Epidermal Nerve Fiber Density

Epidermal Nerve Fiber Density (ENFD) is not a new science, but its value as a diagnostic tool in the assessment of peripheral neuropathy is only now becoming recognized by the medical community. Pioneered by researchers at Johns Hopkins Medical Center and in use by neurologists since the early 1990’s, it is rapidly evolving into the gold standard for establishing a diagnosis of that significant subset of peripheral neuropathy known as small fiber peripheral neuropathy. ENFD boasts distinct advantages over older, more broadly recognized tests such as sural nerve biopsy and nerve conduction studies. Its use has spread into the podiatric, family practice, neurologic and wound management communities, and it now becoming recognized as the most sensitive and specific modality when assessing for the presence and severity of small fiber peripheral neuropathy.

Small Fiber Peripheral Neuropathy

Small fiber peripheral neuropathy describes a very common and distinct subset of peripheral neuropathy affecting the small myelinated (A-delta) or unmyelinated C fibers. This form of neuropathy may be focal, but more often involves peripheral nerves in a length-dependent pattern, meaning that the earliest and most severely affected nerves are located most distally, resulting in a loss of function in a characteristic stocking/glove distribution.

Large fiber neuropathy, on the other hand, is characterized by pathology in the more proximal nerves located close to the dorsal root ganglia. The most common cause is compression neuropathy associated with degenerative disc disease. Other causes are demyelinating neuropathies such as multiple sclerosis, Guillain-Barre syndrome, and chronic inflammatory demyelinating polyneuropathy. Even diabetic neuropathy, considered primarily a small fiber neuropathy, may sometimes involve large nerve fibers, as in large fiber mononeuropathy or polyneuropathy. These large fiber neuropathies may not initially exhibit an associated small fiber component. It is the function of large fibers, not small fibers, that is tested by nerve conduction studies. This explains why some patients with frank clinical evidence of peripheral neuropathy may display normal conduction studies. Such patients suffer from pure small fiber peripheral neuropathy.

It has long been known that the most common causes of small fiber peripheral neuropathy are types I and II diabetes mellitus, and idiopathic. More recently, it has been identified that nearly half of all subjects with idiopathic small fiber neuropathy have abnormal 2-hour glucose tolerance tests and abnormal glucose levels despite normal glycosylated hemoglobin. Many cases formerly classified as idiopathic, therefore, are now labeled as “pre-diabetes” or metabolic syndrome. Other conditions associated with acquired small fiber neuropathy include alcohol abuse, HIV, amyloidosis, pharmacologic toxins (Flagyl), solvent exposure (industrial painting, screen printing, house painting, dry cleaning), chemotherapeutic agents (platinum-containing compounds, vincristine, taxanes), vasculitis, and autoimmune diseases including Sjogren’s Syndrome, Rheumatoid, Lupus, Multiple Sclerosis, and Guillain-Barre Syndrome.

Epidermal Nerve Fiber Density

*(Visualization of Epidermal Nerve Fibers by Light Microscopy)*

Epidermal nerves normally snake a path between the squamous epithelial cells of the epidermis toward the skin surface, giving them a tortuous appearance. The number of epidermal nerves per unit length of epidermis is termed the epidermal nerve fiber density or ENFD. This density varies throughout life, showing a gradual decrease with advancing age. It also exhibits a gender difference, with females having slightly greater density at all ages until the age of 80. Finally, ENFD varies widely depending on the anatomic site being tested. The skin’s
average fiber density decreases as one moves distally from the dorsal root ganglia. Because the normal range of ENFD varies depending on the anatomic location, to accurately assess the meaning of the ENFD at any particular site, there must be an accepted normal range for that particular site. This is the case for the distal leg (10 cm proximal to the lateral malleolus) and proximal thigh (10 cm distal to the greater trochanter of the femur), both of which boast a vast amount of cumulative data concerning normal range of ENFD.

The analysis of epidermal nerve fiber density utilizes the science of immunohistochemistry (IHC). Immunohistochemical studies rely on the detection of specific surface proteins or antigens by the adherence of antibodies. Nerve fibers express a particular surface antigen called protein gene product 9.5 (PGP 9.5), providing a specific target that can be used for the purpose of identification and subsequent analysis. For ENFD testing, clinicians most commonly perform bilateral 3 mm punch biopsies of skin. In the laboratory, the punch biopsy is placed in a protective glycerol-based gel called cryoprotectant and then frozen. Each biopsy is then sliced into 50 μm thick sections using a special cutting device called a freezing sliding microtome, and each is placed in its own testing tray. Rabbit-derived anti-PGP 9.5 antibodies are then applied to each of the tissue sections. The PGP 9.5-specific rabbit antibodies will identify nerve fibers that are present within the tissue sections. The final step consists of the application of goat-derived anti-rabbit antibodies and chromogen (pigment). The goat-derived anti-rabbit antibodies bind to each of the previously applied PGP 9.5-specific rabbit antibodies which are now bound to the PGP 9.5 antigen. Once enough antibodies have bound, they form a thick coat of antibody and pigment around each epidermal nerve fiber, illuminating even the most lilliputian fibers for light microscopy.

Assessment of epidermal nerve fiber density and morphology

Once the fibers are visualized, their density within the epidermis can be calculated. When quantifying epidermal nerve fibers, all fibers are counted across four or five randomly selected 50 um-thick tissue sections using 400X magnification. The number of epidermal nerves from each representative section is summed. To obtain nerve density, the length of the epidermal surface in each tissue section is measured by image analysis software. Dividing the total number of nerve fibers by the aggregate length of the epidermal surface (in millimeters), produces an average density. This density can then be expressed as the number of nerve fibers per millimeter (fibers/mm). Once the density falls below an established normal threshold, the patient is diagnosed as having small fiber peripheral neuropathy. Beyond counting density, nerve fibers are also assessed for structural integrity. Various signs of degeneration can be enumerated, including axonal swellings, fiber varicosities, excessive branching, nerve thinness, nerve segmentation, and beading of nerve fibers.

Not inconsequentially, there are several different methods of counting epidermal nerves, each associated with its own normative range for a specific anatomic location. Amarillo Pathology follows the protocol established at
the Johns Hopkins Cutaneous Nerve Laboratory, counting only those nerves that are clearly seen crossing the epidermal basement membrane. Consistency in counting method must be maintained when monitoring a particular patient, especially when assessing for incremental improvements secondary to therapy.

**Epidermal Nerve Fiber Density (ENFD) Normative Values for Clinical Use**


**For Female Patients**
(n=285)

<table>
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<tr>
<th>Age (years)</th>
<th>Number of subjects</th>
<th>0.05 quantile ENFD values per age span</th>
<th>Median ENFD values per age span</th>
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<tr>
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<td>6.7</td>
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**For Male Patients**
(n=265)

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<td>30-39</td>
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<tr>
<td>80 and older</td>
<td>9</td>
<td>1.7</td>
<td>7.2</td>
</tr>
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**Choosing the appropriate site to biopsy**

The most common site for ENFD biopsy is the calf at 10 cm proximal to the lateral malleolus. First, because it is the most well studied, thus producing a well-established normative range. Secondly, because small fiber peripheral neuropathy is a length-dependent process, the distal anatomic sites are more severely affected than proximal areas. The second most common site for ENFD biopsy is the proximal thigh at 10 cm distal to the greater trochanter of the femur. There is an advantage to taking biopsies from both the proximal thigh and distal leg, when possible. The distal biopsy is most important, however, providing clinicians with an excellent assessment of the extent of the disease process at any point in time. It is there that the fiber density has optimal significance, and where the test is most sensitive. An abnormal distal biopsy and a normal proximal biopsy is evidence of length dependence, and therefore in favor of small fiber neuropathy.
Small fiber neuropathy is often an asymmetric process, as evidenced by ENFD values that are sometimes variable from right to left. For this reason, bilateral punch biopsies taken from 10 cm proximal to the lateral malleolus may be more useful than a unilateral biopsy in reflecting the true severity of the disease.

Obtaining punch biopsies for ENFD

The punch biopsy is performed using a 3 mm circular punch at a depth of 4 mm. Larger punches may be used, but the need for sutures and the increased complications thereof makes them less desirable. When performing punch biopsies for ENFD analysis, it is important to minimize trauma to the specimen, which might artificially alter the nerve fiber density. To avoid such trauma, the punch blade should cut through the skin by rotating the punch back and forth. The punch should never be forced through the skin exclusively using vertical pressure, as it may cause crush artifact along the periphery, likely causing a fictitious decrease in the nerve fiber density. Instead, only slight downward pressure should be applied while rotating the punch blade.

An additional source of crush artifact may be introduced by handling the epidermis when samples are removed from the biopsy site. To avoid this, the sample should be gripped by the dermal soft tissue rather than the epidermis. After the skin has been punched and the punch blade has been removed, the forceps may be pressed down on the skin on either side of the biopsy site causing the punch itself to rise above the adjacent skin and exposing the underlying dermis. It is this deeper tissue which should be grasped with the forceps. Once this has been accomplished, the sample may be lifted out and the connective tissue base severed with curved scissors. Since the skin biopsy is minimally invasive, the biopsy can later be repeated to evaluate the progression of the neuropathy and/or response to therapy.

Fixation of biopsy specimens for ENFD

Although formalin is an excellent fixative for routine anatomic pathology specimens, it is not appropriate for ENFD testing. Zamboni’s fixative stabilizes cellular proteins, is not easily destroyed by tissue fluids, and will not cause tissue deterioration. Another characteristic of Zamboni’s fixative is that it is stable at room temperature for up to one year. A potential drawback to its use is the fact that it is a mild acid with corrosive properties. Therefore, Zamboni’s fixative should NEVER come into contact with the patient. Further, the specimen can only be exposed to Zamboni’s for a maximum time of 24 hours but no less than 6 to 8 hours for adequate fixation of
the specimen. Specimens should be submerged in Zamboni’s fixative immediately and never be allowed to air dry.

If the specimen is allowed to remain in fixative overnight, it should then be rinsed the following morning. To rinse, the Zamboni’s fixative is first poured off. The vial containing the biopsy is then filled with phosphate buffer and gently agitated. The phosphate buffer is then poured off, and the rinsing step is repeated a second time. This ensures removal or neutralization of all Zamboni’s fixative. Finally the vial containing the rinsed skin sample is filled with a glycerol-based cryoprotectant to protect the specimen during transportation. Tissue can be safely stored in cryoprotectant for up to a week, so short delays in shipping should not be a big problem. For clinicians in geographic proximity to APA Laboratory, and who intend to have the specimen transported the same day, the rinsing step can be omitted. Simply call ahead to schedule a same-day pick (the APA Laboratory pick up number is on the biopsy kit), and leave the biopsy specimen in the Zamboni’s fixative. The rinsing step will be performed by staff of APA Laboratory the following morning.

**Transporting the tissue**

Amarillo Pathology supplies clinicians with a biopsy kit package containing all of the materials that are essential for obtaining and shipping the sample. The kit contains a sterile barrier, sterile punch, curved scissor, alcohol and Betadine wipes. For offices located remotely from the Amarillo metropolitan area and who intend to ship the package by UPS or FedEx, a transport cooler, cool-pack, and prepaid overnight shipping labels are included. The cool-pack should be placed in a freezer in advance of performing the procedure, so it can be ready for use during return shipping. This is particularly important during hot summer months.

If planning to ship the same day, the rinsing step can be omitted as described above. In order to minimize the chance of over-exposure to Zamboni’s fixative, biopsies should be obtained during the early afternoon hours, and then shipped to the lab for delivery the following morning. If planning to ship the following day, the sample will first be fixed overnight in Zamboni’s fixative, then rinsed the next day as described above, and packed for shipping. When packing the specimen, place the cool-pack into the cooler first, cover with the Styrofoam divider, and then insert the vial containing the biopsy. Laboratory processing of ENFD specimens is quite a bit more complex than standard processing of H&E slides. H&E slides can be prepared and examined in a matter of several hours, while ENFD slide preparation usually requires two days. Results can generally be reported out within two days of the punch biopsy being received in our laboratory.

**Indications for ENFD testing**

The medical community is only now awakening to the full utility of ENFD analysis in the management of diabetic small fiber peripheral neuropathy. Used mostly in the context of research over the last two decades, ENFD has emerged as the most specific and sensitive method for diagnosing and quantifying small fiber peripheral neuropathy. ENFD testing allows clinicians to approach the management of diabetic patients from three different angles. These are: 1) as a confirmatory diagnostic tool, 2) as a predictor for the evolution of neuropathy, and 3) as a means by which to gauge the effectiveness of medical treatment.

ENFD testing is particularly helpful for patients who have signs and/or symptoms of neuropathy, and test normal on EMG or nerve conduction studies. This occurs when small nerve fibers are predominately affected as these are not detected in the electro-diagnostic studies. Because medical therapies can now target specific patient populations including both diabetic and non-diabetic populations, serial ENFD tests may be used to monitor the efficacy of treatments over time.
Specificity and sensitivity of the ENFD test

The sensitivity of skin biopsy in diagnosing small fiber neuropathy has been reported to be 88%, in comparison to 54% for the clinical examination, and 49% for quantitative sensory testing. The specificity of the test is 98% (Lauria and Devigli, 2007; Devigli et al, 2008, Lauria and Bakkers, 2010).

Conclusion

In summary, epidermal nerve fiber density is a test which allows for the diagnosis of small fiber peripheral neuropathy in an objective manner, based on the analysis of a common punch of skin. The biopsy technique has subtle differences from a standard punch biopsy, some of which are crucial to the integrity of the test. The test is ideal for evaluating the efficacy of treatment in patients who are undergoing medical management. As a predictive modality for progression to symptomatic peripheral neuropathy, ENFD shows great promise and will certainly be a subject of much future research.

Bibliography