

## **Inactive matrix gla protein plasma levels are associated with peripheral neuropathy in Type 2 diabetes**

Anne-Caroline Jeannin, Joe-Elie Salem, Ziad Massy, Carole Elodie Aubert, Cees Vemeer, Chloé Amouyal, Franck Phan, Marine Halbron, Christian Funck-Brentano, Agnès Hartemann, et al.

► **To cite this version:**

Anne-Caroline Jeannin, Joe-Elie Salem, Ziad Massy, Carole Elodie Aubert, Cees Vemeer, et al.. Inactive matrix gla protein plasma levels are associated with peripheral neuropathy in Type 2 diabetes. PLoS ONE, Public Library of Science, 2020, 15 (2), pp.e0229145. 10.1371/journal.pone.0229145 . inserm-02530894

**HAL Id: inserm-02530894**

**<https://www.hal.inserm.fr/inserm-02530894>**

Submitted on 3 Apr 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

RESEARCH ARTICLE

# Inactive matrix gla protein plasma levels are associated with peripheral neuropathy in Type 2 diabetes

Anne-Caroline Jeannin<sup>1,2</sup>, Joe-Elie Salem<sup>1,3,4,5</sup>, Ziad Massy<sup>6</sup>, Carole Elodie Aubert<sup>7,8</sup>, Cees Vemeer<sup>9</sup>, Chloé Amouyal<sup>1,2,5</sup>, Franck Phan<sup>1,2,5,10</sup>, Marine Halbron<sup>1,2,5</sup>, Christian Funck-Brentano<sup>1,3,4,5</sup>, Agnès Hartemann<sup>1,2,5,10</sup>, Olivier Bourron<sup>1,2,5,10\*</sup>

**1** Sorbonne Université, Paris, France, **2** Assistance Publique-Hôpitaux de Paris (APHP), Diabetology Department, Pitié-Salpêtrière Hospital, Paris, France, **3** Department of Pharmacology and CIC-1421, AP-HP, Pitié-Salpêtrière Hospital, Paris, France, **4** INSERM, CIC-1421, Paris, France, **5** Institute of Cardiometabolism and Nutrition ICAN, Paris, France, **6** Division of Nephrology, Ambroise Paré Hospital, AP-HP, Pitié-Salpêtrière Hospital, Université Paris-Saclay, Paris, France, **7** Department of General Internal Medicine, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland, **8** Institute of Primary Health Care (BIHAM), University of Bern, Bern, Switzerland, **9** Cardiovascular Research Institute CARIM, Maastricht University, Maastricht, The Netherlands, **10** INSERM, UMR\_S 1138, Centre de Recherche des Cordeliers, Paris, France

\* [olivier.bourron@aphp.fr](mailto:olivier.bourron@aphp.fr)



**OPEN ACCESS**

**Citation:** Jeannin A-C, Salem J-E, Massy Z, Aubert CE, Vemeer C, Amouyal C, et al. (2020) Inactive matrix gla protein plasma levels are associated with peripheral neuropathy in Type 2 diabetes. PLoS ONE 15(2): e0229145. <https://doi.org/10.1371/journal.pone.0229145>

**Editor:** Rudolf Kirchmair, Medical University Innsbruck, AUSTRIA

**Received:** September 5, 2019

**Accepted:** January 30, 2020

**Published:** February 24, 2020

**Peer Review History:** PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0229145>

**Copyright:** © 2020 Jeannin et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All data from the current study were reported in the manuscript and tables. However, there was not an authorization for making data set publicly available when the study

## Abstract

### Aims/Hypothesis

Diabetic peripheral neuropathy is a frequent and severe complication of diabetes. As Matrix-gla-protein (MGP) is expressed in several components of the nervous system and is involved in some neurological disease, MGP could play a role in peripheral nervous system homeostasis. The aim of this study was to evaluate factors associated with sensitive diabetic neuropathy in Type 2 Diabetes, and, in particular, dephospho-uncarboxylated MGP (dp-ucMGP), the inactive form of MGP.

### Methods

198 patients with Type 2 Diabetes were included. Presence of sensitive diabetic neuropathy was defined by a neuropathy disability score (NDS)  $\geq 6$ . Plasma levels of dp-ucMGP were measured by ELISA.

### Results

In this cohort, the mean age was 64 $\pm$ 8.4 years old, and 80% of patients were men. Peripheral neuropathy was present in 15.7% of the patients and was significantly associated ( $r = 0.51$ ,  $p < 0.0001$ ) with dp-ucMGP levels ( $\beta = -0.26$ ,  $p = 0.045$ ) after integrating effects of height ( $\beta = -0.38$ ,  $p = 0.01$ ), insulin treatment ( $\beta = 0.42$ ,  $p = 0.002$ ), retinopathy treated by laser ( $\beta = 0.26$ ,  $p = 0.02$ ), and total cholesterol levels ( $\beta = 0.3$ ,  $p = 0.03$ ) by multivariable analysis.

protocol was submitted for the Local Ethical Committee. Therefore, data are available upon request to Dr Alban Danset at: "[alban.danset@aphp.fr](mailto:alban.danset@aphp.fr)".

**Funding:** This work was supported by a fund from the Lilly Company. The research activities of C.E.A. were supported by a doctoral research scholarship from the University of Lausanne. A.C.J received a grant from Ministère français des Affaires Sociales et de la Santé (Bourse année recherche 2017/2018) The company was involved neither in the design of the study nor in data collection.

**Competing interests:** A patent has been filed on a method using circulating Matrix Gla protein measurement for diagnosis and treating peripheral neuropathies by Assistance Publique Hôpitaux de Paris - APHP). Olivier Bourron, Joe-Elie Salem and Agnès Hartemann are the inventors. The application number is 18306503.6 – 1118. Status of application: recorded. The patent specific aspect of manuscript covered in patent application: use of circulating Matrix Gla protein measurement for diagnosis and treating peripheral neuropathies. This study was supported by a fund from Lilly Company. The company was involved neither in the design of the study nor in data collection. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

## Conclusions

The association between diabetic neuropathy and the inactive form of MGP suggests the existence of new pathophysiological pathways to explore. Further studies are needed to determine if dp-ucMGP may be used as a biomarker of sensitive neuropathy. Since dp-ucMGP is a marker of poor vitamin K status, clinical studies are warranted to explore the potential protective effect of high vitamin K intake on diabetic peripheral neuropathy.

## Introduction

Diabetic peripheral neuropathy is a frequent complication of diabetes. It affects about 10 to 15% of patients with Type 2 Diabetes at diagnosis and up to 50% after 10 years of disease duration [1]. Diabetic neuropathy is associated with high morbidity and mortality [2], because of increased risk for foot ulceration and amputation [3], and for poor quality of life and depression [4]. So, it is related to high healthcare costs [5]. The main clinical characteristic of diabetic peripheral neuropathy is a decrease of distal sensitivity that represents the most important risk factor of foot ulceration in patients with diabetes. In 2019, ADA guidelines recommended an annual clinical screening to diagnose sensitive diabetic neuropathy [1]. ADA recommendations for screening and diagnosis include a careful history and assessment of either temperature or pinprick sensation (small-fiber function) and vibration sensation using a 128-Hz tuning fork (for large-fiber function). All patients should have annual 10-g monofilament testing to identify feet at risk for ulceration and amputation. Electrophysiological testing or referral to a neurologist is not recommended for screening, except in situations where the clinical features are atypical, the diagnosis is unclear, or a different etiology is suspected [1].

Mechanisms involved in diabetic neuropathy are not clearly understood. The main hypothesis is that chronic glucotoxicity and lipotoxicity lead to oxidative stress, inflammation, and mitochondrial dysfunction and finally to nerve damage with neuron degeneration and demyelination [6, 7].

Matrix gla protein (MGP) is an 84 amino acids protein containing five glutamic acid residues (glu residues) and three serine residues. MGP exists in inactive and active forms [8]. The activation of MGP is obtained after a vitamin K dependent gamma-glutamyl carboxylation of glutamic acid residues (forming gla residues) and a phosphorylation of serine residues [9, 10]. Desphospho-uncarboxylated MGP (dp-ucMGP) represents therefore the inactive form of MGP. MGP was initially isolated from bone tissue but it is also expressed by chondrocytes, vascular smooth muscle cells, endothelial cells but also neurons and glial cells [11, 12]. Moreover, several studies suggest that MGP plays a role in the nervous system. First, in 2005, a novel mutation of MGP associated with high level of inactive dp-ucMGP is described and associated with neurological manifestations, abnormalities of brain's white matter and optic nerve atrophy, in addition to typical manifestations of Keutel syndrome [13, 14], suggesting a link between MGP activity and nervous system pathophysiology. Then, Goritz et al have demonstrated that MGP is expressed by neurons, and is regulated by glial cells [12]. Finally, some studies reveal also that MGP could be implicated in neurological disease, as glioblastoma [15], and Alzheimer disease [16].

Given that the pathogenesis of diabetic neuropathy remains unclear and that MGP could be involved in nervous system pathophysiology, we hypothesize that MGP may be involved in diabetic peripheral neuropathy development. The objective of this study is to evaluate the clinical and biological markers, in particular inactive dp-ucMGP, associated with diabetic peripheral neuropathy on patients with Type 2 Diabetes.

## Material and methods

### Study design

This study is a cross-sectional ancillary study to the prospective DIACART cohort<sup>17</sup>. DIACART cohort was initially designed to study the clinical and biological variables associated with peripheral arterial calcification and other diabetic complications such as diabetic neuropathy. In this cohort, diabetic peripheral neuropathy was accurately assessed with a careful foot examination to calculate the NDS score [7]. 198 Patients were recruited in the diabetes and cardiology departments, in the Pitié-Salpêtrière hospital (APHP, Paris, France), over eight months, from November 2011 to July 2012. They were subsequently prospectively assessed in a cardio-metabolic clinical research center (INSERM, CIC 1421) for clinical phenotyping and bio banking of their blood samples.

### Participants

DIACART cohort was initially designed to study the clinical and biological variables associated with diabetic complications [17]. The study focused on patients with Type 2 Diabetes, at high cardiovascular risk. Inclusion criteria were Type 2 Diabetes with at least one of the following criteria: coronary artery disease or peripheral arterial occlusive disease or age >50 years for men or >60 years for women. Exclusion criteria were an estimated glomerular filtration rate calculated with the modification of diet in renal disease <30ml/min and a history of lower limb angioplasty and/or bypass. The peripheral nerve deficit of nondiabetic origin (e.g. alcohol, neurotoxic medications (e.g., chemotherapy), vitamin B12 deficiency, hypothyroidism, renal disease, malignancies (e.g., multiple myeloma), infections (e.g., HIV, HCV), chronic inflammatory demyelinating neuropathy, inherited neuropathies, compression due to vertebral disk herniation, and vasculitis) was excluded through a careful medical history review, a differential test or both.

### Informed consent and ethical aspect

The study was approved by the local ethics committee (PARIS VI CPP) and registered in ClinicalTrials.gov (Identifier: NCT02431234). All patients were informed on the study objectives and procedure. Participants gave their written informed consent to participation. All methods were carried out in accordance with relevant guidelines and regulations.

### Procedure

Data collection, including a clinical evaluation and blood tests, were realized during a one-day hospitalization in a cardio-metabolic clinical research center.

### Diabetic peripheral neuropathy

In this cohort, diabetic peripheral neuropathy was accurately assessed with a careful foot examination, including several physical tests [7]. Diabetic peripheral neuropathy was assessed by the modified neuropathy disability score (NDS), scoring from 0 to 10 [18]. NDS assesses vibration sensory on the great toe using 128-Hz tuning fork, temperature sensory on dorsum of the foot using tubes of ice or warm water, pinprick sensory applying pin near to big toe nail and Achilles reflex. Each sensory test scores 0 for normal and 1 for abnormal sensation, for each foot. Achilles reflex score 0 if they are present, 1 if they are present with reinforcement and 2 if they are absent, for each foot.  $NDS \geq 6$  allows the diagnosis of diabetic peripheral neuropathy [19]. The NDS was also used as a continuous variable to assess magnitude of peripheral neuropathy because NDS score is a validated and widely used score for detecting neuropathy.

## Clinical data

During the patient interview, the physician collected medical information about personal disease history, comorbidities and treatment. Clinical tests were conducted by a physician blinded to blood tests results.

## Biochemical measures

Blood and urine samples were collected in the morning fasting for the measurement of biochemistry analyses including hemoglobin A1c (HbA1c), high-sensitivity C-reactive protein (hsCRP), estimated glomerular filtration rate (eGFR) by modification of diet in renal disease (MDRD), urinary albumin/creatinine ratio, serum calcium corrected for albumin, serum phosphorus, total cholesterol, triglycerides and IL-6.

Assays were developed to measure dp-ucMGP in plasma [14]. These assays were conducted after the samples freezing, storage at  $-80^{\circ}\text{C}$  and thawing. Dp-ucMGP levels were measured by a dual-antibody ELISA. The capture antibody was directed against the non-phosphorylated MGP sequence 3–15 (mAb-dpMGP; VitaK BV, Maastricht, The Netherlands) and the detecting antibody was directed against the uncarboxylated MGP sequence 35–49 (mAb-ucMGP; VitaK BV). Intra-assay variability was 5.6% for dp-ucMGP. Inter-assay variability was 9.9% for dp-ucMGP. Dp-ucMGP was measured in archived samples of 81 age-matched controls. The mean levels were respectively  $557\pm 277$  pmol/l (median: 522 pmol/l).

## Statistical analyses

Data were described as mean  $\pm$  standard deviation of the mean or frequency, as appropriate. Comparison of quantitative variables was performed using Student's t test or Mann-Whitney test, when variables were normally and non-normally distributed, respectively. Comparison of qualitative variables was performed using  $\chi^2$  test. Pearson's coefficient ( $r$ ) was used to assess association between quantitative variables. A 95% confidence interval for the correlation coefficient was calculated using Fisher's method (Prism 6; GraphPad Software, Inc). Multivariable analyses were performed by ANCOVA (continuous NDS scoring) or logistic regression (Presence/absence of neuropathy defined by  $\text{NDS}\geq 6/\text{NDS}<6$ ). Only covariates with significant univariate association (In bold, in Tables 1 and 2) with NDS were further integrated for multivariate analyses (XLstat-software, Addinsoft®, New-York). For multivariate analysis, beta-coefficients ( $\beta$ ) were calculated to allow for direct comparison of the relative influence of the explanatory variables on the dependent variable, and their significance ( $P\leq 0.05$  considered significant).

In this cohort (n: 198), the study had a power  $\geq 80\%$  to detect a significant correlation (with  $r\geq 0.2$ ,  $\alpha$ -risk: 0.05, Student approximation) between each clinical or biological variable and NDS score.

## Results

### Baseline characteristics

Clinical and biological characteristics at baseline for the total cohort, and for patients with and without neuropathy are described in Table 1. Finally, 198 patients were included in the DIA-CART study, 80% of whom were men. Study participants were young-old ( $64\pm 8.4$  years old) and overweight (mean BMI of  $29.16\pm 5.3$  kg/m<sup>2</sup>) patients. Their mean height was  $1.7\pm 0.08$  meters. Diabetes duration was  $14.6\pm 9.3$  years, and mean HbA1c was  $7.8\pm 1.5\%$  ( $61.8\pm 16.2$  mmol/L). Concerning diabetes comorbidities, 14.1% of patients had a retinopathy treated with laser, 36% had a urinary albumin/creatinine ratio  $>3$  mg/mmol, and mean eGFR

**Table 1. Baseline characteristics of the patients.**

Characteristics	Total cohort	Neuropathy (NDS≥6)	Without neuropathy (NDS<6)	p-value
N (%)	198	31 (15.7)	167 (84.3)	-
Age, years	64±8.4	64±8.6	64±8.4	ns
Male, n(%)	158 (79.8)	26 (83.9)	132(79)	ns
<b>Height (cm)</b>	170±8	173±7	169±8	<b>0.009</b>
BMI (Kg/m <sup>2</sup> )	29,16±5.3	30,23±5.5	28,97±5.2	ns
Diabetes duration, years	14.6±9.3	14.6±10.2	14.6±9.2	ns
Hypertension, n (%)	163 (82.3)	28 (90.3)	135 (80.8)	ns
<b>NDS score, points</b>	2.4±2.4	6.8±1.5	1.6±1.5	<b>&lt;0.0001</b>
<b>Retinopathy treated with laser, n(%)</b>	28 (14.1)	10(32.3)	19(11.3)	<b>0.003</b>
<b>Coronary arterial disease, n(%)</b>	150 (75.8)	28 (90.3)	122 (73.05)	<b>0.04</b>
Ischemic stroke, n(%)	14 (7.1)	2(6.5)	12(7.2)	ns
eGFR calculated by MDRD, mL/min	76±20	72±20	77±20	ns
<b>Urinary albumin /creatinine ratio&gt;3 (mg/mmol), n(%)</b>	71 (35.9)	19(61.3)	52 (31.1)	<b>0.001</b>
<b>Insulin treatment, n(%)</b>	94 (47.5)	24(77.4)	70(41.9)	<b>0.0003</b>
HbA1c, mmol/mol	61.8±16.2	66.6±20.5	60.9±15.2	ns
HbA1c, %	7.8±1.5	8,2±1.9	7.7±1.4	ns
hsCRP, mg/L	2.2±2.5	2.4±2.8	2.2±2.5	ns
IL-6, pg/mL	5±22	4.6±3.4	5.3±24	ns
Corrected calcium, mmol/L	2.3±0.1	2.3±0.3	2.3±0.1	ns
Phosphorus, mmol/L	1.02±0.15	1.02±0.14	1.02±0.16	ns
Triglycerides, mmol/L	1.6±1.1	1.5±0.8	1.6±1.1	ns
<b>Total cholesterol, mmol/L</b>	3.7±0.9	3.4±0.8	3.8±0.9	<b>0.02</b>
<b>dp-ucMGP, pmol/L</b>	627±451	821±703	591±379	<b>0.009</b>

Quantitative variables are represented by mean ± standard deviation. Data are given as the number (percentage) for binary variables. Data are no significant (ns) if p>0.05. Significant differences between patients with and without neuropathy are in bold.

Abbreviations: BMI body mass index, eGFR MDRD estimated glomerular filtration rate calculated with the modification of diet in renal disease formula, HbA1c haemoglobin A1C, hsCRP high sensitivity C-reactive protein, IL-6 interleukin 6, dp-ucMGP dephospho-uncarboxylated matrix gla protein, NDS neuropathy disability score.

<https://doi.org/10.1371/journal.pone.0229145.t001>

calculated by MDRD was 76+/-20 ml/min. Mean NDS was 2.4+/-2.4 points, and 15.7% of subjects had a diabetic peripheral neuropathy, defined by NDS≥6. The mean level of dp-uc MGP was 627 +/-451 pmol/l.

### Factors associated with diabetic neuropathy (defined by NDS≥6)

Patients with neuropathy were significantly taller (173 cm vs 169 cm, p = 0.009) than patients without neuropathy. Cholesterol total was significantly lower in patients with neuropathy compared to patients without neuropathy (3.8 mmol/L vs 3.4 mmol/L, p = 0.02). Retinopathy treated with laser (32 vs 11%, p = 0.003), urinary albumin/creatinine ratio >3 mg/mmol (61 vs 31%, p = 0.001), coronary arterial disease (90 vs 73%, p = 0.04) and insulin treatment (77 vs 42%, p = 0.0003) were significantly more common in patients with neuropathy. Age, sex ratio, diabetes duration and HbA1c were not different between patients with and without neuropathy (Table 1). Dp-ucMGP levels were significantly higher in patients with neuropathy than in those without neuropathy (821 vs 591 pmol/l respectively, p = 0.009). In multivariate analysis integrating all significant covariates (retinopathy treated with laser, urinary albumin/creatinine ratio, coronary arterial disease, insulin treatment and quantitative variables in bold, in Tables 1 and 2), presence of neuropathy defined by NDS score ≥6 was still associated

**Table 2. Univariate analysis: Correlations between clinical and biological variables and NDS.**

	r, [CI 95%]	p-value
Age	0.07 [-0.08; 0.20]	ns
<b>Height</b>	<b>0.25 [0.11; 0.38]</b>	<b>0.0004</b>
<b>Body mass index (kg/m<sup>2</sup>)</b>	<b>0.09 [-0.05; 0.23]</b>	ns
Diabetes duration	0.03 [-0.11; 0.17]	ns
<b>eGFR</b>	<b>-0.16 [-0.29; -0.02]</b>	<b>0.03</b>
<b>HbA1c</b>	<b>0.21 [0.08; 0.34]</b>	<b>0.04</b>
HsCRP, mg/L	0.03 [-0.11; 0.17]	ns
IL-6, pg/mL	0.08 [-0.07; 0.21]	ns
Corrected calcium, mmol/L	0.08 [-0.06; 0.22]	ns
Phosphorus, mmol/L	0 [-0.14; 0.14]	ns
Triglycerides	-0.07 [-0.21; 0.07]	ns
Total cholesterol	-0.11 [-0.25; 0.02]	ns
<b>dp-ucMGP</b>	<b>0.22 [0.08; 0.34]</b>	<b>0.002</b>

Correlations were performed by Pearson’s coefficient (r). 95% confidence interval of the correlation coefficient was assessed using Fisher’s method, and is presented in brackets. Correlations are significant if  $p < 0.05$ . Significant results are presented in bold.

Abbreviations: BMI body mass index, eGFR MDRD estimated glomerular filtration rate calculated with the modification of diet in renal disease formula, HbA1c haemoglobin A1C, hsCRP high sensitivity C-reactive protein, IL-6 interleukin 6, dp-ucMGP dephospho-uncarboxylated matrix gla protein, NDS neuropathy disability score.

<https://doi.org/10.1371/journal.pone.0229145.t002>

( $r = 0.51, p < 0.0001$ ) with dp-ucMGP levels ( $\beta = -0.26, p = 0.045$ ), height ( $\beta = -0.38, p = 0.01$ ), insulin treatment ( $\beta = 0.42, p = 0.002$ ), retinopathy treated by laser ( $\beta = 0.26, p = 0.02$ ), and total cholesterol level ( $\beta = 0.3, p = 0.03$ ) (Table 3).

### Factors associated with continuous NDS scoring

In univariate analysis (Table 2), NDS was positively associated with height ( $r = 0.25, p = 0.0004$ ), HbA1c ( $r = 0.21, p = 0.04$ ) and dp-ucMGP ( $r = 0.22, p = 0.002$ ). NDS was negatively associated with eGFR ( $r = -0.16, p = 0.03$ ). In multivariate analysis integrating all significant covariates (in bold, in Tables 1 and 2), NDS scoring was still associated ( $r = 0.51, p < 0.0001$ ) with dp-ucMGP levels ( $\beta = 0.16, p = 0.025$ ), height ( $\beta = 0.29, p < 0.0001$ ), HbA1c ( $\beta = 0.19, p = 0.006$ ), insulin treatment ( $\beta = 0.19, p = 0.007$ ), retinopathy treated by laser ( $\beta = 0.16, p = 0.015$ ) and urinary albumin/creatinine ratio  $> 3$  mg/mmol ( $\beta = 0.14, p = 0.031$ ) (Table 4).

**Table 3. Multivariate analysis: Correlations between clinical and biological variables and diabetic neuropathy (NDS  $\geq 6$ ).**

	$\beta$ , [95% confidence interval]	p-value
<b>Height</b>	<b>-0.38, [-0.67–0.09]</b>	<b>0.01</b>
<b>Retinopathy treated with laser</b>	<b>0.26, [0.05–0.47]</b>	<b>0.02</b>
<b>Insulin treatment</b>	<b>0.42, [0.15–0.7]</b>	<b>0.002</b>
<b>Total cholesterol</b>	<b>0.3, [0.03–0.57]</b>	<b>0.03</b>
<b>dp-ucMGP</b>	<b>-0.26, [-0.51–0.01]</b>	<b>0.045</b>

Multivariate analysis was performed using ANCOVA. 95% confidence interval of the standardized coefficient is presented in brackets. Correlations are significant if  $p < 0.05$ . Significant results are presented in bold.

Abbreviations:  $\beta$ : standardized coefficient, dp-ucMGP dephospho-uncarboxylated matrix gla protein, NDS neuropathy disability score.

<https://doi.org/10.1371/journal.pone.0229145.t003>

**Table 4. Multivariate analysis: Correlations between clinical and biological variables and continuous NDS scoring.**

	$\beta$ , [95% confidence interval]	p-value
Height	<b>0.29, [0.16–0.41]</b>	<b>&lt;0.0001</b>
Retinopathy treated with laser	<b>0.16, [0.03–0.29]</b>	<b>0.015</b>
Insulin treatment	<b>0.19, [0.05–0.33]</b>	<b>0.007</b>
Urinary albumin/creatinine ratio>3	<b>0.14, [0.01–0.28]</b>	<b>0.031</b>
HbA1c	<b>0.19, [0.06–0.33]</b>	<b>0.006</b>
dp-ucMGP	<b>0.16, [0.02–0.29]</b>	<b>0.025</b>

Multivariate analysis was performed using ANCOVA. 95% confidence interval of the standardized coefficient is presented in brackets. Correlations are significant if  $p < 0.05$ . Significant results are presented in bold.

Abbreviations:  $\beta$ : standardized coefficient, dp-ucMGP dephospho-uncarboxylated matrix gla protein, HbA1c haemoglobin A1C, NDS neuropathy disability score.

<https://doi.org/10.1371/journal.pone.0229145.t004>

## Discussion

This study reveals that peripheral neuropathy, defined by a NDS score  $\geq 6$ , in type 2 diabetic patients is significantly associated with height, insulin treatment, retinopathy treated with laser, total cholesterol and, particularly to dp-ucMGP plasma levels. These factors, HbA1c and urinary albumin/creatinine ratio  $> 3$  mg/mmol are also associated with the magnitude of NDS scoring.

Height, poor glycemic control and dyslipidemia are known risk factors of diabetic neuropathy [4]. We don't find any significant association between BMI and neuropathy in our study, probably because BMI formula includes height, which is maybe a more important marker of diabetic peripheral neuropathy due to the length-dependent presentation of this neuropathy [20]. Furthermore, BMI is not also associated with neuropathy in the DIACART study probably because majority of the patients included were overweight or obese (mean BMI 29.16  $\pm$  5.3 Kg/m<sup>2</sup>). Although insulin is considered as a neurotrophic factor and although low-dose insulin can have beneficial effects on diabetic neuropathy, insulin use is associated with diabetic neuropathy in the DIACART study [21]. Retinopathy and nephropathy are usual comorbidities of diabetic neuropathy, explaining their association in this study [4]. In the same way, age and gender are not related to neuropathy in our population because most of the patients included in this study were male (80%) and elderly (mean age 64  $\pm$  8.4 years old).

The most important and original result is the association between dp-ucMGP plasma levels and diabetic neuropathy. Moreover, dp-ucMGP plasma levels increased with the continuous NDS scoring.

This association could be explained by several hypotheses.

We can suppose that the association between dp-ucMGP and neuropathy could directly result from MGP involvement in pathophysiology of diabetic peripheral neuropathy. Indeed, MGP is a protein from extracellular matrix mainly expressed in osteoarticular and vascular systems, but Goritz et al have shown that MGP is also expressed by neurons and glial cells [12]. The main ligand of MGP is Bone Morphogenetic Protein-2 (BMP-2) [22] and some data show that MGP, via modulation of BMP-2 signaling, could participate in the early differentiation and growth of neurons, in dendrites formation, in the development of mature Schwann cells and in the myelination [23–26]. Furthermore, MGP can also interact with fibronectin, which is involved in axon regeneration by its interaction with Schwann cells [27–29]. Consequently, an excess of inactive form of MGP (i.e., dp-ucMGP), could be associated with nerve damage and a source of axon regeneration loss, two pathological conditions observed in diabetic neuropathy.



However, dp-ucMGP is primarily an inverse marker for vitamin K status, and the association between dp-ucMGP and neuropathy suggests that poor vitamin K status is an independent risk factor for diabetic peripheral neuropathy. Comparison of different MGP assays showed that the dp-ucMGP assay is particularly suited to assess vascular vitamin K status and dp-ucMGP is then considered as the most sensitive biomarker for poor vitamin K status presently known [30]. Poor vitamin K status, estimated by dp-ucMGP, has been before described as associated with increased cardiovascular risk in Type 2 Diabetes [31]. Here we show, for the first time, an association between vitamin K status and diabetic peripheral neuropathy. The role of vitamin K in the nervous system was initially described via observations of microcephaly, optic atrophy and mental retardation resulting from fetal exposure to warfarin [32]. A recent study has shown that vitamin K enhances, during remyelination, the production of brain sulfatides, the sulfated form of galactosylceramides [33]. Decreases in myelin sulfatides content have been implicated as important factors in the disruption of myelin stability and function [34]. Furthermore, vitamin K seems to have survival-promoting effect on neurons [35]. We can therefore hypothesize that low vitamin K status could be associated with myelin alteration and cytotoxic effects on neurons in peripheral nerve tissue. Further studies are needed to confirm the role of vitamin K in peripheral diabetic neuropathy.

Vitamin K is an essential cofactor for the maturation of several proteins, not only for MGP. We cannot therefore exclude that the association of poor vitamin K status with diabetic peripheral neuropathy may also be a marker of the involvement of another vitamin K-dependent protein that is important for the neural system.

Circulating inactive dp-ucMGP could also be useful as a biomarker of diabetic neuropathy. The diagnosis of diabetic neuropathy is mainly clinical, based on sensory tests. But, these tests need to be associated to increase their sensitivity, are operator-dependent and time-consuming. Different surveys revealed that about only 65% of patients with diabetes yearly had a foot examination by a physician [36]. So, biomarker of diabetic neuropathy could be useful for clinical practice. Several biomarkers have been suggested, as neuron-specific enolase, toll-like receptor 4 or tumor necrosis factor alpha (TNF- $\alpha$ ), but they are not specific of diabetic neuropathy [37, 38]. Further studies are needed to clarify if dp-ucMGP could be a good biomarker in this field.

There is some data showing that dp-ucMGP is associated with several micro and macrovascular complications of diabetes, including diabetic nephropathy, retinopathy, vascular stiffness and vascular calcification [39–41]. Although dp-ucMGP has been repeatedly associated with vascular calcification and cardiovascular disease, dp-ucMGP is not associated in our study with coronary arterial disease (S1 Table) [39, 41]. As observed by others, dp-ucMGP is associated negatively with eGFR estimated by MDRD and with albuminuria (S1 Table) [39, 40]. However we don't find any association between dp-ucMGP and retinopathy treated by laser (S1 Table) despite some data suggesting that dp-ucMGP could be a marker of retinal health [42]. Additional studies are needed to explore specifically these associations in patients with diabetes.

Since dp-ucMGP is a vitamin K dependent protein, diabetic peripheral neuropathy in our study is associated with poor vitamin K status. Clinically, this gives possibilities to explore options for treatment with vitamin K supplements and especially to put in place preventive measures in diabetic patients at risk of peripheral neuropathy. The treatment of diabetic neuropathy remains currently mainly symptomatic, based on pain treatment. Targeted therapies have been developed: aldose reductase inhibitors, blocking the polyol pathway, protein kinase C inhibitors, and aminoguanidine, preventing the synthesis of age glycation end products. Despite promising results in pre-clinical animal models, clinical trials haven't demonstrated any benefit in man [43–46]. Vitamin K supplementation is safe in human and interventional

studies are needed to determine if vitamin K supplementation could prevent diabetic peripheral neuropathy [47, 48].

So, further studies are really warranted to better understand the role of MGP or other vitamin K dependent protein in the peripheral nervous system. These studies could be led in larger cohorts of patients with Type 2 Diabetes patients, and in cohorts of patients with Type 1 Diabetes or with other neuropathies, in order to analyze if this association is specific to diabetic peripheral neuropathy or not. Depending on the results of these studies, MGP could be used in diabetic neuropathy for diagnosis, prediction or therapeutic purposes via vitamin K supplementation.

The strengths of this study are the accurate diagnosis of diabetic neuropathy by a validated score (NDS), and by the same physician for all patients. Although electrophysiological tests and pathological tests, that are gold standards for the evaluation of diabetic polyneuropathy, were not performed in the DIACART study, we used NDS score, a largely validated test for neuropathy diagnosis [18, 49–56]. NDS is well correlated with neurophysiological and sural nerve morphometric abnormalities in patients with diabetes [49, 51, 57–59]. So the NDS is a widely used and widely accepted scoring test for diabetic neuropathy. Moreover, in the DIACART study, most of the common risk factors for neuropathy were associated to NDS score. The subject of this study is innovative. Indeed, this is the first study to concomitant evaluate dp-ucMGP (i.e. low vitamin K status) and peripheral neuropathy in patients with Type 2 Diabetes and we describe here, for the first time, their association. The study would have been strengthened by the presence of a control group of non-diabetic participants, so that they could have been compared with diabetic patients. Other limitations of this study are the small number of patients with a neuropathy, the cross-sectional design which allows only association but not causal relationships and the absence of the gold standard test, sural nerve biopsy, to define diabetic neuropathy.

To conclude, this study suggests that that dp-ucMGP and poor vitamin K status are associated with peripheral diabetic neuropathy in Type 2 Diabetes and that dp-ucMGP could be a biomarker of choice to identify subjects at risk of diabetic neuropathy. Further studies are warranted to precise if circulating MGP is a biomarker and/or a causal factor of diabetic neuropathy. Then fundamental experiments and prospective clinical studies are needed to clarify the role of MGP in diabetic neuropathy, and if this protein could be used as biomarker or as therapeutic target in diabetic neuropathy via vitamin K supplementation.

## Supporting information

**S1 Table. Correlations between dp-ucMGP and coronary arterial disease and other microvascular complications of diabetes.**

(DOCX)

## Acknowledgments

We thank Eli Lilly Company, the University of Lausanne, and the clinical staff of the Clinical Investigation Center Paris-Est as well as the Diabetes and Cardiology Departments from the Assistance Publique-Hôpitaux de Paris Pitié-Salpêtrière Hospital in Paris for their participation in this project.

## Author Contributions

**Conceptualization:** Agnès Hartemann, Olivier Bourron.

**Data curation:** Anne-Caroline Jeannin, Joe-Elie Salem, Cees Vemeer, Chloé Amouyal, Olivier Bourron.

**Formal analysis:** Joe-Elie Salem.

**Investigation:** Carole Elodie Aubert, Marine Halbron, Olivier Bourron.

**Methodology:** Olivier Bourron.

**Supervision:** Olivier Bourron.

**Writing – original draft:** Anne-Caroline Jeannin, Olivier Bourron.

**Writing – review & editing:** Joe-Elie Salem, Ziad Massy, Franck Phan, Marine Halbron, Christian Funck-Brentano, Agnès Hartemann, Olivier Bourron.

## References

1. 11. Microvascular Complications and Foot Care: Standards of Medical Care in Diabetes-2019. *Diabetes Care*. 2019 Jan; 42(Suppl 1):S124–S38. <https://doi.org/10.2337/dc19-S011> PMID: 30559237
2. Forsblom CM, Sane T, Groop PH, Totterman KJ, Kallio M, Saloranta C, et al. Risk factors for mortality in Type II (non-insulin-dependent) diabetes: evidence of a role for neuropathy and a protective effect of HLA-DR4. *Diabetologia*. 1998 Nov; 41(11):1253–62. <https://doi.org/10.1007/s001250051062> PMID: 9833930
3. Pecoraro RE, Reiber GE, Burgess EM. Pathways to diabetic limb amputation. Basis for prevention. *Diabetes Care*. 1990 May; 13(5):513–21. <https://doi.org/10.2337/diacare.13.5.513> PMID: 2351029
4. Papanas N, Ziegler D. Risk Factors and Comorbidities in Diabetic Neuropathy: An Update 2015. *Rev Diabet Stud*. 2015 Spring-Summer; 12(1–2):48–62.
5. Alleman CJ, Westerhout KY, Hensen M, Chambers C, Stoker M, Long S, et al. Humanistic and economic burden of painful diabetic peripheral neuropathy in Europe: A review of the literature. *Diabetes Res Clin Pract*. 2015 Aug; 109(2):215–25. <https://doi.org/10.1016/j.diabres.2015.04.031> PMID: 26008721
6. Feldman EL, Nave KA, Jensen TS, Bennett DLH. New Horizons in Diabetic Neuropathy: Mechanisms, Bioenergetics, and Pain. *Neuron*. 2017 Mar 22; 93(6):1296–313. <https://doi.org/10.1016/j.neuron.2017.02.005> PMID: 28334605
7. Aubert CE, Michel PL, Gillery P, Jaisson S, Fonfrede M, Morel F, et al. Association of peripheral neuropathy with circulating advanced glycation end products, soluble receptor for advanced glycation end products and other risk factors in patients with type 2 diabetes. *Diabetes Metab Res Rev*. 2014 Nov; 30(8):679–85. <https://doi.org/10.1002/dmrr.2529> PMID: 24449227
8. Schurgers LJ, Spronk HM, Skepper JN, Hackeng TM, Shanahan CM, Vermeer C, et al. Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification. *J Thromb Haemost*. 2007 Dec; 5(12):2503–11. <https://doi.org/10.1111/j.1538-7836.2007.02758.x> PMID: 17848178
9. Engelke JA, Hale JE, Suttie JW, Price PA. Vitamin K-dependent carboxylase: utilization of decarboxylated bone Gla protein and matrix Gla protein as substrates. *Biochim Biophys Acta*. 1991 May 30; 1078(1):31–4. [https://doi.org/10.1016/0167-4838\(91\)90088-h](https://doi.org/10.1016/0167-4838(91)90088-h) PMID: 2049381
10. Price PA, Rice JS, Williamson MK. Conserved phosphorylation of serines in the Ser-X-Glu/Ser(P) sequences of the vitamin K-dependent matrix Gla protein from shark, lamb, rat, cow, and human. *Protein Sci*. 1994 May; 3(5):822–30. <https://doi.org/10.1002/pro.5560030511> PMID: 8061611
11. Price PA, Urist MR, Otawara Y. Matrix Gla protein, a new gamma-carboxyglutamic acid-containing protein which is associated with the organic matrix of bone. *Biochem Biophys Res Commun*. 1983 Dec 28; 117(3):765–71. [https://doi.org/10.1016/0006-291x\(83\)91663-7](https://doi.org/10.1016/0006-291x(83)91663-7) PMID: 6607731
12. Goritz C, Thiebaut R, Tessier LH, Nieweg K, Moehle C, Buard I, et al. Glia-induced neuronal differentiation by transcriptional regulation. *Glia*. 2007 Aug 15; 55(11):1108–22. <https://doi.org/10.1002/glia.20531> PMID: 17582617
13. Hur DJ, Raymond GV, Kahler SG, Riegert-Johnson DL, Cohen BA, Boyadjiev SA. A novel MGP mutation in a consanguineous family: review of the clinical and molecular characteristics of Keutel syndrome. *Am J Med Genet A*. 2005 May 15; 135(1):36–40. <https://doi.org/10.1002/ajmg.a.30680> PMID: 15810001
14. Cranenburg EC, KY VANS-Z, Bonafe L, Mittaz Crettol L, Rodiger LA, Dikkers FG, et al. Circulating matrix gamma-carboxyglutamate protein (MGP) species are refractory to vitamin K treatment in a new

- case of Keutel syndrome. *J Thromb Haemost*. 2011 Jun; 9(6):1225–35. <https://doi.org/10.1111/j.1538-7836.2011.04263.x> PMID: 21435166
15. Mertsch S, Schurgers LJ, Weber K, Paulus W, Senner V. Matrix gla protein (MGP): an overexpressed and migration-promoting mesenchymal component in glioblastoma. *BMC Cancer*. 2009 Aug 27; 9:302. <https://doi.org/10.1186/1471-2407-9-302> PMID: 19712474
  16. Santa-Maria I, Avila J, Rabano A. Differential gene expression analysis of human entorhinal cortex support a possible role of some extracellular matrix proteins in the onset of Alzheimer disease. *Neurosci Lett*. 2010 Jan 14; 468(3):225–8. <https://doi.org/10.1016/j.neulet.2009.11.002> PMID: 19922771
  17. Bourron O, Aubert CE, Liabeuf S, Cluzel P, Lajat-Kiss F, Dadon M, et al. Below-knee arterial calcification in type 2 diabetes: association with receptor activator of nuclear factor kappaB ligand, osteoprotegerin, and neuropathy. *J Clin Endocrinol Metab*. 2014 Nov; 99(11):4250–8. <https://doi.org/10.1210/jc.2014-1047> PMID: 25013993
  18. Young MJ, Boulton AJ, MacLeod AF, Williams DR, Sonksen PH. A multicentre study of the prevalence of diabetic peripheral neuropathy in the United Kingdom hospital clinic population. *Diabetologia*. 1993 Feb; 36(2):150–4. <https://doi.org/10.1007/bf00400697> PMID: 8458529
  19. Abbott CA, Carrington AL, Ashe H, Bath S, Every LC, Griffiths J, et al. The North-West Diabetes Foot Care Study: incidence of, and risk factors for, new diabetic foot ulceration in a community-based patient cohort. *Diabet Med*. 2002 May; 19(5):377–84. <https://doi.org/10.1046/j.1464-5491.2002.00698.x> PMID: 12027925
  20. Gadia MT, Natori N, Ramos LB, Ayyar DR, Skyler JS, Sosenko JM. Influence of height on quantitative sensory, nerve-conduction, and clinical indices of diabetic peripheral neuropathy. *Diabetes Care*. 1987 Sep-Oct; 10(5):613–6. <https://doi.org/10.2337/diacare.10.5.613> PMID: 3677981
  21. Grote CW, Wright DE. A Role for Insulin in Diabetic Neuropathy. *Front Neurosci*. 2016; 10:581. <https://doi.org/10.3389/fnins.2016.00581> PMID: 28066166
  22. Zebboudj AF, Imura M, Bostrom K. Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2. *J Biol Chem*. 2002 Feb 8; 277(6):4388–94. <https://doi.org/10.1074/jbc.M109683200> PMID: 11741887
  23. Moon JI, Birren SJ. Target-dependent inhibition of sympathetic neuron growth via modulation of a BMP signaling pathway. *Dev Biol*. 2008 Mar 15; 315(2):404–17. <https://doi.org/10.1016/j.ydbio.2007.12.041> PMID: 18272145
  24. Majdazari A, Stubbusch J, Muller CM, Hennchen M, Weber M, Deng CX, et al. Dendrite complexity of sympathetic neurons is controlled during postnatal development by BMP signaling. *J Neurosci*. 2013 Sep 18; 33(38):15132–44. <https://doi.org/10.1523/JNEUROSCI.4748-12.2013> PMID: 24048844
  25. Dore JJ, Crotty KL, Birren SJ. Inhibition of glial maturation by bone morphogenetic protein 2 in a neural crest-derived cell line. *Dev Neurosci*. 2005 Jan-Feb; 27(1):37–48. <https://doi.org/10.1159/000084531> PMID: 15886483
  26. Wang YL, Wang DZ, Nie X, Lei DL, Liu YP, Zhang YJ, et al. The role of bone morphogenetic protein-2 in vivo in regeneration of peripheral nerves. *Br J Oral Maxillofac Surg*. 2007 Apr; 45(3):197–202. <https://doi.org/10.1016/j.bjoms.2006.06.003> PMID: 16876296
  27. Gonzalez-Perez F, Udina E, Navarro X. Extracellular matrix components in peripheral nerve regeneration. *Int Rev Neurobiol*. 2013; 108:257–75. <https://doi.org/10.1016/B978-0-12-410499-0.00010-1> PMID: 24083438
  28. Nishimoto SK, Nishimoto M. Matrix Gla protein C-terminal region binds to vitronectin. Co-localization suggests binding occurs during tissue development. *Matrix Biol*. 2005 Aug; 24(5):353–61. <https://doi.org/10.1016/j.matbio.2005.05.004> PMID: 15982861
  29. Nishimoto SK, Nishimoto M. Matrix gla protein binds to fibronectin and enhances cell attachment and spreading on fibronectin. *Int J Cell Biol*. 2014; 2014:807013. <https://doi.org/10.1155/2014/807013> PMID: 25210519
  30. Cranenburg EC, Koos R, Schurgers LJ, Magdeleyns EJ, Schoonbrood TH, Landewe RB, et al. Characterisation and potential diagnostic value of circulating matrix Gla protein (MGP) species. *Thromb Haemost*. 2010 Oct; 104(4):811–22. <https://doi.org/10.1160/TH09-11-0786> PMID: 20694284
  31. Dalmeijer GW, van der Schouw YT, Magdeleyns EJ, Vermeer C, Verschuren WM, Boer JM, et al. Matrix Gla protein species and risk of cardiovascular events in type 2 diabetic patients. *Diabetes Care*. 2013 Nov; 36(11):3766–71. <https://doi.org/10.2337/dc13-0065> PMID: 23877986
  32. Hall JG, Pauli RM, Wilson KM. Maternal and fetal sequelae of anticoagulation during pregnancy. *Am J Med*. 1980 Jan; 68(1):122–40. [https://doi.org/10.1016/0002-9343\(80\)90181-3](https://doi.org/10.1016/0002-9343(80)90181-3) PMID: 6985765
  33. Popescu DC, Huang H, Singhal NK, Shriver L, McDonough J, Clements RJ, et al. Vitamin K enhances the production of brain sulfatides during remyelination. *PLoS One*. 2018; 13(8):e0203057. <https://doi.org/10.1371/journal.pone.0203057> PMID: 30148869

34. Coetzee T, Fujita N, Dupree J, Shi R, Blight A, Suzuki K, et al. Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. *Cell*. 1996 Jul 26; 86(2):209–19. [https://doi.org/10.1016/s0092-8674\(00\)80093-8](https://doi.org/10.1016/s0092-8674(00)80093-8) PMID: 8706126
35. Nakajima M, Furukawa S, Hayashi K, Yamada A, Kawashima T, Hayashi Y. Age-dependent survival-promoting activity of vitamin K on cultured CNS neurons. *Brain Res Dev Brain Res*. 1993 May 21; 73(1):17–23. [https://doi.org/10.1016/0165-3806\(93\)90041-8](https://doi.org/10.1016/0165-3806(93)90041-8) PMID: 8513553
36. Druet C, Bourdel-Marchasson I, Weill A, Eschwege E, Penfornis A, Fosse S, et al. [Type 2 diabetes in France: epidemiology, trends of medical care, social and economic burden]. *Presse Med*. 2013 May; 42(5):830–8. <https://doi.org/10.1016/j.lpm.2013.02.312> PMID: 23566620
37. Zhu T, Meng Q, Ji J, Lou X, Zhang L. Toll-like receptor 4 and tumor necrosis factor-alpha as diagnostic biomarkers for diabetic peripheral neuropathy. *Neurosci Lett*. 2015 Jan 12; 585:28–32. <https://doi.org/10.1016/j.neulet.2014.11.020> PMID: 25445373
38. Li J, Zhang H, Xie M, Yan L, Chen J, Wang H. NSE, a potential biomarker, is closely connected to diabetic peripheral neuropathy. *Diabetes Care*. 2013 Nov; 36(11):3405–10. <https://doi.org/10.2337/dc13-0590> PMID: 23846809
39. Roumeliotis S, Dounousi E, Eleftheriadis T, Liakopoulos V. Association of the Inactive Circulating Matrix Gla Protein with Vitamin K Intake, Calcification, Mortality, and Cardiovascular Disease: A Review. *Int J Mol Sci*. 2019 Feb 1; 20(3).
40. Puzantian H, Akers SR, Oldland G, Javaid K, Miller R, Ge Y, et al. Circulating Dephospho-Uncarboxylated Matrix Gla-Protein Is Associated With Kidney Dysfunction and Arterial Stiffness. *Am J Hypertens*. 2018 Aug 3; 31(9):988–94. <https://doi.org/10.1093/ajh/hpy079> PMID: 29788226
41. Kurnatowska I, Grzelak P, Masajtis-Zagajewska A, Kaczmarska M, Stefanczyk L, Vermeer C, et al. Plasma Desphospho-Uncarboxylated Matrix Gla Protein as a Marker of Kidney Damage and Cardiovascular Risk in Advanced Stage of Chronic Kidney Disease. *Kidney Blood Press Res*. 2016; 41(3):231–9. <https://doi.org/10.1159/000443426> PMID: 27100101
42. Wei FF, Huang QF, Zhang ZY, Van Keer K, Thijs L, Trenson S, et al. Inactive matrix Gla protein is a novel circulating biomarker predicting retinal arteriolar narrowing in humans. *Sci Rep*. 2018 Oct 10; 8(1):15088. <https://doi.org/10.1038/s41598-018-33257-6> PMID: 30305657
43. Chalk C, Benstead TJ, Moore F. Aldose reductase inhibitors for the treatment of diabetic polyneuropathy. *Cochrane Database Syst Rev*. 2007 Oct 17(4):CD004572.
44. Vinik AI, Bril V, Kempler P, Litchy WJ, Tesfaye S, Price KL, et al. Treatment of symptomatic diabetic peripheral neuropathy with the protein kinase C beta-inhibitor ruboxistaurin mesylate during a 1-year, randomized, placebo-controlled, double-blind clinical trial. *Clin Ther*. 2005 Aug; 27(8):1164–80. <https://doi.org/10.1016/j.clinthera.2005.08.001> PMID: 16199243
45. Thornalley PJ. Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Arch Biochem Biophys*. 2003 Nov 1; 419(1):31–40. <https://doi.org/10.1016/j.abb.2003.08.013> PMID: 14568006
46. Papanas N, Ziegler D. Efficacy of alpha-lipoic acid in diabetic neuropathy. *Expert Opin Pharmacother*. 2014 Dec; 15(18):2721–31. <https://doi.org/10.1517/14656566.2014.972935> PMID: 25381809
47. Inoue T, Fujita T, Kishimoto H, Makino T, Nakamura T, Sato T, et al. Randomized controlled study on the prevention of osteoporotic fractures (OF study): a phase IV clinical study of 15-mg menatetrenone capsules. *J Bone Miner Metab*. 2009; 27(1):66–75. <https://doi.org/10.1007/s00774-008-0008-8> PMID: 19082528
48. Vissers LE, Dalmeijer GW, Boer JM, Monique Verschuren WM, van der Schouw YT, Beulens JW. Intake of dietary phylloquinone and menaquinones and risk of stroke. *J Am Heart Assoc*. 2013 Dec 10; 2(6):e000455. <https://doi.org/10.1161/JAHA.113.000455> PMID: 24326161
49. Dyck PJ. Detection, characterization, and staging of polyneuropathy: assessed in diabetics. *Muscle Nerve*. 1988 Jan; 11(1):21–32. <https://doi.org/10.1002/mus.880110106> PMID: 3277049
50. Dyck PJ, Bushek W, Spring EM, Karnes JL, Litchy WJ, O'Brien PC, et al. Vibratory and cooling detection thresholds compared with other tests in diagnosing and staging diabetic neuropathy. *Diabetes Care*. 1987 Jul-Aug; 10(4):432–40. <https://doi.org/10.2337/diacare.10.4.432> PMID: 3622200
51. Dyck PJ, Kratz KM, Lehman KA, Karnes JL, Melton LJ 3rd, O'Brien PC, et al. The Rochester Diabetic Neuropathy Study: design, criteria for types of neuropathy, selection bias, and reproducibility of neuropathic tests. *Neurology*. 1991 Jun; 41(6):799–807. <https://doi.org/10.1212/wnl.41.6.799> PMID: 2046920
52. Dyck PJ, Karnes JL, O'Brien PC, Litchy WJ, Low PA, Melton LJ, 3rd. The Rochester Diabetic Neuropathy Study: reassessment of tests and criteria for diagnosis and staged severity. *Neurology*. 1992 Jun; 42(6):1164–70. PMID: 1603343

53. Feki I, Lefaucheur JP. Correlation between nerve conduction studies and clinical scores in diabetic neuropathy. *Muscle Nerve*. 2001 Apr; 24(4):555–8. <https://doi.org/10.1002/mus.1040> PMID: 11268029
54. Roustit M, Loader J, Deusenberg C, Baltzis D, Veves A. Endothelial Dysfunction as a Link Between Cardiovascular Risk Factors and Peripheral Neuropathy in Diabetes. *J Clin Endocrinol Metab*. 2016 Sep; 101(9):3401–8. <https://doi.org/10.1210/jc.2016-2030> PMID: 27399351
55. Andreassen CS, Jakobsen J, Andersen H. Muscle weakness: a progressive late complication in diabetic distal symmetric polyneuropathy. *Diabetes*. 2006 Mar; 55(3):806–12. <https://doi.org/10.2337/diabetes.55.03.06.db05-1237> PMID: 16505247
56. Weintrob N, Amitay I, Lilos P, Shalitin S, Lazar L, Josefsberg Z. Bedside neuropathy disability score compared to quantitative sensory testing for measurement of diabetic neuropathy in children, adolescents, and young adults with type 1 diabetes. *J Diabetes Complications*. 2007 Jan-Feb; 21(1):13–9. <https://doi.org/10.1016/j.jdiacomp.2005.11.002> PMID: 17189869
57. Dyck PJ, Sherman WR, Hallcher LM, Service FJ, O'Brien PC, Grina LA, et al. Human diabetic endoneurial sorbitol, fructose, and myo-inositol related to sural nerve morphometry. *Ann Neurol*. 1980 Dec; 8(6):590–6. <https://doi.org/10.1002/ana.410080608> PMID: 7212646
58. Dyck PJ, Karnes JL, Daube J, O'Brien P, Service FJ. Clinical and neuropathological criteria for the diagnosis and staging of diabetic polyneuropathy. *Brain*. 1985 Dec; 108 (Pt 4):861–80.
59. Dyck PJ, Kratz KM, Karnes JL, Litchy WJ, Klein R, Pach JM, et al. The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study. *Neurology*. 1993 Apr; 43(4):817–24. <https://doi.org/10.1212/wnl.43.4.817> PMID: 8469345