OBJECTIVE—To explore the relationship between serum neuron-specific enolase (NSE) levels and diabetic neuropathy.

RESULTS—Serum NSE levels increased slightly in diabetic subjects compared with normal subjects (9.1 [1.5] vs. 8.7 [1.7], \( P = 0.037 \)), and the levels increased greatly in diabetic subjects with neuropathy compared with those without (10.8 [2.8] vs. 9.1 [1.5], \( P = 0.000 \)). The association of NSE with diabetic neuropathy was independent of the hyperglycemic state (fasting blood glucose, HbA1c, duration, and the type of diabetes) and other potential confounders affecting NSE levels (e.g., age, sex, and renal status) (odds ratio 1.48 [1.13–1.94], \( P = 0.001 \)). In addition, NSE levels increased with and were closely correlated to the stages of neuropathy (r = 0.63 [0.52–0.74], \( P = 0.000 \)). The optimal cutoff point for serum NSE levels to distinguish patients with diabetic neuropathy from those without was 10.10 \( \mu \)g/L, with a sensitivity of 66.3% and a specificity of 72.5%.

CONCLUSIONS—Serum NSE levels are closely associated with peripheral neuropathy in patients with diabetes. Future studies are warranted to clarify the relationship.

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NEUROPATHY IS ONE OF MOST COMMON CHRONIC COMPLICATIONS IN PATIENTS WITH DIABETES. A TIMELY AND ACCURATE DIAGNOSIS OF DIABETIC NEUROPATHY IS ESSENTIAL TO EARLY INTERVENTIONS FOR DECREASING THE RATE OF THE ASSOCIATED DISABILITY AND DEATH.

A range of diagnostic tools are available (1). However, a biomarker specific to neural damage is still not known. In this regard, neuron-specific enolase (NSE), which catalyzes the conversion of 2-phosphoglycerate into phosphoenolpyruvate, may act as a new emerging biomarker of peripheral neuropathy in diabetes.

\( \gamma \)-Enolase, also known as enolase 2 (ENO2) or NSE (2), is a highly soluble intracellular enzyme normally located in the cytoplasm in neuroendocrine cells. The protein is principally located in neuronal tissues. After tissue injury, NSE is readily released into the cerebrospinal fluid and blood where it has been shown to have a biological half-life of 48 h (3). Neurons of the peripheral nervous system reside in the ganglia around the spine or in close proximity to their target organs. Peripheral nerves are bundled along with blood vessels supplying the organs. Surrounding each fiber of these peripheral nerves is the endoneurium, analogous to the blood–brain barrier to some extent. In this respect, endoneurial fluid is similar to cerebrospinal fluid in the central nervous system. During the development of peripheral nerve damage, the amount of endoneurial fluid may increase at the site due to irritation or other deleterious stimuli (4). However, unlike the central nervous system with the protection of the blood–brain barrier, the peripheral nervous system is more vulnerable and readily exposed to toxins (4). Hyperglycemic and ischemic or hypoxic environments induce oxidative stress in the nervous system (1–3). Oxidation inactivates several glycolytic enzymes, including enolase, in neurons (4). To meet the fairly high-energy requirements under such conditions, the glycolytic enzymes are compensatively upregulated to increase survival of the neurons (5). Chronic exposure to hyperglycemia or its related ischemia/hypoxia with oxidative stress leads to an increased risk for peripheral neuropathy (6), which is characterized by neurodegeneration that is often concomitant with neuroregeneration (7). During this process, the rate of synthesis of the enolase in the affected neurons may change, and it is likely to cause the NSE to leak into the endoneurial fluid and serum. We postulated that in this circumstance, NSE levels in circulation may be indicative of diabetic peripheral neuropathy (DPNP).

Previous studies on NSE in association with DPNP are limited (8). There are no meticulous analyses of this relationship. In addition, few data are available for the Chinese who are plagued by an increasing incidence of diabetes (9). For this reason, we evaluated the relationship between the concentration of NSE and diabetic neuropathy among subjects with diabetes.

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NSE and peripheral neuropathy

The authors conducted a study to assess the prevalence and characteristics of peripheral neuropathy in a population of patients, primarily focusing on diabetic neuropathy. The study involved 3406 patients who were randomly selected from seven districts in Nanjing, China. The participants were divided into three groups based on their diabetes status: patients with diabetes, patients with prediabetes, and controls.

**Neuropathy assessment**

Testing was performed on each participant by the same experienced physician according to standard procedures. The results were evaluated using age-related reference values. For somatic neuropathy, symptoms were documented, such as numbness, asleep feeling, burning, deep aching, and unsteadiness in walking. For cardiac autonomic neuropathy, symptoms were documented, including unexplained resting tachycardia and postural fainting. A score $\geq 1$ was positive for a symptom(s).

**Distal peripheral neuropathy quantitative tests**

Quantitative thermal sensory test. A quantitative sensory test was performed on each subject using a thermoregulator. The test was performed by placing a $30 \times 30$ mm thermode over the skin at the site on both sides of the body (e.g., the dorsolateral border of the foot or the hypothenar eminence) to be tested. The laboratory temperature was maintained at 23–24°C. A cluster of stimuli for each condition (cold sensation, warm sensation, heat-induced pain, and cold-induced pain) was tested. All thresholds were obtained with ramped stimuli (1°C/s) that were terminated when the subject pressed a button. Cutoff temperatures were 0 and 50°C. The baseline temperature was 32°C. Three consecutive recordings were averaged. Based on the values obtained from the control reference, the mean threshold temperature ±2.5 SD for each modality was considered as the upper or lower limit of normal.

**Nerve conduction velocity tests**

Ulnar, median, sural, and peroneal nerve conduction velocities (NCVs) were performed on the right side of each subject using EMG (key point, medronet 4, Minneapolis, MN). All stimulations were performed supramaximally. The threshold for the slowed NCV was set at >2.5 SD of the control NCV. When two or more nerves were tested abnormal, nerve conduction (NC) was considered abnormal.

**Cardiac autonomic neuropathy tests**

Testing was carried out based on standard procedures. The Valsalva maneuver was performed three times. The mean value was the ratio of the longest R-R interval to the shortest R-R interval after breathing against a resistance of 40 mmHg for 15 s. The expiration-inspiration ratio was the ratio of the mean of the three longest R-R intervals on expiration to the mean of the three shortest R-R intervals on inspiration during 1 min of deep breathing at six breaths per minute. The lying-to-standing ratio was the ratio of the longest R-R interval around the 30th beat after standing to the shortest R-R interval around the 15th beat after standing. The blood pressure response was tested. The blood pressure was measured in the supine position at 1 min after standing up and at 1-min intervals thereafter for a 5-min period. A decrease in systolic pressure of more than at least 20 mmHg was considered abnormal. The results were evaluated using age-related reference values. Two or more abnormal tests were required for a confirmed diagnosis of cardiac autonomic neuropathy (CAN).

**Diagnosis and stages of polyneuropathy**

Diabetic neuropathy was classified according to the American Diabetes Association recommendation. The peripheral nerve deficit of nondiabetic origin (e.g., compression due to vertebral disk herniation, vitamin B deficiency, alcoholism, or primary amyloidosis) was excluded through a careful medical history review, a differential test, or both.

Polyneuropathy was further staged according to components of composite scores similar to that previously described by Dyck (14): 1) NC, 2) neurological examination (NE), 3) quantitative thermal sensory test (QTST) or autonomic examination (QAE), and 4) neuropathic symptoms (NS). Stage 0 (no neuropathy) was defined as fewer than two abnormalities among the above parameters. Stage 1 (asymptomatic neuropathy) was two or more abnormalities among 1) NC, 2) NE,
Clinical feature measurement

Body weight was measured on the same scales in light clothing and no shoes before breakfast, and upright height was measured on the same wall-mounted stadiometer. Individual BMI was then calculated as weight (kg)/height (m)^2. The right-arm blood pressure of each seated subject was obtained after 10 min of rest using a mercury sphygmomanometer. The last two of three consecutive readings with 1-min intervals were averaged as the blood pressure.

Retinal conditions were assessed by ophthalmologists using a combination of clinical examination, stereoscopic retinal photographs, and fluorescein angiography.

Blood NSE and other parameter measurement

Fasting serum NSE concentration was collected and measured using electrochemiluminescence immunoassay automatic analyzer (Analytics E170; Roche, Basel, Switzerland) according to the instruction of the manufacturer. Hemolysis was avoided during the procedure. Fasting plasma glucose, plasma cholesterol and triacylglycerol, and serum creatinine were measured using an automatic analyzer (AU5400; Olympus, Shinjuku, Japan). HbA_1c was measured using high-performance liquid chromatography (D10; Bio-Rad, Berkeley, CA). Values of HbA_1c were first reported as percent, followed by the mmol/mol equivalent. Serum vitamin B12 was determined using automated test assays (Access; Beckman, Brea, CA). Urinary albumin concentration was measured using immunoelectrophelometry (DCA 2000; Bayer, Leverkusen, North Rhine-Westphalia, Germany).

Urinary creatinine concentrations were measured using the alkaline picrate method. The individual urinary albumin-to-creatinine ratio (ACR) was then calculated as albumin (mg)/creatinine (g). The endogenous Ccr was calculated to estimate the glomerular filtration rate according to the Cockcroft equation: Ccr = [(140 – age (years)) × body weight (kg)]/0.818 × serum creatinine (Scr, μmol/L)] for male and the result × 0.85 for female. All measurements were repeated twice.

Statistical analysis

We used SPSS version 13 for Windows (SPSS Inc., Chicago, IL) software for statistical analysis. The data were expressed as median with 25th and 75th quartiles for skewed data or as the mean (SD) for normally distributed data. Nonnormally distributed variables were log transformed before analysis. The multiple comparisons among groups were assessed using ANOVA for variables. Percentages were compared using the x^2 test. The variables, including NSE, were first assessed in univariate analyses. Serum NSE was later added to a logistic regression model, controlling for possible confounders for serum NSE. The relation of the NSE levels to the stages of neuropathy was also examined. Receiver operating characteristic (ROC) analysis was conducted with MedCalc Software version 12.0 for Windows (Mariakerke, Belgium) to assess the accuracy of serum NSE levels in distinguishing between patients with diabetic neuropathy and without. The optimal cutoff point was identified by calculating the area under the curve (AUC). P < 0.05 was considered statistically significant.

All procedures were conducted according to the 19th revision of the Declaration of Helsinki.

RESULTS — A total of 17 subjects withdrew from the study for the following reasons: withdrawal of consent, medical care affordability, deterioration of medical conditions, or other difficulties completing the required tests. The study was completed by 568 subjects, 21–77 years of age, including 136 healthy control subjects, 218 diabetic subjects without neuropathy, and 214 diabetic subjects with neuropathy (Table 1). Among the three groups of subjects, there were no obvious differences between any given two groups in the following variables: age, sex ratio, diabetes type ratio, BMI, blood pressure, lipid profile (total cholesterol, HDL cholesterol, and triglycerides), vitamin B12, and renal status (urinary ACR and Ccr) (insignificant P values not shown). Retinopathy (46 non-proliferative diabetic retinopathy and 12 proliferative diabetic retinopathy) or nephropathy was identified in 58 and 49 diabetic patients, respectively. Neuropathy was identified in diabetic subjects with increased levels of blood glucose and HbA_1c, longer duration of diabetes, increased prevalence of retinopathy, and increased use of antidiabetics, antihypertensives, and lipid-regulating agents compared with the patients without neuropathy. Serum NSE levels increased slightly in the diabetic subjects, and the levels were significantly elevated in diabetic subjects with neuropathy in contrast to those without (10.8 [2.8] vs. 9.1 [1.5], P = 0.000) (Table 1). In addition, NSE was shown to be higher in neuropathic patients with retinopathy than those without (11.5 [1.7] vs. 9.6 [1.4], P = 0.034). The two sexes had similar NSE levels (9.0 [1.6] [male] vs. 9.2 [1.7] [female], P = 0.14). The serum NSE level in relation to neuropathy was further assessed in a multivariate model (Table 2), controlling for retinopathy and other co-variables that may potentially influence the NSE level or neuropathy, which included age, sex, the type of diabetes, blood glucose, HbA_1c, duration of diabetes, vitamin B12, and renal status. After adjustment, the serum NSE level was still independently associated with diabetic neuropathy (odds ratio 1.48 [1.13–1.74], P = 0.001). The diabetic neuropathy identified included 172 distal sensorimotor peripheral neuropathies (DSMPNs) without CANs, 14 CANs without DSMPNs, and 28 DSMPNs mixed with CANs. The overall severity of the neuropathies was stage 1, 2, or 3 in 95, 75, and 44 patients, respectively. The serum NSE levels increased with the neuropathy stages from the asymptomatic to the disabling (stage 1–3) when compared with diabetic subjects without neuropathy (stage 0) (P = 0.004–0.000, respectively) (Table 3). Correspondingly, the NSE levels were closely correlated to the stages of neuropathy (r = 0.63 [0.52–0.74], P = 0.000). Using ROC analysis, the optimal cutoff point for the serum NSE level in order to distinguish patients with diabetic neuropathy from those without was 10.10 μg/L, with a sensitivity of 66.3%, a specificity of 72.5%, and a highest AUC equal to 0.73 (0.68–0.77, P = 0.000) (Fig. 1).

CONCLUSIONS — The results of this study demonstrated that serum NSE is a potential biomarker of DPNP. In this study,
NSE levels were increased in diabetic subjects. More important, NSE was observed to be significantly elevated in those with neuropathy. The elevated NSE levels were closely related to diabetic neuropathy, and this relationship was independent of covariables. In addition, enolase levels increased with stages of neuropathy, and they were significantly correlated. The threshold of the serum NSE level for the presence of DPNP was found to be ~10.10 μg/L. This cutoff value was higher than the mean value of NSE in either diabetic or healthy control subjects of the study but did not exceed the normal upper limit (16.5 μg/L) of the reference range for the general population in our hospital laboratory. This result suggests that the upper limit, if applicable as a reference value, may have been too high, at least for these subjects with diabetic neuropathy. This is most likely attributed to subject selection. A larger study is needed to confirm our findings.

High glucose levels have been reported to be linked to elevated serum enolase concentrations in stroke patients (19). In addition to central nervous system (CNS) disorders, hyperglycemia-induced pericyte loss contributes to blood–brain barrier disruption (20). We found a slightly increased level of serum enolase in diabetic patients without detectable CNS disorders and peripheral neuropathies. Like the blood–brain barrier, the permeability of the structurally similar endothelium may also be increased in patients with diabetes. Further increased levels of NSE were shown to be highly associated with confirmed DPNPs. In our study, neuropathy severity was assessed using the composite components: quantitative neuroelectrophysiological tests, quantitative somatic and autonomic tests, and clinical manifestations. Therefore, elevated NSE levels may be indicative of neuropathy and the level of involved nerve fiber damage, which are likely to be associated with changes in the synthesis and release of the enolase.

During the process of the pathological changes, including demyelination and remyelination, associated with DPNP, NSE may be released not only from affected neurons but also from affected Schwann cells forming myelin, as it was suggested in one report that NSE was detected in oligodendrocytes as well as in neurons (21). This may explain why NSE levels were higher in large-fiber neuropathy than small-fiber neuropathy in the spectrum of neuropathy, although both were closely correlated to NSE from our observation (data not shown). Interestingly, NSE was shown to be more elevated in neuropathy with retinopathy than without in our study. Even early diabetic retinopathy

### Table 1—The demographic, clinical, and laboratory profiles of all the subjects

| Variables                      | Healthy control | Diabetes without neuropathy | Diabetes with neuropathy |
|-------------------------------|-----------------|-----------------------------|--------------------------|
| Age (years)                   | 52.5 (37.9–61.9) | 51.4 (43.3–60.6)           | 52.3 (40.6–64.8)        |
| Sex, male/female (%)          | 65.4            | 64.7                        | 67.2                     |
| Type 1/type 2 diabetes (%)    |                 |                             |                          |
| Fasting glucose (mmol/L)      | 5.3 (3.5)       | 7.4 (2.3)                   | 7.9 (2.6)                |
| HbA1c (%) (mmol/mol)          | 5.4 (36 [42])   | 6.8 (51 [64])              | 7.5 (58 [93])           |
| Duration of diabetes (years)  |                 |                             |                          |
| Retinopathy (%)               |                 | 5.9 (2.4)                   | 7.2 (3.1)                |
| BMI (kg/m²)                   | 22.8 (3.3)      | 23.0 (2.1)                  | 23.4 (2.5)              |
| Systolic blood pressure (mmHg)| 130 (12)        | 129 (11)                    | 130 (14)                |
| Diastolic blood pressure (mmHg)| 78 (10)        | 78 (9)                      | 79 (12)                 |
| Plasma total cholesterol (mmol/L)| 5.0 (0.8)    | 5.1 (1.0)                   | 5.0 (0.7)               |
| Plasma HDL cholesterol (mmol/L)| 1.06 (0.10)  | 1.05 (0.10)                 | 1.06 (0.08)             |
| Plasma triglycerides (mmol/L) | 1.52 (0.19)     | 1.52 (0.26)                 | 1.51 (0.28)             |
| Serum B12 (pmol/L)            | 389 (106)       | 402 (108)                   | 390 (112)               |
| Urinary ACR (mg/g)            | 24 (0.1)        | 24 (6.2)                    | 25 (7.2)                |
| Ccr (ml/min)                  | 84.1 (10.4)     | 81.9 (7.3)                  | 82.5 (18.4)             |
| Serum NSE (μg/L)              | 8.7 (1.7)       | 9.1 (1.5)                   | 10.8 (2.8)              |

All values are mean (SD) for normally distributed data and median (interquartile range 25–75%) for skewed data. HbA1c is first reported as percent, followed by the mmol/mol equivalent in parentheses. Sex, retinopathy, and type 1/type 2 diabetes ratio data are percentage (%). N, insignificant, P > 0.05. 1P, healthy control vs. diabetes without neuropathy. 2P, diabetes without neuropathy vs. healthy control. 3P, diabetes with neuropathy vs. diabetes without neuropathy.

### Table 2—Multiple regression analysis of the relation of NSE to neuropathy

| Covariables         | OR (95% CI) | P value |
|---------------------|-------------|---------|
| Age (years)         | 1.02 (0.97–1.08) | 0.461   |
| Male/female (%)     | 0.83 (0.54–1.10) | 0.145   |
| Type 1/type 2 diabetes (%) | 0.97 (0.32–2.61) | 0.953   |
| Fasting glucose (mmol/L) | 1.01 (0.89–1.11) | 0.890   |
| HbA1c (%)           | 1.13 (1.02–1.24) | 0.041   |
| Duration of diabetes (years) | 1.31 (1.06–1.50) | 0.005   |
| Retinopathy (%)     | 0.57 (0.28–1.13) | 0.107   |
| Serum B12 (pmol/L)  | 0.95 (0.87–1.05) | 0.768   |
| Urinary ACR (mg/g)  | 0.98 (0.92–1.03) | 0.613   |
| Ccr (ml/min)        | 0.93 (0.78–1.14) | 0.832   |
| Serum NSE (μg/L)    | 1.48 (1.13–1.74) | 0.001   |

Sex, retinopathy, and type 1/type 2 diabetes ratio data are percentage (%). N, insignificant, P > 0.05. Negelkerke R² = 0.23. OR, odds ratio.
Involves a retinal neurodegenerative change (22). Therefore, a fraction of this NSE may be due to nerve damage in the retina. After retinopathy was considered, NSE was still correlated to peripheral neuropathy.

Apart from retinopathy, the NSE level associated with neuropathy was independent of other variables. Hemolysis, a major interfering factor, was avoided so that the analysis would not be affected during the study. We excluded CNS disorders and other diseases reported to cause elevated serum enolase (23–27). Potential confounders that may influence the serum enolase levels were also considered, such as age, sex, and renal status, as suggested in previous reports on Alzheimer disease and cancer markers (28,29). In our subjects, we did not observe obvious differences in the serum NSE levels between the two sexes. Enolase levels increased slightly but not significantly with age. There was no significant enolase level correlation with renal function (by Ccr >50 mL/min). In addition, type 1 and 2 diabetes had similar NSE levels at the given levels of severity of neuropathy. These variables were considered among the studied groups during the evaluation of the relation of NSE to neuropathy. Furthermore, we did not find that antidiabetics, antihypertensives, and lipid-regulating agents (used in the subjects) affected NSE levels significantly (data not shown). In general, the coefficient of variation for inrasubject day-to-day reproducibilities of NSE analysis for our normal control and diabetic subjects was 7.9 and 9.5%, respectively.

There were some limitations of our study. We assessed the relationship between NSE levels and overall diabetic neuropathy, and the result may be influenced by the number and ethnicity of the subjects enrolled in this study. In addition, the difference in the level of NSE between diabetic patients without neuropathy and with asymptomatic neuropathy as opposed to the later stages of neuropathy was relatively small although significant. This, along with a relatively low sensitivity of the cutoff value of NSE (through ROC analysis) for the indication of neuropathy, may be attributed to the detection limit of the NSE assay currently used by our hospital. This low sensitivity for NSE may limit the clinical and research usefulness of the test. Future, larger-scale studies that include more subcategories of diabetic neuropathy along with possible improvements in the detection of NSE are warranted. This may clarify the NSE-neuropathy relationship observed in this cross-sectional study, thus providing an additional approach to guide the management of this diabetes complication.

In conclusion, we have observed for the first time that serum NSE levels are elevated in diabetes and are related to diabetic neuropathy. This may provide a potential blood biomarker for diabetic neuropathy. If future studies confirm our results, an increase of serum NSE as an indicator of diabetic neuropathy would aid the timely prediction, diagnosis, and treatment of the diabetic population.

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J.L. designed the study, researched data, and wrote, reviewed, and edited the manuscript. H.Z., M.X., and L.Y. collected and researched data. J.C. reviewed the manuscript.

H.W. collected and researched data and contributed to discussion. L.Y. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 3—The relation of levels of NSE to stages of neuropathy

| Neuropathy stage | n  | NSE | P value |
|------------------|----|-----|---------|
| 0                | 218| 9.1 | 1.7     |
| 1                | 95 | 9.7 | 2.1     |
| 2                | 75 | 10.9| 4.1     |
| 3                | 44 | 11.4| 3.2     |

Stage 0, diabetes with no neuropathy; stage 1, diabetes with asymptomatic neuropathy; stage 2, diabetes with symptomatic neuropathy; stage 3, diabetes with disabling neuropathy. 1 P stage 1 vs. stage 0. 2 P stage 2 vs. stage 0. 3 P stage 3 vs. stage 0.

Figure 1—ROC plot. Serum NSE levels were shown in distinguishing between patients with and without diabetic neuropathy. The optimal cutoff point of the serum NSE levels was 10.10 μg/L, with a sensitivity of 66.3%, a specificity of 72.5%, and the highest AUC equal to 0.73 (0.68–0.77, P = 0.000).
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