CLINICAL STUDY

Intraepidermal nerve fiber density reduction as a marker of preclinical asymptomatic small-fiber sensory neuropathy in hypothyroid patients

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Abstract

Aim: To evaluate, by using skin biopsy technique, the intraepidermal nerve fiber (IENF) density in a group of untreated patients with hypothyroidism, either overt (OH) or subclinical (SH), who did not complain of neurologic symptoms.

Methods: We evaluated 18 neurologically asymptomatic patients newly diagnosed with OH or SH. Fifteen healthy, age-matched, controls were also studied. A nerve conduction study was performed. Skin biopsy was carried out from the skin of upper thigh and distal leg. Nerve fiber density was measured using an immunofluorescence technique. The density of innervation was calculated by counting only fibers crossing the basement membrane.

Results: Electroneurographic parameters were similar in patients and controls. When compared with healthy controls, patients with OH or SH showed a significantly lower IENF density. As assessed by the proximal/distal fiber density ratio, the hypothyroid neuropathy was length dependent. When individually considered, an abnormally reduced IENF was observed in 60% of patients with OH at the distal leg and in 20% at the proximal site. In patients with SH, an abnormal IENF density was found at the distal leg in 25% of cases and at the proximal thigh in 12.5% of cases.

Conclusions: Our study provides the first direct demonstration of reduced IENF density in patients with OH or SH. In all patients, the IENF density reduction was length dependent. These findings suggest that a considerable number of untreated hypothyroid patients may have preclinical asymptomatic small-fiber sensory neuropathy.

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Introduction

Neurologic complications, including polyneuropathy, are well-known findings in overt hypothyroidism (OH), with a prevalence ranging from 42 to 72% (1, 2). In neurologic series, the prevalence of hypothyroidism as a cause of polyneuropathy is reported to be around 2–4% (3, 4). Alterations in peripheral nerves are well documented in myxedema (2), but few data are available in subclinical hypothyroidism (SH) (5).

In the past, patients with long-standing, untreated hypothyroidism commonly complained of symptoms suggesting small-fiber sensory neuropathy (SFSN), such as paresthesias and burning extremity pain (6). Hypothyroid patients also experienced a sensory–motor polyneuropathy with a distal–proximal progression, first involving lower limbs and then the upper ones (7). It is generally accepted that the severity of the peripheral nervous system involvement is

significantly related to the duration of hypothyroidism rather than to the degree of thyroid failure (1, 7). Thus, long-standing biochemical abnormalities might lead to neurologic alterations even in SH. Recently, painful neuropathy was reported in a patient with SH who recovered after hormone replacement therapy (8).

Skin biopsy is a safe and validated technique to assess intraepidermal nerve fiber (IENF) density. IENF density is a reproducible marker of SFSN, showing a sensitivity of more than 90% in the diagnosis of this type of sensory neuropathy (9, 10).

The aim of the current study was to evaluate the IENF density in a group of untreated patients with hypothyroidism, either OH or SH, who did not complain of neurologic symptoms. Our data indicate that a reduction of IENF density, suggestive of SFSN, is a relatively common finding in OH, but it is also detected in subclinical thyroid failure.

Materials and methods

The study enrolled 18 patients (mean age 51.2 ± 13.1 years) newly diagnosed with hypothyroidism. Ten patients presented with OH, defined as raised serum levels of TSH associated with free thyroxine (FT₄) values below the normal range. Eight patients were classified as having SH because their serum levels of TSH were high (> 4 mIU/l), while FT₄ concentrations were in the normal range.

All patients were neurologically asymptomatic, and did not display motor symptoms, alterations of pin prick, light touch, vibration, and joint position sensation. Tendon reflexes were also normal. Diabetes mellitus (according to the American Diabetes Association Diagnostic Criteria (11), neurologic disorders, alcoholism and/or the use of neurotoxic/neuroactive drugs, low levels of vitamin B12 and folic acid (mean values $\pm$ s.d. 493 ± 180 pg/ml, normal range 191–663, and 6.14 ± 3.2 ng/ml, normal range 3.8–16 respectively), were mandatory exclusion criteria.

In the majority of patients (14/18), hypothyroidism was caused by autoimmune thyroiditis. Three patients suffered from post-ablative hypothyroidism resulting from radioiodine therapy or total thyroidectomy. In two patients, radioiodine therapy for Graves' disease had been performed 60 and 12 months before inclusion in the study. One patient was diagnosed with central hypothyroidism (Table 1). The duration of hypothyroidism, calculated as the time elapsed from the biochemical diagnosis or from the surgical or radiometabolic procedure not followed by hormonal substitution, ranged from 3 to 60 months (median 12 months). Fifteen age-matched (53.3 ± 17.3 years) controls with no neurologic/systemic diseases and not taking nervous system interfering drugs were also studied.

A nerve conduction study was performed in hypothyroid patients and controls to evaluate the presence of subclinical large fiber polyneuropathy. Both patients and controls gave their informed consent to participate in the study, which was performed in accordance with the guidelines of the Declaration of Helsinki and with the Institution Ethics Committee on human experimentation.

Serum assay

Serum concentrations of FT₄ (normal range 8–19 pg/ml) and TSH (third generation TSH assay; normal range 0.4–4 mIU/l) were measured using immunochemiluminiscent assays by an automated analyser (Immulite 2000, DPC Cirrus, Los Angeles, CA, USA) employing commercial kits (Diagnostic Products Corporation, Los Angeles, CA, USA). Serum concentrations of anti-thyroglobulin antibody (Tg-Ab; normal range < 60 U/ml) and anti-thyroid peroxidase antibody (TPO-Ab; normal range < 60 U/ml) were measured using immunochemiluminiscent assays employing commercial kits (Brahams, Hennigsdorf, Germany).

Nerve conduction study

Electrophysiological examination was performed bilaterally on lower limbs with constant monitoring of skin and room temperature. Skin temperature was maintained between 32 and 34 °C, and room temperature was maintained between 22 and 24 °C. Motor nerve conduction velocities of deep peroneal nerves were measured with standardized surface stimulating and recording techniques. Sensory nerve conduction velocities in superficial peroneal nerves were measured antidromically. Motor and sensory action potential amplitude (MAP and SAP) and distal latency (DL)

Table 1 Clinical and laboratory features of patients with overt or subclinical hypothyroid (overt hypothyroidism (OH) and subclinical hypothyroidism (SH) respectively).

	Age	Sex	Cause of hypothyroidism	TSH (mIU/l) (0.4–4)	FT ₄ (ng/dl) (0.8–1.9)	Tg-Ab (U/ml) (< 60)	TPO-Ab (U/ml) (< 60)
OH	50	F	Post-thyroidectomy for multinodular goiter	40	0.3	10	10
OH	54	M	Hashimoto's thyroiditis	164	0.3	15	10 740
OH	74	M	Central hypothyroidism	0.26	0.49	12.4	10
OH	58	F	Hashimoto's thyroiditis	169	0.3	10	4823
OH	51	F	Hashimoto's thyroiditis	152	0.6	64.8	10
OH	32	F	Post-radioiodine therapy for Graves' disease	268	0.3	123.2	774.1
OH	63	F	Post-radioiodine therapy for Graves' disease	40	0.33	12.2	10
OH	79	F	Hashimoto's thyroiditis	21.7	0.63	43.8	3167
OH	30	F	Hashimoto's thyroiditis	289	0.3	207	60
OH	56	M	Hashimoto's thyroiditis	112	0.35	10	588
SH	46	F	Hashimoto's thyroiditis	8.44	0.83	74.4	77
SH	45	F	Hashimoto's thyroiditis	23	1	176.9	11 470
SH	45	F	Hashimoto's thyroiditis	16.8	0.95	176.4	1004
SH	31	F	Hashimoto's thyroiditis	9.65	1.02	70	42
SH	41	F	Hashimoto's thyroiditis	6.1	1.15	284	11 870
SH	42	M	Hashimoto's thyroiditis	9.6	1.27	10	5748
SH	52	M	Hashimoto's thyroiditis	45.2	1.01	9348	2188
SH	56	F	Hashimoto's thyroiditis	13.4	0.81	152	212

of MAP were measured in the same nerves. All electrophysiological parameters (conduction velocity (CV), distal motor latency, and potential amplitude) were compared with reference values obtained in age-matched controls. An electrophysiological diagnosis of polyneuropathy was made when CV and/or DL and/or potential amplitude were abnormal bilaterally.

Skin biopsy

Using a 3-mm punch tool, two skin biopsies were carried out from the skin of the upper thigh (20 cm below the iliac spine) and distal leg (10 cm proximal to lateral malleolus). We will refer to these procedures as proximal and distal skin biopsies. Biopsy specimens were immediately fixed in cold Zamboni's solution and cryoprotected in PBS. Specimens were then cut into 50- μ m sections using a freezing sliding microtome. Nerve fibers were identified by indirect immunofluorescence. The primary antibody was directed against PGP 9.5 (Biogenesis, Poole, UK; 1:1000), a non-specific neuronal marker, and then detected using a secondary antibody conjugated to the cyanine dye fluorophor Cy3 (Jackson Immuno Research, West Grove, PA, USA). In order to identify the basement membrane, another primary antibody was directed against type IV collagen (Chemicon International, Temecula, CA, USA) and detected using a secondary antibody conjugated to the cyanine dye fluorophor Cy2 (Jackson Immuno Research). Sections were finally adhered to coverslips with agar and dehydrated via an alcohol gradient. At the end of the processing, they were cleared with methyl salicylate and mounted in Dibutyl Phtalate Xylene (DDK, Milan, Italy).

Using a fluorescence microscope system (magnification 40 $\times$, Axioskop 40 FL, Zeiss, Göttingen, Germany), the nerve fiber density was calculated by counting only fibers crossing the basement membrane. The density of IENF was expressed as the number of IENF per millimeter of epidermis (IENF/mm) (12).

Statistical analysis

Data were expressed as mean $\pm$ s.d. We also calculated the proximal/distal (P/D) fiber density ratio in controls and hypothyroid patients. Between-group comparisons

were performed by Student's *t*-test for unpaired data for both proximal and distal skin biopsies. Furthermore, due to skewed distribution of the numbers of small fiber detected by immunohistochemistry, nonparametric Mann–Whitney *U* test was also used, providing the same conclusions. To indicate how many s.d.s the number of small fiber were above or below the mean, we calculated the Z scores for each subject. A significant Z score was set at ± 2 .

Results

Using the conventional electroneurographic approach, the mean values of the parameters evaluated were not different in patients with hypothyroidism, both SH and OH, and in controls (Table 2). When individually considered, only two patients with OH showed electrophysiological findings indicative of polyneuropathy. In both cases, a mild reduction of motor and sensory CV (30–35 m/s) and a reduction of SAP amplitude were observed, suggesting a sensory–motor neuropathy.

The results of skin biopsy are summarized in Fig. 1. When compared with healthy controls, patients with OH or SH showed a significantly lower IENF density, both at the proximal (P) thigh and at the distal (D) leg. A representative visualization of nerve fibers by indirect immunofluorescence on skin biopsy specimens from overt (A), subclinical (B) hypothyroid patients, and healthy control (C) is shown in Fig. 2. As assessed by the P/D fiber density ratio, the neuropathy observed in our hypothyroid patients was length dependent.

When individually considered, eight patients showed an abnormally reduced IENF (Z scores < 2). In particular, six out of ten patients (60%) with OH and two out of eight (25%) patients with SH had a reduced IENF density. Among patients with OH, six (60%) showed a reduction in the IENF at the distal leg and two (20%) also at the proximal thigh. In patients with SH, an abnormal IENF density was found at the distal leg in two cases (25%) and also at the proximal thigh in one case (12.5%). Z scores of proximal and distal IENF/mm in both groups of patients are reported in Fig. 3.

When individually considered, the P/D fiber density ratio indicated a length-dependent neuropathy (Z scores > 2) in four (40%) patients with OH and in two

Table 2 Electroneurographic data of hypothyroid patients and control subjects (mean $\pm$ s.d.).

	Controls	Subclinical hypothyroidism	Overt hypothyroidism
Conduction velocity of left external sciatic popliteal nerve (m/s)	46.61 $\pm$ 2.72	47.51 $\pm$ 4.62	45.85 $\pm$ 4.73
Conduction velocity of right external sciatic popliteal nerve (m/s)	46.46 $\pm$ 2.21	47.09 $\pm$ 3.02	46.39 $\pm$ 5.39
Conduction velocity of left superior peroneal nerve (m/s)	39.13 $\pm$ 3.14	39.28 $\pm$ 4.98	36.65 $\pm$ 3.98
Conduction velocity of right superior peroneal nerve (m/s)	38.84 $\pm$ 2.55	38.71 $\pm$ 4.11	37.05 $\pm$ 5.34
Amplitude of left external sciatic popliteal nerve (mV)	13.72 $\pm$ 13.6	9.41 $\pm$ 4.34	10.44 $\pm$ 3.57
Amplitude of right external sciatic popliteal nerve (mV)	8.31 $\pm$ 1.98	9.53 $\pm$ 3.97	11.29 $\pm$ 4.16
Amplitude left superior peroneal nerve (mV)	15.24 $\pm$ 8.27	19.19 $\pm$ 10.4	11.84 $\pm$ 7.51
Amplitude right superior peroneal nerve (mV)	14.35 $\pm$ 8.43	17.89 $\pm$ 9.61	13.28 $\pm$ 8.72

Figure 1 Intraepidermal nerve fiber density (means ± S.D.; * versus Controls).

(25%) patients with SH, confirming that the neuropathy observed in our hypothyroid patients was length dependent.

Both patients with electroneurographic findings suggesting large fibers neuropathy had also reduced IENF density (Fig. 3).

No significant relationship between IENF density and the thyroid hormonal profile (serum FT₄ and serum TSH) or duration of hypothyroidism was found.

Discussion

Hypothyroidism, particularly when subclinical, is the most common endocrinological disorder, with a prevalence ranging from 4 to 10% of the adult population (13, 14). Hypothyroidism may induce a wide range of neurologic abnormalities. In the past, neurologic complaints were described in up to 80% of patients with hypothyroidism (15). Most of these patients, having a severe long-standing hypothyroidism, complained of major neurologic symptoms, such as burning and lancinating extremity pain, usually related to a small-fiber damage (6). These major neurologic

complaints are nowadays rarely reported, probably due to earlier diagnosis and adequate correction of hypothyroidism with levothyroxine (L-T₄).

In neurologically asymptomatic patients with hypothyroidism, abnormalities in nerve conduction studies were found in one (16), but not in another study (7). Recently, a small-fiber dysfunction, as assessed by a screening test such as the quantitative sensory testing, was suspected in some hypothyroid patients, who, in spite of treatment with L-T₄, complained of painful of extremities (17). Skin biopsy with IENF fiber density assessment is more sensitive than clinical examination and quantitative sensory testing in detecting abnormalities in small-nerve fibers, although a consensus reference standard for small-fiber neuropathy is still lacking (18). A reduced IENF density, as assessed by skin biopsy, was recently reported in hypothyroid patients treated with L-T₄, who were at the time of the neurologic evaluation euthyroid, subclinically hypothyroid or even thyrotoxic (19). Thus, the groups of patients included in this study were clinically and biochemically different, and could not be considered as one category (20).

In our series of untreated hypothyroid patients, an asymptomatic polyneuropathy was demonstrated by conventional neurophysiological studies in only two cases (11.1%). On the other hand, using the skin biopsy technique, a significant reduction of IENF density was observed in patients with both OH and SH. The prevalence of individually evaluated abnormal IENF density ranged from 60% in OH to 25% in subclinical thyroid failure. In all affected patients, the IENF density reduction was length dependent. These findings suggest that in hypothyroid patients with no signs or symptoms of nerve damage, neurologic abnormalities can be

Figure 2 Representative visualization of nerve fibers by indirect immunofluorescence on skin biopsy specimens from patients with overt (A and B) and subclinical (C and D) hypothyroidism and in normal subject (E and F). A, C, and E are samples from distal leg, B, D, and F are samples from thigh. Space bar: 50 μm.

Figure 3 Intraepidermal nerve fiber (IENF) density. A Z score <2 indicates a significant reduction in IENF density when patients were considered individually. The IENF results of the two patients with sensory-motor polyneuropathy are indicated in black type, as assessed by conventional neurophysiological study.

detected using the skin biopsy technique. Moreover, preliminary data obtained in a subgroup of our patients indicate that after 1 year on L-T₄ treatment, the nerve fiber density does improve, especially at distal site, in both OH and SH (data not shown). The lack of correlation between IENF density and the duration of the hypothyroid state may be due to the small number of studied subjects and to the difficulty in determining the duration of hypothyroidism. The nonsignificant relationship between IENF density and the severity of thyroid failure is in agreement with previous data in the literature showing no direct relation between peripheral and central nervous system alterations and FT₄ or TSH serum concentrations (21).

The relationship between IENF density and pain is still debated. However, there is evidence suggesting that the degree of IENF density loss is related to the severity of the neuropathy (22). On the other hand, in sensory neuropathy, an increase in IENF density is associated with a reduction in neuropathic pain (23). Thus, based on the present results, we can assume that even SH can be considered a potential cause of symptomatic peripheral neuropathy.

The pathophysiological mechanisms leading to SFSN in hypothyroid patients are still unclear. Because in the majority of hypothyroid patients, the cause of thyroid failure was autoimmune thyroiditis, the possible involvement of immune mechanisms in the development of SFSN may be speculatively taken into account. Such an immune neuropathy has been described in patients with systemic lupus erythematosus, Sjögren syndrome, and rheumatoid arthritis (24).

The question of whether SH should always be treated is a matter of discussion (25, 26). Several panels of experts examined this question and reached different conclusions, recommending either in favor or against L-T₄ treatment (27). Our findings, suggesting the presence of a preclinical SFSN not only in OH but also in SH, although limited to a small number of cases, might represent a further argument favoring the treatment with L-T₄ of patients with subclinical thyroid failure.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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