Identification of Factors Associated With Sural Nerve Regeneration and Degeneration in Diabetic Neuropathy

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OBJECTIVE—Patients with diabetic neuropathy (DN) demonstrate variable degrees of nerve regeneration and degeneration. Our aim was to identify risk factors associated with sural nerve degeneration in patients with DN.

RESEARCH DESIGN AND METHODS—Demographic, anthropometric, biochemical, and anatomical data of subjects with DN from a 52-week trial of acetyl-L-carnitine were retrospectively examined. Based on the change in sural nerve myelinated fiber density (ΔMFD%), subjects were divided into three groups: regenerator (top 16 percentiles, $n = 67$), degenerator (bottom 16 percentiles, $n = 67$), and intermediate ($n = 290$), with dramatically increased, decreased, and steady ΔMFD%, respectively. ANOVA, Fisher exact test, and multifactorial logistic regression were used to evaluate statistical significance.

RESULTS—ΔMFD%s were 35.6 ± 17.4 (regenerator), −4.8 ± 12.1 (intermediate), and −39.8 ± 11.0 (degenerator). HbA_{1c} at baseline was the only factor significantly different across the three groups ($P = 0.01$). In multifactorial logistic regression, HbA_{1c} at baseline was also the only risk factor significantly different between regenerator (8.3 ± 1.6%) and degenerator (9.2 ± 1.8%) (odds ratio 0.68 [95% CI 0.54–0.85]; $P < 0.01$). Support Vector Machine classifier using HbA_{1c} demonstrated 62.4% accuracy of classifying subjects into regenerator or degenerator. A preliminary microarray experiment revealed that upregulated genes in the regenerator group are enriched with cell cycle and myelin sheath functions, while downregulated genes are enriched in immune/inflammatory responses.

CONCLUSIONS—These data, based on the largest cohort with ΔMFD% information, suggest that HbA_{1c} levels predict myelinated nerve fiber regeneration and degeneration in patients with DN. Therefore, maintaining optimal blood glucose control is likely essential in patients with DN to prevent continued nerve injury.

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Twenty-five million Americans, or >8% of the population, have diabetes, and 1.9 million new cases were diagnosed in 2010 (1). In the U.S., the total cost for management of diabetes in 2007 was 218 billion USD (1). Complications of diabetes, including diabetic neuropathy (DN), nephropathy, and retinopathy, often have a significant impact on quality of life. DN is the most common diabetes complication; 60–70% of diabetic patients develop DN (2). DN is responsible for >60% of nontraumatic lower-limb amputations (1,3). Management of DN-related complications accounts for an estimated 27% of the total cost of diabetes treatment (3).

The most common type of DN is distal symmetric polyneuropathy. It affects the longest axons in the extremities first and progresses proximally in a stocking-glove pattern with increasing severity and duration of diabetes (2). The sural nerve is one of the most frequently affected nerves in DN. Although overall sural myelinated fiber density (MFD) decreases with age, the nerve itself can regenerate, making the grafting of sural nerves into other injured nerves possible (4). Axonal regeneration is a natural response of the body to compensate for damage caused by diabetes, but incomplete or unsuccessful regeneration may constitute a critical component in DN progression (5).

Our laboratory maintains a unique repository of human sural nerve biopsies harvested as part of a double-blind placebo-controlled clinical trial testing acetyl-L-carnitine (ALC) efficacy for DN (6,7). ALC treatment alleviated pain symptoms but had no effect on sural nerve conduction velocities (NCVs), amplitudes, or MFD (6). Our initial demographic analyses of these participants revealed that elevated serum triglycerides measured at trial onset correlated with DN progression after correcting for baseline DN severity and clinical factors, such as sex, age, duration and types of diabetes, insulin treatment, ALC treatment, and HbA_{1c} (7). A subsequent study identified 532 differentially expressed genes (DEGs) between progressive and non-progressive DN, highly enriched with immune response and lipid metabolism (8). Our previous studies focused on the loss of absolute MFD over the course of a 52-week clinical trial, resulting into two groups of patients (a progressor group with ≥500 fibers/mm^2 MFD loss and a non-progressor group with ≤100 fibers/mm^2 MFD loss). While reexamining these data, we observed that ~43% of the subjects gained MFD over 52 weeks. Although modest regeneration has been documented in DN (9), no study has investigated critical factors affecting nerve regeneration in DN.

In the current study, we reexamined this DN cohort with MFD data available to identify critical factors that may impact sural nerve regeneration, focusing on subjects with the greatest gain or loss of MFD. As patients at different ages and

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Table 1—*Characteristics of subjects and statistical evaluation results*

				Fisher exact test or *t* test *P* value			
	Degen	Inter	Regen	Three groups	Degen vs. Regen	Inter vs. Degen	Inter vs. Regen
Total subjects	67	290	67				
ALC treatment				0.69	1	1	1
ALC	43	201	47				
Placebo	24	89	20				
Sex				0.12	0.21	1	0.35
Female	18	94	29				
Male	49	196	38				
Insulin treatment				0.06	1	0.11	0.64
Yes	32	181	36				
No	35	109	31				
Diabetes type				0.31	0.82	0.43	1
Type 1	10	68	16				
Type 2	57	222	51				
Age (years)	55.0 ± 9.7	52.7 ± 10.8	53.6 ± 10.0	0.25	0.25	1	1
BMI (kg/cm^2)	30.2 ± 5.7	29.5 ± 5.8	29.5 ± 5.1	0.66	1	1	1
Diabetes duration (years)	10.5 ± 8.1	12.7 ± 8.1	12.6 ± 9.5	0.15	0.15	0.50	1
HbA_{1c}, % (mmol/mol)	9.2 ± 1.8 (76.7 ± 20.0)	8.8 ± 1.7 (73.1 ± 18.9)	8.3 ± 1.6 (66.9 ± 17.7)	0.01*	0.01*	0.54	0.04*
Triglyceride (mmol/L)	3.6 ± 6.6	2.5 ± 2.7	2.6 ± 2.1	0.07	0.70	0.57	1
Cholesterol (mmol/L)	5.7 ± 1.7	5.6 ± 1.3	5.6 ± 1.3	0.86	1	1	1
Albumin (mmol/L)	42.6 ± 2.6	42.2 ± 2.8	42.5 ± 3.3	0.41	1	0.59	1
Hematocrit (fraction)	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.82	1	1	1
O'Brien neuropathy score	2,839.8 ± 1,167.5	3,563.4 ± 1,119.7	3,427.6 ± 1,132.7	1.88E-05*	0.01*	3.77E-05*	1
MFD baseline (fibers/mm^2)	2,949.2 ± 1,504.5	4,057.4 ± 1,762.8	3,100.0 ± 1,634.9	8.34E-08*	1	2.16E-06*	0.00*
MFD 52 weeks (fibers/mm^2)	1,792.4 ± 971.0	3,850.0 ± 1,700.7	4,054.6 ± 1,950.6	9.96E-19*	7.04E-13*	1.10E-27*	1
MFD change (fibers/mm^2)	−1,156.8 ± 670.1	−207.4 ± 539.4	954.6 ± 442.9	2.86E-72*	1.51E-41*	2.75E-17*	5.66E-36*
MFD percent change (%)	−39.8 ± 11.0	−4.8 ± 12.1	35.6 ± 17.4	3.89E-121*	9.51E-55*	1.35E-42*	9.80E-30*

Degen, degenerator; Inter, intermediate; Regen, regenerator. *Significant, $P < 0.05$.

duration of diabetes tend to have different levels of baseline MFD (4), we opted to investigate MFD percent change (ΔMFD%), rather than an absolute change in MFD to identify the clinical factors closely associated with MFD change over the specific duration of the disease course. With use of this classification approach, biomarkers and differential gene expression distinguishing patients exhibiting regeneration were examined and identified.

RESEARCH DESIGN AND METHODS

For all subjects, demographic, anthropometric, biochemical, and anatomical data included in the current study were reported previously (6,7). Briefly, human sural nerve biopsies were obtained during a double-blind, placebo-controlled, 52-week trial of ALC. The trial included both type 1 and 2 diabetic patients—all with existing neuropathy. A sural nerve biopsy (week 0, baseline) and a blood sample were collected at the time of patient enrollment, and the following measures were recorded: HbA_{1c}, hematocrit, serum triglycerides, cholesterol, and albumin. After 52 weeks of treatment, measures of DN were reassessed and a second sural nerve biopsy was harvested (week 52) from the opposite leg. All the harvested biopsies were processed at the Nerve Biopsy Laboratory, University of Michigan, according to the published protocols (10). No blood sample was collected at the end of the trial. Among the 748 participants in the trial, 427 participants had two sural nerve biopsies and complete blood chemistry analyses.

Outcome measures

The primary outcome measure was ΔMFD% at week 52. Three outlier subjects with >200% increase in MFD were excluded from any further analyses. In the remaining 424 subjects (ΔMFD% range −78.6 to 87.7%), 183 subjects (42.9%) demonstrated positive ΔMFD%s. Based on ΔMFD%, the subjects were divided into three groups: regenerator (top 16 percentiles equivalent to beyond 1 SD from the mean); degenerator (bottom 16 percentiles), and intermediate (remaining subjects).

Neuropathy evaluations

Electrophysiological measurements, including bilateral sural NCV and amplitude, peroneal NCV, and amplitude on the dominant side and median motor and

Table 2—*Factor analysis using multivariate logistic regression*

Factor	*P*	OR (95% CI)
ALC treatment (treated)	0.58	1.23 (0.58–2.61)
Sex (female)	0.08	2.01 (0.92–4.36)
Insulin treatment (no)	0.90	0.95 (0.41–2.21)
Diabetes type (type 1)	0.97	0.97 (0.26–3.68)
Age (years)	0.32	0.98 (0.94–1.02)
BMI	0.67	0.99 (0.92–1.06)
Diabetes duration (years)	0.25	1.03 (0.98–1.09)
HbA_{1c} (%, mmol/mol)	0.00**	0.68 (0.54–0.85)
Triglyceride (mmol/L)	0.30	0.94 (0.82–1.06)
Cholesterol (mmol/L)	0.22	1.25 (0.87–1.78)
Albumin (mmol/L)	0.74	0.98 (0.86–1.12)
Hematocrit (fraction)	0.79	0.29 (0.00–2,506.07)

**Significant, $P < 0.01$.

sensory NCV and amplitude on the nondominant side, were performed the baseline and completion of the trial to generate an O'Brien neuropathy score (6). These measurements were done in triplicate, and the median value was used.

Computational classifier for regenerator and degenerator

Computational classifiers of regenerator and degenerator were generated and evaluated using ORANGE (http://orange.biolab.si/), an open-source, component-based data-mining and machine learning software suite (11). Seven classification algorithms (Naïve Bayes, Logistic Regression, k Nearest Neighbors, Classification Tree, CN2 rules, Support Vector Machine [SVM], and Random Forest) available for binary class prediction were used with 20-fold cross-validation sampling to classify the subjects as regenerator or degenerator based on the demographic, anthropometric, and biochemical data.

Microarray data analysis

Based on the new grouping, we reanalyzed the previously published microarray data set (8) and an additional batch of unpublished microarray data ($n = 35$ and $n = 33$, respectively). These 68 microarrays included samples from 14 degenerators, 7 regenerators, and 45 intermediates. Microarrays were normalized using Robust Multiarray Average (12), and the batch effect was corrected using the distance-weighted discrimination method (13). Intensity-based moderated *T* statistics (14) was used to determine the DEGs among the groups. Owing to the small number of available microarrays (7 regenerators), a nominal *P* value of 0.05 without multiple testing corrections was used as the cutoff for DEGs.

The identified DEGs were further analyzed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) (15,16), to determine overrepresented biological functions in terms of Gene Ontology (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). LRpath (http://lrpath.ncibi.org/), a logistic regression-based gene set enrichment testing tool, was also used in our analysis. LRpath accepts statistical significance values from all genes on the tested array and does not require a predefined DEG set (17).

Correlation between regeneration cluster density and MFD change

Electron microscopy (EM) was performed on the baseline and/or 52-week biopsies of approximately half of the subjects ($n = 219$) immediately after the termination of the 52-week trial. The number of regenerating nerve clusters were counted, and the density of regenerating clusters was calculated (6). In the current study, we correlated the ΔMFD% over 52 weeks with changes in the density of regenerating clusters for subjects with both baseline and 52-week biopsies examined by EM ($n = 168$).

Statistical analysis

Variable differences between the groups were analyzed with the Fisher exact test for categorical variables and ANOVA with Bonferroni post hoc tests for continuous variables. Multifactorial logistic regression between the regenerator and degenerator groups was performed to evaluate the effect of multiple factors including age, sex, ALC treatment, diabetes type, diabetes duration, HbA_{1c}, insulin treatment, BMI, triglyceride, cholesterol, albumin, hematocrit, and O'Brien neuropathy rank-sum score (7,18). The statistical significance level was set at 0.05. For statistical analyses, R, version 2.15.2 (http://cran.r-project.org/), was used. Data are means ± SD or percentage unless otherwise stated.

RESULTS

Group classification

Based on the ΔMFD%, subjects were divided into three groups: regenerator ($n = 67$), degenerator ($n = 67$), and intermediate ($n = 290$). The mean ΔMFD%s ± SD were 35.6 ± 17.4 (regenerator), −4.8 ± 12.1 (intermediate), and −39.8 ± 11.0 (degenerator). Table 1 summarizes the demographic, anthropometric, and biochemical characteristics for all subjects, comparing subjects from regenerator, degenerator, and intermediate groups by ANOVA and Fisher exact tests.

At baseline, there were no differences between groups in age, sex, diabetes duration or type, BMI, triglyceride, or total cholesterol. There were also no significant differences between groups in the number of subjects randomized to ALC or subjects treated with insulin. In addition, there were no differences between MFD at baseline between degenerator and regenerator, but MFD was significantly higher at baseline in the intermediate group ($P = 8.34E-08$). The groups differed in the O'Brien neuropathy rank-sum score, based on the electrophysiological measurements, with the lowest score observed in the degenerator group (2,839.8 ± 1,167.5) and the highest score in the regenerator (3,427.6 ± 1,132.7) and intermediate (3,563.4 ± 1,119.7) groups ($P = 1.88E-05$). Among the other evaluated risk factors shown in Table 1, HbA_{1c} at baseline was the only risk factor significantly different across the three groups: regenerator (8.3 ± 1.6%), degenerator (9.2 ± 1.8%), and intermediate (8.8 ± 1.7%) ($P = 0.01$).

To further understand potential reasons that would drive regeneration or degeneration, we next compared the two extreme groups (degenerator vs. regenerator). Again, HbA_{1c} level was the only significantly different biochemical factor (Bonferroni-corrected $P = 0.01$) between the two groups, while all other variables listed were not

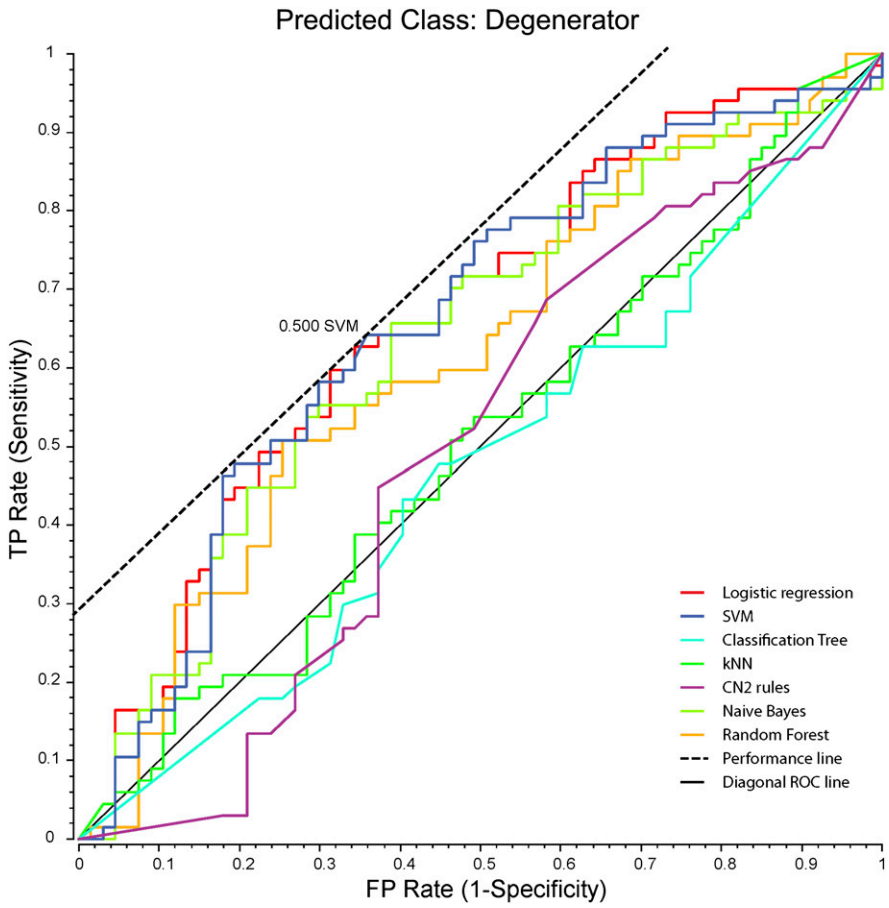

Figure 1—*Receiver operating characteristic of the classifiers. The seven machine learning classification algorithms available in ORANGE were evaluated for classifying DN patient regenerator and degenerator groups. Classifiers were trained on the HbA$_{1c}$ levels of the subjects from these two groups, and testing was performed in 20-fold cross-validation. CN2, Clark Niblett 2; FP, false positive; kNN, k-nearest neighbor; TP, true positive.*

significantly different (Table 1). The multivariable logistic regression analysis also confirmed that the HbA$_{1c}$ level at baseline was the only significantly different factor (odds ratio [OR] 0.68 [95% CI 0.54–0.85]; $P < 0.01$) with other variables adjusted (Table 2). The regenerator group included more females and more patients with type 1 diabetes; however, the differences were not statistically significant. Interestingly, although these two groups had similar baseline MFD (2,949.2 ± 1,504.5 [degenerator] and 3,100.0 ± 1,634.9 [regenerator]; $P = 1$), the baseline O'Brien score was significantly lower in the degenerator group compared with the regenerator group (Table 1) (2,839.8 ± 1,167.5 vs. 3,427.6 ± 1,132.7 respectively, $P = 0.01$).

Computational classifier for regenerator and degenerator

A machine-learning approach was used to test whether risk factors may predict the classification category outcome of participants with DN. Among the seven machine learning algorithms using a 20-fold cross-validation, two algorithms (SVM and logistic regression) achieved a classification accuracy (CA) >60%, with logistic regression being the best classifier (CA = 62.7%). Figure 1 illustrates receiver operating characteristic curves of the evaluated classifiers and indicates that SVM and logistic regression are the best classifiers. The addition of other factors resulted in degraded classification performance; however, O'Brien neuropathy score slightly improved the classifiers, with SVM achieving the highest CA of 64.2%.

Microarray data analysis

To examine the gene expression profiles that are significantly different between the two extreme groups, two batches of human sural nerve microarray datasets were combined (one published [8]). Intensity-based moderated *T* statistics identified a total of 490 DEGs between regenerator ($n = 7$) and degenerator ($n = 15$) at a nominal *P* value of 0.05 without multiple testing corrections. Supplementary Table 1 lists the 10 most upregulated and 10 most downregulated DEGs. Multiple immune-related genes such as CD177 molecule (CD177) (19), human leukocyte antigen (HLA) complex group 4 (HCG4), and chemokine (C-X-C motif) ligand 10 (CXCL10) (20) are upregulated in regenerator, indicating possible activation of multiple immune cell types such as neutrophils (19) and natural killer cells (20).

Table 3 lists the top 20 concepts (gene sets defined by biological functional terms such as Gene Ontology terms) identified by LRpath that have a significantly lower false discovery rate (FDR) for differential gene expression. Although some immune activation gene markers (CD177 and CXCL10) were markedly upregulated in regenerator, LRpath suggests that genes associated with immune response (FDR = 2.23E-08), defense response (FDR = 3.73E-07), and inflammatory response (FDR = 6.16E-04) were generally downregulated in regenerator. The top concepts upregulated in regenerator included condensed chromosome (FDR = 4.47E-04) and transmission of nerve impulse (FDR = 5.44E-04). Supplementary Table 2 lists all the significant genes in these 20 top concepts. DAVID, another gene set enrichment analysis tool, identified biological functional terms significantly overrepresented in the 490 DEG set. A heat map (Supplementary Fig. 1) was generated to summarize the most significant functions and indicates that the genes upregulated in regenerators were highly enriched with cell cycle, suggesting active regeneration.

Correlation with regeneration clusters

Approximately one-third ($n = 168$) of the study subjects had their baseline and 52-week biopsies examined by EM, and the Pearson correlation coefficient between ΔMFD% and the regeneration fiber density change was 0.33 ($P < 0.001$). When the analysis is limited only to the regenerator and degenerator groups, the correlation coefficient was 0.35 ($P = 0.014$ with $n = 48$). Although the correlation is not strong, the data suggest that the change in MFD is partially reflected in the decreased level of nerve regeneration. We also

Table 3—*Top 20 most differential biological functions between degenerator and regenerator identified by LRpath*

Name	Concept type	#Genes	#SigGenes	OR	FDR	Direction
Immune response	GO BP	518	12	0.21	2.23E-08	Down
Regulation of immune system process	GO BP	348	12	0.17	5.62E-08	Down
Defense response	GO BP	500	11	0.24	3.73E-07	Down
Myeloid cell differentiation	GO BP	135	8	0.10	8.52E-06	Down
Regulation of immune response	GO BP	195	7	0.14	1.13E-05	Down
External side of plasma membrane	GO CC	111	7	0.09	2.24E-05	Down
Ribonucleoprotein complex	GO CC	418	13	0.28	5.04E-05	Down
Regulation of response to stimulus	GO BP	428	10	0.27	8.71E-05	Down
Hematopoietic cell lineage	KEGG	62	4	0.05	1.34E-04	Down
Mitochondrial part	GO CC	530	12	0.34	1.69E-04	Down
Condensed chromosome	GO CC	113	5	6.91	3.36E-04	Up
Condensed chromosome kinetochore	GO CC	61	4	10.99	4.47E-04	Up
Condensed chromosome, centromeric region	GO CC	66	4	9.95	4.47E-04	Up
Outer kinetochore of condensed chromosome	GO CC	10	1	64.73	4.47E-04	Up
Mitochondrial lumen	GO CC	202	7	0.21	4.47E-04	Down
Mitochondrial matrix	GO CC	202	7	0.21	4.47E-04	Down
Positive regulation of immune system process	GO BP	222	6	0.20	5.44E-04	Down
Myeloid leukocyte differentiation	GO BP	64	4	0.06	5.44E-04	Down
Transmission of nerve impulse	GO BP	286	6	3.90	5.44E-04	Up
Inflammatory response	GO BP	295	6	0.25	6.16E-04	Down

Direction indicates that the significant genes are upregulated (up) or downregulated (down) in regenerator. BP, Biological Process; CC, Cellular Component; GO, Gene Ontology; MF, Molecular Function; #Genes, number of genes associated with the given concept; #SigGenes, number of significantly DEGs associated with the given concept.

examined whether HbA_{1c} level is correlated with the absolute density and the change of the regenerating clusters. The correlation between baseline HbA_{1c} and the changes in regenerating clustering density was 0.02 for all 168 subjects; however, this correlation became −0.15 with the analysis limited to the regenerator and degenerator groups (n = 48). Although this does not reach the statistical significance cutoff, the negative correlation values suggest that there is a trend for decreased regeneration cluster density over 52 weeks with a higher baseline HbA_{1c} level. However, no linear correlation was observed between the baseline HbA_{1c} level and the baseline regeneration cluster density (correlation coefficient = 0.01 in both sets using all subjects and the two extreme sets). These results suggest that the HbA_{1c} level at a certain time point may not be predictive of the absolute level of the regenerating cluster density but may partially predict the changes in the regenerating cluster and MFD over time.

CONCLUSIONS—Previous analyses by our group of human sural nerve biopsies harvested as part of a double-blind, placebo-controlled, 52-week trial of ALC for DN (6,7) revealed that elevated serum triglycerides measured at trial onset correlate with DN progression (7) and that the alterations in immune response and lipid metabolism genes are also associated with progressive DN (8). Further examination of these data, however, revealed that MFD improved in ~43% of the subjects, and although modest regeneration has been documented in DN (9), no study has investigated critical factors affecting nerve regeneration in DN. In the current study, we examined demographic, anthropometric, and biochemical data of these subjects to identify the potential risk factors that correlate with myelinated nerve fiber regeneration and degeneration. We found that HbA_{1c} was the only factor significantly associated with regeneration and degeneration. In fact, the baseline HbA_{1c} level alone was able to correctly classify 62.7% of the subjects as a regenerator or degenerator.

This study is an extension of the previously published analysis of the same cohort and was pursued in an attempt to better understand factors contributing to nerve fiber degeneration associated with DN. The previous study used the absolute loss of MFD over the course of the 52-week clinical trial as the classifier, resulting in three groups of patients (progressor group with ≥500 fibers/mm^2 of MFD loss, nonprogressor group with ≤100 fibers/mm^2 of MFD loss, and intermediate group for the remainders) (7,8). Therefore, the nonprogressor group in the previous study comprised all of the regenerator subjects from the current study, as well as a large portion of the intermediate subjects. Furthermore, the present subject groups were selected using the ΔMFD% as the classifier rather than absolute MFD change, as patients at different ages and with different durations of diabetes tend to have substantially variable levels of baseline MFD. Thus, the percent change, rather than absolute change of MFD, was used to evaluate the effects of several important clinical factors shown to contribute to DN progression.

Peripheral nerves undergo spontaneous regeneration upon injury; however, the risk factors in diabetes affecting nerve regeneration and degeneration are not clearly understood. Although the overall MFD decreases with age, the nerve itself may regenerate either spontaneously or in response to external stimuli (21). Axonal regeneration actively takes place as a natural compensatory response to damage caused by diabetes, but incomplete or unsuccessful regeneration may constitute a critical component of DN progression (5). We anticipated that genes related to axonal regeneration or cell growth would be more actively expressed in the

regenerator compared with the degenerator group.

Microarray analyses confirmed that those genes involved in cell cycle functions are highly upregulated in regenerator compared with degenerator. Biological functions associated with neuron projection and myelin sheath were highly enriched in those genes upregulated in regenerator compared with the intermediate group. These functions were not significantly overrepresented in the DEGs between degenerator and regenerator, which is probably due to the low power of detecting differential expression with a limited number of samples. The results should be only considered as preliminary, and more samples need to be processed to increase the statistical power. It should also be noted that Schwann cells are major contributors to the mRNA in the sural nerve biopsies, with a small contribution coming from axons, epineural fibroblasts, adipocytes, vascular endothelial cells, and immune cells such as macrophages. Therefore, the gene expression changes observed by microarray are most likely to represent the changes in Schwann cells in response to diabetes.

Another interesting finding is the fact that in spite of similar MFD at baseline in the degenerator and regenerator groups, the degenerator group had a much lower O'Brien neuropathy score. The O'Brien scores are accepted methods to quantify multiple electrophysiological measurements obtained from nerve conduction studies (NCSs). NCSs assess mostly large myelinated nerve fiber function and are still considered by most as the gold standard end point for DN in clinical trials (22). Our findings suggest that nerve fiber function as assessed by NCS may be decreased before an anatomical loss of myelinated fibers and can be used in combination with other factors (such as HbA_{1c}) to predict nerve fiber degeneration.

Fiber regeneration delays have been observed in both the tibial (largely motor) and sural (sensory) distal sciatic branches after both sciatic nerve crush injury and complete sciatic nerve transection in streptozocin-treated mice, a type 1 diabetes animal model (23). Interestingly, macrophage invasion was associated with the delay in this model, supporting a potential mechanism for impaired regeneration due to abnormal macrophage participation in nerve repair (23); however, the role of macrophages in nerve repair is still controversial (24–26). According to our microarray data, macrophage differentiation had a FDR of 0.018. Although only one gene, THO complex 5 (*THOC5*), was deemed a significant gene according to this concept, the overall changes of genes related to macrophage differentiation and other similar terms, such as macrophage activation, were found to be downregulated in regenerator by LRpath. More studies on the potential role of macrophages will be necessary to elucidate the exact mechanisms.

Study limitations include that blood chemistry data were only available at baseline and no subsequent measuring was done during or at the end of the trial. Therefore, controlling for in-trial changes in the covariates analyzed was not possible. It is possible that the overall HbA_{1c} levels changed during the trial and data regarding lifestyle or diet changes were not available. In addition, even though the study cohort is the largest one available to date with ΔMFD% information, we may have lacked sufficient power to detect meaningful effects for all risk factors. Although diabetes type did not have a statistically significant effect on regenerator and degenerator classification in the current study, future separate analyses of type 1 and 2 diabetic subjects may be informative and will be pursued.

In the current study, we evaluated potential biomarkers and gene expression profiles of the sural nerve biopsies from the largest available DN patient cohort with ΔMFD% information in order to ascertain factors associated with nerve regeneration and degeneration in DN. The data suggest that HbA_{1c} levels are significantly associated with the nerve regeneration and degeneration and may be predictive of future sural peripheral nerve regeneration. The microarray data suggest that immune and inflammatory responses may play a crucial role in nerve regeneration and degeneration. Although the exact mechanisms must still be elucidated, these data indicate that optimal blood glucose control in patients with DN is likely to impact sural nerve regeneration and that the immune response may play an important role in this process.

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