)	Currently under investigation for diabetic gastroparesis in phase III trials. May be used off-label in selected cases
Granisetron	5-HT_3 receptor agonist	3.1 mg per 24 hours (patch)	Evidence from controlled trials is lacking in diabetic gastroparesis

myomectomy of the pylorus, but at present, there are no sufficient data to support these procedures outside protocol settings [77].

7.1.5. Surgical Procedures. Gastric electrical stimulation (GES) has been approved by the FDA as a Humanitarian Device Exemption in patients with refractory symptoms of diabetic or idiopathic gastroparesis [87]. It shows most promising results in patients with predominance of nausea and vomiting, where response rates up to 60% are shown in uncontrolled studies. These results should, however, be interpreted cautiously because a controlled trial failed to show any difference in symptom scores between the on and off phases in patients treated for refractory diabetic gastroparesis [88]. The trial did, however, show a significant reduction in vomiting episodes during all phases compared with the preimplantation period as well as favourable long-term clinical outcome [89].

Other surgical options include total or subtotal gastrectomy, which generally should be reserved as a last resort treatment in patients with severe treatment refractory symptoms after thorough evaluation in a multidisciplinary setting. Importantly, the surgical reports reporting favourable outcomes of these procedures have all been performed in uncontrolled settings, with relatively short follow-up. Taken together, more studies are needed to elucidate the underlying mechanisms and potentially identify the patients that may benefit from surgical intervention.

7.2. Abnormal Bowel Function. The treatment options available for bowel dysfunction in patients with diabetic enteropathy follow the recommendations used for other functional GI disorders.

7.2.1. Diarrhoea. In the presence of diarrhoea, patients should be evaluated for secondary causes including infectious and inflammatory bowel diseases, coeliac disease, exocrine pancreatic insufficiency, and small bowel intestinal overgrowth [90]. Fibre supplementation might be helpful in some cases but can also worsen symptoms of gastroparesis. In most cases, loperamide is an effective and safe treatment for chronic diarrhoea, although not formally evaluated in the context of diabetic enteropathy [91].

7.2.2. Constipation. Treatment of constipation is based on conventional laxatives that all have been shown to be relatively efficient and safe [92]. In treatment of refractory cases, a more detailed workup may be needed which ideally should include assessment of intestinal transit time, endoscopy, proctography, and anorectal-physiological evaluation. In the presence of slow-transit constipation, osmotic laxatives are preferred over fibre supplementation and bulking agents, because they stimulate the intestines to absorb excessive amounts of fluid from the body. Novel treatment options include prucalopride [93] that may also improve symptoms of gastroparesis, and linaclotide may be particularly helpful in patients with concomitant symptoms of irritable bowel syndrome [94]. Gastric symptoms in diabetes may be caused—at least in part—by vagal neuropathy, and therefore, there is a theoretical background to use a neuromodulation treatment option and this is the rationale for gastric pacing. Several studies have shown that the method may be effective to alleviate nausea and vomiting and is cost-effective [95]. However, most studies have been small and suffered from methodological problems (i.e., no sham arm), and recent guidelines have not recommended this modality outside protocol studies [96]. In idiopathic faecal incontinence, emerging areas such as neuromodulation, e.g., sacral nerve stimulation, have shown promising results in other GI functional diseases [97]. Within diabetes enteropathy, data is sparse and currently, no randomized sham-controlled studies exist, but such studies will undoubtedly contribute with knowledge in the upcoming years.

7.3. Abdominal Pain. Treatment of abdominal pain secondary to diabetic enteropathy is complex and involves a multidisciplinary approach including diabetologist, gastroenterologist, pain specialist, and psychologists. An active

screening for psychiatric comorbidity, including anxiety and depression, should be done, and treatment initiated if present. There is a paucity of studies investigating pharmacological therapies for pain associated with diabetic enteropathy. However, as the pain may be of neuropathic origin, drugs, which have been evaluated for this indication in other diseases, may be helpful. These include antidepressants (tricyclic antidepressants, selective serotonin reuptake inhibitors, and selective serotonin-noradrenaline reuptake inhibitors) as well as the gabapentoids (gabapentin and pregabalin) that can be used in combination depending on the clinical situation [98]. However, in many patients, the pain is secondary to transit problems, bacterial overgrowth, and constipation and shall be treated accordingly. Side effects to medications can also give abdominal pain, and if patients are treated with opioids on other indications, these may give bowel dysfunction and abdominal pain [99].

8. Concluding Remarks

Symptoms from the GI tract, including dysmotility and abdominal pain, are frequent in diabetes. Traditionally, diabetes-induced gastrointestinal complications are focusing solely on gastroparesis and symptoms of the upper gastrointestinal tract. However, accumulating evidence supports the presence of structural and functional alterations in the ENS of the entire GI tract and the interconnections with enteric glial cells and interstitial cells of Cajal. This explains the biochemical, immune-mediated, and inflammatory pathophysiological mechanisms, which coalesce in the development and maintenance of cell death, altered secretion of neurotransmitters, dysmotility, and concomitant symptoms in the entire length of the GI tract. Taken together, increased recognition of diabetic enteropathy may allow earlier diagnosis and intervention. This gives rise to hope for the recognition of diabetic enteropathy at an earlier time and specific diagnoses in the future. Finally, more targeted nonpharmacological and pharmacological treatments and interventions can be individually tailored based on pathophysiological findings, in order to improve patient outcomes.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Markers of Local Inflammation and Bone Resorption in the Acute Diabetic Charcot Foot

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Objective. Due to the localized nature of Charcot foot, systemically altered levels of inflammation markers can be difficult to measure. The aim of this study was to investigate whether it is possible to detect an arteriovenous (A-V) flux in any locally produced inflammatory biomarkers from an acute Charcot foot by comparing local and systemic measurements. *Methods*. We included patients with acute diabetic Charcot foot. Blood was sampled from the vena saphena magna on the distal part of the crus bilaterally as well as from the arteria radialis. To minimize the A-V shunting effect, the feet were externally cooled with ice water prior to resampling. *Results*. Both before and after cooling, the A-V flux of interleukin-6 (IL-6) between the Charcot feet and the arterial level was significantly higher than the flux between the healthy feet and the arterial level ($\Delta value_{before}$: 7.25 versus 0.41 pg/mL, resp., $p = 0.008$; $\Delta value_{after}$: 10.04 versus 1.68 pg/mL, resp., $p = 0.032$). There were no differences in the fluxes for other markers of inflammation. *Conclusion*. We have found an increased A-V flux of IL-6 in the acute diabetic Charcot foot compared to the healthy foot in the same patients.

1. Introduction

Charcot osteoarthropathy is a rare disorder manifesting with aseptic inflammation and hyperemia in and around load-bearing bones and tissues. The process is normally unilateral and leads to progressive, uncontrolled resorption and degeneration of bone mass, resulting in spontaneous fatigue bone fractures [1–4]. While different locations have been described, the most common is in the feet (Charcot foot (CF)) [5, 6], where the process can cause deformity, ulcerations, and amputations. The Charcot inflammation can be located at different sites in the affected foot, most prominently in the midfoot [2, 7].

The precise pathological mechanisms underlying Charcot foot are still not fully understood. However, it is dependent on relatively unimpaired lower limb blood flow and established peripheral neuropathy [8–11]. It can be triggered by a number of diseases, although today most cases occur in individuals with diabetes mellitus [1, 2, 12].

Recent evidence suggests that the initial inflammation is provoked by repeated local microtrauma and dysregulated bone resorption [13–16], which in turn initiates the inflammatory process.

Several studies have reported changes in biomarkers of bone resorption and inflammation in individuals with

Charcot foot [17–24], and it seems that the inflammation leads to microstructural changes in the affected bones [25, 26].

Related to this, studies have explored the possible relationship between interleukin levels and acute Charcot foot [17, 27–29] and found increased levels of interleukin-1 receptor antagonist (IL-1RA), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and interleukin-17 subtypes A, E, and F (IL-17A/E/F), as well as decreased levels of interleukin-1β (IL-1β) and interleukin-8 (IL-8).

In addition, many individuals with Charcot foot also seem to have a degree of vascular calcification and inflammation [30, 31]. This is of particular interest due to the connection between vascular calcification, neuropathy, and the nuclear factor-κB (NF-κB) system, as described by Petrova and Shanahan [32]. A possible way to assess this could be through the system of advanced glycation end products (AGEs) and their soluble receptors (sRAGE) [33–35].

However, the biomarkers in question might only be produced locally around the inflamed bones in the foot, which means that the signal on a systemic level can be difficult to register. Furthermore, a general systemic release of these biomarkers might happen in response to a number of inflammatory processes unrelated to the Charcot foot.

Therefore, it is plausible that a stronger and more specific signal from an acute Charcot foot might be achieved by measuring the flux of a specific marker between the local venous concentration in the foot and the arterial concentration. Local sampling from the dorsal venous arch of the foot in acute Charcot feet has previously been done by Gough et al. and Pearson et al. [21, 23], although neither measured all the markers discussed here. To our knowledge, local fluxes of inflammatory biomarkers across an acute Charcot foot have not been measured previously.

The aim of this study was to investigate whether it is possible to detect a flux in any locally produced biomarkers from an acute Charcot foot by measuring the arteriovenous (A-V) difference.

2. Materials and Methods

2.1. Participants. We included participants with acute Charcot foot, recruited at the Copenhagen Wound Healing Center at Bispebjerg Hospital, Denmark, and at the Steno Diabetes Center, Gentofte, Denmark. The participants were referred by specialists after thorough physical examination, full blood panel, X-ray, bone scintigraphy, and/or MRI. All participants were examined as close as possible to the reported outbreak of the acute Charcot symptom (<3 months).

Exclusion criteria included no diabetes mellitus, temperature difference < 2°C between the feet, duration > 3 months, foot ulcers, prior foot surgery, new objective foot deformities, bilateral Charcot foot, infection in the foot, antiosteoporotic medication, arterial insufficiency, or foot or toe amputation on either side.

To confirm that the Charcot foot still had a high activity on the day of examination as assessed by a locally elevated blood flow, this was measured in the feet with venous occlusion plethysmography [36]. Foot temperature and foot somatosensoric neuropathy as assessed by biothesiometry were measured on the study day as well.

The study was approved by the Regional Ethical Committee for Copenhagen.

2.2. Arteriovenous Flux. To measure the fluxes in biomarker production in the acute Charcot foot, blood was sampled from the vena saphena magna on the distal part of the crus above the ankle. This was done on both the affected (Charcot) side and the healthy side. Arterial blood was sampled from the a. radialis (or from the a. brachialis if the a. radialis was inaccessible).

The venous drainage of the foot happens primarily through the veins saphena magna and parva, while the deep veins only play a minor role. The saphena veins connect through the dorsal venous arch on the dorsal side of the foot. The dorsal venous arch collects blood from both superficial and deep veins in the foot, as well as from the networks rete venosus plantare and rete venosus dorsale pedis. The superficial and deep veins of the foot are linked by communicating perforant veins. The few valves present in these perforants are turned so that blood can only run from the deep to the superficial veins, thus helping with thermoregulation and pressure absorption. This means that parts of the drainage of the deep foot happen through the superficial veins, which can thus be sampled from a superficial vein on the lower leg [37–39].

A portion of the blood flow in the feet bypasses normal microcirculatory exchange by shunting directly through A-V anastomoses. This is in part a thermoregulatory effect and is thus more prevalent at higher skin temperatures [40]. As the shunted blood will not be exposed to any biomarkers produced in the deep foot tissue, this A-V shunting in effect dilutes the signal of any inflammatory biomarkers in a mixed venous sample. To minimize this shunting effect in our setup, we cooled down the feet externally with cold water prior to the final sampling (t_{ice}).

2.3. Experimental Setup. All three sites were sampled simultaneously (t_{start}) (Figure 1). Fluxes between the arterial and venous concentrations were calculated as vein − artery to get a positive gradient if the Charcot foot produced the biomarker in question. After sampling, both feet were cooled down for approximately 10 minutes in an icy water bath, while foot temperature was measured. The three sites were then sampled again while the participants kept their feet in the water (t_{ice}).

In the following, venous samples from the acute Charcot foot are denoted as CF(v) and venous samples from the non-Charcot foot are denoted as non-CF(v).

The blood samples were centrifuged at 4°C and stored at −80°C. All samples were analyzed together at the end of the study.

2.4. Biomarkers. To estimate the existing interleukin profile and the highest relative concentrations, t_{start} samples were analyzed on a Bio-Plex System multiplex immunoassay screening panel (Bio-Rad Laboratories Inc., 4000 Alfred Nobel Drive, Hercules, California 94547, USA). The panel

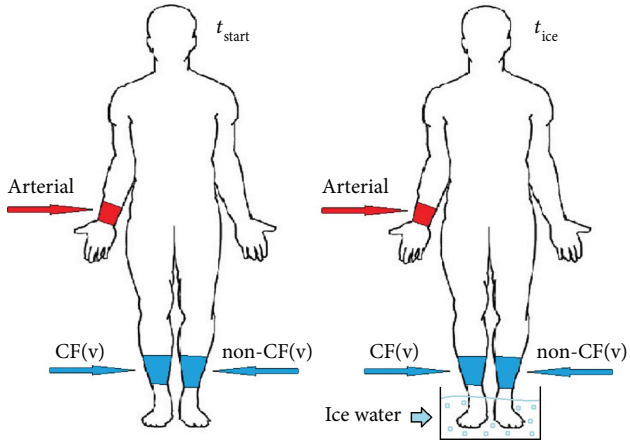

FIGURE 1: Sites of arterial (red) or venous (blue) blood samples before (t_{start}) and after (t_{ice}) external cooling of the feet. Arterial samples were taken from the a. radialis, or from the a. brachialis if the a. radialis was inaccessible. Venous samples were taken from a large superficial vein at the third distal part of the crus both on the side with a Charcot foot (CF(v)) and on the side without a Charcot foot (non-CF(v)).

used screened for IL-1β, IL-1RA, IL-6, IL-8, IL-17A, and TNF-α, with the best signals detected for IL-6 and IL-8.

All analyses were performed by Biolab, Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Denmark. Special ELISA setups were used for IL-6, IL-8, free soluble receptor activator of nuclear factor kappa-*B* ligand (fsRANK-L), osteoprotegerin (OPG), IL-17F, sRAGE, and AGEs. The remaining samples were analyzed as part of the daily hospital sample routines. The accepted intraindividual sample CV for all assays was 20%.

(i) AGEs were measured with a Human sandwich ELISA AGE kit (Nordic BioSite AB, Propellervägen 4A, 183 62 Täby, Sweden) (kit serial number LS-F10641-1; range 0.78–50 ng/mL).

(ii) IL-6 was measured with an IL-6 Quantikine HS ELISA kit (Bio-Techne Ltd., 614 McKinley Place NE, Minneapolis, MN 55413, USA) (kit serial number HS600B).

(iii) IL-8 was measured with a Human CXCL8 Quantikine kit (Bio-Techne Ltd., 614 McKinley Place NE, Minneapolis, MN 55413, USA) (kit serial number D8000C).

(iv) IL-17F was measured with a Human IL-17F Platinum ELISA kit (AH-Diagnostics A/S, Runetoften 18, DK-8210 Aarhus V, Denmark) (kit serial number BMS2037/2).

(v) Assays for fsRANK-L, osteoprotegerin, and sRAGE were performed as previously described [41].

2.5. Statistical Analysis. Data are presented as mean ± 1 SD or range unless otherwise noted. An α-level of <0.05 was considered significant. Normal distribution in data was tested using Shapiro-Wilks tests. No transformations were used. *t*-tests or paired *t*-tests were used for variance analysis between groups in normally distributed data sets. For data sets not normally distributed, nonparametric tests in the form of the Mann-Whitney rank-sum test were used, while Wilcoxon signed-rank tests were used for comparing paired samples.

Statistics and general data handling were done using IBM SPSS Statistics v. 23 by IBM Corporation, SIGMAPLOT v. 11.0.0.77 by Systat Software Inc., Microsoft Excel 2000 v. 9.0.2812 by Microsoft Corporation, and Apache OpenOffice 4.0.1 by the Apache Software Foundation.

3. Results

3.1. Participants. We included 5 patients with acute Charcot foot. In total, 22 patients were screened for inclusion. Of these, 7 patients were excluded due to having foot ulcers or receiving foot surgery and/or ulcer debridement before the examinations. Another 7 did not want to participate or were unable to participate due to personal reasons, while 3 patients had had their Charcot foot for too long to be considered acute (duration > 3 months).

The average time from the reported onset of symptoms to examination was 7.2 weeks. The Charcot feet were on average 2.6°C warmer than the contralateral and had a 3 times increased blood flow. All 5 patients had recently started off-loading treatment with an AirCast® removable walker boot before measurements. All 5 patients were diagnosed with stage 0 Charcot foot.

Anthropometric data for the participants are listed in Table 1, along with the results for biothesiometry, venous occlusion plethysmography, and arterial samples of markers of bone health taken prior to the cooling of the feet (t_{start}). There were a significant higher temperature and blood flow in the acute Charcot feet compared to the healthy feet.

3.2. Multiplex Data. For IL-1β, IL-1RA, IL-17A, and TNF-α, almost all measured values were below the multiplex limit of detection in all samples. This was tested with both serum and plasma. The detection limits were 1.32 pg/mL for IL-1, 29.64 pg/mL for IL-1RA, 7.99 pg/mL for IL-17A, and 12.72 pg/mL for TNF-α. There was a single signal in one patient in IL-1RA and IL-17A (not the same patient for both markers).

The average level of IL-6 detected was 10.6 pg/mL, and for IL-8, it was 12.5 pg/mL.

3.3. Measurements before Cooling. Measurements from all three sites (arterial, CF(v), and non-CF(v)) before and after cooling are listed in Table 2. At t_{start}, there were no differences in the levels of fsRANK-L, OPG, IL-6, IL-8, sRAGE, or AGEs—neither between arterial and CF(v) nor between CF(v) and non-CF(v) samples. The highest relative numerical difference was for IL-6 in arterial versus CF(v) levels (7.31 versus 14.56) ($p = 0.109$). It was not possible to measure IL-17F as it was below the assay detection limit of 7.8 pg/mL for all samples at t_{start} and t_{ice}.

The venous-arterial flux of IL-6 between the Charcot feet and the arterial level was significantly higher than the flux

Table 1: Anthropomorphic data for diabetes patients with acute Charcot foot (CF). Test results are from arterial sampling.

(a)

	Data listed as mean; range or n
Age (years)	48.6; 26.0
Sex (m/f)	3/2
Affected foot (left/right)	1/4
Diabetes type (I/II)	2/3
Diabetes duration (years)	19.2; 31.0
HbA1c (mmol/mol) (31–44 mmol/mol)	73; 53
Ca^{2+}(free, ionized) (mmol/L) (1.18–1.32 mmol/L)	1.24; 0.14
PTH (pmol/L) (1.6–6.9 pmol/L)	4.5; 4.3
CRP (mg/L) (<10 mg/L)	9.8; 15.0
25-OH-vitamin D (nmol/L) (50–160 nmol/L)	36.7; 52.6
Alkaline phosphatase (bone specific) (μg/L) (<20 μg/L)[$]	20.3; 7.3
CTX (ng/L) (<630 ng/L)[$]	240; 0.5
P1NP (μg/L) (22–87 μg/L)[$]	48.3; 53.8
Osteocalcin (μg/L) (9–42 μg/L)	25.3; 42.1

(b)

	Charcot foot	Contralateral foot	Difference, p value
Foot temperature (CF/non-CF) (°C)	33.7	31.1	Δ2.6, $p = 0.004$*
Biothesiometry (CF/non-CF) (V)	42	39	Δ3, $p = 0.648$
Plethysmography (CF/non-CF) (mL/(100 g·min))	6.9	1.8	Δ5.1, $p = 0.045$*

*Significant at the chosen α-level of 0.05. [$]Reference range listed for 50 y.o. male where ranges differ with age and/or sex.

Table 2: Levels of inflammation markers in local venous samples in the acute Charcot foot (CF(v)), the healthy foot (non-CF(v)), and arterial samples from the a. radialis. Measurements listed before (t_{start}) and after (t_{ice}) external cooling of both feet with ice water.

	Sampling site	t_{start}	t_{ice}
fsRANK-L (pmol/L)	Arterial	0.14 ± 0.12	0.13 ± 0.11
	CF(v)	0.13 ± 0.11	0.14 ± 0.11
	non-CF(v)	0.13 ± 0.11	0.14 ± 0.13
OPG (pmol/L)	Arterial	6.5 ± 5.4	6.6 ± 5.1
	CF(v)	6.4 ± 5.8	7.3 ± 5.9
	non-CF(v)	6.3 ± 5.5	7.5 ± 6.1
IL-6 (pg/mL)	Arterial	7.31 ± 6.88	6.25 ± 5.21
	CF(v)	14.56 ± 14.27	16.29 ± 11.45
	non-CF(v)	7.71 ± 7.07	7.93 ± 5.70
IL-8 (pg/mL)	Arterial	15.6 ± 7.9	13.1 ± 6.3
	CF(v)	13.4 ± 4.4	12.1 ± 6.3
	non-CF(v)	14.5 ± 9.6	11.5 ± 4.7
sRAGE (ng/L)	Arterial	845 ± 266	860 ± 247
	CF(v)	833 ± 292	878 ± 298
	non-CF(v)	827 ± 252	911 ± 293
AGEs (ng/mL)	Arterial	6.2 ± 7.7	5.4 ± 7.4
	CF(v)	5.7 ± 6.8	7.9 ± 7.1
	non-CF(v)	5.9 ± 7.2	8.4 ± 8.4

Data listed as mean ± 1 SD. fsRANKL = free soluble receptor activator of nuclear factor-κB; OPG = osteoprotegerin; IL-6/IL-8 = interleukin 6/interleukin 8; sRAGE = soluble receptor for advanced glycation end products; AGEs = advanced glycation end products.

between the healthy feet and the arterial level (Δvalues: 7.25 versus 0.41 pg/mL, resp.) ($p = 0.008$). There were no differences in fsRANK-L, OPG, IL-8, sRAGE, or AGEs.

3.4. Measurements after External Cooling. The ice bath used for cooling maintained an average temperature of 7.7°C, and it was used for cooling for an average of 11.6 min. The ice bath cooled the Charcot feet at an average of 11.0°C (from 33.7 to 22.7°C), and the non-Charcot feet were cooled at an average of 12.9°C (from 31.1 to 18.2°C). Temperatures in each foot before and after cooling are listed in Table 3.

At t_{ice}, there was a significantly elevated level of IL-6 (Δvalue: 10.04 pg/mL) in the Charcot feet compared to the arterial value ($p = 0.049$) (Figure 2). There was also a significantly elevated level of AGEs (Δvalue: 2.5 ng/mL) ($p = 0.002$) (Table 2). There were no differences in fsRANK-L, OPG, IL-8, or sRAGE.

The venous-arterial flux for IL-6 at t_{ice} was still significantly increased in the Charcot feet (CF(v)-arterial) compared to the healthy feet (non-CF(v)-arterial) (Δvalues: 10.04 versus 1.68 pg/mL) ($p = 0.032$). There were no differences in the fluxes for fsRANK-L, OPG, IL-8, sRAGE, or AGEs.

The fsRANK-L/OPG ratio at t_{ice} was 3.7 in the arterial sample, 4.0 in the CF(v) sample, and 3.8 in the non-CF(v) sample and did not differ in a one-way ANOVA on ranks ($p = 0.970$).

Table 3: Temperature measurements on the feet of each individual patient during the study day.

Patient number	Charcot foot temperature (°C)		Non-Charcot foot temperature (°C)	
	Before cooling (t_{start})	After cooling (t_{ice})	Before cooling (t_{start})	After cooling (t_{ice})
1	32.0	20.0	29.7	18.0
2	33.8	25.9	31.7	18.6
3	33.7	20.2	32.3	20.6
4	34.8	21.8	31.0	17.0
5	34.4	25.7	30.7	17.0

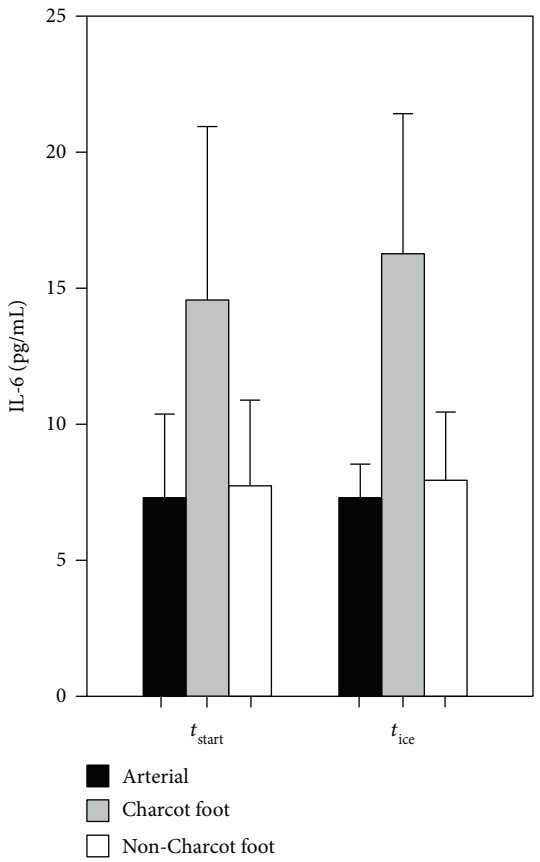

Figure 2: Levels of interleukin 6 (IL-6) in arterial and local venous samples in both feet (CF(v) and non-CF(v)) before (t_{start}) and after (t_{ice}) external cooling with ice water. Bars = mean; error bars = SEM.

4. Discussion

In this study, we have tested a novel approach to evaluating the local inflammatory activity in an acute Charcot foot by measuring the venous-arterial flux across the Charcot foot while lowering the possible dilution from A-V shunting by externally cooling the foot.

The data show a difference in the venous-arterial flux of IL-6 both before and after external cooling. We also saw a two-fold elevated level of IL-6 in the Charcot foot compared to the arterial level after cooling, indicating a local production of IL-6. It is interesting that both IL-6 and AGEs only show a significant difference between the Charcot foot and arterial level after cooling, thus possibly indicating an effect of limiting A-V shunting in the feet before sampling.

The results are mostly in line with what other groups have found. Divyateja et al. indicated an increased median IL-6 level in the Charcot foot [24], while both Petrova et al. [17] and Folestad et al. [29] have suggested increased levels of IL-6 systemically (although Folestad et al. did not find an initially increased level of IL-6).

Unlike Folestad et al., we have been unable to demonstrate high levels of IL-17F systemically in acute Charcot patients [27]. However, they did show an initial low level of IL-17F (corresponding to the time where we performed our sampling), and additionally, they used high-sensitivity ECL as opposed to the ELISA that we used.

A finding of locally increased levels of IL-6 is of particular interest for several reasons. As a proinflammatory cytokine, its presence supports the theory regarding the pathogenesis of acute Charcot foot as put forth by, for instance, Jeffcoate et al. [13]. Furthermore, IL-6 is involved in bone resorption through osteoclastic differentiation and activation [42–45]. Thus, the finding further supports local osteoclastic hyperactivation as a central element in the Charcot foot bone metabolism and confirms the findings of IL-6 in osteoclasts in bone samples from Charcot feet as seen by Baumhauer et al. [46]. The source of this local production of IL-6 remains unknown.

Recently, Petrova et al. reported that OPG was elevated in patients with Charcot foot without a corresponding elevation in RANK-L [17] and that osteoclasts from patients with Charcot foot can be modulated by TNF-α through RANK-L [47]. It is important to note however that elevated OPG levels could be associated with neuropathy in itself [48].

Ndip et al. have indicated that individuals with Charcot foot have an increased RANK-L/OPG ratio and suggest that this could play a role in medial vascular calcification [22]. We have also previously shown a higher RANK-L/OPG ratio in patients with acute Charcot foot than non-Charcot diabetic controls [41]. In the current setup, we did not find a difference in the venous-arterial flux or a locally elevated RANK-L/OPG ratio. However, this was not to be expected either as both markers only circulate in very small quantities and furthermore have half-lives sufficient to recirculate the vascular system many times, making it difficult to detect a local difference.

Regarding the increased level of AGEs after cooling, it is unclear whether this is an expression of a local increase in production of AGEs due to cooling, a by-product of the Charcot inflammation, or merely a random sampling variation.

The presence of tissue-bound receptors for AGEs (RAGE) has been associated with impaired bone matrix mineralization and enhanced osteoclast formation [49]. AGEs have been linked to a negative modification of collagen integrity and fragile bones in general [50–52]. Thus, if there is indeed an increased level of AGEs present in Charcot feet, this might account for a further weakening of the bones. Furthermore, there is a link between increased levels of RAGE and activation of the NF-κB system and several associated cytokines [53, 54]. As such, it shares a common pathway of influence of osteoclastic activation with RANK-L/OPG and by extension IL-6.

4.1. Strengths and Limitations. To our knowledge, this is the first time that the local venous-arterial flux across an acute Charcot foot has been studied. Furthermore, we are unaware of other studies that have limited the local A-V shunting effect prior to measuring a Charcot foot.

The study was limited by the number of available participants. In total, we screened 22 patients and most of these were excluded due to foot ulcers or extended time from the symptom onset to diagnosis. Thus, part of the recruitment issue was the rigorous exclusion criteria needed to ensure that any possible findings were not clouded by infections, surgery, or prolonged Charcot inflammation.

Furthermore, most of the assays we have used have a limited accuracy and substantial intraindividual variations, and thus it was difficult to register any possible differences. These variations might be the reason why we saw an increase in AGEs after cooling in the Charcot foot compared to the arterial level. Unless more accurate assays are developed, future tests in a similar setup could be performed with multiple samples from each site and each time point to help alleviate this issue.

5. Conclusion

In conclusion, we have found an increased venous-arterial flux of IL-6 in the acute diabetic Charcot foot compared to the healthy foot. We also found an increased level of IL-6 and AGEs in the acute Charcot foot compared to the arterial level after, but not before, externally cooling the feet.

Data Availability

All data, in anonymised form, are available upon contact to the corresponding author.

Disclosure

The foundations had no influence on the design, execution, results, or conclusions of the study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Appendix 1 contains the primary output from the multiplex assay. The strength of the responses was used to evaluate the most likely markers for the final assay panel. *(Supplementary Materials)*

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Research Article

Changes in Immunoreactivity of Sensory Substances within the Enteric Nervous System of the Porcine Stomach during Experimentally Induced Diabetes

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One of the most frequently reported disorders associated with diabetes is gastrointestinal (GI) disturbance. Although pathogenesis of these complications is multifactorial, the complicity of the enteric nervous system (ENS) in this respect has significant importance. Therefore, this paper analysed changes in substance P- (SP-), calcitonin gene-related peptide- (CGRP-), and leu5-enkephalin- (L-ENK-) like immunoreactivity (LI) in enteric stomach neurons caused by chemically induced diabetes in a porcine model. Using double immunofluorescent labelling, it was found that acute hyperglycaemia led to significant changes in the chemical coding of stomach enteric neurons. Generally, the response to artificially inducted diabetes depended on the "kind" of enteric plexus as well as the stomach region studied. A clear increase in the percentage of neurons immunoreactive to SP and CGRP was visible in the myenteric plexus (MP) in the antrum, corpus, and pylorus as well as in the submucosal plexus (SmP) in the corpus. For L-ENK, an increase in the number of L-ENK-LI neurons was observed in the MP of the antrum and SmP in the corpus, while in the MP of the corpus and pylorus, a decrease in the percentage of L-ENK-LI neurons was noted.

1. Introduction

Diabetes mellitus is actually considered an epidemic in the 21st century. The diabetic population has systematically increased worldwide, and it is expected to rise to 592 million patients/cases by the year 2035 [1]. Improper treatment of diabetes leads to long-term increased blood glucose level, which is considered to be a major pathophysiological factor in the development of severe diabetic complications [2, 3]. It has been evaluated that up to 75% of patients with type 1 or type 2 diabetes will have the complication of neuropathy. One of the clinical signs of neuropathy is gastrointestinal (GI) disorder [4–6]. Regardless of their etiology, it is possible that the molecular mechanisms of pathological changes within the GI tract are partly due to changes within the enteric nervous system (ENS) [4].

The enteric nervous system regulates gastrointestinal functions, which is independent of the central nervous system and is embedded in the wall of the gastrointestinal tract [7]. The ENS is comprised approximately of millions of sensory, motor, and interneurons formed in intramural ganglionated plexuses interconnected with a very dense network of nerves [8]. In the porcine stomach, it is formed by the myenteric plexus (MP) located between the longitudinal and circular muscle layers, which mainly regulates the proper gastric motility, both after and between the meals, and the submucosal plexus (SmP) is situated in the inner side of the circular muscle layer, primarily regulating fluid secretion and resorption [9, 10].

Gastrointestinal complications in the course of diabetes mellitus appear to be multifactorial [11]. Several possibilities have been suggested, such as diabetic autonomic neuropathy

altering the parasympathetic and sympathetic nerve functions. Moreover, other possibilities have been proposed. Namely, enteric neurons may undergo changes caused by adaptive and/or neuroprotective processes during many gastrointestinal and extraintestinal diseases, and these changes are mainly manifested in the modification of neuronal chemical phenotyping [12, 13]. Proper sensory perception is conditioned by a functional balance between nociceptive substances such as substance P (SP) or calcitonin gene-related peptide (CGRP) and, on the other hand, an appropriate level of antinociceptive factors, particularly the opioid family, for example, leu5-enkephalin (L-ENK) [14–17].

SP is an undecapeptide and belongs to the tachykinin/neurokinin family. The neurokinin family consists of (in addition to SP) neurokinin A (NKA) and neurokinin B (NKB) [18]. SP and NKA are encoded by the preprotachykinin I gene, while NKB is encoded by the preprotachykinin II gene [19]. SP is widely distributed in the ENS. Till now, SP-like immunoreactivity has been described in different fragments of the GI tract, like the oesophagus, stomach, duodenum, jejunum, and descending colon [20]. SP in the GI tract is first of all considered to be a neurotransmitter for primary sensory afferent nerve fibres, which are involved in pain signal transmission. Furthermore, SP is engaged in the regulation of blood flow, both by relaxation and by contraction of muscular cells of blood vessels [21–24]. The expression of SP within the enteric nervous system, as well as their changes, has been described in various species under different pathological conditions, including inflammatory bowel disease [22], bisfenol A intoxication [25], parasite infection [26], *Bacteroides fragilis* infection [27], or carcinoma [28].

Calcitonin gene-related peptide (CGRP) is a member of the calcitonin family of peptides [29]. CGRP is one of the most abundant peptides, produced in both the peripheral and central nervous systems [28]. One of the better-known functions of CGRP in the GI tract is its participation in nociception signal transmission. It is also known that CGRP plays a role as a neuromediator and/or neuromodulator, participating in the regulation of motor activity, blood flow, and gastric acid secretion [27]. CGRP is also considered to be a marker of intrinsic primary afferent neurons. This class of neurons plays an essential role in short reflexes in the intestinal wall excluding the central nervous system. This mentioned function corresponds with wide distribution of CGRP in the GI tract. Namely, CGRP-immunoreactive nerve structures were described in the wall of the GI tract from the oesophagus to the rectum [30]. Multiple functions of CGRP in the GI tract were confirmed by numerous studies regarding pathological conditions resulting in changes in expression of CGRP [30].

In turn, met-enkephalin, leu-enkephalin, β-endorphin, α-neo-endorphin, dynorphin, and nociceptin/orphanin are opioid peptides. Leu-enkephalin (L-ENK) was the first time isolated from the porcine brain in 1975. Opioid peptides in the stomach originate from both enteroendocrine cells and ENS neurons. LENK is a factor that most often is present in sensory neurons and its participation in sensory and pain transmission is well known [31, 32].

Therefore, this experiment was designed to determine the possible alterations in expression of sensory substances in the stomach enteric neurons under the influence of acute hyperglycaemia. Streptozotocin-induced hyperglycaemic/diabetic porcine model was used in this study. In many publications on diabetic complications, much attention is paid to issues that affect life-threatening conditions or are threatened with irreversible crippling. As high as 75% of patients with diabetes may experience gastrointestinal symptoms. These complications represent a major cause of morbidity and have a negative impact on healthcare (leads to a significant impairment in the quality of life) and costs in diabetes. Taking this into account, it seems highly important to find an appropriate animal model that allows learning the exact etiology of changes occurring in diabetes in the gastrointestinal tract. Especially in the area of diabetes, the swine in the last time has gained considerable interest [33, 34]. It is due to the fact that biochemical and pathophysiological responses to diabetes remain in part like those observed in people. It has been documented that blood flow of the pig pancreas is similar to that of the human pancreas and the number of insulin producing cells is within a similar number as that observed in humans [35]. Moreover, pigs, in contrast to rodents, are omnivorous and active during the day. It all makes the pig a much better model for studying diabetic complications than rodents. The current study for the first time focused on the examination of SP, CGRP, and leu5-enkephalin expression in stomach neurons during streptozotocin-inducted diabetes. The choice of these neuropeptides was dictated by the fact that these substances are involved in sensory signal modulation. It is well documented that abdominal pain is one of the most serious GI diabetic complications. The current results may help to create the foundation for further, more detailed, and clinically oriented studies, involving these neuropeptides and their roles in gastrointestinal diabetic symptom treatment.

2. Materials and Methods

2.1. Animals. We followed the methods of Bulc et al. [36]. This study was conducted on ten juvenile female pigs of the white large Polish breed, weighing from 17.0 kg to 20 kg. At the beginning of the experiment, the animals were randomly distributed into two groups: the diabetic group (D, $n = 5$) and the control group (C, $n = 5$) and were housed in cages suitable for pigs. The animals were given one week of acclimatization to observe their general health, to minimize physiological stress, and to ensure the proper conduct of the study. The treatment of animals was conducted in compliance with the instructions of the Local Ethical Committee in Olsztyn (Poland) (decision number 13/2015/DTN) with special attention paid to minimizing any stress reaction.

2.2. Chemical Induction of Diabetes. After acclimatization, hyperglycaemia was induced as previously described [36]. Streptozotocin (STZ) (Sigma-Aldrich, St Louis, MO, USA, S0130), 150 mg/kg of body weight, was dissolved in a freshly prepared disodium citrate buffer solution (pH = 4.23, 1 g streptozotocin/10 mL solution). For this purpose, pigs were

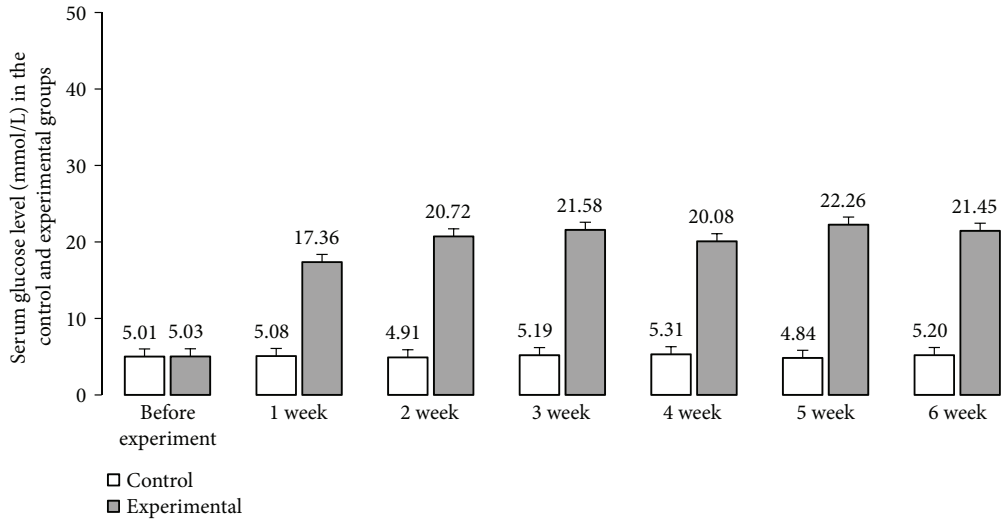

Figure 1: Serum glucose levels. Serum glucose levels after induction of diabetes and glucose concentration after streptozotocin administration (1 to 6 weeks).

anesthetized and the solution was administrated via an intravenous needle inserted into an ear with continuous infusion for approximately 5 minutes. To avoid gastrointestinal complications such as nausea and vomiting after streptozotocin injection, animals were fasted for 18 h before the experiment and the control pigs were injected with equal amounts of vehicle (citrate buffer).

The pigs were continuously observed for 24 h after streptozotocin injection. Because streptozotocin often causes temporary hypoglycaemia, 250 mL of 50% glucose solution per animal was administered. The pigs received a normal diet throughout the experiment twice a day and tap water ad libitum. The blood glucose level was measured to confirm hyperglycaemia. The blood glucose concentration was estimated using an Accent-200 (Germany) biochemical analyser, with the colorimetric measurement at a wavelength of 510 nm/670 nm. For this purpose, capillary blood from the ear was collected. The plasma glucose level was measured prior to the experiment initiation in both control and experimental groups. The next measurement was made 48 hours after the induction of diabetes. Subsequent measurements of glucose levels were monitored weekly until the end of the experiment.

2.3. Tissue Collection. Six weeks after streptozotocin injection, pigs were deeply anesthetized via intravenous administration of pentobarbital (Vetbutal, Biowet, Poland) and perfused transcardially via the ascending aorta with 4% paraformaldehyde in a 0.1 M phosphate buffer (PB, pH 7.4). The samples were postfixed by immersion in the same fixative for 1 h, rinsed several times with (PB), and then transferred into a 30% sucrose solution and stored at 4°C until sectioning. The frozen tissue blocks were cut in frontal or sagittal planes using a Microm HM 560 cryostat (Carl Zeiss, Germany) at a thickness of 12 μm and mounted on chrome-coated slides.

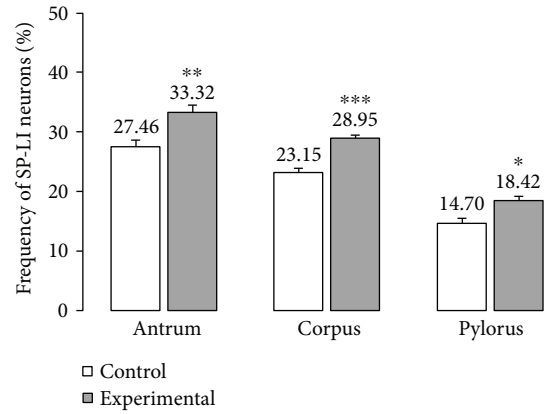

Figure 2: The average percentage of MP neurons immunoreactive to SP. The mean of SP-like immunoreactive (SP-LI) neurons in the myenteric plexus (MP) of the stomach antrum, corpus, and pylorus regions in the control and streptozotocin-inducted diabetes groups. Data are presented as mean ± SEM; statistically significant data ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

2.4. Immunofluorescence Procedure. The sections were processed for double immunofluorescence staining. Briefly, after air drying at room temperature for 45 min and rinsing in 0.1 M phosphate-buffered saline (PBS; pH 7.4; 3 × 10 min), the sections were incubated in a blocking buffer containing 10% normal goat serum (MP Biomedicals, USA), in 0.1 M PBS, 0.1% donkey serum (Abcam, UK), 1% Triton X-100 (Sigma-Aldrich, USA), 0.05% Thimerosal (Sigma-Aldrich, USA), and 0.01% NaN_3 for 1 h at room temperature to reduce nonspecific background staining. Subsequently, after another wash in PBS (3 × 10 min), the sections were incubated overnight at 4°C with primary antibodies raised in different species and directed towards general neuronal marker Hu C/D proteins (mouse polyclonal: Invitrogen USA; code A-212711:1.000; working dilution 1 : 1000), anti-

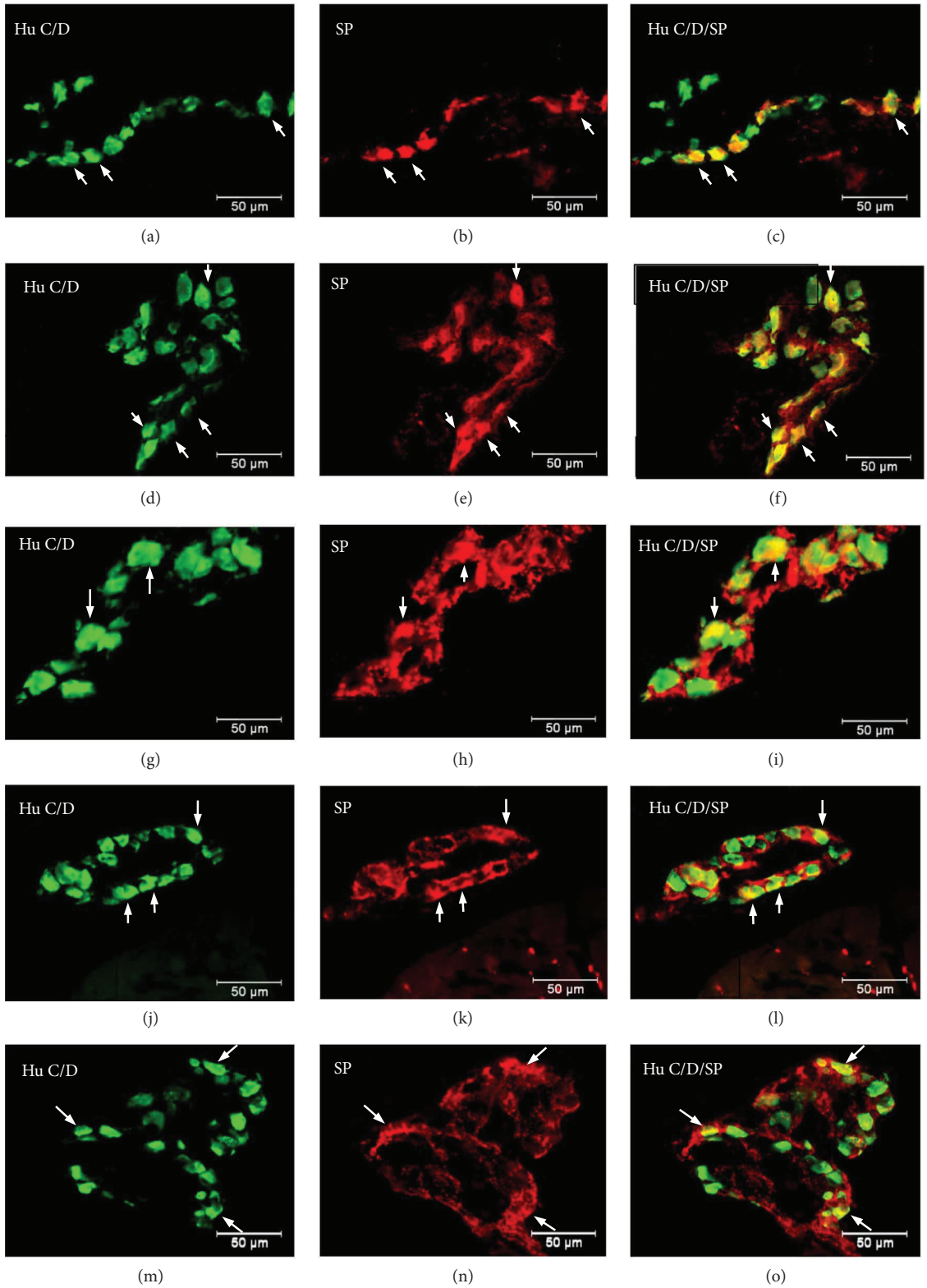

Figure 3: Continued.

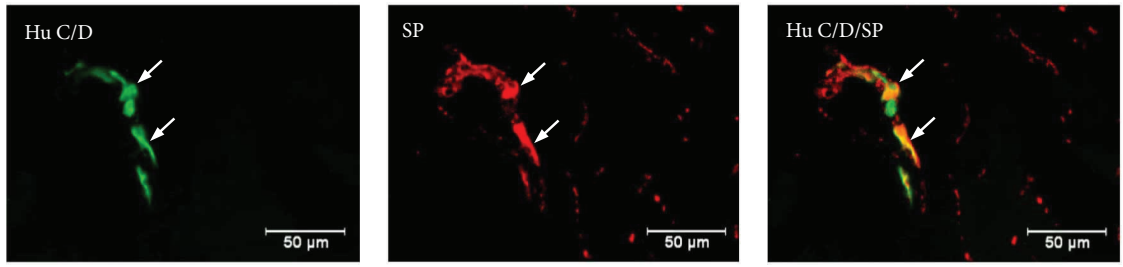

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Figure 3: Myenteric ganglion of the porcine stomach immunoreactive to SP. Myenteric ganglion of the porcine antrum under physiological condition (a–c) and during experimentally inducted diabetes (d–f); myenteric ganglion of the porcine corpus under physiological condition (g–i) and during experimentally inducted diabetes (j–l); and myenteric ganglion of the porcine pylorus under physiological condition (m–o) and during experimentally inducted diabetes (p–r), immunostained for Hu C/D (green/arrows) and SP (red/arrows). The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies is indicated with arrows.

nSP antibodies (rat monoclonal: AbD Serotec, cat. number 8450-0505; working dilution 1 : 150), CGRP (rabbit polyclonal; Millipore, cat. number AB15360; working dilution 1: 4000), and L-ENK (rabbit polyclonal: Abcam, cat. number ab85798; working dilution 1 : 1000). All antibodies were diluted in PBS containing 0.3% Triton X-100 and 1% BSA. On the following day, the sections were rinsed (PBS, 3 × 15 min) and incubated with secondary antibodies (donkey anti-mouse Alexa Fluor 488, 1 : 1000 Invitrogen USA, code A21202; donkey anti-rabbit Alexa Fluor 546 1 : 1000 Invitrogen, USA, code A10040 diluted in PBS containing 0.25% BSA and 0.1% Triton X-100; and donkey anti-rat Alexa Fluor 546, 1 : 1000 Invitrogen, USA; code A21208 diluted in PBS containing 0.25% BSA and 0.1% Triton X-100) for 4 hours. The sections were then rinsed three times (PBS, 3 × 5 min) and mounted in fluorescent mounting medium (DAKO, Carpinteria, CA, USA). The prepared specimens were viewed and photographed using an Olympus BX51 microscope equipped with epifluorescence and appropriate filter sets, coupled with a digital monochromatic camera (Olympus XM 10) connected to a PC, and analysed with Cell Dimension software (Olympus, Tokyo, Japan). Standard controls, that is, preabsorption of the neuropeptide antisera with appropriate antigen, omission, and replacement of the primary antisera by nonimmune sera, were performed to test the antibodies and specificity of the method. The test was performed as follows: sections of the stomach were incubated with a "working" dilution of the primary immunoserum, which had been previously preabsorbed for 18 h at 37°C with 20 μg of appropriate purified protein SP (ab120170, Abcam), CGRP (ab158017, Abcam), and L-ENK (ab159087, Abcam). Additional negative controls, such as the omission and replacement of all primary antisera with nonimmune sera, were also performed. This procedure completely eliminated specific staining.

2.5. Counting of the Nerve Structures and Statistical Evaluation. The number of SP-, CGRP-, and L-ENK-like immunoreactive (LI) enteric neurons was expressed as a percentage of the total number of Hu C/D-positive perikarya. At least 700 Hu C/D-labelled cell bodies of intramural ganglia of each part of the stomach were examined. Only neurons with well-visible nucleus were counted. To prevent the double counting of Hu C/D-immunoreactive neurons, the sections were located at least 100 μm apart. The data pooled from all animal groups were statistically analysed using Statistica 10 software (StatSoft Inc., Tulsa, OK, USA) and expressed as a mean ± standard error (SE) of mean. Significant differences were evaluated using Student's t-test for independent samples ($^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P > 0.001$).

Evaluation of the density of nerves within the muscular or mucosal layers was based on counting all the nerve fibres which were immunoreactive to SP, CGRP, and L-ENK per microscopic observation and was assessed under ×40 objective (0.55 mm2) by subjective observation (two independent researchers). Nerve profiles with clearly visible varicosities were counted in four sections per animal (in five fields per section) and the obtained data were pooled and presented as a mean ± SEM.

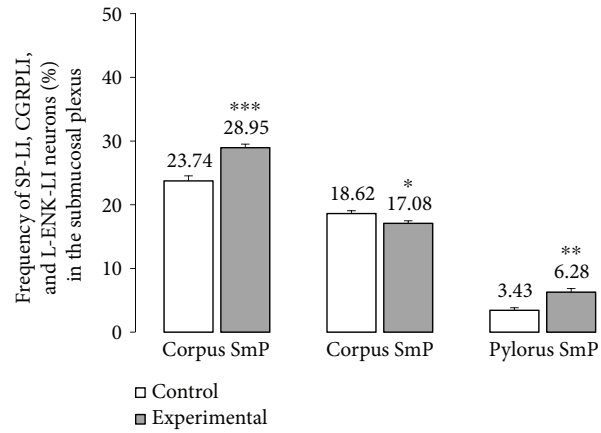
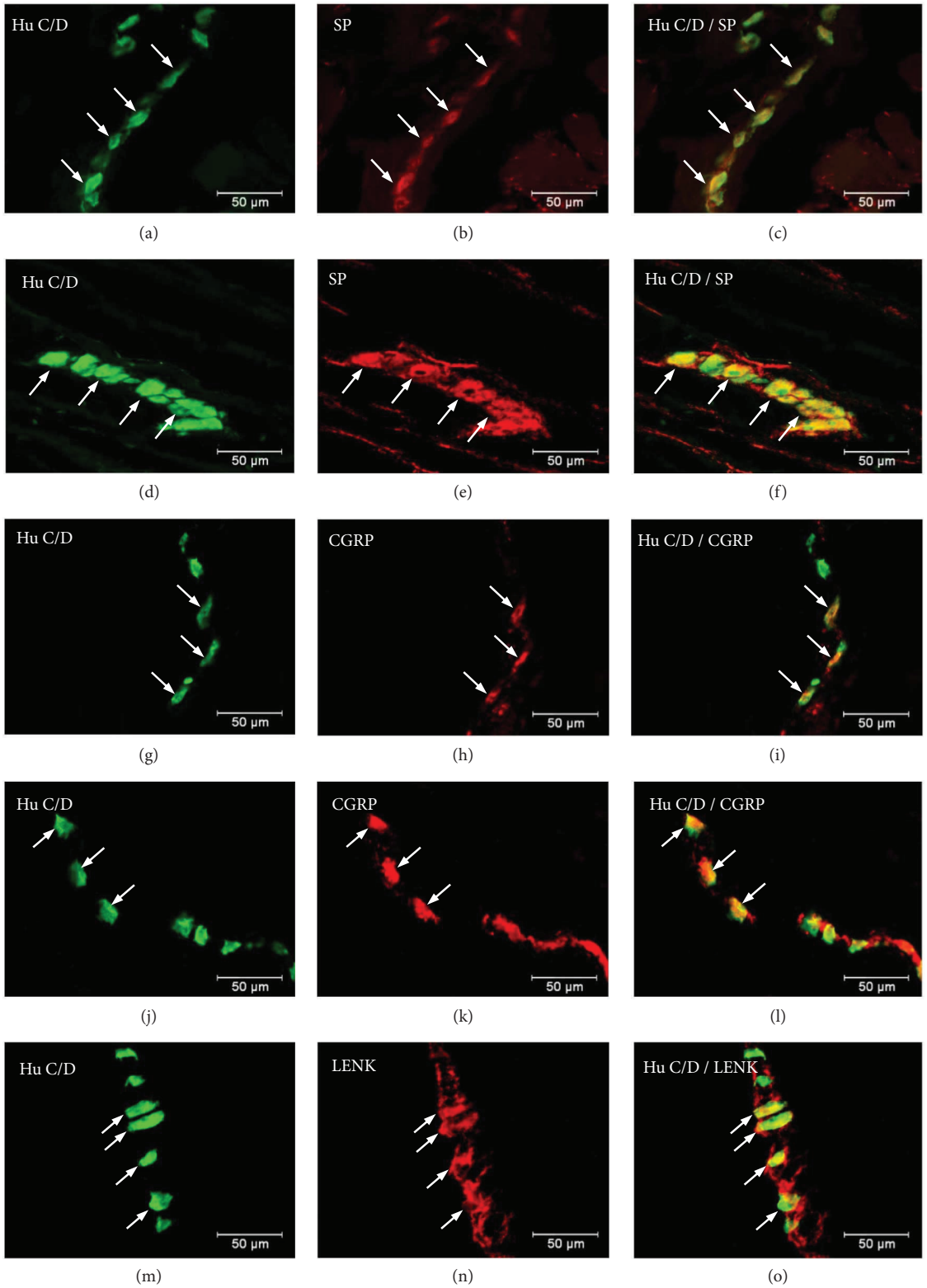


Figure 4: The average percentage of SmP neurons immunoreactive to SP, CGRP, and L-ENK. The mean of SP-like immunoreactive (SP-IL), CGRP-like immunoreactive (CGRP-LI), and L-ENK-like immunoreactive (LENK-LI) neurons in the submucosal plexus (SmP) of the stomach corpus in the control and streptozotocin-inducted diabetes groups. Data are presented as mean ± SEM; statistically significant data ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).



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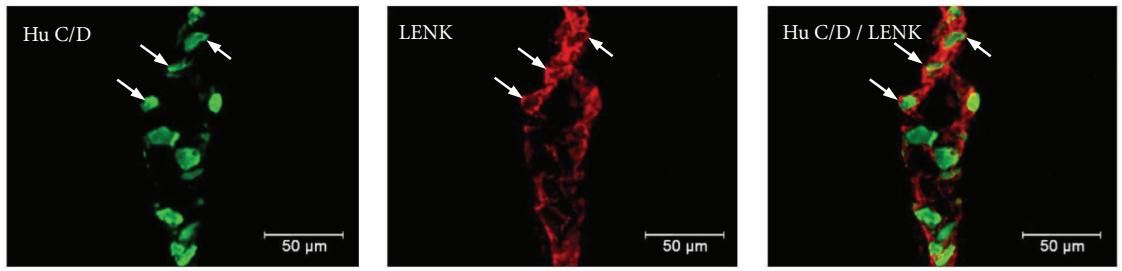

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Figure 5: Submucosal ganglion of the porcine stomach immunoreactive to SP, CGRP, and L-ENK. Submucosal ganglion of the porcine corpus under physiological condition (a–c) and during experimentally inducted diabetes (d–f) immunoreactive to SP; submucosal ganglion of the porcine corpus under physiological condition (g–i) and during experimentally inducted diabetes (j–l) immunoreactive to CGRP; and submucosal ganglion of the porcine corpus under physiological condition (m–o) and during experimentally inducted diabetes (p–r) immunoreactive to L-ENK, immunostained for Hu C/D (green/arrows) and SP,CGRP, and L-ENK (red/arrows). The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies is indicated with arrows.

Table 1: SP-, CGRP-, and L-ENK-immunoreactive nerve fibres in various parts of the porcine stomach under physiological conditions (control group) and during experimentally induced diabetes (experimental group).

Stomach part	Control group			Experimental group		
	Antrum	Corpus	Pylorus	Antrum	Corpus	Pylorus
SP						
Circular muscle layer[a]	4.82 ± 0.16	3.65 ± 0.16	6.07 ± 0.13	7.42 ± 0.37**	5.63 ± 0.15***	10.75 ± 0.4***
Submucosal/mucosal layer[a]	2.8 ± 0.28	5.19 ± 0.20	2.41 ± 0.18	6.72 ± 0.16***	9.46 ± 0.41***	2.89 ± 0.25
CGRP						
Circular muscle layer[a]	6.10 ± 0.08	6.20 ± 0.18	6.24 ± 0.33	10.44 ± 0.26***	11.38 ± 0.34***	8.85 ± 0.19
Submucosal/mucosal layer[a]	2.58 ± 0.12	2.08 ± 0.14	3.40 ± 0.18	2.88 ± 0.18	2.45 ± 0.09	3.14 ± 0.26
L-ENK						
Circular muscle layer[a]	1.63 ± 0.21	3.11 ± 0.22	1.78 ± 0.15	1.98 ± 0.12	1.47 ± 0.37**	2.10 ± 0.24
Submucosal/mucosal layer[a]	1.81 ± 0.24	1.43 ± 0.09	2.25 ± 0.10	1.62 ± 0.51	1.58 ± 0.11	2.55 ± 0.25

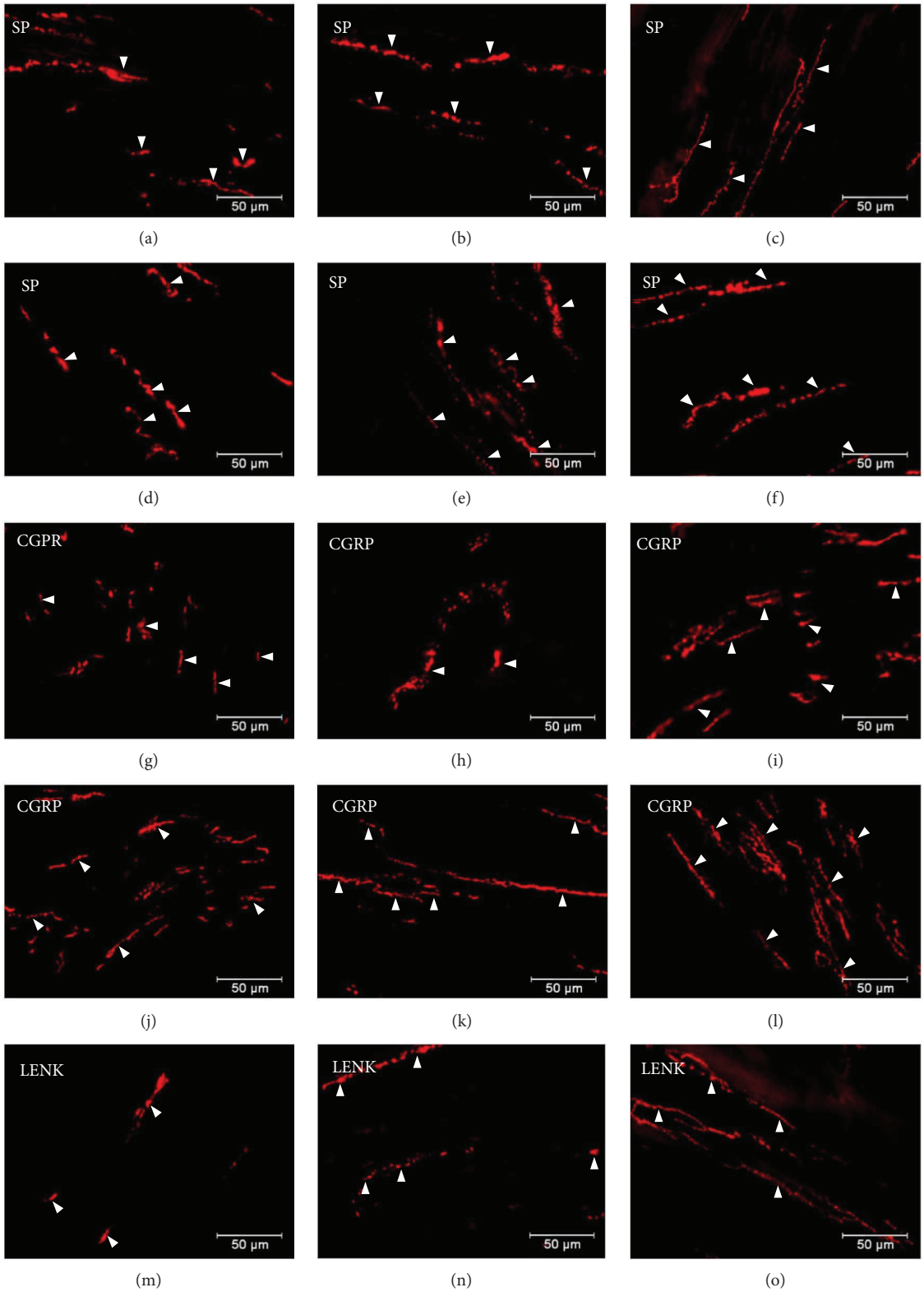
Statistically significant data (** $P > 0.01$ and *** $P > 0.001$). [a]Average number of nerve profiles per area (0.55 μm^2) studied (mean ± SEM).

3. Results

The mean glycaemia level in pigs before streptozotocin injection was within standard reference values for the pig (5.01 mmol/L ± 0.10 mmol/L) (Figure 1) and did not differ between individual animals. Following the STZ induction, a consistent increase in plasma glucose concentration was observed (Figure 1). A significant (17.36 mmol/L ± 0.38 mmol/L) increase in glucose level was observed on the 7th day after STZ injection. The highest increase in blood glucose concentration was detected 4-5 weeks after the STZ injection (22.26 mmol/L ± 1.21 mmol/L) (Figure 1). In the last week of the experiment, the mean serum glucose level increased slightly, reaching 21.24 mmol/L ± 1.11 mmol/L (Figure 1). It should be noted that although chronic glycaemia in experimental animals was significantly higher than that in controls, all pigs which received streptozotocin survived the duration of the experiment in a good general condition and none of the animals required exogenous insulin injection.

Double labelling immunohistochemistry revealed that in the control group, the SP distribution in the ENS neurons was varied and clearly depended on the analysed area of the stomach (Figure 2). In the myenteric ganglia of the antrum, the SP-positive cell bodies constitute 27.46 ± 1.09% of all HuC/D neurons studied (Figure 2, Figures 3(a)–3(c)). In turn, inside the MP of the corpus, the quantity of SP-positive cell bodies was slightly lower (23.15 ± 0.75%) (Figure 2, Figures 3(g)–3(i)). A very similar number of SP-LI neurons were observed in the submucosal plexus (SmP) of the corpus (23.74 ± 0.80%) (Figure 4, Figures 5(a)–5(c)). The smallest number of SP-LI perikarya was found in the MG of the pylorus (14.70 ± 0.80%) (Figure 2, Figures 3(m)–3(o)).

In the diabetic group, a statistically significant increase in the number of SP-positive cell bodies was observed in all investigated area of the stomach. In the MG of the antrum, the population of SP neurons amounted to 33.32 ± 1.21%. (Figure 2, Figures 3(d)–3(f)). The highest increase in SP expression was observed in the MG of the corpus, where the number of SP-LI neurons was 36.58 ± 0.64% (Figure 2, Figures 3(j)–3(l)). A slightly lower increase in SP-positive neurons (to 28.5 ± 0.56%) in the submucosal ganglia of the corpus was noted (Figure 4, Figures 5(d)–5(f)). With regard



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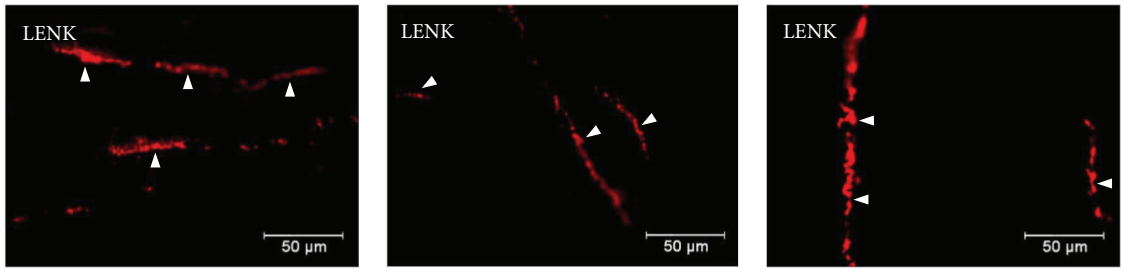

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Figure 6: Distribution pattern of nerve fibres (arrow) immunoreactive to SP, CGRP, and L-ENK within the circular muscle layer. Distribution pattern of nerve fibres (arrows) within the circular muscle layer immunoreactive to SP under physiological condition: antrum (a), corpus (b), and pylorus (c) and under experimentally inducted diabetes: antrum (d), corpus (e), and pylorus (f); for CGRP under physiological condition: antrum (g) corpus (e), and pylorus (f) and under experimentally inducted diabetes: antrum (j), corpus (k), and pylorus (l); and for L-ENK under physiological condition: antrum (m), corpus (n), and pylorus (o) and under experimentally inducted diabetes: antrum (p), corpus (q), and pylorus (r).

to the MG in the pylorus, changes in chemical coding were relativity smaller and amounted to 18.42 ± 0.82% (Figure 2, Figures 3(p)–3(r)).

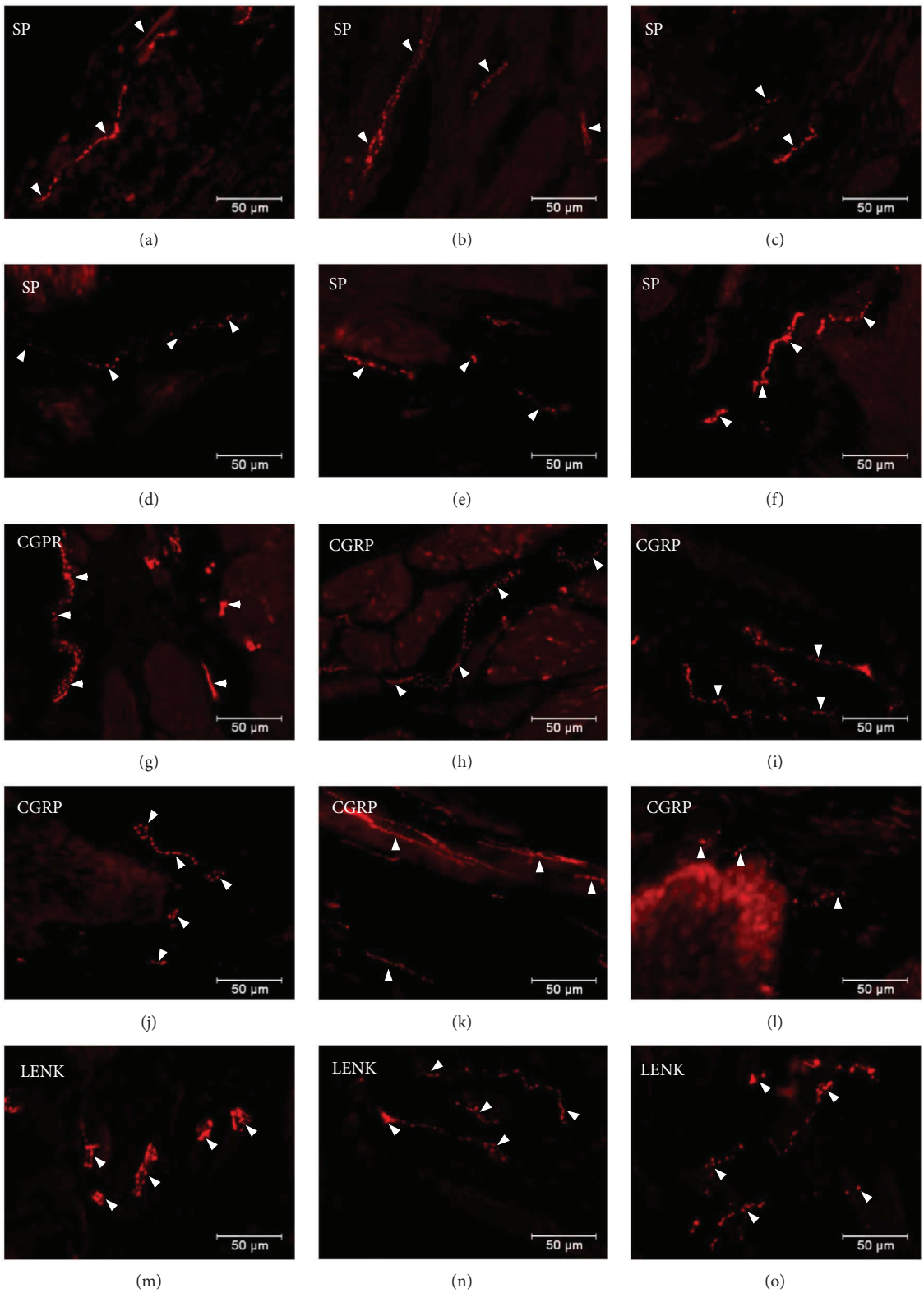
Moreover, in addition to cell bodies, the density of nerve fibres was also investigated. Namely, SP-positive nerve fibres were present in the circular and submucosal muscle layers. In the control group, the SP-immunoreactive nerve fibres were more numerous in the circular muscle layer and they constitute 4.82 ± 0.16 in the antrum, 3.65 ± 0.16 in the corpus, and 6.07 ± 0.13 in the pylorus (Table 1, Figures 6(a)–6(c)). In the diabetic group, a statistically significant increase was observed in the density of SP-LI nerve fibres in the circular muscle layer in all areas of the stomach. In the antrum, SP-positive nerve fibres constituted 7.42 ± 0.37, 5.63 ± 0.16 in the corpus, and 10.75 ± 0.40 in the pylorus (Table 1, Figures 6(d)–6(f)). In the submucosal layer, the SP-positive nerve fibres in the control group were more numerous in the corpus 5.19 ± 0.20, while in the antrum and pylorus, they constituted only 2.8 ± 0.28 and 2.41 ± 0.18, respectively (Table 1, Figures 7(a)–7(c)). Under hyperglycaemia conditions, a statistically significant increase in the density of SP nerve fibres in the antrum and corpus was observed (6.72 ± 0.16 and 9.46 ± 0.41, resp.) (Table 1, Figures 7(d) and 7(e)), while in the pylorus, no statistically significant changes were observed (Table 1, Figure 7(f)).

The other investigated substance was CGRP. Similar to SP, CGRP-positive neurons were presented in all studied areas, but clear differences were noted between various regions of the stomach. In the control group, the highest population of CGRP was noted in the antrum (33.07 ± 1.10%) (Figure 8, Figures 9(a)–9(c)), while in the other regions of the stomach, the number of CGRP-immunoreactive cell bodies was relatively lower and amounted to 23.95 ± 0.64% within the MG in the corpus (Figure 8, Figures 9(g)–9(i)) and 16.15 ± 0.80% in the pylorus (Figure 8, Figures 9(m)–9(o)) and 18.62 ± 0.44% in the submucosal ganglia in the corpus (Figure 4, Figures 5(g)–5(h)). In the diabetic group, statistically significant changes were observed in all investigated areas. The number of CGRP-positive neurons within the MG was estimated at 41.6 ± 0.99% in the antrum (Figure 8, Figures 9(d)–9(f)), 38.61 ± 0.81% in the corpus (Figure 8, Figures 9(j)–9(l)), and 22.33 ± 0.86% in the pylorus (Figure 8, Figures 9(p)–9(r)). In turn, in the submucosal ganglia inside the corpus, the numbers of GGRP-positive neurons were 17.08 ± 0.40% (Figure 4, Figures 5(j)–5(l)). Nerve fibres immunoreactive to CGRP were observed in the circular muscle layer and in the submucosal layer (Table 1). In the control group, the density of CGRP-LI neuronal processes was similar in all investigated areas. In the circular muscle layer, an average of 6.10 ± 0.37 of CGRP-positive fibres were noted in the antrum (Figure 6(g)), 6.20 ± 0.18 in the corpus (Figure 6(h)), and 6.24 ± 0.33 in the pylorus (Figure 6(i), Table 1).

In the diabetic group, a statistically significant increase in the density of CGRP-LI fibres within the circular muscle layer was observed. In the antrum, the density of CGRP-containing nerve fibres was estimated at 10.44 ± 0.23 (Figure 7(j)), whereas in the corpus and pylorus, it was 11.38 ± 0.32 (Figure 7(k)) and 8.85 ± 0.19, respectively (Table 1, Figure 7(l)). Within the submucosal layer, the density of CGRP-positive nerve fibres was scarce. In the antrum of control animals, 2.58 ± 0.12 of CGRP-positive nerve fibres was observed, while in the corpus, it was 2.08 ± 0.14 and it was 3.40 ± 0.18 in the pylorus (Table 1, Figures 7(g)–7(i)). Moreover, in the experimental pigs, no statistically significant changes in the density of CGRP-immunoreactive nerve fibres were observed (Table 1, Figures 7(j)–7(l)).

Another substance studied was L-ENK. The population of neurons containing L-ENK was the least numerous among investigated peptides. In the control group, the population of L-ENK-positive neurons was the highest in the MG of the corpus (15.06 ± 0.47%) (Figure 10, Figures 11(g)–11(i)), a bit lower in the antrum (8.84 ± 0.54%) (Figure 10, Figures 11(a)–11(c)), and the lowest in the pylorus (5.43 ± 0.32%) (Figure 10, Figures 11(m)–11(o)). In turn, in the submucosal ganglia within the corpus, this value was calculated at 3.43 ± 0.40% (Figure 4, Figures 5(m)–5(o)).

Hyperglycaemia triggered the following changes in the chemical phenotyping of L-ENK-positive enteric neurons. In the MG of the antrum, an increase was noted in L-ENK-LI neurons (to 11.45 ± 0.44%) (Figure 10,



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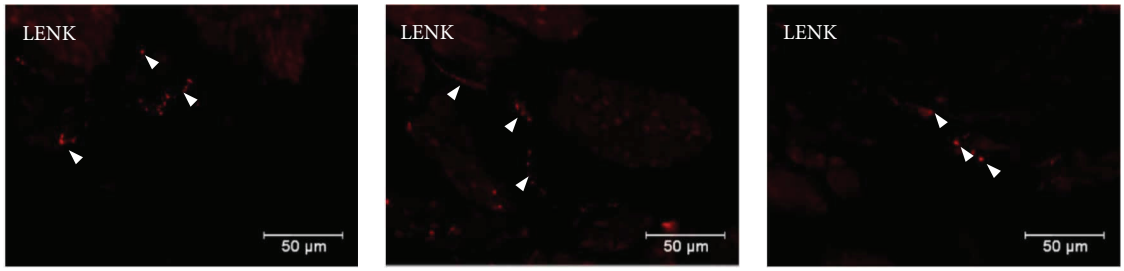

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Figure 7: Distribution pattern of nerve fibres (arrow) immunoreactive to SP, CGRP, and L-ENK within the submucosal muscle layer. Distribution pattern of nerve fibres (arrows) within the submucosal muscle layer immunoreactive to SP under physiological condition: antrum (a), corpus (b), and pylorus (c) and under experimentally inducted diabetes: antrum (d), corpus (e), and pylorus (f); for CGRP under physiological condition: antrum (g), corpus (e), and pylorus (f) and under experimentally inducted diabetes: antrum (j), corpus (k), and pylorus (l); and for L-ENK under physiological condition: antrum (m), corpus (n), and pylorus (o) and under experimentally inducted diabetes: antrum (p), corpus (q), and pylorus (r).

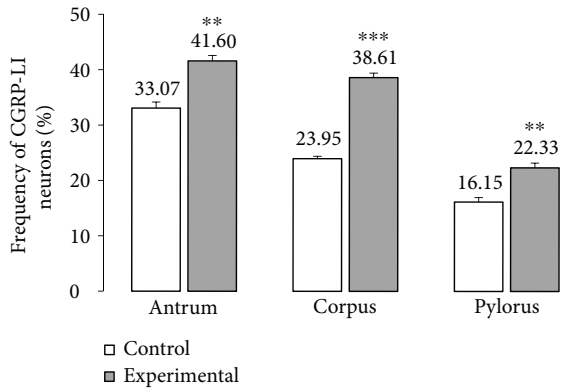


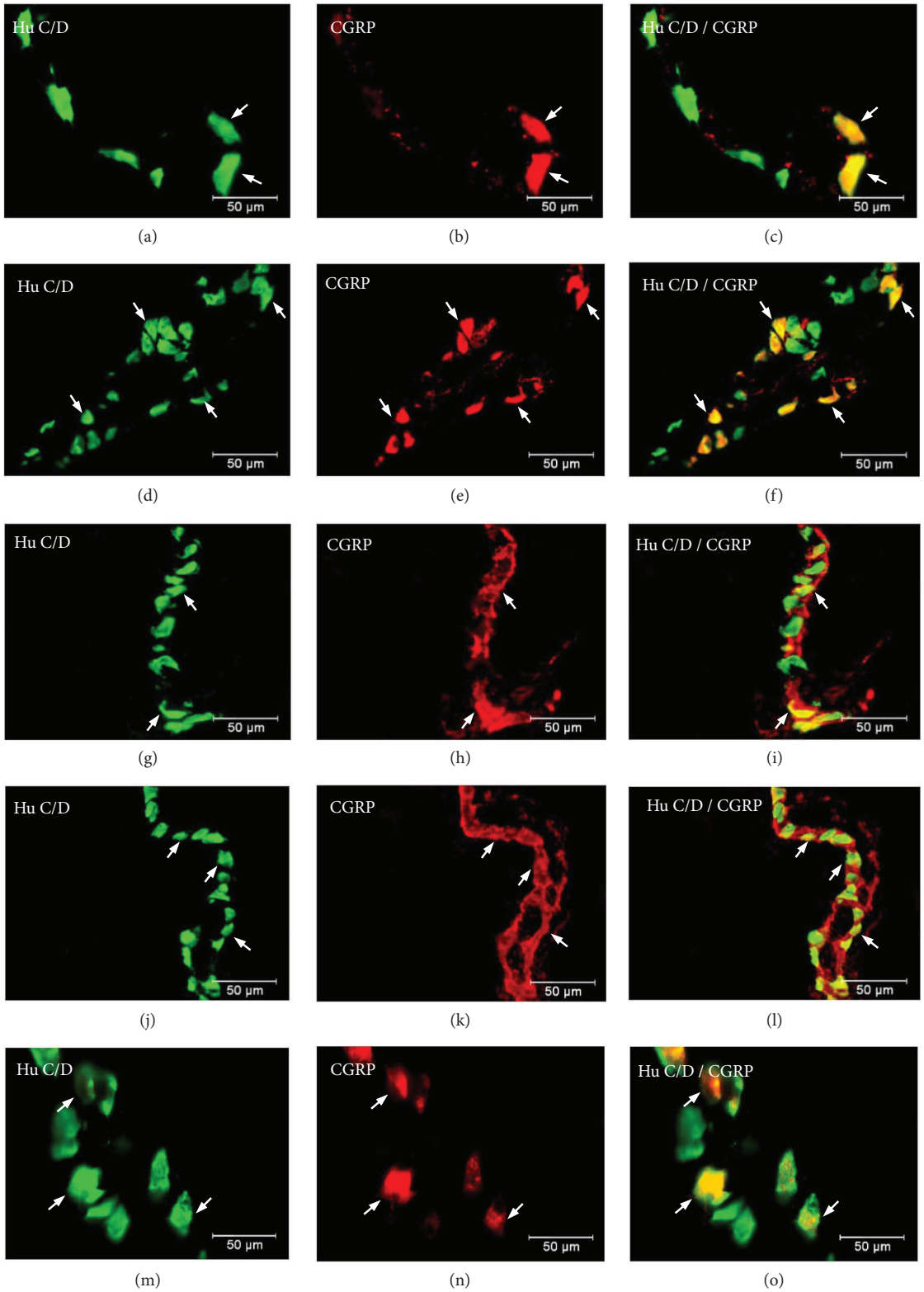
Figure 8: The average percentage of MP neurons immunoreactive to CGRP. The mean of CGRP-like immunoreactive (CGRP-LI) neurons in the myenteric plexus (MP) of the antrum, corpus, and pylorus regions of the stomach in the control and streptozotocin-inducted diabetes groups. Data are presented as mean ± SEM; statistically significant data ($^{**}P < 0.01$ and $^{***}P < 0.001$).

Figures 11(d)–11(f)), while in the corpus, there was a decrease in L-ENK-LI neurons (to 6.94 ± 0.42%) (Figure 10, Figures 11(j)–11(l)), while in the pylorus, the number of L-ENK-immunopositive cell bodies was decreased (1.90 ± 0.16%) (Figure 10, Figures 11(p)–11(r)). In turn, in the submucosal ganglion of the corpus, the population of L-ENK-LI neurons was increased (to 6.28 ± 1.67%) (Figure 4, Figures 5(p)–5(r)).

On the other hand, L-ENK-positive nerve fibres were visible in both the circular and submucosal muscle layers. In the control group, the density of nerve fibres within the circular muscle layer amounted to 1.63 ± 0.21 in the antrum, 3.11 ± 0.22 in the corpus, and 1.78 ± 0.15 in the pylorus (Table 1, Figures 6(m)–6(o)). In a diabetes condition, statistically significant changes were observed only in the corpus, that is, a decrease was noted in L-ENK-positive nerve fibres (to 1.47 ± 0.37) (Table 1, Figures 6(p)–6(r)). In the submucosal layer, the density of nerve fibres in the control group was as follows: 1.81 ± 0.24 in the antrum, 1.43 ± 0.09 in the corpus, and 2.25 ± 0.01 in the pylorus (Table 1, Figures 7(m)–7(o)). In the diabetic group, the density of L-ENK-containing nerves fibres changed only in a statistically insignificant manner compared to that in the control group (Figures 7(p)–7(r)). In the preabsorbtion test positive reaction was not observed (Figure 12).

4. Discussion

The present study showed that the porcine gastric enteric neurons, after six weeks of sustained hyperglycaemia, exhibit alterations in the chemical phenotyping of the ENS neurons situated in the porcine stomach. Hitherto, data describing chemical changes in the intramural enteric neurons in response to hyperglycaemia were limited to studies on small animal models, especially rats [12, 37, 38]. The population of the SP-IR neurons has been described in a different region of the gastrointestinal tract [20, 24, 25]. Previous studies clearly showed that the level of SP expression in the GI tract is dependent on diabetes duration. In the human stomach, after 15 years of diabetes condition, a decreased concentration of SP in Cajal cells was described [39]. However, in rats, 48 weeks after the induction of diabetes, significant changes in SP expression in both the stomach and small intestine were not observed [40]. The results obtained in the present experiment, performed on immature gilts, are different than those presented above. Namely, a statistically significant increase was observed in the number of SP-positive neurons in all investigated areas of the stomach. It is hypothesized that the observed augmentation in SP expression in diabetic pigs may be explained by the time of hyperglycaemia duration. Moreover, throughout the entire time of the experiment, all animals in the diabetic group exhibited an approx. fourfold increase in glucose serum compared to those in the control group. Additionally, none of the pigs in the diabetic group received exogenous insulin. The supplementation of exogenous insulin seems to be an important factor to restore an appropriate level of SP [41]. In turn, with respect to differentiation of SP distribution due to the region of the gastrointestinal tract, previous studies demonstrated differences between the stomach and small and large intestines, as well as among each ganglion [42–44]. For example, in diabetic rats, an increase in SP



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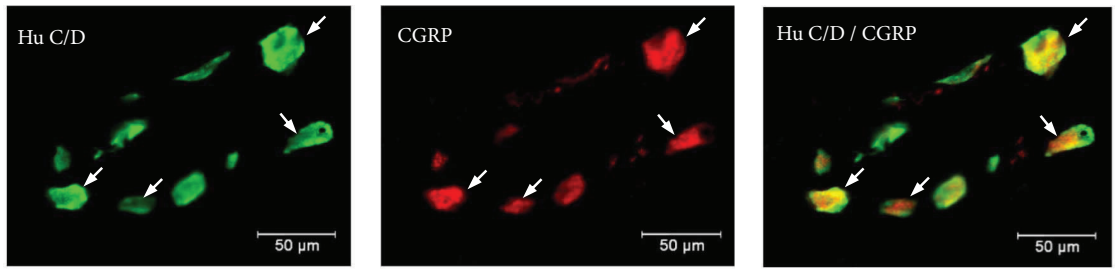


Figure 9: Myenteric ganglion of the porcine stomach immunoreactive to CGRP. Myenteric ganglion of the porcine antrum under physiological condition (a–c) and during experimentally inducted diabetes (d–f); myenteric ganglion of the porcine corpus under physiological condition (g–i) and during experimentally inducted diabetes (j–l); and myenteric ganglion of the porcine pylorus under physiological condition (m–o) and during experimentally inducted diabetes (p–r), immunostained for Hu C/D (green/arrows) and CGRP (red/arrows). The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies is indicated with arrows.

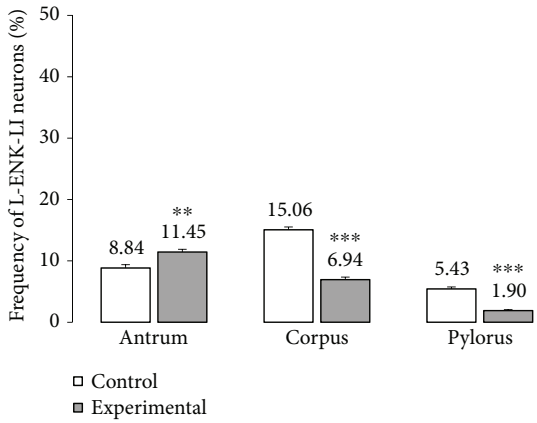


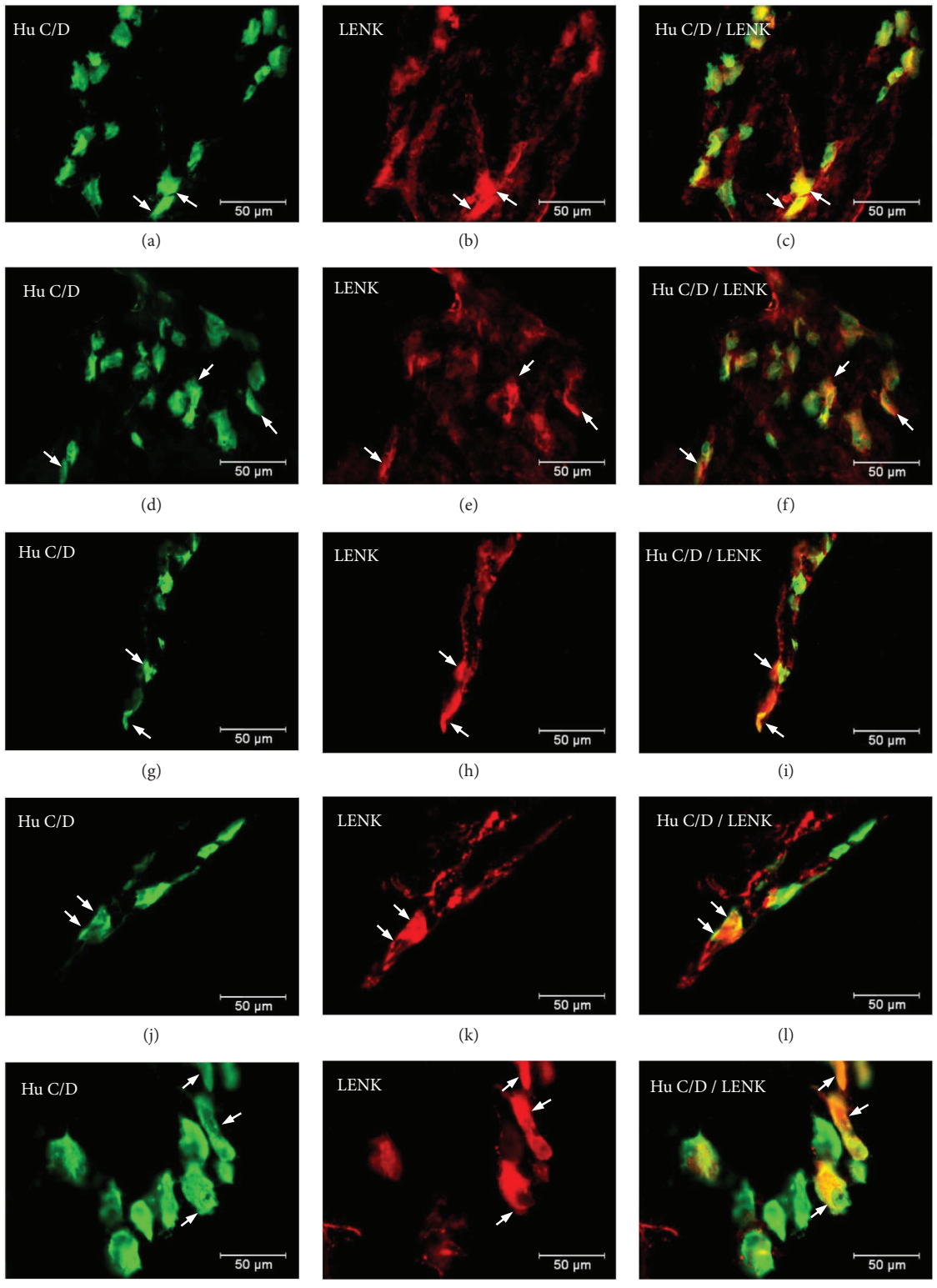
Figure 10: The average percentage of MP neurons immunoreactive to L-ENK. The mean of L-ENK-like immunoreactive (LENK-LI) neurons in the myenteric plexus (MP) of the antrum, corpus, and pylorus regions of the stomach in the control and streptozotocin-inducted diabetes group. Data are presented as mean ± SEM; statistically significant data ($^{**}P < 0.01$ and $^{***}P < 0.001$).

expression has been noted in the myenteric ganglia of the duodenum and colon, as well as within the ileum [12]. Moreover, we have observed an increase in the number of SP-positive nerve fibres. A similar process was also observed in other pathological conditions [20]. But it should be noted that the exact origin of these fibres (extrinsic/intrinsic) is unclear and its elucidation requires application of retrograde tracing techniques.

Furthermore, CGRP immunoreactivity was found to be significantly reduced after 8 and 12 weeks of diabetes in the myenteric and submucosal plexuses of rat ileum and colon [43, 44]. On the other hand, there is a lack of information about changes in CGRP expression in the stomach. The current study indicates that in the myenteric ganglia of the antrum, corpus, and pylorus in diabetic pigs, the expression level of CGRP was higher than the number of CGRP-LI neurons observed in the healthy group. In the submucosal ganglia in the corpus, statistically significant changes in the CGRP expression were also noted. It should be emphasized that previous studies were performed on rats using the streptozotocin diabetes model, while the current study, for the first time, used pigs as a model to focus studying on a phenotype of stomach enteric neurons. The rate of metabolism in swine is similar to humans [45]. In addition, pigs are increasingly used as experimental models in diseases with metabolic disorders [46].

The role of SP and CGRP in the gastrointestinal tract is well established [15, 16]. One of the best-known functions of SP and CGRP, not only in the gastrointestinal tract, is sensory and nociceptive information transmission [20]. Both SP and CGRP are markers of primary afferent neurons whose main role is to participate in short reflexes within the gastrointestinal wall. These neurons also take part in the transmission of nociceptive stimuli from the lamina mucosa and muscular layer of the gastrointestinal tract [30]. This observation strongly suggests that SP and CGRP may be actively involved in the pathophysiological mechanisms of abdominal pain during diabetes. This function seems to be very important because pain episodes are one of the most often disturbances in people with long-term diabetes [2]. Our studies show that an increase in the number of SP and CGRP neurons can be correlated with pain episodes. In order to fully answer of this question, further research should be carried out using analogues or agonists/antagonists of these substances to confirm the role of SP and CGRP in this process.

It should also be added that in the course of many gastrointestinal symptoms, gastrointestinal motility is impaired. Gastrointestinal symptoms including dysfunction of motor activity appear more commonly in diabetes [47]. The most important manifestation of gastrointestinal autonomic neuropathy is gastroparesis [4, 5]. The molecular mechanism underlying the above symptoms is not fully understood. The receptor for advanced glycation end products (RAGE) has recently gained attention as a potential contributor to neuropathy including autonomic neuropathy [48]. The mechanism triggering RAGE-related neurodegenerative



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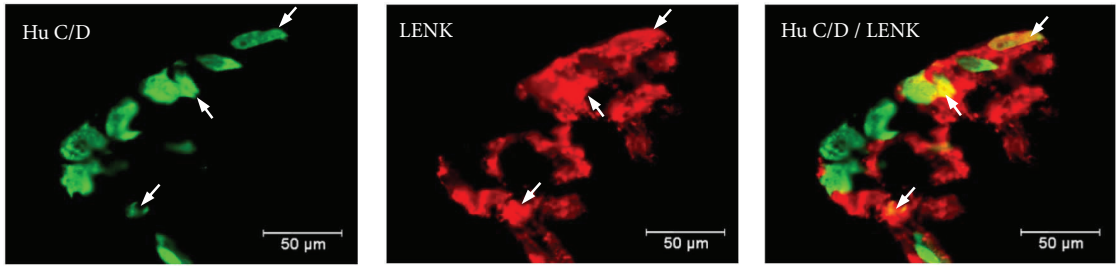
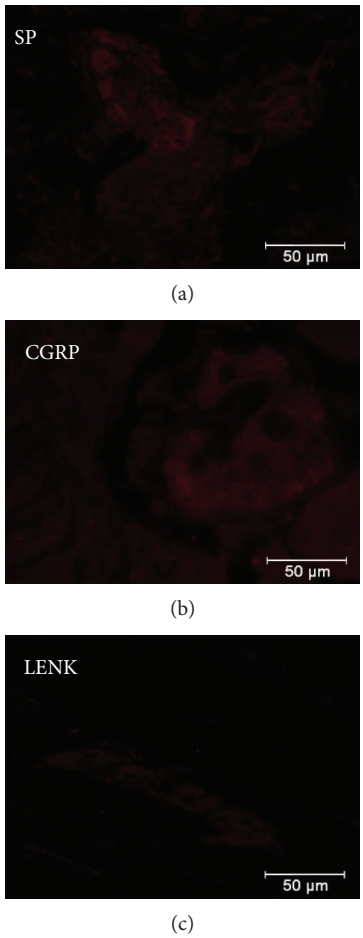


(p) (q) (r)

Figure 11: The average percentage of MP neurons immunoreactive to L-ENK. The mean of L-ENK-like immunoreactive (LENK-LI) neurons in the myenteric plexus (MP) of the antrum, corpus, and pylorus regions of the stomach in the control and streptozotocin-inducted diabetes group. Data are presented as mean ± SEM; statistically significant data ($^{**}P < 0.01$ and $^{***}P < 0.001$).



(a)

(b)

(c)

Figure 12: Negative controls for SP (a), CGRP (b), and LENK (c).

processes is likely related to oxidative stress caused by an increase in reactive oxygen species through an NF-κB-activated inflammatory pathway [49]. In the above described processes, SP and CGRP can be at least partially involved. Inflammatory and immune modulatory actions of SP are relatively well known [50]. Namely, SP is considered to be an important proinflammatory factor, which inducts cytokine release [51], as well as an agent sending information between the nervous and immune systems [50].

Leu5-enkephalin, which was also investigated in the present study, belongs to a group of opioid peptides [31]. The sources of opioid peptides in the GI tract include both endocrine cells and enteric neurons. Enkephalins are predominantly located in submucosal and myenteric neurons. They are also present in stomach muscle membranes, as well as in the muscle layer of the small and large intestines. These peptides exhibit various effects on the function of the gastrointestinal tract. Generally, they show a modulatory effect on the secretion of other peptides and are able to enhance or inhibit the contractility of particular parts of the gastrointestinal tract [17, 52, 53].

The current results, for the first time, describe changes in the expression of leu5-enkephalin in stomach enteric neurons under acute hyperglycaemia. In contrast to SP and CGRP, an increase in the number of neurons containing leu5-enkephalin in investigated areas of the stomach was not observed. With regard to myenteric ganglia, in the corpus and pylorus, a decrease in the expression of leu5-enkephalin was observed. To date, changes of leu5-enkephalin in the course of diabetes in enteric neurons have not been investigated. Till now, the expression and distribution of leu5-enkephalin were described in the human large intestine in the course of drug-resistant colitis [30]. Moreover, the distribution of opiate receptors in the gastrointestinal tract were also described [54]. It is well known that endogenous opioids have strong antinociceptive properties. Based on the current results, it can be concluded that changes in the amount of leu5-enkephalin neurons may be at least partially responsible for increasing pain reactions in the course of long-lasting hyperglycaemia in diabetes patients. Also, like in the case of CGRP and SP utilization of agonists or antagonists of opioids, receptors can provide more information concerning engagement of investigated substances in gastrointestinal complications in the course of diabetes.

5. Conclusion

In conclusion, the obtained results proved that hyperglycaemia causes significant changes in the neurochemical profile of the porcine stomach enteric neurons with respect to SP, CGRP, and L-ENK. It also seems that the participation of investigated substances in the development

of gastric complications is significant. Undoubtedly, they are involved in the process of transmitting and modulating sensory information and may also influence the immune response, inflammatory processes, and regulate motor activity. It cannot be excluded that, in the future, research on the role of these peptides may result in the development of effective pharmacotherapy of gastrointestinal disorders. In addition, it seems that the pig is an appropriate model for studying the effect of hyperglycaemia on the neurochemical coding of enteric neurons.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Research Article

Effect of Dietary Content of Menhaden Oil with or without Salsalate on Neuropathic Endpoints in High-Fat-Fed/Low-Dose Streptozotocin-Treated Sprague Dawley Rats

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In this study, we wanted to extend our investigation of the efficacy of fish oil with or without salsalate on vascular and neural complications using a type 2 diabetic rat model. Four weeks after the onset of hyperglycemia, diabetic rats were treated via the diet with 3 different amounts of menhaden oil with or without salsalate for 12 weeks. Afterwards, vascular reactivity of epineurial arterioles and neuropathy-related endpoints were examined. The addition of salsalate to high-fat diets enriched with 10% or 25% kcal of menhaden oil protected vascular reactivity to acetylcholine and calcium gene-related peptide, motor and sensory nerve conduction velocity, thermal nociception, intraepidermal nerve fiber density, and cornea sensitivity to a greater extent than 10% or 25% menhaden oil alone. Vascular and neural function was maximally protected with diet containing 45% kcal as menhaden oil, and adding salsalate did not provide any additional benefit. Salsalate alone in the high-fat diet of diabetic rats provided minimal protection/improvement of vascular and neural dysfunction. These studies imply that dietary salsalate in combination with lower amounts of menhaden oil can provide greater benefit toward diabetes-induced vascular and neural impairment than menhaden oil alone.

1. Introduction

Peripheral neuropathy is one of the most common complications of diabetes and has been reported to affect about 50% of the diabetic population, and up to 30% of the population reported to be prediabetic with insulin resistance [1, 2]. After many years of research, there is no treatment for diabetic peripheral neuropathy other than good glycemic control but for patients with the most common form of diabetes, referred to as late onset or type 2 diabetes, good glycemic control provides minimal benefit [1]. Therefore, there is a critical need for a treatment that will at the minimum slow progression.

Previously, we have demonstrated that increasing the dietary content of fish oil alone or in combination is an effective treatment for vascular and neural complications in diabetic rodents [3, 4]. We have also shown that cotreatment of type 1 diabetic mice with menhaden (fish) oil and salsalate is more effective toward neuropathy than menhaden oil alone and that circulating resolvin D1 levels are increased when salsalate is combined with menhaden oil [5]. Lastly, we have demonstrated that treating either type 1 or type 2 diabetic mice with daily injections of resolvin E1 or D1, metabolites of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively, most abundant omega-3 polyunsaturated fatty acids found in fish oil, improves peripheral neuropathy [6, 7].

Increasing consumption of omega-3 (n-3) polyunsaturated fatty acids, commonly found in marine mammals, through the diet or by supplements has been shown to

improve cardiovascular disease [8, 9]. Decreasing the n-6 to n-3 ratio of polyunsaturated fatty acids in circulation has been linked to reducing inflammatory stress [10, 11]. Furthermore, several metabolites of EPA and DHA including neuroprotectin D1 and E and D series resolvins have been shown to have anti-inflammatory and neuroprotective properties [6, 12, 13]. There have been very few side effects reported following increased natural consumption of n-3 polyunsaturated fatty acids. Individuals increasing fish oil consumption via capsules have reported a fishy taste or breath, and no adverse events were reported in a large multicenter clinical trial [14]. Therefore, increasing intake of n-3 polyunsaturated fatty acids is widely considered to be safe and potentially beneficial toward different metabolism-related disorders. Moreover, it has been reported that a higher n-3 polyunsaturated fatty acid index is associated with statistically significant, clinically relevant lower systolic and diastolic blood pressure levels in normotensive young and healthy individuals [15]. From studies in animal models, it would seem that increasing the formation of metabolites of n-3 polyunsaturated fatty acids would also be an advantageous approach to improving health benefits of increased intake of EPA and DHA. To that end, we have investigated the potential for salsalate to increase production of resolvins and its effect on vascular reactivity and neural endpoints related to diabetic peripheral neuropathy.

Salicylsalicylic acid (salsalate), a nonacetylated salicylate, is a nonsteroidal anti-inflammatory agent that inhibits the synthesis of prostaglandins by inactivating cyclooxygenase enzymes [16]. Salsalate is insoluble in gastric juice and moves to the small intestine where it is hydrolyzed into two salicylic acid molecules. In clinical trials, the main side effects of salsalate were tinnitus, headache, dizziness, and gastrointestinal discomfort, which occurred at higher doses and subsided following withdrawal of treatment [17, 18]. Salsalate produces less gastrointestinal bleedings when compared to acetylsalicylic acid due to less mucosal prostaglandin inhibition [19].

In this study, we have used a rat model of type 2 diabetes and examined the singular and combined effect of salsalate with 3 different concentrations of menhaden oil on vascular reactivity by epineurial arterioles, blood vessels that provide circulation to the sciatic nerve and multiple endpoints associated with peripheral nerve function.

2. Materials and Methods

Chemicals used in this study came from Sigma-Aldrich chemical company (St. Louis, MO, USA), unless otherwise stated.

2.1. Animals. Sprague Dawley (Envigo, Indianapolis, IN) male rats 10–11 weeks of age were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited animal care facility, and food (Envigo, number 7001, 4.25% kcal as fat, 3.0 kcal/g, Madison, WI) and water were provided ad libitum. These studies were approved by the University of Iowa Animal Care and Use Committee (number 5071450). All institutional and NIH guidelines for use of animals were followed. At 12 weeks of age, rats were divided into 8 groups. Rats in seven of these groups were designated to become diabetic and were placed on a high-fat diet (D12451 [45% kcal as fat, 4.7 kcal/g], Research Diets, New Brunswick, NJ) with lard as the primary source of the fat. The remaining set of rats were designated to be the control group and remained on the base diet throughout the study. Eight weeks later, the high-fat-fed rats were treated with streptozotocin (30 mg/kg in 0.1 M citric acid buffer, pH 4.5, ip, EMD/Millipore, Billerica, MA) to induce hyperglycemia. Control rats were treated with vehicle. Blood glucose was evaluated 96 h later using glucose-oxidase reagent strips (Aviva Accu-Chek, Roche, Mannheim, Germany), and rats having a blood glucose level of 250 mg/dl (13.8 mM) or greater were considered to be diabetic. Four weeks after the confirmation of hyperglycemia, treatments were initiated. Rats in the untreated group continued to receive the 45% kcal high-fat diet. One treatment group received the 45% kcal high-fat diet supplemented with 2 g/kg salsalate. Three treatment groups received a high-fat diet with 22%, 56%, or 100% of the kcal derived from lard replaced with menhaden oil. These groups were referred to as 10%, 25%, and 45% menhaden oil-treated rats, respectively. The three remaining treatment groups received the three different menhaden oil-enriched diets supplemented with 2 g/kg salsalate. These modified high-fat diets were prepared by Research Diets. The final fat content of all the high-fat diets was maintained at 45% kcal through a mixture of lard and/or menhaden oil as the source of the fat. The treatment period was 12 weeks.

2.2. Nerve Function Endpoints. Thermal nociceptive response was assessed in rats one week prior to euthanasia using the Hargreaves method with an IITC Life Science Inc. device (Woodland Hills, CA, model 390G) as previously described [20]. Prior to measurement, rats were allowed 15 min to assimilate in the device. Six recordings were made using both hindpaws with a 5-minute delay between recordings, the initial recording was discarded, and the remaining five were averaged, presented in seconds, and served as the thermal nociceptive response latency. An automatic termination of the operation was set for 25 sec to avoid tissue injury to the rat due to severe decrease of sensation.

Corneal sensation was determined by a Cochet-Bonnet filament esthesiometer (Luneau Ophtalmologie, France) [21]. Rats were gently restrained by hand and the 6 cm filament advanced to touch the eye. If the rat blinked, the length of the filament was recorded. If the rat did not blink, the filament was shortened by 0.5 cm and the procedure was repeated. This process was continued until the rat blinked. Each eye was evaluated. The data were reported in cm.

On the day of terminal studies, rats were anesthetized with Nembutal (50 mg/kg, ip, Diamondback Drugs, Scottsdale, AZ) and motor and sensory nerve conduction velocities were assessed in the sciatic-posterior tibial conducting system and digital nerve, respectively, as in previous experiments [20]. Motor nerve conduction velocity was calculated by using the stimulus artifact of the evoked potential, subtracting the latency measurement (in milliseconds) from the sciatic notch from the latency measurement of the

Table 1: Effect of menhaden oil with or without salsalate on weight, chow consumed, serum triglycerides, free fatty acids, and cholesterol in type 2 diabetic Sprague Dawley rats.

Condition	Start weight (g)	End weight (g)	Chow consumed (g/kg body weight/day)	Blood glucose (mg/dl)	Serum triglycerides (mg/ml)	Serum-free fatty acids (mmol/L)	Serum cholesterol (mg/ml)
Control (12)	336 ± 3	494 ± 13	50.5 ± 3.0	147 ± 6	32.7 ± 5.8	0.13 ± 0.02	0.91 ± 0.02
Diabetic (12)	335 ± 2	439 ± 9	58.0 ± 3.8	478 ± 35[a]	192.3 ± 18.4[a]	0.37 ± 0.05[a]	1.95 ± 0.06[a]
Diabetic + salsalate (12)	333 ± 3	451 ± 12	48.0 ± 3.5	443 ± 40[a]	125.1 ± 25.9	0.36 ± 0.03[a]	1.73 ± 0.20[a]
Diabetic + menhaden oil 10% (12)	322 ± 3	420 ± 9	48.9 ± 3.3	469 ± 20[a]	246.4 ± 30.2[a]	0.42 ± 0.06[a]	1.53 ± 0.17[a]
Diabetic + menhaden oil 10% + salsalate (12)	326 ± 2	439 ± 21	50.9 ± 3.2	396 ± 43[a]	102.4 ± 25.0	0.32 ± 0.05[a]	1.55 ± 0.17[a]
Diabetic + menhaden oil 25% (12)	309 ± 4	426 ± 21	64.5 ± 4.6	500 ± 16[a]	316.9 ± 37.5[a]	0.41 ± 0.04[a]	2.81 ± 0.22[a]
Diabetic + menhaden oil 25% + salsalate (12)	305 ± 4	415 ± 14	58.2 ± 3.8	478 ± 39[a]	119.4 ± 26.2	0.35 ± 0.03[a]	1.78 ± 0.15[a]
Diabetic + menhaden oil 45% (12)	335 ± 3	459 ± 18	57.9 ± 4.3	426 ± 42[a]	82.6 ± 13.0[a,b]	0.40 ± 0.06[a]	1.96 ± 0.14[a]
Diabetic + menhaden oil 45% + salsalate (12)	315 ± 5	438 ± 14	67.2 ± 5.7	431 ± 35[a]	68.9 ± 18.6[b]	0.17 ± 0.04	1.33 ± 0.18

Data are presented as the mean ± SEM. [a]$P < 0.05$ compared to control rats. [b]$P < 0.05$ compared to diabetic rats. Parentheses indicate the number of experimental animals.

Achilles tendon, and dividing the difference by the distance between the two stimulating electrodes (measured in millimeters). Sensory nerve conduction velocity equaled the distance between stimulating and recording electrodes over the latency to initial peak negative deflection. Both motor and sensory nerve conduction velocity was reported in meters per second.

Subepithelial corneal nerves were imaged using the Rostock cornea module of the Heidelberg Retina Tomograph confocal microscope (Heidelberg Engineering, Heidelberg, Germany) [21]. The investigator acquiring these images was masked with respect to identity of the animal condition. Anesthetized rats were secured to a stereotaxic rat head holder mounted on a platform that allows for three-dimensional adjustments. GenTeal eye lubricant gel (Alcon; Fort Worth, TX) was applied to the lens, and the lens was advanced until the lubricant came into contact with the rat cornea epithelium. A minimum of six images was acquired for each animal by adjusting the platform to focus on a different region of the cornea. The data is presented as corneal nerve fiber length defined as the total length of all nerve fibers and branches (in millimeters) present in the acquired images standardized for area of the image (in square millimeters). The corneal fiber length for each animal was the mean value obtained from the acquired images and expressed as mm/mm^2.

Immunoreactive nerve fiber profiles innervating the skin from the hindpaw were visualized using standard confocal microscopy combined with immunohistochemistry [20]. The primary antibody used was anti-tubulin B3 (BioLegend, San Diego, CA), and the secondary antibody was Alexa Fluor 546 goat anti-mouse (Life Technologies, Carlsbad, CA). Profiles were imaged and counted using a Zeiss LSM710 confocal microscope (Oberkochen, Germany) with a 40x objective by two individual investigators that were masked to the sample identity and normalized to length. Data are presented as profiles/mm.

Videomicroscopy was used to investigate in vitro vasodilatory responsiveness of epineurial arterioles, blood vessels that provide circulation to the region of the sciatic nerve [20]. Following isolation, suspension of the vessels in organ chambers, and verification of reactivity, cumulative concentration-response relationships were evaluated for acetylcholine (10^{-8}–10^{-4} M) and calcitonin gene-related peptide (10^{-11}–10^{-8} M). At the end of each dose response curve, papaverine (10^{-5} M) was added to determine maximal vasodilation.

2.3. Serum Determinations. Rat blood was collected from the heart's right ventricle, and serum was analyzed for triglycerides, free fatty acids, cholesterol, and resolvin D1 using commercial kits as reported in previous experiments [20].

2.4. Data Analysis. Results are presented as mean ± SEM. Comparisons between the treatment groups and control and nontreated diabetic rats were conducted using one-way ANOVA and Bonferroni posttest comparison (Prism software 7; GraphPad, San Diego, CA). Concentration response curves for acetylcholine and calcitonin gene-related peptide were compared using a two-way repeated-measures analysis of variance with autoregressive covariance structure using PROC MIXED program of SAS [20]. Log EC_{50} were generated using Prism software. A P value less than 0.05 was considered significant.

3. Results

The weight of all rats at the start of the study was statistically the same (Table 1). All rats gained weight over the study

period. There was a trend for the diabetic rats to gain less weight than the control rats, but the difference was not significant between any groups. There was no statistical difference in the amount of chow consumed by the rats in any of the groups. All diabetic rats were hyperglycemic at the end of the study, and no treatments changed the nonfasting blood glucose levels. Serum triglyceride levels were significantly increased in the untreated diabetic rats and diabetic rats treated with menhaden oil compared to control rats. The serum triglyceride levels in diabetic rats treated with 45% menhaden oil were significantly less than the serum triglyceride levels in untreated diabetic rats. Treating diabetic rats with salsalate with or without menhaden oil caused serum triglyceride levels to be lower at the end of the study compared to untreated diabetic rats. Serum triglyceride levels in diabetic rats treated with salsalate were higher than those observed in control rats, but the comparison was not statistically different. Serum-free fatty acid and cholesterol levels were significantly increased in untreated diabetic rats and diabetic rats treated with or without menhaden oil and/or salsalate compared to control rats with the exception of diabetic rats treated with 45% menhaden oil and salsalate. Serum resolvin D1 levels were measured in all groups of rats at the end of the study period. Data in Figure 1 demonstrate that resolvin D1 levels were significantly increased in diabetic rats treated with 25% and 45% menhaden oil with salsalate compared to control rats.

At the end of the study period, several endpoints related to peripheral nerve activity and density were examined (Table 2). Motor and sensory nerve conduction velocity was significantly decreased in untreated diabetic rats and diabetic rats treated with salsalate alone or with 10% or 25% menhaden oil compared to control rats. Treating diabetic rats with 10% menhaden oil and salsalate significantly improved motor nerve conduction velocity but not sensory nerve conduction velocity compared to untreated diabetic rats. Treating diabetic rats with 25% menhaden oil with salsalate significantly improved both motor and sensory nerve conduction velocity compared to untreated diabetic rats. Treating diabetic rats with 45% menhaden oil with or without salsalate significantly improved both motor and sensory nerve conduction velocity.

Thermal nociception was significantly impaired in untreated diabetic rats, and the difference was not statistically improved in diabetic rats treated with salsalate alone compared to control rats (Table 2). Treating diabetic rats with 10% menhaden oil moderately improved thermal nociception compared to control and untreated diabetic rats. However, treating diabetic rats with 10% menhaden oil with salsalate or with 25% or 45% menhaden oil with or without salsalate significantly improved thermal nociception compared to untreated diabetic rats. Intraepidermal nerve fiber density was significantly decreased in untreated diabetic rats, diabetic rats treated with salsalate alone, diabetic rats treated with 10% menhaden oil with or without salsalate, or diabetic rats treated with 25% menhaden oil. Treating diabetic rats with 25% menhaden oil with salsalate or with 45% menhaden oil with or without salsalate significantly improved intraepidermal nerve fiber density compared to untreated diabetic rats.

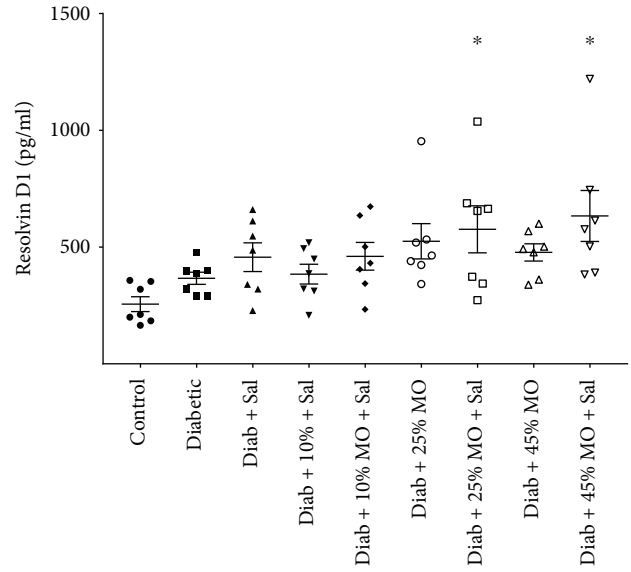

Figure 1: Scatter plot of serum resolvin D1 levels. Effect of menhaden oil concentration with or without salsalate on serum levels of resolvin D1 in type 2 diabetic rats are presented. Serum levels are presented as mean ± SEM and are in pg/ml serum. Each symbol per group represents a different animal, $n = 7$ per condition. $^{*}P < 0.05$ compared to control rats.

Determination of changes in corneal nerve sensitivity and subepithelial corneal nerve density is being promoted as an early marker of peripheral nerve damage in human subjects with diabetes [22, 23]. We have also been examining diabetes-induced changes in corneal nerve sensitivity and subepithelial corneal nerve density in diabetic rodents and found changes to correlate with other markers of peripheral neuropathy [24, 25]. In this study, we found that corneal sensitivity was significantly decreased in untreated diabetic rats, diabetic rats treated with salsalate alone, diabetic rats treated with 10% menhaden oil with or without salsalate, and diabetic rats treated with 25% menhaden oil compared to control rats (Table 2). In contrast, treating diabetic rats with 25% menhaden oil with salsalate or 45% menhaden oil with or without salsalate significantly improved corneal nerve sensitivity compared to untreated diabetic rats. Subepithelial corneal nerve fiber length was significantly decreased in untreated diabetic rats, diabetic rats treated with salsalate alone, and diabetic rats treated with 10% menhaden oil with or without salsalate compared to control rats. In contrast, subepithelial corneal nerve fiber length was protected significantly when diabetic rats were treated with 25% or 45% menhaden oil with or without salsalate compared to untreated diabetic rats.

We have previously demonstrated that decreased vascular reactivity by epineurial arterioles, resistance size vessels that provide circulation to the sciatic nerve, to acetylcholine precedes decrease in motor nerve conduction velocity [26]. We have also shown that epineurial arterioles are innervated by sensory nerves expressing calcitonin gene-related peptide and that the calcitonin gene-related peptide is a very potent

Table 2: Effect of menhaden oil with or without salsalate on motor and sensory nerve conduction velocity, thermal nociception, intraepidermal nerve fiber density, corneal sensitivity, and cornea nerve fiber length in type 2 diabetic Sprague Dawley rats.

Condition	Motor nerve conduction velocity (m/sec)	Sensory nerve conduction velocity (m/sec)	Thermal nociception (sec)	Intraepidermal nerve fiber density (profiles/mm)	Corneal sensitivity (cm)	Cornea nerve fiber length (mm/mm^2)
Control (12)	55.7 ± 2.1	31.9 ± 0.5	11.9 ± 0.3	20.7 ± 0.7	5.77 ± 0.08	9.5 ± 0.5
Diabetic (12)	40.4 ± 1.4^a	26.7 ± 0.6^a	18.4 ± 0.5^a	13.1 ± 0.8^a	4.67 ± 0.22^a	4.9 ± 0.4^a
Diabetic + salsalate (12)	45.0 ± 1.9^a	27.8 ± 0.4^a	16.0 ± 1.0^a	15.9 ± 0.4^a	4.65 ± 0.20^a	6.6 ± 0.5^a
Diabetic + menhaden oil 10% (12)	44.1 ± 1.8^a	27.3 ± 0.6^a	15.2 ± 1.0	14.5 ± 0.9^a	4.88 ± 0.19^a	6.4 ± 0.5^a
Diabetic + menhaden oil 10% + salsalate (12)	51.0 ± 2.0^b	28.7 ± 0.6^a	13.4 ± 1.2^b	16.2 ± 0.06^a	5.03 ± 0.22^a	6.4 ± 0.5^a
Diabetic + menhaden oil 25% (12)	47.2 ± 1.4^a	28.3 ± 0.5^a	12.3 ± 0.9^b	15.6 ± 1.1^a	4.81 ± 0.16^a	8.1 ± 0.4^b
Diabetic + menhaden oil 25% + salsalate (12)	49.2 ± 1.6^b	29.7 ± 0.4^b	11.2 ± 0.7^b	18.9 ± 1.0^b	5.58 ± 0.10^b	8.5 ± 0.7^b
Diabetic + menhaden oil 45% (12)	51.6 ± 1.4^b	30.0 ± 0.4^b	11.3 ± 0.4^b	19.0 ± 1.2^b	5.54 ± 0.13^b	9.1 ± 0.6^b
Diabetic + menhaden oil 45% + salsalate (12)	52.3 ± 1.2^b	30.3 ± 0.5^b	11.2 ± 0.7^b	19.1 ± 0.6^b	5.88 ± 0.06^b	9.4 ± 0.6^b

Data are presented as the mean ± SEM. $^aP < 0.05$ compared to control rats. $^bP < 0.05$ compared to diabetic rats. Parentheses indicate the number of experimental animals.

vasodilator of these vessels and this vasodilation is impaired by diabetes [27]. In this study, like in previous studies, we found that diabetes causes a decrease in vasodilation to acetylcholine (Figure 2 and Table 3). Treating diabetic rats with salsalate alone or with 10% menhaden oil did not significantly improve vascular relaxation to acetylcholine compared to untreated diabetic rats. However, treating diabetic rats with 10% menhaden oil with salsalate or 25% or 45% menhaden oil with or without salsalate significantly improved vascular relaxation. Vascular relaxation of epineurial arterioles to calcitonin gene-related peptide at 5×10^{-10} and 10^{-9} M doses was significantly decreased in untreated diabetic rats and was not improved when diabetic rats were treated with salsalate alone or with 10% menhaden oil (Figure 3 and Table 3). In contrast, treating diabetic rats with 10% menhaden oil with salsalate tended to improve vascular relaxation to the calcitonin gene-related peptide, and treating diabetic rats with 25% or 45% menhaden oil with or without salsalate significantly improved vascular relaxation to calcitonin gene-related peptide compared to untreated diabetic rats.

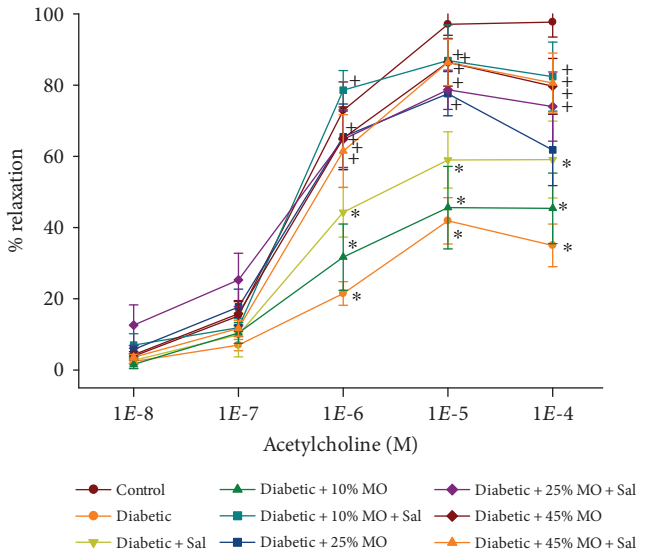

Figure 2: Effect of menhaden oil concentration with or without salsalate on vascular relaxation by acetylcholine in epineurial arterioles of the sciatic nerve in Sprague Dawley type 2 diabetic rats. Pressurized arterioles (40 mmHg and ranging from 60 to 100 μm luminal diameters) were constricted with phenylephrine (30–50%), and incremental doses of acetylcholine were added to the bathing solution while recording steady-state vessel diameter. The number of rats in each group was the same as shown in Table 1. Data are presented as the mean of % relaxation ± SEM. $^*P < 0.05$ compared to control rats; $^+P < 0.05$ compared to diabetic rats.

4. Discussion

These studies were performed in a high-fat diet fed rats treated 8 weeks later with a low dose of streptozotocin. This diabetic rat models late-stage type 2 diabetes. It has been reported to simulate the human syndrome and to be suitable for testing the effect of antidiabetic compounds [28, 29]. Furthermore, my laboratory previously has characterized the progression of peripheral neuropathy in this model including decrease in corneal nerve sensitivity and density [20, 21, 24]. Using this model, we have been seeking potential new treatments for diabetic vascular and neural complications that would be safe for human use and have minimal side effects. In the present experiments, we examined the

Table 3: Effect of menhaden oil with or without salsalate on log EC 50 for acetylcholine and calcitonin gene-related peptide-induced relaxation of epineurial arterioles of the sciatic nerve in type 2 diabetic Sprague Dawley rats.

Condition	Acetylcholine	Calcitonin gene-related peptide
Control (12)	-6.35 ± 0.11	-9.69 ± 0.04
Diabetic (12)	-4.04 ± 0.20^{a}	-9.11 ± 0.08^{a}
Diabetic + salsalate (12)	-5.12 ± 0.27^{a}	-9.13 ± 0.07^{a}
Diabetic + menhaden oil 10% (12)	-4.61 ± 0.44^{a}	-9.11 ± 0.11^{a}
Diabetic + menhaden oil 10% + salsalate (12)	-6.03 ± 0.36^{b}	-9.38 ± 0.07
Diabetic + menhaden oil 25% (12)	-5.93 ± 0.34^{b}	-9.23 ± 0.11
Diabetic + menhaden oil 25% + salsalate (12)	-6.14 ± 0.31^{b}	-9.58 ± 0.10^{b}
Diabetic + menhaden oil 45% (12)	-5.93 ± 0.26^{b}	-9.46 ± 0.11^{b}
Diabetic + menhaden oil 45% + salsalate (12)	-5.94 ± 0.25^{b}	-9.56 ± 0.11^{b}

Data are presented as the mean ± SEM. $^{a}P < 0.05$ compared to control rats. $^{b}P < 0.05$ compared to diabetic rats. Parentheses indicate the number of experimental animals.

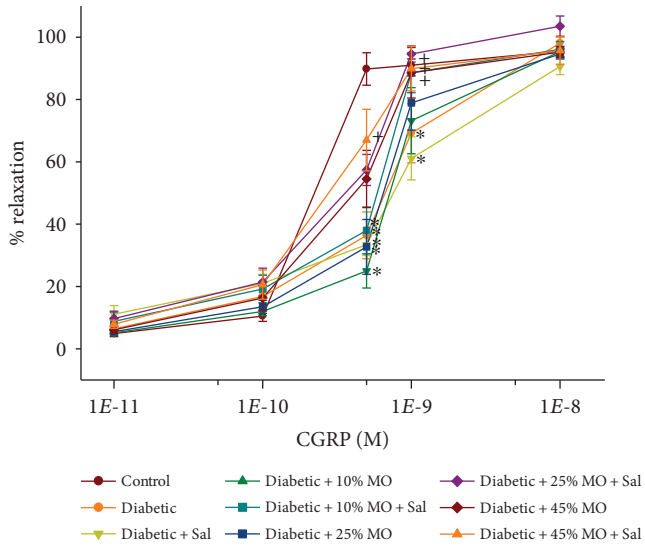

Figure 3: Effect of menhaden oil concentration with or without salsalate on vascular relaxation by calcitonin gene-related peptide in epineurial arterioles of the sciatic nerve in Sprague Dawley type 2 diabetic rats. Pressurized arterioles (40 mmHg and ranging from 60 to 100 μm luminal diameters) were constricted with phenylephrine (30–50%), and incremental doses of calcitonin gene-related peptide were added to the bathing solution while recording steady-state vessel diameter. The number of rats in each group was the same as shown in Table 1. Data are presented as the mean of % relaxation ± SEM. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to diabetic rats.

therapeutic potential of salsalate with and without menhaden oil on diabetes-induced vascular and neural deficits. Both of these compounds have been shown to be safe for human use.

In humans with type 2 diabetes, salsalate, 3.5 g/day, improved glycemia and decreased inflammatory markers [17]. In obese, insulin-resistant, nondiabetic subjects, salsalate reduced the atherogenicity of the lipid, lipoprotein, and apoprotein profile and insulin sensitivity [30, 31]. In animal studies, salsalate has been shown to reduce vascular injury in Zucker fatty rats and activate skeletal muscle thermogenesis and protect C57Bl/6J mice from a high-fat diet [32, 33]. In rodents, the effects of salsalate are thought to be due primarily to reducing inflammation; however, studies have shown that salsalate has metabolic effects beyond suppressing inflammation [34]. In diabetic mice, we have shown that salsalate alone partially improves peripheral neuropathy [5]. In this study, rats treated with salsalate alone or in combination with menhaden oil consumed about 40–50 mg salsalate per rat. Treatment of diabetic rats with salsalate alone tended to improve vascular reactivity of epineurial arterioles to acetylcholine and several neuropathy-related endpoints, but all these remained significantly impaired compared to control rats. Thus, it appears that salsalate alone was not as efficacious in diabetic rats compared to diabetic mice on improving peripheral neuropathy [5]. However, this study demonstrated for the first time that combining salsalate with menhaden oil provided greater efficacy toward improving vascular reactivity by epineurial arterioles to acetylcholine and calcitonin gene-related peptide.

Fish oils, such as menhaden oil, contain a high concentration of eicosapentaenoic and docosahexaenoic acids, and their consumption elevates the plasma concentration of proresolving inflammatory lipid mediators like resolvins E1 and D1, respectively [5]. This study extends our previous findings by demonstrating the effectiveness of different concentrations of menhaden oil with or without salsalate on resolvin D1 formation and alleviation of diabetic vascular and neural dysfunction in a type 2 diabetic rat model [5]. Data in Table 1 demonstrated that salsalate alone or in combination with menhaden oil reduces serum triglyceride levels. A similar outcome has been reported in humans with type 2 diabetes treated with salsalate [17]. Even though salsalate and 10% menhaden oil alone were minimally effective toward neuropathy-related endpoints, when combined, there was a significant improvement in motor nerve conduction velocity and thermal nociception (Table 2). Examining the effect of salsalate and menhaden oil on other neural endpoints impacted by diabetes, we found that treating diabetic rats with 25% menhaden oil alone significantly improved motor nerve conduction velocity and thermal nociception similar to the combination of salsalate and 10% menhaden oil, but sensory nerve conduction velocity, intraepidermal

nerve fiber density, and corneal sensitivity remained significantly impaired. However, when salsalate was combined with 25% menhaden oil, all three of these neural endpoints were significantly improved (Table 2). Corneal nerve fiber length was significantly improved with 25% menhaden oil alone, and salsalate did not provide any additional benefit when combined with either 10% or 25% menhaden oil. Treating diabetic rats with 45% menhaden oil with or without salsalate provided maximal benefit toward these different neural endpoints.

Our study provides the first evaluation of the potential effects of salsalate alone or in combination with menhaden oil on diabetes-induced vascular dysfunction. Disrupted vascular function and reduced blood flow to peripheral nerves have long been considered a potential mechanism for diabetic peripheral neuropathy [35]. Our studies in both type 1 and type 2 diabetic rats have demonstrated that impaired vascular reactivity of epineurial arterioles, microvessels that provide blood flow to the sciatic nerve, precedes the development of nerve dysfunction, as identified by reduced nerve conduction velocity [26, 36]. Like in previous studies, vascular relaxation to acetylcholine or calcitonin gene-related peptide was decreased in diabetic rats. Treating diabetic rats with salsalate or 10% menhaden oil alone did not significantly improve vascular relaxation to acetylcholine. However, when diabetic rats were treated with salsalate and 10% menhaden oil, vascular relaxation to acetylcholine was significantly improved (Figure 2 and Table 3). A similar outcome was observed when vascular relaxation to calcitonin gene-related peptide was studied. Treating diabetic rats with salsalate or 10% menhaden oil alone did not improve vascular relaxation to calcitonin gene-related peptide (Figure 3 and Table 3). When diabetic rats were treated with the combination of salsalate and 10% menhaden, oil vascular relaxation to calcitonin gene-related peptide was moderately improved and the difference was not significantly different to control rats. Likewise, treating diabetic rats with 25% menhaden oil alone moderately improved vascular relaxation to calcitonin gene-related peptide but combining salsalate with 25% menhaden oil significantly improved vascular relaxation.

Some of the beneficial results of salsalate could be attributed to increased formation of resolvins when salsalate is combined with menhaden oil. Combining salsalate with 25% and 45% menhaden oil caused a significant increase in serum resolvin D1 levels (Table 1). Our studies demonstrated a significant improvement in some neural endpoints and vascular relaxation to acetylcholine when salsalate was combined with 10% menhaden oil. However, combining salsalate with 10% menhaden oil did not result in a significant increase in resolvin D1 levels in serum. This highlights some of the limitations of this study. We were limited to examining only resolvin D1 levels in serum in this study. We do not currently have the ability to measure resolvin E1 or neuroprotectin D1 in our serum samples. These determinations would require GC/MS, and we do not have access to this equipment, although a new core at the University of Iowa is being created that will provide this important capability in the near future. It is possible that resolvin E1 and/or neuroprotectin D1 are increased in diabetic rats treated with 10% menhaden oil and salsalate. To answer this question, it will be important to determine the combined levels of resolvins E1 and D1 as well as neuroprotectin D1 under the conditions used in this study. Previous studies have shown that treating diabetic mice with exogenous resolvin E1 or D1 improves peripheral neuropathy [5–7]. It has also been shown that resolvin D1 and neuroprotectin D1 can stimulate neurite outgrowth by dorsal root ganglion or trigeminal ganglion neurons in culture [6, 37]. These studies suggest that biological properties of resolvins and neuroprotectins toward inflammatory resolution and neural regeneration likely contribute to the beneficial effects of fish oil with or without salsalate on diabetes-induced vascular dysfunction and peripheral neuropathy. However, we cannot rule out other potential mechanisms that may also be contributing to the improved outcome of diabetic rats treated with menhaden oil and salsalate.

5. Conclusions

The principle finding of this study is that the beneficial dietary effects of fish oil on diabetic neurovascular dysfunction and peripheral neuropathy can be enhanced with the inclusion of salsalate in the diet. The beneficial effects of salsalate were most apparent at lower doses of dietary menhaden oil. Whether fish oil in combination with salsalate can provide benefit to patients suffering from diabetic peripheral neuropathy will require further investigation.

Data Availability

Upon written request from a responsible source, data pertaining to this study will be made available through the website for my laboratory. Work pertaining to this article was done in part as part of the official duties of the contact author to the federal government. Thus, data release will require a specific request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the work presented in this manuscript.

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Clinical Study

In Vivo Corneal Confocal Microscopy Detects Improvement of Corneal Nerve Parameters following Glycemic Control in Patients with Type 2 Diabetes

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Aim. This study aimed to investigate whether in vivo corneal confocal microscopy (CCM) can detect the improvement of corneal nerve parameters following glycemic control in patients with type 2 diabetes in natural history. *Methods.* Thirty-two patients with diabetes complicated by DPN and 12 age-matched control subjects underwent detailed clinical examination and were assessed per the Toronto Clinical Scoring Scale for DPN, nerve conduction studies, and IVCCM at baseline and after approximately one year from the first visit. *Results.* At follow-up, 16 diabetic patients had improved glycemic control (group A, HbA1c < 7.0%, 7.78 ± 1.62% versus 6.52 ± 0.59%, $P = 0.005$), while the remainder continued to have elevated HbA1c levels (group B, HbA1c ≥ 7.0%, 8.55 ± 1.57% versus 8.79 ± 1.05%, $P = 0.527$). For patients in group A, corneal nerve fiber density (CNFD) (18.55 ± 5.25 n/mm^2 versus 21.78 ± 6.13 n/mm^2, $P = 0.005$) and corneal nerve fiber length (CNFL) (11.62 ± 2.89 mm/mm^2 versus 13.04 ± 2.44 mm/mm^2, $P = 0.029$) increased significantly compared to baseline. For patients in group B, sural sensory nerve conduction velocity (47.93 ± 7.20 m/s versus 44.67 ± 6.43 m/s, $P = 0.024$), CNFD (17.19 ± 5.31 n/mm^2 versus 15.67 ± 4.16 n/mm^2, $P = 0.001$), corneal nerve branch density (19.33 ± 12.82 n/mm^2 versus 14.23 ± 6.56 n/mm^2, $P = 0.033$), and CNFL (11.16 ± 2.57 mm/mm^2 versus 9.90 ± 1.75 mm/mm^2, $P = 0.011$) decreased significantly. *Conclusions.* The results of this study suggest that morphological repair of corneal nerve fibers can be detected when glycemic control improves. In vivo CCM could be a sensitive method that can be applied in future longitudinal or interventional studies on DPN.

1. Introduction

Diabetic polyneuropathy (DPN) is one of the most common chronic complications of diabetes. Over 50% of diabetic patients develop DPN as their disease course progresses [1]. Diabetic neuropathy leads to morbidity in diabetic patients in the form of painful neuropathy and foot ulceration with consequent lower limb amputation [2]. It accounts for reduced quality of life and imposes a significant economic burden on both individuals and society [3]. Prospective studies on diabetic neuropathy have revealed that neuropathy progresses gradually with time. A long-term follow-up study on type 2 diabetes reported that the rate of abnormal nerve conduction velocity (NCV) results was 8% at baseline, and it increased to 16% and 42% after 5 years and 10 years, respectively [4].

Metabolic factors including blood glucose, blood lipids, blood pressure, and body mass index (BMI) have been identified as risk factors for DPN [5]. However, clinical intervention studies correlating stricter control of such metabolic factors have rarely shown a corresponding improvement in DPN. The Epidemiology of Diabetes Interventions and Complications (EDIC) study showed that improved glycemic control in patients with type 1 diabetes yielded immediate and long-term benefits for DPN [6]. However, the Veterans Affairs Diabetes Trial (VADT) showed no obvious

improvement in diabetic neuropathy resulting from glycemic control in patients with type 2 diabetes; further, it revealed a trend towards deterioration of autonomic neuropathies in the study cohort [7]. In the Steno-2 study, although targeted interventions aimed at multiple risk factors in patients with type 2 diabetes improved diabetic retinopathy, diabetic nephropathy, and cardiovascular autonomic neuropathy, similar improvements were not observed for somatic neuropathy [8].

The prevailing "mainstream opinions" in the medical community, therefore, reflect the belief that the neurologic impairments caused by diabetes are difficult to reverse. However, inconclusive results regarding neurological improvement of DPN may not reflect irreversibility but rather may stem from the lack of appropriately sensitive and effective evaluation methods that can be utilized to reveal the effect of glycemic control on DPN. Growing evidence supports a prominent association between corneal nerve morphology measured using corneal confocal microscopy (CCM) and DPN [9, 10]. As a quick, noninvasive, and reiterative technique, CCM has demonstrated the capacity to detect early small nerve fiber damage in diabetic patients [10, 11] and to diagnose [11] and classify the severity of DPN [12, 13].

Our study sought to determine whether improved glycemic control at one-year follow-up improved corneal nerve morphology and DPN in patients with type 2 diabetes.

2. Materials and Methods

2.1. Subjects. From September 2015 to March 2016, 60 patients (age range 30–80 years) with type 2 diabetes and HbA1c $\geq$ 7.0% were recruited. Type 2 diabetes was diagnosed according to World Health Organization criteria [14]. All patients received NCV testing; 50 were diagnosed with DPN owing to abnormal NCV results and symptoms or signs of DPN [15]. Thirty-two patients with DPN completed one-year follow-up, and 18 patients were lost to follow-up.

All patients were provided medical recommendations for antidiabetic, antihypertensive, and lipid-lowering therapy at the beginning of the study. During the next year, there were no limitations imposed on the patients regarding diabetic care. After one year had elapsed, the 32 diabetic patients who were followed up were divided into two groups (groups A and B) according to their HbA1c status relative to the control goal suggested by the Chinese Diabetes Society (CDS), which recommends an HbA1c < 7.0%. Sixteen individuals achieved an HbA1c < 7.0% and were assigned to group A, whereas group B was comprised of 16 patients who had not reached the HbA1c goal (i.e., HbA1c $\geq$ 7.0%) at one-year follow-up. Twelve age-matched, healthy volunteers without diabetes mellitus, prediabetes, and/or clinical or paraclinical signs or symptoms of polyneuropathy were recruited to form a control group.

Exclusion criteria were diseases affecting the central or peripheral nervous systems, malignant tumors, connective tissue diseases, acute and chronic hepatic or renal diseases, thyroid diseases, endocrinopathies, metabolic derangements, psychological conditions, diabetic foot ulcers, active oculopathy, history of ocular operation, glaucoma, acute and chronic corneal diseases, and an extended history of corneal contact lens use. Both diabetic and control participants were comprehensively examined before recruitment into the study to ascertain health status and ensure that no exclusion criteria were met.

The study was approved by the ethics committee of Beijing Hospital, and written informed consent was obtained according to the Declaration of Helsinki.

2.2. Clinical Assessment. All study participants underwent medical and neurologic assessments at baseline and at one-year follow-up (15.2 ± 1.6 months). Medical assessments included the measurement of systolic (SBP) and diastolic blood pressure (DBP), HbA1c (%), and lipid fractions (concentrations of total cholesterol (TC) (mmol/L), high- (HDLC) and low-density lipoprotein cholesterol (LDLC) (mmol/L), and triglycerides (TG) (mmol/L)).

2.3. Peripheral Neuropathy Assessment. The Toronto Clinical Scoring System (TCSS) for DPN was used [16]. Electrodiagnostic studies were performed using a Medoc "Key point" system (Medoc Dynamics Ltd., Bristol, UK). Tibial (TMNCV) and peroneal motor nerve conduction velocity (PMNCV), as well as sural (SSNCV) and superficial peroneal sensory nerve conduction velocity (SPSNCV), was measured in the left lower limb (calf-to-ankle) by a consultant neurophysiologist.

2.4. In Vivo Corneal Confocal Microscopy. All study subjects underwent examination with the Heidelberg retina tomograph-II in vivo corneal confocal microscope. The subjects' eyes were anesthetized using one drop of 0.4% benoxinate hydrochloride, and Viscotears were applied to the front of the eye for lubrication. One drop of viscoelastic gel was placed on the tip of the objective lens, and a sterile disposable Perspex cap was placed over the lens allowing optical coupling of the objective lens to the cornea. The patient was instructed to fixate the eye not being examined on a target. Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backwards and forwards for ~2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provided en face two-dimensional images with a lateral resolution of ~2 mm/pixel and final image size of 400×400 pixels of the subbasal nerve plexus of the cornea from each patient and control subject.

One examiner masked from patients' HbA1c results selected and analyzed 3 to 6 high-clarity images from the central subbasal nerve plexus. Criteria for image selection were depth, focus, position, and contrast. The examiner quantified the images with semiautomated, purpose-written, proprietary software (ACCMetrics, M. A. Dabbah, Imaging Science Biomedical Engineering, University of Manchester, Manchester, UK). Three corneal nerve parameters were quantified: (1) corneal nerve fiber density (CNFD), calculated as the total number of major nerves per square millimeter of corneal tissue (n/mm^2); (2) corneal nerve branch density (CNBD), calculated as the number of branches emanating from all major nerve trunks per square millimeter of

Table 1: Clinical characteristics of control subjects and patients with diabetes at baseline and one-year follow-up.

Parameter	Baseline		One-year follow-up		*P* value		
	Control, Ia ($n = 12$)	Diabetes, IIa ($n = 32$)	Control, Ib ($n = 12$)	Diabetes, IIb ($n = 32$)	Ia versus IIa*	Ib versus Ia†	IIb versus IIa‡
Age, y	54.4 ± 12.7	56.9 ± 14.7	55.3 ± 12.8	58.1 ± 14.6	0.488	≤0.001	≤0.001
Sex (M/F)	6/6	18/14			—	—	—
Duration of diabetes, y	—	11.2 ± 9.2	—	12.4 ± 9.0			≤0.001
Weight, kg	63.8 ± 7.4	71.0 ± 16.5	63.4 ± 8.1	70.1 ± 14.5	0.155	0.692	0.318
BMI, kg/m^2	23.15 ± 2.40	25.80 ± 4.78	22.9 ± 2.20	25.50 ± 4.14	0.075	0.631	0.328
SBP, mmHg	122.3 ± 11.8	137.8 ± 16.3	122.8 ± 11.4	133.3 ± 14.2	0.005	0.564	0.175
DBP, mmHg	73.5 ± 5.6	82.6 ± 8.0	74.0 ± 5.5	81.3 ± 10.6	0.001	0.551	0.396
TC, mmol/L	4.88 ± 0.67	4.52 ± 1.07	4.71 ± 0.59	4.59 ± 1.14	0.407	0.296	0.902
TG, mmol/L	1.25 ± 0.56	1.94 ± 1.82	1.21 ± 0.51	1.78 ± 1.57	0.177	0.819	0.483
LDLC, mmol/L	2.78 ± 0.64	2.69 ± 0.72	2.67 ± 0.61	2.56 ± 0.83	0.884	0.457	0.326
HDLC, mmol/L	1.51 ± 0.28	1.22 ± 0.33	1.42 ± 0.28	1.30 ± 0.31	0.008	0.107	0.058
HbA1c, %	5.36 ± 0.12	8.22 ± 1.67	5.38 ± 0.11	7.58 ± 1.47	≤0.001	0.275	0.058
TCSS	0.6 ± 0.7	5.2 ± 4.5	0.7 ± 0.7	5.4 ± 4.8	0.001	0.674	0.338
PMNCV, m/s	50.20 ± 2.84	44.67 ± 4.07	49.34 ± 2.36	43.43 ± 3.80	≤0.001	0.201	0.496
TMNCV, m/s	50.56 ± 3.15	44.24 ± 4.60	50.03 ± 2.39	42.46 ± 4.76	≤0.001	0.341	0.439
SPSNCV, m/s	55.44 ± 3.99	48.71 ± 7.36	54.98 ± 3.23	46.76 ± 7.20	≤0.001	0.491	0.049
SSNCV, m/s	53.69 ± 3.05	48.34 ± 7.18	52.63 ± 2.00	44.92 ± 6.21	0.017	0.156	0.146
CNFD, n/mm^2	29.31 ± 4.31	18.71 ± 4.73	28.29 ± 3.38	19.12 ± 5.99	≤0.001	0.093	0.643
CNBD, n/mm^2	42.19 ± 13.91	21.80 ± 14.67	39.11 ± 18.11	20.78 ± 12.98	0.003	0.279	0.667
CNFL, mm/mm^2	17.96 ± 2.40	11.81 ± 2.46	17.15 ± 2.44	11.63 ± 2.72	≤0.001	0.191	0.737

Results are expressed as mean ± SD or counts for categorical variables. *Independent-sample *t*-test. †Paired-sample *t*-test. ‡χ^2 test.

corneal tissue (n/mm^2); and (3) corneal nerve fiber length (CNFL), calculated as the total length of all nerve fibers and branches within the area of the corneal tissue (mm/mm^2).

2.5. Statistical Methods. SPSS 23.0 for Windows was used to compute the results. Analysis included descriptive and frequency statistics. All data are expressed as means ± standard deviation (SD). Independent-sample *t*-tests were used to test whether a sample mean differed between control subjects and diabetic patients. Paired-sample *t*-tests were used for the comparison between baseline and follow-up data. Nonparametric data were analyzed using χ^2 tests. Pearson correlation analysis and linear regression were adapted for the correlation between corneal nerve parameters and other indexes. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Comparison of Control and Diabetes Groups. Table 1 shows the clinical characteristics of control subjects and patients with diabetes at baseline and one-year follow-up. Notable differences in baseline characteristics between the groups included a higher HbA1c, SBP, and DBP in diabetic patients. Conversely, controls had significantly higher HDLC. When surveying TCSS, nerve conduction, and corneal nerve parameters, there were also significant differences between the two groups. TCSS was significantly lower in diabetic patients. All motor and sensory nerve conduction velocities were significantly slower in diabetic patients than in controls; similarly, diabetics' corneal parameters were significantly lower.

The control group showed no significant changes between examinations at baseline and follow-up. In the diabetic cohort, a significant improvement in glycemic control was demonstrated from baseline to follow-up HbA1c. Conversely, there were no significant changes in SBP and DBP, as well as levels of TC, TG, LDLC, and HDLC (Table 1). TCSS did not show a significant change at one-year follow-up. There was a significant decrease in conduction velocity seen in the PMNCV, TMNCV, and SPSNCV examinations; SSNCV examination did not yield a significant change at one-year follow-up.

3.2. Comparison of Diabetes Groups A and B. Table 2 shows the clinical characteristics of group A and group B at baseline and one-year follow-up. At baseline, there were no significant differences among the groups across all parameters.

At one-year follow-up, group A showed a significant decrease in HbA1c compared to baseline (from 7.78 ± 1.62% to 6.52 ± 0.59%, $P = 0.005$). No significant changes were seen for metabolic indexes (weight, SBP, DBP, TC, TG, LDLC, and HDLC) at the follow-up. For DPN assessment, no significant changes were observed for TCSS (4.4 ± 4.4 to 4.5 ± 4.8, $P = 0.544$) and NCV (TMNCV (44.15 ± 4.86 m/s to 43.56 ± 4.86 m/s, $P = 0.269$), PMNCV (44.47 ± 4.10 m/s to 44.46 ± 4.33 m/s, $P = 0.984$), SSNCV (48.87 ± 7.89 m/s to

Table 2: Clinical characteristics of patients with type 2 diabetes with improved HbA1c at one-year follow-up (group A) and consistently poor glycemic control at one-year follow-up (group B).

Parameter	Baseline		One-year follow-up		*P* value		
	Group A0 ($n = 16$)	Group B0 ($n = 16$)	Group A1 ($n = 16$)	Group B1 ($n = 16$)	A0 versus B0*	A1 versus A0†	B1 versus B0‡
Age, y	56.1 ± 17.5	59.4 ± 11.	57.3 ± 17.4	60.8 ± 11.1	0.521	≤0.001	≤0.001
Sex (M/F)	9/7	9/7			—	—	—
Duration of diabetes, y	11.3 ± 11.1	10.4 ± 7.5	12.6 ± 10.9	11.5 ± 7.3	0.780	≤0.001	≤0.001
Weight, kg	72.0 ± 19.0	69.8 ± 15.7	70.3 ± 16.2	68.9 ± 14.1	0.233	0.201	0.940
BMI, kg/m^2	27.36 ± 5.49	24.12 ± 7.69	26.73 ± 4.50	24.10 ± 3.25	0.063	0.207	0.909
SBP, mmHg	135.6 ± 15.4	137.4 ± 17.2	134.9 ± 13.5	134.8 ± 15.4	0.307	0.867	0.059
DBP, mmHg	82.7 ± 9.3	82.5 ± 8.6	81.4 ± 11.3	82.2 ± 10.8	0.846	0.552	0.486
TC, mmol/L	4.46 ± 1.23	4.51 ± 1.07	4.44 ± 1.15	4.54 ± 1.11	0.896	0.954	0.208
TG, mmol/L	1.83 ± 1.93	1.92 ± 1.71	1.31 ± 1.16	1.74 ± 1.47	0.671	0.190	0.367
LDLC, mmol/L	2.55 ± 0.78	2.70 ± 0.73	2.45 ± 0.80	2.54 ± 0.80	0.424	0.739	0.677
HDLC, mmol/L	1.26 ± 0.34	1.21 ± 0.31	1.39 ± 0.33	1.29 ± 0.30	0.996	0.073	0.491
HbA1c, %	7.78 ± 1.62	8.55 ± 1.57	6.52 ± 0.59	8.79 ± 1.05	0.268	0.005	0.527
TCSS	4.4 ± 4.4	6.1 ± 4.5	4.5 ± 4.8	6.3 ± 4.7	0.293	0.544	0.468
PMNCV, m/s	44.47 ± 4.10	44.62 ± 4.15	44.46 ± 4.33	43.39 ± 3.84	0.895	0.984	0.124
TMNCV, m/s	44.15 ± 4.86	44.12 ± 4.35	43.56 ± 4.86	42.54 ± 4.66	0.793	0.269	0.951
SPSNCV, m/s	50.42 ± 6.81	48.66 ± 7.48	50.14 ± 7.19	46.64 ± 7.21	0.261	0.287	0.056
SSNCV, m/s	48.87 ± 7.89	47.93 ± 7.20	47.28 ± 6.05	44.67 ± 6.43	0.982	0.293	0.024
CNFD, n/mm^2	18.55 ± 5.25	17.19 ± 5.31	21.78 ± 6.13	15.67 ± 4.16	0.070	0.005	0.001
CNBD, n/mm^2	21.76 ± 16.10	19.33 ± 12.82	26.19 ± 13.87	14.23 ± 6.56	0.349	0.122	0.033
CNFL, mm/mm^2	11.62 ± 2.89	11.16 ± 2.57	13.04 ± 2.44	9.90 ± 1.75	0.137	0.029	0.011

Results are expressed as mean ± SD or counts for categorical variables. *Independent-sample t-test. †Paired-sample t-test. ‡χ^2 test.

47.28 ± 6.05 m/s, $P = 0.293$), and SPSNCV (50.42 ± 6.81 m/s to 50.14 ± 7.19 m/s, $P = 0.287$)), otherwise corneal nerve showed a significant improvement (CNFD (18.55 ± 5.25 n/mm^2 to 21.78 ± 6.13 n/mm^2, $P = 0.005$, change of 2.35 ± 3.53), CNBD (21.76 ± 16.10 n/mm^2 to 26.19 ± 13.87 n/mm^2, $P = 0.122$, change of 3.06 ± 12.31), and CNFL (11.62 ± 2.89 mm/mm^2 to 13.04 ± 2.44 mm/mm^2, $P = 0.029$, change of 0.90 ± 2.42)) (Figures 1 and 2).

Group B showed no significant change at one-year follow-up and remained with an average HbA1c ≥ 7.0% (from 8.55 ± 1.57% to 8.79 ± 1.05%, $P = 0.527$). In group B, metabolic indexes (weight, SBP, DBP, TC, TG, LDLC, and HDLC) were similarly unchanged. For DPN assessment, TCSS showed no significant change. SSNCV was significantly lower at one-year follow-up than baseline (47.93 ± 7.20 m/s to 44.67 ± 6.43 m/s, $P = 0.024$), while all other nerve conduction velocities remained unchanged. All three corneal nerve parameters measured (CNFD (17.19 ± 5.31 n/mm^2 to 15.67 ± 4.16 n/mm^2, $P = 0.001$, change of −2.92 ± 2.96), CNBD (19.33 ± 12.82 n/mm^2 to 14.23 ± 6.56 n/mm^2, $P = 0.033$, change of −6.82 ± 11.65), and CNFL (11.16 ± 2.57 mm/mm^2 to 9.90 ± 1.75 mm/mm^2, $P = 0.011$, change of −1.71 ± 2.37)) decreased at one-year follow-up in group B (Figures 1 and 3).

We have retrospect all the patient's outpatient records; patients in group A had 152 times of outpatient records for diabetes in the past year in all, while patients in group B had 78 times of outpatient records for diabetes in all. This suggested that patients in group A have higher compliance (Table 3).

3.3. Factors Affecting the Changes of Corneal Nerve Parameters. Multiple linear regression and correlative analysis showed that the changes of corneal nerve parameters (CNFD, CNBD, and CNFL) had not shown significant correlations with the duration of diabetes, changes of weight, blood pressure, blood lipids, and HbA1c ($P > 0.05$).

4. Discussion

Longitudinal data from the Rochester cohort support the contention that the duration and severity of exposure to hyperglycemia are related to the progression and hence severity of neuropathy [17]. Interventional studies have examined large-fiber neuropathy in the evaluation of DPN; however, interventions assessed in these studies have failed to demonstrate improvement of DPN. It is currently unclear whether glucose control can improve diabetic neuropathy and affect its natural history. Recently, there has been increasing interest in the assessment of small-fiber neuropathy both as a method of detection of early-stage DPN and as an indicator of potentially regenerable nerves. A lifestyle intervention study [18] in patients with impaired glucose tolerance showed improvements in weight, blood lipid levels, blood pressure, and blood glucose levels after one year of

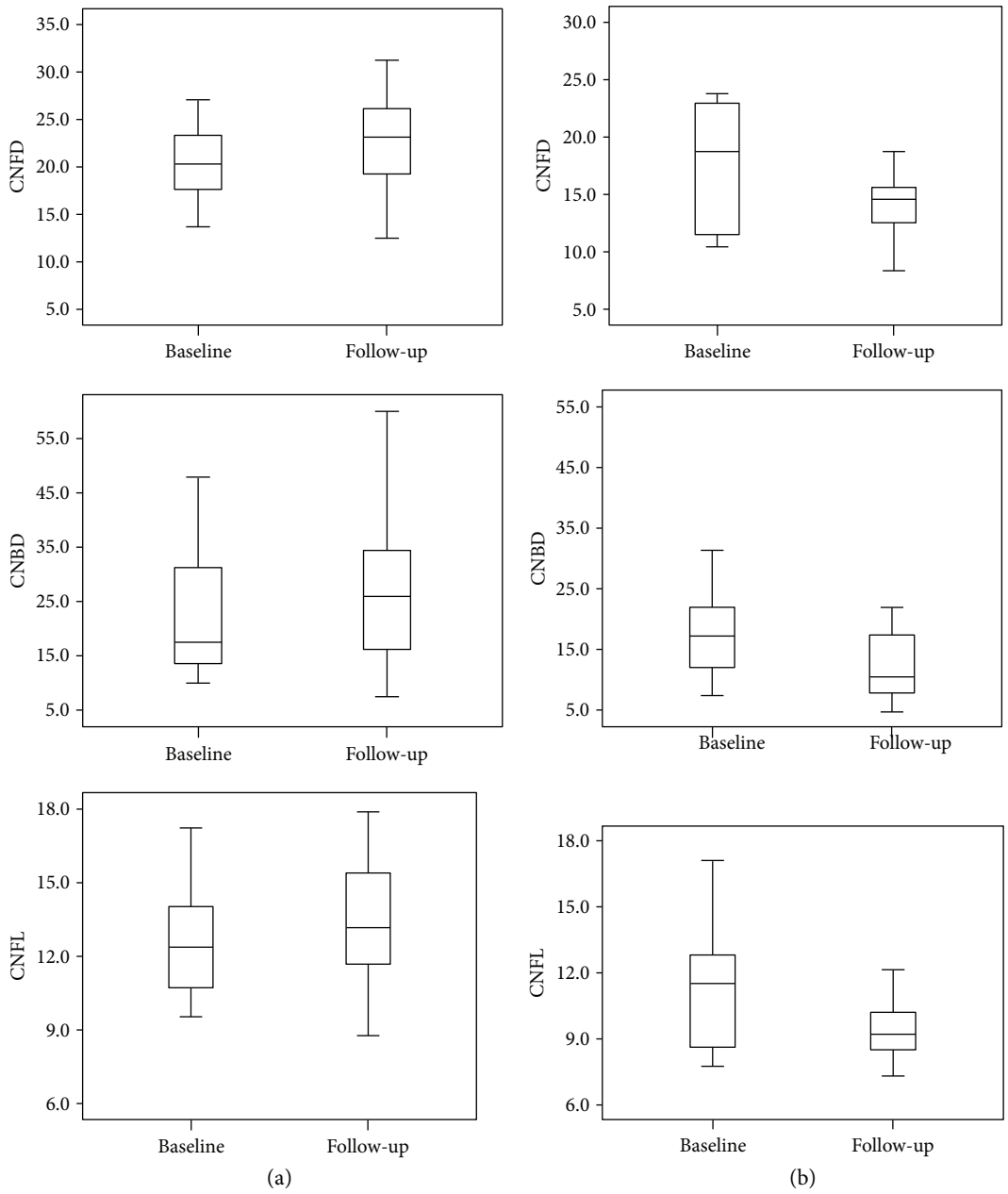
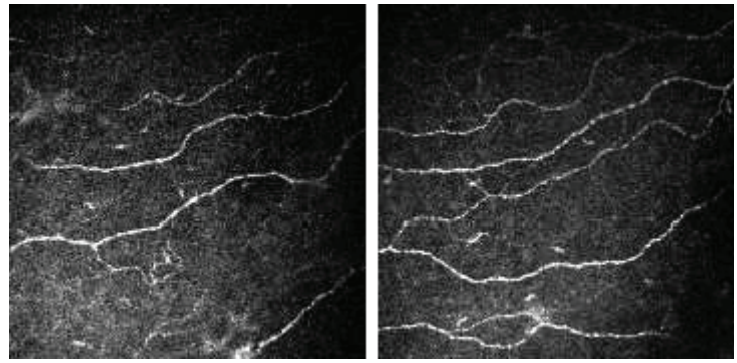


Figure 1: Changes of CCM (from top to bottom are CNFD, CNBD, and CNFL) from baseline to follow-up in group A (a) and group B (b).

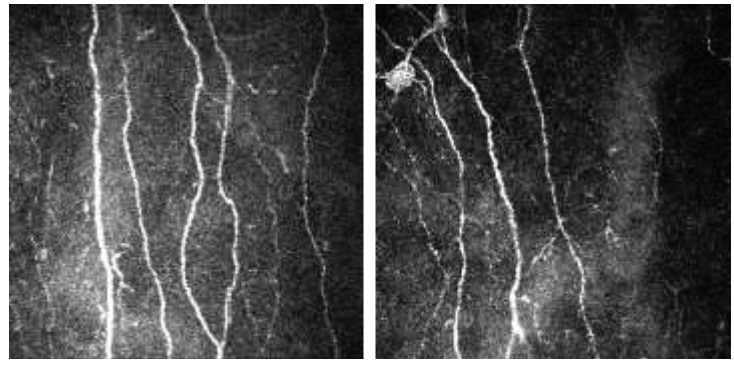
intervention but failed to show improvements in vibration sense and electrophysiology. In the aforementioned study, the quantitative sudomotor axon reflex test and intraepidermal nerve fiber density (IENFD) test, which detect small-fiber nerve function and structure, revealed improvements after one year. IENFD is the gold standard for the diagnosis of small-fiber neuropathy; however, it is difficult to clinically adopt nerve or skin biopsies due to their invasive nature. As a noninvasive diagnostic method for small-fiber neuropathy, CCM has multiple advantages, including high sensitivity [19, 20], good repeatability [21, 22], and easy operation.

Studies of DPN in people with type 2 diabetes often have confusing results [7, 23] because metabolic risk factors, such as glucose, lipids, blood pressure, and BMI, have been shown to be related to the development of DPN [5]. Therefore, we sought to utilize CCM in a noninterventional study to observe the influence of glucose control on the natural history of DPN. At one year follow-up, 50% of diabetic patients had achieved good glucose control with HbA1c < 7% (group A), with the remainder failing to meet this mark (group B). At follow-up, although there were positive trends towards the improvement in blood pressure and blood lipids, there were no significant changes observed in metabolic indexes other than HbA1c in all diabetic patients. In group A, the assessment of neuropathy showed significant improvements compared to baseline only in CNFD and CNFL; there were no significant changes observed in TCSS and lower extremity NCV. Assessment of neuropathy in group B showed a



(a) (b)

Figure 2: CCM images from a diabetic patient of group A at baseline (a) and follow-up (b).



(a) (b)

Figure 3: CCM images from a diabetic patient of group B at baseline (a) and follow-up (b).

significant decrease in SSNCV. Corneal nerve parameters examined in this group including CNFD, CNBD, and CNFL also showed a decrease at one-year follow-up.

Diabetic neuropathy is a complication of diabetic microangiopathy that is specifically related to glucose control but also related to multiple metabolic factors especially in type 2 diabetes. We observed improvement of corneal nerve parameters but not TCSS or NCV in patients with type 2 diabetes with good glucose control, indicating that the corneal nerve, which is reflective of small-fiber neuropathy, can highly sensitively detect the improvement of neuropathy. In addition to glucose control, positive trends towards the improvement in blood pressure and blood lipids especially in group A also contribute to the improvement of corneal nerve morphology. In patients with type 1 diabetes who underwent combined pancreas and kidney transplantation, one year following transplantation, among all diagnostic parameters used to evaluate DPN, only CNFL, CNFD, and CNBD showed improvements [21]. In a study comparing continuous subcutaneous insulin infusion (CSII) and multiple injections of insulin in type 1 diabetes, although glycemic control was similar among the two treatment groups after 24 months, improvements were observed in CNFL, CNFD, and CNBD only in the CSII group [24].

We did not find any correlations between changes in corneal nerve parameters and the disease course of type 2 diabetes, weight, SBP, DBP, blood lipids, or HbA1c. In a previous 24-month observational study, the decrease in HbA1c value was significantly associated with an increase in CNFD [25].

Table 3: Correlation coefficients between corneal nerve parameters and other indexes.

Correlation coefficients (r)	Change in CNFD	Change in CNBD	Change in CNFL
Age	0.085	−0.220	−0.056
Duration of diabetes	0.036	−0.047	0.206
Change of Weight	−0.203	−0.199	
Change of SBP	−0.006	−0.126	−0.006
Change of DBP	−0.241	0.121	0.064
Change of TC	−0.146	0.000	−0.061
Change of TG	−0.031	0.01	0.034
Change of LDLC	−0.138	−0.084	−0.119
Change of HDLC	−0.042	0.180	0.110
Change of HbA1c	−0.127	0.200	0.077

Limitations of the current study include the small size of the study sample and the lack of randomization. Larger randomized studies with active intervention are required to confirm our findings. Another limitation is that HbA1c only reflects the glycemic status of 3 months; in this observational study, we could not get HbA1c values of every person every 3 months, so we do not know how long were the patients in

good glycemic control before there were changes in the corneal nerve morphology. Nevertheless, the present data suggest that CCM may be a convenient, noninvasive technique to assess the progression of nerve damage and potentially to assess the effects of therapeutic intervention in future clinical trials of human diabetic neuropathy.

5. Conclusion

The results of this study suggest that morphological repair of corneal nerve fibers can be detected when glycemic control improves. In vivo CCM could be a sensitive method that can be applied in future longitudinal or interventional studies on diabetic neuropathy.

Data Availability

All data generated or analyzed during this study are included in this article and available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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