Biotechnological Advances for Diagnosis of Peripheral Diabetic Neuropathy

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Abstract
Diabetic neuropathy is a major challenge for the healthcare system, being associated with multiple local and general complications which involve increased medical and socio-economic costs and an important reduction of the patient’s quality of life.

In this paper we review the relevant aspects regarding the micro-morphological and pathophysiological changes in peripheral diabetic neuropathy, we summarize the main techniques used for diagnosis and staging of diabetic neuropathy and we discuss the biotechnological advances that were achieved in this field. Thus, recent developments in proteomics and in vivo investigation of cutaneous nerve structures provides promising data and could lead to the achievement of new biotechnological diagnostic strategies easy to implement, with a greater accuracy of results, which allow the diagnosis of early changes in diabetic neuropathy, in a stage in which the preventive and therapeutical measures have maximum efficiency, thus being able to contribute to the diminishing of the morbidity associated with the disease.

Keywords: diabetic neuropathy, biotechnology, diagnosis, staging, reflectance confocal microscopy

1. Introduction
Diabetic neuropathy is a severe chronic complication of diabetes mellitus and is the most frequent form of neuropathy in the developed countries [1-8]. It affects 40-60% of the diabetic patients [6, 9], representing a significant cause of morbidity and mortality [2, 5, 7, 10]. Diabetic neuropathy is associated with a whole range of local and general complications that inflict important health care costs and reduce the patient’s quality of life, with an overall major socio-economic impact. The local and general complications include the neuropathic chronic pain, the development of neuro-osteoarthropathy lesions leading to the aspect of diabetic Charcot foot, or the appearance of recurrent ulcers of the distal extremities, localized infections that can spread and that can lead to amputation. [11, 12].

The most frequent form of diabetic neuropathy is the distal symmetrical polineuropathy [2, 7, 10], which is a gradual, diffuse, symmetrical impairment of the peripheral nerve fibers of the extremities [8, 13]. Sensory, motor but also autonomic nerve fibers can be involved [6, 14]. However, the most frequent form of peripheral diabetic neuropathy is the sensory one [5].
The nerve fibers impairment can appear early in diabetes mellitus [15, 16], some authors emphasizing as well as an association between the polineuropathic changes and the impaired glucose tolerance [17]. The disease can be triggered by various pathophysiological mechanisms, having a heterogeneous symptomatology associated with a variable evolution [6, 14].

The clinical manifestations of diabetic neuropathy include disorders of the thermor-algesic, tactile, vibratory and pressure sensitivity. Changes of pain sensitivity may range from hyperalgesia and allodynia to an important decrease of the perception of nociceptive stimuli. Moreover, the decrease of the sudomotor activity can be accompanied by consecutive cutaneous xerosis. The symptoms appear and are more severe at the distal regions, having a “gloves and stockings” distribution, but evolve with a proximal progression [4-6, 18-20].

The distal regions are characterized by a high density of skin nerve fibers and an increased variety of their types [4]. The diverse symptomatology including changes of tactile, thermic and pain-perception sensitivity may suggests impairment of the thin myelinated A-delta nerve fibers or unmyelinated C fibers as well as at the level of large diameter myelinated A-beta nerve fibers; furthermore, the vasoregulatory and sudomotor disorders indicate the involvement of autonomic innervation [4, 5, 13, 21-26].

The diagnosis of diabetic neuropathy, especially in an early stage where treatment has maximum efficacy, is a major challenge in clinical practice. The biotechnological advances in this field, particularly the recent developments in proteomics and in vivo investigation of cutaneous nerve structures, are very promising and could contribute to the early diagnosis and the reduction of the morbidity associated with the disease.

2. Micro-morphological and pathophysiological changes in peripheral diabetic neuropathy

Previous research has shown that the first changes in peripheral diabetic neuropathy usually affect the thin myelinated A-delta nerve fibers or unmyelinated type C fibers [22, 27]. Most of the studies concerning the changes of nerve fibers in diabetic neuropathy performed on human subjects have noted a reduction in the density of cutaneous nerve fibers, in patients diagnosed with diabetes mellitus type 1 and 2 [23, 28, 29] and in patients with impaired glucose tolerance [30]; in this cases a significant reduction of the density of intraepidermal nerve fibers being noticed [31-35]. Previous research [21, 31] has also highlighted a decreased expression of neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP), prevalent in the small diameter sensory nerve fibers, as well as of vasoactive intestinal peptide (VIP) and neuropeptide Y (NYP), prevalent in the autonomic nerve fibers [36]. Studies carried out on diabetic patients have emphasised in addition a decreased number of dermal and epidermal nerve fibers positive for the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) but also a reduced expression of TRPV1 in the remaining nerve fibers.

TRPV1 is a ligand-gated, nonselective cation channel that integrates various noxious stimuli, being involved in the transmission and modulation of pain. It is predominantly expressed in the unmyelinated type C sensory nerve fibers or the thin myelinated A-delta nerve fibers [37], and this reduction of the immunoreactivity for TRPV1, possibly triggered by a decrease of nerve growth factor (NGF), seems to precede the reduction in the density of these nerve fibers [38]. The diminution of TRPV1 expression can trigger sensitivity disorders specific for diabetic neuropathy, the TRPV1 receptor being activated by physical triggers like high temperatures (>43°C), by the considerable increase in the concentration of H+ ions.
(pH<6), as well as by endocannabinoids (anandamide), by capsaicin and other vanilloid substances [37, 39-41].

Studies carried out on non-human primates have highlighted the association of diabetes with a significant remodeling of skin innervation of the extremities, involving all types of nerve fibers [4]. Thus, an accelerated decrease of the intraepidermal thin nerve fibers density has been noticed, together with the reduction of the expression of neuropeptides like CGRP and of TRPV1 receptor. Likewise, a hypertrophy and an increased number of Meissner corpuscles innervated by thick A-beta nerve fibers have been highlighted [4].

Studies performed on murine models of diabetes revealed the early occurrence of some functional changes including thermal hyperalgesia and mechanical allodynia [15, 42-44] caused by the hyperactivity and increased sensitivity of both unmyelinated type C nerve fibers [15, 45, 46] and myelinated thin A-delta and thick A-beta fibers [42]. The results of previous studies have shown that the impairment of nociceptive sensitivity in diabetic neuropathy could be associated with changes in the expression and activity of TRPV1 receptor. Thus, in dorsal root ganglia of diabetic rats it has been shown an increase of TRPV1 expression in the origin neurons of large diameter myelinated A-beta fibers, and a reduction of TRPV1 expression in the origin neurons of small diameter sensory fibers; however, the activation of TRPV1 receptor by capsaicin or low pH triggers a higher response, suggesting the involvement of modulation of expression and activity of the capsaicin-sensitive TRPV1 receptor in phenomena such as hyperalgesia and allodynia in diabetic neuropathy [16]. Other studies performed on diabetic mice have also emphasized an increase in TRPV1 sensitivity associated with allodynia and hyperalgesia [45].

Moreover, in diabetic neuropathy, in peripheral nerve fibers subcellular disturbances have been reported, such as reduction of ATPases activity, mitochondrial dysfunctions and other metabolic disorders associated with decreased nerve conduction and axo-glial morphological changes, all these phenomena leading to atrophy and loss of nerve fibers [47-49]. The mechanisms that lie at the root of these changes are still incompletely revealed [5], yet the impairment of the nerve fibers is associated with a precarious control of glycemia, changes in lipid profile, accumulation of advanced glycation end products and oxidative stress generated products [6, 50-52]. Microvascular changes and disorders of endothelial function associated with diabetes could also be involved in the development of neuropathy [53, 54]. The endoneurial microvessels of diabetic patients show striking changes of the vessel walls with pericyte degeneration and reduplicated basement membranes [53]. The damage of vascular endothelium may be induced by the metabolic changes in diabetes with increased levels of oxygen free radicals. This also may lead to neutralization of nitric oxide (NO) inducing a reduced vasa nervorum vasodilation response with a consecutive decrease of nerve perfusion leading to impaired nerve function [54].

3. Diagnosis and staging of diabetic neuropathy

The diagnosis and staging of diabetic neuropathy can be achieved through various methods assessing the sensory, motor and autonomic fibers. The assessment also refers to the changes which appear both in small and large diameter nerve fibers [55].

The clinical examination still remains fundamental in the diagnosis of diabetic neuropathy [2, 10]. The vibratory, pressure, proprioceptive, tactile and thermo-algesic perception are investigated and the myotatic reflexes and the muscular force are evaluated. The presence of clinical signs that are suggestive for neuropathy such as cutaneous xerosis, infections, ulcers or even deformities of the extremities are also considered [56, 57]. Usually a
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standardized clinical examination which allows the calculation of a neuropathy score is undertaken; systems that are frequently used being the Michigan Neuropathy Screening Instrument (MNSI) [56] and the Neuropathy Disability Score (NDS) [57].

The quantitative and semi-quantitative sensory evaluation can be achieved using various types of devices specific for each type of sensitivity. Thus, in order to test vibratory sensitivity a tuning fork of 64/128 Hz [56-59] or devices like Neurothesiometer, Biothesiometer, Vibratron or Vibratron II can be used [60-64]. Tactile sensitivity can be tested with a 10-g monofilament [63], while the pressure sensitivity can be tested with the Neuphen [9, 63, 64]. Thermal sensitivity can be investigated using devices equipped with automatic cooling or heating probes or by devices like Therm Tip [65-67]. The nociceptor function can be evaluated using a device like Algometer [12, 59, 68, 69] or like Neurotip [9, 63, 64]. Complex, digitally-controlled systems have been developed in order to detect the sensitivity threshold for various types of sensations [9].

Nevertheless, the clinical evaluation and the quantitative sensory determinations using various devices are largely biased by subjective reports of the patients and/or even by the investigators [9, 12, 70, 71].

Nerve conduction studies can emphasize the dysfunctions of diabetic neuropathy [6], and enable the early diagnosis of neuropathic changes [72, 73]. However, the laborious character of the investigation makes it difficult to use this technique as a screening test for diabetic patients [2, 7].

The impairment of the autonomic nerve fibers in distal diabetic neuropathy can be assessed through different tests of the sudomotor function, as well as through the quantitative sudomotor axon reflex test, which, however, requires complex equipment and a laborious methodology [7].

During the last years, investigation of the morphology of cutaneous nerve fibers and evaluation of their density became more and more important in the early identification of peripheral neuropathic impairment associated with diabetes mellitus. The histological evaluation of a skin sample harvested by biopsy allows the investigation of various types of nerve fibers, ranging from large diameter myelinated fibers to thin unmyelinated fibers and enabling the investigator with an exact framing of the disease together with the evaluation of its progress [4, 9, 19, 32, 38]. Immunohistochemistry allows the identification of intraepidermal nerve fibers, together with the study of dermal peptidergic or non-peptidergic nerve fibers using antibodies targeted against various parts of neural structures [21, 30, 36, 74-76]. One of the most used markers in immunohistochemistry is PGP 9.5 (protein gene product 9.5), which allows the labelling of every nervous structure in a certain tissue. Using antibodies targeted against specific structures, various subpopulations of nerve fibers can be highlighted. Thus, by means of this technique, especially in the nerve endings of thin sensory fibers, the presence of a multitude of neuropeptides and neurohormons such as substance P, CGRP, neurokinin A, galanin or α-MSH (α-melanocyte-stimulating hormone) [77, 78] was demonstrated. Immunoreactivity for NPY and atrial natriuretic peptide (ANP) was observed in the autonomic fibers, this labeling being able to differentiate them from sensory nerve fibers [36, 79]. Another marker of cutaneous autonomic nerve fibers is tyrosine hydroxylase [80].

The evaluation of intraepidermal nerve fibers density represents an efficient way to emphasize the impairment of small-diameter nerve fibers. Also, highlighting of morphological changes, such as diffuse swellings of intraepidermal nerve fibers may be a predictive factor for the progression of neuropathy [81]. Moreover, in the diabetic patients the histological evaluation of the density of fibers which innervate the sweat glands is
correlated with the sudomotor function and the neuropathic symptomatology [82]. Thus, the histological evaluation of cutaneous innervation is an essential element in the diagnostic strategy of diabetic neuropathy. However, the information provided with regard to the functionality of nerve fibers is limited, and the invasive character, the technical complexity and the increased costs of the investigation do not allow the large scale use of this method.

Another way to evaluate the morphological changes of nerve fibers in diabetic neuropathy is the nerve biopsy, usually performed at the sural nerve, but the technique is invasive and quite laborious, which significantly limits its applicability in clinical practice [6, 83].

Information regarding the state of the thin nerve fibers can be obtained by investigating the corneal nerve endings through confocal microscopy [84, 85]. Although the impairment of the corneal nerve fibers is correlated with the lowering of the density of intraepidermal nerve fibers [85], their investigation provides only indirect information concerning the changes of the cutaneous innervation [6].

4. Biotechnological advances for the assessment of diabetic neuropathy

In spite of the great number of evaluation techniques, in clinical practice diabetic neuropathy is still underdiagnosed [7, 86]. Developing new methods or protocols useful for the assessment of diabetic neuropathy represents a major challenge for the scientific research [7, 8].

4.1. Early markers - cutaneous nerve fibers evaluation

One of the main topics of interest is the evaluation of the functionality of cutaneous small diameter nerve fibers as their alteration constitutes an early marker of neuropathy in diabetes mellitus [9, 85, 87]. At the moment the available techniques have limited applicability, are biased and involve a great variability [88]. This is why identification of new methods for evaluation of thin nerve fibers injury is a major challenge.

The vasodilatory response induced by axon reflex expresses directly the activation of the small diameter nociceptive nerve fibers; previous studies have suggested the possibility of using the evaluation of this neurovascular response in order to appreciate the functionality of the nociceptive nerve fibers [89, 90]. Recent research has validated the measurement of neurovascular cutaneous response as an objective evaluation method of diabetic neuropathy. The alterations of this vasodilatory response appear in the initial stages of diabetic neuropathy, when quantitative sensory tests are still unchanged. Thus, the cutaneous neurovascular response could be an early marker of the thin sensory nerve fibers dysfunction, even in the subclinical stage of neuropathy [88, 9, 91].

4.2. Models of cutaneous neurovascular response

In order to trigger the neurovascular response, various methods have been used, such as the local administration of acetylcholin through iontophoresis [88] or the application of thermic stimuli [9, 92], the evaluation of the local vasodilatory response being achieved with laser Doppler flowmetry evaluation.

However, one of the best known and intensely studied models of cutaneous neurovascular response is the one triggered by the local administration of capsaicin, the hot ingredient of chilli pepper (Capsicum annuum). Capsaicin directly activates the vanilloid TPRV1 receptor [37], which, as we have previously mentioned, is predominantly localized in the thin sensory fibers and plays an important role in the pathogenesis of diabetic neuropathy. The stimulation of nerve endings by capsaicin activates an orthodromic signal and causes a sensation of burning pain. In the mean while, by generating an axon reflex, it triggers the release of
proinflammatory neuropeptides, mainly SP and CGRP [93] and the onset of an inflammatory process which includes cutaneous vasodilatation, the increase of vascular permeability, plasmatic extravasation and edema [80, 94].

A recent study of our research group [95] has highlighted the possibility of using in vivo reflectance confocal microscopy (RCM) to assess the cutaneous neurogenic vasodilatory reaction triggered by capsaicin. This technique allows the investigation of the skin structures to a depth of around 250 μm, its resolution being comparable with the classical histological examination. Thus, RCM allows an in vivo study of skin structures and the real time observation of the manner in which various micro-morphological parameters may vary [95-98].

Figure 1. RCM images of the same dermal papillae (white asterisk) acquired (A) before and (B) 40 min after the application of 1% capsaicin solution, showing an increased diameter of the capillaries (arrows) after the application of the active substance.

RCM allows the investigation of cutaneous microvascularization both from a structural and a functional point of view, being considered an excellent assessment method especially for capillaries localized at the dermo-epidermal junction [95, 99]. Capillary loops in the dermal papillae appear in transversal section as dark discs representing the vascular lumina. They are disposed in areas surrounded by bright cells, representing the keratinocytes and melanocytes from the basal layer of the epidermis which circumscribes the connective tissue of the papillary dermis (see Figure 1). The real time investigation technique with the capacity to record video sequences allows the observation and the analysis of blood cells dynamics through cutaneous vessels. One essential characteristic of this technique is the non-invasive character which offers the possibility of a serial assessment, at various time intervals, of the same cutaneous region.

In our previously mentioned study the technique of capsaicin administration dissolved in the immersion oil has enabled a regular distribution of the active substance on the investigated skin area and also the strict control of the quantity of capsaicin per tegumentary surface unit. It has also facilitated the investigation of the same cutaneous region at each experimental stage. These characteristics have laid the foundation for a new test able to investigate the functionality of cutaneous small diameter nerve fibers, with obvious clinical applicability [95].
Moreover, both previous published research [100] and our own preliminary data have shown that in vivo RCM allows an objective assessment of Meissner corpuscles density and morphology in glabrous skin, thus suggesting the possibility to estimate the change of this parameters in diabetic neuropathy (see Figure 2).

Figure 2 A,B. RCM images showing Meissner corpuscles (arrows) as bright, roundish structures with a heterogeneous, lobulated internal architecture, located inside the dermal papillae, disposed in parallel rows on each side of epidermal ridges of the palm skin.

4.3. Proteomic approaches in diabetic neuropathy

Another promising topic in modern research of diabetic neuropathy is the investigation of proteomic changes. Published studies do not abound in the description of proteomic approaches to diabetic neuropathy, but they allow drawing an outline in the field of proteomics. A recent study searching for serial biomarkers by using mass-spectrometry technology MALDI-TOF-MS (matrix-assisted-laser-desorption/ionisation time of flight mass spectrometry) and bioinformatics analysis flexAnalysisTM and Clin-ProtTM has identified peaks relevant to this disease. Among these relevant peaks, a 6631 Da peptide was identified as a fragment of precursor Apolipoprotein C-I. The identified peptides together with this precursor can represent good candidates for the study of biomarkers in diabetic neuropathy [101]. Another research direction was focused on the pathology of sensory neurons in diabetic neuropathy. In this respect, disorders have been identified in the regulation of thousands of proteins associated to various cellular pathways, oxidative phosphorylation, detoxification, mitochondrial dysfunction and so on. Mitochondrial respiration is affected by hyperglycemia and can lead to remodelations at the level of Schwann cells and thus to diabetes associated neuropathy [102].

5. Conclusions

The development of new methods for assessing peripheral diabetic neuropathy is a major interest topic in research. New advances in proteomics could lead to discoveries in biomarkers that can indicate the early onset of the disease and/or improve diagnostic and evolution of the disease monitoring. In vivo morphological and functional investigation of cutaneous nerve structures provides promising data and could lead to the development of new biotechnological
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tests for the assessment of diabetic peripheral neuropathy, that are easy to use, minimally invasive, with a higher sensitivity and specificity than the current methods. Their implementation in clinical practice could enable an early diagnosis of neurodegenerative changes of the peripheral nervous system, in a stage where treatment has maximum efficacy, could facilitate the therapeutic monitoring of patients and reduce the risk for comorbidities.

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