Original Article

Brunei Int Med J. *2017; 13 (6): 180-193*

Expression of skin Glyoxalase-I, advanced glycation end products (AGEs) and receptor (RAGE) in patients with long-term type 1 diabetes and diabetic neuropathy.

Ahmed T. Alahmar[1,2], Ioannis N. Petropoulos[2,3], Maryam Ferdousi[2], Wendy Jones[2], Hassan Fadavi[2], Shazli Azmi[2], Uazman Alam[2], Omar Asghar[2], Aisha Meskiri[2], Ahmad Kheyami[2], Georgios Ponirakis[2,3], Andrew Marshall[4], Andrew J.M. Boulton[2,5], Mitra Tavakoli[2], Maria Jeziorska[2], Rayaz A. Malik [2,5]

[1]College of Pharmacy, University of Babylon, Iraq, [2]Institute of Human Development, Centre for Endocrinology and Diabetes, University of Manchester, UK, [3]Research division, Weill Cornell Medical College, Doha, Qatar, [4]Department of Neurophysiology and [5]Manchester Diabetes Centre, Central Manchester NHS Foundation Trust, Manchester, UK.

ABSTRACT

Background: Certain group of diabetic patients have been shown to remain free of diabetic complications despite having had diabetes for longer periods. Advanced glycation end products (AGEs), their receptor (RAGE) and Glyoxalase-I (GLO-I) have been implicated in the development of diabetic neuropathy. **Objective:** To assess the effect of long-term type 1 diabetes mellitus on skin distribution and expression of AGEs, RAGE and GLO-I and to correlate these expressions with measures of small and large nerve fibre damage. **Methods:** Sixty-seven patients with type 1 diabetes mellitus of shorter (<15 years, n=20), intermediate (15 -40 years, n=25) and longer (>40 years, n=22) duration and 34 non-diabetic controls underwent diabetic neuropathy assessment: Neuropathy disability score (NDS), quantitative sensory testing (QST) including vibration pressure and thermal thresholds, nerve conduction studies (NCS), deep breathing heart rate variability (DB-HRV), corneal confocal microscopy (CCM) and intra-epidermal nerve fibre density (IENFD) and AGEs, RAGE and GLO-I expression in foot skin biopsies. **Results:** Compared to controls, type 1 diabetes mellitus patients showed progressively increased skin expression of AGEs, RAGE but progressively lower GLO-I expression with increasing duration of diabetes. Thus patients with longer-duration diabetes demonstrated significantly higher skin AGEs and RAGE but lower GLO-I expression than both shorter and intermediate-duration diabetic groups. In patients with longer-duration diabetes who developed diabetic neuropathy, the skin expression of AGEs and RAGE were significantly higher but GLO-I were significantly lower than those who did not develop diabetic neuropathy. These expressions also correlated with IENFD, CCM and NCS measures. **Conclusion:** Patients with type 1 diabetes mellitus showed progressively increased skin expression of AGEs, RAGE but progressively lower GLO-I expression with increasing duration of diabetes. Patients with longer-duration diabetes who developed diabetic neuropathy have significantly higher skin AGEs and RAGE and decreased GLO-I expression suggesting a potential role for these macromolecules as aetiological, marker of the disease as well as therapeutic target for diabetic neuropathy.

Keywords: Advanced glycation end products, diabetes mellitus, type 1, diabetic neuropathy, receptor for advanced glycation end products, GLO-I protein, human.

Correspondence: Ahmed T Alahmar, MBChB, MSc, College of Pharmacy, University of Babylon, Iraq.
Tel: +9647808180900
Email: ahmed.t.alahmar@gmail.com

INTRODUCTION

Higher morbidity and mortality in patients with diabetes mellitus (DM) have been linked to several factors such longer duration of DM,

inadequate glycaemic control, impaired renal function, hypertension, and dyslipidaemia.[1,2] Combination of longer duration of DM with inadequate glycaemic control predisposes patients to development of long-term diabetic complications. Nevertheless, a proportion of diabetic patients have been shown to survive long period of DM and remained free of complications such as diabetic neuropathy (DN).[3–5] These patients constitute a unique group who possesses the endogenous protective factors that mediate longevity, resistance to developing diabetic complications and maintenance of endogenous β-cells function.[6] Studies on patients with long duration of DM are limited and interest in these long survivors of DM founded medal programmes in the US and UK for these survivors.[5]

DN is a common complication of DM, affecting around 50% of diabetic patients and a leading contributing factor for foot ulceration and subsequent amputation. Different questionnaires and tools are available to assess symptoms, signs and neurological deficit of DN. Techniques to evaluate large nerve fibre damage in DN encompass vibration perception threshold (VPT) and nerve conduction studies (NCS).[7] Small nerve fibre damage can be assessed using quantitative sensory testing, nerve biopsy, the less invasive skin biopsy and recently corneal confocal microscopy (CCM) measurements which has been shown to correlate with intra-epidermal nerve fibre (IENFD) loss.[8]

The pathogenesis of DN is complex. Mechanisms that linked hyperglycaemia with neurovascular damage include hexosamine pathway flux, enhancement of polyol pathway, excessive reactive oxygen species formation, protein kinase C, abnormal endothelial nitric oxide (NO) activity, enhanced formation of vascular endothelial factor (VEGF), and advanced glycation end products (AGEs) and their receptor (RAGE).[9,10] AGEs are formed under the influence of sustained hyperglycemia via non-enzymetic condensation reaction called Millard reaction which is initiated by the reaction of reducing sugars with free amino group of proteins. The most widely studied receptor for AGEs is RAGE and AGE-RAGE interaction leads to a series of inflammatory reactions mediated by Phosphatidylinositol-3 Kinase (PI-3K), Ki-Ras, and Mitogen-Activated Protein Kinase.[11] AGEs can be detoxified in vivo by the glyoxalase system, the physiological line of defence against reactive dicarbonyls. The rate-limiting enzyme of the system is Glyoxalase-I (GLO-I) which converts methylglyoxal into D-lactate and reduced glutathione and thus prevents AGEs formation.[12]

There is increasing evidence linking AGEs, RAGE and recently GLO-I to the development and progression of diabetic complications including DN and recently to β-cells apoptosis.[11,13-16] In a study of 216 patients of the Diabetes Control and Complications Trial (DCCT) trial, skin collagen AGEs were lower in the intensive treatment group but not for Carboxymethyllysine (CML) and acid soluble collagen and independently associated with DN, retinopathy and nephropathy.[17] A recent study demonstrated that specific methylglyoxal concentration can differentiate between diabetic patients with painful and painless DN.[18] RAGE has been shown to be upregulated in DN.[15] Very recently, studies have reported that Glyoxalase-I activity was reduced in patients with DN and GLO-I upregulation reduced AGEs and RAGE expression and counteracted accompanying mitochondrial dysfunction.[16,19,20] The influence of DM duration on RAGE and GLO-I in DN is less clear. AGEs and RAGE have been assessed in tissues, plasma and via skin auto-fluorescence. However, plasma levels do not necessarily reflect tissue levels and skin auto-fluorescence has limitations.[21,22] This paper assessed the distribution and expression of skin AGEs, RAGE and GLO-I in the unique group of patients with more than 40 years of

DM with and without DN and correlated these expressions with small and larger nerve fibre damage measures.

METHODS

Patients

This prospective cross-sectional study recruited type 1 DM patients with duration of DM from <15 year to >40 years, from the Manchester Diabetes Centre, UK. Patients were excluded if they have non-diabetic causes of peripheral neuropathy, systemic disease like cancer, Addison's disease, heart failure, history of previous corneal trauma or surgery. A total of 67 type 1 DM patients were recruited and divided into 3 study groups based on duration of DM: shorter-duration of <15 years (n=20), intermediate-duration of 15-40 years (n=25) and longer-duration of >40 years (n=22). Thirty-four healthy non-diabetic volunteers were recruited as controls. These controls were either relative of patients or recruited from the same centre. The study was approved by Central Manchester Ethics Committee (2011/06/22, 354CMEC), and all participants gave informed consent upon recruitment.

Assessment of Neuropathy

DN diagnosis was based on the Toronto consensus as the presence of abnormal personal motor nerve conduction velocity (<42m/sec) and the presence of abnormal symptoms and signs of DN (Neuropathy Disability Score (NDS score) >2).[7] Symptoms of DN were evaluated in study participants using neuropathy symptom profile (NSP) and McGill visual analogue pain scale (McGill VAS).[23,24] Neurological functional impairments were assessed using NDS, quantitative sensory testing (QST) in form of warm (WT) and cold (CT) thresholds assessed by Medoc Neuro Sensory Analyser TSA-II (Medoc Ltd., Ramat, UK).[25] Vibration perception threshold (VPT) was evaluated by using a Neuroaesthesiometer (Horwell, Scientific Laboratory, Wilford, UK).[25] Autonomic nervous system function was quantified by obtaining deep breathing heart rate variability (DB-HRV) using a CASE IV machine (WR Medical Electronics, Inc, MN, USA). Nerve conduction studies (NCS) were undertaken by a consultant neurophysiologist using a Medtronic Keypoint™ EMG system and limb temperature was maintained constantly in a range of 32-35°C. Left Peroneal motor and Sural sensory nerves amplitudes (LSA and LPA) and conduction velocities (LPV and PMNCV) were quantified using silver-silver chloride electrodes according to the approved protocol. All participants were scanned with a laser CCM (HRT III-RCM Heidelberg Engineering GmBH, Heidelberg, Germany) using previously published method.[25] Three parameters were quantified using CCM: Corneal Nerve Fibre Density (CNFD) - total number of major nerves/mm^2; Corneal Nerve Fibre Length (CNFL) - total length of all nerve fibres and branches (mm/mm^2) and Corneal Nerve Branch Density (CNBD) - number of branches emanating from major nerve trunks/mm^2.

Immunohistochemistry

Skin biopsies were taken using 3-mm punch from the dorsum of the foot, 2cm proximal to the second metatarsal head with the use of 1% lidocaine anaesthesia. The samples were fixed in PBS-buffered (4%) paraformaldehyde for 18-24 hours immediately after collection. One sample was used for IENFD assessment on frozen sections while the second one was routinely processed to paraffin block for immunohistochemistry evaluation. Following washing in TBS buffer and graded solutions of sucrose for 2-4 h for cryoprotection, the specimen was frozen in liquid nitrogen, stored at -80°C and cut into 50 μm sections on a cryostat microtome (Microm HM450, Microm Int GmbH, Germany). Four floating sections from each patient were subjected to further processing. The non-specific staining and endogenous peroxidase activity were blocked by incubation in 5% goat serum and 0.3% hy-

drogen peroxide respectively. The sections were incubated with polyclonal rabbit anti-human PGP 9.5 neuronal marker IgG (Serotec Ltd, Oxford, England) followed by goat anti-rabbit secondary antibody and then by Horseradish Peroxidase (HRP)-Streptavidin (both from Vector Laboratories, Peterborough, UK). Immunoreactivity was revealed by using SG chromogen (Vector Laboratories, Peterborough, UK). IENFD was calculated as the number of nerve fibres crossing the basement membrane of the epidermis per millimetre length of the epidermis.

Formalin-fixed paraffin-embedded tissue blocks were cut at 5μm thickness on a microtome (Leica Biosystems, Peterborough, UK) for AGEs, RAGE and GLO-I assessment. Following deparaffinisation of sections with xylene and rehydration in graded alcohols, antigens were unmasked with 0.1M citrate buffer pH 6.0 for antigen retrieval. To quench endogenous peroxidase activity, sections were immersed in Dako Peroxidase-Blocking Solution (Dako Ltd, Denmark) and nonspecific binding was blocked by the incubation of sections in 5% Normal Horse Serum (NHS) for AGEs and GLO-I or normal goat serum (NGS) for RAGE. Sections were incubated with primary antibodies goat IgG anti-RAGE (Millipore, CA, USA), rabbit polyclonal anti-AGEs IgG (Abcam, Cambridge, USA) or rabbit polyclonal anti-GLO-I IgG (GeneTex, CA, USA), all diluted in 5% respective sera at 4°C overnight in a humidified chamber. Sections were then incubated with secondary antibodies: biotinylated horse anti-goat IgG antibody (BA-9500, Vector Laboratories, Inc., CA, USA) for RAGE and biotinylated horse anti-rabbit IgG (Vector Laboratories, Inc., CA, USA) for AGEs and GLO-I (all diluted in 5% respective sera). Samples were incubated with HRP-Avidin (Vector Laboratories, Inc., CA, USA) in a humidified chamber. Immunoreactivity was revealed by incubation of sections with SG Chromogen (Vector Laboratories, Inc., CA, USA).

Negative controls comprised substituting the primary antibody with non-immune immunoglobulin at a concentration parallel to that of the primary antibody (DakoCytomation) which showed lack of immunostaining. In each experiment, five new slides from five cases immunostained in the previous experiment were re-stained and compared with the current experiment's staining. Only when the five pairs of sections showed identical staining then the latest experiment was accepted, otherwise the entire series was repeated. A semi-quantitative method was applied to quantify AGEs, RAGE and GLO-I expression using a light microscope under 400x magnification and identical light intensity. A score form 0-5 for staining intensity was used in which 0 represents lack of immunostaining and 5 represents 80-100% immunostaining. The best representation of scores in different anatomical locations was used as a visual aid. Immunostaining of AGEs, RAGE and GLO-I was assessed in skin epithelium, microvessels and extracellular matrix (ECM). Before commencing the semi-quantitative assessment, all sections were reviewed and the best representation of scores for each antigen in different skin structures was selected and used as a visual aid for the final assessment. All sections underwent blind assessment three times in random sequence by the investigator (ATA) to estimate intra-observer repeatability. The sections were also subsequently assessed blindly by an expert pathologist (MJ) to obtain inter-observer repeatability. The final results used for statistical assessment were reconciled scores between the two observers (ATA and MJ).

Statistical analysis. StatsDirect Version 2.7.8 (StatsDirect Ltd., Cheshire, UK) and SPSS 22.0 for Windows (SPSS, Chicago, IL) software were used to perform statistical analysis. Normality of data was assessed with Shapiro-Wilk test and relevant histograms. Normally distributed data were expressed as

Table 1: Demographics and clinical neuropathy assessment.

Variables	Control (n=34)	Type 1 DM		
		Shorter-duration (n=20)	Intermediate-duration (n=25)	Longer-duration (n=22)
Age (years) ‡	39.17±14.25	22.41±3.85¶§	35.51±8.16§	50.07±4.16¶
Duration of DM (years)		9.59±3.12§	27.78±6.07§	45.11±2.12
With DN No. (%)		5 (25%)	9 (34.4%)	12 (54.5%)
HbA1c (Mean ± SD) †	5.32±1.29	8.68±2.37¶	8.19±1.16¶	8.12±1.11¶
NSP(0-37) †	0.10±0.41	2.88±6.2	4.32±6.12¶	5.25±5.28¶
McGill VAS (0-10) *	0.15±0.49	2.25±3.41	2.7±3.62¶	2.29±2.91
NDS(0-10))‡	0(0-1)	0(0-0) §	4(2-5) ¶§	6.5(5.5-8.5) ¶
VPT R(V))‡	3.5(3-5)	4.35(3-8.25) §	8.83(6-13.5) ¶§	25.16(22.83-29.5)¶
CT(°C) ‡	28.75(27.9-29.7)	28.1(23.9-29.7) §	26.2(24.4-28.3)¶§	18.91(7.4-22.1) ¶
WT(°C) ‡	36.55(35.1-37.8)	39.2(37-42.4) §	38.9(37.3-42) ¶§	44.31(41.3-45.8) ¶
DB-HRV (beats per min) ‡	25.73±8.17	28.16±13.44§	20.24±12.01¶§	11.3±6.79¶
LSA (uV) ‡	18.52±7.26	13.24±5.34§	9.37±6.58¶§	7.82±3.74¶
LSV (m/s) ‡	50.5±4.07	44.86±2.03§	43±4.94¶§	35.19±6.92¶
LPA (m/s) ‡	5.82±2.07	5.29±2.43§	3.15±1.84¶§	1.24±1.32¶
PMNCV (m/s) ‡	49.0±3.92	41.11±3.43¶§	39.54±7.59¶§	34.26±9.56¶
CNFD (no/mm^2) ‡	37.27±6.06	28.45±5.71§	23.85±8.7¶§	16.24±9.58¶
CNBD (no/mm^2) ‡	94.44±3.98	58.93±32.5¶§	57.31±32.35¶§	42.63±33.23¶
CNFL(mm/mm^2) ‡	37.27±6.06	19.95±4.08§	18.77±6.36¶§	13.64±7.79¶
IENFD(no/mm^2) (mm) ‡	10.33±2.31	6.04±2.01¶§	4.05±1.81¶§	2.75±1.42¶
Cholesterol (mmol/l)	5.04±0.78	4.41±1.1	4.45±0.94	4.34±1.12
HDL(mmol/l) †	1.56±0.37	1.46±0.34§	1.41±0.44	1.7±0.95¶
TRIG(mmol/l)	1.43±0.68	1.64±0.79	1.01±0.44	1.7±0.43
LDL(mmol/l)	2.4±0.92	2.22±1.09	2.2±0.82	2.17±1.1

Results are expressed as mean ± SD or median (interquartile range). Statistically significant differences using ANOVA or Kruskal Wallis test: * P<0.05, † P<0.01, ‡ P<0.001, ¶ Post hoc (Tukey or Conover Inman test) results significantly different from control subjects, § Post hoc results significantly different from long duration group. DPN, diabetic peripheral neuropathy; NSP, Neuropathy Symptom Profile; McGill VAS, McGill Visual Analogue Scale; NDS, Neuropathy Disability Score; VPT, Vibration Perception Threshold; WT, Warm Threshold; CT, Cold Threshold; CIP, Cold Induced Pain; HRV, Hear Rate Variability; HRV-DB, Heart Rate Variability to Deep Breathing; LSA, Left Sural Amplitude; LSV, Left Sural Velocity; LPA; Left Peroneal Amplitude; PMNCV; Peroneal Motor Nerve Conduction Velocity; HDL, High-Density Lipoproteins; TRIG, Triglycerides; LDL, Low-Density Lipoproteins; CNFD (Corneal Nerve Fibre Density); CNBD (Corneal Nerve Branch Density); CNFL (Corneal Nerve Fibre Length); IENFD (Intra-Epidermal Nerve Fibre Density).

mean ± SD. Analysis of variance (ANOVA) was used to compare the means among the groups with Tukey test as a post hoc test. Non-normally distributed data were presented as median and interquartile range with Kruskal-Wallis test to compare groups and Conover-Inman test as a post hoc test. Correlations between variables were performed using Pearson correlation coefficient. Intra-observer and inter-observer repeatability was estimated using repeatability coefficient. P<0.05 was considered as statistically significant.

RESULTS

The prevalence of DN was higher in patients with longer-duration DM (54.5%) than those with shorter-duration (25%) and intermediate -duration (34.3%) DM groups (Table 1). The levels of HbA1c were comparable between groups of different duration of diabetes but higher than controls. DM patients with longer-duration diabetes demonstrated significantly higher NDS, VPT (P<0.001, P<0.01, P<0.001), WT (P<0.001, P<0.01, P<0.01) and lower CT (P<0.001, P<0.01, P<0.001) as compared to controls, shorter-duration and intermediate-duration DM groups respectively. NCS findings were consistent with small fibres measures and patients with longer-duration DM have significantly lower sural and peroneal

Table 2: Skin AGEs, RAGE and GLO-I in controls and patients with short to long duration of diabetes.

	Variables	Control (n=34)	Type 1 DM		
			Shorter-duration (n=20)	Intermediate-duration (n=25)	Longer-duration (n=22)
AGEs	Epidermis ‡	2.17±0.38	2.40±0.75§	2.82±0.98¶§	3.58±1.40¶
	Microvessels ‡	2.20±0.55	2.72±0.70¶§	2.86±0.57¶§	3.51±1.18¶
	Endothelium ‡	1.26±0.44	2.00±0.78¶§	2.20±0.84¶§	3.09±0.14¶
	Basement Membrane ‡	2.05±0.54	2.13±0.64§	3.03±0.71¶§	3.71±1.22¶
	Papillary ECM ‡	1.87±0.84	1.70±0.53§	2.03±0.61§	3.00±1.21¶
	Reticular ECM ‡	1.93±0.59	2.57±0.9¶§	2.60±0.76¶§	3.63±1.18¶
RAGE	Epidermis ‡	2.07±0.72	2.93±0.74¶§	3.32±1.1¶§	4.09±0.94¶
	Microvessels ‡	2.15±0.57	2.73±0.78¶§	2.87±0.67¶§	3.85±1.28¶
	Endothelium ‡	2.09±0.59	2.40±0.84§	2.97±0.93¶	3.71±1.27¶
	Basement Membrane ‡	1.92±0.73	2.50±0.67¶§	2.62±0.65¶§	3.54±0.96¶
	Papillary ECM ‡	2.02±0.28	2.24±0.78§	2.61±1.24¶§	3.28±1.08¶
	Reticular ECM ‡	1.99±0.64	2.68±1.06¶§	3.10±0.72¶§	4.00±0.81¶
GLO-I	Epidermis ‡	3.64±1.22	2.90±1.08§	2.45±0.68¶	1.62±0.69¶
	Microvessels ‡	3.50±1.37	2.79±0.68¶§	2.57±0.75¶§	1.69±0.54¶
	Endothelium ‡	3.41±1.33	2.89±0.93§	2.31±0.75¶	2.08±0.56¶
	Basement Membrane ‡	3.25±1.06	2.29±0.62¶§	2.40±0.63¶§	1.58±0.7¶
	Papillary ECM ‡	2.33±1.40	1.91±1.12	1.29±0.61¶	1.50±0.90
	Reticular ECM ‡	2.94±1.57	2.53±0.84§	2.00±0.96	1.42±0.64¶

Results are expressed as mean ± SD or median (interquartile range). Statistically significant differences using ANOVA or
Results are expressed as mean ± SD or median (interquartile range). Statistically significant differences using ANOVA or
Kruskal Wallis test: † P<0.01, ‡ P<0.001, ¶ Post hoc (Tukey or Conover Inman test) results significantly different from control subjects, § Post hoc results significantly different from long duration group. ECM; Extracellular Matrix.

nerves amplitudes (P<0.001, P<0.001, P<0.01) and nerve conduction velocities (P<0.001, P<0.001, P<0.01) as compared to controls, short and intermediate duration groups respectively.

Lipid profile values were comparable between study groups apart from HDL, which was significantly higher in the longer-duration group than controls (P<0.01). As for IENFD, there was progressive reduction in IENFD as compared to controls (P<0.001), shorter-duration (P<0.01) and intermediate-duration (P<0.01) DM groups with increasing duration of DM and these differences were statistically significant. CM metrics namely CNFD, CNBD and CNFL also decreased significantly with increasing duration of DM (P<0.001, P<0.01, P<0.01 as compared to controls, short and intermediate duration groups respectively).

Intra-observer repeatability coefficients for immunohistochemical scores of AGE, RAGE and GLO-I in epidermis were 0.90, 0.87 and 0.85 respectively. Inter-observer repeatability coefficients for immunohistochemical scores of AGE, RAGE and GLO-I in epidermis were 0.88, 0.86 and 0.82 respectively.

Skin AGEs expression was significantly higher (P<0.001) in all patients compared to control in all skin structures assessed (epidermis, microvessels, endothelium, basement membrane and reticular ECM) apart from papillary ECM (Table 2). There was progressive increase of skin AGEs with increasing duration of DM in the epidermis (P<0.01, P<0.05), microvessels (P<0.05,P<0.05), endothelium (P<0.01,P<0.01), basement membrane (P<0.001, P<0.01), papillary ECM (P<0.001,P<0.01) and reticular ECM (P<0.001,P<0.01) in longer-duration DM group compared to shorter-duration and inter-

mediate-duration DM groups.

Similarly, skin RAGE expression was higher in patients than controls in all skin structures (P<0.001) (Table 2). There was progressive increase of skin RAGE with increasing duration of DM in epidermis (P<0.001, P<0.01), microvessels (P<0.001, P<0.001), basement membrane (P<0.001, P<0.01), papillary ECM (P<0.001, P<0.01) and reticular ECM (P<0.001, P<0.01) in longer-duration DM group compared to shorter-duration and intermediate-duration DM groups.

Skin expression of GLO-I was the inverse of those seen with AGEs and RAGE expression. Skin expression of GLO-I was significantly lower in patients than controls (P<0.001) (Table 2). Skin GLO-I progressively decreased with increasing duration of DM in epidermis (P<0.001) in longer-duration DM group compared to shorter-duration DM group, microvessels (P<0.01, P<0.01) and BM (P<0.01, P<0.01) in longer-duration group compared to both shorter-duration and intermediate-duration DM groups.

Patients with longer-duration DM were further stratified into those with and without DN and analysed (Table 3). Patients with longer-duration DM without DN exhibited significantly lower NSP, NDS, VPT, WT, DB-HRV and higher CT as compared to those with DN. Sural and peroneal nerves amplitudes and conduction velocities were also higher in patients without DN as compared to those with DN. CCM metrics were also consistent with other small nerve fibres measure. Scores of IENFD, CNFD, CNBD and CNFL were higher in patients without DN as compared to those with DN.

Skin expression of AGEs, RAGE and GLO-I in controls and Patients with longer-duration DM with and without DN are summarized in table 4 and figure 1. Patients with longer-duration DM without DN had significantly lower skin AGEs expression in the epidermis (P<0.01), microvessels (P<0.05), endothelium (P<0.01), basement membrane (P<0.01), papillary ECM (P<0.05) and reticular ECM (P<0.01) as compare to those with DN. Skin RAGE expression in patients without DN was significantly lower in epidermis (P<0.05), microvessels (P<0.01), endothelium (P<0.01), and basement membrane (P<0.01). Skin GLO-I expression was higher in patients without DN in epidermis (P<0.01), microvessels (P<0.01) and basement membrane (P<0.05) as compared to those with DN.

Skin AGEs and RAGE expression in multiple skin structures correlated directly (Supplementary page: Appendix I and II) and skin GLO-I expression correlated inversely (Supplementary page: Appendix III) with IENFD, CCM metrics and NCS findings.

DISCUSSION

In the current study, Patients with longer-duration (>40 years) DM exhibited more advanced large fibre damage as evidenced by their NDS, VPT and NCS findings as well as more advanced small fibre damage as indicated by their CT, WT, CCM and DB-HRV results. Interestingly, HDL-cholesterol was higher in patients with longer-duration DM which is in agreement with another study and this could provide one explanation for the long survival of these patients.[5]

This study is the first to report skin distribution and expression of the combined set of AGEs, RAGE and GLO-I in patients with longer-duration type 1 DM who has undergone detailed assessment of neuropathy. Skin AGEs expression was higher in patients with longer-duration DM as compared to control subjects and shorter-duration or intermediate -duration DM. There was progressive increase of AGEs expression with increasing DM dura-

Table 3: Demographics and clinical neuropathy assessment in longer-duration (>40 years) DM patients with or without DN.

Variables	Control (n=34)	Longer-duration (>40 years) Type 1 DM	
		NO NP (n=10)	NP (n=12)
Age (years) ‡	39.17±14.25	48.15±3.82¶	51.87±4.24¶
Duration of DM (years)		44.11±2.32	46.01±2.98
HbA1c (Mean ± SD) †	5.32±1.29	8.22±0.98¶	46.01±2.98
NSP(0-37) †	0.10±0.41	2.23±1.11¶	5.86±5.65¶§
McGill VAS (0-10) *	0.15±0.49	1.50±1.75¶	2.63±2.86¶
NDS(0-10))‡	0(0-1)	2(0-3)	7(4.5-8.5) ¶§
VPT R(V))‡	3.5(3-5)	7.33(5-11)¶	30.16(13.85-38.1)¶§
CT(°C) ‡	28.75(27.9-29.7)	26(22.6-28.5)	14.11(8.4-26.7) ¶§
WT(°C) ‡	36.55(35.1-37.8)	38.2(36.41-39.73)	46.5(40.5-49.4)¶§
DB-HRV (beats per min) ‡	25.73±8.17	18.3±3.79¶	8.25±3.46¶§
LSA (uV) ‡	18.52±7.26	10.66±3.82¶	4.47±2.22¶§
LSV (m/s) ‡	50.5±4.07	43.7±4.72¶	32.6±6.27 ¶§
LPA (m/s) ‡	5.82±2.07	2.72±1.55¶	0.68±0.78¶§
PMNCV (m/s) ‡	49.0±3.92	44.5±3.98¶	31.6±7.70¶§
CNFD (no/mm^2) ‡	37.27±6.06	27.74±8.02¶	15.67±8.94¶§
CNBD (no/mm^2) ‡	94.44±3.98	63.47 31.22¶	31.59±22.04¶§
CNFL(mm/mm^2) ‡	37.27±6.06	20.81±5.31¶	12.36±6.70¶§
IENFD(no/mm^2) (mm) ‡	10.33±2.31	6.73±3.38¶	3.38±4.79¶§
Cholesterol (mmol/l)	5.04±0.78	4.67±1.08	4.26±0.97
HDL(mmol/l) †	1.56±0.37	1.98±0.46¶	1.69±0.55§
TRIG(mmol/l)	1.43±0.68	1.63±0.43	1.71±0.55
LDL(mmol/l)	2.4±0.92	2.01± 1.06	2.20±0.92

Results are expressed as mean ± SD or median (interquartile range). Statistically significant differences using ANOVA or Kruskal Wallis test: * P<0.05, † P<0.01, ‡ P<0.001, ¶ Post hoc (Tukey or Conover Inman test) results significantly different from control subjects, § Post hoc results significantly different from no neuropathy group. DN, diabetic neuropathy; NSP, Neuropathy Symptom Profile; McGill VAS, McGill Visual Analogue Scale; NDS, Neuropathy Disability Score; VPT, Vibration Perception Threshold; WT, Warm Threshold; CT, Cold Threshold; CIP, Cold Induced Pain; HRV, Hear Rate Variability; HRV-DB, Heart Rate Variability to Deep Breathing; LSA, Left Sural Amplitude; LSV, Left Sural Velocity; LPA; Left Peroneal Amplitude; PMNCV; Peroneal Motor Nerve Conduction Velocity; HDL, High-Density Lipoproteins; TRIG, Triglycerides; LDL, Low-Density Lipoproteins; CNFD (Corneal Nerve Fibre Density); CNBD (Corneal Nerve Branch Density); CNFL (Corneal Nerve Fibre Length); IENFD (Intra-Epidermal Nerve Fibre Density).

tion in epidermis, micro-vessels, endothelium, basement membrane and ECM. Evidence shows that AGEs have been previously localized in skin, skin collagen, epidermal and peripheral nerves, dorsal root ganglia, blood vessels, heart, renal and retinal tissues and serum in both animal models of diabetes and patients.[13,15, 18,26,27] Our findings are also in keeping with those of previous studies which reported an association between AGEs expression and DM duration in patients with DN.[13,28] However, recent studies have reported independent association between AGEs and DN even after adjustments for age, DM duration and HbA1c. [13,17,26,29 30]

We have detected higher RAGE expression in longer-duration DM group as compared with control subjects and patients with shorter-duration or intermediate-duration DM groups in the same skin structures, which exhibited higher AGEs expression. Furthermore, there was a progressive increase in RAGE expression in these structures with increasing duration of DM. These results are consistent with reports of previous studies that observed higher RAGE expression in epidermal nerves, peripheral nerves, renal and retinal tissues,

Table 4: Skin AGEs, RAGE and GLO-I expression in patients with 40 years of diabetes with and without diabetic neuropathy.

Variables		Control (n=34)	Longer-duration (>40 years) Type 1 DM	
			NO NP (n=10)	NP (n=12)
AGEs	Epidermis ǂ	2.17±0.38	3.11±1.21¶	3.95±1.55¶§
	Microvessels ǂ	2.20±0.55	3.14±1.23¶	3.86±1.38¶§
	Endothelium ǂ	1.26±0.44	1.81±0.20	3.90±0.44¶§
	Basement Membrane ǂ	2.05±0.54	3.24±1.11¶	4.11±1.34¶§
	Papillary ECM ǂ	1.87±0.84	2.46±1.35	2.46±1.35
	Reticular ECM ǂ	1.93±0.59	3.10±1.18¶	4.22±1.15¶§
RAGE	Epidermis ǂ	2.07±0.72	3.61±0.76¶	4.28±1.04¶§
	Microvessels ǂ	2.15±0.57	2.45±1.43	4.06±1.62¶§
	Endothelium ǂ	2.09±0.59	3.02±1.37¶	4.36±1.18¶§
	Basement Membrane ǂ	1.92±0.73	2.51±0.74	3.87±0.96¶§
	Papillary ECM ǂ	2.02±0.28	3.04±1.21¶	3.42±1.18¶
	Reticular ECM ǂ	1.99±0.64	2.48±0.65	3.85±0.44¶
GLO-I	Epidermis ǂ	3.64±1.22	2.26±0.86¶	1.11±0.52¶§
	Microvessels ǂ	3.50±1.37	2.38±0.78¶	1.28±0.43¶§
	Endothelium ǂ	3.41±1.33	2.31±0.46¶	1.56±0.68¶
	Basement Membrane ǂ	3.25±1.06	1.93±0.82¶	1.25±0.74¶§
	Papillary ECM ǂ	2.33±1.40	1.80±1.11	1.38±0.61¶
	Reticular ECM ǂ	2.94±1.57	1.36±0.66¶	1.42±0.42¶

Results are expressed as mean ± SD or median (interquartile range). Statistically significant differences using ANOVA or Kruskal Wallis test: † P<0.01, ǂ P<0.001, ¶ Post hoc (Tukey or Conover Inman test) results significantly different from control subjects, § Post hoc results significantly different from no neuropathy group. ECM; Extracellular Matrix.

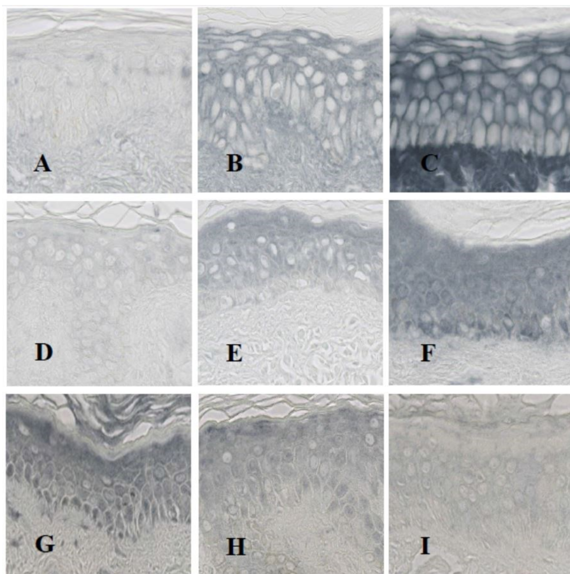

Figure 1: Immuno-localization of AGEs, RAGE and GLO-I in controls and diabetic patients with 40 years of diabetes mellitus. Upper row: Immuno-localization of AGEs in the epidermis (A-C) in control, diabetic patient without neuropathy and diabetic patient with neuropathy. Middle row: Immuno-localization of RAGE in the epidermis (D-F) in control, diabetic patient without neuropathy and diabetic patient with neuropathy. Lower row: Immuno-localization of GLO-I in the epidermis (G-I) in control, diabetic patient without neuropathy and diabetic patient with neuropathy.400x Magnification. (Click to enlarge)

blood vessels and serum in DM patients.[9,15,29,31,32,33] Higher RAGE expression could be explained by upregulation of RAGE in response to increased AGEs expression and this process is more enhanced in longer-duration DM patients. However, the relationship between the duration of DM and RAGE expression have not been fully investigated and whether this upregulation is a response to increased AGEs expression in these sites, other ligands, reactive oxygen species or a response to combined factors remains to be investigated.

In combination with increased AGEs and RAGE expression, we have detected decreased GLO-I expression in longer-duration DM group as compared with control subjects and with shorter-duration DM patients in the epidermis, micro-vessels, endothelium and basement membrane. Moreover, GLO-I expression gradually decrease with increasing

duration of DM. GLO-I is a key enzyme that detoxifies the precursor of AGEs and limits AGEs production. Thus, lower GLO-I expression in our longer-duration DM group could partially explains the increased AGEs expression detected in these patients. These findings are in keeping with the findings of more recent studies which demonstrated reduced GLO-I activity in blood and peripheral nerves and dorsal root ganglia in experimental DM and in patients.[16,34,35] The reason for reduced GLO-I activity in DM patients, however, is unknown although genetic variability of the enzyme activity have been reported in murine models and a recent study reported an association between single nucleotide polymorphism (SNP) of minor alleles rs1130534 and rs1049346 and decreased GLO-I activity in type 1 and type 2 DM patients.[34,36]. Our results indicate that AGEs, RAGE and GLO-I levels cannot explain how these longer-duration DM group have survived long duration of the disease therefore genetic or other factor like higher HDL may play a role in their longevity.

Longer-duration (>40 years) DM patients without DN demonstrated lower skin AGEs expression in the epidermis, micro-vessels, endothelium, basement membrane and reticular ECM in comparison with those with DN. A mechanistic role for AGEs in the development and progression of diabetic microvascular complications including DN has been proposed with a link to neuronal structural changes of DN.[14,18,37,38,39] Moreover, AGEs have been reported recently as a marker of DN and linked to β-cell apoptosis, decreased insulin synthesis.[11,13] Evidence shows that the association between AGEs and DN remained significant even after controlling for HbA1c.[17,30] AGEs were shown to correlate with age and diabetes duration but recent studies showed significant association between certain AGEs and DN after adjustment for these factors.[13,28,29,40] Data about AGEs expression in this unique group with longer-duration DM are rare but one recent study which have assessed serum AGEs in 351 type-1 DM patients with longer-duration of DM and interestingly have reported a dual predisposing and protective effect for AGEs combinations in relation to DN and other complications.[1] Yet, in that study, AGEs were assessed in the serum and serum AGEs levels does not necessarily reflect tissues levels [21].

Skin expression of RAGE in the unique protected group was also lower in the same skin structures that showed low AGEs expression apart from ECM as compared to patients withlonger-duration of DM and DN. The increased AGEs and RAGE expression in longer-duration DM group in the same skin structures adjacent to the small epidermal fibres together with higher prevalence of DN in this group points to a potential mechanistic role of AGEs and RAGE interaction in DN. Our observations are in agreement with previous observations of similar co-localization of these macromolecules in the target tissues for DM microvascular complications and link with these complications including DN.[15,31] The exact mechanism of RAGE-mediated neural damage, however, remains to be identified

As for skin expression of GLO-I, DM patients who did not develop DN demonstrated higher levels in epidermis, micro-vessels and basement membrane. These data and in combination with higher AGEs and RAGE expression and high prevalence of DN also suggest that GLO-I under expression induces detrimental effects that may lead to DN in these patients. Emerging reports are also linking reduced GLO-I to the development DN and painful DN.[12,16,36] A study investigated different GLO-I expressions in STZ-induced diabetic mice on DN revealed that lower GLO-I expression is associated with the behavioural changes of neuropathy, reduced IENFD and reduced mitochondrial oxidative phosphorylation while GLO-I overexpressing mice were protected against neuropathy and showed opposite changes.[35] A more recent study in 108 type-

1 and 109 type-2 DM patients demonstrated lower blood GLO-I in patients with painful DN as compared to non-painful DN.[16]

Skin AGEs and RAGE expression in multiple skin structures correlated directly and skin GLO-I expression correlated inversely with measures of small (IENFD and CCM measures) and large nerve fibre (NCS measures) damage in our study and these correlations were significant. These findings suggest that AGE, RAGE and GLO-I axis contribute to both structural and functional neuronal damage in DM patients which ultimately lead to DN. In a small study in DM patients with DN, AGEs were detected in 90% of sural and femoral nerve biopsies and correlated with morphological alterations of nerve damage including reduced numbers of nerve fibres.[39] Similarly, lower GLO-I expression was shown to reduce IENFD and higher expression to increase IENFD in animal models of DN.[35] We demonstrated very significant correlations between these three macromolecules and IENFD and CCM measures, adding to the data supporting the notion that CCM is a robust surrogate marker of DN.

There are some limitations in our study. We cannot provide measures for the variability of AGE, RAGE and GLO-I over time due to the cross-sectional nature of the study. Another limitation is the semi-quantitative nature of the scoring system albeit we undertook rigorous blinded assessment with excellent reproducibility. Of course other techniques such as liquid chromatography/ mass spectrometry (LC/MS) and polymerase chain reaction could be utilized to measure the levels of these molecules but these techniques cannot localize AGE, RAGE and GLO-I in their natural anatomical locations. Furthermore, the sample size of patients with >40 years of DM is small because it was challenging to recruit these longer-duration DM patients and to obtain skin biopsy from them.

CONCLUSION

Findings from our study provide further supporting evidence regarding the progressive increased skin expression of AGEs and RAGE, and a progressive decrease in GLO-I expression with increasing duration of DM. Our study also showed here for the first time in patients with >40 years of DM but without DN, the expression of skin AGEs and RAGE, although in general significantly higher than patients with <40 years of DM, the levels are still significantly lower than those with DN. This is also true of GLO-I expression which was significantly higher in patients with >40 years DM without DN compared with those with DN. Skin AGEs, RAGE and GLO-I expression also correlated significantly with small and large fibre damage measures which augments the notion that these macromolecules induce detrimental structural and functional effects that culminate in the development of DN which suggests potential role as a marker of the disease and therapeutic target. Further studies are required to consolidate the findings of this study.

Acknowledgments

This work was carried out in collaboration between all authors. ATA researched and analysed data and wrote manuscript. ION, MF, WJ, HV, SA, UA, OA, AM, AK, GP, AM, AJMB and MT researched data. MJ and RAM designed the study and reviewed work.

Financial Disclosure

This research was funded by awards from the Juvenile Diabetes Research Foundation International (27-2008-362) and the Higher Committee for Education Development (HCED) in Iraq.

Conflict of interest

The authors declare that he has no conflict of interest.

Human and Animal Rights

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Informed Consent

Informed consent was obtained from all patients for being included in the study

REFERENCES

1: Sun JK, Keenan HA, Cavallerano JD et al. Protection from retinopathy and other complications in patients with type 1 diabetes of extreme duration: the joslin 50-year medalist study. Diabetes Care. 2011;34(4):968–974.

2: Tavakoli M, Kallinikos P, Iqbal A et al. Corneal confocal microscopy detects improvement in corneal nerve morphology with an improvement in risk factors for diabetic neuropathy. Diabet Med. 2011;28(10):1261–1267.

3: Keenan HA, Costacou T, Sun JK et al. Clinical factors associated with resistance to microvascular complications in diabetic patients of extreme disease duration: the 50-year medalist study. Diabetes Care. 2007;30:1995–1997.

4: Oakley WG, Pyke DA, Tattersall RB & Watkins PJ. Long-term diabetes. A clinical study of 92 patients after 40 years. Q J Med. 1974;43:145 –156.

5: Bain SC, Gill GV, Dyer PH, Jones AF, Murphy M, Jones KE, Smyth C, Barnett AH. Characteristics of Type 1 diabetes of over 50 years duration (the Golden Years Cohort). Diabet Med. 2003;20:808–811.

6: Keenan HA, Sun JK, Levine J, Doria A, Aiello LP, Eisenbarth G, Bonner-Weir S, King GL. Residual insulin production and pancreatic ss-cell turnover after 50 years of diabetes: Joslin Medalist Study. Diabetes. 2010;59(11):2846–2853.

7: Tesfaye S, Boulton AJM, Dyck PJ et al and on behalf of the Toronto Diabetic Neuropathy Expert Group. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care. 2010;33(10):2285–2293.

8: Tavakoli M, Quattrini C, Abbott C, Kallinikos P, Marshall A, Finnigan J, Morgan P, Efron N, Boulton AJ, Malik RA. Corneal confocal microscopy: a novel noninvasive test to diagnose and stratify the severity of human diabetic neuropathy. Diabetes Care. 2010;33(8):1792–1797.

9: El-Mesallamy HO, Hamdy NM, Ezzat OA & Reda AM. Levels of soluble advanced glycation end product-receptors and other soluble serum markers as indicators of diabetic neuropathy in the foot. J Investig Med. 2011;59:1233–1238.

10: Kerkeni M, Saidi A, Bouzidi H et al. Pentosidine as a biomarker for microvascular complications in type 2 diabetic patients. Diab Vasc Dis Res. 2013;10:239–245.

11: Costal F, Oliveira E, Raposo A et al. Dual effect of advanced glycation end products in pancreatic islet apoptosis. Diabetes Metab Res Rev. 2013;29:296–307.

12: Rabbani N & Thornalley PJ. Glyoxalase in diabetes, obesity and related disorders. Semin Cell Dev Biol. 2011;22:309–317.

13: Monnier VM, Sell DR, Strauch C, Sun W, Lachin JM, Cleary PA, Genuth S, and the DCCT Research Group. The association between skin collagen glucosepane and past progression of microvascular and neuropathic complications in type 1 diabetes. J Diabetes Complicat. 2013;27:141–149.

14: Genuth S, Sun W, Cleary P, Sell DR, Dahms W, Malone J, Sivitz W, Monnier VM and for the DCCT Skin Collagen Ancillary Study Group. Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the diabetes control and complications trial and epidemiology of diabetes interventions and complications p. Diabetes. 2005;54:3103–3111.

15: Juranek JK, Kothary P, Mehra A, Hays A, Brannagan TH, and Schmidt AM. Increased expression of the receptor for advanced glycation end-products in human peripheral neuropathies. Brain Behav. 2013;3(6):701–709.

16: Skapare E, Konrade I, Liepinsh E, Strele I, Makrecka M, Bierhaus A, Lejnieks A, Pirags V, Dambrova M. Association of reduced glyoxalase 1 activity and painful peripheral diabetic neuropathy in type 1 and 2 diabetes mellitus patients. J Diabetes Complicat. 2013;27:262–267.

17: Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S. Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glycated collagen products versus HbA1c as markers of diabetic complications. DCCT Skin Co. Diabetes. 1999;48(4):870–880.

18: Bierhaus A, Fleming T, Stoyanov S et al. Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy. Nat Med.

2012;18:926–933.

19: Liu YW, Zhu X, Zhang L, Lu Q, Wang JY, Zhang F, Guo H, Yin JL and Yin XX. Up-regulation of glyoxalase 1 by mangiferin prevents diabetic nephropathy progression in streptozotocin-induced diabetic rats. Eur J Pharmacol. 2013;721:355–364.

20: Brouwers O, Niessen PM, Ferreira I, et al. Overexpression of glyoxalase-I reduces hyperglycemia-induced levels of advanced glycation end products and oxidative stress in diabetic rats. J Biol Chem. 2011;286:1374–1380.

21: Dorrian CA, Cathcart S, Clausen J, Shapiro D & Dominiczak MH. Factors in human serum interfere with the measurement of advanced glycation endproducts. Cell Mol Biol. 1998;44:1069–1079.

22: Bos DC, de Ranitz-Greven WL & de Valk HW. Advanced glycation end products, measured as skin autofluorescence and diabetes complications: a systematic review. Diabetes Technol Ther. 2011;13:773–779.

23: Meijer JW, Smit AJ, Sonderen EV, Groothoff JW, Eisma WH, Links TP. Symptom scoring systems to diagnose distal polyneuropathy in diabetes: the Diabetic Neuropathy Symptom score. Diabet Med. 2002;19:962–965.

24: Melzack R. The short-form McGill Pain Questionnaire. Pain. 1987;30:191–197.

25: Tavakoli M, Mitu-Pretorian M, Petropoulos IN, Fadavi H, Asghar O, Alam U, Ponirakis G, Jeziorska M, Marshall A, Efron N, Boulton AJ, Augustine T, Malik RA. Corneal confocal microscopy detects early nerve regeneration in diabetic neuropathy after simultaneous pancreas and kidney transplantation. Diabetes. 2013;62(1):254–260.

26: Hu H, Han CM, Hu XL, Ye WI, Huang WJ, and Smit AJ. Elevated skin autofluorescence is strongly associated with foot ulcers in patients with diabetes: a cross-sectional, observational study of Chinese subjects. J Zhejiang Univ Sci B. 2012;13: 372–377.

27: Karachalias N, Babaei-Jadidi R, Ahmed N & Thornalley PJ. Accumulation of fructosyl-lysine and advanced glycation end products in the kidney, retina and peripheral nerve of streptozotocin-induced diabetic rats. Biochem Soc Trans. 2003;31:1423–1425.

28: Lutgers HL, Graaff R, Links TP, Ubink-Veltmaat LJ, Bilo HJ, Gans RO, Smit AJ. Skin autofluorescence as a noninvasive marker of vascular damage in patients with type 2 diabetes. Diabetes Care. 2006;29(12):2654–2659.

29: Aubert CE, Michel PL, Gillery P, Jaisson S, Fonfrede M, Morel F, Hartemann A, Bourron O. 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;30 (8):679-85. doi:10.1002/dmrr.2529.

30: Conway BN, Aroda VR, Maynard JD, Matter N, Fernandez S, Ratner RE, Orchard TJ. Skin intrinsic fluorescence correlates with autonomic and distal symmetrical polyneuropathy in individuals with type 1 diabetes. Diabetes Care. 2011;34:1000–1005.

31: Del Carro U, Fiorina P, Amadio S et al. Evaluation of polyneuropathy markers in type 1 diabetic kidney transplant patients and effects of islet transplantation: neurophysiological and skin biopsy longitudinal analysis. Diabetes Care. 2007;30:3063–3069.

32: Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam BG, Murray-McIntosh RP, Cooper ME. Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. Diabetologia. 1997;40(6):619–628.

33: Tanji N, Markowitz GS, Fu C, Kislinger T, Taguchi A, Pischetsrieder M, Stern D, Schmidt AM, D'Agati VD. Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. J Am Soc Nephrol. 2000;11 (9):1656–1666.

34: Peculis R, Konrade I, Skapare E, Fridmanis D, Nikitina-Zake I, Lejnieks A, Pirags V, Dambrova M, Klovins J. Identification of glyoxalase 1 polymorphisms associated with enzyme activity. Gene. 2012;515(1):140–143.

35: Jack MM, Ryals JM & Wright DE. Protection from diabetes-induced peripheral sensory neuropathy --a role for elevated glyoxalase I? Exp Neurol. 2012;234:62–69.

36: Jack MM, Ryals JM & Wright DE. Characterisation of glyoxalase I in a streptozocin-induced mouse model of diabetes with painful and insensate neuropathy. Diabetologia. 2011;54:2174–2182.

37: Jack M & Wright D. Role of advanced glycation endproducts and glyoxalase I in diabetic peripheral sensory neuropathy. Transl Res. 2012;159:355–365.

38: Vouillarmet J, Maucort-Boulch D, Michon P &

Thivolet C. Advanced glycation end products assessed by skin autofluorescence: a new marker of diabetic foot ulceration. Diabetes Technol Ther. 2013;15:601–605.

39: Misur I, Zarković K, Barada A, Batelja L, Milicević Z, Turk Z. Advanced glycation endproducts in peripheral nerve in type 2 diabetes with neuropathy. Acta Diabetol. 2004;41(4):158–166.

40: Schiel R, Franke S, Appel T, Voigt U, ross IA, Kientsch-Engel R, Stein G, Muller UA. Improvement in quality of diabetes control and concentrations of AGE-products in patients with type 1 and insulin-treated type 2 diabetes mellitus studied over a period of 10 years (JEVIN). J Diabetes Complicat. 2003;17(2):90–97.