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Biochemical use of neurofilament light polypeptide and vitamin B_{12} in relation to diabetic polyneuropathy in Danish adolescents with type 1 diabetes: a cross-sectional study

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ABSTRACT

Introduction The aim of this study was to investigate serum Neurofilament Light polypeptide (NfL) as a biomarker for diabetic polyneuropathy (DPN) in adolescents with type 1 diabetes (T1D). Secondarily, to investigate vitamin B_{12} (B_{12}) deficiency as a cause for DPN in adolescents with T1D.

Research design and methods Cross-sectional study. Sixty Danish adolescents with T1D (age 15–18 years, diabetes duration >5 years) and 23 age-matched control subjects were included. Based on nerve conduction studies (NCS), intraepidermal nerve fibre density (IENFD) and neurological examination, patients were divided into three groups: (1) T1D without DPN, (2) T1D with subclinical DPN and (3) T1D with confirmed DPN. Blood levels of NfL, B_{12}, B_{12}-binding protein holotranscobalamin (HoloTC) and methylmalonic acid (MMA) were determined.

Results Twenty-four of the adolescents were without DPN, twenty-one had subclinical DPN and eight had confirmed DPN. NCS was not conducted in three participants and four patients did not have blood samples taken. There were no significant differences in NfL levels or any of the B_{12} parameters between any of the groups.

Conclusions NfL used in a cross-sectional manner was not found useful to distinguish between the adolescents with DPN and those without. Vitamin B_{12} deficiency did not contribute to neuropathy in Danish adolescents with T1D.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ The use of gold standard tests performed by the same healthcare professionals to assess the prevalence of DPN is a main strength.
⇒ The use of best evidence definitions for categorisation is a main strength.
⇒ The small sample size, as well as the small control group providing the cut-off for abnormality, is the major limitation.
⇒ No questionnaire used to standardise the assessment of possible symptoms of DPN among participants is a limitation.

INTRODUCTION

The global prevalence of type 1 diabetes (T1D)[1] in children and adolescents (0–20 years) is approximately 1.5 million. Diabetic polyneuropathy (DPN) is a common complication of diabetes, with a prevalence of 12–62% in adolescents with T1D.[2]

Bedside screening tests for detecting DPN in individuals with T1D include neurological examination and biothesiometry.[3] Nerve conduction studies (NCS) require equipment, time and trained health professionals, and are therefore not routinely performed. The screening programme for diabetic neuropathy might therefore benefit from being reinforced by a reliable and easily accessible biomarker for early detection of neuropathy, such as blood samples. However, the literature lacks evidence and research on possible biological markers for neuropathy in individuals with T1D, especially in adolescents.[4]

A possible biomarker for diabetic neuropathy could be Neurofilament Light polypeptide (NfL). NfL is an axonal cytoskeleton protein released into plasma and cerebrospinal fluid (CSF) when any nerve degeneration occurs.[5] It is released into the extracellular environment in proportional quantities to the nerve damage that transpires.[6] The correlation between high serum and CSF levels of NfL and neurodegenerative diseases in the central nervous system is well established.[7] However, further research on NfL as a possible biomarker for peripheral nerve damage is still needed.[8]

Furthermore, lower vitamin B_{12} (B_{12}) levels among patients with T1D are reported.[9 10]

B_{12} deficiency would theoretically diminish the methylation of the myelin sheaths and neurotransmitters.[11] This creates a need to investigate the association between B_{12} levels and neuropathy status in adolescents with T1D, to investigate B_{12} deficiency as a cause of the occurrence of DPN. Solely measuring blood levels of B_{12} itself has been considered insufficient to investigate B_{12} status[12]; hence, to get a more accurate B_{12} profile, blood levels of transcobalamin-bound B_{12}, called holotranscobalamin,[13 14] as well as methylmalonic acid (MMA)[15] can be included.

The primary aim of this study was to investigate NfL as a biomarker for DPN in adolescents with T1D. The secondary aim was to investigate B_{12} deficiency as a cause for DPN in adolescents with T1D.

METHODS

Study population

This study was based on data and blood samples collected as part of the T1DANES study[16] performed at Aarhus University Hospital, Denmark. In this cross-sectional study, 60 Danish adolescents with T1D (age 15–18 years, diabetes duration >5 years) and 23 Danish control subjects were included between September 2020 and December 2021. As described elsewhere,[16] exclusion criteria were adolescents who were taking medication or had diseases that could impact the central or peripheral nervous system. However, the presence of associated well-treated autoimmune disorders (such as thyroid disease and coeliac disease) or complications to diabetes (such as microalbuminuria) was allowed. Inclusion and exclusion procedures are reported in online supplemental appendix figure 1.

All procedures in the study protocol were approved by the local Ethics Committee (VEK j.nr. 1-10-72-211-19) and the Danish Data Protection Agency (j.nr. 1-16-02-42-21). Written informed consent was obtained from all participants or from their legal guardian if the participant was younger than 18 years.

Diagnostic tests for DPN

The clinical neurological examination was performed to assess the signs of DPN in length-dependent areas. As described in detail previously,[16] the tests included examination of muscle power in arms and legs, deep Achilles and patellar tendon reflexes, proprioception at the first toes, vibration sense (128 Hz tuning fork on the first interphalangeal joint), touch sensation (cotton wool, sharp wooden end of a broken cotton swab, monofilament 10 g on the first toes), pain sensation (using Neuropen with NeuroTip (Owen Mumford, Oxford, UK) and test for allodynia using brush on lower legs) and temperature sensation (Rolltemp II, Somedic, Sweden) at predetermined temperature levels of 25 and 38°C. All participants were orally asked if they experienced symptoms in the length-dependent area, which none of them stated. The diagnostic confirmatory test for large fibre neuropathy was NCS.[17] Both sensory and motor NCS were performed unilaterally on the right leg using transcutaneous stimulation and standardised neurophysiological procedures with electromyography equipment (Keypoint Medtronic, Skovlunde, Denmark). A sufficiently warm skin temperature was ensured using heat lamps. The measured attributes were conduction velocity, sensory and motor action potential amplitude, distal motor latency and minimum F-wave latency. In total, five nerves were tested: motor NCS of the peroneal and tibial nerves and sensory NCS of the superficial peroneal, sural and lateral dorsal cutaneous nerves. The diagnostic confirmatory test for small fibre neuropathy was skin biopsy from 10 cm above the right lateral malleolus, with calculation of intraepidermal nerve fibre density (IENFD).

Based on the NCS, IENFD and results from the neurological examination, the participants were categorised according to their neuropathy status using definitions convened by the Toronto expert panel.[18] They were categorised as not having DPN if they had normal NCS and IENFD. If they had an abnormal NCS (one or more abnormal attributes in two or more separate nerves)[17] and/or IENFD, but no symptoms or bilateral signs of neuropathy, they were categorised as having subclinical neuropathy.[19] If they had an abnormal NCS and/or IENFD as well as positive symptoms or bilateral signs of respectively large-fibre and small-fibre neuropathy at the neurological examination, they were categorised as having confirmed neuropathy.

Hence, the study population with diabetes was divided into three groups: (1) T1D without DPN (noDPN), (2) T1D with subclinical DPN (subDPN) and (3) T1D with confirmed DPN (conDPN) (online supplemental appendix, figure 1).

Measurement of biomarkers

The blood was collected in fasting participants in different test tubes (serum and lithium-heparin plasma), centrifuged and stored at −80°C in smaller tubes (500 µL).

All blood samples were analysed in the internationally accredited laboratory at Aarhus University Hospital, Aarhus, Denmark (DS/EN ISO/IEC 15189).

Serum NfL was measured by the NF-light assay using the ultrasensitive Simoa HD-1 platform (Quanterix, Lexington, MA). This is a digital immunoassay with dedicated hardware and software that uses singulated capture and reading of immunocomplexes on microbeads to quantify analyte concentrations. According to the manufacturer, the limit of detection and limit of quantification for NfL are 0.038 pg/mL and 0.174 pg/mL, respectively. The calibrator range is 0–500 pg/mL with linearity from 4 to 128 times dilution. On the level of 10–20 pg/mL, the intra-assay and inter-assay coefficients of variation were 4.3% and 6.4%, respectively.

NfL levels are confirmed to be stable through at least three freeze-thaw cycles. Serum samples were thawed on ice before being batch analysed.[20]

Plasma B_{12} was estimated by competitive immunoassay using the ADVIA Centaur instrument (Siemens Healthcare Diagnostics Products, Sudbury, UK). Serum HoloTC was estimated with the commercially available ELISA kit, Active-B_{12} (holotranscobalamin) EIA (Axis-Shield, Dundee, UK). Serum MMA was estimated by high-pressure liquid chromatography using liquid chromatography–tandem mass spectrometry, AB SCIEX Triple Quad 5500 System (AB SCIEX LLC, Framingham, MA).

Statistical analysis

Data were analysed using R (R Core Team (2022), Vienna, AT). The Shapiro-Wilk test was performed to determine the distribution of all variables in the groups. To test for differences in demographic variables between groups, the Welch two sample t-test was applied for parametric continuous data; while the Wilcoxon rank sum test was applied for non-parametric continuous data, and for categorical variables, Fisher's exact test was used. To evaluate the statistical correlation between biomarkers and neuropathy status between the three groups, the ANOVA test was used; and to further elaborate any statistical differences, Tukey's HSD post hoc test was applied. In the assessment of the statistical correlation between two parametric variables, Spearman's rank correlation was applied. P values <0.05 were considered significant. Descriptive data are presented as mean (SD) for parametric continuous variables, median (range) for non-parametric continuous variables, and number (%) for categorical variables (table 1). Missing data were handled by omission.

Based on NCS from the control subjects, the 5th percentile for conduction velocities and amplitudes, and the 95th percentile for distal motor latencies and F-wave latencies were determined. These percentiles were used as thresholds for abnormality in the evaluation of each attribute from NCS performed on participants with T1D. Based on IENFD from the healthy control subjects, an abnormal IENFD was defined as being below the 5th percentile of normal.

Patient and public involvement

Patients and/or the public were not involved in this study.

RESULTS

Demographic and clinical information of the participants with T1D are shown in table 1. Information about the control cohort is previously published.[16]

NCS was not possible to be carried out in three patients. Furthermore, four individuals with T1D did not get their blood samples taken due to needle phobia. Online supplemental appendix (figure 1) illustrates the selection process. Only one participant reported having consumed B_{12} supplements on the test day after having been directly asked. This participant needed B_{12} supplements due to having methylmalonic acidaemia. Hence, blood from this participant was included in the NfL analysis, but not in the B_{12} analysis.

Association between NfL and DPN

Serum levels of NfL in the groups are presented in table 2. No significant differences in NfL levels between any of the groups were found.

CLSI reference interval

Using the Clinical and Laboratory Standards Institute (CLSI) guideline C28-A39,[21] the NfL values of the present control cohort (n=23) were compared with the reference interval for individuals aged 18–20 years reported by Schjørring *et al*.[22] Only one subject fell outside the 97.5th percentile threshold of 7.4 ng/L for this age group, indicating that the NfL levels in the control cohort are representative for this adolescent subpopulation.

Association between vitamin B_{12} parameters and DPN

Levels of the B_{12} parameters in the groups are presented in table 3. There were no significant differences in any of the B_{12} parameters between any of the groups.

Three participants had probable B_{12} deficiency according to the diagnostic combination of cut-off for low vitamin B_{12} level and the upper reference limit for MMA for age 12–17 years of P-B_{12} ≤250 pmol/L + MMA >0.35 µmol/L.[23 24] All of them were in the noDPN group and had B_{12}+ MMA levels of, respectively, 205 pmol/L + 0.61 µmol/L, 212 pmol/L + 0.36 µmol/L and 239 pmol/L + 0.42 µmol/L.

DISCUSSION

In the present study, we found no difference in serum NfL levels between adolescents with T1D with and without DPN independent of the subtype: subclinical or confirmed DPN. Furthermore, we did not find any differences in blood levels of any of the B_{12} parameters between the groups.

There could be two main explanations for the unusability of serum NfL for detecting DPN in adolescents in our study: (1) slow or too little nerve degeneration, and (2) high inter-individual variation of NfL.

If the nerve degeneration occurs too slowly or in too small an amount, serum NfL levels will not be detectable because it requires excessive or quickly accelerated nerve damage to measure notable increases in NfL levels.[25] Longitudinal measurements of NfL as a predictor of chemotherapy-induced peripheral neuropathy, an often quickly developed type of neuropathy, have been well supported.[26–28] The prevalence of peripheral neuropathy in adolescents with T1D has been found to rise from 5% to 13% as the duration of diabetes progresses from 5 to 10 years to more than 10 years.[29] This implies a generally slow progression of neuropathy over several years.

Remarkably, it is known that NfL exhibits high interindividual variability, whereas the intraindividual variability is low.[25] This means that although we did not find NfL useful in a cross-sectional manner, it might still have potential as a biomarker for monitoring the severity of DPN over time. This is further supported by a recent follow-up study

Table 1 Participant demographics

Characteristic	noDPN, n=24*	noDPN vs subDPN subDPN, n=21*	P value†	noDPN vs conDPN conDPN, n=8*	P value†
Gender (female)	11 (45.8%)	10 (47.6%)	1.00	3 (37.5%)	1.00
Age (years)	17.1 (1.1)	16.5 (1.2)	0.09	16.8 (0.8)	0.38
Diabetes duration (years)	8.9 (5.6–16.6)	8.4 (5.0–14.2)	0.49	8.9 (5.3–17.4)	0.83
HbA1c (mmol/mol)	58 (10)	60 (8)	0.50	67 (16)	0.18
HbA1c mean 5 years (mmol/mol)	57 (9)	60 (10)	0.09	61 (12)	0.40
Time in range (%)	54 (13)	49 (17)	0.27	64 (16)	0.35
Time in hypoglycaemia (%)	5.7 (4.0)	5.5 (4.0)	0.90	10.0 (NA)	NA
Basal insulin (units)	26 (7)	29 (11)	0.69	30 (12)	0.48
Total daily insulin (units/kg/day)	0.83 (0.19)	0.80 (0.24)	0.19	0.90 (0.31)	0.59
BMI (kg/m^2)	23.4 (2.8)	22.6 (2.8)	0.35	23.0 (3.2)	0.75
BMI-SDS	0.6 (0.7)	0.5 (0.9)	0.63	0.5 (0.7)	0.78
Height (cm)	171 (8)	175 (9)	0.14	182 (8)	**0.00**
Hip circumference (cm)	98 (7)	97 (7)	0.51	102 (6)	0.17
Waist circumference (cm)	74 (53–90)	77 (67–97)	0.67	77 (73–100)	0.27
Tanner (stage)			0.76		0.39
4	8 (33.3%)	8 (38.1%)		1 (12.4%)	
5	16 (66.7%)	13 (61.9%)		7 (87.5%)	
SBP (mmHg)	114 (101–129)	112 (94–130)	0.59	115 (103–145)	0.91
DBP (mmHg)	68 (6)	67 (5)	0.52	72 (11)	0.33
HR (BPM)	75 (13)	78 (15)	0.35	70 (11)	0.34
Retinopathy (yes)	2 (8.3%)	0 (0.0%)	0.54	0 (0.0%)	1.00
Nephropathy (yes)	0 (0.0%)	1 (4.8%)	0.31	0 (0.0%)	1.00
Cholesterol (mmol/L)	4.12 (0.86)	4.31 (0.83)	0.48	4.20 (0.83)	0.82
LDL (mmol/L)	2.05 (1.09–4.00)	1.99 (0.50–3.70)	0.28	2.10 (1.90–4.10)	0.47
HDL (mmol/L)	1.53 (0.32)	1.60 (0.65)	0.12	1.40 (0.28)	0.28
Triglycerides (mmol/L)	0.90 (0.60–1.70)	0.70 (0.30–3.80)	0.41	1.00 (0.70–1.50)	0.33
Alcohol (units/week)			0.73		0.71
0	1 (4.2%)	3 (14.3%)		0 (0.0%)	
1–3	12 (50.0%)	9 (42.9%)		6 (75.0%)	
4–7	8 (33.3%)	5 (23.8%)		1 (12.5%)	
8–14	2 (8.3%)	2 (9.5%)		1 (12.5%)	
>15	1 (4.2%)	2 (9.5%)		0 (0.0%)	
Smoking (status)			0.67		0.66
Never	18 (75.0%)	19 (90.5%)		5 (62.5%)	
Previous	2 (8.3%)	0 (0.0%)		2 (25.0%)	
Smoke	3 (12.5%)	2 (9.5%)		1 (12.5%)	
NI	1 (4.2 %)	0 (0.0%)		0 (0.0%)	
Activity (hours/week)			0.97		0.52
0	2 (8.4%)	2 (9.5%)		1 (12.5%)	
1–3	6 (25.0%)	5 (23.8%)		0 (0.0%)	
4–7	8 (33.3%)	6 (28.6%)		4 (50.0%)	
>7	8 (33.3%)	8 (38.1%)		3 (37.5%)	

noDPN, Participants with T1D without polyneuropathy.
subDPN, subclinical polyneuropathy, Participants with T1D with abnormal nerve conduction study and/or intraepidermal nerve fibre density and normal neurological examination.
conDPN, confirmed polyneuropathy, Participants with T1D with abnormal nerve conduction study and/or intraepidermal nerve fibre density and bilateral sign of DPN in the neurological examination.
Definitions: HbA1c mean 5 years, mean of haemoglobin A1c levels from the past 5 years; Time in range, Per cent of time with blood glucose level between 3.9 and 10 mmol/L the past 14 days before the test day; Time in hypoglycaemia, Percent of time with blood glucose level <3.9 mmol/L the past 14 days before the test.
*Mean (SD) for parametric continuous data; median (range) for non-parametric continuous data; n (%) for categorical data.
†Welch two sample t-test; Wilcoxon rank sum test; Fisher's exact test.
BMI, body mass index; BMS-SDS, body mass index SD score; BPM, beats per minute; DBP, diastolic blood pressure; HbA1c, haemoglobin A1c; HDL, high-density lipoproteins; LDL, low-density lipoproteins; N, numbers; NA, not applicable due to insufficient amount of values; NI, not indicated meaning not stated.; SBP, systolic blood pressure.

Table 2 Serum concentration of NfL in adolescents with type 1 diabetes without neuropathy, with subclinical neuropathy, with confirmed neuropathy and control subjects

Biomarker	noDPN (n=24)	subDPN (n=21)	conDPN (n=8)	Control (n=23)	noDPN vs subDPN (p*)	noDPN vs conDPN (p*)	subDPN vs conDPN (p*)
NfL (pg/mL)	5.9 (3.1–14.3)	5.4 (2.1–13.2)	4.8 (3.6–7.0)	5.2 (3.1–8.0)	0.85	0.61	0.93

Median (range)
noDPN, Participants with T1D without polyneuropathy.
subDPN, subclinical polyneuropathy, Participants with T1D with abnormal nerve conduction study and/or intraepidermal nerve fibre density and normal neurological examination.
conDPN, confirmed polyneuropathy, Participants with T1D with abnormal nerve conduction study and/or intraepidermal nerve fibre density and bilateral sign of DPN in the neurological examination.
Control, Control subjects.
*Tukey's HSD post hoc test.
NfL, Neurofilament light polypeptide.

in patients with type 2 diabetes that reported significantly greater longitudinal increase in serum NfL in patients with DPN compared with those without,[30] suggesting the potential utility of serum NfL trajectories as a biomarker for DPN.

In data on NfL obtained from presumed healthy adults, it is shown that age is the most important factor influencing serum NfL concentration, and at an age below 60 years, BMI and blood volume further influence serum levels of NfL.[31] In our study, BMI did not vary between the subgroups with diabetes. Blood volume depends on many factors and is highly variable, but it can be estimated based on height, weight and gender. Remarkably, the adolescents with confirmed DPN were taller than those without DPN, which is of importance both due to its potential impact on blood volume and because nerve conduction velocity is known to vary inversely with height.[32]

In adults, a higher serum NfL level has been found to be associated with slower motor and sensory nerve conduction velocities, which emphasises its potential as a biomarker of neuropathy.[33] Furthermore, NfL has been found to be higher in adults with diabetes, and is not only promising for monitoring peripheral sensorimotor neuropathy,[33] but also cognitive function[34] and detection of individuals with impaired awareness of hypoglycaemia.[35]

Therefore, in the future, it would be of interest to follow-up on this sample population to investigate the possible association between continuous values of both NfL and neurological status.

Additionally, further longitudinal studies of NfL in larger paediatric populations are needed for the investigation of the usefulness of NfL for different types of neuropathies. Supplementary research in this field is especially relevant in order to detect neuropathy early and prevent further progression, which is shown possible with optimised glycaemic control.[36]

Our findings of similar levels of B_{12}, HoloTC and MMA in our study groups indicate that there is no difference in intracellular B_{12} status between adolescents with and without DPN. Although being a secondary outcome, it suggests that B_{12} deficiency does not frequently contribute to the development of DPN in Danish adolescents with T1D. Hence, if there is no concern about unhealthy or restricted diet or high alcohol consumption, it could be considered to omit this measurement from a health-economic perspective.

The main strengths of this study are the use of confirmatory tests as well as definitions and criteria based on best available evidence in the diagnosis of DPN. The tests were performed by the same healthcare professionals using the same equipment throughout the study, and all

Table 3 Concentration of B_{12} parameters in participants with T1D without neuropathy, with subclinical neuropathy, with clinical neuropathy and control subjects

Biomarker	noDPN (n=23)	subDPN (n=21)	conDPN (n=8)	Control (n=23)	noDPN vs subDPN (p*)	noDPN vs conDPN (p*)	subDPN vs conDPN (p*)
B_{12} (pmol/L)	359 (205–567)	386 (201–973)	459 (212–1046)	310 (196–446)	0.98	0.56	0.81
HoloTC (pmol/L)	94 (24–226)	112 (50–444)	111 (43–225)	77 (26–190)	0.89	0.97	1.00
MMA (µmol/L)	0.16 (0.07–0.61)	0.15 (0.06–0.33)	0.11 (0.07–0.21)	0.15 (0.12–0.19)	0.95	0.31	0.65

Median (range)
noDPN, Participants with T1D without polyneuropathy.
subDPN, subclinical polyneuropathy, participants with T1D with abnormal nerve conduction study and/or intraepidermal nerve fibre density and normal neurological examination.
conDPN, confirmed polyneuropathy, participants with T1D with abnormal nerve conduction study and/or intraepidermal nerve fibre density and bilateral sign of DPN in the neurological examination.
Control, control subjects.
*Tukey's HSD post hoc test.
B_{12}, vitamin B_{12}; HoloTC, holotranscobalamin; MMA, methylmalonic acid.

blood samples were analysed in the same internationally accredited laboratory.

The main limitation of this study is the small sample size. Additionally, the small control group provided the cut-off for abnormality in NCS and IENFD, which could contribute to inaccurate categorisation. However, the prevalence of DPN in our study was found to be comparable with previously published prevalences.[2] Moreover, the cross-sectional design of this study prevents us from investigating the association between DPN and NfL trajectories. We did not use a questionnaire to clarify whether the participants had symptoms of polyneuropathy. A questionnaire could have been advantageously used. Furthermore, the results of the neurological examination can be affected by some degree of subjectivity, that is, when assessing the reflexes, adding some diagnostic uncertainty to the examination.

In conclusion, the present study found similar blood levels of NfL and B_{12} parameters in adolescents with T1D with and without DPN. This suggests that NfL solely measured in a cross-sectional manner does not seem to be a useful biomarker of the early stage of DPN in adolescents with T1D. Future longitudinal studies are needed to investigate NfL as a possible biomarker for monitoring diabetic neuropathy. Furthermore, the results indicate that vitamin B_{12} deficiency does not contribute to neuropathy in Danish adolescents with T1D.

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