ORIGINAL ARTICLE

SKIN ADVANCED GLYCATION END PRODUCTS (AGES), RAGE AND GLYOXALASE-I (GLO-I) ARE ASSOCIATED WITH DIABETIC NEUROPATHY IN PATIENTS WITH TYPE 1 DIABETES MELLITUS

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ABSTRACT

Introduction: Advanced Glycation End Products (AGEs), their receptor (RAGE) and their detoxifying enzyme Glyoxalase-I (GLO-I) have been implicated in the development of experimental diabetic peripheral neuropathy (DPN). However, few studies have assessed their role in the tissues of diabetic patients.

Aim: We have assessed the relationship between skin expression of AGEs, RAGE, GLO-I and diabetic neuropathy in patients with type 1 diabetes.

Materials and Methods: Sixty-two patients with type 1 diabetes mellitus (16 with and 46 without DPN) and 30 age-matched control subjects underwent detailed assessment of neurologic deficits, quantitative sensory testing, electrophysiology, corneal confocal microscopy (CCM) , intraepidermal nerve fibre density (IENFD) and AGEs, RAGE and GLO1-I expression in foot skin biopsies.

Results: Skin AGEs and RAGE expression was significantly higher and GLO-I was significantly lower in the epidermis, microvessels and reticular extracellular matrix ofpatients with diabetic neuropathy as compared to diabetic patients without neuropathy and control subjects.Skin AGEs and RAGE expression was also moderately but significantly increased and GLO-I expression was decreased in some skin structures in patients without diabetic neuropathy as compared to control subjects. Skin AGEs and RAGE expression correlated negatively and GLO-I expression correlated positively with sural nerve amplitude and velocity, IENFD and corneal nerve pathology.

Conclusion: These findings suggest that AGEs, RAGE and GLO-I may play an important role in the etiology of human diabetic neuropathy.

Keywords: Diabetic neuropathy, Advanced Glycation Endproducts (AGEs), RAGE, Glyoxalase-I

INTRODUCTION

Diabetic Peripheral Neuropathy (DPN) is one of the most common chronic complications of diabetes which affects around 50% of all diabetic patients and the main contributing factor for foot ulceration and amputation in diabetic patients.[1] Several mechanisms have been suggested linking hyperglycemia with DPN and include activation of the polyol pathway, oxidative/nitrosative stress, increased protein kinase C activity, enhancement of poly ADP-ribose polymerase, and particularly the increased expression of advanced glycation end products (AGEs) and their receptor (RAGE).[2]Several tests have been advocated to assess nerve damage in DPN including nerve conduction studies, quantitative sensory testing and nerve biopsy.[3] Recently, the less invasive skin biopsy have been used to assess intraepidemal nerve fibre (IENF) damage and Corneal Confocal Microscopy (CCM) have been used to assess corneal nerve fibres damage which correlated well with IENF findings.[3]

AGEs are heterogeneous molecules derived from non-enzymatic reaction between sugar moieties and amine residues of proteins, lipids and nucleic acids, as a consequence of sustained hyperglycemia and their production is accelerated in diabetes mellitus (DM).[4,5] The main and best characterized receptor of AGEs is RAGE, a member of the immunoglobulin superfamily. AGE-RAGE binding triggers intracellular signalling, NF-kB activation and an inflammatory response accompanied by the release of cytokines,

inflammatory cells and generation of reactive oxygen species.[6] The glyoxalase system is the physiological dicarbonyl-detoxifying system where Glyoxalase-I (GLO-I), the rate-limiting enzyme, converts precursors of reactive AGEs into D-Lactate, thuspreventing AGEs formation.[7]

AGEs and RAGE have been linked to the chronic complications of diabetes including DPN, their progression [8] and recently to β-cells apoptosis.[9] There are also emerging data that connect the AGEs detoxifying enzyme, GLO-I with DPN [6] and its activity has been shown to be significantly lower in type 1 and type 2 DM patients with painful DPN.[10]Thus, alterations in AGEs, RAGE and GLO-I expression has been reported predominantly in animal tissues and there are only limited translational data in human plasma [5] and using human skin autofluorescence [11] to suggest they may play a significant role in the pathogenesis of DPN. However, plasma levels do not reflect tissue levels [12] and assessment of skin autofluorescence has limitations.[13]

We have had the unique opportunity to assess the expression of AGEs, RAGE and GLO-I in skin biopsies from the feet of diabetic patients with and without neuropathy. We have also explored the relationship of the expression of these proteins with the severity of neuropathy, which was quantified using a range of established and novel measures of human diabetic neuropathy.

METHODOLOGY

Ninety-two participants were enrolled in this study. Sixty-two patients with Type 1 diabetes mellitus and 30 healthy control subjects participated in this study. Neuropathy was defined according to the Toronto consensus [14]. DPN was defined as the presence of abnormal personal motor nerve conduction velocity (<42m/sec) and the presence of abnormal symptoms and signs of DPN (NDS score >2). Exclusion criteria includes non-diabetic causes of peripheral neuropathy, sever DPN (NDS>8), history of corneal traumatic injury or corneal surgery or systemic disease like cancer and congestive heart failure (grade III or VI). The study was approved by the Central Manchester Ethics Committee (Ref. no. 09/H1006/38) and written informed consent was obtained according to the declaration of Helsinki.

Assessment of Neuropathy: Symptoms were evaluated using the Neuropathy Symptom Profile [15]and painful symptoms were assessed by the short form McGill pain questionnaire [16]. Neurological deficits were assessed using the Neuropathy Disability Score (NDS), quantitative sensory testing (QST) involving the quantification of cold and warm thresholds using the Neuro Sensory Analyzer TSA-II (Medoc Ltd., Ramat Yishai 30095, Israel) [17]. Vibration perception threshold (VPT) was evaluated using a Neuroaesthesiometer (Horwell, Scientific Laboratory Supplies, Wilford, UK) [17]. Autonomic function (deep breathing heart rate variability DB-HRV) was assessed using a CASE IV automated system (WR Medical Electronics, Inc, Stillwater, MN, USA). Nerve conduction studies were performed by a consultant neurophysiologist using Medtronic Keypoint™ EMG system equipped with temperature regulator to maintain limb temperature in a range of 32-35°C. Peroneal motor and sural sensory nerves amplitudes and conduction velocities were assessed on the right foot.

All participants had both eyes scanned with a laser CCM (HRT III-RCM Heidelberg GmBH, Heidelberg, Germany) by purpose trained optometrists to obtain several scans of the entire corneausing previously published method[3]. Three parameters were quantified in each IVCCM image: 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: Two 3 mm punch skin biopsies were taken from the dorsum of the feet, approximately 2 cm proximal to the second metatarsal head under local subcutaneous anesthesia (1% lidocaine). Immediately after collection, the biopsies were fixed in 4% buffered paraformaldehyde for 18-24 hours. One sample was used for IENFD assessment (frozen sections) using previously published method [18] while the second one was routinely processed to paraffin block for immunohistochemistry assessment. Intraepidermal nerve fibre density (IENFD) was calculated as the number of nerve fibres crossing the basement membrane of the epidermis per millimeter length of the epidermis.

The formalin-fixed paraffin-embedded tissue blocks were cut at 5μm thickness on a microtome (Leica Biosystems, Peterborough, UK). The deparaffinised and rehydrated sections were subjected to antigen retrieval in 0.1M citrate buffer pH 6.0.. Endogenous peroxidase was quenched with Dako Peroxidase-Blocking Solution (Dako Ltd, Denmark) and non-specific binding was blocked with 5% normal horse serum (NHS) for AGEs and GLO-I or normal goat serum (NGS) for RAGE. Consecutive sections were incubated with primary antibodies: goat anti-RAGE IgG (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) in a humidified chamber at 4°C overnight. Sections were then incubated with biotinylated secondary antibodies: horse anti-goat IgG (- for RAGE and horse anti-rabbit IgG for AGEs and GLO-I (diluted in 5% respective sera)

followed by avidin conjugated to HRP and finally (Vector Laboratories, Peterborough, UK) SG chromogen (Vector Laboratories, Peterborough, UK).

Negative controls comprised substituting the primary antibody with non-immune immunoglobulin (Dako-Cytomation) in addition to the biotinylated secondary antibodies as above and showed negative staining. To ensure comparability of immunostaining in each new experiment, sections from five cases immunostained in the previous experiment were re-stained and compared with the current experiment's results. Only when the five pairs of sections showed identical intensity of immunostaining the latest experiment was accepted, otherwise the entire run was repeated. Quantification of AGEs, RAGE and GLO-1 was performed semi-quantitatively using a light microscope under 400x magnification and identical light intensity[19]. The quantification of immunostaining was performed for skin epithelium, microvessels, and extracellular matrix. The intensity of immunostaining was assessed using a semi-quantitative method on a scale 0-5 where 0 is lack of immunostaining and 5 is the highest. 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 were blindly assessed three times in random sequence by the investigator (AA) to establish intra-observer repeatability. The sections were also assessed blindly by an expert pathologist (MJ) to establish inter-observer repeatability. The final results used for statistical assessment were reconciled scores between the two observers (AA & MJ).

Statistical analysis: Statistical analysis was performed using Stats Direct Version 2.7.7 (StatsDirect Ltd., Cheshire, UK) and SPSS 20.0 for Windows (SPSS, Chicago, IL) software. The data were assessed for normality using relevant histograms and Shapiro-Wilk test where appropriate. For normally distributed data, the results were expressed as mean±SD. Analysis of variance (ANOVA) was used to compare the means among the groups. Tukey test was used as a post hoc test to determine the significance of difference between pairs of groups. Non-normally distributed data were expressed as median (interquartile range). Kruskal-Wallis test was used to compare groups and Conover-Inman test was used as a post hoc test. Pearson correlation coefficient was used to assess correlations. Intra-observer and inter-observer repeatability was estimated using repeatability coefficient. $P<0.05$ was considered as statistically significant.

RESULTS

Clinical demographics (Table 1)

Age and duration of diabetes were well-matched between groups. HbA1c was higher in patients than controls ($P<0.001$) but was comparable between patients with and without neuropathy.

Table 1: Demographics and neuropathy assessment in control subjects and diabetic patients without with Diabetic Peripheral Neuropathy (DPN)

Variables	Control (n=30)	No DPN (n=46)	DPN (n=16)
Age(years)	42.55±15.63	42.31±12.94	46.79±11.6
Duration of Diabetes (years)		26.15±13.91	31.02±13.25
HbA1c (%)‡	5.36±1.13	8.11±1.16¶	8.7±1.86¶
IFCC (mmol/mol)‡	36.06±7.98	61.17±22.21¶	71.59±20.38¶
NSP (0-37)‡	0(0-0)	1(0-2) ¶	5(1-18) ¶§
McGill VAS 0-10)†	0(0-0)	0(0-0)	5(0-8) ¶§
NDS (0-10)‡	0(0-1)	2.5(0-4) ¶	6(4.5-8.5) ¶§
VPT (V)‡	6.2±6.03	7.88±5.73	27.05±14.01¶§
CT (°C)‡	28.3±2.03	26.78±2.95	15.84±11.87¶§
WT (°C)‡	36.7±2.22	38.78±3.41¶	44.52±4.7¶§
SA (uV)‡	20.34±8.68	12.17±6.79¶	3.98±3.69¶§
SNCV (m/s)‡	50.45±4.07	45.27±4.29	36.91±7.59¶§
PA (m/s)†	5.82±2.07	6.16±8.3	1.05±1.26¶§
PMNCV (m/s)‡	49.01±3.92	43.86±3.01¶	29.31±9.07¶§
DB-HRV (beats per min)†	34.13±13.45	34.17±18.24	17.54±15.39¶§
CNFD (no/mm^2)‡	37.85±5.83	28.65±6.76¶	16.7±8.24¶§
CNBD (no/mm^2)‡	94.67±37.34	62.39±29.08¶	33.43±22.9¶§
CNFL (mm/mm^2)‡	26.84±3.87	21.0±4.62¶	11.64±7.01¶§
IENFD (no/mm)‡	8.5±4.6	6.37±3.83¶	3.67±4.89¶§

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 no neuropathy group. NSP (Neuropathy Symptom Profile); McGill VAS (McGill Visual Analogue Scale); NDS (Neuropathy Disability Score); VPT (Vibration Perception Threshold); CT (Cold Threshold); WT (Warm Threshold); DB-HRV (Deep Breathing- Heart Rate Variability); SA (Sural Amplitude); SNCV (Sural Nerve Conduction Velocity); PA (Peroneal Amplitude); PMNCV (Peroneal Motor Nerve Conduction Velocity), CNFD (Corneal Nerve Fibre Density); CNBD (Corneal Nerve Branch Density); CNFL (Corneal Nerve Fibre Length); IENFD (Intra-Epidermal Nerve Fibre Density).

Neuropathy Symptoms and Deficits

Neuropathy symptoms profile, McGill visual analogue and NDS were higher in patients than controls and were also higher in patients with DPN as compared to patients without DPN(P<0.001).

Quantitative Sensory Testing and Electrophysiology

VPT was increased in patients with neuropathy compared to controls (P<0.001) and those without neuropathy (P<0.001). CT was lower in patients with neuropathy compared with controls (P<0.001) and those without neuropathy (P<0.001). WT was higher in patients with (P<0.001) and without (P<0.001) neuropathy compared with controls and was also higher in patients without neuropathy compared to controls (P<0.001). SA was lower in patients with and without neuropathy (P<0.0001) compared to controls and was also lower in patients with compared to those without neuropathy (P<0.01). SNCV was lower in patients with neuropathy compared to controls (P<0.001) and patients without neuropathy (P<0.01). PA was lower in patients with neuropathy compared to controls (P<0.01) and patientswithout neuropathy (P<0.01). PMNCV was lower in patients with neuropathy compared to controls (P<0.001) and patients without neuropathy (P<0.001) and was also lower in patients without neuropathy compared to controls (P<0.01)

Small Fibre Testing

DB-HRV was lower in patients with neuropathy compared to controls (P<0.01) and those without neuropathy (P<0.01). CNFD, CNBD and CNFL were lower in patients with (P<0.001, P<0.001, P<0.001 respectively) and without (P<0.01, P<0.001, P<0.01 respectively) neuropathy compared to control subjects and were also significantly reduced in those with neuropathy compared to those without neuropathy (P<0.01, P<0.001, P<0.01 respectively). IENFD was lower in patients with (P<0.001) and without (P<0.01) neuropathy compared with controls and was also reduced in patients with neuropathy compared to those without neuropathy (P<0.01).

Intra-observer and Inter-observer repeatability

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

Skin AGEs expression (Table 2, Fig. 1)

There was a significantly increased expression of AGEs in patients with neuropathy compared to controls and patients without neuropathy in the epidermis (P<0.001, P<0.01), microvessels (P<0.001, P<0.01), endothelium (P<0.001, P<0.05), basement membrane (P<0.01, P<0.01) and reticular ECM (P<0.01, P<0.01) respectively. AGEs expression was also significantly increased in diabetic patients without neuropathy compared to control subjects in the epidermis (P<0.01), endothelium (P<0.001) and reticular ECM (P<0.01)

Table 2: Skin AGEs expression in control subjects and diabetic patients without and with Diabetic Peripheral Neuropathy (DPN)

Skin structure	Controls	No DPN	DPN
N	30	46	16
Epidermis‡	2.06±0.70	2.47±0.73¶	3.33±1.20¶§
Microvessels ‡	2.27±0.76	2.44±0.78	3.21±0.94¶§
Endothelium‡	1.90±0.72	2.19±0.78¶	2.85±0.94¶§
Basement Membrane†	2.40±0.79	2.45±0.78	3.27±0.96¶§
Papillary ECM*	3.32±0.86	3.40±0.58	3.88±0.72¶
Reticular ECM †	2.80±0.86	3.07±0.71¶	3.79±0.71¶§

Results are expressed as Mean ± SD. Statistically significant differences using ANOVA: * P<0.05, † P<0.01, ‡ P<0.001, ¶ Post hoc (Tukey) results significantly different from control subjects, § Post hoc results significantly different from no neuropathy group. ECM (Extracellular Matrix)

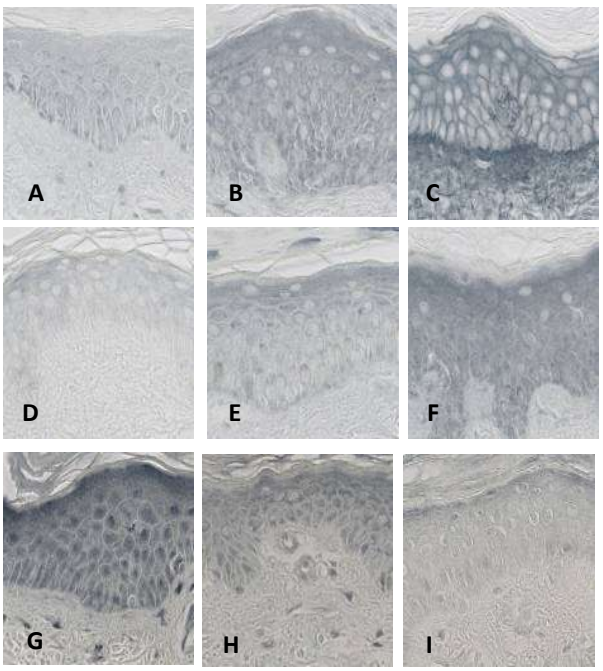

Fig. 1: Upper row: Immunolocalization of AGE in the epidermis (A-C) in control, diabetic patient without neuropathy and diabetic patient with neuropathy. Middle row: Immunolocalization of RAGE in the epidermis (A-C) in control, diabetic patient without neuropathy and diabetic patient with neuropathy. Lower row: Immunolocalization of GLO-I in the epidermis (G-I) in control, diabetic patient without neuropathy and diabetic patient with neuropathy.400x Maginification

Skin RAGE expression (Table 3, Fig. 1)

RAGE expression was significantly increased in patients with neuropathy compared to controls and patients without neuropathy in the epidermis (P<0.01, P<0.05), microvessels (P<0.001, P<0.05), endothelium (P<0.001, P<0.01) and basement membrane (P<0.001, P<0.05). RAGE expression was significantly increased in the epidermis (P<0.01) and microvessels (P<0.001) of those without neuropathy compared to control subjects.

Table 3: Skin RAGE expression in control subjects and diabetic patients without and with Diabetic Peripheral Neuropathy (DPN)

Skin structure	Controls	No DPN	DPN
N	30	46	16
Epidermis†	2.87±0.84	3.59±0.81¶	3.78±0.87¶§
Microvessels ‡	2.57±0.87	3.18±10¶	3.92±0.95¶§
Endothelium‡	2.55±0.77	3.09±0.98	3.90±0.91¶§
Basement Membrane‡	2.62±0.92	3.15±0.94	3.85±1.04¶§
Papillary ECM	2.87±0.94	3.19±0.96	3.33±0.72
Reticular ECM†	2.26±0.70	2.53±0.51	3.02±0.63¶

Results are expressed as Mean ± SD. Statistically significant differencesANOVA: † P<0.01, ‡ P<0.001, ¶ Post hoc (Tukey) results significantly different from control subjects, § Post hoc results significantly different from no neuropathy group. ECM (Extracellular Matrix).

Skin GLO1-I expression (Table 4, Fig. 1)

GLO-I expression was significantly reduced in patients with neuropathy compared to controls and patients without neuropathy in the epidermis (P<0.001, P<0.01), microvessels (P<0.001, P<0.01), endothelium (P<0.001, P<0.01) and basement membrane (P<0.001, P<0.01), respectively. There was also a reduction in GLO-I expression in the epidermis (P<0.001), microvessels (P<0.001), endothelium (P<0.001), basement membrane (P<0.001) and both papillary ECM (P<0.01 and reticular ECM (P<0.001) of patients without neuropathy compared to control subjects.

Table 4: Skin GLO-I expression in control subjects and diabetic patients without and with Diabetic Peripheral Neuropathy (DPN)

Skin structure	Controls	No DPN	DPN
N	30	46	16
Epidermis‡	3.69±0.83	3.10±0.82¶	2.73±0.88¶§
Microvessels‡	3.92±0.9	3.19±0.88¶	2.46±0.89¶§
Endothelium‡	3.88±10	3.10±0.99¶	2.47±0.65¶§
Basement Membrane‡	3.87±0.89	3.17±0.91¶	2.50±0.93¶§
Papillary ECM†	2.96±0.73	2.40±0.83¶	2.19±0.70¶
Reticular ECM ‡	2.98±0.83	2.50±0.8¶	2.09±0.47¶

Results are expressed as Mean ± SD. Statistically significant differencesANOVA:† P<0.01, ‡ P<0.001, ¶ Post hoc (Tukey) results significantly different from control subjects, § Post hoc results significantly different from no neuropathy group. ECM (Extracellular Matrix).

Correlation between skin AGEs expression and measures of neuropathy (Table 5)

There was a significant inverse correlation between skin AGEs expression in the epidermis and IENFD, CNFD, CNFL and SNCV. There was a significant inverse correlation between skin AGEs expression in the microvessels, endothelium and basement membrane with IENFD, CNFD, CNBD, CNFL, SA and SNCV. Papillary ECM AGEs expression correlated inversely with IENFD, CNFD and SA and reticular ECM expression correlated inversely with IENFD, CNFD, CNBD, CNFL and SA.

Table 5: Relationship between skin AGEs and measures of Diabetic Peripheral Neuropathy (DPN)

Skin structure	IENFD	CNFD	CNBD	CNFL	SA	SNCV
	r	r	r	r	r	r
	p	p	p	p	p	p
Epidermis	-0.634	-0.582	-0.209	-0.616	-0.205	-0.462
	0.000	0.000	0.119	0.000	0.149	0.000
Microvessels	-0.593	-0.546	-0.468	-0.666	-0.412	-0.531
	0.000	0.000	0.000	0.000	0.003	0.000
Endothelium	-0.561	-0.523	-0.609	-0.551	-0.402	-0.511
	0.000	0.000	0.000	0.000	0.003	0.000
Basement Membrane	-0.630	-0.434	-0.468	-0.527	-0.426	-0.532
	0.000	0.001	0.000	0.000	0.002	0.000
Papillary ECM	-0.467	-0.458	-0.161	-0.210	-0.408	-0.216
	0.000	0.000	0.231	0.117	0.003	0.116
Reticular ECM	-0.509	-0.442	-0.337	-0.504	-0.431	-0.168
	0.000	0.001	0.010	0.000	0.002	0.224

r- Pearson's correlation coefficient, significant correlations are in bold. ECM (Extracellular Matrix); IENFD (Intra-Epidermal Nerve Fibre Density); CNFD (Corneal Nerve Fibre Density); CNBD (Corneal Nerve Branch Density); CNFL (Corneal Nerve Fibre Length); SA (Sural Amplitude); SNCV (Sural Nerve Conduction Velocity)

Table 6: Relationship between skin RAGE and measures of Diabetic Peripheral Neuropathy (DPN)

Skin structure	IENFD	CNFD	CNBD	CNFL	SA	SNCV
	r	r	r	R	r	r
	p	p	p	P	p	p
Epidermis	-0.525	-0.591	-.0490	-0.560	-0.264	-0.512
	0.000	0.000	0.000	0.000	0.061	0.000
Microvessels	-0.568	-0.484	-0.517	-0.537	-0.474	-0.487
	0.000	0.000	0.000	0.000	0.000	0.000
Endothelium	-0.444	-0.547	-0.425	-0.528	-0.244	-0.523
	0.001	0.000	0.001	0.000	0.084	0.000
Basement Membrane	-0.504	-0.441	-0.267	-0.426	-.0494	-0.481
	0.000	0.001	0.044	0.001	0.000	0.000
Papillary ECM	-0.363	-0.425	-0.120	-0.140	-0.394	-0.217
	0.006	0.001	0.375	0.299	0.004	0.114
Reticular ECM	-0.408	-0.214	-0.020	-0.426	-0.186	-0.372
	0.002	0.121	0.882	0.001	0.190	0.006

r-Pearson's correlation coefficient, significant correlations are in bold. ECM (Extracellular Matrix); IENFD (Intra-Epidermal Nerve Fibre Density); CNFD (Corneal Nerve Fibre Density); CNBD (Corneal Nerve Branch Density); CNFL (Corneal Nerve Fibre Length); SA (Sural Amplitude); SNCV (Sural Nerve Conduction Velocity)

Table 7: Relationship between skin GLO-I and measures of Diabetic Peripheral Neuropathy (DPN)

Skin structure	**IENFD**	**CNFD**	**CNBD**	**CNFL**	**SA**	**SNCV**
	r	r	r	r	r	r
	p	p	p	p	p	p
Epidermis	0.464	0.482	0.252	0.503	0.408	0.491
	0.000	0.000	0.059	0.000	0.003	0.000
Microvessels	0.498	0.446	0.397	0.489	0.363	0.462
	0.000	0.000	0.002	0.000	0.009	0.000
Endothelium	0.442	0.464	0.356	0.502	0.311	0.456
	0.000	0.000	0.006	0.000	0.026	0.000
Basement Membrane	0.464	0.431	0.331	0.460	0.354	0.388
	0.000	0.001	0.012	0.000	0.011	0.003
Papillary ECM	0.228	0.238	0.119	0.351	0.145	0.382
	0.094	0.082	0.375	0.007	0.309	0.006
Reticular ECM	0.390	0.358	0.055	0.417	0.374	0.341
	0.003	0.007	0.684	0.001	0.007	0.015

r- Pearson's correlation coefficient, significant correlations are in bold. ECM (Extracellular Matrix); IENFD (Intra-Epidermal Nerve Fibre Density); CNFD (Corneal Nerve Fibre Density); CNBD (Corneal Nerve Branch Density); CNFL (Corneal Nerve Fibre Length); SA (Sural Amplitude); SNCV (Sural Nerve Conduction Velocity)

Correlation between skin RAGE expression and measures of neuropathy (Table 6)

There was a significant inverse correlation between skin RAGE expression in the epidermis and IENFD, CNFD, CBND, CNFL and SNCV. There was a significant inverse correlation between skin RAGE expression in the microvessels, endothelium and basement membrane with IENFD, CNFD, CNBD, CNFL, SA and SNCV. Papillary ECM RAGE expression correlated inversely with IENFD, CNFD and SA and reticular ECM expression correlated inversely with IENFD, CNFL and SNCV.

Correlation between skin GLO-I expression and measures of neuropathy (Table 7)

There was a significant direct correlation between skin GLO-I expression in the epidermis and IENFD, CNFD, CNFL, SA and SNCV. There was a significant direct correlation between skin GLO1 expression in the microvessels, endothelium and basement membrane with IENFD, CNFD, CNBD, CNFL, SA and SNCV. Papillary ECM GLO-I expression correlated with CNFL and SNCV and reticular ECM correlated inversely with IENFD, CNFD, CNFL, SA and SNCV.

DISCUSSION

This study is the first to report on the expression of the triplicate set of AGEs, RAGE and GLO-I in foot skin of type 1 diabetic patients and control subjects who had undergone detailed assessment of neuropathy. Skin AGEs expression was increased in patients with DPN in multiple skin structures, namely the epidermis, microvessels (endothelium and basement membrane) as well as the reticular ECM. Interestingly, skin AGEs expression was also moderately but significantly increased in patients without DPN

in the epidermis, endothelium and reticular ECM. Previous studies have shown increased expression of AGEs in skin collagen[20], nerves and blood vessels predominantly in animal models [21] compared to controls. Our findings therefore build on a robust link between AGEs and DPN in experimental diabetic neuropathy and indirect evidence from plasma levels of AGEs as well as skin autofluorescence[11,22]. In substudies of the Diabetes Control and Complications Trial (DCCT), AGEs were associated with DPN [23] and predicted the progression of microvascular complications, even after adjustment for HbA1c [8]. Glucospane, a major AGE has recently been proposed as a robust marker for diabetic microvascular complications including DPN [20]. Our findings suggest increased AGEs production as well as decreased detoxification in diabetic patients with DPN. Furthermore, we also show that this process is already operative in the early stages of neuropathy, in particular in relation to small fibre damage as evidenced by the correlation with IENFD and CCM abnormalities.

In combination with increased AGEs expression, we have also shown increased skin RAGE expression in patients without DPN and in particular those with DPN. In relation to the microvascular complications RAGE has been detected on epidermal nerves [19], peripheral nerves and their blood vessels [21], renal glomeruli and podocytes[24] and in the retina.[5] Therefore, our results provide translational support for previous reports showing increased RAGE expression in experimental DPN.[25] Moreover, in the same study RAGE knockout mice were protected against DPN. Recently, RAGE knockout diabetic mice exhibited enhanced post-injury nerve regeneration.[26] In the current study, AGEs and RAGE were co-localized in the same skin structures, which is in keeping with previous experimental reports demonstrating AGEs and RAGE co-localization.[21,27] The higher expression of RAGE suggests upregulation of the receptor in target structures, particularly in relation to the severity of DPN. Whether RAGE upregulation is a direct consequence of hyperglycemia, higher AGEs or other ligands, ROS, or a response to combined factors remains to be identified.

Emerging reports link the AGEs-detoxifying enzyme GLO-I, with diabetic microvascular complications, including DPN.[28] We show lower skin GLO-I expression in patients with DPN which was immunolocalized to the same structures where AGEs and RAGE expression were increased. Thus our data are consistent with recent studies which have related reduced GLO-I activity to DPN[4] including painful DPN.[10] Increased expression of GLO-I in the DRG of diabetic mice conveyed protection against small fibreloss.[4] Indeed GLO-I overexpression has been shown to reduce AGEs, RAGE, oxidative stress markers as well as increase mitochondrial oxidative phosphorylation[7] while under-expression generates the opposite effects.[28,29] GLO-I overexpressing mice were protected against IENFD loss.[4] This could also partially explain increased AGE expression as a consequence of reduced detoxification.

The combined data showing altered AGE/RAGE/GLO-I expression in the same skin structures and the significant correlations with measures of both large and small fibre neuropathy leads us to speculate that these three factors play a key mechanistic role in DPN. We also show significant correlations between the expression of AGE/RAGE/GLO-I in foot skin and both intraepidermal and corneal nerve fibre loss, which is comparable to or better than neurophysiology, adding to the data supporting the notion that CCM is a robust surrogate marker of DPN.

We acknowledge that due to the cross sectional nature of this study we can only provide association and not a cause effect relationship between AGEs/RAGE/GLO-I and DPN. Another limitation is the semi-quantitative nature of the scoring system, albeit we undertook rigorous blinded assessment with excellent reproducibility. Of course alternative techniques such as liquid chromatography/ mass spectrometry (LC/MS) could be used to interrogate the content of the biopsy material, however, this does not allow anatomical localization of AGEs/RAGE/GLO-I expression.

CONCLUSION

In conclusion, we report for the first time higher expression of AGEs/RAGE and lower expression of GLO-I in the foot skin of type 1 diabetic patients with DPN. Moreover, skin AGE/RAGE/GLO-I correlated significantly with small and large fibre damage. These findings suggest a potential role for AGE/RAGE/GLO-I as a marker and therapeutic target for DPN. Further clinical and experimental studies are warranted to consolidate the evidence provided in this study.

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