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The predictive role of markers of Inflammation and endothelial dysfunction on the course of diabetic retinopathy in Type 1 Diabetes

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Abstract

Aims—This study was undertaken to determine whether levels of inflammation and endothelial dysfunction biomarkers in serum samples collected at baseline in the Diabetes Control and Complications Trial (DCCT) cohort could predict the development of retinopathy.

Methods—Levels of clotting/fibrinolysis, inflammation and endothelial dysfunction biomarkers were measured in 1391 subjects with type 1 diabetes to determine whether their levels predicted increased risk to develop or accelerated progression of retinopathy during 16 years of follow-up.

Results—Using regression models adjusted for DCCT treatment group, duration of diabetes, baseline retinopathy scores, HbA1c and albumin excretion rate, the baseline levels of sE-selectin and PAI-1 (active) were significantly associated with increased risk of a 3-step progression in retinopathy score in the Primary Prevention Cohort (PPC). After adjusting for additional covariates (e.g., ACE/ARB and statin therapy), this association persisted. Levels of active and total PAI-1 in the same group were also significantly associated, after similar adjustments, with the time to progress to severe non-proliferative retinopathy during the follow-up period (54% and

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29%, respectively of increased risk). No associations were observed in the Secondary Intervention Cohort for any of the outcomes.

Conclusions—High levels of sE-selectin and PAI-1 are associated with the development of retinopathy in patients with uncomplicated type 1 diabetes.

Keywords

Type 1 diabetes; diabetes complications; retinopathy; biomarkers; E-selectin; Plasminogen activator inhibitor type 1

INTRODUCTION

Diabetic retinopathy (DR), a frequent microvascular complication of diabetes, is the leading cause of preventable blindness in working-age adults (Mohamed et al., 2007). Inflammation and endothelial dysfunction have been considered as contributing factors to the development of diabetic retinopathy and other microvascular complications in patients with diabetes (Cheung et al., 2010; Goldberg, 2009; Adamis, 2002; Joussen et al., 2004). Several groups have reported an association between high levels of pro-inflammatory cytokines and the development of diabetic retinopathy. Serum levels of soluble TNF receptors 1 and 2 have been reported to correlate with the severity of diabetic retinopathy in Hispanics (Kuo et al., 2012). In children with type 1 diabetes mellitus (DM), those with non-proliferative diabetic retinopathy (NPDR) had significantly higher circulating TNF levels than those without retinopathy (Mysliwska, et al., 2007). Another study performed in type 1 diabetes mellitus also showed a significant association between serum TNF levels and proliferative diabetic retinopathy (PDR) (Gustavsson et al., 2008). This observation has been confirmed and expanded to vascular endothelial growth factor (VEGF) and IL-6 by Mysliwiec et al. (2007). Therefore, while some studies strongly suggest that inflammation markers predict the development of diabetic retinopathy, other groups have failed to find significant associations between the levels of inflammation markers and the development of diabetic nephropathy (Altinova et al., 2005; Klein, Knudson, Tsai et al., 2009; Nguyen et al. 2009).

Nowak et al. (2008) showed correlations between the levels of soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin) levels and the presence of retinopathy, thus suggesting that endothelial dysfunction, assessed by increased expression of cellular adhesion molecules, is linked to the presence of diabetic retinopathy. Similar findings have been obtained by Soedamah-Muthu et al. (2006) who reported that sVCAM-1 and sE-selectin levels are positively associated with retinopathy thus suggesting that adhesion molecules are important in the pathogenesis of vascular complications in Type 1 diabetes.

Hemostatic abnormalities have also been found to be associated with PDR. The concentrations of tissue-type plasminogen activator (t-PA) and of its fast-acting inhibitor PAI-1 were measured in the plasma collected from eight patients with type 1 diabetes and PDR, and from eight patients with type 1 diabetes and background or no retinopathy. The ratio of t-PA to PAI-1 in plasma was significantly higher in patients with proliferative retinopathy than in those without (Simpson et al., 1999).

In summary, the published data, obtained mainly in studies carried out with small cohorts, reflects simple associations between the presence of retinopathy and high levels of inflammatory markers and endothelial cell dysfunction markers, although with some inconsistencies. Therefore, in order to obtain a more definitive conclusion about the importance of these biomarkers in the development of retinopathy, it was necessary to carry out this investigation in a larger cohort. The measurement of these biomarkers in plasma/serum samples collected from 1391 type 1 diabetes patients at the time of enrollment on the Diabetes Control and Complications Trial (DCCT) when the patients had either mild or moderate retinopathy or albuminuria or were completely free of complications and the fact that these patients have been carefully followed for the development/progression of complications for more than 20 years provided us not only a critical mass of patients but the ability of determining which measurements were able to predict the development / progression of retinopathy as well of identifying biomarkers able to identify patients at high risk of prematurely developing severe retinopathy and therefore facilitate early intervention to prevent the disease.

RESEARCH DESIGN AND METHODS

Our study included 1391 subjects from the DCCT/EDIC cohort who had biomarkers measured in samples obtained at entry into the DCCT study as well as Early Treatment Diabetic Retinopathy Study (ETDRS) scores performed throughout the DCCT/EDIC study. This study was performed to test the hypothesis that DCCT baseline values of markers of inflammation and endothelial dysfunction would be associated with the subsequent development and progression of diabetic retinopathy. The DCCT was a randomized controlled trial of 1,441 subjects who were 13–39 years of age and had Type 1 diabetes for 1–15 years at study entry (DCCT, 1993). Subjects were randomized to intensive or conventional diabetes therapy. The DCCT included two cohorts, subjects in the primary prevention cohort were retinopathy-free (ETDRS=1), had diabetes duration between 1–5 years, and normal albumin excretion rates (AER<40 mg/24hrs). The subjects in the secondary intervention cohort had mild to moderate non-proliferative diabetic retinopathy (ETDRS=2–9), had diabetes for 1–15 years, and AER ≤ 200 mg/24 hrs. At DCCT Baseline (1983–1989), none of the patients had hypertension (defined as 140 (systolic) and/or 90 mmHg (diastolic) or dyslipidemia (defined as total cholesterol >200 and/or LDL >160 mg/dl). Subjects were followed for an average of 6.5 years before the study was terminated early, in 1993, because of its obvious positive impact on microvascular complications. In 1994, approximately 95% of the DCCT participants was enrolled into the Epidemiology of Diabetes Interventions and Complications (EDIC) study. The goal of EDIC was to assess the development of macrovascular disease in Type 1 diabetes and the progression of microvascular disease (EDIC, 1999). During EDIC, subjects were under the care of their personal physicians and encouraged to practice intensive insulin therapy. Each EDIC participant underwent a standardized annual history, physical examination, resting ECG, and routine laboratory analysis that included HbA_{1c} levels (DCCT, 1987; EDIC, 1999). Lipid profiles and 4-hour urine collections to measure albumin excretion rates (AER) were obtained in alternate years (DCCT, 1993; EDIC 1999) and retinopathy assessment was performed yearly in 25% of the cohort and in the whole cohort at years 4 and 10 of EDIC.

Samples

Fasting serum samples obtained during DCCT/EDIC were sent to the DCCT/EDIC central laboratory for standard lipid analysis. Aliquots of these samples were archived for future research purposes. In 1999–2000, as part of Medical University of South Carolina Program Project Grant funded by the NIH/JDF, serum samples collected during DCCT were provided by the DCCT/EDIC Coordinating Center and NIDDK to complement the serum samples collected during EDIC. The serum samples had been stored at -70°C and refreezing effects were minimized by preparing aliquots of the serum when thawed for the first time and using a new frozen aliquot for each new test performed. The IRB at Medical University of South Carolina and all participating DCCT/EDIC centers approved the sample collection procedures. Written informed consent was obtained from all participants.

Assessment of Diabetic Retinopathy

During DCCT, diabetic retinopathy was assessed every six months in all subjects. During EDIC, retinopathy was assessed in approximately one quarter of the cohort during each follow-up year and the entire cohort was assessed at EDIC years 4 and 10. Severity of retinopathy was determined using stereoscopic seven-field fundus photographs and graded according to the ETDRS protocol using methods standardized by the DCCT/EDIC group (DCCT, 1993; ETDRS, 1991). This study used the abbreviated final version of the ETDRS scale of diabetic retinopathy severity (ETDRS, 1991), which provides a composite score on a scale of 1 to 23 for both eyes on each subject. Retinopathy severity levels were defined as follows: ETDRS score 1–3=None-minimal retinopathy; ETDRS score 4–9=mild-moderate non-proliferative retinopathy; ETDRS score 10–23=severe pre-proliferative and proliferative retinopathy (SNPDR) (Lyons et al, 2004). Two pre-defined primary retinal outcomes of interest were established for this study: 1) time to significant progression of diabetic retinopathy beyond what was measured at DCCT baseline (defined as a three-step or greater increase in ETDRS from that obtained at DCCT baseline), and 2) time to develop severe non-proliferative diabetic retinopathy (SNPDR; ETDRS score ≥ 10). Patients having any scatter laser photocoagulation performed during the study were included in the groups determined to have progression of retinopathy. Measurements were collected from DCCT baseline through EDIC study year 10.

Measurement of Markers of Inflammation

Serum levels of C-reactive Protein (CRP), PAI-1 total and PAI active, sICAM-1, sVCAM-1, sE-Selectin, Interleukin 6 (IL-6), and of soluble tumor necrosis factor receptor 1 and 2 (sTNFR-1 and sTNFR-2) were assayed using the Signature Plus Protein Array imaging and Analysis System (Aushon BioSystems) using ArrayVision™ software for data analysis. Inter-assay coefficients of variation were respectively 2.6% for CRP, 3.4% for PAI- total, 5.9% for PAI-1 active, 3% for sICAM-1, 4% for sVCAM-1, 4% for sE-selectin, 7.5% for IL-6, 5.9% for sTNFR-1 and 2.7% for sTNFR-2. Plasma concentrations of fibrinogen were determined using a commercially available assay (Fibrinogen SPQ Test System, Diasorin Inc., Stillwater MN).

Other methods

At the baseline DCCT examination each participant completed a physical examination, medical history, electrocardiogram and laboratory testing including serum creatinine, lipid profile and hemoglobin A1c (HbA_{1c}) (DCCT, 1987; EDIC, 1999). Four-hour urine collections for measurement of AER and creatinine clearance were also obtained during EDIC in alternate years. Baseline covariates for the current analyses were obtained from DCCT baseline history, physical examination and laboratory data (fasting lipids and renal function). The methodology used to perform all the routine measurements used as conventional risk factors in this study has been described elsewhere (DCCT, 1986; DCCT, 1987; EDIC, 1999).

Statistical Analysis

The concentrations of markers of inflammation and endothelial dysfunction from serum samples collected at DCCT baseline were used to determine whether increases in the levels of these biomarkers could predict elevated risk to develop or induce accelerated progression of retinopathy during 16 years of follow up in the DCCT/EDIC study. All biomarker levels were assessed for normality and transformed when necessary (i.e., CRP, IL-6, sTNF-R1, sTNF-R2 and sE-selectin required natural log transformation). Following data normalization, all biomarkers were standardized and the analysis results represent the association between a change of one standard deviation in each biomarker and the hazard ratio associated with progression of retinopathy. Composite biomarker scores were created to assess the combined impact of multiple biomarkers believed to be acting on the same pathway. Specifically, three composite scores were created by combining standardized-scores of individual biomarkers: acute phase reactants (i.e., fibrinogen and CRP); cytokines and cytokine receptors/adipokines (i.e., sTNFR 1 and 2, PAI-I active, PAI-1 total and IL-6); and thrombosis/fibrinolysis (i.e., fibrinogen, PAI-I active and PAI-1 total). Baseline demographic and clinical variables were stratified by retinopathy cohort (primary prevention; retinopathy-free) vs. secondary intervention (mild-moderate baseline retinopathy) and progression to severe retinopathy during the follow up study. A two-sample Wilcoxon rank-sum test was used to compare groups for ordinal/continuous variables while a Pearson's chi square test was used to compare categorical characteristics. Univariate comparisons of levels of markers were compared across baseline retinopathy cohorts as well as within cohort across outcomes using a two-sample Wilcoxon rank-sum test.

Due to varying time intervals between fundus photo visits for each participant, parametric hazard models for interval-censored event/survival times were used to assess the effect of the increases in biomarker levels and of clinical and demographic variables on the risk of progression of retinal disease (Odelet al., 1992; Sparling et al., 2006). Preliminary adjusted models accounted for DCCT treatment assignment, presence of baseline retinopathy, duration of diabetes, and baseline measures of AER, HbA_{1c}, and ETDRS score. Additionally, evidence has shown a strong and consistent protective effect of HMG-CoA reductase inhibitors ('statins') and angiotensin-converting-enzyme inhibitors (ACE) on the risk of cardiovascular complications of diabetes as well as promise in reducing the risk of diabetic retinopathy progression (Al-Shabrawey et al., 2008; Chaturvedi, 2000). In light of this, final models were additionally adjusted for effects of ACE/ angiotensin II receptor

blockers (ARB) and lipid-lowering therapy (the use of these drugs increased as the DCCT/EDIC study progressed; as such, their use at any time leading up to an event or censor time (t) is entered into the model as time-varying covariate). The final models were also adjusted for DCCT treatment group, gender, smoking, DCCT baseline retinopathy status and baseline levels of HDL-Cholesterol, LDL-Cholesterol, triglycerides, systolic blood pressure, and age. Interval timing was defined as the number of days since the baseline fundus for the photo sessions both before and after progression of disease as we don't have exact disease progression dates.

All statistical analyses were performed using SAS System version 9.3 (SAS Institute, Cary, NC, USA). No adjustments for multiple testing were made, as they are known to reduce statistical power and increase the probability of accepting a null hypothesis that is truly false. A type 1 error rate of 5% was used as a nominal value for statistical significance.

RESULTS

Demographics and Retinopathy Outcomes

In both the primary prevention (free of retinopathy at baseline) and the secondary intervention (mild to moderate retinopathy at baseline) cohorts, baseline levels of HbA1c % and the duration of diabetes were higher in those that progress to severe retinopathy (table 1). Additionally, those who progress to severe retinopathy are less likely to be in the intensively treated study arm, and more likely to have been treated with lipid lowering drugs and ACE/ARB drugs during the study period. Of the 1391 study participants, 260 (18.7%) progressed to SNPDR and 831 (59.7%) experienced progression of retinopathy from their ETDRS baseline score (3 step change or greater). In the primary prevention cohort, 63 (8.9%) participants progressed to SNPDR and 455 (64.4%) had a 3-step increase from their baseline score. In the secondary intervention cohort, 197 (28.8%) progressed to SNPDR and 376 (54.9%) experienced a 3-step increase from baseline.

Baseline Levels and Cohort Differences

Baseline levels of makers of inflammation were compared across baseline retinopathy cohorts as well as by SNPDR outcome within each cohort. In general, participants with no retinopathy at DCCT baseline (Primary Prevention Cohort) had significantly lower levels of CRP ($p=0.042$) and sTNFR 1 and 2 ($p=0.001$ & $p=0.005$, respectively) and higher levels of sVCAM-1 ($p=0.024$) than subjects who had retinopathy at baseline (secondary intervention cohort, data not shown). Within the cohort of those without retinopathy at baseline, those that progressed to SNPDR had significantly lower levels of sVCAM-1 than those who did not progress (Table 2; $p=0.002$). In subjects with mild to moderate diabetic retinopathy at baseline, those that progressed to SNPDR had significantly higher levels of sE-Selectin as compared to those that did not progress ($p=0.005$). In the group of subjects without retinopathy at baseline the levels of sE-selectin in those who progressed to SNPDR were also higher but the difference was not statistically significant.

Biomarker Association with Progression of Retinopathy

In design-adjusted models (DCCT Treatment Group, Duration of Diabetes, Baseline retinopathy scores and Baseline measures of AER and HbA_{1c}), increased baseline levels of both sE-selectin and PAI-1 (active) were significantly associated with a 3-step progression in retinopathy score from baseline in the Primary Prevention cohort (Hazard Ratio (95% CI): 1.18 (1.05–1.32) $p=0.004$, aROC=0.770 and 1.21(1.07–1.40) $p=0.003$, aROC=0.771), but not the Secondary Intervention cohort (1.06 (0.963–1.20) $p=0.361$, aROC=0.737 and 0.93 (0.832–1.05) $p=0.245$, aROC=0.730, data not shown). After adjusting for additional covariates of interest and the time varying effect of ACE/ARB and Statin drug use, changes of one standard deviation in sE-Selectin and PAI-1 (active) in the primary prevention cohort were significantly associated with a 16% and 21% increased risk of a 3 step increase in retinopathy scores, respectively (Table 3; 1.16 (1.03–1.30) $p=0.011$, aROC=0.772 and 1.21 (1.05–1.38) $p=0.006$, aROC=0.775 respectively). The composite measures of acute phase reactants, cytokines/adipokines, and thrombosis were not significantly associated with increased risk of progression of diabetic retinopathy.

Biomarker Association with Progression to Severe Non-Proliferative Retinopathy

Progression to SNPDR was defined as the time to reach an ETDRS score 10. In the design adjusted models, participants with no retinopathy present at baseline who had increased levels of both active PAI-1 and total PAI-1 were at increased risk to progress to severe retinopathy during the follow up [HR (95% CI): 1.49 (1.12–1.99) $p=0.006$, aROC=0.821 and 1.28 (1.02–1.61) $p=0.031$, aROC=0.813, respectively]. After adjusting for additional covariates of interest and the time varying effect of ACE/ARB and Statin drug use, a change of one standard deviation in both active and total PAI-1 levels in the primary prevention cohort were significantly associated with a 54% and 29% increased risk to progress to severe NPDR during the follow up period, respectively (Table 4; 1.54 (1.15–2.07) $p=0.004$, aROC=0.820 and 1.29 (1.02–1.64) $p=0.034$, aROC=0.821 respectively). However, in the design-adjusted model, increased levels of sVCAM in subjects without baseline retinopathy were not associated with a significant decreased risk of the progression to severe retinopathy [HR=0.64 (95% CI: 0.40–1.03) $p=0.067$, aROC=0.827]. This relationship was moderately associated in the covariate-adjusted model [HR=0.58 (95% CI: 0.35–0.97) $p=0.037$, aROC=0.827]. Subjects who already had mild to moderate retinopathy at DCCT baseline (Secondary Intervention Cohort) did not have a significant association present between any of the measured markers or composite scores and progression to severe NPDR.

DISCUSSION

In our present study, serum levels of CRP, PAI-1 total and PAI active, sICAM-1, sVCAM-1, sE-Selectin, IL-6, sTNFR-1 and sTNFR-2 were assayed. At the DCCT baseline visit, patients without retinopathy had significant lower levels of inflammatory markers (CRP and sTNFR 1 and 2) than those with mild to moderate retinopathy. Additionally, patients with mild to moderate retinopathy who progressed to SNPDR had significantly higher levels of sE-selectin at baseline. The same was true in patients without retinopathy at baseline (primary prevention cohort) but due to wide individual variation of sE-selectin among the patients studied, the difference did not reach statistical significance.

Increased levels of sE-selectin and PAI-1 (active), at baseline, were associated with a 3 step change in the development of retinopathy in patients with normal retinal examination at baseline (Primary Prevention cohort). Progression to SNPDR, however, was only related to increased levels of PAI-1 (active and total). None of our biomarkers was or were associated with further progression of retinopathy in subjects who entered the trial with mild to moderate retinopathy (Secondary Prevention Cohort).

The findings of the present study are interesting and shed light on some important associations of retinopathy with inflammation, endothelial dysfunction and clotting/fibrinolysis. Conventional inflammation markers although increased in patients with retinopathy, as clearly demonstrated in the secondary prevention cohort, had no predictive value for the development/ progression of retinopathy. This is interesting since, regardless of some conflicting data (Klein et al., 2009; Nguyen; et al. 2009), there is significant evidence supporting the association of inflammation with diabetic retinopathy (Adamis, 2002; Altinova et al., 2005; Fasching et al., 1996; Gustavsson et al., 2008; Joussen et al., 2004; Kuo et al., 2012; Lopes-Virella et al., 2013; Lopes-Virella et al., 2014; Mysliwska et al., 2007; Mysliwiec et al., 2007; Nowak et al, 2008; Olson et al, 1997; Soedamah-Muthu et al., 2006; Spijkerman et al., 2007). Furthermore, the role of chronic inflammation in diabetes extends well beyond retinopathy. Several groups have reported increased levels of systemic inflammatory markers in subjects with uncomplicated diabetes and diabetes with both micro and macrovascular complications (Nowak et al, 2008; Soedamah-Muthu et al., 2006; UKPDS, 1998). Our data seems however to suggest that inflammation is associated with the presence of retinopathy but may not contribute to the initiation of the disease. In other words inflammation is a component of the disease but not one of its causal factors. Therefore the classical serum markers of inflammation do not predict retinopathy progression or development, just identify its presence.

In contrast both markers of endothelial dysfunction and decreased fibrinolysis seem to be the presence of its development and progression. Endothelial dysfunction, associated with inflammation, may result in increased vascular permeability, alteration of blood flow, oxidative stress, angiogenesis, and has been characterized by elevated levels of sVCAM-1 and sICAM-1 in diabetes (Schram et al. 2003). One of the earlier events in diabetic retinal inflammation is the adhesion of leukocytes to the microvasculature. Increased leukocyte adhesion results in loss of endothelial cells and breakdown of the blood-retinal barrier (Abiko et al., 2003; Ai & Song, 2012; Barouch et al, 2000; Fashing et al., 1996; Olson et al., 1997; Spijkerman et al., 2007). The leukocyte adhesion in the diabetic retina is facilitated by the increased expression of adhesion molecules such as sICAM-1 and sE-selectin, and sVCAM on the endothelium and its leukocyte counter-receptor CD18 (Ai & Song, 2012). The inhibition of leukocyte adhesion prevents the loss of pericytes and the formation of acellular capillaries, leading to breakdown of the blood-retinal barrier (BRB) in animal models of diabetic retinopathy. Breakdown of the BRB leads to increased retinal leukostasis within days of developing diabetes and correlates with the increased expression of retinal ICAM-1 and CD18 (Barouch, Miyamoto, Akport et al, 2000). Mice deficient in the genes encoding for the leukocyte adhesion molecules CD18 and ICAM-1 demonstrate significantly fewer adherent leukocytes in the retina (Joussen et al., 2004).

Our study shows that levels of sE-selectin may be the initial development of retinopathy but does not correlate with the progression to SNPDR. In contrast, the increased expression of any of the other adhesion molecules could not predict the initiation/progression of retinopathy in this cohort and surprisingly we found that elevated levels of sVCAM-1 were associated with reduced progression to SNPDR.

The findings for sVCAM-1, appear to be paradoxical in light of the findings for sE-selectin and PAI-1 in this study and the findings of other studies that have looked at the expression and release of inflammatory markers in diabetic patients (Nowak et al. 2008; Soedamah-Muthu et al. 2006). Our results have shown that the subjects without retinopathy at DCCT baseline (Primary Prevention Cohort) have increased levels of sVCAM-1 ($p=0.024$), and within the group of patients from the DCCT cohort without retinopathy at baseline, those who progressed to SNPDR had significantly lower levels of sVCAM-1 than those who did not progress. Significantly lower levels of sVCAM-1 were also observed in the group of patients of the same cohort with normal AER at baseline who did not advance to macroalbuminuria (Lopes-Virella et al, 2013; Lopes-Virella et al., 2014). This fact is not easy to explain and certainly does not agree with data in previously published studies showing that VCAM-1 may play a role in diabetic endothelial activation as suggested by increased sVCAM-1 along with other endothelial activation markers in serum of diabetic subjects with different stages of retinopathy. (Fasching et al., 1996; Nowak et al 2008; Soedamah-Muthu, et al. 2006). This unexpected finding was not secondary to sample deterioration or methodology problem, as previously reported (Lopes-Virella et al., 2014), but it may be related either to a particular set of characteristics in these patients or to the stage of disease at the time of sample collection.

Adhesion molecule families are functionally distinct and their impact may be seen at different stages of disease. In one study (Klein et al., 2009), high levels of sVCAM-1, TNF, and homocysteine were found to be associated with increased risk of more severe retinopathy in patients with kidney disease, but not in subjects without kidney disease. Like our study, however, this particular study had also a paradoxical finding: elevated levels of sICAM-1 were associated with decreased risk of severe retinopathy. In the same study, there was also a decreased risk for progression of retinopathy in patients with increased levels of soluble IL-6, TNF- α , and homocysteine in those with chronic kidney disease, and for sICAM-1 in those without kidney disease. In contrast, a large study by Soedamah-Muthu et al. (2006) in patients with type 1 diabetes, has shown that sVCAM-1 and sE-selectin have positive associations with retinopathy, albuminuria, and cardiovascular disease. Nowak et al (2008) have also shown that serum concentration of sICAM-1 and sELAM-1 were significantly elevated while the concentration sVCAM-1 was elevated but not significantly in diabetic retinopathy patients when compared with control subjects.

Thus, there is a considerable amount of variability between the results of several studies, suggesting that other factors, not yet identified, may impact the increase in adhesion molecules observed in diabetic retinopathy. Another possibility is lack of correlation between the endothelial cell expression of adhesion molecules and their soluble levels, which may depend on differences in blood collection time. Whatever the reason, our results do not resolve or explain the variability of results found in previous published work but they

clearly point to the need to prevent insults which may induce increased expression of adhesion molecules, an important first step in the development of nephropathy.

Finally our study has shown that PAI-1, a well-known adipokine and inhibitor of fibrinolysis, has a role in the development and progression of retinopathy. Plasma PAI-1 levels in diabetic patients with microvascular complications have been reported as being significantly higher than those of the diabetic patients without microvascular complications (Erem et al., 2005). Increased plasma concentrations of PAI-1 and other clotting/fibrinolysis factors were significantly higher in the diabetic patients with retinopathy, nephropathy, and neuropathy than in diabetic patients without neuropathy (Erem et al., 2005). Plasma levels of PAI-1 were also found to be an independent predictor of end-stage PDR in Northern Chinese Han population with type 2 diabetes (Zhing & Chen, 2012). In these patients the plasma levels of PAI-1 in patients with end-stage proliferative diabetic retinopathy were significantly higher than those observed in patients without end stage proliferative diabetic retinopathy or in normal subjects.

Due to the dual effect of PAI-1 in inflammation and in the clotting/fibrinolytic system it was important to examine which one of these roles was likely responsible to for its role in predicting the development/progression of retinopathy. As an adipokine, PAI-1 is associated with insulin resistance and metabolic syndrome. However as shown by Kilpatrick et al (2007), although, in the DCCT/EDIC cohort, the prevalence of metabolic syndrome and visceral adiposity increased markedly during DCCT mainly in the group intensively treated with insulin, the progression /development of retinopathy in the intensively treated sub-group was markedly reduced not increased, leading us to conclude that the association of PAI-1 with retinopathy found in our studies was likely related with the anti-fibrinolytic function of PAI-1 not to its pro-inflammatory properties. This conclusion is supported by the fact that neither IL6, sTNF receptors or CRP, all well recognized biomarkers of inflammation, could predict the development of retinopathy in this cohort.

Large landmark studies have demonstrated the importance of good glucose and blood pressure control to reduce the risk of development and progression of diabetic retinopathy (DCCT, 1993, UKPDS, 1998). Both hyperglycemia and hypertension may influence both oxidative stress and the consequent inflammatory reaction thus influencing inflammatory markers, endothelial function and the clotting/fibrinolytic system. Therefore the marked reduction in the development and progression of microvascular complications in diabetes induced by tight glucose control may result from its down-regulating effect in oxidative stress and inflammatory pathways.

Since optimal glucose control although markedly reducing the development of retinopathy is not able to completely eliminate its development in some patients, an effort to identify other markers predictive of a high risk to develop retinopathy needs to be further pursued. Diabetic retinopathy is the leading cause of adult vision loss and blindness in adults (Mohamed, Gillies & Wong, 2007) and, worldwide, vision loss from diabetic retinopathy is predicted to double over the next 30 years (Cheung, Mitchel & Wong, 2010).

Our study shows that although markers of altered endothelial cell dysfunction and fibrinolysis are able to predict patients at high risk to develop retinopathy prior to the appearance of the disease, none of the biomarkers studied was able to predict progression of disease once the disease is established. Interestingly it has also shown that composites of different biomarkers do not have a higher predictive value than individual markers. In other words, although inflammation, endothelial dysfunction and clotting/fibrinolysis should be intimately related, it seems obvious that our understanding of the precise interactions and correlations between the three pathogenic processes is less than perfect and that they may be strongly influenced by other factors not yet identified.

Therefore, further research aiming to define markers able to identify patients at high risk to progression to severe retinopathy once the disease is present is essential. Until better biomarkers are available, other therapies besides optimal glucose control, aimed at reducing endothelial dysfunction and preventing abnormalities in the clotting/fibrinolytic system as well as reducing the inflammatory reaction, such as those successfully implemented for the treatment of rheumatoid arthritis and other autoimmune diseases should be evaluated for their capacity to normalize risk factors associated with the development of retinopathy in diabetic patients and whether such normalization would result into more effective prevention of the development of retinopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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H.A.R. helped with data analysis and wrote and reviewed the manuscript. N.L.B. and K.J.H. analyzed data and wrote, reviewed, and edited the manuscript. P.A.C. provided the clinical data from the EDIC cohort and assisted in the writing and revision of the manuscript. R.K. supervised the performance of all of the biomarker assays performed in this study and assembled the resulting data. J.L. and P.A.C. helped with the statistical analysis, the writing, reviewing, and editing of the manuscript. G.V. wrote, reviewed, and edited the manuscript. The DCCT/EDIC group provided samples, reviewed, and edited the manuscript. M.F.L.-V. supervised the performance of all the biomarker assays performed in this study, assembled the resulting data, and wrote, reviewed, and edited the manuscript. M.F.L.-V. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. This work was supported by the Grant R01-DK-081352 (M.F. L.-V) funded by the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and by the Research Service of the Ralph H. Johnson Department of the Veterans Affairs Medical Center. The DCCT/EDIC was sponsored through research contracts from the Division of Diabetes, Endocrinology and Metabolic Diseases (NIDDK) of the National Institutes of Health.

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Table 1

Baseline Clinical and Demographic Characteristics by Retinopathy Status during the Follow-up period.

Characteristic	Primary Prevention Cohort n=706		Secondary Intervention Cohort n=685	
	Overall N=1391	None, Mild, or Moderate Retinopathy N=646	None, Mild, or Moderate Retinopathy N=488	SNPD Retinopathy N=197
Age (yrs)	26.8 ± 7.1	26.5 ± 7.3	26.2 ± 8.3	26.1 ± 6.8
Duration of T1D (yrs)	5.6 ± 4.1	2.5 ± 1.3	3.1 ± 1.8	9.3 ± 3.6
Experimental Trt % (n)	49.3 (686)	50.6 (327)	22.2 (14)	30.5 (60)
Caucasian	96.5 (1342)	96.1 (621)	95.2 (60)	94.4 (186)
Male	52.9 (736)	51.4 (332)	58.7 (37)	52.8 (104)
Body Mass Index	23.4 ± 2.8	23.1 ± 2.8	23.9 ± 3.1	23.5 ± 3.0
Systolic Blood Pressure	113.9 ± 11.5	113.1 ± 11.3	115.3 ± 10.0	114.9 ± 11.7
Diastolic Blood Pressure	72.5 ± 8.8	72.1 ± 8.7	72.3 ± 8.4	73.4 ± 8.9
Mean Blood Pressure	86.3 ± 8.7	85.7 ± 8.6	86.6 ± 7.8	87.3 ± 8.8
Total Cholesterol	176.5 ± 33.3	173.8 ± 33.5	180.7 ± 38.3	182.4 ± 30.9
HDL – Cholesterol	50.7 ± 12.3	51.8 ± 12.9	51.2 ± 13.8	49.9 ± 11.9
LDL – Cholesterol	109.6 ± 29.1	106.9 ± 29.4	113.6 ± 32.2	113.8 ± 26.8
Triglycerides	81.1 ± 47.9	75.7 ± 51.4	79.3 ± 38.1	93.6 ± 43.4
AER	15.8 ± 18.5	11.6 ± 7.5	13.9 ± 13.9	23.5 ± 25.0
Serum Creatinine	0.80 ± 0.15	0.80 ± 0.15	0.80 ± 0.13	0.79 ± 0.16
Creatinine Clearance	128.3 ± 30.0	127.7 ± 29.1	122.8 ± 27.3	127.5 ± 29.0
HbA1c % (mmol/mol)	8.9 ± 1.6 74±12	8.7 ± 1.6 72±13	10.0 ± 2.0 86±16	9.6 ± 1.5 81±11

Characteristic	Primary Prevention Cohort n=706		Secondary Intervention Cohort n=685		P Value
	Overall N=1391	None, Mild, or Moderate Retinopathy N=646	None, Mild, or Moderate Retinopathy N=488	SNPD Retinopathy N=197	
Retinopathy Score	2.2 ± 1.6	1.0 ± 0.0	1.0 ± 0.0	4.2 ± 1.7	<0.001
Use of ACE/ARB Drugs during Study	40.7 (566)	34.2 (221)	68.3 (43)	62.9 (124)	<0.001
Use of Lipid Lowering Drugs during Study	31.6 (439)	27.4 (177)	42.9 (27)	41.6 (82)	0.012

Table 2

Baseline levels of measured biomarkers.

Biomarker	Overall n=1391	Primary Prevention Cohort n=706		Secondary Intervention Cohort n=685	
		None, Mild, or Moderate Retinopathy n=646	SNPD Retinopathy n=63	None, Mild, or Moderate Retinopathy n=488	SNPD Retinopathy n=197
CRP (mg/dl)	0.42 ± 1.04	0.38 ± 0.80	0.30 ± 0.63	0.42 ± 1.07	0.59 ± 1.62
sE-Selectin (ng/ml)	63.4 ± 57.0	60.9 ± 55.3	77.2 ± 80.7	61.0 ± 53.8	73.1 ± 60.6*
IL-6 (pg/ml)	9.7 ± 18.0	10.7 ± 21.6	7.0 ± 6.3	8.9 ± 15.2	9.0 ± 13.3
sICAM 1 (ng/ml)	363.7 ± 132.3	361.9 ± 122.6	350.8 ± 115.4	369.0 ± 147.5	362.0 ± 130.1
sVCAM 1 (ng/ml)	1020.3 ± 440.9	1067.0 ± 417.3	868.0 ± 315.7*	992.9 ± 378.3	982.3 ± 648.0
PAI-1 Active (ng/ml)	8.8 ± 5.9	8.7 ± 5.5	10.1 ± 6.8	8.8 ± 5.9	9.2 ± 7.0
PAI-1 Total (ng/ml)	188.2 ± 107.5	186.5 ± 103.6	212.4 ± 138.3	187.0 ± 107.8	189.1 ± 109.8
sTNF-R1 (ng/ml)	1.45 ± 0.67	1.39 ± 0.57	1.38 ± 0.47	1.49 ± 0.82	1.55 ± 0.65
sTNF-R2 (ng/ml)	1.46 ± 0.60	1.41 ± 0.52	1.42 ± 0.55	1.52 ± 0.69	1.49 ± 0.55
Fibrinogen (mg/dl)	196.1 ± 59.1	197.1 ± 55.7	195.2 ± 69.4	193.9 ± 61.1	197.9 ± 63.9

All subjects in the primary prevention cohort have baseline ETDRS scores of 1.

* P<0.05 as compared to those who do not progress to SNPD within the cohort; Statistical analysis done using unadjusted Wilcoxon Rank-Sum test statistic.

Table 3

Hazard ratio's and 95% confidence intervals for a one Standard deviation increase in marker levels on a 3 step change in ETDRS score from DCCT Baseline.

Biomarker	Overall (831 Events / 560 Censored)	Baseline Retinopathy Cohort	
		Primary Prevention 455/251	Secondary Intervention 376/309
Individual Markers			
CRP (mg/dl)	0.99 (0.92–1.07)	0.94 (0.85–1.04)	1.06 (0.94–1.19)
sE-Selectin (ng/ml)	1.07 (0.99–1.16)	1.16 (1.03–1.30)	1.06 (0.94–1.20)
IL-6 (pg/ml)	0.98 (0.92–1.05)	0.98 (0.89–1.08)	1.00 (0.90–1.11)
sICAM 1 (ng/ml)	1.00 (0.93–1.08)	1.02 (0.91–1.13)	0.99 (0.90–1.10)
sVCAM 1 (ng/ml)	0.96 (0.88–1.04)	0.93 (0.81–1.06)	0.95 (0.84–1.08)
PAI-1 Active (ng/ml)	1.01 (0.92–1.11)	1.21 (1.05–1.38)	0.92 (0.81–1.04)
PAI-1 Total (ng/ml)	1.00 (0.94–1.07)	1.06 (0.97–1.16)	0.96 (0.87–1.05)
sTNF-R1 (ng/ml)	0.96 (0.89–1.04)	0.97 (0.87–1.08)	0.95 (0.85–1.06)
sTNF-R2 (ng/ml)	0.95 (0.88–1.03)	1.05 (0.94–1.17)	0.89 (0.80–1.01)
Fibrinogen (mg/dl)	0.90 (0.81–1.01)	0.88 (0.75–1.03)	0.92 (0.77–1.09)
Composite Scores			
Acute	0.91 (0.80–1.04)	0.85 (0.70–1.03)	0.96 (0.80–1.16)
Cytokines	1.00 (0.88–1.12)	1.13 (0.96–1.32)	0.91 (0.76–1.09)
Thrombosis	0.97 (0.84–1.12)	1.06 (0.86–1.32)	0.94 (0.76–1.16)

All subjects in the primary prevention cohort have baseline ETDRS scores of 1. Results are shown adjusted for DCCT treatment group, baseline retinopathy cohort, baseline measures of duration of diabetes, ETDRS score (secondary cohort), AER, HbA1c %, LDL-Cholesterol, HDL-Cholesterol, Triglyceride levels, age, systolic blood pressure, smoking status, and the time varying effects of treatment with lipid lowering drugs and ACE/ARB therapy. Data are shown as the adjusted hazard for one SD unit change in marker levels with associated Wald 95% CI.

Table 4

Hazard ratio's and 95% confidence intervals for a one Standard deviation increase in marker levels on Progression to severe non-proliferative diabetic retinopathy.

Biomarker	Overall (260 Events / 1127 Censored)	Baseline Retinopathy Cohort	
		Primary Prevention 63/646	Secondary Intervention 197/488
Individual Markers			
CRP (mg/dl)	1.00 (0.88–1.14)	0.96 (0.75–1.22)	0.96 (0.83–1.12)
sE-Selectin (ng/ml)	1.06 (0.91–1.23)	1.19 (0.87–1.62)	0.97 (0.81–1.16)
IL-6 (pg/ml)	0.94 (0.83–1.06)	0.90 (0.69–1.17)	0.95 (0.82–1.10)
sICAM 1 (ng/ml)	0.89 (0.79–1.01)	0.89 (0.64–1.24)	0.91 (0.79–1.04)
sVCAM 1 (ng/ml)	0.97 (0.82–1.15)	0.58 (0.35–0.97)	1.00 (0.89–1.20)
PAI-1 Active (ng/ml)	1.03 (0.88–1.20)	1.54 (1.15–2.07)	0.91 (0.76–1.09)
PAI-1 Total (ng/ml)	1.03 (0.92–1.16)	1.29 (1.02–1.64)	1.03 (0.94–1.14)
sTNF-R1 (ng/ml)	0.97 (0.84–1.11)	1.13 (0.81–1.58)	0.93 (0.79–1.09)
sTNF-R2 (ng/ml)	0.93 (0.81–1.06)	1.18 (0.86–1.62)	0.86 (0.74–1.01)
Fibrinogen (mg/dl)	1.01 (0.80–1.28)	1.02 (0.59–1.76)	0.99 (0.77–1.27)
Composite Scores			
Acute	1.00 (0.79–1.27)	0.96 (0.51–1.79)	1.04 (0.86–1.25)
Cytokines	0.91 (0.75–1.12)	1.47 (0.97–2.24)	0.79 (0.62–1.00)
Thrombosis	0.91 (0.76–1.09)	1.04 (0.85–1.26)	0.92 (0.74–1.14)

All subjects in the primary prevention cohort have baseline ETDRS scores of 1. Results are shown adjusted for DCCT treatment group, baseline retinopathy cohort, baseline measures of duration of diabetes, ETDRS score (secondary cohort), AER, HbA1c %, LDL-Cholesterol, HDL-Cholesterol, Triglyceride levels, age, systolic blood pressure, smoking status, and the time varying effects of treatment with lipid lowering drugs and ACE/ARB therapy. Data are shown as the adjusted Hazard Ratios for one SD unit change in marker levels with associated Wald 95% Confidence interval.