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RESEARCH ARTICLE

Distinctive low epidermal nerve fiber density in schwannomatosis patients provides a major parameter for diagnosis and differential diagnosis

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Keywords
IEND, neurofibromatosis type 2, schwannomatosis, skin biopsy, small fiber neuropathy.

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Abstract

Schwannomatosis and neurofibromatosis type 2 (NF2) are two distinct neuro-genetic tumor predisposition disorders, which, however, share some clinical and genetic features. While germline mutations in the *NF2* gene are only found in NF2, a majority of schwannomatosis patients have germline mutations in the *SMARCB1* or *LZTR1* genes. The overlapping clinical phenotypes pose a serious challenge in differential diagnosis and in risk stratification of these two entities which is further complicated by frequent mosaicism in both disorders. Chronic neuropathic pain which is a typical consequence of small fiber neuropathy, is characteristic for schwannomatosis. By contrast, NF2 patients do not have chronic pain but may have moderate to severe sensory deficits and paresis which are not characteristic for schwannomatosis. In the present study, we determined intraepidermal nerve fiber density (IEND) in skin biopsies of 34 clinically ascertained schwannomatosis and 25 NF2 patients. In the NF2 group, 11/25 (44%) presented with IEND below the age- and gender-matched bottom 5% normative reference IEND. In contrast, nearly all (33/34 = 97%) schwannomatosis patients showed IEND below or on the bottom 5% normative reference. The reduction of IEND in schwannomatosis patients was age-independent. Paired *t*-test revealed no difference between the NF2-IEND and the corresponding bottom 5% normative reference ($P = 0.98$). By contrast, IEND in the schwannomatosis patients were highly significantly lower than the corresponding 5% normative reference IEND ($P < 0.0001$). In addition, the difference between the IEND of our patients and the 5% lowest normative reference IEND was highly significantly larger in schwannomatosis patients than in NF2 patients ($P < 0.0001$). IEND of our patients did not correlate with neither the presence nor types of germline mutations in neither the NF2 nor the LZTR1 gene. In conclusion, schwannomatosis patients have marked low IEND which provides a major parameter for diagnosis and differential diagnosis.

INTRODUCTION

Schwannomatosis and neurofibromatosis type 2 (NF2) are two different tumor suppressor gene syndromes, which, however, share some clinical and genetic features (7). Clinically, both syndromes are characterized by multiple schwannomas. However, schwannomas of the peripheral nerves are frequent in schwannomatosis, while non-vestibular cranial schwannomas are more frequent in NF2. Unilateral vestibular schwannomas are also present in schwannomatosis patients, although less frequent than in NF2 patients (4, 12), adding to the clinical overlap. Though bilateral vestibular schwannomas are considered the hallmark of NF2, bilateral vestibular schwannomas are not sufficient for an a priori diagnosis of NF2, especially in the elderly, as bilateral vestibular schwannomas can rarely occur as two random events also in non-NF2 patients including schwannomatosis patients (15). Finally, both genetic syndromes can manifest spinal tumors and peripheral nerve schwannomas.

Somatic mosaicism in NF2 is frequent, and is found at least in 33% of *de novo* NF2 cases with bilateral vestibular schwannomas and in up to 60% of *de novo* NF2 patients with unilateral vestibular schwannomas (1, 8, 13). Mosaic NF2 patients may even lack vestibular schwannomas while still develop multiple schwannomas of the peripheral nerves.

Genetically, NF2 patients exhibit germline mutations in the *NF2* gene while a majority of schwannomatosis patients have germline mutations in the *SMARCB1* or *LZTR1* genes (6, 7), all located on chromosome 22q. However, the two disorders share common somatic alterations in associated

tumors which is the biallelic inactivation of the *NF2* gene. Though there are differences in pattern and profile of genetic alterations in the tumors, and consequently, mutation analysis is essential for and contributes greatly to the differential diagnosis; interpretation of the data is often tricky and demands extensive experience and thorough understanding of the two disorders. In addition, a considerable portion of patients do not have detectable germline mutations in *NF2*, *SMARCB1* nor *LZTR1* and tumors are often not available for genetic analysis (7). However, the definite differentiation between NF2 and schwannomatosis is crucial for risk stratification, follow-up and treatment strategy.

Intraepidermal nerve fiber density (IEND) analysis is a widely used clinical tool to assess quality and quantity of small fiber peripheral neuropathy (9, 10). Neuropathy is within the clinical spectra of both NF2 and schwannomatosis (7, 12). However, clinical expression of neuropathy differs in these two disorders. Schwannomatosis patients frequently suffer from chronic neuropathic pain which is a typical clinical symptom of small fiber neuropathy. By contrast, NF2 patients exhibit a mild to moderate sensory motor axonal neuropathy, which may lead to sensory deficits and weakness rather than pain. Indeed, we frequently observe small fiber neuropathy predominantly of unmyelinated C-fibers, faster laser-evoked potentials latencies and decreased IEND in schwannomatosis patients. These observations raised the hypothesis that distinctive low IEND is a specific feature of schwannomatosis which may explain the disease-specific small fiber neuropathy and provide a valuable parameter for diagnosis for this disorder and further for differential diagnosis from NF2. In this study, we systematically evaluated IEND of 25 NF2 and 34 schwannomatosis patients by comparing them to the normative reference and between the two disorders.

PATIENTS AND METHODS

A total of 25 NF2 and 34 schwannomatosis patients were included in this study. The study was approved by the ethical board of the Medical Association Hamburg (PV4421), Germany, and performed in accordance with the declaration of Helsinki of 1964 and its later amendments. Written informed consent was obtained from all study participants. Inclusion criteria were fulfillment of the clinical diagnostic criteria for the respective disorders and absence of any other disease with potential impact on central or peripheral nervous system function such as chronic infections, autoimmune diseases, vitamin deficiencies, malignancies, diabetes or alcoholism. Detailed neurological examination was performed by a physician (VFM) with 30 years of experience in diagnosis and treatment of neurofibromatosis and schwannomatosis. The clinical diagnosis was based on the revised consensus criteria for schwannomatosis (11, 14) and the modified National Institutes of Health criteria for definite NF2 (5) respectively. All NF2 patients underwent high-resolution contrast-enhanced brain MRI and had bilateral vestibular schwannomas which is the hallmark of NF2. One NF2 patient from this cohort is known to be genetically mosaic. All patients underwent genetic analysis for the *NF2, SMARCB1* and *LZTR1* genes in blood and—if available—in tumor tissue as previously reported (8).

All 59 patients received skin biopsy for IEND analysis for diagnostic purpose regarding pain or neuropathic conditions. An 8mm punch biopsy from the skin of the calf was taken 10 cm above the lateral malleolus according to the standardized protocol for diagnosis of small fiber neuropathies (10). Fourteen of the schwannomatosis patients have already undergone skin biopsy for another study aiming at clinical pain classification (unpublished). After fixation with a Zamboni's fixative, 50 micron-thick sections were cut and subjected to immune-staining with antibody against PGP9.5 (9). The intraepidermal nerve fibers were counted under a microscope by two experienced neuropathologists (JM, MG) to obtain the density in fibers/mm.

The raw IEND data were plotted against age, separately for male and female patients. The resulting graphs were superposed onto the normative reference IEND graphic (9). In addition, the 5% low normative reference IEND in each age group from Lauria *et al* were plotted against middle age of each group. Using a polynomial curve fitting of order 2, an equation for each age obtained as

$$(5\%\ \text{lownormative}) = 0.0007\text{age}^2 - 0.2\text{age} + 13 \text{ for female and} \quad (1)$$

$$(5\%\ \text{lownormative}) = 0.0004\text{age}^2 - 0.12\text{age} + 8.9 \text{ for male.} \quad (2)$$

Subsequently, percentage difference from the 5% low normative reference IEND was calculated for each patient as following:

$$\text{Difference} = 100 \times \left[\text{IEND} - (5\%\ \text{low normative reference})\right] / (5\%\ \text{low normative reference})$$

Whereas the 5% low normative reference is calculated using the age of the patient as in the above Equations (1) and (2).

Unpaired and two-tailed *t*-test was used to compare the IEND in NF2 patients and in schwannomatosis patients with age- and gender-matched 5% low normative reference IEND. Significant level was set at 0.05.

RESULTS

Intraepidermal nerve density (IEND)

IEND was generally low in both NF2 and schwannomatosis patients compared to the normative reference IEND (Figure 1). All female NF2 patients had IEND below the 5% lower normative reference value (Figure 2). Male NF2 patients had somehow better values with half above the 5% lower reference though still below the 50% reference. Taken together, all NF2 patients had IEND below the 50% normative reference IEND while approximately half (12/25) had IEND below the 5% lowest normative reference.

Nearly all schwannomatosis patients (33/34 = 97%) had IEND below or on the 5% normative reference. Among them, 31 (91%) had IEND below and only two (6%) had IEND slightly above the 5% normative reference. Reduction of IEND in schwannomatosis patients is rather age-independent.

Only one single schwannomatosis patient had IEND above the 50% normative reference. However, no special clinical feature was found in this patient nor germline mutations in any of the three genes *NF2*, *SMARCB1* nor *LZTR1*.

IEND itself did not differ significantly between male and female patients, for both NF2 and schwannomatosis ($P > 0.4$). However, as the normative reference IEND is higher in females, the difference between the IEND of our patient and the 5% lower normative reference IEND was larger in female patients than in male patients, both for NF2 and schwannomatosis with the significances of unpaired *t*-test being 0.01 and 0.03, respectively.

Paired *t*-test revealed that the IEND in the NF2 group did not differ from the bottom 5% normative reference ($P = 0.98$). By contrast, IEND in the schwannomatosis patients was highly significantly lower than the age- and gender-matched 5% lower normative reference ($P < 0.0001$).

To interpret the IEND more precisely by taking age and gender in account, we next constructed an equation for calculating the gender-specific 5% lowest normative reference values for each age based on the dataset of the world-wide normative reference (9). We took the middle age of each age group (Table 1), and plotted them against the 5% lower IEND normative reference values. Using polynomial curve fitting, satisfactory match was reached. The curves themselves also matched the given curves of 5% lower reference IEND nearly perfectly (9). The resulting equation was used to

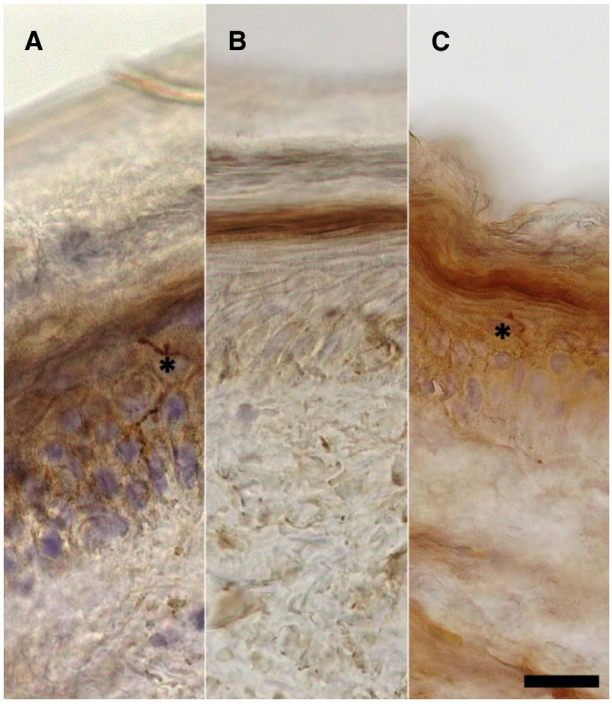

Figure 1. *Intraepidermal nerve fibers visualized by immunohistochemical staining with PGP9.5 antibodies.* **A.** A 51-year-old female healthy control with small nerve fiber density of 7.1/mm. **B.** A 48-year-old female schwannomatosis patient with severely reduced small nerve fiber density of 1.0/mm. **C.** A 42-year-old female NF2 patient with reduced small nerve fiber density of 5.0/mm. Asterisks indicate nerve fibers. Scale bar = 20 µm.

Table 1. Normative reference data from Lauria *et al* (9).

		5% lower normative reference IEND	
Age group	Age†	Female	Male
20–29	25	8.4	6.1
30–39	35	7.1	5.2
40–49	45	5.7	4.4
50–59	55	4.3	3.5
60–69	65	3.2	2.8
70–79	75	2.2	2.1
≥80	85	1.6	1.7

†Set in this study to obtain the curves and curve fitting equations.

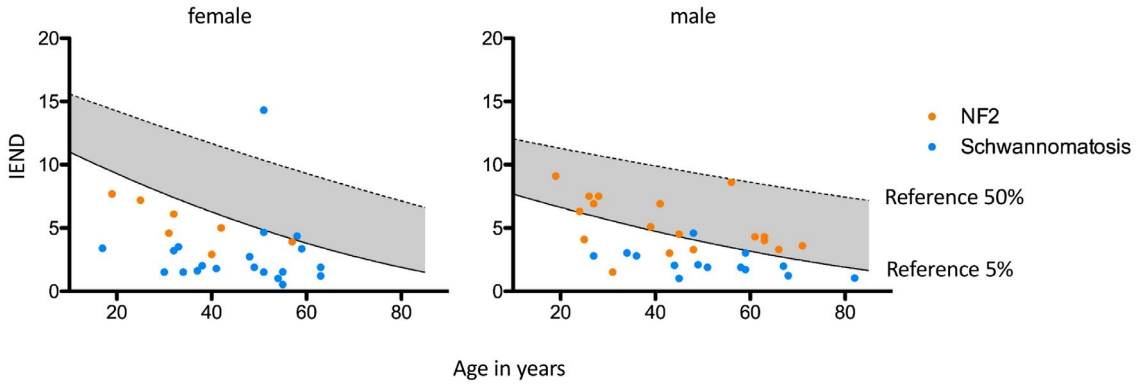

Figure 2. Original IEND data in fibers/mm of the 34 schwannomatosis patients (blue dots) and the 25 NF2 patients (orange dots) where separated according to gender and plotted directly on the respective normative reference charts 10 with indicated 5% normative value (solid line) and 50% normative value (dashed line).

calculate gender-specific IEND for each age. Subsequently, difference between the IEND of each patient and the corresponding 5% normative reference IEND was calculated and plotted against age of the patient (Figure 3). Visibly, this IEND-difference was larger (lower from the 0 line) in the schwannomatosis patients than in the NF2 patients. Unpaired *t*-test confirmed a high level of significance of this difference ($P < 0.001$).

Whereas clinical phenotypes of NF2 patients and schwannomatosis patients overlap, and in addition, both disorders frequently have mosaicism, we further selected only genetically ascertained cases (see the next subsection) and repeated the above analysis. The 10 genetically ascertained schwannomatosis patients all had identified pathogenic mutations in the *LZRT1* or the *SMARCB1* gene. The 11 genetically ascertained NF2 patients all had identified pathogenic mutations in the *NF2* gene. Missense mutations and other mutations with uncertain pathogenicity were not included. Using these selected cases, same result was obtained: difference between the IEND of our patient and the 5% normative reference IEND was significantly larger in the genetically ascertained schwannomatosis patients than in the genetically ascertained NF2 patients (Figure 4; $P = 0.001$).

Genetic finding

Germline mutation of the *NF2* gene was found in 12 (63%) out of the 19 NF2 patients analyzed including nonsense, frameshifting and splicing mutations as well as one missense mutation and two exon deletions. No difference in the IEND values was detectable for different types of *NF2* mutations.

Out of the 34 schwannomatosis patients, 32 were screened for mutations in the *LZTR1* gene. Truncating mutations including nonsense, frameshifting and splicing mutations were found in nine cases (31%), missense mutations with unclear pathogenicity in five cases (16%), intron mutation in one case. In one case, pathogenic mutation was found in the *SMARCB1* gene. For 16 (50%) patients, no *LZTR1* mutations were found in the blood. The IEND did not differ in different types or lack of *LZTR1* mutations. None of the schwannomatosis patients had germline *NF2* mutations.

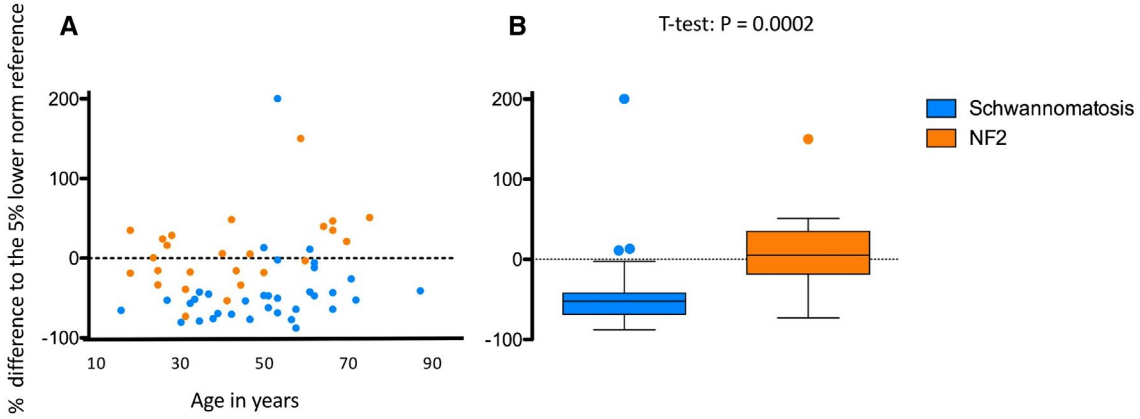

Figure 3. *Normalized difference of IEND from age- and gender-matched 5% normative value.* **A.** scattered plot showing each schwannomatosis (blue) and NF2 (orange) cases. **B.** box plot.

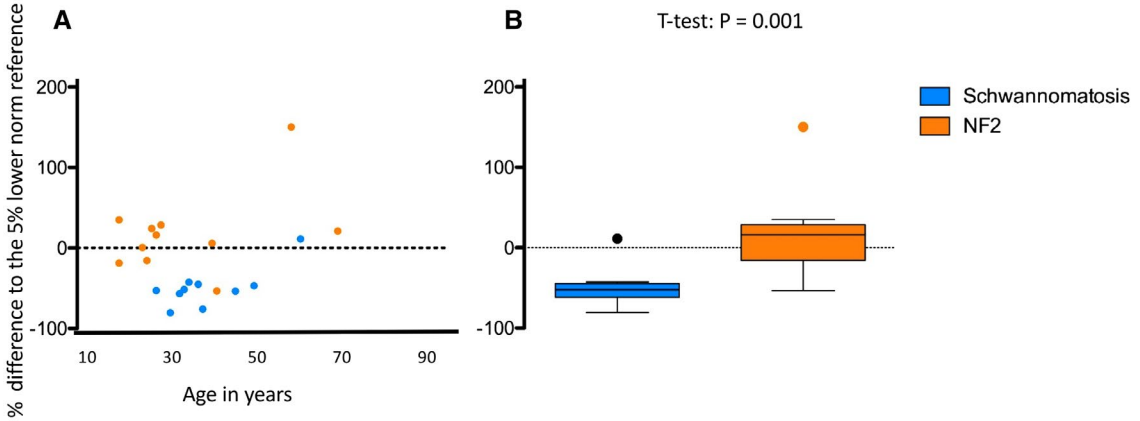

Figure 4. *Normalized difference of IEND from age- and gender-matched 5% normative value.* Only patients with genetically ascertained schwannomatosis or NF2 were included. **A.** scattered plot showing each schwannomatosis (blue) and NF2 (orange) cases. **B.** box plot.

DISCUSSION

In this study, we present data showing that 11/25 (44%) NF2 and 33/34 (97%) schwannomatosis patients had IEND below or on the bottom 5% normative reference IEND. In addition, the IEND reduction from the 5% normative reference was significantly larger ($P < 0.001$) in schwannomatosis patients than in NF2 patients. Identical results were obtained for patients with genetically ascertained schwannomatosis or NF2.

Only one schwannomatosis patient and one NF2 patient had IEND above/on the 50% normative reference, respectively. However, no special clinical features were recognizable in these two patients. Considering frequent mosaicism in both of the two disorders, it is possible that these patients do not carry the genetic alteration in all tissues including the biopsied tissue for measuring IEND because of mosaicism. Determination of IEND is currently considered as the gold standard tool for the diagnosis of small fiber neuropathies (9, 10). In the present study, we used an antibody for PGP9.5 to stain and quantify the nerve fibers. Since C-fiber is major type of nerve fibers in the epidermal layer of the skin, the reduced IEND in our patients may be considered as reduction in C-fibers. Indeed, our preliminary investigation found convincing evidence for small fiber neuropathy in majority of schwannomatosis patients. In concordance, chronic neuropathic pain which is a typical consequence of small fiber neuropathy, is characteristic for schwannomatosis (11, 12). It is worth to note that the IEND in schwannomatosis patients is rather constantly low, also in young patients. This age independent feature of the marked reduction of IEND in schwannomatosis patients is therefore unlikely caused by a degenerative process, but rather represents some intrinsic manifestation caused by genetic alteration.

Though IEND is also reduced in NF2 patients, the extent is significantly less than in schwannomatosis patients. In concordance, small fiber neuropathy was found also in NF2 patient, but less frequent when compared with schwannomatosis patients. NF2 patients do not have chronic pain but may have moderate to severe sensory deficits and paresis which are not characteristic for schwannomatosis. NF2-neuropathy may therefore be more generalized including small fiber and thicker myelinated fibers. IEND in the NF2 patients of the present study exhibits a slight age-dependence trend, more than schwannomatosis but less than the normative reference. However, because of the limited sample size and the large variability of the data, reliable interpretation is not possible at this time. Nevertheless, considering all findings together, the pathomechanism of neuropathy in NF2 and schwannomatosis seems to be different.

Early diagnosis and differential diagnosis are essential for treatment and management of NF2 and schwannomatosis patients because their clinical courses differ. For NF2 patients, more severe complications like spinal ependymomas, sensory motor neuropathy and bilateral cataracts have to be anticipated. For schwannomatosis patients, adequate pain management is of greater importance. Unfortunately, differential diagnosis of NF2 and schwannomatosis is still challenging for a considerable number of cases because of the wide clinical overlap, especially for young patients in the early stage of the diseases. Since mutations can only be found in approximately half of the patients, diagnostic classification often cannot rely on genetic evidence.

Table 2. Clinical, genetical and pathological characteristics of NF2 and schwannomatosis.

Aspect	NF2	Schwannomatosis
Clinic		
Bilateral vestibular schwannoma	Hallmark (>90%)	extreme rare
Meningioma	45%–58%	Rare (5%)
Ependymoma	18%–58%	No
Skin plaques	41%–48%	No
Intradermal tumor	27%	No
Retinal hamartoma	6%–22%	No
Epiretinal membrane	12%–40%	No
Subcapsular cataract	60%–81%	No
Unilateral vestibular schwannoma	overlap	
Spinal tumors	overlap	
Peripheral nerve schwannoma	overlap	
Subcutaneous tumors	overlap	
Genetics		
Germline *SMARCB1*	No	50%
Germline *LZTR1* mutation	No	50%
Germline *NF2* mutation	100% in inherited cases	No
Somatic *NF2* mutation	overlap	
Somatic *NF2* LOH	overlap	
Pathology		
Histology	overlap (schwannoma or hybrid tumors)	
Mean IEND normalized against the lower 5% normative	−42 ± 50	7 ± 45

Besides high resolution magnetic resonance peripheral neurography (2) and dorsal root ganglia volume (3) the marked reduction of IEND therefore provides a third major surrogate parameter to be considered in differential diagnosis of schwannomatosis. However, despite the highly significant difference, schwannomatosis and NF2 patients still overlap regarding this feature and consequently—without a clear cut-off value—the IEND alone is not sufficient for differential diagnosis. A detailed and comprehensive assessment case by case considering clinical, genetic and neuropathological features is therefore mandatory (Table 2).

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AUTHOR CONTRIBUTIONS

S.F., C.H. and V.-F.M. contributed to conception and design of the study; S.F., L.K., R.F., J.M., M.G. and C.H.

contributed to acquisition and analysis of data; S.F., L.K. and V.-F.M. contributed to drafting the text or preparing the figure. C.H. and V.-F.M. contributed equally to the publication.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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