ORIGINAL

Longitudinal analysis of low-molecular weight fluorophores in type 1 diabetes mellitus

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Abstract : *Objectives :* Circulating low molecular weight (<10 kDa) fluorophores (LMW-F) measured by non-specific fluorescence spectroscopy may detect small advanced glycation end-products (AGEs) not recognized by other assays. This longitudinal study assessed correlates of LMW-F and predictive power of LMW-F levels for vascular health in Type 1 diabetes (T1DM) patients. *Methods :* Fasting patients with T1DM (n=37) were studied twice at intervals of 12-60 months (mean±SD, 33±15 months). LMW-F levels were also measured once in 112 healthy control subjects. *Results :* Relative to controls, LMW-F levels were higher in diabetic subjects at initial and final time points (mean±SD), 5.4±1.9 AU/ml and 4.5±1.8 AU/ml respectively vs. 3.8±2.1 AU/ml ; p=0.0001 and p=0.06). Baseline LMW-F levels predicted subsequent hs-CRP and oxLDL/LDL values. LMW-F levels decreased significantly over time in diabetes (5.4 ± 1.9 vs. 4.5 ± 1.8 AU/ml ; p=0.02). Rises in LMW-F levels in individual diabetic subjects correlated significantly with worsening renal function (BUN), glycemia (HbA1c) and with vascular dysfunction (systemic vascular resistance). *Conclusions :* LMW-F levels predict levels of inflammation and oxidation in T1DM. Changes in LMW-F levels in T1DM reflect variations in glycemia and renal function. Biochemical characterization of LMW-F would facilitate understanding of the potential utility of LMW-F as a therapeutic target. J. Med. Invest. 55 : 29-36, February, 2008

Keywords **:** *advanced glycation end products, low molecular weight fluorophores, type 1 diabetes, renal function*

BACKGROUND

Advanced glycation-end products (AGEs) are a heterogeneous group of fluorescent and non-fluorescent compounds implicated in the pathogenesis of the micro- and macro-vascular complications of diabetes, and in renal disease and atherosclerosis in the non-diabetic population. AGEs can form in short- and long-lived proteins such as albumin, immunoglobulins, lipoproteins, skin collagen, ocular, renal and vascular tissues (1). AGEs may be both a cause and effect of inflammation, oxidative stress and dyslipoproteinemia (2, 3). Recent animal and human studies show benefit of AGE-inhibitors and AGE-breakers in ameliorating renal and cardiovascular damage (4, 5). Therefore AGE levels may have a role in the identification of Type 1 diabetic patients at high risk for complications and also provide a tool for moni-

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toring therapeutic interventions.

Circulating low molecular weight (<10 kDa) AGEs (LMW-AGEs or AGE-peptides) can be measured by relatively simple non-specific fluorescence spectroscopy, which may otherwise only be detected by sophisticated and laborious assays (6). Given the current lack of biochemical characterization of these compounds, an alternative less specific name is low molecular weight fluorophores (LMW-F). Relative to healthy subjects, serum LMW-F levels have been reported to be elevated in diabetes (7-9), in (diabetic and non-diabetic) renal failure (10) and to be lowered by hemodialysis (11). In a large cross-sectional study of Type 2 diabetes patients LMW-AGEs were inversely related to renal function and to hemoglobin levels (independent of renal function) (8). Using a fluorescence spectroscopy assay with potential clinical utility, we previously reported that plasma LMW-F levels were elevated in patients with Type 1 diabetes (T1DM) and vascular complications, and that levels did not differ between complication-free T1DM patients and healthy non-diabetic subjects (12). In this cross-sectional study LMW-F levels were correlated with renal function, but not with age, diabetes duration, body habitus, smoking, glycemia, lipid levels or arterial elasticity (12).

We now present the behavior over time of LMW-F levels in T1DM patients, and their relationships with renal and vascular health, and measures of glycemia, inflammation and oxidative damage.

METHODS

Subject characterization : The study was approved by the St Vincent's Hospital Human Research Ethics Committee and each subject gave written informed consent. Thirty-seven patients with T1DM (13 with and 24 without complications) with (mean ± SD) diabetes duration of 21 ± 13 years from the St. Vincent's diabetes clinics volunteered for the study. Patients attended at least twice at an interval of 12-60 months ; long enough for changes in glycemia to be reflected by the concurrent HbA1c level. We compared results with data obtained (on one occasion) from 112 healthy control subjects. These control subjects did not have clinically evident macrovascular disease, hypertension, diabetes, renal disease or dyslipidemia requiring drug treatment. They were not taking any medications (other than the oral contraceptive pill). No subject was taking antioxidant vitamin supplements. Smokers were not excluded from the study.

Blood pressure and arterial elasticity were measured in fasted supine subjects by pulse-wave analysis (HDI Inc, Egan, MN) as previously described (13), including measures of vascular elasticity and systemic vascular resistance (SVR). Venous blood was taken after an overnight fast and a single void urine sample collected. Routine clinical chemistry was performed by the St.Vincent's Hospital Pathology Department. For research analyses plasma (1 mM EDTA) and serum were prepared (2000 g, 15 min, 4°C) and stored (-80°C) until LMW-F, cystatin C and measures of inflammation (high sensitivity C-reactive protein, (hs-CRP) and ESR) and oxidative damage (Oxidized LDL, (OxLDL)) were assayed. Levels of LMW-fluorophores in (first-thaw) plasma were determined in duplicate by fluorescence spectroscopy as described (8), with intra- and inter-assay coefficients of variation (CV) of 5.2% and 7.3% respectively. In preliminary studies there were no statistically significant differences between LMW-F in serum and EDTA plasma from the same subjects, and LMW-fluorescence was stable through two, but not three, freeze-thaw cycles. For the analysis of yearly changes a subset of subjects (n=37) with one year or more between-visit intervals were studied (see below). Creatinine clearance was calculated using the Cockroft-Gault formula (14, 15). Normal range is defined as 90 - 150 ml/min. Cystatin C was measured in serum by nephelometry using Dade Boehring, Germany instrument. The reference interval is 0.53 - 0.95 mg/l. Intra- and interassay CV's were <3.3%. Oxidative stress : Oxidized LDL was measured in serum by ELISA (Mercodia AB, Uppsala, Sweden). All intra- and inter-assay CVs were <6.3% and <12.0% respectively.

To control for the potential influence of the different time intervals at which patients were studied and reduce variance, all changes were normalized, using the formula below, as the percentage change per annum, with initial visit values being taken as 100%. Thus, a positive or negative percent change at last visit reflects both change in magnitude and direction.

$$Percent\ change = 100\frac{(\sqrt[Years]{Last\ visit} - \sqrt[Years]{First\ visit})}{\sqrt[Years]{First\ visit}}$$

Years = time between 1st and last assessment in years

Data were analyzed using Statistica for Windows (StatSoft, Inc. (2007) Tulsa, OK. STATISTICA (data analysis software system), version 8.0 www.statsoft.

com). Non-normally distributed variables : hsCRP, triglycerides (TG), urinary albumin and albumin to creatinine ratio (ACR) values, were transformed logarithmically. Differences between diabetic and control subjects were analyzed using Student-*t*-test and between control subjects and diabetic patients subdivided into those with and without complications (CX[+] and CX[-] respectively) - using one way analysis of variance (ANOVA). For correlation analyses Pearson's correlation coefficient was calculated and multiple regression analysis done. Statistical significance was taken at $p<0.05$.

RESULTS

Subject characteristics are reported in Table 1. Thirty-seven subjects (20 women and 17 men, 13 with and 24 without diabetes complications) with T1DM studied at an interval of at least 1 year were evaluated. Mean ± SD follow-up time was 33 ± 15 months, with a range of 12-60 months. Data are given for controls (at baseline) and for all diabetic subjects at their first and final visit. There was no statistically significant change in any clinical or biochemical parameter analyzed over the study duration.

Longitudinal measures of LMW-F in diabetes and its complications. The baseline characteristics of the group studied longitudinally did not differ from that of the whole larger diabetic group (n=148) in the previously reported cross-sectional study (12), and also did not differ significantly from those diabetic subjects not restudied (data not shown). Relative to healthy controls, plasma LMW-F were increased in patients with T1DM at the initial but not at the final visit (5.4 ± 1.9 AU/ml at the initial visit and 4.5 ± 1.8 AU/ml at the final visit vs. 3.8 ± 2.1 AU/ml ; p= 0.0001 and p=0.06 respectively). Levels of LMW-F were significantly higher at the beginning of the study relative to those at the final visit (5.4 ± 1.9 AU/ml vs. 4.5 ± 1.8 AU/ml, p=0.02). In 19 subjects in whom LMW-F levels were measured more than twice, mean LMW-F levels between first and final visit correlated with values obtained at the last visit (r=0.79 ; $p<0.00001$). However, when data from the first visit were compared with mean levels obtained at all other visits (including the last one) the statistical significance disappeared (5.4 ± 1.9 AU/ml vs. 4.9 ± 1.8 AU/ml, p=0.24).

Table 1. Clinical characteristics of Control and Type 1 diabetic (T1DM) subjects. Data are mean ± SEM.

	Control subjects	Type 1 DM First visit	Type 1 DM Last visit
n (M/F)	112 (52/60)	37 (17/20)	37 (17/20)
Age (years)	37 ± 12	34 ± 14	37 ± 14
BMI (kg/m^2)	25.0 ± 4.1	25.6 ± 4.4	26.0 ± 4.9
SBP (mmHg)	120 ± 13	128 ± 13*	128 ± 15*
DBP (mmHg)	68 ± 10	73 ± 10*	73 ± 10*
MAP (mmHg)	87 ± 12	94 ± 11*	93 ± 12*
Pulse pressure (mmHg)	52 ± 8	57 ± 14*	55 ± 10
LAE (ml/mmHg × 10)	17.6 ± 4.9	16.4 ± 4.6	16.9 ± 6.0
SAE (ml/mmHg × 100)	8.3 ± 3.4	6.1 ± 2.7*	6.9 ± 3.5*
SVR (dyne × sec × cm^{-5})	1246 ± 230	1336± 268	1296± 270
Fasting serum glucose (mmol/l)	4.9 ± 0.5	11.7 ± 4.6*	11.5 ± 5.6*
HbA1c (%)	5.1 ± 0.3	8.1 ± 1.5*	8.1 ± 1.9*
Total Cholesterol (mmol/l)	5.1 ± 1.0	5.0 ± 1.0	4.6 ± 1.0*
Triglycerides (mmol/l)	1.11 ± 0.68	1.16 ± 0.82	0.97 ± 0.56
HDL-Cholesterol (mmol/l)	1.52 ± 0.38	1.49 ± 0.53	1.36 ± 0.33
OxLDL/LDL ratio (U/mmol)	33.2 ± 12.3	35.5 ± 6.9	33.8 ± 13.9
Hemoglobin (g/l)	140 ± 16	137 ± 20	137 ± 15
ESR (mm/h)	14.3 ± 47.9	9.6 ± 9.2	12.4 ± 11.3
Urinary Albumin/Creatinine (mg/mmol)	0.8 ± 1.2	9.9 ± 21.7*	19.8 ± 69.7*
Serum BUN (mg/l)	4.9 ± 1.2	6.2 ± 2.3*	7.2 ± 3.9*
Cystatin C (mg/l)	0.78 ± 0.10	0.89 ± 0.36	1.03 ± 0.58*
GFR (ml/s)	1.75 ± 0.54	1.71 ± 0.53	1.95 ± 1.92
Hs-CRP (mg/l)	1.7 ± 2.2	2.4 ± 2.5	2.7 ± 2.6*
LMW-F (AU/ml)	3.8 ± 2.1	5.4 ± 1.9*	4.5 ± 1.8*
Current smokers (n/%)	15/13	7/19	10/27*
Lipid lowering drugs (n/%)	0	22	30

*) $p<0.04$ vs. Controls

There was no statistically significant difference between LMW-F levels between women and men at the beginning (mean ± SD : females 5.9 ± 2.2 AU/ml vs. males 4.8 ± 1.3 AU/ml ; p=0.08) or end of the study (mean ± SD : females 5.0 ± 2.0 AU/ml vs. males 3.9 ± 1.3 AU/ml ; p=0.07). However in men LMW-F levels decreased significantly during the study (4.8 ± 1.3 AU/ml at the initial visit vs. 3.9 ± 1.3 AU/ml at the final visit ; p=0.03) whereas levels in women did not change significantly (5.9 ± 2.2 AU/ml at study beginning vs. 5.0 ± 2.0 AU/ml at the end ; p=0.18). There was no statistically significant difference between LMW-F levels in subjects stratified by diabetes complication status. In the complication-free group baseline LMW-F levels were 5.6 ± 2.0 AU/ml and 4.5 ± 1.3 AU/ml at the end of the study. In the diabetic group with complications baseline LMW-F levels were 5.1 ± 1.9 AU/ml and 4.6 ± 2.5 AU/ml at the end of the study. This change did not reach statistical significance in either group. The time intervals at which subjects were studied did not correlate with differences between LMW-F levels.

In univariate analysis of cross-sectional data, initial LMW-F levels correlated with baseline hs-CRP (r=0.51 ; p=0.005) values, with ACR (r=0.48 ; p=0.03), and with calculated GFR levels (r=0.50 ; p=0.007), as previously reported In the larger diabetes group (12). Baseline LMW-F levels predicted final visit hs-CRP (r=0.57 ; p=0.002) and OxLDL/LDL ratio (r=0.69 ; p=0.001) (Figure 1). In this respect they were better predictors than HbA1c and TG levels as neither baseline level correlated with final visit hs-CRP or OxLDL/LDL ratio. Final LMW-F correlated with : final mean arterial pressure (also systolic and diastolic blood pressure) (r=0.52 ; p=0.002), systemic vascular resistance (r=0.38 ; p=0.03) serum creatinine (r=0.45 ; p=0.006), BUN (r=0.48 ; p=0.004), cystatin C (r=0.62 ; p=0.002), and urinary ACR (r=0.69 ; p=0.0001), serum billirubin (an antioxidant) (r=-0.51 ; p=0.04) and ESR (r=0.66 ; p=0.0001). Serum BUN and ACR values at baseline correlated significantly with end-point LMW-F (r=0.38 ; p=0.03 and r=0.46 ; p=0.03 respectively). There was a significant negative correlation between initial hemoglobin levels and final LMW-F levels (r=-0.54 ; p=0.009).

In multivariate analysis after adjustment for gender, age, diabetes complications, glycemic control, renal function (GFR), lipids (TG, HDL-C, non-HDL-C) and inflammation the strongest predictors of LMW-F levels at the initial visit were gender (p=0.006), renal function (p=0.002) and TG levels (p=0.03). In the same model applied at the final visit the only statistically significant predictor of LMW-F levels was renal function (p=0.002). Similarly when the model was applied to combined data from baseline and final visits the significant predictors of LMW-F levels were diabetes complication status (p=0.04) and renal function (ACR) (p=0.0002).

In control subjects significant correlations between LMW-F and albuminuria (but not ACR) (r=0.22 ; p=0.03) and TG (r=0.21 ; p=0.03) were noted. There was no significant difference between LMW-F levels in males and females in the control group (3.9 ± 0.4 vs. 3.8 ± 0.2 AU/ml ; p >0.05 respectively). In multivariate analysis after the adjustment for gender, age, glycemia, renal function, lipids and inflammation the only significant LMW-F predictor in healthy subjects was TG level (p=0.007).

In T1DM subjects, the annual percentage change in LMW-F between visits correlated with the yearly change in BMI (r=0.43 ; p=0.02), systolic blood pressure (r=0.54 ; p=0.004), pulse pressure (r=0.54 ; p=0.004), systemic vascular resistance (SVR) (r=0.39 ; p=0.04), BUN (r=0.38 ; p=0.03), HbA1c (r=0.38 ; p=

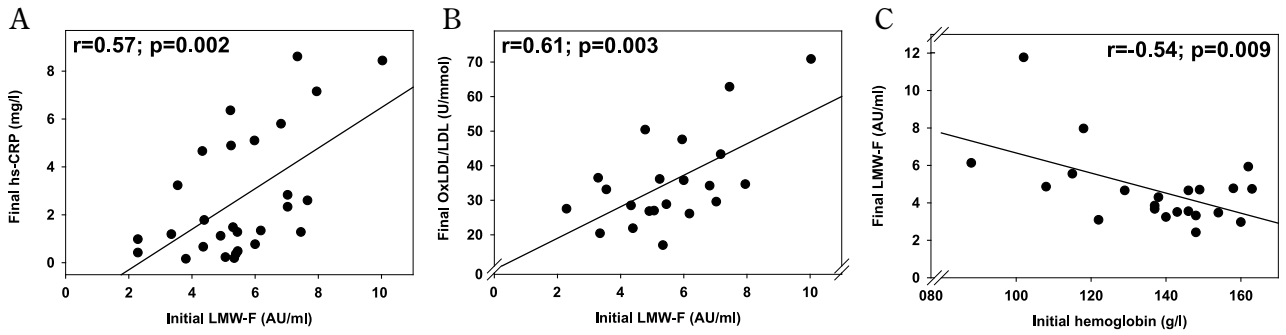

Figure 1. Baseline LMW-F levels correlated significantly with inflammation (hs-CRP) (panel A) and OxLDL/LDL ratio (panel B) at the end of the study. Baseline hemoglobin level correlated significantly with LMW-F levels at the end of the study (panel C).

0.04) and hemoglobin (r=0.65 ; p=0.005) (Figure 2). Mean HbA1c levels measured during the study correlated with SVR yearly percentage changes (r= 0.44 ; p=0.03). Similarly, mean TG levels correlated with hs-CRP yearly percentage changes (r=0.56 ; p= 0.009). Annual percentage changes in TG levels correlated with the yearly changes in hs-CRP levels (r= 0.51 ; p=0.02) and the OxLDL/LDL ratio (r=-0.67 ; p=0.02).

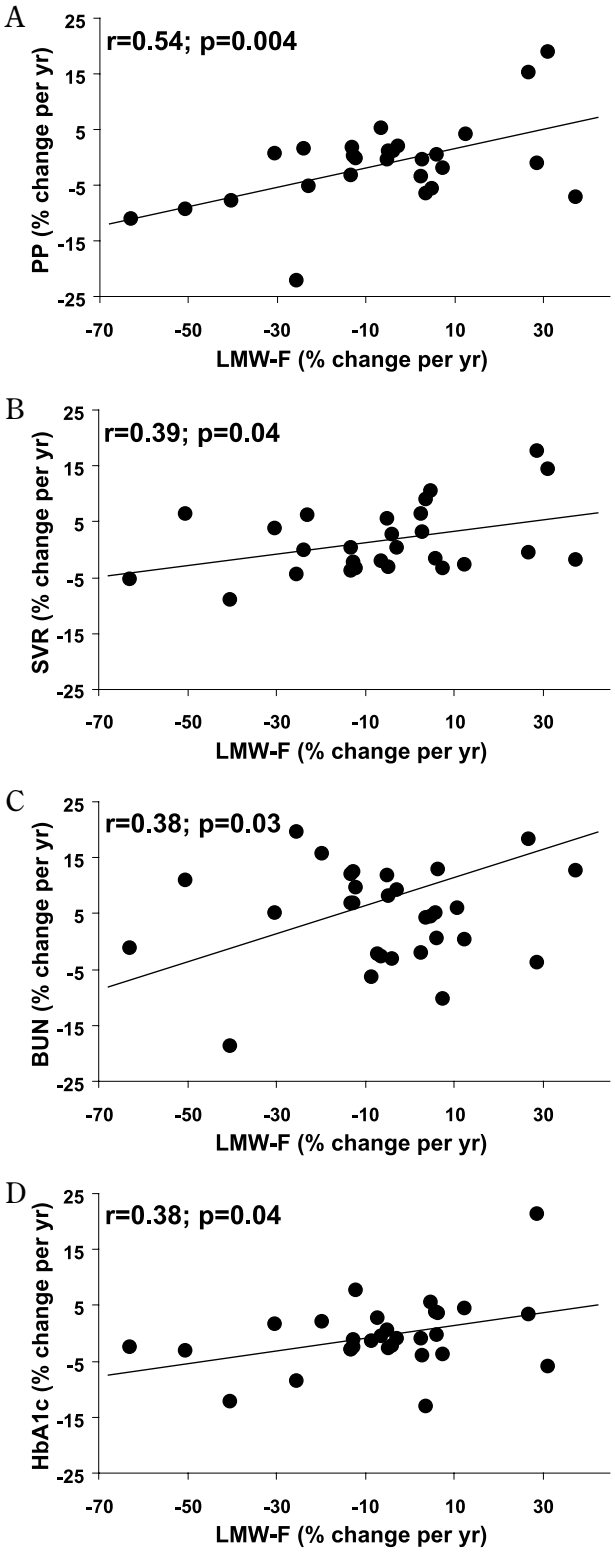

Figure 2. Percentage change of LMW-F between first and last visit normalized per year correlated significantly with values of the yearly change of pulse pressure (panel A), systemic vascular resistance (panel B), BUN (panel C) and HbA1c (panel D).

DISCUSSION

We report results of a longitudinal study of plasma LMW-F levels measured by a relatively simple technique in Type 1 diabetic patients. Relative to healthy subjects LMW-F levels were increased in diabetes regardless of complication status. There was a significant decrease in LMW-F levels over the study period in the diabetic group as a whole, however not as a result of amelioration of renal function, as serum creatinine, BUN, GFR and ACR did not change significantly. Also, when individual changes were expressed as percentage change per annum they correlated with changes in BMI, HbA1c, vascular health, as measured by pulse-wave analysis, BUN and hemoglobin. Baseline LMW-F levels correlated with inflammation and renal function parameters and were predictive of oxidation stress and inflammation. In multivariate analysis LMW-F levels were determined by renal function and lipids.

In a cross-sectional analysis LMW-F were related to inflammation (hs-CRP), renal function (ACR, calculated GFR, serum creatinine, cystatin C), vascular status (blood pressure, systemic vascular resistance). No associations between age, diabetes duration, body habitus, HbA1c or oxidative stress and LMW-F levels were noted. These findings are similar to large cross-sectional studies of subjects with Type 2 diabetes (8) and a mixed group of subjects with Type 1 and Type 2 diabetes, in which levels in T1DM (20% of the group) were not reported separately (7). In both studies levels of LMW-AGEs (as these compounds are referred to in these reports) were significantly higher in patients with diabetes than in non-diabetic subjects. Limited data are available regarding LMW-F levels in T1DM. Using fluorescence spectroscopy Galler, *et al.* found increased levels of fluorescent AGEs in a pediatric group with T1DM, which were positively correlated with HbA_{1c} and TG levels (9). We previously reported a cross-sectional analysis of 148 T1DM patients in which LMW-F levels correlated with serum creatinine and BUN, and creatinine clearance, and these relationships were statistically significant even within the normal range of renal function. In addition, LMW-F

levels correlated with urinary ACR. There were no statistically significant relationships between LMW-F and diabetes duration, age or HbA1c in this cross-sectional study (12), as in this present study. Here in a longitudinal study we showed a correlation between annual percentage changes of HbA1c and LMW-F levels. In multivariate analysis TG were the third strongest predictor (after renal function and gender) of LMW-F levels. Blood samples were fasting, which would reduce the potential influence of dietary AGEs (16). The finding of a significant association between LMW-F and lipids in our study supports the notion that AGEs can form within and be derived from lipoproteins - Advanced Lipoxidation End products (ALEs) (3, 17).

T1DM with microvascular complications was associated with higher LMW-F (in our earlier larger cross-sectional study (12), and in keeping, in this current study renal function (expressed as GFR or ACR) was the strongest predictor of LMW-F levels. Our results support previous research that LMW-F levels are related to renal function (8, 12). In addition, studies by others investigating low- and high-molecular weight AGEs demonstrate elevated levels in renal failure (10, 18) with a reduction by high-flux hemodialysis (19, 20). It is uncertain if the elevated level of LMW-F or other AGEs in renal disease relates to impaired removal, increased production, or both. ACE inhibitors, a common treatment for increased urinary albumin loss and for hypertension in diabetes can lower AGEs (21). However in this present study and in our earlier larger cross-sectional series, LMW-F levels did not differ between diabetic subjects taking ACE-inhibitors or angiotensin receptor blocking agents and those not on these medications (data not shown). We found a strong negative correlation between initial hemoglobin levels and final LMW-F. A similar observation was previously reported by Thomas, *et al.* (8). They reported that anemic patients with T2DM had higher levels of LMW-F than non-anemic T2DM patients. In our study no patients were anemic ; however we did find significant correlations between hemoglobin and LMW-F levels, and also between the annual changes of both measures. This may reflect a more subtle effect of renal dysfunction on hemoglobin levels. It may also reflect that reduced tissue oxygenation contributes to AGE formation, or it may reflect increased oxidative stress as the result of disturbed protein metabolism or inflammation, or both.

In this present study age and diabetes duration were not related to LMW-F levels. In contrast Sharp, *et al.* found that age correlated positively with LMW-AGEs levels in both their older control and (predominantly T2DM) diabetic groups (7). This may be related to age-related renal function decline. A correlation between age, diabetes duration and fluorescent AGEs was observed in children and adolescents with T1DM (9). By dividing subjects into two age subgroups (9-13 and 13-16 years) the authors showed a 15% increase in LMW-AGE levels. This may reflect a pubertal effect, and the relevance of this finding to our non-pediatric population is unclear.

Circulating AGE levels have been correlated with endothelial dysfunction measured by flow-mediated dilatation (FMD) (22). In our studies final circulating LMW-F levels were related to measures of vascular health (mean arterial pressure, systolic and diastolic blood pressure and SVR, but no such correlations were observed at the initial visit. Changes in LMW-F levels, expressed as percentage per year, correlated with similarly expressed changes in systolic blood pressure, pulse pressure and SVR. In this regard LMW-F was a comparable vascular health predictor to HbA1c.

AGE formation may be promoted by inflammation, and conversely AGEs can induce inflammation, including vascular endothelial cell adhesion molecule expression (23, 24). In keeping, we noted significant correlations between baseline LMW-F and ESR and CRP levels. We also noted a correlation between mean and yearly percent changes of TG levels with percent changes per year of hs-CRP levels. The strength of these correlations was comparable with that between LMW-F and hs-CRP but the statistical significance in each case was lower than that between LMW-F and hs-CRP. Similarly AGE formation may induce and be promoted by oxidative stress (17, 25) and we identified a relationship between initial LMW-F and final OxLDL/LDL ratio. The changes in the OxLDL/LDL ratio also correlated significantly with changes in TG levels, but the statistical strength of this correlation was lower than that between LMW-F and the OxLDL/LDL ratio.

In conclusion, our results suggest that levels of LMW-F rise early with a decline in renal function, at a time when an effective therapeutic intervention could still preserve renal, retinal, and macrovascular health. Changes in LMW-F levels follow changes in glycemic control and vascular health. Additionally, LMW-F levels can predict levels of inflammation and oxidative stress. Apart from renal function,

inflammation and lipids levels are the strongest predictors of LMW-F levels. The exact chemical moieties measured by this non-specific fluorescence-based assay, the relationship between these products and well-defined AGEs and their biological sources *in vivo* are not fully elucidated, but studies are in progress (26). In addition to better biochemical characterization of the LMW-fluorophores detected by this potentially clinically applicable assay, longitudinal studies relating these fluorophores to tissue fluorescence, vascular complications and mortality are warranted. Intervention studies, including evaluation of effects of AGE inhibitors, AGE breakers, antioxidants, and various renal replacement regimens are also merited.

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