Gastric mucosal nerve density

A biomarker for diabetic autonomic neuropathy?

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ABSTRACT

Background: Autonomic neuropathy is a frequent diagnosis for the gastrointestinal symptoms or postural hypotension experienced by patients with longstanding diabetes. However, neuropathologic evidence to substantiate the diagnosis is limited. We hypothesized that quantification of nerves in gastric mucosa would confirm the presence of autonomic neuropathy.

Methods: Mucosal biopsies from the stomach antrum and fundus were obtained during endoscopy from 15 healthy controls and 13 type 1 diabetic candidates for pancreas transplantation who had secondary diabetic complications affecting the eyes, kidneys, and nerves, including a diagnosis of gastroparesis. Neurologic status was evaluated by neurologic examination, nerve conduction studies, and skin biopsy. Biopsies were processed to quantify gastric mucosal nerves and epidermal nerves.

Results: Gastric mucosal nerves from diabetic subjects had reduced density and abnormal morphology compared to control subjects ($p < 0.05$). The horizontal and vertical meshwork pattern of nerve fibers that normally extends from the base of gastric glands to the basal lamina underlying the epithelial surface was deficient in diabetic subjects. Eleven of the 13 diabetic patients had residual food in the stomach after overnight fasting. Neurologic abnormalities on clinical examination were found in 12 of 13 diabetic subjects and nerve conduction studies were abnormal in all patients. The epidermal nerve fiber density was deficient in skin biopsies from diabetic subjects.

Conclusions: In this observational study, gastric mucosal nerves were abnormal in patients with type 1 diabetes with secondary complications and clinical evidence of gastroparesis. Gastric mucosal biopsy is a safe, practical method for histologic diagnosis of gastric autonomic neuropathy. **Neurology® 2010;75:973-981**

GLOSSARY

CGRP = calcitonin gene-related peptide; **ENF** = epidermal nerve fiber; **ENFD** = epidermal nerve fiber density; **MNF** = mucosal nerve fiber; **MNFD** = mucosal nerve fiber density; **PGP-ir** = protein gene product 9.5-immunoreactive; **SP** = substance P; **VIP** = vasoactive intestinal peptide.

Autonomic neuropathy is commonly cited as the underlying cause of the gastrointestinal symptoms, postural hypotension, hypohidrosis, and impotence experienced by many patients with longstanding diabetes. The diagnosis is usually based on clinical criteria and physiologic tests of cardiorespiratory reflexes and sweating; however, histologic evidence of a neuropathic etiology of these symptoms is limited.^{1,2} Reviews of diabetic neuropathy,³ autonomic nervous system disorders,⁴ and gastrointestinal pathology⁵ lack detailed discussions of autonomic nerve fiber pathology in diabetes. Early studies of the vagus nerve in diabetes reported segmental demyelination and axonal degeneration,^{6,7} whereas another report showed no pathology.⁸ A description⁹ of distended neurons in sympathetic and celiac ganglia, loss of myelinated nerves in the vagus nerve and sympathetic trunks, and inflammatory changes in autonomic ganglia remains

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unconfirmed. Full-thickness stomach specimens from subjects with type 2 diabetes with gastroparesis have fewer interstitial cells of Cajal^{10,11} and inhibitory neurons in the myenteric plexus¹¹ but these are not easily available for analysis by routine endoscopic biopsy.

We hypothesized that a diagnosis of gastrointestinal autonomic neuropathy is possible by histologic examination of nerves in the mucosal layer of the gastrointestinal tract. This required that we develop a method to quantify the complex innervation of gastric mucosa. We chose to study stomach mucosa because this tissue can be safely acquired by standard biopsy techniques during routine endoscopy. We also hypothesized that a relationship exists between gastric mucosal nerve fiber density (MNFD) and the symptoms associated with gastroparesis.

We enrolled diabetic candidates for pancreas transplantation because similar patients have been shown to have a high prevalence of secondary complications.¹² Enrollment was restricted to diabetic patients who had a prior physician diagnosis of gastroparesis, preferably with gastric emptying studies, and a history of symptoms associated with gastroparesis.^{13,14} We examined the associations of MNFD with gastric symptoms, epidermal nerve fiber density (ENFD), and results of neurologic examination.

METHODS **Study subjects.** Thirteen type 1 diabetic candidates for pancreas transplantation and 15 control subjects participated. Participants answered a health questionnaire and were excluded if they had known or suspected gastrointestinal disease (such as ulcerative colitis, Crohn disease, scleroderma, gastric or duodenal ulcer), substance abuse, pregnancy, or systemic disease associated with neuropathy. Diabetic participants with a prior diagnosis of gastroparesis and symptoms associated with gastroparesis were selected.

Standard protocol approvals, registrations, and patient consents. The study was approved by the University of Minnesota's Institutional Review Board in accordance with the ethical guidelines for conducting research on human subjects. All subjects gave informed written consent to participate in the research study and Health Insurance Portability and Accountability Act authorization.

Study protocol. A neurologic examination designed to detect signs of neuropathy¹² was performed together with nerve conduction studies of 1 sensory (sural) and 2 motor (peroneal and tibial) nerves, cardiorespiratory autonomic reflexes (heart rate variation during deep breathing and Valsalva ratio), and quantitative sensory testing on the right thigh and calf for touch (von

Frey-like filaments) and mechanical pain (pin). Two 3-mm punch skin biopsies were removed from the sensory test sites.

Upper endoscopy was performed under conscious sedation following overnight fasting. Mucosal biopsies were removed from the stomach antrum and fundus and pinned mucosa up in cold Zamboni fixative.¹⁵ Stomach and skin biopsies were fixed for 12 hours and then cryoprotected.¹⁶

Laboratory procedures and quantification. All biopsies were sectioned at 60- μ m using a freezing sliding microtome (Leica 2000R, Germany) and labeled for double and triple immunofluorescent staining with a panel of antibodies following a standard protocol¹⁶ (table e-1 on the *Neurology*[®] Web site at www.neurology.org).

Gastric MNFD was determined using confocal microscopy (Olympus BX51-DSU, Olympus America, Inc., Melville, NY) with integrated x, y, z stage movements and design-based stereology (StereoInvestigator[®] software, MicroBrightField Bioscience, Williston, VT). Criteria for data collection were set to achieve a coefficient of error of less than 10%.¹⁷ Analyses were performed on a 3-mm length of mucosa for fundus and 1.5-mm for antrum. Quantification was performed using a 20 $\times$ objective lens on 5 sections per biopsy taken at 2 section intervals, starting with a systematic random¹⁷ section. We computed 2 density measures for each gastric mucosal biopsy. Nerve length density was determined using the isotropic spherical probe¹⁸ (using 30- μ m radius hemispheres). The probe estimates total length of MNFs across the sections examined. To avoid artifacts, a 5- μ m guard zone was maintained above and below the sampled locations (z-plane). Nerve length was converted to length density with units μ m/ μ m³. Nerve volume density was determined using the area fraction fractionator probe.¹⁹ The counting frame was 130- μ m by 130- μ m; point spacing was 15- μ m by 15- μ m. Markers were placed at the point spaces, to discriminate nerve from tissue. This probe estimates the fraction volume occupied by MNFs at the anatomic site examined (fundus or antrum). Nerve volume density units are μ m³/ μ m³. A fundus sample was unavailable for one subject.

Epidermal nerve fibers (ENFs) were measured in 2 60- μ m-thick skin sections. From each section, 2 confocal (CARV, ATTO Bioscience, Rockville, MD) images, 16 optical sections (z-series), 2- μ m apart (20 $\times$ objective) were used to quantify ENFD as the number of ENFs per mm length of epidermis using Neurolucida[®] software (MicroBrightField Bioscience). Subjects with ENFD above the 5th percentile (5% cutoff), based on established normal data,²⁰ were considered normal.

Statistical analysis. Numeric summaries are expressed as average $\pm$ standard error. We compared control and diabetic groups for differences in gastric mucosal and epidermal (skin) innervation using multiple linear regression. Specifically, the dependent variable was a nerve measure, and the independent variables were an indicator of diabetic status (yes vs no) and age, treated as a continuous measure. In effect, this is an age-adjusted *t* test, with 2-sided *p* values. Otherwise, control and diabetic groups were compared using 2-sample *t* tests or Fisher exact test, as appropriate.

RESULTS **Subjects.** Demographic and clinical data with results of neurologic testing for the study groups are summarized in table 1. Diabetic subjects were older (range 31–62 years) than control subjects (range 21–57 years) but the ages overlapped. Our diabetic subjects had a high prevalence of secondary complications,

Table 1 Characteristics of the study groups^a

	Control (n = 15)	Diabetic (n = 13)	p Value
Demographics and past history			
Age, y	31 ± 2.9	45 ± 2.7	0.001
Sex, M/F	6/9	5/8	0.93
Duration of diabetes, y	—	29 ± 2.2	—
Retinopathy	—	13 (100)	—
Nephropathy	—	12 (92)	—
History of skin ulcers in feet	—	13 (100)	—
History of postural hypotension	—	10 (77)	—
History of hypoglycemic unawareness	—	11 (85)	—
Gastrointestinal symptoms			
Dyspepsia	0 (0)	12 (92)	<0.001
Bloating	1 (7)	12 (92)	<0.001
Nausea	0 (0)	12 (92)	<0.001
Vomiting	0 (0)	8 (62)	<0.001
Neurologic sensory examination, abnormal			
Vibration	0	12 (92)	<0.001
Pinprick	0	12 (92)	<0.001
Light touch	0	8 (62)	<0.001
Position sense	0	2 (15)	0.12
Motor examination, strength (Medical Research Council score [0–5]²⁷)			
Right extensor hallucis longus	5 ± 0	4 ± 0.5	0.049
Reflex examination, abnormal			
Achilles reflex	0 (0)	13 (100)	<0.001
Nerve conduction studies			
Sural amplitude, μ V (normal ≥ 8)	23.2 ± 4.6	0.4 ± 0.3 ^b	<0.001
Sural conduction velocity, m/s (normal ≥ 38)	49 ± 1.5	35.9 ± 0.9 ^b	<0.001
Peroneal amplitude, μ V (normal ≥ 2.5)	5.6 ± 0.5	1.1 ± 0.4 ^c	<0.001
Peroneal conduction velocity, m/s (normal ≥ 38)	51 ± 1.3	32.9 ± 1.6 ^c	<0.001
Tibial amplitude, μ V (normal ≥ 5)	13.1 ± 1.4	2.3 ± 0.9 ^d	<0.001
Tibial conduction velocity, m/s (normal ≥ 38)	51 ± 1.7	35.4 ± 1.6 ^d	<0.001
Autonomic evaluation			
Valsalva ratio	1.8 ± 0.1	1.2 ± 0.1	<0.001
Heart rate variation during deep breathing ²⁸	23.9 ± 1.8	2.6 ± 1.0	<0.001

^a Data are mean ± SE or n (%).^b A total of 11 diabetic subjects had no response.^c A total of 6 diabetic subjects had no response.^d A total of 5 diabetic subjects had no response.

gastrointestinal symptoms, and abnormal findings on neurologic examination that were comparable to complications in similar patients.¹² All diabetic subjects had a clinical diagnosis of gastroparesis; 6 were diagnosed with delayed gastric emptying by previous gastric emptying studies (time for 50% of ingested meal to exit the stomach exceeded 90 minutes). The nerve conduction studies and at least one cardiorespiratory reflex were abnormal in all diabetic subjects. In control subjects, neurologic examination, cardiorespiratory reflex testing,

nerve conduction studies, and quantitative sensory testing were normal except for one subject with below normal cardiorespiratory reflexes, who also had the lowest gastric antrum MNFD of the control group.

Endoscopy. The stomach mucosa appeared normal by endoscopy in control and diabetic subjects. Eleven of 13 diabetic subjects had remnants of food in their stomach.

Stomach samples. Mucosal nerve fibers (MNFs) of control subjects formed a dense network of horizontal and vertical nerve fibers that encompassed glands, blood, and lymphatic vessels (figure 1A). This complex protein gene product 9.5-immunoreactive (PGP-ir) network originates from ganglia in deep neuronal plexi (not present in mucosal biopsies) whose nerves penetrate the muscularis mucosa and extend through the lamina propria to the luminal surface of the mucosa. Below the mucosal luminal border, fine nerve endings branching from proximal larger fibers extended to just below the basement membrane of the surface epithelium (figure 1A). MNF neuropeptide expression colocalized with the PGP-ir network, but the amount differed depending on biopsy location. In the antrum, most PGP-ir fibers appeared to coexpress similar amounts of vasoactive intestinal peptide (VIP) and substance P (SP) (figure 2, A and C). In contrast, the fundus showed similar VIP-ir nerve fibers (figure 2G), but fewer SP-ir fibers (figure 2E). Calcitonin gene-related peptide (CGRP)-ir fibers were infrequent in the lamina propria of the antrum and fundus; a few CGRP-positive fibers were present in the muscularis mucosa (figure 2I).

The morphology and density of the MNFs in biopsies from diabetic subjects were abnormal throughout the mucosa. In subjects with less deficiency, the MNF network was discontinuous in the superficial mucosa (figure 1, B and C), while in other subjects innervation extended to only half the mucosal height (figure 1, D and E). Nerve varicosities (figure 1B) and areas of patchy nerve loss (figure 1C) were common. In samples with greater nerve deficiency the MNF network was disorganized (figure 1D) and contained thicker, more tortuous MNFs (figure 1E). VIP (figure 2, D and H) and SP-ir (figure 2, B and F) nerve loss followed the pattern of the PGP-ir nerve deficiency. CGRP-ir nerve fibers were absent from the lamina propria and rare in the muscularis mucosa (figure 2J).

Gastric MNFs in the fundus had an average length density of $0.0025 \mu\text{m}/\mu\text{m}^3 \pm 0.00017$ for the control group and $0.0018 \mu\text{m}/\mu\text{m}^3 \pm 0.00022$ for the diabetic group ($p = 0.013$) (figure 3 and table e-2). The average volume density in the fundus was $8.20 \mu\text{m}^3/\mu\text{m}^3 \pm 0.35$ for the control group and

(A) Control subject's antrum mucosal innervation with a well-organized network of unmyelinated nerve fibers oriented vertically and horizontally within the mucosa but never extending into the epithelium. Green and yellow: pan-neuronal marker, PGP 9.5-immunoreactive mucosal nerve fibers (MNFs); red: type IV collagen-immunoreactive basement membrane around glands, blood vessels, lymphatics, and thin muscle layer of the muscularis mucosa, separating mucosa from submucosa. (B-E) Diabetic antrum showing grades of MNF loss. (B) Morphologic changes as nerve thickening and tortuosities (arrows) with minimal MNF loss. (C) Patchy moderate nerve loss forming innervation gaps (*), with few MNFs reaching the top of the mucosa (arrows). (D, E) More diffuse and extensive loss with disappearance of the network orientation. Scale bar = 200 μm .

$3.43 \mu\text{m}^3/\mu\text{m}^3 \pm 0.45$ for the diabetic group ($p < 0.001$). The MNFD values of the 2 groups overlapped for length density (figure 3A), but were separate for volume density (figure 3B).

Gastric MNFD in the antrum had an average length density of $0.0028 \mu\text{m}/\mu\text{m}^3 \pm 0.00021$ for the control group and $0.0019 \mu\text{m}/\mu\text{m}^3 \pm 0.00025$ for the diabetic group ($p = 0.015$) (figure 3 and table e-2). The average volume density was $9.10 \mu\text{m}^3/\mu\text{m}^3 \pm 0.59$ for the control group and $4.49 \mu\text{m}^3/\mu\text{m}^3$ for the diabetic group ($p < 0.001$).

Again, diabetic and normal values overlapped for length density (figure 3C), but were separate for volume density (figure 3D).

Skin samples. ENF morphology in biopsies of control subjects was normal, as previously described²¹ (figure 4A). Neuropeptide expression in cutaneous nerves was plentiful compared to skin from diabetic subjects. Specifically, in the papillary dermis CGRP-ir fibers were

Figure 2 Neuropeptide localization and distribution in confocal images of stomach mucosa in control and diabetic subjects

The left side of the image panel represents control subjects and the right side diabetic subjects. Neuropeptides colocalized with the pan-neuronal marker, PGP 9.5-immunoreactive (ir) fibers, not shown in this panel of images. (A) An abundance of substance P (SP-ir) fibers and (C) vasoactive intestinal peptide (VIP-ir) fibers in the mucosa of the antrum. (E) The fundus of control subjects showed less SP-ir fibers compared to (G) the abundant VIP-ir fibers. (I) Calcitonin gene-related peptide (CGRP)-ir was very sparse in either antrum or fundus of control subjects and was usually located in proximity to the muscularis mucosa (MM) and infrequently within the lamina propria (mucosa). Gastric mucosa of diabetic subjects showed an apparent decrease in SP and VIP-ir fibers (arrowheads) in both the antrum (B, D) and fundus (F, H) with (J) no CGRP-ir fibers found. Scale bar = 100 μ m.

abundant (figure 4D) and more frequent than SP-ir fibers (figure 4F). VIP-ir fibers were absent. The diabetic group had fewer CGRP-ir (figure 4E) and SP-ir (figure 4G) fibers while the PGP-ir subepidermal neural plexus was generally sparse. ENFs were absent in 10 skin biopsies (figure 4B); when present, they often had an irregular distribution with many branch points (figure 4C) and axonal swellings.

The mean ENFD was within the normal range²⁰ for the control subjects, 21.4/mm $\pm$ 2.12 for calf and 39.1/mm $\pm$ 4.3 for thigh. In diabetic subjects, ENF densities were reduced in a pattern consistent with a length-dependent sensory neuropathy. The ENFD was significantly below control values in the calf (2.36/mm $\pm$ 2.54; p < 0.001), but not for thigh (15.7/mm $\pm$ 5.11;

Figure 3 Differences between diabetic and control subjects for gastric, fundus, and antrum mucosal nerve fiber density using design-based stereology

(A) Fundus length density, LD, $p = 0.013$. (B) Fundus volume density, VD, $p < 0.0001$. (C) Antrum LD, $p = 0.015$. (D) Antrum VD, $p < 0.0001$. Plots are box-and-whisker plots, in which highest and lowest bars show the largest and smallest measure within each group, while the top, middle, and bottom of the box are the 75th, 50th, and 25th percentile. p Values are from age-adjusted tests.

$p = 0.068$) after adjustment for age (table e-3).

Associations of MNFD with other measures of diabetic neuropathy. Mucosal nerve length density and volume density correlated well for fundus and antrum (Pearson $r = 0.8$ in diabetic patients and $r = 0.7$ in controls; $p < 0.001$). Diabetic subjects with longer disease duration tended to have lower MNFD, with the antrum innervation correlating better with disease duration ($r = -0.6$; $p = 0.04$) than fundus ($r = -0.4$; $p = 0.29$).

The best predictor for MNFD of the neurologic examination measures was the nerve conduction studies, especially results for amplitudes of tibial compound muscle action potentials, even though several diabetic subjects had no response (sural 11, peroneal 6, and tibial 5). However, no combination of measures reliably predicted MNFD. For example, among diabetic subjects, the best correlation was between tibial amplitude and MNF volume density of the antrum ($r = 0.6$, $p = 0.07$). Among the cardiorespiratory reflexes, Valsalva ratio correlated with antrum MNF volume density ($r = 0.5$, $p = 0.07$), as did heart rate variation with deep breathing ($r = 0.5$, $p = 0.09$). None of these reached significance, however.

Within the diabetic group, considering all Pearson correlations between cutaneous and MNFD, the best correlation was for thigh ENFD and antrum volume density ($r = 0.7$, $p = 0.01$), but calf ENFD correlated poorly. Among gastroparesis symptoms, bloating (on a visual analog score) was negatively correlated with MNFD, specifically, length density of antrum and fundus ($r = -0.5$, $p = 0.13$). However, the trend did not reach significance.

DISCUSSION This study provides histologic confirmation of autonomic neuropathy affecting nerves in the gastric mucosa of type 1 diabetic candidates for pancreas transplantation who had clear evidence of secondary complications due to diabetes, including neuropathy, retinopathy, and nephropathy, and had a clinical diagnosis of gastroparesis. The gastric neuropathic features are deficiency of MNF length density and volume density plus altered MNF innervation pattern and morphology. The MNF loss was associated with evidence of somatic peripheral neuropathy affecting myelinated and unmyelinated nerves. MNFD correlated inversely with the duration of diabetes, particularly in the antrum. Among the symptoms of gastroparesis, bloating showed a trend toward associa-

Figure 4 Confocal images of skin from the thigh of a control and diabetic subject

The left side of the panel represents a control subject's thigh while the right side represents a diabetic subject's thigh. (A) Regular distribution of epidermal nerve fibers (ENFs) in a control subject. Green and yellow are pan-neuronal marker, PGP 9.5-immunoreactive nerve fibers; red: type IV collagen-immunoreactive basement membrane, at the basement membrane zone (BMZ) and around capillaries and blood vessels (BV). In contrast, (B) example of severe loss of ENFs in the skin of a diabetic subject with a scanty subepidermal neural plexus (SNP). (C) Example of irregular distribution of ENFs in the skin of a diabetic subject with nerve clustering as well as adjacent areas lacking ENFs (*). (D) Numerous neuropeptide containing nerve fibers (control) as calcitonin gene-related peptide (CGRP) and (F) substance P (SP). (E) A decreased expression of CGRP and (G) SP in diabetic skin. Scale bar = 100 μ m.

tion with the MNFD. However, none of the measures of gastric symptoms, results of neurologic testing, or ENFD could predict gastric MNF deficiency. In particular, the severity of abnormalities found on autonomic cardiorespiratory reflex tests did not correlate well with the severity of gastric MNF loss.

The deficiency of MNFs in diabetic subjects was more apparent in the superficial mucosa closer to the epithelial surface than in the more heavily innervated deeper mucosa containing the gastric glands. Future software enhancements that permit distinction between MNFD in the superficial vs deep mucosa should increase diagnostic sensitivity. Of the 2 mea-

surements for gastric mucosal nerves, volume density was less laborious to measure and more clearly discriminated between the control and diabetic groups, whereas the length density values of the 2 groups overlapped. We tentatively attribute this to the presence of many thin nerves, presumed to be compensatory collateral branches that contribute more to measurements of length than of volume.

We anticipated that unmyelinated ENFs in skin biopsies of our diabetic subjects would mirror changes in the unmyelinated nerves of the gastric mucosa since a previous study of 290 candidates for pancreas transplantation showed that, with rare ex-

ception, all had a peripheral neuropathy that affected myelinated and unmyelinated nerves.¹² Although ENFs and MNFs were both reduced, ENFs did not provide a reliable surrogate for gastric mucosal innervation in this group. However, ENFs were absent in the calf biopsies of 7 diabetic subjects and 4 others had counts below the 5th percentile.²⁰ This narrow distribution of ENFD reduces the ability to find a substantial correlation with gastric innervation. The same is true for results of motor and sensory-evoked potentials in nerve conduction studies and for symptom severity assessment. A cohort of more moderately affected diabetic subjects may provide better associations.

Mucosal neuropeptide distribution differed in the fundus and antrum of control subjects. In the fundus, VIP-ir nerve fibers were abundant compared to fewer SP-ir fibers. In contrast, the antrum had similar amounts of VIP and SP-ir fibers. CGRP-ir fibers were infrequent in the fundus and antrum of normal and diabetic subjects. Both VIP and SP containing mucosal nerves appeared decreased in diabetic patients.

Resolving the relationship of deficient mucosal innervation to delayed gastric emptying and prolonged retention of undigested food is complex. The contribution of mucosal nerves to normal gastric motility is probably minimal because, unlike the small intestine, gastric submucosa contains few intrinsic primary afferent neurons^{22,23} and the intrinsic reflexes are poorly developed,²⁴ except for possible augmentation of contraction intensity. In contrast, mucosal nerves in the small intestine are mainly sensory terminals of intrinsic primary afferent neurons^{22,23} and function as the afferent limb of the peristaltic reflex to move intestinal contents in the aboral direction, whereas gastric emptying occurs in response to antral peristaltic waves that are coordinated by slow waves initiated by the interstitial cells of Cajal.²⁴ In type 2 diabetes, the interstitial cells of Cajal and neurons in the myenteric plexus that express nitric oxide synthase and SP were reduced in the stomach antrum, but mucosal nerves were not described.¹¹ Because of the severity of the peripheral neuropathy and the MNF deficiency in our patients it is probable that these deeper structures which are so important to gastric motility were also abnormal; however, they are not available for examination in mucosal biopsies.

The undigested nature of food retained after an overnight fast is possibly related to mucosal nerve deficiency. Reduction of food into small particles for propulsion through the pyloric sphincter relies on mechanical milling or trituration aided by secretion of pepsin and acid produced by gastric mucosal

glands. These functions are less productive in patients with diabetic gastroparesis who are known to have hypergastrinemia,²⁵ and deficient secretion of acid.^{25,26}

This study shows that evaluation of gastric MNFs in endoscopic biopsies is a promising clinical method to histologically verify the presence of gastric autonomic neuropathy. Future studies that compare mucosal findings to functional motility studies will elucidate further the role of these MNFs. Similar involvement of the other segments of the gastrointestinal tract is probable, but is yet to be proven. While objective quantification requires confocal microscopy with stereology, for many patients it is possible to take the practical approach and perform a pathologic rating directly from the immunostained sections. Examination of MNFs in the stomach and other portions of the gastrointestinal tract of type 1 and type 2 diabetic patients who have a broad spectrum and severity of gastrointestinal complications will further define the role of mucosal nerve analysis as a biomarker to diagnose and stage gastrointestinal autonomic neuropathy.

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