Temporal trend of small nerve fibre degeneration in people with and without type 2 diabetes mellitus

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Abstract
Aims: We investigated the long-term temporal trend of intraepidermal nerve fibre density (IENFD) and the association between changes in IENFD and metabolic factors in individuals with and without type 2 diabetes.

Methods: A total of 66 participants were enrolled in this longitudinal population-based study, at baseline consisting of 35 individuals (median 61 years) without diabetes and 31 individuals with type 2 diabetes mellitus. Participants underwent clinical and electrophysiological examinations, as well as a skin biopsy both at baseline and at the follow-up visit (mean 8.1 ± 0.5 years). IENFD was assessed in thin sections of 5 μm, stained with the protein gene product 9.5-antibody and compared between the groups.

Results: IENFD decreased during the period in both groups, with a greater decline in the group without diabetes than in type 2 diabetes (−2.3 and −0.6 fibres/mm respectively; p < 0.001). While IENFD at baseline was significantly reduced in type 2 diabetes relative to people without (p < 0.001), no difference in IENFD was found between groups at the follow-up (p = 0.183). Linear mixed model analysis indicated that age, weight and HbA1c were associated with decrease in IENFD in the total population (p < 0.007). IENFD also decreased with increasing age and weight, but not with HbA1c, in the separate groups (p < 0.049).

Conclusions: Despite lower IENFD levels at baseline in type 2 diabetes, IENFD was equal between the groups at follow-up. A decrease in IENFD is to a limited extent affected by body weight, and HbA1c, but age seems to be the long-term determinant of IENFD in an elderly population.

Keywords
diabetes mellitus, intraepidermal nerve fibre density, longitudinal study, peripheral neuropathy, skin biopsy
1 | INTRODUCTION

Peripheral neuropathy is a common long-term complication of diabetes mellitus and one of the primary risk factors for foot ulceration.\(^1\) Diabetic peripheral neuropathy is a symmetrical polyneuropathy, where the main characteristics may include motor and sensory dysfunction as well as muscle atrophy with loss of axons.\(^2\) Axonal degeneration affects both myelinated and non-myelinated nerve fibres, whereas the small fibres seem to be the first to be affected.\(^3\) Small nerve fibre degeneration has been found in both symptomatic and asymptomatic diabetes,\(^4\) and has been reported in healthy elderly individuals when assessed using the intraepidermal nerve fibre density (IENFD) parameter.\(^5\)

Assessment of IENFD in skin biopsies is considered a useful and sensitive method for assessing small nerve fibre dysfunction in upper and lower extremities.\(^6\)–\(^9\) Associations have been found between decreased IENFD and several metabolic factors, such as obesity, Hba\(_{1c}\), hypertriglyceridemia and an advanced glycation end product (AGE).\(^9\)–\(^11\) Previous longitudinal studies of IENFD have shown that nerve fibres deteriorate over time in both healthy individuals as well as in people with type 1 and type 2 diabetes.\(^3\)\(^,\)\(^12\) However, these studies were performed on skin sections of 50 \(\mu\)m and the participants were followed during a short period of time. We evaluated IENFD in thin 5 \(\mu\)m sections as these are easier to handle in an ordinary diagnostic laboratory situation.\(^7\)–\(^9\) The thin sections allow for an immunohistochemical staining that is reproducible and stable over time, whereas thick sections generally require immunofluorescence, which is time-consuming, costly and does not permit long-term storage or later re-evaluation.

In the present study, we followed a population-based cohort with and without type 2 diabetes mellitus. We aimed to investigate the long-term temporal trend of IENFD and to evaluate potential differences in IENFD change between the two groups. Second, we assessed the association between metabolic factors and changes in IENFD.

2 | METHODS

2.1 | Ethics statement

The regional ethical review board of Umeå University, Umeå, Sweden, approved the study (ethical application no. Dnr 2013-21-31 M). The study was conducted in accordance with the Declaration of Helsinki and all participants provided written informed consent prior to inclusion. The study adheres to the STROBE guidelines.

Novelty statement

- People with diabetes may suffer from diabetic neuropathy, affecting both large and small nerve fibres, but there is a need for longitudinal data regarding nerve fibre degeneration.
- Analysis of the intraepidermal nerve fibre density (IENFD) is a valuable diagnostic tool to detect small nerve fibre degeneration.
- At an age of 70 years, people with type 2 diabetes and healthy individuals presented with equal levels of IENFD, despite a lower IENFD in type 2 diabetes at baseline 8 years earlier.
- Age seems to be the major long-term determinant of IENFD, together with body weight, and Hba\(_{1c}\) to a limited extent.

2.2 | Study design and participants

Individuals with and without type 2 diabetes were consecutively recruited from a cohort in northern Sweden for a longitudinal study, including an initial baseline investigation (November 2004–March 2007) and a follow-up in 2014 (mean difference 8.1 ± 0.5 years). The baseline study included 119 participants from a cohort originally recruited within the Västerbotten Invention Programme (VIP) in northern Sweden,\(^13\) which has been described previously.\(^14\) Participants underwent physical and electrophysiological examinations, as well as skin biopsies.\(^8\)\(^,\)\(^14\) At the follow-up in 2014, 87 participants were included, as six individuals were deceased and 26 declined further participation.\(^15\) From this group, 83 participants agreed to provide a skin biopsy sample. Of these, a further three were excluded, due to either missing oral glucose tolerance test data at follow-up (two participants) or incomplete skin biopsy sample at baseline (one participant). Finally, 80 participants (42 men and 38 women) with full skin biopsy data at both baseline and follow-up were included. The baseline group consisted of 35 individuals with normal glucose tolerance (NGT; 15 men, 20 women), 14 with impaired glucose tolerance (IGT; 9 men, 5 women) and 31 participants with type 2 diabetes (18 men, 13 women). The IGT group was excluded from further analysis due to the low number of individuals.

2.3 | Measurements

Study participants were examined by the same research nurse and physician using the same questionnaires, measurements and tests at both baseline and follow-up,
conducted at the Skellefteå Hospital in Sweden.\textsuperscript{6,14} Blood pressure, weight and height were measured and BMI (kg/m\textsuperscript{2}) was calculated. Blood samples were drawn and measured for cholesterol, triglycerides, creatinine and HbA\textsubscript{1c}. The glycaemic status, that is, fasting and 2 h plasma glucose, in the individuals without diabetes was assessed by an oral glucose tolerance test (OGTT).\textsuperscript{14}

2.4 | Neurophysiological examinations

An experienced neurophysiologist performed standardized nerve conduction studies (NCS) at the clinical neurophysiology laboratory at Umeå University, Sweden, at baseline and at the follow-up in 2014. The neurophysiologist was blinded to the participant group identity, that is, if individuals had type 2 diabetes or not. Amplitude and conduction velocity of the sural nerve, as well as conduction velocity of the peroneal nerve, were measured at the right leg. Thermal threshold tests were performed with Thermotest equipment (Somedic AB, Hörby, Sweden) by using the method of limits.\textsuperscript{16}

2.5 | Clinical signs and symptoms

A modified version of Dyck’s original Neuropathy Disability Score (NDS) and Neuropathy Symptom Score (NSS) was used to define incidence as well as severity of peripheral neuropathy in the extremities.\textsuperscript{17,18}

2.6 | Skin biopsy

All punch skin biopsies were performed by the same person, using a 3-mm disposable circular needle. The samples were obtained from the distal right leg, approximately 10 cm proximal to the lateral malleolus, harvested without any residual problems, according to published guidelines.\textsuperscript{6} The punch wound was allowed to heal without application of any sutures.

2.7 | Immunohistochemistry

Immunohistochemistry procedures were developed and revised in Lund, Sweden,\textsuperscript{7,19} as a modification to the published guidelines.\textsuperscript{6,20} Biopsies were immediately fixed in 4% buffered formaldehyde solution for at least 24 h, then dehydrated and paraffin embedded. Sections with the thickness of 5 \( \mu \text{m} \) each (spaced 15 \( \mu \text{m} \) apart) were mounted on glass for immunohistochemical staining and dried 1 hour at 60\textdegree C. Sections were de-waxed, rehydrated and microwave pre-treated in 10 mM citrate buffer (pH 6.0) for 19 min at 750 W to achieve antigen retrieval. Immunohistochemical staining was performed with a rabbit polyclonal Protein Gene Product (PGP) 9.5 antibody (Cell Marque), in a 1:3000 dilution with an automated immunostainer (TechMate 500 Plus; Dako). Baseline samples were sectioned and stained continuously during the years of study inclusion. Twenty-four samples from 2014 were prepared in 2015, whereas the remaining were processed in 2018. However, all samples were sectioned and stained in the same laboratory (Lund) using an identical procedure.

2.8 | Assessment of intraepidermal nerve fibre density (IENFD)

Intraepidermal nerve fibre density denotes the number of nerve fibres per millimetre of epidermal length. In order to assess IENFD, counting of nerve fibres and measuring of epidermal length were carried out in two central and consecutive sections of the biopsy from each participant. Both procedures were performed manually using light microscopy at a magnification of 200X and 400X. PGP 9.5-positive nerve fibres were counted if they met certain criteria, which were modified from the standard recommendations in order to optimize the count in thin sections.\textsuperscript{6,7,9} The number of individual fibres in the epidermis was counted if the length measured at least half the width of the epidermal layer in the actual area. If the fibre branched within the epidermis only a single fibre was counted. Because of the thin sections used, even well-defined fibres not visibly crossing the junction were counted if the remaining criteria were deemed satisfactory. Sections from baseline and follow-up were all investigated in 2019 since the procedure and criteria had been developed over the years. Additionally, all sections were re-counted on a second occasion to assess the intra-rater reliability. In order to obtain the inter-rater reliability, a second independent observer determined the IENFD of all sections. Sections were blinded in order to prohibit identification of the participant group, and observers were unaware of initially calculated results when re-counting. The IENFD for each biopsy was calculated using the highest count among the two sections.

2.9 | Statistical analyses

Normality tests were performed prior to the choice of statistical methods. Data are presented as numbers (n) and proportions (%) for categorical variables. Data are also presented as mean ± SD or median and interquartile
range (IQR) for quantitative variables. Differences between the groups with and without type 2 diabetes were analysed using Mann–Whitney U-test or Chi²-test, separately at baseline and at follow-up. Pairwise testing with the Wilcoxon signed-rank test was used to identify significant changes between baseline and follow-up investigations for the groups respectively. Linear mixed model analyses were performed in order to investigate if metabolic factors were associated with changes in IENFD. In total, three models with IENFD as the dependent factor were analysed (all participants: type 2 diabetes; normal glucose tolerance). The first model, with all participants, included age, sex, height, weight, blood pressure, statin treatment and HbA₁c levels as independent factors. When analysing the group of type 2 diabetes, the independent factors were age, weight, statin treatment and HbA₁c. In the third model, the factors age, weight and HbA₁c were applied to the group with normal glucose tolerance. Data from both baseline and follow-up were applied in the models. Data from two participants were excluded from the repeated measurement analyses (Wilcoxon signed-rank test) and subgroup analyses (linear mixed model; model 2 and 3) since these individuals were reclassified regarding glycaemic status during the follow-up. Results were considered significant at a two-tailed level of α = 0.05. Analyses were performed using SPSS (IBM SPSS Statistics version 27).

3 | RESULTS

3.1 | Oral glucose tolerance test

At follow-up in 2014, the number of participants with normal glucose tolerance were 33 whereas 31 individuals had type 2 diabetes. Thus, two participants developed type 2 diabetes during the follow-up period.

3.2 | Temporal trend of IENFD

IENFD was reduced at follow-up in both the group with normal glucose tolerance as well as in the group with type 2 diabetes, relative to baseline (Table 1). However, the amount of decrease in IENFD was smaller in the individuals with type 2 diabetes compared to those without \( p < 0.001 \), Figure 1). In fact, 5/31 (16%) of the individuals with type 2 diabetes appeared to have no deterioration over time, as their IENFD was already zero at baseline. At follow-up, 5/33 (15%) individuals with normal glucose tolerance and 11/33 (33%) with type 2 diabetes presented with zero counts. In contrast to the observation at baseline \( p < 0.001 \), there was no difference in IENFD between the people with our without type 2 diabetes at follow-up \( (p = 0.183, \text{Table 1}) \).

3.3 | Changes in clinical and electrophysiological parameters over time

HbA₁c values increased within the group of normal glucose tolerance \([3.4 (1.4–3.4) \text{ mmol/mol}; \ p < 0.001] \), but not within type 2 diabetes \( (p = 0.147) \), whereas blood pressure increased over time in both groups (Table 1). Creatinine levels were normal (local reference values; men: 60–105 \( \mu \text{mol/L}; \) women: 45–90 \( \mu \text{mol/L} \)) for both groups at follow-up, although increased over time in the type 2 diabetes group \( (p = 0.041) \). Individuals with normal glucose tolerance had minimally, yet higher total cholesterol levels at follow-up \( (p = 0.003) \), whereas individuals with type 2 diabetes had low levels of total cholesterol both at baseline and follow-up. Triglyceride levels increased \( (p = 0.027; \ p = 0.002) \) whereas LDL was lowered \( (p = 0.010; \ p = 0.035) \) over time for both individuals with normal glucose tolerance and type 2 diabetes. We observed a small deterioration of sural nerve amplitude and peroneal conduction velocity over time for both groups (for all comparisons, see Table 1).

3.4 | Glycaemic group comparisons at follow-up

Comparing the groups at follow-up, we observed that individuals with type 2 diabetes had higher BMI \( (p = 0.001) \), HbA₁c \( (p < 0.001) \) and triglycerides \( (p < 0.001) \), as well as lower total cholesterol \( (p < 0.001) \), HDL \( (p = 0.018) \) and LDL \( (p < 0.001) \) as compared to the people with normal glucose tolerance (Table 1).

3.5 | Associations of temporal trend in IENFD with changes in metabolic factors

In a linear mixed model analysis, IENFD decreased over time with increasing weight for all participants \( (−0.02 \text{ fibres/mm per kg}; \ p = 0.005) \), that is individuals with and without type 2 diabetes (Table 2, Figure 2). HbA₁c was associated with changes in IENFD levels when analysing all participants, but the association was not found when the groups were investigated separately.

3.6 | Intraclass correlation coefficient

A high degree of both inter- and intra-rater reliability was found in the assessment of nerve fibre counts. The
<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Normal glucose tolerance</th>
<th>Type 2 Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diabetes duration [years]</strong></td>
<td>n/a</td>
<td>3 (2–12)</td>
</tr>
<tr>
<td><strong>Sex (men/women)</strong></td>
<td>15/20</td>
<td>18/13</td>
</tr>
<tr>
<td><strong>Age [years]</strong></td>
<td>61 (61–62)</td>
<td>61 (61–61)</td>
</tr>
<tr>
<td><strong>Height [m]</strong></td>
<td>1.69 (1.61–1.77)</td>
<td>1.72 (1.63–1.79)</td>
</tr>
<tr>
<td><strong>Weight [kg]</strong></td>
<td>74 (62–87)</td>
<td>83 (79–99)#</td>
</tr>
<tr>
<td><strong>BMI [kg/m²]</strong></td>
<td>25.7 (22.3–27.7)</td>
<td>29.4 (26.6–32.4)#</td>
</tr>
<tr>
<td><strong>HbA₁c [mmol/mol] [%]</strong></td>
<td>35 (33–38)</td>
<td>54 (46–62)#</td>
</tr>
<tr>
<td><strong>Systolic blood pressure [mm Hg]</strong></td>
<td>122 (115–138)</td>
<td>128 (122–137)#</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure [mm Hg]</strong></td>
<td>75 (70–80)</td>
<td>76 (70–81)#</td>
</tr>
<tr>
<td><strong>Creatinine [mol/L]</strong></td>
<td>75 (61–83)</td>
<td>76 (63–78)</td>
</tr>
<tr>
<td><strong>Total cholesterol [mmol/L]</strong></td>
<td>5.8 (5.0–6.4)b</td>
<td>4.8 (4.4–5.3)#</td>
</tr>
<tr>
<td><strong>Triglycerides [mmol/L]</strong></td>
<td>1.1 (0.8–1.4)a</td>
<td>1.5 (1.1–2.0)#</td>
</tr>
<tr>
<td><strong>HDL [mmol/L]</strong></td>
<td>1.4 (1.2–1.7)</td>
<td>1.1 (1.0–1.4)#</td>
</tr>
<tr>
<td><strong>LDL [mmol/L]</strong></td>
<td>3.7 (3.3–4.3)b</td>
<td>2.9 (2.6–3.4)#</td>
</tr>
<tr>
<td><strong>Statin treatment, n (%)</strong></td>
<td>2 (5.7)</td>
<td>15 (48.4)#</td>
</tr>
<tr>
<td><strong>Neuropathy symptom score</strong></td>
<td>0.0 (0.0–1.0)</td>
<td>2.0 (0.0–4.0)#</td>
</tr>
<tr>
<td><strong>Neuropathy disability score</strong></td>
<td>4.0 (1.8–7.3)a</td>
<td>7.0 (2.8–13.3)a</td>
</tr>
<tr>
<td><strong>Sural nerve</strong></td>
<td><strong>Conduction velocity [m/s]</strong></td>
<td>45 (42–48)#</td>
</tr>
<tr>
<td><strong>Conduction velocity [µV]</strong></td>
<td>10 (7–14)a</td>
<td>46 (42–49)#</td>
</tr>
<tr>
<td><strong>Peroneal nerve</strong></td>
<td><strong>Conduction velocity [m/s]</strong></td>
<td>45 (40–50)#</td>
</tr>
<tr>
<td><strong>Quantitative sensory testing</strong></td>
<td><strong>Cold thresholds [C]</strong></td>
<td><strong>Heat thresholds [C]</strong></td>
</tr>
<tr>
<td><strong>Right</strong></td>
<td>29.7 (26.5–30.4)a</td>
<td>38.8 (36.5–40.7)a</td>
</tr>
<tr>
<td><strong>Left</strong></td>
<td>28.9 (25.9–29.9)a</td>
<td>39.5 (37.2–42.0)a</td>
</tr>
<tr>
<td><strong>IENFD [fibres/mm]</strong></td>
<td>3.2 (2.5–4.3)</td>
<td>0.8 (0.4–1.4)</td>
</tr>
</tbody>
</table>

Data are given as median (IQR = Q1–Q3) or numbers. Significant values (p < 0.05) are highlighted in bold given by repeated follow-up measurements as compared to baseline measurements, for people with (n = 31) and without diabetes (n = 33) respectively (Wilcoxon signed-rank test). Significant values (p < 0.05) between no diabetes and type 2 diabetes are denoted by # for baseline comparison and by ## for follow-up comparison respectively (Mann–Whitney U test or Chi²-test). †Two participants with normal glucose tolerance were reclassified as having type 2 diabetes mellitus at follow-up, these are not included in the repeated measurement analyses (Wilcoxon signed-rank test). IENFD, intraepidermal nerve fibre density.

a1 missing value.
b2 missing values.
c3 missing values.
d4 missing values.
e5 missing values.
average ICC measures were 0.908 and 0.933 ($p < 0.001$) respectively.

4 | DISCUSSION

For both the people with and without diabetes, IENFD was significantly reduced in 2014 relative to baseline, while the groups had similar mean IENFD at follow-up. In comparison to previously published data on individuals of a corresponding age, IENFD for participants with type 2 diabetes were comparable (0.8 fibres/mm in both studies), whereas individuals with normal glucose tolerance in the present study presented with lower densities (0.7 vs. 1.3 fibres/mm). This difference may be influenced by the fact that HbA1c levels were noticeably higher among individuals without diabetes in our current study, compared to previously published studies, as high HbA1c levels have been shown to be associated with lower IENFD. The fact that IENFD levels for both groups were different at baseline, but equivalent at follow-up, could be a result of a floor effect. As nerve depletion due to diabetes may have occurred in an earlier stage, perhaps even prior to diagnosis, the participants with type 2 diabetes already had low IENFD levels at baseline and could not deteriorate as much and not further than to zero. Thus, neuropathy in type 2 diabetes could be more sensitive to the cumulative time period over which IENFD is pathologically reduced, rather than to the absolute level of IENFD at a given time. Additional controlled, longitudinal studies assessing IENFD are therefore warranted to further investigate this hypothesis.

Diabetic peripheral neuropathy is thought to be caused by metabolic insults such as hyperglycaemia,
With respect to implementation of IENFD diagnostics in clinical practice, one should consider the impact of age and weight on the nerve fibre density, as noted in both individuals with and without type 2 diabetes.

This study has some limitations. First, the study population in our study was small due to a high drop-out of participants over the years. The small sample size could have affected the power to assess the association between metabolic factors, for example, HbA1c and IENFD in the subgroups. Second, our participants with T2DM were rather healthy as they were well-treated in terms of metabolic control, which may have led to a healthy participant bias. Third, a selection bias emerged as participants denied further participation to the follow-up mainly in the group with type 2 diabetes. Consequently, the final population with type 2 diabetes was relatively healthy. On the other hand, the strengths of this study were the multiple well-defined examinations that were carried out by the same staff in an identical manner at both baseline and at a long follow-up. Additionally, to our knowledge, there has been no previous study of IENFD assessment with a long-term follow-up.

5 CONCLUSION

In this longitudinal study, we observed that individuals both with and without type 2 diabetes presented with low levels of IENFD over 8 years of follow-up, where weight was a contributing factor to the decrease in both groups, while HbA1c was only associated with decreasing IENFD in the total population. Although deterioration of IENFD was brought out earlier in the type 2 diabetes, levels at follow-up were equal between people with normal glucose tolerance and type 2 diabetes. Ageing seems to be the long-term determinant of IENFD in an elderly population with healthy participants and individuals with type 2 diabetes.

ACKNOWLEDGEMENTS

The authors thank Dr. Sigbritt Rasmark, RN Karin Nilsson and biomedical scientist Anette Broberg for their skilful assessment of the participants. A special thanks to the late Professor Göran Sundqvist, who was one of the initiators of the study. The work was supported by grants from Västerbotten County Council and Umeå University, Sweden, funds from Skåne University Hospital, Lund University and the Swedish Diabetes Foundation. The funders played no part in study design, data collection and analysis, decision to publish or preparation of the manuscript.

CONFLICT OF INTEREST

Nothing to declare.
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How to cite this article: Ekman L, Pourhamidi K, Englund E, Lagali N, Rolandsson O, Dahlin LB. Temporal trend of small nerve fibre degeneration in people with and without type 2 diabetes mellitus. Diabet Med. 2021;00:e14691. https://doi.org/10.1111/dme.14691