LUND UNIVERSITY

Molecular and pathological studies in the posterior interosseous nerve of diabetic and non-diabetic patients with carpal tunnel syndrome

Mojaddidi, Moaz A.; Ahmed, Mohammed S.; Ali, Razwan; Jeziorska, Maria; Al-Sunni, Ahmed; Thomsen, Niels; Dahlin, Lars; Malik, Rayaz A.

Published in:
Diabetologia

DOI:
10.1007/s00125-014-3271-3

2014

Document Version:
Peer reviewed version (aka post-print)

Link to publication

Citation for published version (APA):
Mojaddidi, M. A., Ahmed, M. S., Ali, R., Jeziorska, M., Al-Sunni, A., Thomsen, N., Dahlin, L., & Malik, R. A. (2014). Molecular and pathological studies in the posterior interosseous nerve of diabetic and non-diabetic patients with carpal tunnel syndrome. *Diabetologia*, *57*(8), 1711-1719. https://doi.org/10.1007/s00125-014-3271-3

Total number of authors:
8

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Article

Molecular and pathological studies in the posterior interosseous nerve of diabetic and non-diabetic patients with carpal tunnel syndrome

Moaz A. Mojaddidi[1,2], Mohammed S. Ahmed[1], Razwan Ali[1], Maria Jeziorska[1], Ahmed Al-Sunni[1,3], Niels O. B. Thomsen[4], Lars B. Dahlin[4,5] and Rayaz A. Malik[1]

1. Centre for Endocrinology and Diabetes, Institute of Human Development, Faculty of Medical and Human Sciences, The University of Manchester, AV Hill Building, Oxford Road, Manchester M13 9PT, UK
2. Department of Physiology, Taibah University, Almadinah Almunawwarah, Saudi Arabia
3. Department of Physiology, University of Dammam, College of Medicine, Dammam, Saudi Arabia
4. Department of Hand Surgery, Malmö University Hospital, Malmö, Sweden
5. Department of Clinical Sciences Malmö – Hand Surgery, Lund University, Malmö, Sweden

Corresponding author

R. A. Malik, Centre for Endocrinology and Diabetes, Institute of Human Development, Faculty of Medical and Human Sciences, The University of Manchester, AV Hill Building, Oxford Road, Manchester M13 9PT, UK

E-mail: Rayaz.a.Malik@man.ac.uk

Keywords

Carpal tunnel syndrome, Diabetes, HIF-1α, Microangiopathy, Myelinated fibre, Neuropathy, Unmyelinated fibre, VEGF

Received: 13 February 2014 / Accepted: 22 April 2014

Abstract

Aims/hypothesis We sought to establish the molecular and pathological changes predisposing diabetic and non-diabetic patients to the development of carpal tunnel syndrome (CTS).

Methods The posterior interosseous nerve (PIN) was biopsied in 25 diabetic and 19 non-diabetic patients undergoing carpal tunnel decompression for CTS. Detailed morphometric and immunohistological analyses were performed in the nerve biopsy.

Results In diabetic patients median nerve distal motor latency was prolonged ($p < 0.05$ vs non-diabetic patients), PIN myelinated fibre density ($p < 0.05$), fibre area ($p < 0.0001$) and axon area ($p < 0.0001$) were reduced, the percentage of unassociated Schwann cell profiles ($p < 0.0001$) and unmyelinated axon density ($p < 0.0001$) were increased and the axon diameter was reduced ($p < 0.0001$). Endoneurial capillary basement membrane area was increased ($p < 0.0001$) in diabetic patients, but endothelial cell number was increased ($p < 0.01$) and luminal area was reduced ($p < 0.05$) in non-diabetic patients with CTS. There was no difference in the expression of hypoxia-inducible factor 1α between diabetic and non-diabetic patients with CTS. However, the expression of vascular endothelial growth factor A (VEGF) ($p < 0.05$) and its receptors VEGFR-1 ($p < 0.01$) and VEGFR-2 ($p < 0.05$) was significantly increased in diabetic patients, particularly those with type 1 diabetes, and related to the severity of nerve fibre pathology.

Conclusions/interpretation This study demonstrates increased nerve fibre and microvascular pathology in relation to enhanced expression of VEGF and its receptors in a non-compressed nerve in diabetic compared with non-diabetic patients with CTS. It therefore provides a potential molecular and pathological basis for the predisposition of diabetic patients to the development of CTS.

Abbreviations

Ab	Antibody
CTS	Carpal tunnel syndrome
DCTS	Diabetic patients with CTS
DML	Distal motor latency
fSNCV	Fractionated measurement of antidromic sensory conduction velocity
HIF-1α	Hypoxia-inducible factor 1α
MNFD	Myelinated nerve fibre density
NCTS	Non-diabetic patients with CTS

PIN	Posterior interosseus nerve
USCP	Unassociated Schwann cell profile
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor

Introduction

Carpal tunnel syndrome (CTS) is the most frequent peripheral nerve disorder encountered in clinical practice [1], with a reported prevalence in the general population ranging between 2.7% and 5.8% [2, 3]. Patients with diabetes are at significantly greater risk of developing CTS when compared with the general population [4], but the mechanistic basis for this is not known. There are limited data suggesting that reductions in carpal tunnel volume occur due to connective tissue proliferation and fibrosis [5, 6], leading to nerve compression. Swelling of the median nerve is seen on ultrasound and MRI in patients with CTS and has been attributed to nerve oedema [7, 8], but the underlying cause has not been established. The tenosynovial swelling [7–9] has been attributed to oedema, fibrosis and de novo angiogenesis, in part due to vascular endothelial growth factor (VEGF) A [10, 11]. VEGF-A (thereafter called VEGF) is the best characterised and the most studied of the VEGF family members and is a potent vasoactive molecule, which increases vascular permeability and angiogenesis [12, 13]. It mediates its biological functions via Flt-1 (VEGF receptor 1 [VEGFR-1]) and Flk-1/KDR (VEGFR-2) [12]. Hypoxia-inducible factor 1α (HIF-lα) is central to VEGF expression through binding of the hypoxia-responsive element in the VEGF gene promoter region [14] and it also upregulates VEGFR-1 expression via an HIF-1α-dependent mechanism [15].

Experimental studies have suggested that nerves in patients with diabetes may be more vulnerable to entrapment [16] due to localised endoneurial microcirculatory changes [17]. Furthermore, diabetic microangiopathy [18] may enhance endoneurial hypoxia [19], leading to upregulation of angiogenic factors such as HIF-lα and VEGF. The response mediated by these cytokines may be of particular relevance within peripheral nerves subjected to localised compression in CTS, due to the lack of autoregulation of the endoneurial vascular bed [20]. We have had the unique opportunity to undertake detailed neuropathological and quantitative immunohistological analysis in the non-compressed posterior interosseous nerve (PIN) lying on the dorsum of the forearm at the same anatomically distal aspect as the median nerve in the adjacent carpal tunnel, which was biopsied at the time of carpal tunnel decompression.

Nerve fibre and endoneurial capillary pathology was quantified and compared in relation to the expression of HIF-1α, VEGF and its receptors (VEGFR-1 and VEGFR-2) between diabetic (DCTS) and non-diabetic (NCTS) patients with CTS.

Methods

Participants Patients with clinically and electrophysiologically confirmed CTS, undergoing carpal tunnel release and simultaneous PIN biopsy participated in this study. Exclusion criteria included previous carpal tunnel release in the hand under study, clinical signs of focal nerve entrapment other than CTS, cervical radiculopathy, inflammatory joint disease, renal failure, thyroid disorders, previous wrist fracture on the affected side, daily long-term exposure to vibrating tools, pregnancy or age under 18 years. PIN biopsies were obtained from 25 diabetic (DCTS) and 19 non-diabetic (NCTS) age-matched patients using our established surgical technique [21]. The DCTS group consisted of patients with type 1 (n = 11) and type 2 (n = 14) diabetes mellitus. The study was approved by the Regional Ethical Review Board, Lund University (LU 508/03).

Symptoms and electrophysiology The severity of symptoms related to CTS, were established using the Symptom Severity Score from the Boston carpal tunnel questionnaire [22]. Examination was performed using a Viking Select Electromyograph (Viasys, Madison, WI, USA). All studies were conducted with surface electrodes (skin temperature kept above 30°C). The diagnosis of CTS was based on a fractionated measurement of antidromic sensory conduction velocity (fSNCV) at the carpal tunnel segment and distal motor latency (DML) for the median nerve [23]. An abnormality in one or more of the following was used to diagnose peripheral neuropathy: sural sensory nerve conduction velocity, sural sensory nerve action potential, peroneal nerve motor conduction velocity or peroneal nerve compound muscle action potential.

Biopsy A 3 cm PIN biopsy was harvested from the dorsum of the distal forearm and divided into two pieces, which were processed and fixed into Epon (Agar Scientific. Stansted, UK) for light and electron microscopy and paraffin blocks for immunohistology, as previously described [24, 25].

Light microscopy Semi-thin Epon sections (0.5 µm) were cut for light and electron microscopy, stained with thionin and counter-stained with acridine orange. Fascicles were photographed (magnification ×200) using a Leitz Diaplan light microscope (Ernst Leitz, Wetzlar, Germany) and digital images of all fascicles were captured from sections of each biopsy. The Image Pro-Plus 4.1 (Media Cybernetics, Rockville, MD, USA) image analysis system was employed to measure fascicular area (mm^2). Endoneurial capillaries and myelinated nerve fibres were counted directly from the images and the endoneurial capillary and myelinated fibre densities (No./mm^2) were derived by dividing the number of endoneurial capillaries and myelinated nerve fibres relative to the fascicular area, respectively [26]. All fascicles were examined in each nerve and mean values were derived.

Electron microscopy Electron microscopy of nerve fibres and endoneurial capillaries was undertaken using our previously described methodology [26]. Ultrathin (< 0.1 µm) sections stained with methanolic uranyl acetate and lead citrate were used to prepare electron micrographs with a Philips EM201 electron microscope (NV Philips, Eindhoven, the Netherlands). Electron micrographs (magnification ×3,000) of at least 200 myelinated fibres were prepared for each biopsy specimen and the axonal and fibre perimeter were manually digitised to assess the myelinated axon and fibre area. For unmyelinated fibres a minimum of 25 electron micrographs per biopsy were prepared at a final magnification of ×10,000. The number of unassociated Schwann cell profiles (USCPs) was counted directly from all micrographs and expressed as a percentage of total Schwann cell profiles. The unmyelinated axons were identified using established criteria to differentiate axons from Schwann cell cytoplasm [27, 28]. The number of axons was derived by a direct count and the density was derived from the total endoneurial area assessed. Bands of Büngner and axons associated with this structure were excluded from the analysis. Micrographs (magnification ×6,000) of at least ten endoneurial capillaries were prepared per biopsy. Vessel, basement membrane and luminal areas and endothelial cell profile number per capillary were quantified using our established methodology [26].

Immunohistochemistry Formalin-fixed, paraffin-embedded tissue blocks containing PIN nerves were cut into 5 µm sections and mounted on positively charged slides (three per slide). The sections were dewaxed in xylene and then gradually rehydrated through decreasing ethanol dilutions. Optimal visualisation was obtained by retrieving antigen through

microwaving in citrate buffer pH 6.0. The following anti-human primary antibodies (Abs) were applied overnight at 5°C: mouse monoclonal Abs to HIF-1α (1:150) (Dako, Ely, UK) and rabbit polyclonal Abs to VEGF-A (1:500), VEGFR-1 (1:250) and VEGFR-2 (1:100) (Dako). Subsequently, human anti-mouse IgG secondary Ab was applied to HIF-1α (1:500) (Dako) and biotinylated goat anti-rabbit IgG secondary Abs were applied to VEGF, VEGFR-1 and VEGFR-2 (1:200) (Dako) stained sections for 45 min at room temperature. Negative controls comprised sections in the same run with the primary antibody omitted.

In every run, sections were processed synchronously and developing time was kept consistent. Immunohistochemistry sections were examined blinded using light microscopy (Leica DM RB microscope, Leica Microsystems, Wetzlar, Germany) and digital images of the endoneurium from each fascicle were captured using a Nikon digital camera (Nikon, Tokyo, Japan) (magnification ×268). Endoneurial area and percentage staining were then calculated using the Image Pro-Plus 6.2 image analysis system (Media Cybernetics, Bethesda, MD, USA). Endoneurial blood vessels were not included in expressional analysis. All images from each section were examined and the total endoneurial area was calculated to determine percentage area immunostained (expression).

Statistical analysis Data are presented as mean ± SD unless stated otherwise. The Kolmogorov–Smirnov test was used to compare distributions. Differences between groups were determined using the unpaired *t* test and one-way ANOVA (with the post hoc Tukey test). Pearson's coefficient (*r*) was used to correlate variables. Statistical analysis was performed using SPSS 16.0 software package (SPSS, Chicago, IL, USA). Results were considered significant at $p \leq 0.05$.

Results

Clinical and electrophysiological findings The clinical and electrophysiological characteristics of the patients with and without diabetes, matched for age, are shown in Table 1. HbA_{1c} was significantly greater in the DCTS group ($p < 0.0001$), though this group showed good overall glycaemic control. Patients with type 1 diabetes had a significantly lower BMI compared with NCTS and, as expected, patients with type 2 diabetes had a significantly higher BMI compared with NCTS and patients with type 1 diabetes (Table 2). There was no significant difference in the symptom severity scale between NCTS and DCTS (Table 1). The duration of CTS was shorter and the median sensory nerve conduction

velocity was lower, though not significantly, while the median DML was significantly ($p < 0.05$) more prolonged in patients with diabetes (Table 1). Twelve out of 25 patients in the DCTS group had neuropathy, whereas none of the NCTS group had evidence of neuropathy.

Nerve morphology The fascicular area was increased in the DCTS group compared with the NCTS group but this was not statistically significant (Table 1). However, myelinated nerve fibre density (MNFD) ($p = 0.05$), fibre area ($p < 0.0001$) and axon area ($p < 0.0001$) were significantly reduced in DCTS (Table 1, Fig. 1 g, h). The size frequency distribution showed a significant shift to the left for both myelinated fibre area (Fig. 2a) and axon area (Fig. 2b) in DCTS. The percentage of USCPs ($p < 0.0001$) and the unmyelinated axon density ($p < 0.0001$) were increased, with a significant reduction in axon diameter ($p < 0.0001$) (Table 1, Fig. 1 i, j) and a shift of the size frequency distribution in DCTS (Fig. 2c), consistent with unmyelinated fibre degeneration with regeneration. Endoneurial capillary density did not differ between DCTS and NCTS (Table 1). Endoneurial capillary vessel ($p < 0.0001$) and basement membrane area ($p < 0.0001$) were significantly increased in the DCTS group. However, the luminal area ($p <0.05$) was significantly reduced and endothelial cell profile number ($p < 0.01$) was significantly increased in NCTS vs DCTS (Table 1, Fig. 1 k, l).

Cytokine expression HIF-1α expression (%) was observed in both groups of patients but did not differ between patients with and without diabetes (Table 1). The expression of VEGF, VEGFR-1 and VEGFR-2 was increased in DCTS compared with NCTS (Fig. 1 a–f). Quantitative image analysis demonstrated a significant increase in the expression (%) of VEGF ($p < 0.05$), VEGFR-1 ($p < 0.02$) and VEGFR-2 ($p < 0.05$) in DCTS compared with NCTS (Table 1, Fig. 3).

Type 1 vs type 2 diabetes CTS patients with type 1 diabetes were significantly younger, had a lower BMI, higher HbA_{1c} and longer duration of diabetes compared with those with type 2 diabetes (Table 2). Electrophysiological findings were comparable between type 1 and type 2 diabetes. The expression of VEGF, VEGFR-1 and VEGFR-2 was increased in CTS patients with both type 1 diabetes and type 2 diabetes relative to the NCTS group; however, this was significant only in patients with type 1 diabetes (respectively, $p < 0.05$, $p < 0.02$ and $p < 0.02$) (Table 2, Fig. 3).

The fascicular area was comparable between CTS patients with type 1 diabetes and those with type 2 diabetes (Table 3). The myelinated nerve fibre density did not differ between type 1 and type 2 diabetes but myelinated fibre area ($p < 0.05$) and axon area ($p < 0.01$) were significantly lower in patients with type 1 diabetes than in those with type 2 diabetes. The percentage of USCPs was significantly increased ($p < 0.05$), but there was no significant difference in unmyelinated fibre axon density or diameter in patients with type 1 compared with type 2 diabetes. Endoneurial capillary vessel area ($p < 0.01$) and basement membrane area ($p < 0.001$) were significantly greater in CTS patients with type 1 diabetes compared with type 2 diabetes. There was no difference in capillary luminal area or endothelial cell profile number between patients with type 1 diabetes and type 2 diabetes.

Correlations The expression of HIF-1α correlated significantly with VEGF ($r = 0.34$; $p < 0.05$) and VEGFR-1 ($r = 0.42$; $p < 0.01$). VEGF expression correlated significantly with the expression of VEGFR-1 ($r = 0.78$; $p < 0.0001$) and VEGFR-2 ($r = 0.56$; $p < 0.0001$) and VEGFR-1 and VEGFR-2 expression correlated highly significantly with each other ($r = 0.79$; $p < 0.0001$). VEGF ($r = 0.33$; $p < 0.05$), VEGFR-1 ($r = 0.43$; $p < 0.01$) and VEGFR-2 ($r = 0.42$; $p < 0.01$) expression correlated with diabetes duration but not with age, duration of CTS or HbA_{1c} level.

Correlation between neurophysiology, nerve morphology and immunohistology Median fSNCV correlated significantly with USCP density ($r = -0.30$, $p < 0.05$), endoneurial capillary endothelial cell area ($r = -0.32$; $p < 0.05$), basement membrane area ($r = -0.31$; $p < 0.05$) and vessel area ($r = -0.35$; $p < 0.05$). DML correlated significantly with endoneurial capillary endothelial cell area ($r = 0.36$; $p < 0.05$) and vessel area ($r = 0.35$; $p < 0.05$). There was no correlation between median fSNCV and the expression of HIF-1α, VEGF or its receptors and DML was inversely correlated only with the expression of HIF-1α ($r = -0.36$; $p < 0.05$).

Correlation between immunohistology and nerve morphology Cytokine and receptor expression was compared with nerve morphology (Table 4). VEGF expression correlated with axon area ($r = -0.38$; $p < 0.05$), percentage of USCPs ($r = 0.39$; $p < 0.01$), endothelial cell profile number ($r = -0.36$; $p < 0.05$), endoneurial capillary vessel area ($r = 0.33$; $p < 0.05$) and basement membrane area ($r = 0.32$; $p < 0.05$). VEGFR-1 expression correlated with

myelinated fibre area ($r = -0.47$; $p < 0.01$), myelinated axon area ($r = -0.39$; $p < 0.05$), percentage of USCPs ($r = 0.41$; $p = 0.01$), unmyelinated axon density ($r = 0.46$; $p < 0.01$), endoneurial capillary vessel area ($r = 0.41$; $p = 0.01$) and basement membrane area ($r = 0.43$; $p = 0.01$). VEGFR-2 expression correlated with unmyelinated axon density. ($r = 0.37$; $p < 0.05$), basement membrane area ($r = 0.37$; $p < 0.05$) and endothelial cell profile number ($r = -0.34$; $p < 0.05$). Fascicular area, MNFD, endoneurial capillary density, endothelial cell area and luminal area did not correlate with the expression of any immunostained variable. HIF-1α expression did not correlate with any morphological variable except luminal area ($r = 0.37$; $p < 0.05$).

Discussion

CTS is the most common entrapment neuropathy, occurring six times more frequently in patients with type 1 diabetes and four times more frequently in patients with type 2 diabetes than in the general population [29]. These observations suggest that there are predisposing factors for CTS in patients with diabetes. Given the demonstration of nerve oedema in the median nerve in those with CTS [30], altered VEGF expression/action provides a potential mechanistic link [31]. We demonstrate significant nerve fibre and endoneurial capillary pathology in the PIN biopsy, suggesting that if a similar degree of pathology is present in the median nerve, this will lead to enhanced dysfunction and damage should compression occur. Furthermore, increased expression of endoneurial VEGF, VEGFR-1 and VEGFR-2 in DCTS, and the relationship with severity of neuropathology, provides a potential pathophysiological basis for CTS. These findings may even provide a therapeutic rationale for anti-VEGF therapy as an adjunct or alternative to the surgical treatment of CTS.

Hirata et al [11] showed increased tenosynovial expression of VEGF, but concluded that this was dependent on the clinical phase of CTS (there was evidence for low expression early in CTS development—contesting a causative link). All nerve biopsies in this study were taken in patients with established CTS at the time of carpal tunnel release, therefore any alterations would be considered relevant to the pathophysiology of CTS. The nerve under study (PIN) was not subject to compression. However, its close proximity to the median nerve makes it an ideal nerve for interrogating alterations predisposing to CTS [18]. In support of underlying endoneurial hypoxia in patients with CTS we confirm the expression of HIF-1α in the PIN of both DCTS and NCTS [32]. The significant correlations between the expression of HIF-1α and both VEGF and VEGFR-1, provide a potential link for the development of endoneurial

hypoxia and increased cytokine expression leading to increased vascular permeability and potential neovascularisation. Interestingly increased vascular permeability is also present after nerve compression in animals [33]. Furthermore, this relationship follows the recognised HIF-1α expressional modulation of VEGF and VEGFR-1 [15]. Additionally, strong correlations between the expression of VEGF and its receptors were demonstrated, consistent with reported VEGF-mediated positive autoregulation of VEGFR-1 and -2 [34, 35].

We have previously suggested that endoneurial hypoxia may occur in dDCTS on the basis of a reduction in PIN endoneurial capillary density and endoneurial capillary basement membrane thickening in DCTS compared with NCTS and controls [18]. However, in the present study HIF-1α expression did not differ and there was no difference in endoneurial capillary density between DCTS and NCTS. Although oxygen tension has been proposed to be a key modulator of VEGF expression [36] due to endoneurial hypoxia in diabetic nerves [17], the present study cannot attribute higher VEGF expression in patients with diabetes to either morphological (endoneurial capillary density) or immunohistological (HIF-1α) evidence of hypoxia. VEGF expression can be induced by hyperglycaemia [37], but we did not find a correlation with HbA_{1c} levels [38]. However, a single recent HbA_{1c} may not be representative of lifetime glycaemic control. Additionally, AGEs, known to be elevated in patients with diabetes, also upregulate *VEGF* mRNA levels through activation of the receptor for AGEs (RAGE) [39].

Enhanced nerve fibre damage due to ischaemia is likely to play an important role in the development and progression of CTS [40] and may also contribute to a lesser resolution of symptoms and electrophysiological resolution after surgery for CTS in patients with diabetes [41, 42]. Our morphometric studies confirm more advanced myelinated and unmyelinated fibre pathology in DCTS. We also demonstrate more advanced endoneurial microangiopathy, which correlated with increased expression of VEGF and its receptors and median nerve electrophysiology. As endoneurial capillary basement membrane thickening predisposes to endoneurial hypoxia [43–45], the association of VEGF and its receptors with this pathology provides a possible mechanistic link to hypoxia-mediated increases in VEGF expression.

CTS has been shown to occur more commonly in type 1 diabetes than in type 2 diabetes [29, 46–48], but the underlying mechanism for this is not clear. The enhanced expression of endoneurial VEGF and its receptors in patients with type 1 diabetes, compared with type 2 diabetes, may provide an explanation. Indeed patients with type 1 diabetes are at higher risk

of developing proliferative retinopathy [49] and VEGF has been shown to be strongly expressed in the vitreous and neovascular tufts of those with type 1 but not type 2 diabetes [50]. This increased propensity for the development of CTS could be further accounted for by the more advanced endoneurial capillary microangiopathy and more severe myelinated and unmyelinated nerve fibre pathology in type 1 diabetes than in type 2 diabetes.

A major limitation of this study is of course the fact that the PIN does not undergo compression and we should therefore be cautious in the interpretation of our findings to the actual pathophysiological changes that may occur in the median nerve. We also have a small sample size, especially when stratifying for type of diabetes, although in support of our data the differences in endoneurial expression between type 1 and type 2 diabetes and between diabetic and non-diabetic patients with CTS were highly significant. Another limitation of the study is the lack of a comparison with PIN biopsies from a control group unaffected by CTS, which would have enabled us to define pathological and immunohistological alterations in CTS per se. However, it was not ethically feasible to perform PIN biopsy in individuals without CTS, and the use of post-mortem samples was not appropriate due to the significant morphological change that can occur after death, rendering significant inaccuracies in morphometric and immunohistological studies.

In summary, this study provides molecular and pathological insights into factors that may predispose diabetic patients to CTS. Further studies are warranted to help fully elucidate the role of VEGF in the development of CTS.

Acknowledgements

The study was supported by the Swedish Research Council (Medicine; No. 5188), the Medical Faculty, Lund, Diabetesföreningen i Malmö med omnejd, Skåne University Hospital, Malmö and Region Skåne (ALF).

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement

MAM, MA, RA, MJ and AA made substantial contributions to the acquisition, analysis and interpretation of data and drafted the article. NOBT and LBD made substantial contributions to the conception and design of the study and the acquisition, analysis and interpretation of data and revised the article critically for important intellectual content. RAM made

substantial contributions to the conception and design of the study, analysis and interpretation of data and drafting and revising the article critically for important intellectual content. All authors gave final approval for the version to be published. RAM is the guarantor of this work.

References

[1] Bickel KD (2010) Carpal tunnel syndrome. J Hand Surg Am 35: 147-152

[2] de Krom MC, Knipschild PG, Kester AD, Thijs CT, Boekkooi PF, Spaans F (1992) Carpal tunnel syndrome: prevalence in the general population. J Clin Epidemiol 45: 373-376

[3] Atroshi I, Gummesson C, Johnsson R, Ornstein E, Ranstam J, Rosen I (1999) Prevalence of carpal tunnel syndrome in a general population. JAMA 282: 153-158

[4] Geoghegan JM, Clark DI, Bainbridge LC, Smith C, Hubbard R (2004) Risk factors in carpal tunnel syndrome. J Hand Surg Br 29: 315-320

[5] Burner TW, Rosenthal AK (2009) Diabetes and rheumatic diseases. Curr Opin Rheumatol 21: 50-54

[6] Kim RP, Edelman SV, Kim DD (2001) Musculoskeletal complications of diabetes mellitus. Clinical Diabetes 19: 132-135

[7] Mesgarzadeh M, Schneck CD, Bonakdarpour A, Mitra A, Conaway D (1989) Carpal tunnel: MR imaging. Part II. Carpal tunnel syndrome. Radiology 171: 749-754

[8] Buchberger W, Judmaier W, Birbamer G, Lener M, Schmidauer C (1992) Carpal tunnel syndrome: diagnosis with high-resolution sonography. AJR Am J Roentgenol 159: 793-798

[9] Richard R (1999) Carpal tunnel syndrome and the myth of El Dorado. Muscle Nerve 22: 1165-1167

[10] Donato G, Galasso O, Valentino P, et al. (2009) Pathological findings in subsynovial connective tissue in idiopathic carpal tunnel syndrome. Clin Neuropathol 28: 129-135

[11] Hirata H, Nagakura T, Tsujii M, Morita A, Fujisawa K, Uchida A (2004) The relationship of VEGF and PGE2 expression to extracellular matrix remodelling of the tenosynovium in the carpal tunnel syndrome. J Pathol 204: 605-612

[12] Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25: 581-611

[13] Bates DO, Harper SJ (2002) Regulation of vascular permeability by vascular endothelial growth factors. Vascul Pharmacol 39: 225-237

[14] Pugh CW, Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9: 677-684

[15] Gerber HP, Condorelli F, Park J, Ferrara N (1997) Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 272: 23659-23667

[16] Kim WK, Kwon SH, Lee SH, Sunwoo IN (2000) Asymptomatic electrophysiologic carpal tunnel syndrome in diabetics: entrapment or polyneuropathy. Yonsei Med J 41: 123-127

[17] Dahlin LB, Meiri KF, McLean WG, Rydevik B, Sjostrand J (1986) Effects of nerve compression on fast axonal transport in streptozotocin-induced diabetes mellitus. An experimental study in the sciatic nerve of rats. Diabetologia 29: 181-185

[18] Thomsen NO, Mojaddidi M, Malik RA, Dahlin LB (2009) Reduced myelinated nerve fibre and endoneurial capillary densities in the forearm of diabetic and non-diabetic patients with carpal tunnel syndrome. Acta Neuropathol 118: 785-791

[19] Low PA (1987) Recent advances in the pathogenesis of diabetic neuropathy. Muscle Nerve 10: 121-128

[20] Cameron NE, Mathias CJ (2003) Structure and function of the nervous system. In: Gries FA, Cameron NE, Low PA, Ziegler D (eds), Textbook of diabetic neuropathy. Thieme, Stuttgart, Germany. p. 50

[21] Thomsen NO, Mojaddidi M, Malik RA, Dahlin LB (2009) Biopsy of the posterior interosseous nerve: a low morbidity method for assessment of peripheral nerve disorders. Diabetic Medicine 26: 100-104

[22] Thomsen NO, Cederlund R, Bjork J, Dahlin LB (2010) Health-related quality of life in diabetic patients with carpal tunnel syndrome. Diabet Med 27: 466-472

[23] Rosen I (1993) Neurophysiological diagnosis of the carpal tunnel syndrome: evaluation of neurographic techniques. Scand J Plast Reconstr Surg Hand Surg 27: 95-101

[24] Malik RA, Veves A, Walker D, et al. (2001) Sural nerve fibre pathology in diabetic patients with mild neuropathy: relationship to pain, quantitative sensory testing and peripheral nerve electrophysiology. Acta Neuropathol 101: 367-374

[25] Quattrini C, Jeziorska M, Boulton AJ, Malik RA (2008) Reduced vascular endothelial growth factor expression and intra-epidermal nerve fiber loss in human diabetic neuropathy. Diabetes Care 31: 140-145

[26] Malik RA, Tesfaye S, Newrick PG, et al. (2005) Sural nerve pathology in diabetic patients with minimal but progressive neuropathy. Diabetologia 48: 578-585

[27] Ochoa J (1978) Recognition of unmyelinated fiber disease: morphologic criteria. Muscle Nerve 1: 375-387

[28] Jacobs JM, Love S (1985) Qualitative and quantitative morphology of human sural nerve at different ages. Brain 108: 897-924

[29] Chammas M, Bousquet P, Renard E, Poirier J-L, Jaffiol C, Allieu Y (1995) Dupuytren's disease, carpal tunnel syndrome, trigger finger, and diabetes mellitus. J Hand Surg Am 20: 109-114

[30] Schmid AB, Elliott JM, Strudwick MW, Little M, Coppieters MW (2012) Effect of splinting and exercise on intraneural edema of the median nerve in carpal tunnel syndrome--an MRI study to reveal therapeutic mechanisms. J Orthop Res 30: 1343-1350

[31] Wirostko B, Wong TY, Simo R (2008) Vascular endothelial growth factor and diabetic complications. Prog Retin Eye Res 27: 608-621

[32] Carmeliet P, Storkebaum E (2002) Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. Semin Cell Dev Biol 13: 39-53

[33] Rydevik B, Lundborg G (1977) Permeability of intraneural microvessels and perineurium following acute, graded experimental nerve compression. Scand J Plast Reconstr Surg 11: 179-187

[34] Wilting J, Birkenhäger R, Eichmann A, et al. (1996) VEGF121 Induces proliferation of vascular endothelial cells and expression of flk-1 without affecting lymphatic vessels of the chorioallantoic membrane. Developmental Biology 176: 76-85

[35] Barleon B, Siemeister G, Martiny-Baron G, Weindel K, Herzog C, Marme D (1997) Vascular endothelial growth factor up-regulates its receptor fms-like tyrosine kinase 1 (FLT-1) and a soluble variant of FLT-1 in human vascular endothelial cells. Cancer Res 57: 5421-5425

[36] Minchenko A, Bauer T, Salceda S, Caro J (1994) Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. Lab Invest 71: 374-379

[37] Hoshi S, Nomoto K, Kuromitsu J, Tomari S, Nagata M (2002) High glucose induced vegf expression via PKC and ERK in glomerular podocytes. Biochemical and Biophysical Research Communications 290: 177-184

[38] Chiarelli F, Spagnoli A, Basciani F, et al. (2000) Vascular endothelial growth factor (VEGF) in children, adolescents and young adults with type 1 diabetes mellitus: relation to glycaemic control and microvascular complications. Diabetic Medicine 17: 650-656

[39] Okamoto T, Yamagishi S, Inagaki Y, et al. (2002) Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin. FASEB J 16: 1928-1930

[40] Hart AM, Wiberg M (2001) Nerve compression or mechanical neuropathy: neuropathology. Current Orthopaedics 15: 245-248

[41] Ozkul Y, Sabuncu T, Kocabey Y, Nazligul Y (2002) Outcomes of carpal tunnel release in diabetic and non-diabetic patients. Acta Neurol Scand 106: 168-172

[42] Wilbourn A (1999) Diabetic entrapment and compression neuropathies. In: Dyck P, Thomas P (eds), Diabetic neuropathy. WB Saunders, Philadelphia, pp 481-508

[43] Sugimoto K, Shoji M, Yasujima M, Suda T, Yagihashi S (2004) Peripheral nerve endoneurial microangiopathy and necrosis in rats with insulinoma. Acta Neuropathologica 108: 503-514

[44] Powell HC, Rosoff J, Myers RR (1985) Microangiopathy in human diabetic neuropathy. Acta Neuropathologica 68: 295-305

[45] Malik RA, Newrick PG, Sharma AK, et al. (1989) Microangiopathy in human diabetic neuropathy: relationship between capillary abnormalities and the severity of neuropathy. Diabetologia 32: 92-102

[46] Renard E, Jacques D, Chammas M, et al. (1994) Increased prevalence of soft tissue hand lesions in type 1 and type 2 diabetes mellitus: various entities and associated significance. Diabete Metab 20: 513-521

[47] Cagliero E, Apruzzese W, Perlmutter GS, Nathan DM (2002) Musculoskeletal disorders of the hand and shoulder in patients with diabetes mellitus. The American Journal of Medicine 112: 487-490

[48] Dyck PJ, Kratz KM, Karnes JL, et al. (1993) The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study. Neurology 43: 817-824

[49] Henricsson M, Nilsson A, Groop L, Heijl A, Janzon L (1996) Prevalence of diabetic retinopathy in relation to age at onset of the diabetes, treatment, duration and glycemic control. Acta Ophthalmol Scand 74: 523-527

[50] Kinnunen K, Puustjärvi T, Teräsvirta M, et al. (2009) Differences in retinal neovascular tissue and vitreous humour in patients with type 1 and type 2 diabetes. British Journal of Ophthalmology 93: 1109-1115

Table 1 Clinical, electrophysiological, morphological and immunohistological findings in non-diabetic and diabetic patients with CTS

Variable	NCTS	DCTS
n	19	25
Age (years)	54.7 ± 11.1	55.8 ± 12.7
Diabetes duration (years)	–	18.6 ± 12.4
HbA_{1c} (%)	4.5 ± 0.3	7.1 ± 1.3***
HbA_{1c} (mmol/mol)	25.7 ± 3.2	54.2 ± 13.9***
CTS duration (months)	44.1 ± 41.6	27.6 ±19.36
fSNCV (m/s)	22.8 ± 12.8	17.4 ± 12.5
DML (ms)	5.5 ± 1.3	6.4 ± 1.6*
Fascicular area (mm^2)	0.09 ± 0.04	0.12 ± 0.05
Myelinated fibres		
Density (No./mm^2)	6,501 ± 1,948	5,139 ± 1,262*
Fibre area (μm^2)	36.2 ± 6.3	22.8 ± 5.0****
Axon area (μm^2)	12.1 ± 1.6	7.9 ± 2.1****
Unmyelinated fibres		
USCP (%) cell profiles	13.6 ± 3.8	33.6 ± 12.0****
Axon diameter (μm)	0.76 ± 0.12	0.57 ± 0.10****
Axon density (No./$mm^2 \times 10^3$)	48.0 ± 11.1	134.0 ± 31.9****
Endoneurial capillaries		
Density (No./mm^2)	62.5 ± 24.9	56.5 ± 20.7
Luminal area (μm^2)	9.7 ± 9.0	18.6 ± 14.2*
Vessel area (μm^2)	89.9 ± 31.4	372.4 ± 167.8****
Basement membrane area (μm^2)	27.3 ± 13.3	278.4 ± 134.8****
Endothelial cell profile No.	6.9 ± 1.5	5.0 ± 1.4***
HIF-1α (%)	30.8 ± 14.2	33.1 ± 19.0
VEGF (%)	46.0 ± 28.5	62.2 ± 19.5*
VEGFR-1 (%)	49.1 ± 25.7	69.2 ± 20.5†

VEGFR-2 (%)	51.9 ± 31.3	68.8 ± 19.2*

*p<0.05, †p<0.02,**p<0.01, ***p<0.001, ****p<0.0001 vs NCTS

Table 2 Clinical, electrophysiological and immunohistological findings in CTS patients, comparing type 1 diabetes with type 2 diabetes

Variable	NCTS	DCTS	
		Type 1 diabetes	Type 2 diabetes
n	19	11	14
Age (years)	54.7 ± 11.1	46.7 ± 10.7*	60.7 ± 10.6‡
Diabetes duration (years)	–	28.6 ± 10.9	11.1 ± 7.5‡‡‡‡
BMI (kg/m^2)	27.0 ± 3.7	23.7 ± 3.9*	31.1 ± 2.4**‡‡‡‡
Symptom severity scale	2.76 ± 0.72	2.91 ± 0.86	3.17 ± 0.94
HbA_{1c} (%)	4.5 ±0.3	7.6 ± 1.1****	6.7 ± 1.2****‡
HbA_{1c} (mmol/mol)	25.7 ± 3.2	60.1 ± 12.5****	49.7 ± 13.6****‡
CTS duration (months)	44.1 ± 41.6	21.2 ± 9.8	32.7 ± 23.6
fSNCV (m/s)	22.8 ± 12.8	17.4 ± 12.5	22.8 ± 12.8
DML (ms)	5.5 ± 1.3	6.4 ± 1.8	6.4 ± 1.5
HIF-1α (%)	30.8 ± 14.2	33.8 ± 17.2	32.6 ± 21.1
VEGF (%)	46.0 ± 28.5	69.8 ± 15.6*	54.7 ± 20.7
VEGFR-1 (%)	49.1 ± 25.7	79.3 ± 18.3†	60.0 ± 18.4
VEGFR-2 (%)	51.9 ± 31.3	80.4 ± 16.4†	58.2 ± 15.4

*p<0.05, †p<0.02, **p<0.01, ****p<0.0001 vs NCTS; ‡p<0.05; ‡‡‡‡p<0.0001, type 1 vs type 2 diabetes

Table 3 Differences in PIN morphology in CTS patients

Variable	NCTS	DCTS	
		Type 1 diabetes	Type 2 diabetes
n	19	11	14
Fascicular area (mm^2)	0.09 ± 0.04	0.12 ± 0.05	0.12 ± 0.06
Myelinated fibres			
Density (No./mm^2)	6,501 ± 1,948	4,913 ± 1,149	5,310 ± 1,366
Fibre area (μm^2)	36.2 ± 6.3	19.3 ± 3.8****	25.8 ± 3.7****†
Axon area (μm^2)	12.1 ± 1.6	6.3 ± 1.3****	9.2 ± 1.6****††
Unmyelinated fibres			
USCP (%)	13.6 ± 3.8	38.9 ± 17.1****	29.8 ± 4.0****†
Axon diameter (μm)	0.76 ± 0.12	0.55 ± 0.10****	0.59 ± 0.10****
Axon density (No./mm^2 × 10^3)	48.0 ± 11.1	140.8 ± 28.5****	129.2 ± 34.2****
Endoneurial capillaries			
Density (No./mm^2)	62.5 ± 24.9	51.6 ± 18.4	60.2 ± 22.3
Luminal area (μm^2)	9.7 ± 9.0	16.3 ± 10.9	20.7 ± 16.9*
Vessel area (μm^2)	89.9 ± 31.4	462.0 ± 194.0****	291.1 ± 84.7****††
Basement membrane area (μm^2)	27.3 ± 13.3	361.2 ± 145.9****	203.1 ± 63.9****†††
Endothelial cell profile No.	6.9 ± 1.5	5.2 ± 0.6***	5.9 ± 1.7***

*$p<0.05$, ***$p<0.001$ and ****$p<0.0001$ vs NCTS group; †$p<0.05$, ††$p<0.01$, †††$p<0.001$, type 1 vs type 2 diabetes

Table 4 Correlation between PIN cytokine/receptor expression and morphology

Fibre/vessel	VEGF	VEGFR-1	VEGFR-2
Myelinated fibre			
Axon area	$r = -0.38$; $p = 0.03$[a]	$r = -0.39$; $p = 0.03$[a]	*NS*
Fibre area	$r = -0.32$; $p = 0.06$	$r = -0.47$; $p < 0.01$[a]	$r = -0.33$; $p = 0.07$
Unmyelinated fibre			
Axon density	NS	$r = 0.46$; $p < 0.01$[a]	r = 0.37; $p = 0.02$[a]
USCP	$r = 0.39$; $p = 0.01$[a]	$r = 0.41$; $p = 0.01$[a]	$r = 0.31$; $p = 0.06$
Endoneurial capillaries			
Vessel area	$r = 0.33$; $p < 0.05$[a]	$r = 0.41$; $p = 0.01$[a]	$r = 0.32$; $p = 0.06$
Basement membrane area	$r = 0.32$; $p = 0.05$[a]	$r = 0.43$; $p = 0.01$[a]	$r = 0.37$; $p = 0.03$[a]
Endothelial cell No.	$r = -0.36$; $p = 0.03$[a]	NS	$r = -0.34$; $p < 0.05$[a]

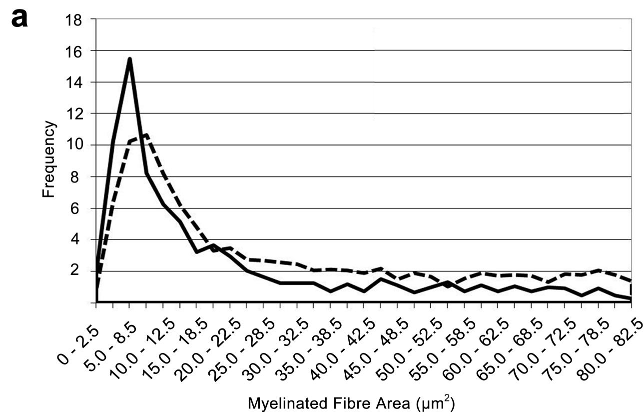
[a]Significant correlations

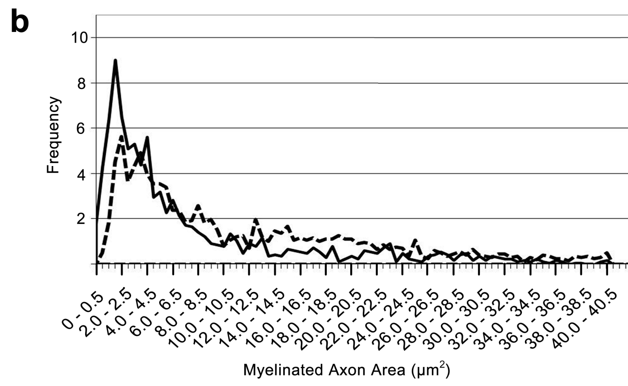
Figure legends

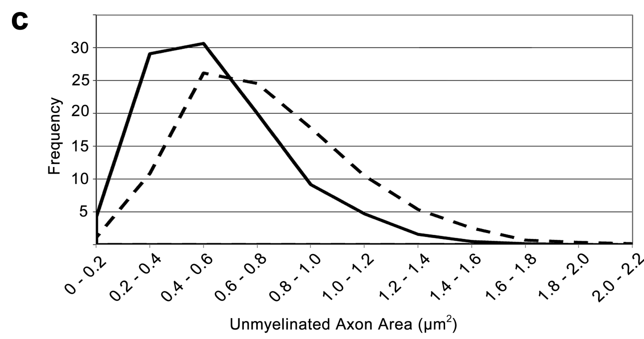
Fig. 1 (**a**–**f**) PIN sections, from representative cases of non-diabetic (**a**, **c**, **e**) and diabetic patients (**b**, **d**, **f**), immunostained for VEGF (**a**, **b**), VEGFR1 (**c**, **d**) and VEGFR2 (**e**, **f**), showing more intense expression of all three proteins in diabetic patients with CTS. (**g**, **h**) Light micrographs showing moderate loss of myelinated fibres in diabetic (**h**) compared with non-diabetic (**g**) patients with CTS. (**i**–**l**) Electronmicrographs showing unmyelinated nerve fibre degeneration with regenerative sprouts and more pronounced endoneurial microangiopathy in diabetic (**j**, **l**) compared with non-diabetic patients (**i**, **k**) with CTS, respectively. (a-f scale bar = 10μm, g and h scale bar = 1 μm, and k and l scale bar =2 μm)

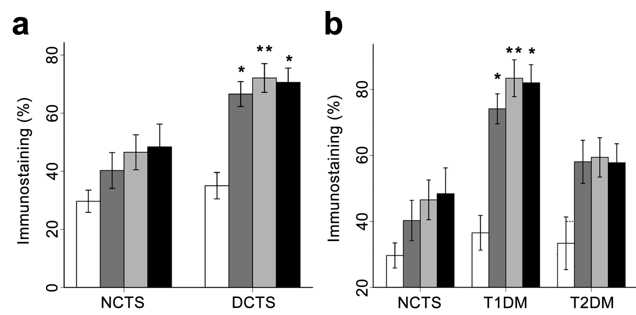
Fig. 2 Composite size frequency distribution diagrams for myelinated fibre area (**a**), axon area (**b**) and unmyelinated axon diameter (**c**), showing an increase in the percentage of myelinated fibres and axons and unmyelinated axons of smaller size in DCTS (solid vs dashed line)

Fig. 3 Endoneurial cytokine/receptor immunostained area (%), expressed as mean ± SE. (a) control subjects versus all patients with diabetes, (b) control subjects versus patients with T1DM and T2DM; $^{*}p<0.05$ and $^{\dagger}p<0.02$ vs NCTS group; White bars, HIF-1α; dark-grey bars, VEGF; light-grey bars, VEGFR-1; black bars, VEGFR-2; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus


a
Frequency
Myelinated Fibre Area (μm2)


b
Frequency
Myelinated Axon Area (μm2)


c
Frequency
Unmyelinated Axon Area (μm2)


a
b
Immunostaining (%)
0
20
40
60
80
*
**
*
NCTS
DCTS
Immunostaining (%)
20
40
60
80
*
**
*
NCTS
T1DM
T2DM