

ORIGINAL ARTICLE

Lower serum extracellular superoxide dismutase levels are associated with polyneuropathy in recent-onset diabetes

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Increased oxidative stress is implicated in the pathogenesis of experimental diabetic neuropathy, but translational evidence in recent-onset diabetes is scarce. We aimed to determine whether markers of systemic oxidative stress are associated with diabetic sensorimotor polyneuropathy (DSPN) in recent-onset diabetes. In this cross-sectional study, we measured serum concentrations of extracellular superoxide dismutase (SOD3), thiobarbituric acid reactive substances (TBARS), and reduced glutathione (GSH) in 107 type 1 and 215 type 2 diabetes patients from the German Diabetes Study baseline cohort and 37 glucose-tolerant individuals (controls). DSPN was defined by electrophysiological and clinical criteria (Toronto Consensus, 2011). SOD3 and GSH concentrations were lower in individuals with type 1 and type 2 diabetes compared with concentrations in controls ($P < 0.0001$). In contrast, the TBARS concentration was higher in participants with type 1 diabetes and type 2 diabetes compared with levels in controls ($P < 0.0001$). In addition, the SOD3 concentration was higher in participants with type 1 diabetes compared to concentrations in those with type 2 diabetes ($P < 0.0001$). A low SOD3 concentration was associated with DSPN in individuals with type 1 diabetes ($\beta = -0.306$, $P = 0.002$), type 2 diabetes ($\beta = -0.164$, $P = 0.017$), and in both groups combined ($\beta = -0.206$, $P = 0.0003$). Lower SOD3 concentrations were associated with decreased motor nerve conduction velocity (NCV) in men and, to a lesser degree, with reduced sensory NCV in women with diabetes. In conclusion, several biomarkers of oxidative stress are altered in recent-onset diabetes, with only a lower SOD3 concentration being linked to the presence of DSPN, suggesting a role for reduced extracellular antioxidative defense against superoxide in the early development of DSPN.

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INTRODUCTION

Diabetic sensorimotor polyneuropathy (DSPN) is one of the most common and severe diabetes complications, associated with substantial morbidity, increased mortality and reduced quality of life.¹ A decreased cellular protection through antioxidants can result in oxidative stress and cell death. Reactive oxygen species, including mitochondrial overproduction of superoxide, have been linked to the development of diabetic microvascular complications, including experimental neuropathy.² Superoxide dismutases (SODs) are the primary antioxidant defense system, catalyzing dismutation of superoxide into O_2 and H_2O_2 .³ In mammals, there are cytosolic (SOD1), mitochondrial (SOD2) and extracellular (SOD3)

isoforms that are all products of distinct genes.⁴ Reduced glutathione (GSH) is another important antioxidant, which functions, among other roles, as a co-substrate for glutathione peroxidase activity.⁵ Thiobarbituric acid reactive substances (TBARS) represent an indirect measure of early-stage lipid peroxidation triggered by oxidative stress.^{6,7} A range of experimental studies indicate that oxidative stress is a major factor contributing to deficits in nerve conduction and nerve blood flow in diabetic neuropathy.⁷ In line with experimental evidence, we demonstrated previously that increased plasma superoxide generation is associated with the severity of DSPN⁸ and may predict the progression of nerve conduction slowing and cardiac autonomic dysfunction over six years in longer-

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term diabetes patients.⁹ In recently diagnosed type 2 diabetes, we found increased dermal expression of mitochondrial SOD2 in the lower limbs.¹⁰ In the present study, we aimed to determine whether markers of systemic oxidative stress and antioxidative defense are altered in relation to the presence of early DSPN in individuals with recently diagnosed type 1 or type 2 diabetes.

MATERIALS AND METHODS

Participants

Volunteers with type 1 diabetes ($n=107$) or type 2 diabetes ($n=215$) were consecutive participants from the prospective German Diabetes Study (GDS), which evaluates the long-term course of diabetes and its sequelae (ClinicalTrials.gov Identifier: NCT01055093). Inclusion criteria for entry into the GDS were type 1 diabetes or type 2 diabetes, known diabetes duration of ≤ 1 year, and age ≥ 18 years. Exclusion criteria were secondary diabetes, pregnancy, severe diseases such as cancer, psychiatric disorders, immunosuppressive therapy, limited cooperation ability or neuropathy from causes other than diabetes. The baseline cohort profile has been published elsewhere.¹¹ Glucose-tolerant individuals were recruited as controls also at the German Diabetes Center ($n=37$; sex (male): 54.1%; age: 40.4 ± 2.5 years; BMI: 25.2 ± 0.6 ; smokers: 13.5%). Inclusion criteria for this group were age ≥ 18 years, while exclusion criteria corresponded to those defined in the persons with diabetes or an abnormal oral glucose tolerance test.

This cross-sectional study was approved by the local ethics committee (Heinrich Heine University, Düsseldorf). All participants provided a written informed consent according to the Declaration of Helsinki.

Peripheral nerve function and neurological examination

Peripheral nerve function tests were performed as previously described.¹² Motor nerve conduction velocity (MNCV) was measured in the median, ulnar and peroneal nerves, while sensory nerve conduction velocity (SNCV) was determined in the median, ulnar and sural nerves at a skin temperature of 33–34 °C using surface electrodes (Nicolet VikingQuest, Natus Medical, San Carlos, CA, USA). Quantitative sensory testing was evaluated by vibration perception threshold (VPT) at the second metacarpal bone and medial malleolus using the method of limits (Vibrometer, Somedic, Stockholm, Sweden) and by thermal detection thresholds (TDT) including warm and cold thresholds at the thenar eminence and dorsum of the foot using the method of limits (TSA-II NeuroSensory Analyzer, Medoc, Ramat Yishai, Israel).¹² Neurological examination was performed using the Neuropathy Disability Score (NDS) and Neuropathy Symptom Score (NSS).¹³

Stages of DSPN were defined according to modified Toronto Consensus criteria.¹⁴ *Subclinical DSPN (Stage 1a)*: NDS ≤ 2 points, NSS ≤ 2 points, and peroneal motor nerve conduction velocity (MNCV) < 2.5 th percentile and sural sensory nerve conduction velocity (SNCV) and/or sural sensory nerve amplitude (SNAP) < 2.5 th percentile; *confirmed asymptomatic DSPN (Stage 1b)*: NDS ≥ 3 points and NSS ≤ 2 points and peroneal MNCV < 2.5 th percentile and sural SNCV < 2.5 th percentile and/or sural SNAP < 2.5 th percentile; *confirmed symptomatic DSPN (Stage 2)*: NSS ≥ 3 points and peroneal MNCV < 2.5 th percentile and sural SNCV < 2.5 th percentile and/or sural SNAP < 2.5 th percentile.

Markers of oxidative stress

Extracellular superoxide dismutase (SOD3) concentration in serum samples was measured using the human Enzyme-linked Immunosorbent Assay kit for SOD3 (Hözel Diagnostika, Germany) according to

Table 1 Anthropometric, demographic, clinical, and neurophysiological data of the individuals with diabetes studied

| | Type 1 diabetes (n = 107) | Type 2 diabetes (n = 215) | P-value |
|---|---------------------------|---------------------------|---------|
| Age (years) | 34.4 \pm 1.3 | 52.8 \pm 0.7 | <0.0001 |
| Sex (% male) | 57.0 | 66.0 | NS |
| BMI (kg m ⁻²) | 24.6 \pm 0.4 | 31.7 \pm 0.4 | <0.0001 |
| Height (cm) | 175 \pm 1.0 | 172 \pm 0.6 | 0.014 |
| Heart rate (b.p.m.) | 79.3 \pm 1.4 | 80.8 \pm 0.9 | NS |
| Systolic BP (mm Hg) ^a | 130 \pm 1.5 | 142 \pm 1.3 | NS |
| Diastolic BP (mm Hg) ^a | 77.8 \pm 1.0 | 85.0 \pm 0.8 | 0.05 |
| Regular smokers (%) | 19.6 | 18.1 | NS |
| Diabetes duration (months) | 6.62 \pm 0.30 | 5.55 \pm 0.20 | 0.005 |
| HbA _{1c} (mmol mol ⁻¹) | 50.6 \pm 1.5 | 47.4 \pm 0.7 | NS |
| HbA _{1c} (%) | 6.78 \pm 0.14 | 6.48 \pm 0.06 | NS |
| Peroneal MNCV (m s ⁻¹) ^a | 46.4 \pm 0.4 | 44.4 \pm 0.3 | NS |
| Sural SNCV (m s ⁻¹) ^a | 45.5 \pm 0.5 | 43.9 \pm 0.4 | NS |
| Malleolar VPT (μ m) ^a | 0.62 \pm 0.03 | 2.19 \pm 0.20 | NS |
| Warm TDT foot (°C) ^a | 38.2 \pm 0.3 | 40.4 \pm 0.3 | NS |
| Cold TDT foot (°C) ^a | 28.6 \pm 0.2 | 27.0 \pm 0.3 | NS |
| NSS ^a | 0.22 \pm 0.10 | 1.00 \pm 0.15 | NS |
| NDS ^a | 0.45 \pm 0.12 | 1.72 \pm 0.15 | NS |
| DSPN (%) | 21.5 | 30.2 | NS |

Abbreviations: DSPN, diabetic sensorimotor polyneuropathy; MNCV, motor nerve conduction velocity; NDS, Neuropathy Disability Score; NSS, Neuropathy Symptom Score; NS, not significant; SNCV, sensory nerve conduction velocity; TDT, thermal detection threshold; VPT, vibration perception threshold.

Data are expressed as the mean \pm s.e.m. except for sex, regular smokers and DSPN variables, which are expressed as %. Mann-Whitney-U-test was used where appropriate to calculate differences between groups. $P < 0.05$ was considered significant.

^aGroup comparisons were adjusted for age, sex, BMI, smoking, known diabetes duration, and HbA_{1c}.

the manufacturer's instructions. All samples were analyzed in duplicate. TBARS were assessed, as described before.¹⁵ In brief, the quantification of malonaldehyde in serum samples was performed using a TBARS assay (Sigma Aldrich, Germany) according to the manufacturer's instructions. Serum concentration of reduced glutathione (GSH) was assessed by the enzymatic recycling method using the Sensolyte Glutathione assay kit (Anaspec, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Categorical variables were compared using the χ^2 test. Continuous data were analyzed using the Nonparametric Mann–Whitney U test. Categorical data were expressed as percentages of participants with 95% confidence intervals (CI) while continuous data were expressed

as the mean \pm s.e.m. Correlation and multiple linear regression analyses were performed to determine associations between two variables. Dependent variables were assessed for normality and transformed (natural logarithm or square root) where appropriate to obtain normal distribution. The full models were adjusted for sex, age, BMI and HbA1c. All statistical tests were performed two-sided. The level of significance was set at $\alpha=0.05$. All analyses were performed using the SPSS v22.0 (Chicago, IL, USA).

RESULTS

Anthropometric, demographic, clinical and functional data of the two diabetes groups are shown in Table 1. Participants with type 1 diabetes were younger, taller, and had lower BMI, lower diastolic blood pressure, and longer known diabetes duration than type 2 diabetes individuals (all $P<0.05$). There were no differences in neurophysiological function between both groups. DSPN was present in 21.5% of type 1 diabetes (subclinical: 17.8%; confirmed asymptomatic: 0.9%; confirmed symptomatic: 2.8%) individuals and 30.2% (16.3/7.0/6.9%) of those with type 2 diabetes.

The mean concentrations of SOD3, TBARS and GSH are also shown in Figure 1. After adjustment for age, sex, BMI and HbA1c, SOD3 concentration was reduced in both type 1 (38.6 ± 1.3 ng ml⁻¹) and type 2 diabetes (31.5 ± 0.8 ng ml⁻¹) patients compared with controls (53.6 ± 2.5 ng ml⁻¹), as well as in type 2 diabetes patients compared with type 1 diabetes patients ($P<0.0001$). GSH was also decreased in both type 1 diabetes (1.61 ± 0.06 μ mol) and type 2 diabetes (1.63 ± 0.05 μ mol) patients compared with controls (2.62 ± 0.19 μ mol). In contrast, TBARS concentration was higher in type 1 (2.85 ± 0.15 μ mol) and type 2 diabetes (2.98 ± 0.10 μ mol) patients compared with controls (0.88 ± 0.05 μ mol) ($P<0.0001$ for type 1 and type 2 diabetes vs controls for both markers).

The entire study population showed a positive correlation of SOD3 with peroneal, median and ulnar MNCV, sural, median and ulnar SNCV, median and ulnar SNAP, and an inverse correlation of SOD3 with NDS and DSPN (all $P<0.05$), which remained significant after adjustment for age, sex, BMI and HbA1c for all parameters, except for median MNCV and SNAP (Table 2). In the type 1 diabetes group, SOD3 concentrations showed positive correlations with peroneal MNCV, sural, median and ulnar SNCV, and median SNAP and an inverse association with DSPN (all $P<0.05$), which remained significant after adjustment for all parameters, except for median SNCV. In the type 2 diabetes group, only a positive correlation of SOD3 concentrations with ulnar MNCV and an inverse correlation with DSPN were observed (both $P<0.05$), which remained significant after adjustment. No associations of GSH and TBARS with neurophysiological parameters were found. SOD3 concentrations were markedly higher in patients without DSPN compared to those with DSPN.

Gender-specific correlations of serum SOD3 concentrations with neurophysiological and clinical parameters and DSPN were also observed (Table 3 and Figure 2). In men, SOD3 correlated positively with peroneal, median, and ulnar MNCV,

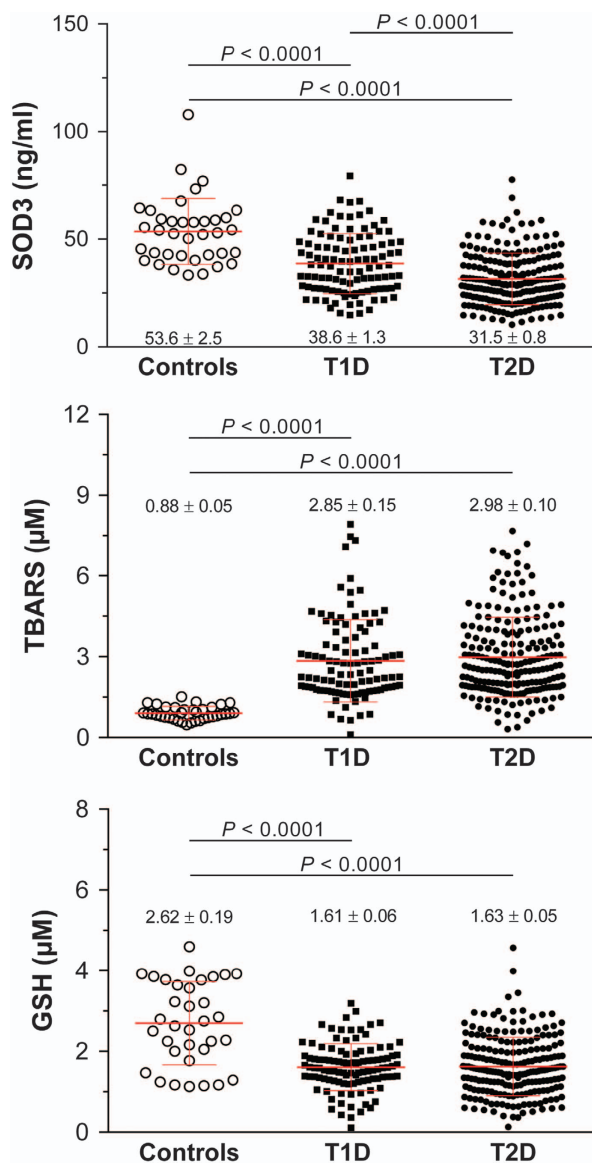


Figure 1 Serum concentrations of SOD3, GSH and TBARS in recently diagnosed diabetes patients and individuals with normal glucose tolerance (controls). Each spot represents one individual. The horizontal line represents the mean, while the whiskers show the SD. *P* values adjusted for age, sex, BMI and HbA1c are given. (T1D, type 1 diabetes; T2D, type 2 diabetes).

Table 2 Correlations of serum SOD3 concentration with neurophysiological and clinical measures and diabetic sensorimotor polyneuropathy (DSPN).

| | All participants | | Type 1 diabetes | | Type 2 diabetes | |
|---------------|------------------|---------------------------|-----------------|--------------------------|-----------------|--------------------------|
| | <i>r</i> | <i>P</i> -value | <i>r</i> | <i>P</i> -value | <i>r</i> | <i>P</i> -value |
| Peroneal MNCV | 0.153 | 0.006^a | 0.213 | 0.028^a | 0.073 | 0.287 |
| Median MNCV | 0.188 | 0.001 | 0.162 | 0.096 | 0.103 | 0.133 |
| Ulnar MNCV | 0.176 | 0.002^a | 0.091 | 0.351 | 0.171 | 0.012^a |
| Sural SNCV | 0.114 | 0.040^a | 0.204 | 0.035^a | 0.040 | 0.561 |
| Sural SNAP | 0.048 | 0.388 | 0.159 | 0.101 | -0.104 | 0.130 |
| Median SNCV | 0.174 | 0.002^a | 0.219 | 0.024 | 0.071 | 0.304 |
| Median SNAP | 0.161 | 0.004 | 0.295 | 0.002^a | 0.001 | 0.993 |
| Ulnar SNCV | 0.162 | 0.004^a | 0.231 | 0.018^a | 0.124 | 0.071 |
| Ulnar SNAP | 0.162 | 0.004^a | 0.142 | 0.148 | 0.050 | 0.467 |
| NSS* | -0.081 | 0.146 | -0.116 | 0.234 | -0.015 | 0.830 |
| NDS* | -0.109 | 0.05 | -0.021 | 0.827 | -0.022 | 0.744 |
| DSPN* | -0.216 | 0.0001^a | -0.296 | 0.002^a | -0.143 | 0.036^a |

Abbreviations: MNCV, motor nerve conduction velocity; NDS, Neuropathy Disability Score; NSS, Neuropathy Symptom Score; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity; TDT, thermal detection threshold; VPT, vibration perception threshold.

Pearson correlation coefficients were used to determine correlations between two variables (*Spearman correlation coefficients were applied). *P*<0.05 (bold) was considered significant.

^aAssociation remained significant after multiple linear regression analyses with adjustments for age, sex, BMI, smoking, known diabetes duration and HbA_{1c}.

Table 3 Gender-specific correlations of serum SOD3 concentrations with neurophysiological and clinical parameters and DSPN

| | Male (n = 203) | | Female (n = 119) | |
|---------------|----------------|---------------------------|------------------|--------------------------|
| | <i>r</i> | <i>P</i> -value | <i>r</i> | <i>P</i> -value |
| Peroneal MNCV | 0.231 | 0.001^a | 0.107 | 0.248 |
| Median MNCV | 0.269 | 0.0001^a | 0.074 | 0.422 |
| Ulnar MNCV | 0.245 | 0.0004^a | 0.112 | 0.224 |
| Sural SNCV | 0.090 | 0.199 | 0.234 | 0.010^a |
| Sural SNAP | -0.009 | 0.895 | -0.171 | 0.063 |
| Median SNCV | 0.197 | 0.005 | 0.151 | 0.105 |
| Median SNAP | 0.170 | 0.016 | 0.171 | 0.066 |
| Ulnar SNCV | 0.130 | 0.065 | 0.246 | 0.008^a |
| Ulnar SNAP | 0.212 | 0.003 | 0.148 | 0.113 |
| NSS* | -0.122 | 0.083 | -0.015 | 0.869 |
| NDS* | -0.143 | 0.041 | -0.071 | 0.443 |
| DSPN* | -0.225 | 0.001^a | -0.199 | 0.030^a |

Abbreviations: MNCV, motor nerve conduction velocity; NDS, Neuropathy Disability Score; NSS, Neuropathy Symptom Score; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity; TDT, thermal detection threshold; VPT, vibration perception threshold.

Pearson correlation coefficients were used to determine correlations between two variables (*Spearman correlation coefficients were applied). *P*<0.05 (bold) was considered significant.

^aAssociation remained significant after multiple linear regression analyses with adjustments for age, sex, BMI, smoking, known diabetes duration and HbA_{1c}.

median SNCV, median SNAP and ulnar SNAP and inversely with NDS and DSPN (all *P*<0.05). In contrast, women showed only positive correlations of SOD3 with sural and ulnar SNCV and an inverse correlation with DSPN (all *P*<0.05). For all three parameters, the associations observed in women remained significant after adjustment, while in men, the associations remained significant for peroneal, median, and

ulnar MNCV and DSPN. There was no difference between men and women in mean SOD3 concentration (34.6 ± 0.9 vs 32.6 ± 1.2 ng ml⁻¹).

DISCUSSION

This study demonstrates that patients with well-controlled, recent-onset type 1 or type 2 diabetes exhibit markedly decreased serum SOD3 and GSH concentrations and increased TBARS levels, but only SOD3 levels are associated with early changes in motor and sensory nerve conduction attributes and the presence of DSPN defined by electrophysiological criteria. This novel finding is in line with the hypothesis suggesting that a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain plays a pivotal role in the pathogenesis of experimental diabetic neuropathy.⁶ Since these associations were found despite favorable glycemic control, with mean HbA_{1c} levels of 6.8 and 6.5% in type 1 and type 2 diabetes, respectively, it is conceivable that only mild degrees of hyperglycemia are sufficient to trigger the neuropathic process.

SOD3 is a secretory extracellular isoform and the major SOD located in the vascular extracellular space, where it plays an important role in maintaining endothelial function.¹⁶ *In vitro*, SOD3 secreted in response to inflammatory cytokines by human mesenchymal stem cells promotes neuronal survival.¹⁷ Therefore, decreased SOD3 concentration could lead to endothelial dysfunction and neurotoxicity mediated by inflammatory cytokines¹⁸ and be, at least partially, responsible for the development of early DSPN in recent-onset type 1 and type 2 diabetes patients.

Interestingly, SOD3 concentrations were more strongly associated with electrophysiological parameters in men than in women. Moreover, after adjustment for potential

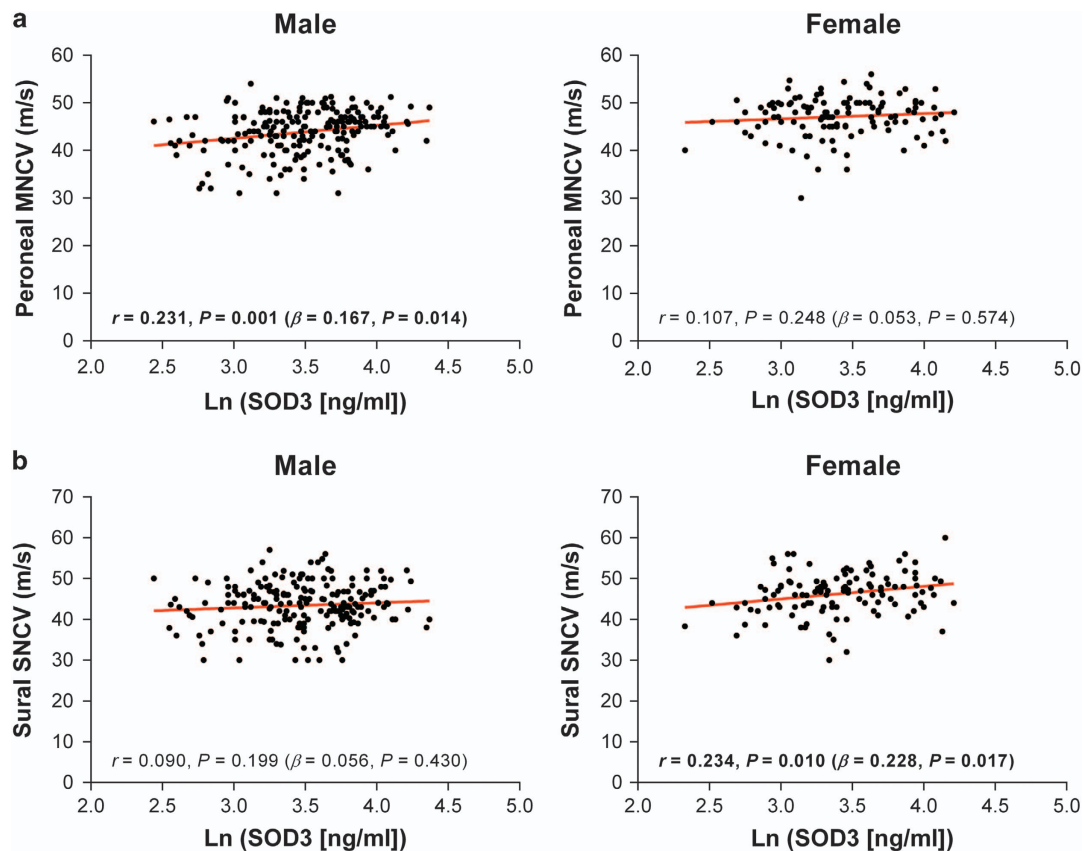


Figure 2 Examples of gender-specific correlations of SOD3 with electrophysiological parameters in recently diagnosed diabetes patients. (a) Correlation of SOD3 concentrations with peroneal motor nerve conduction velocity (MNCV) in male, but not female, recently diagnosed diabetes patients. (b) Correlation of SOD3 concentrations with sural sensory nerve conduction velocity (SNCV) in female, but not male, patients. Each spot represents one individual. Analyses were adjusted for age, sex, BMI, smoking and HbA1c.

confounders, lower SOD3 levels were associated with lower MNCV in men and lower SNCV in women. These findings are in line with other studies demonstrating gender differences in the risk factors associated with diabetes complications including DSPN.^{19,20} The reasons for the differences between the genders are still unclear. Although HDL cholesterol levels were considerably higher in women than men (1.51 ± 0.40 vs 1.23 ± 0.35 mmol l⁻¹; $P < 0.0001$), this finding cannot explain the difference, since higher HDL cholesterol was not associated with higher NCV. Future studies are needed to determine the mechanisms (for example, hormone composition or metabolic status) responsible for these discrepancies.

Insufficient cellular antioxidative defense mechanisms could be involved in processes of neuronal damage. Nerve damage and neuronal apoptosis are associated with reduced GSH concentrations and elevated oxidized lipoprotein concentrations.⁷ There are no published studies assessing the association between oxidative stress and DSPN in recent-onset diabetes. Previous studies showed that among patients with long-term type 2 diabetes, those with DSPN had lower cellular GSH and SOD levels compared with those without DSPN,^{21,22} whereas other studies showed higher erythrocyte SOD levels in individuals with type 1 and type 2 diabetes compared with control subjects.^{23,24} Thus, our study including recently

diagnosed diabetes patients does not confirm these reports. Reasons for these discrepancies could be the different study populations and samples analyzed. While these studies used circulating mononuclear cells to measure the levels of oxidative stress markers, we used serum samples.

The strengths of the present study are the large study sample size and the use of electrophysiological criteria to confirm the presence of DSPN used in addition to scores for clinical symptoms and signs. However, due to the cross-sectional nature of the present analysis, it was not possible to assess the predictive value of reduced antioxidative defense for incident DSPN.

In conclusion, patients with recently diagnosed type 1 and type 2 diabetes show evidence of systemic oxidative stress despite good glycemic control. Reduced SOD3 concentration was associated with diminished nerve conduction attributes and DSPN, pointing to a role of impaired extracellular antioxidative defense of superoxide in the evolution of early DSPN. Prospective studies should determine the long-term predictive value of SOD3 serum levels in the development and progression of DSPN.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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