Higher Total Insulin Dose Has Positive Effect on Corneal Nerve Fibers in DM1 Patients

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PURPOSE. Neuropathies are among the most common long-term complications of diabetes mellitus (DM) and good glycemic control is essential in prevention of this complication. DM patients with similar mean glucose levels or HbA1c levels often exhibit differences in glucose variability. We tested for possible associations between parameters of glycemia compensation and corneal sub-basal nerve fiber status.

METHODS. The study included 20 patients with DM type 1 treated using an intensified insulin regimen. The corneas of both eyes were examined using in vivo corneal confocal microscopy. Corneal nerve fiber density (NFD), nerve fiber length (NFL), and nerve branch density (NBD) were evaluated. Possible associations between parameters of glycemia compensation (HbA1c, glycemias SD, and insulin dose) and other clinical factors were analyzed.

RESULTS. NBD was the highest in those with higher glycemic variability (P = 0.025). HbA1c had a negligible effect on corneal nerve parameters. NFD, NFL, and NBD were statistically significantly higher in those with higher total insulin per kilogram (P = 0.02, P = 0.01, and P = 0.012, respectively). Among other factors, a positive correlation between free thyroxine (fT4) levels and NFD and NBD was also found (P = 0.041 and P = 0.015, respectively).

CONCLUSIONS. Total insulin dose per kilogram may be an important factor influencing nerve fiber status and needs to be considered in future studies of diabetic neuropathy pathophysiology and its progression. Also, more attention must be paid to other possible factors when elucidating the development of diabetic complications.

Keywords: corneal nerve fiber, diabetes mellitus, neuropathy, confocal microscopy

Neuropathies are among the most common long-term complications of diabetes mellitus (DM), affecting up to 50% of patients.1,2 The cornea is the most innervated organ in the body, and we are now able to image corneal nerve fibers using in vivo corneal confocal microscopy (IVCM). IVCM is an easy, reproducible, and noninvasive technique that provides optical sections of the cornea.3 A close association between damage to peripheral nerves due to diabetes and alterations in the corneal sub-basal nerve plexus, detected using IVCM, has been demonstrated.4,5

Neuropathy is widely classified as a microvascular complication of DM. Good glycemic control is essential in prevention of chronic complications of DM, including neuropathy. However, DM patients with similar mean glucose levels or HbA1c levels often exhibit differences in terms of both the number and degree of glucose excursions.8 This phenomenon, termed glucose variability (GV), may be responsible for differences in microvascular outcomes between DM patients with the same mean HbA1c. Furthermore, new evidence suggests that the process is more complex and may involve a repertoire of molecular alterations and abnormalities of insulin signaling that accompany neurodegeneration.9

Currently diabetologists have an excellent tool, continuous glucose monitoring (CGM), to quantify and closely monitor GV. CGM systems can be operated in blinded or in open mode (real time [RT-CGM]), which enables the patient to react immediately. CGM consists of a small glucose sensor placed in the subcutaneous tissue with an attached transceiver. In the case of RT-CGM patients, they carry the receiver with them. It can be a special device, such as an insulin pump or even a smart mobile phone. CGM systems register glycemias (i.e., glucose concentrations in interstitial tissue) at 5-minute intervals. This provides 288 values per day for analysis. From these values, different parameters reflecting GV can be calculated. The standard deviation (SD) is accepted among them as an easily calculable and still useful parameter.10 We can of course also calculate these parameters from standard glycemias profiles measured with personal glucometers.10,11

In our study, we looked for possible associations between glycemias compensation (i.e., HbA1c, glycemias SD, and insulin dose) and corneal sub-basal nerve fiber status.

METHODS

The study enrolled men and women 24 years or older, who were willing and able to participate in the study. All subjects were examined from 2012 to 2017. We approached patients
with type 1 DM (T1DM) who had been followed by our outpatient clinic for several years. We primarily focused on highly motivated T1DM patients on continuous subcutaneous insulin infusion (CSII) therapy.

Exclusion criteria included patients with a history of ocular surgery or laser treatment, eye trauma, use of chronic ophthalmic topical medication, contact lens wearers, or any systemic diseases that could adversely affect the ocular surface (e.g., Sjögren syndrome, or other rheumatologic diseases).

Informed consent was obtained from each patient after an explanation of the nature and possible consequences of the study. The study was approved by the Motol University Hospital Ethics Committee. The study protocol followed the tenets of the Declaration of Helsinki for research involving human participants.

Complete data sets were available from 20 T1DM patients: 12 females and 8 males. All 20 patients were treated using an intensified insulin regime, 17 (17/20) using CSII. Nine (9/20) were without chronic diabetic complications at the time of their ophthalmological examination, five had already developed one chronic diabetic complication, and six suffered from multiple chronic diabetic complications by the time they entered into the study. One patient suffered from solely diabetic neuropathy, three from solely diabetic retinopathy, and one patient from diabetic nephropathy. Four patients had diabetic neuropathy accompanied by diabetic retinopathy and two patients had all three types of microvascular complications developed (e.g., neuropathy, nephropathy, as well as retinopathy). Eleven patients (11/20) had body mass index (BMI) < 25. Six (6/20) were smokers (3 had BMI < 25), 2 were classified as ex-smokers (1 had BMI < 25), and 12 had never smoked (7 had BMI < 25). Nine (9/20) were diagnosed with thyroid gland disease (of an autoimmune origin). Five (5/9) were taking thyroid hormone substitution therapy and four (4/9) were on iso-hormonal therapy. There were six patients treated by angiotensin-converting enzyme inhibitors (ACEI: four patients by perindopril and the other two by ramipril). The other medications used by the patients within the study group should not interfere with the analysis. Other patient characteristics are presented in Table 1.

### In Vivo Corneal Confocal Microscopy

In vivo confocal microscopy images of the cornea were obtained from all participants. Topical anesthesia (oxybuprocaine hydrochloride 0.4%) was used before IVCM examinations. A corneal confocal microscope (scanning slit corneal microscope, Confoscan 3.0; NIDEK Technologies, Albignasego, Italy), equipped with a nonapplanating ×40 immersion objective lens designed for full-thickness examination of the cornea, with a working distance of 1.92 mm (Achromplan; Zeiss, Oberkochen, Germany), was used to scan the central corneal region. Before use, the objective lens of the confocal microscope was disinfected (isopropyl alcohol 70% vol/vol, swabs), and one drop of viscous isotonic gel (Vidisic gel; Bausch & Lomb, Rochester, NY, USA) was applied to the tip of the lens. Images of the patient’s central cornea, starting from the anterior chamber, were recorded. The image interval was set to 5 μm and an automatic or semiautomatic scanning mode was used. Typically, at least two scanning cycles were performed, and at least 700 images of the cornea of each eye were captured, and both eyes were scanned. There were usually 20 to 30 images captured containing sub-basal nerves in each eye.

### Table 1. Study Group Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean Value</th>
<th>Median Value</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>39.5</td>
<td>41.6</td>
<td>13.1</td>
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<tr>
<td>Diabetes duration, y</td>
<td>15.5</td>
<td>16.2</td>
<td>10.7</td>
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<tr>
<td>HbA1c, mmol/mol</td>
<td>68.0</td>
<td>71.4</td>
<td>14.2</td>
</tr>
<tr>
<td>Glyceria SD,* mmol/L</td>
<td>3.5</td>
<td>3.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Total 24-h ID, IU</td>
<td>37.5</td>
<td>42.9</td>
<td>15.5</td>
</tr>
<tr>
<td>Total 24-h insulin dose/kg, IU/kg</td>
<td>0.5</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.6</td>
<td>25.1</td>
<td>2.7</td>
</tr>
<tr>
<td>25-OH vitamin D, nmol/L</td>
<td>56.1</td>
<td>52.7</td>
<td>15.9</td>
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<tr>
<td>LDL, mmol/L</td>
<td>2.3</td>
<td>2.4</td>
<td>0.7</td>
</tr>
<tr>
<td>25-OH vitamin D, nmol/L</td>
<td>24.6</td>
<td>25.1</td>
<td>2.7</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>129.0</td>
<td>126.8</td>
<td>14.0</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>79.0</td>
<td>78.9</td>
<td>6.5</td>
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</table>

* Calculated from CGM profiles.

### Table 2. Nonparametric Correlation Analyses (Using Spearman’s Rho Test; n = 20)

<table>
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<tr>
<th>Characteristic</th>
<th>NFD</th>
<th>NFL</th>
<th>NBD</th>
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<tr>
<td>DBP, mmol/mol</td>
<td>0.071</td>
<td>0.109</td>
<td>0.071</td>
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<tr>
<td>LDL, cholesterol</td>
<td>0.765</td>
<td>0.649</td>
<td>0.766</td>
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<tr>
<td>Total 24-h ID, IU</td>
<td>-0.144</td>
<td>-0.091</td>
<td>-0.082</td>
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<tr>
<td>Total 24-h insulin dose/kg, IU/kg</td>
<td>0.544</td>
<td>0.704</td>
<td>0.731</td>
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<tr>
<td>BMI, kg/m²</td>
<td>0.185</td>
<td>0.239</td>
<td>0.079</td>
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<tr>
<td>25-OH vitamin D, nmol/L</td>
<td>0.439</td>
<td>0.310</td>
<td>0.740</td>
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<tr>
<td>Correlation coefficient</td>
<td>0.460</td>
<td>0.321</td>
<td>0.535</td>
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<td>Correlation coefficient</td>
<td>0.041</td>
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<td>0.015</td>
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<td>Correlation coefficient</td>
<td>0.266</td>
<td>0.292</td>
<td>0.400</td>
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<td>0.257</td>
<td>0.212</td>
<td>0.080</td>
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<tr>
<td>Correlation coefficient</td>
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<td>-0.060</td>
<td>0.091</td>
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<tr>
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<td>0.845</td>
<td>0.801</td>
<td>0.704</td>
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<td>-0.359</td>
<td>-0.339</td>
<td>-0.250</td>
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<tr>
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<td>0.144</td>
<td>0.289</td>
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<td>0.429</td>
<td>0.513</td>
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<td>Correlation coefficient</td>
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<td>0.059</td>
<td>0.021</td>
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<td>Correlation coefficient</td>
<td>0.502</td>
<td>0.568</td>
<td>0.509</td>
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<tr>
<td>Correlation coefficient</td>
<td>0.024</td>
<td>0.009</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Sig., significance. * Correlation is significant at the 0.01 level. † Correlation is significant at the 0.05 level.
Analysis of Corneal Images

We used manual identification of nerve fibers and branching, as well as manual tracing of nerve fibers. Custom image analysis software (NIS-Elements; Laboratory Imaging, Praha, Czech Republic) was used for nerve fiber analysis. Because the Confoscan 3 produces images that blur at the edges, these were cut out and only the well-focused central area (0.01 mm²) was used for nerve fiber assessment. Four of the most representative images (two from each eye with the best captured nerve fibers), according to the recommendation of Smith et al.,¹² were selected for nerve fiber measurements, which included mean values of the total nerve length (NFL, in mm per mm²), total number of nerves (nerve fiber density [NFD], per mm²), and branching point density (NBD, per mm²). All the images were evaluated by one investigator (GM), at the end of the study, who was blinded relative to patient identity.

Statistical analysis was carried out using SPSS SW v.24 software (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA). Nonparametric correlation analyses were done on all parameters (Spearman’s rho). The Mann-Whitney test was used for two group comparisons, and the Kruskal-Wallis test was used for three or more group comparisons.

For categorization, we used values according to the recommendation of the Czech Diabetological Society for therapeutic goals. The following parameters were used as group categories: sex, BMI (patients having BMI ≤25 were compared with patients with BMI >25), smoker status (smoker/ex-smoker/nonsmoker), HbA1c (those with HbA1c ≤60 IFCC versus patients having HbA1c >60 mmol/mol), glycemic variability (reference value GV = 3.5 mmol/L), insulin dose (reference value ID = 0.6 IU/kg), low density lipoprotein (LDL) cholesterol (reference value was 2.5 mmol/L), vitamin D level (reference value was 50 mmol/L), the thyroid gland function (thyroid-stimulating hormone [TSH] and free thyroxine [fT4] hormone levels) and high blood pressure (systolic blood pressure [SBP] ≥140 mm Hg, diastolic blood pressure [DBP] ≥90 mm Hg, or antihypertensive drug therapy). As patients used two different drugs and in different doses, this factor (ACEI treatment) was not used as a grouping variable. Other variables (which could have potentially been used as grouping variables) could not be used in our analysis due to the small variability within the groups (for example, nobody suffered from ischemic disease of the lower extremities, Ankle Brachial Index [ABI], or Intima Media Thickness [IMT]; high-density lipoprotein cholesterol and triglycerides were normal in most patients).

RESULTS

The Effect of Other Clinical Factors on Corneal Nerve Fiber Parameters

We did not observe any influence relative to age, sex, BMI, or smoker status on the studied corneal nerve parameters. Other parameters, which were used as category (grouping) variables (vitamin D levels, lipids – LDL cholesterol, SBP and DBP) also revealed no significant relationships with the corneal nerve status (Table 2).

As already mentioned, factors such as triglycerides, IMT, or ABI were not used as category (grouping) variables due to the small variability of our study sample within these categories. However, an unexpected relationship between thyroid gland function and corneal nerve parameters was observed. Namely, although there was no association between the TSH levels and corneal nerve parameters (Table 2), there was a positive correlation between fT4 levels and NFD (r = 0.46, P = 0.041) and between fT4 and NBD (r = 0.535, P = 0.015). These findings are also presented in Figures 1 and 2.

Three (3/6) of the ACEI-treated patients had GV <3.5 mmol/L and three (3/6) patients GV >3.5 mmol/L; two (2/6) patients had ID <0.6 IU/kg and four (4/6) patients had ID >0.6 IU/kg.

Figure 1. Correlation between fT4 levels (pmol/L) and corneal NFD (per mm²). fT4 values positively correlate with NFD.
The Effect of Diabetes-Related Factors on Corneal Nerve Parameters

GV expressed as glycemia SD calculated from CGM profiles was associated with all nerve parameters analyzed (i.e., NFD, NFL, and NBD). Surprisingly, these parameters were higher in those with higher glycemic variability (Table 2, Figs. 3–5). The most obvious connection was observed between GV and NBD, where patients having higher GV (shown as group 2) had a higher NBD ($P = 0.023$; Fig. 3). Surprisingly, the association between HbA1c and corneal nerve parameters was negligible.

Total ID per kilogram was also correlated with GV ($r = 0.531$, $P = 0.016$). NFD, NFL, and NBD were statistically significantly higher in those with a higher total dose of insulin per kilogram ($P = 0.02$, $P = 0.01$, $P = 0.012$, respectively; Table 2, Figs. 6–8).

DISCUSSION

Over the past several years, corneal confocal microscopy has started to be widely used for corneal sub-basal nerve fiber

![Figure 2. Correlation between fT4 levels (pmol/L) and corneal NBD (per mm²). fT4 values positively correlate with NFD.](image)

![Figure 3. The effect of GV (reference value GV = 3.5 mmol/L) on corneal NBD (number per mm²). The NFD was higher in patients with higher GV ($P = 0.023$).](image)
status assessment. Corneal nerve status has been found to correlate with diabetic neuropathy.

The Diabetes Control and Complications Trial conclusively demonstrated the risk of microvascular complications increases as HbA1c increases. However, even within the same mean HbA1c levels, individuals can vary in the number and degree of glycemic excursions. More recently, studies suggested that this may be an additional risk factor for development of retinopathy and nephropathy. However, results from studies assessing the association between GV or HbA1c fluctuations and the risk of diabetic neuropathy are controversial. Virk et al. found an association between GV and cardiac autonomic neuropathy. Additionally, the impact of glycemic variability was considerably greater than that of mean HbA1c. In contrast, peripheral neuropathy has not been associated with either mean HbA1c or HbA1c variability. In view of the large sample size and high event rate of the above-mentioned study (i.e., the study had the power to detect even a weak association), the authors suggested that the results may reflect additional unknown pathogenetic factors. Although some authors have found HbA1c levels to be an independent predictor of corneal NFL and NFD in T1DM patients, other authors have failed

**Figure 4.** The effect of GV (reference value GV=3.5 mmol/L) on corneal NFD (number per mm²). The difference between the groups was not statistically significant ($P = 0.063$).

**Figure 5.** The effect of GV (reference value GV=3.5 mmol/L) on corneal NFL (mm/mm²). The difference between the groups was not statistically significant ($P = 0.052$).
to find confirmation for this association, or they have found connections with other parameters.

We did not find an association between HbA1c values and corneal nerve fiber status in our study. Surprisingly, we found higher NFL and NBD values in patients with higher GV. An association between GV and ID was previously described by Felicio et al.; adding to this, we found an even stronger association between corneal nerve fiber parameters and total ID per kilogram. Thus, the total ID per kilogram may be the additional pathogenetic factor mentioned by Virk et al.

It has been recognized for some time that insulin possesses actions beyond glucose regulation. In the peripheral nervous system, insulin has been shown to facilitate nerve regeneration. Even low doses of intrathecal insulin were able to reverse electrophysiological as well as structural changes in experimental diabetic neuropathy. Moreover, insulin applied to the cornea of diabetic mice prevented axonal loss in the sub-basal plexus detected using IVCM. Thus, the higher total dose in patients with higher GV may explain the better status of the corneal nerve fibers and may also explain the confounding results of studies assessing the association between neuropathy and parameters of glycemic control.

ACEIs were documented to possess a potentially neuroprotective effect. As the number of patients treated with ACEIs

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**Figure 6.** The effect of total ID (reference value ID = 0.6 IU/kg) on corneal NFD (number per mm²). The NFD was higher in patients with higher ID per kilogram (P = 0.02).

**Figure 7.** The effect of total ID (reference value ID = 0.6 IU/kg) on corneal NBD (number per mm²). The NBD was higher in patients with higher ID per kg (P = 0.012).
was almost equal in the both GV and ID groups, we believe this factor did not interfere with the results.

We also found a possible association between free thyroid hormone levels (fT4) and corneal nerve parameters. Serum thyroid hormone abnormalities are common in patients with DM.\textsuperscript{30–32} It was shown that thyroxine increases nerve growth factor and augments nerve regeneration.\textsuperscript{33} In the developing chick, exogenous thyroxine increases nerve elongation.\textsuperscript{34} Thyroid hormone receptors are present in all layers of the cornea.\textsuperscript{35} However, the effects of thyroxine on corneal nerve regeneration are unknown.\textsuperscript{36} One possible effect of an imbalance in deiodinase activity was recently suggested in patients with DM. Higher blood glucose and/or insulin resistance may decrease deiodinase activity and reduce peripheral conversion of T4 to T3. Alternatively, poor glycemic control could act through the action of increased proinflammatory mediators.\textsuperscript{30} Taking this into account, the observed association between fT4 and corneal nerve fiber parameters may be a false association without a real causal link.

The main possible limitation of our study is a relatively small number of patients enrolled. We tried to select patients with all possibly relevant clinical data available, which of course limits the number of study subjects. Despite the small study group, we believe our data are of importance.

To our knowledge, the possible effect of total ID has not been tested in previous studies. However, total ID per kilogram may be an important factor influencing nerve fiber status in T1DM patients and needs be taken into account in future studies of diabetic neuropathy pathophysiology and its progression. Additionally, more attention needs to be paid to the other possible factors, as well as differences between the T1DM and type 2 DM patients, when elucidating the development of diabetic complications.

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**References**


