

Innervation of the Skin of the Forearm in Diabetic Patients: Relation to Nerve Function*

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Complications of diabetes include sensory and autonomic neuropathy. The aim of the present paper was to study the degree of sensory and autonomic neuropathy and correlate these findings with the distribution and density of neuropeptidergic nerve fibers in the skin of the forearm of diabetic patients and healthy controls. We investigated 30 diabetics (24 type 1 and 6 type 2) and compared them with 13 healthy controls. There were no differences between the groups with respect to density and distribution of nerve fibers displaying immunoreactivity to the pan-neuronal marker PGP 9.5 and sensory and parasympathetic neuropeptides (substance P, calcitonin gene-related peptide and vasoactive intestinal peptide). By contrast, nerve fibers containing neuropeptide Y, a marker of sympathetic neurons, were reduced in number in the diabetic patients. C-fiber function (measured as the axon-reflex-evoked flare response) became impaired with increasing age in all subjects. The diabetic patients, however, showed a reduced flare compared to age-matched healthy controls. The reduction was particularly prominent in the younger patients (20–50 years). There was a greater reduction of the flare in neuropathic patients than in non-neuropathic patients, but there was no correlation between the degree of functional impairment and the duration of the disease.

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Diabetic neuropathy causes high morbidity and a reduced quality of life (1). Not only sensory but also motor and autonomic nerve functions are impaired. Although its pathogenesis remains unknown, diabetic neuropathy is considered to be secondary to long-term insulin deficiency and/or hyperglycemia (2). Detection, characterization and staging of sensory and autonomic neuropathy are important in monitoring the progression of the disease. Careful testing can be used to grade the function and state of sensory (unmyelinated or myelinated) nerve fibers in these patients. Symptoms of sensory neuropathy have been classified as "positive" or "negative". The former symptoms (feelings of prickling, tightness, crawling, thermal sensations and various types of pain) reflect neural hyperactivity, thought to arise from ectopic generators in damaged fibers or sprouts (3). The latter symptoms reflect impaired or abolished neural function. For the evaluation of autonomic dysfunction it is useful to

assess the function of unmyelinated nerve fibers. Such nerves play an important role in the regulation of blood pressure, skin temperature and perspiration. Morphological studies of nerve biopsies may give insight into the degree of axon loss and nerve lesion. A major obstacle in these studies is the poor healing observed after biopsies in diabetic patients. Skin biopsies taken from the lower arm offer a relatively safe way to examine peripheral nerves in such patients.

Slowly conducting, unmyelinated C-fibers are thought to convey much of the sensory perception of pain from the periphery to the spinal cord (4). Free nerve endings located in the upper dermis have been found to contain the neuropeptide substance P (SP) and calcitonin gene-related peptide (CGRP) (5–8), both of which are associated with C-fibers. Neuropeptide Y (NPY) is a constituent of sympathetic (adrenergic fibers), while vasoactive intestinal peptide (VIP) occurs in many parasympathetic (cholinergic) fibers (7, 9). Antibodies to a general neuronal marker, referred to as PGP 9.5 (protein gene product) (10), can be used to demonstrate nerve fibers collectively.

The aim of the study was to examine whether the degree of sensory and autonomic neuropathy was correlated to the distribution and density of neuropeptidergic nerve fibers in the skin of the forearm of diabetic patients. The functional capacity of dermal C-fibers can be studied by monitoring the axon reflex measured as the area of the histamine- or SP-evoked flare. The response is thought to reflect the release of neuropeptides from C-fibers (11, 12). The possibility that the release of neuropeptides from C-fibers is reduced in the presence of peripheral neuropathy, and the fact that this alteration is manifested clinically as a reduced flare area has been discussed (13). Healthy controls were studied for comparison.

PATIENTS AND METHODS

Thirty diabetic patients from the Central Institute of Diabetes in Karlsruhe, Germany, and 13 healthy volunteers participated after giving their informed consent. The study was approved by the ethics committees in Lund and Greifswald.

Twenty-four patients had type 1 diabetes (IDDM) and 6 had type II diabetes (NIDDM). All patients were treated with insulin. The age of the patients ranged from 24 to 71 years and the time since diagnosis was 4–50 years. The mean duration of the disease was 19.2 years.

None of the patients had any untreated endocrine, metabolic or neurological disease that might affect peripheral nerve function beside the diabetes. Individuals with a history of alcohol abuse were excluded from the study. Glycated hemoglobin (HBA_{1c}) reflecting the mean blood glucose value during 2–6 weeks prior to sampling was at range 5.8–11.7% (mean value 8.6%).

The control group consisted of 13 healthy subjects, 5 males, 8 females, age range 22–71 years (mean value 50.7 ± 4.9 years).

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Table I. Details of the antibodies used (raised in rabbits)

SP (SP8):	raised against protein-conjugated, synthetic bovine SP, working dilution – 1:320**;
CGRP (8427):	raised against protein-conjugated synthetic rat CGRP, working dilution – 1:1280**;
VIP (7852):	raised against unconjugated pure natural porcine VIP, working dilution – 1:640**;
NPY (8404):	raised against protein-conjugated synthetic porcine NPY, working dilution – 1:320**;
PGP 9.5:	raised against unconjugated human brain PGP 9.5, working dilution – 1:200***

Sources: *PC. Emson, MRC, Cambridge, UK, ** Milab, Malmö, Sweden, *** Ultracelone, Cambridge, UK.

Clinical evaluation of polyneuropathy

Sensory neuropathy was graded in three stages as follows:

Stage 1. Slight (at least 2 of the following symptoms): a) weakness of tendon reflexes, b) reduced sensibility, c) subjective complaints (pain, tingling, numbness).

Stage 2. Moderate (at least 2 of the following symptoms): a) absence of tendon reflexes, b) markedly reduced sensibility, c) several subjective complaints (pain, tingling, numbness).

Stage 3. Severe (at least 3 of the following symptoms): a) absence of tendon reflexes, b) severe reduction of sensibility in feet and lower legs, c) skin lesions on the legs, d) muscle weakness, fasciculations, spasms, cramps, muscle waste or palsy, and e) several and frequent or constant subjective complaints (pain, tingling, numbness).

The patients were classified accordingly: Stage 0: 6 patients, all males, age 24–51 y (\bar{x} = 33.2), all type I diabetes, duration of diabetes 6–23 y (\bar{x} = 13.5). Stage 1: 8 patients, 3 males, age 31–89 y (\bar{x} = 45.8), 6 type I diabetes, duration of diabetes 4–50 y (\bar{x} = 22.8 y). Stage 2: 10 patients, 6 males, age 49–71 y (\bar{x} = 55.7 y), 7 type I diabetes, duration of diabetes 4–30 y (\bar{x} = 24.3 y). Stage 3: 3 patients, one male, age 35–41 y, \bar{x} = 38 y, all type I diabetes, duration of diabetes 19–27 y (\bar{x} = 22.3 y).

Clinical evaluation of autonomic neuropathy

The staging of autonomic function was based on the presence of the following manifestations according to Ewing's test battery for the detection of cardiovascular autonomic dysfunction (14): a) trophic disturbances (skin lesions), b) pathologic cardiovascular reflex, and c) absence of light reflexes. Stage 1: one of a, b, c. Stage 2: two of a, b, c. Stage 3: three of a, b, c, or two of a, b, c together with several clinical manifestations (history of postural fainting, impotence in male, loss of micturition control, night diarrhea, obstipation, sweating disturbances, sometimes gustatory sweating).

The patients were classified accordingly: Stage 0: 8 patients, 6 males, age 24–43 y (\bar{x} = 31.6), all type I diabetes, duration of diabetes 7–39 y (\bar{x} = 17 y). Stage 1: 10 patients, 7 males, age 31–71 y (\bar{x} = 53.3 y), 7 type I diabetes, duration of diabetes 4–50 y (\bar{x} = 22.8 y). Stage 2: 5 patients, 3 males, age 38–80 y (\bar{x} = 51.2 y), 4 type I diabetes, duration of diabetes 13–39 y (\bar{x} = 25.4 y). Stage 3: 4 patients, one male, age 35–69 y (\bar{x} = 48.5 y), 3 type I diabetes, duration of diabetes 4–33 y (\bar{x} = 21.2 y).

Biopsies and immunocytochemistry

Full-thickness skin specimens (punch biopsies 3–4 mm in diameter) were taken from the upper part of the volar aspect of the forearm of both patients and controls. These areas did not show any symptoms of neuropathy. In order to visualize all nerve fibers in the skin, we used antibodies to a general neuronal marker referred to as PGP 9.5 (10).

All specimens were fixed by immersion overnight in ice-chilled 4% buffered formaldehyde, pH 7.2 (solution of Stefanini). They were then rinsed repeatedly in Tyrode's solution containing sucrose (100 g/l) and frozen on dry ice.

Cryostat sections (10 μ m thickness) were processed for immunocyto-

chemistry using antibodies against SP, CGRP, VIP and NPY and against PGP 9.5. Preadsorption studies using pure antigens were performed as controls. Details on the antibodies are given in Table I. Three non-serial sections from each specimen were used for each antiserum. The density of immunoreactive fibers in each specimen was assessed visually and the results graded from score 0 (0 nerve fibers), score 1 (1–3 nerve fibers), score 2 (3–5 nerve fibers) to score 3 (>5 nerve fibers) (the number of nerve fibers refers to a visual field at $\times 250$ magnification). All sections were assessed by an observer, who was unaware of the diabetic and neuropathic state of the patients. The variation between sections from the same patient was invariably small (not exceeding one score).

Axon reflexes

SP (50 pmol) in physiological saline was injected intracutaneously in a volume of 0.05 ml on the volar aspect of the forearm. Healthy volunteers (5 in each age group, medical staff and patients from the department of audiology) served as controls. The area of the flare was evaluated planimetrically after 5 min (16).

Statistical method

Wilcoxon's rank sum test $*-p < 0.05$; $**p < 0.01$ was used for evaluation of the nerve density. In order for us to eliminate the influence of age when comparing the flare response in different groups, the analysis of covariance was applied. The assumption is that the regressions are linear and parallel. The analysis of covariance tests whether the regression lines of the compared groups have the same relative position, i.e. if the intercepts on the ordinate differ when the influence of age is fixed (controlled).

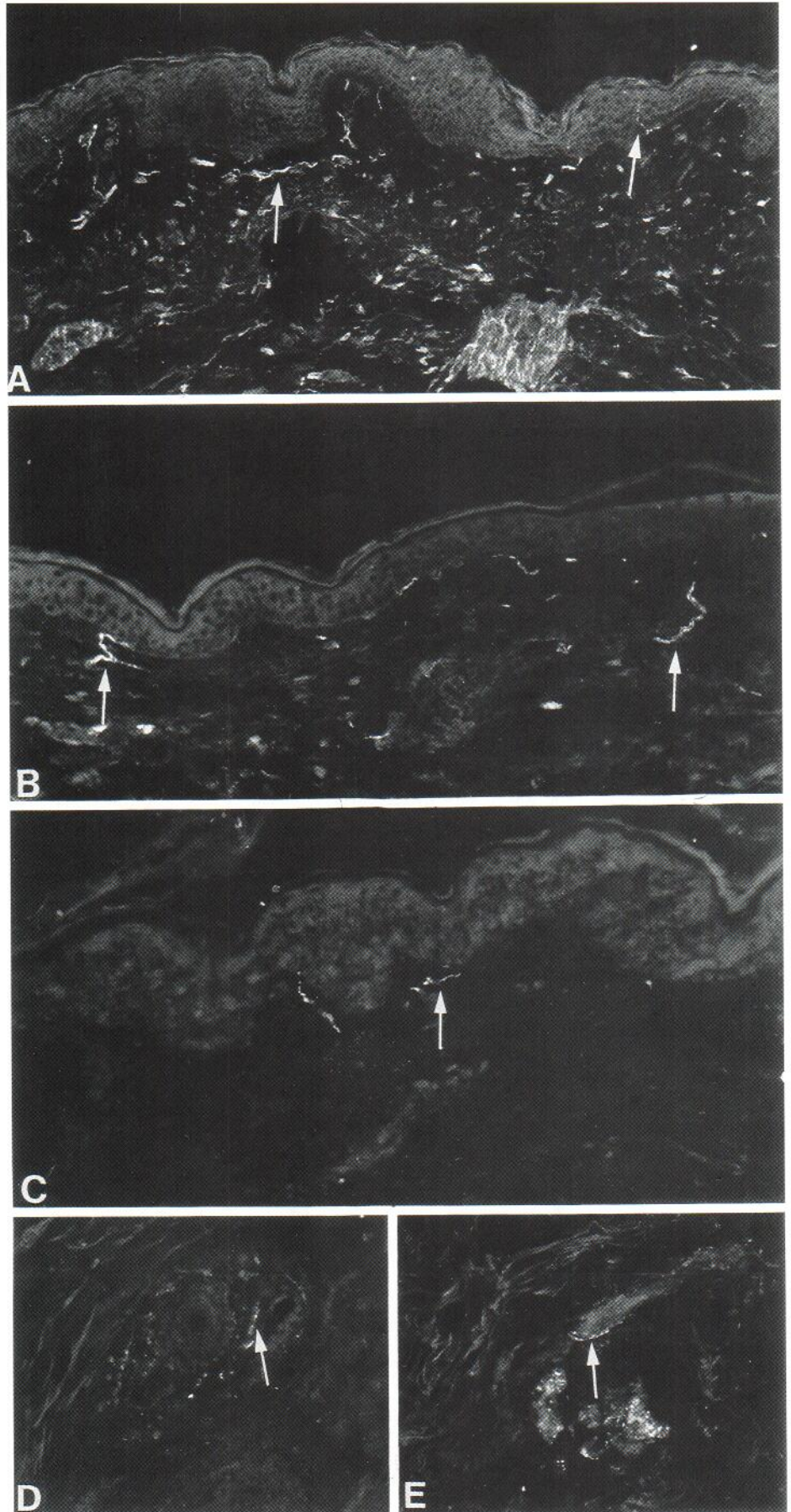
RESULTS

PGP-immunoreactive (IR) nerve fibers occurred as free nerve endings in the upper dermis. In the deeper parts of the dermis they occurred around sweat glands and blood vessels and as bundles of fibers in the connective tissue. CGRP- and SP-IR fibers appeared as beaded fibers in the upper dermis, sometimes penetrating into the epidermis. CGRP-IR fibers were more numerous than SP-IR fibers. NPY-IR fibers were usually found in the deeper dermis around blood vessels and VIP-IR fibers around sweat glands. NPY-IR fibers were rather few (Fig. 1). There were no apparent age-related changes in the frequency and distribution of neuropeptide-containing fibers (not shown).

Screening with antibodies to SP, CGRP, VIP and PGP revealed no difference in the number of immunopositive fibers between diabetics and controls (Fig. 2), and there was no difference between patients with or without neuropathy. There was no difference in nerve fiber density between short or long duration (more than 20 years) of the disease, except for a reduced number of NPY-IR nerve fibers in patients with long-standing ($p < 0.05$) and short-standing ($p < 0.01$) diabetes (Fig. 3).

SP-evoked flare reactions were negatively correlated with age both in patients and in healthy controls ($p < 0.01$). The diabetic patients showed a reduced flare compared to age-matched healthy controls ($p < 0.001$). The reduction was particularly prominent in younger (20–50 years) patients with neuropathy (Fig. 4A). The patients (20–50 years) with moderate neuropathy had a smaller flare response ($p < 0.05$) than those with slight or no neuropathy (Fig. 4B). The duration of the diabetes did not seem to be a factor contributing to the impairment of the flare reaction.

Fig. 1. Skin biopsies immunostained for PGP 9.5 (A), CGRP (B), SP (C) and VIP (D and E). PGP-immunoreactive nerve fibers, comprising the total innervation, are distributed in both superficial and deeper layers. CGRP- and SP-immunoreactive fibers predominate in the superficial and deeper layers, just beneath the epithelium. VIP-immunoreactive fibers occur around hair follicles (D) and around the acini of sweat glands (E). Magnification $\times 175$ (A), $\times 175$ (B), $\times 250$ (C) and $\times 250$ (D-E). The counted structures are exemplified with arrows.



NERVE FIBER DENSITY

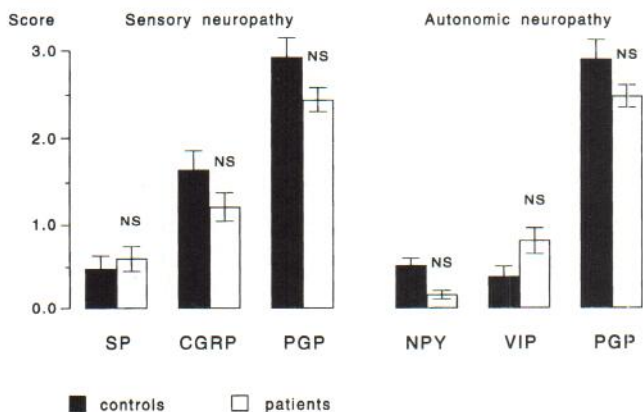


Fig. 2. Nerve fiber density in the skin of diabetic patients ($n=27$) and healthy controls ($n=13$). Statistical evaluation by Wilcoxon's rank sum test; * $p<0.05$; ** $p<0.01$.

DISCUSSION

One aim of the present study was to examine whether the degree of sensory and autonomic neuropathy can be correlated with alterations in the distribution and density of peptide-containing nerve fibers in skin biopsies collected from the upper part of the volar aspect of the forearm. This biopsy site was chosen because skin in this region is known to heal well in diabetics. This may reflect the slower development of diabetic neuropathy in forearms compared with legs. Even when a patient exhibits distinct pathological changes in the lower legs, the forearms often appear normal.

On the whole, nerve fibers in the skin of the forearm demonstrated by antibodies to PGP 9.5 showed the same distribution pattern and density in patients with diabetes (regardless of the degree of neuropathy) as in healthy controls. Sensory nerve fibers (those that contain SP and/or CGRP) had the same distribution and density in the skin of diabetics as in the skin of healthy controls. These findings contrast to other observations (17–19) where fewer SP-, CGRP- and PGP-containing fibers

were found in diabetic patients than in normal healthy controls. These workers collected biopsies mostly from areas of the lower legs that showed symptoms of neuropathy and peripheral vascular disease, while our biopsies were taken from an area not displaying such symptoms.

Faerman et al. (20) compared forearm skin with the skin of the lower leg in patients with evidence of anhidrosis of the lower leg. They found abnormal Minor test (diagnostic for anhidrotic skin) and abnormal histology of nerve fibers (beaded, spindle-shaped thickening and fragmentation adjacent to the sweat glands) in the skin from the legs, while forearm skin revealed no abnormalities.

The number of VIP-IR fibers did not differ between diabetic patients and controls (Fig. 3). In contrast, Levy et al. (18, 19) observed fewer dermal VIP fibers in diabetic patients than in healthy individuals (biopsy site: lower legs).

In our study, the NPY-IR fibers were reduced in number in the diabetic patients, regardless of the duration of the disease (Fig. 3). The reduced number of NPY-positive nerve fibers might be involved in the degeneration of small blood vessels, which is a common feature of diabetes.

We found no correlation between the neurophysiological test results and the immunohistochemical findings, and we were not able to detect pathological changes in nerve fibers in the tissue samples examined. The standardized biopsy site on the forearm and the analysis of subepidermal unmyelinated nerves (SP, CGRP, PGP) or nerve structures around the blood vessels (mostly NPY) and sweat glands (mostly VIP) gave no guidance for the evaluation of either sensory or autonomic neuropathy in diabetic patients.

The functional capacity of unmyelinated C-fibers in the skin deteriorates with increasing age, as suggested by the reduced flare response to SP (Fig. 4). Our results suggest that the age-dependent reduction of the axon flare is more important than any effect caused by diabetes. However, in the younger individuals (20–50 years) we found a reduction of the axon flare ($p=0.0081$) in patients with clinical signs of moderate to severe neuropathy compared to healthy controls. There was also a difference between diabetic patients with or without neuropathy in this age group ($p=0.0141$). It must be emphasized that the axon flare was evoked in skin that did not display histological signs of neuropathy and that we do not know what the axon flare response would have been in skin displaying symptoms of neuropathy.

The consensus statement (15) on the classification of diabetic neuropathy recommends the use of the flare reaction for the assessment of neuropathic involvement of unmyelinated nociceptor fibers. Our finding emphasizes the need for such data to be correlated to an age-matched control population. Possibly, the axon flare can be used to monitor the progress of the disease on an individual basis, provided age is corrected for. Our data are in accordance with a previous report (21) showing that the flare response to histamine was reduced in patients with diabetes. We did not observe any correlation between the magnitude of the axon flare response in the skin of the lower arm on one hand and the duration of the diabetes or the degree of the neuropathy on the other.

In conclusion, histological examination of standardized biop-

NERVE FIBER DENSITY

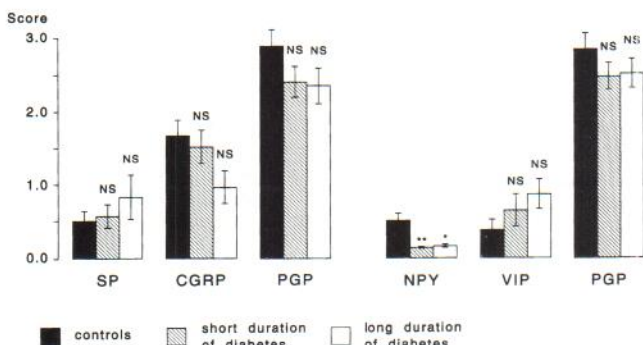
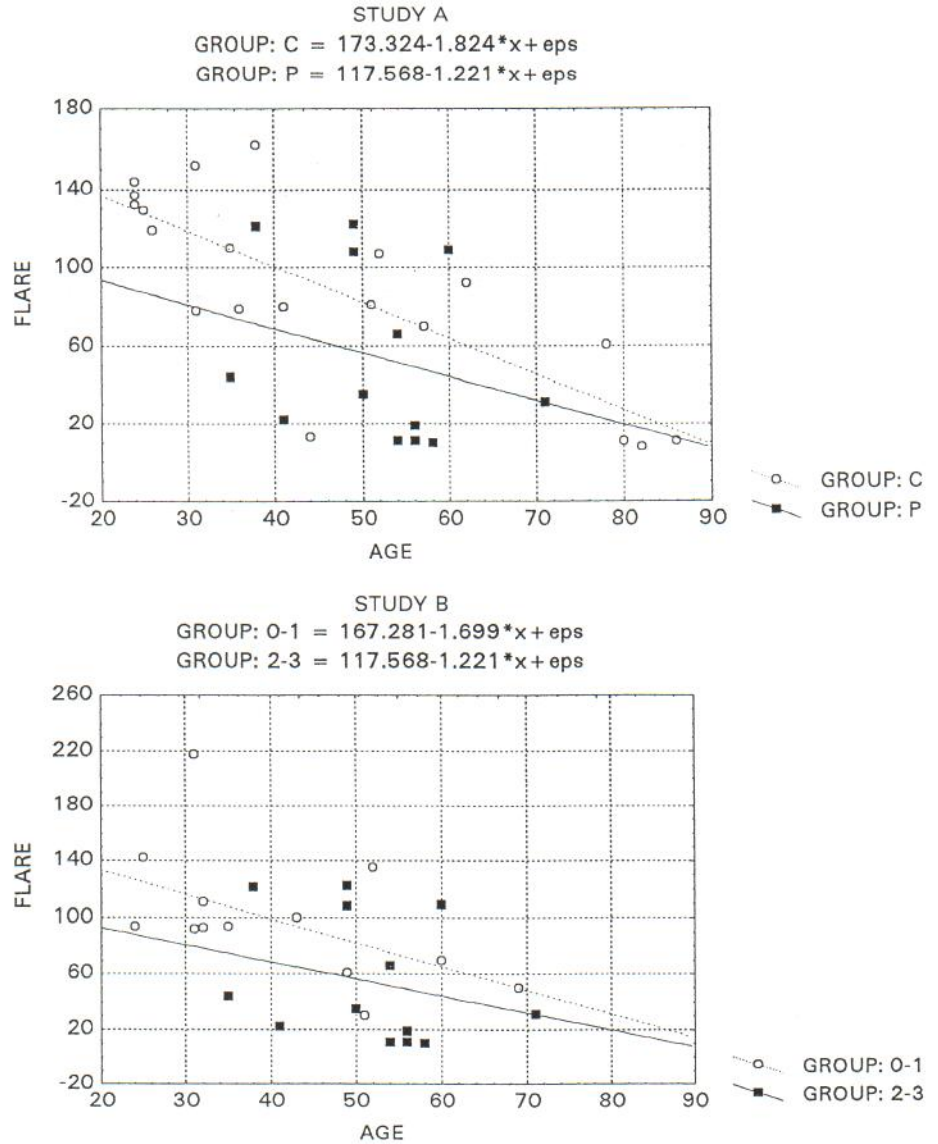


Fig. 3. Density of SP- and CGRP-, NPY-, VIP- and PGP-positive nerve fibers in patients with short ($n=13$) or long duration of diabetes ($n=14$) and in healthy controls ($n=13$). Statistical evaluation by Wilcoxon's rank sum test; * $p<0.05$; ** $p<0.01$.

Fig. 4. SP-induced flare area (as an indicator of C-fiber function) versus age in diabetic patients ($n=27$) and in age-matched healthy controls ($n=20$). Analysis of covariance has been applied. A. Comparison between patients with neuropathy (2-3) and healthy controls. ($p=0.0081$). B. Comparison between patients without neuropathy or with slight neuropathy (0-1) on one hand and with moderate neuropathy (2-3) on the other ($p=0.0141$).



sies from the lower arm cannot be used to diagnose or evaluate sensory and autonomic neuropathy. Axon reflexes induced in a standardized manner at this site can be used to assess C-fiber impairment in diabetic patients aged 20-50 years and to monitor the progress of the diabetes neuropathy in the individual.

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