

# Oxidative Stress and Response in Relation to Coronary Artery Disease in Type 1 Diabetes

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**OBJECTIVE**—Although oxidative stress (OxS) is thought to contribute to atherosclerosis and coronary artery disease (CAD), little is known about the variability in an individual's ability to respond to OxS. Therefore, we assessed potential indices of response to OxS and evaluated whether they modify the association between OxS and CAD.

**RESEARCH DESIGN AND METHODS**—We evaluated plasma  $\alpha$ - and  $\gamma$ -tocopherol per unit cholesterol (potential response markers); urinary 15-isoprostane  $F_{2t}$  per milligram creatinine (isoprostane [IsoP], a potential stress marker); and the  $\alpha$ -tocopherol-to-IsoP ratio (as a measure of response to stress), measured three times during 20 years of follow-up, in relation to CAD incidence in a cohort with childhood-onset type 1 diabetes ( $n = 658$ ; mean age at baseline, 28 years; duration of diabetes, 19 years). Participants with three samples (blood and either 24-h or overnight urine) available before the onset of CAD or the end of follow-up ( $n = 356$ ) were selected for study.

**RESULTS**—In multivariable mixed models,  $\alpha$ -tocopherol over time was inversely associated with CAD ( $\beta = -0.27$ ;  $P = 0.02$ ), whereas a direct association was observed for IsoP ( $\beta = 0.0008$ ;  $P = 0.06$ ). Moreover, the  $\alpha$ -tocopherol-to-IsoP ratio was strongly and inversely related to CAD incidence ( $\beta = -0.72$ ;  $P = 0.003$ ), whereas in a separate model including  $\alpha$ -tocopherol and IsoP, both biomarkers maintained statistical significance. No association was observed for  $\gamma$ -tocopherol ( $\beta = -0.22$ ;  $P = 0.54$ ).

**CONCLUSIONS**—These data suggest that a greater potential capability ( $\alpha$ -tocopherol) to respond to OxS (urinary IsoP) relates to CAD incidence.

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Although the generation of reactive oxygen species is a natural and essential feature of human physiology, free radicals can also be cytotoxic, attacking and altering the structure and function of lipids, proteins, and nucleic acids, thereby compromising normal cellular function (1). To maintain normal cellular physiology, a balance is required between the pro- and antioxidant forces, while a relative excess production of free radicals, and the resulting oxidative stress (OxS), has been implicated in both the development as well as the progression of diabetes-associated complications (2). Unfortunately, the lack of sensitivity, specificity, or both in the methodologies

commonly used to assess OxS in vivo, the requirement for invasive methods, and/or the influence of external factors on measurements obtained have prohibited definitive conclusions in human studies (3). Furthermore, little effort has been made to quantify response potentials (i.e., antioxidant defenses) relative to the stress encountered by individuals.

Antioxidant vitamins comprise one of the defense mechanisms available to living systems to counteract the damaging effects of excess free radical production (4). However, despite early encouraging findings from prospective cohort studies, clinical trials of antioxidant supplementation for the prevention of

cardiovascular disease have generally provided null results (5). Our own observation of a protective effect of plasma concentrations of  $\alpha$ -tocopherol on the incidence of coronary artery disease (CAD) among individuals with type 1 diabetes also was puzzling because it seemed to be confined to those using antioxidant vitamin supplements but occurred at concentrations similar to those of non-supplement users (6).

More recent discoveries, such as the ability to measure isoprostanes, may help to better quantify the level of OxS and permit analyses related to antioxidative defenses in humans. Indeed, isoprostanes are currently considered the gold standard biomarker of OxS and lipid peroxidation in vivo (3) and may provide a better opportunity to evaluate this important lipid peroxidation pathway in the pathophysiology of vascular diseases. Thus, we aimed to revisit the OxS hypothesis to evaluate whether the degree to which an individual with type 1 diabetes is potentially able to respond to OxS (e.g., plasma levels of antioxidant vitamins) is associated with cardiovascular risk and whether this varies with levels of such stress (e.g., urinary isoprostanes).

## RESEARCH DESIGN AND METHODS

The Pittsburgh Epidemiology of Diabetes Complications (EDC) study is a historical, prospective investigation of incident cases of childhood-onset type 1 diabetes diagnosed, or seen within 1 year of diagnosis, at the Children's Hospital of Pittsburgh between 1950 and 1980. A total of 658 patients who were eligible and willing to participate in the study had an initial clinical assessment between 1986 and 1988 (mean age 28 years; mean duration of diabetes 19 years) and have been subsequently reexamined or surveyed biennially for up to 20 years. The University of Pittsburgh Institutional Review Board approved the study protocol. For the analyses in this study, EDC study participants who were free of CAD at study entry and had three stored samples (blood and either 24-h or overnight urine) available before the development of CAD (for

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incident cases) or the end of follow-up were selected for study ( $n = 356$ ). Given availability, samples were chosen to reflect an early, midpoint, and late assessment during a participant's follow-up. Thus, biologic determinations were approximately 6 years apart for noncases, whereas, given the shorter available follow-up for those developing CAD, biomarker measurements were approximately 4 years apart for incident cases.

Demographic, health care, diabetes self-care, and medical history information were ascertained via self-administered questionnaires before each clinic visit. Blood pressure was measured with a random zero sphygmomanometer after a 5-min rest (7), and hypertension was defined as blood pressure  $\geq 140/90$  mmHg or use of antihypertensive medications. Although the definition of hypertension has been altered in recent years, given that the majority of the follow-up within the EDC study, which was initiated in 1986, falls within the time period during which hypertension was still defined as 140/90 mmHg, we felt that it would not be appropriate to reclassify the cohort retrospectively. Nevertheless, statistical analyses were repeated using 130/80 mmHg as the cutoff points for the definition of hypertension. Stable glycosylated hemoglobin (HbA<sub>1c</sub>) was measured by ion exchange chromatography (Isolab, Akron, OH) for the first 18 months and by automated high-performance liquid chromatography (Diamat, BioRad, Hercules, CA) for the subsequent 10 years; the two assays were highly correlated ( $r = 0.95$ ). For follow-up beyond 10 years, HbA<sub>1c</sub> was measured with the DCA 2000 analyzer (Bayer, Tarrytown, NY). The DCA 2000 and Diamat assays also were highly correlated ( $r = 0.95$ ). Original HbA<sub>1</sub> (1986–1998) and later HbA<sub>1c</sub> values (1998–2004) were converted to standard HbA<sub>1c</sub> values aligned with the Diabetes Control and Complications Trial (DCCT) using regression formulae derived from duplicate assays [DCCT HbA<sub>1c</sub> =  $(0.83 \times \text{DIAMAT HbA}_1) + 0.14$  and DCCT HbA<sub>1c</sub> =  $(\text{DCA HbA}_{1c} - 1.13) / 0.81$ ]. HDL cholesterol was determined enzymatically after precipitation with heparin and manganese chloride, using a modified version of the Lipid Research Clinics method (8). Cholesterol and triglycerides were measured enzymatically (9,10). Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol. White blood cell count was obtained using a counter S-plus intravenous line. Urinary albumin was measured by immunonephelometry (11), and creatinine was assayed using an Ectachem 400 Analyzer (Eastman Kodak

Co., Rochester, NY). Glomerular filtration rate was estimated using the equation used in the Chronic Kidney Disease Epidemiology Collaboration study (12).

CAD was defined as myocardial infarction confirmed by Q-waves on electrocardiogram (Minnesota code 1.1, 1.2) or hospital records, angiographic stenosis  $\geq 50\%$ , revascularization, or death due to CAD.

Plasma levels of  $\alpha$ - and  $\gamma$ -tocopherol as well as urinary 15-isoprostane F<sub>2t</sub> (IsoP) were measured from samples collected at the clinical visit at three time points during the 20-year follow-up of the EDC study and stored at  $-70^\circ\text{C}$  until the present analyses. The concentrations of  $\alpha$ - and  $\gamma$ -tocopherol in plasma samples anticoagulated with EDTA were measured under subdued lighting, as previously reported (13,14). The coefficient of variation between runs is 6.0 and 2.8% for  $\alpha$ -tocopherol and  $\gamma$ -tocopherol, respectively. The effect of prolonged storage on serum antioxidant samples was addressed within the Multiple Risk Factor Intervention Trial (MRFIT); no appreciable degradation was seen in samples stored at  $-50$  to  $-70^\circ\text{C}$  (15). Urine samples were obtained in 24-h ( $\sim 58\%$ ) or overnight ( $\sim 42\%$ ) collections. The same type of timed sample was used at all time points for a given individual. Levels of IsoP were measured using a competitive ELISA procedure developed by Oxford Biomedical Research (Oxford, MI), which has a correlation ( $r^2$ ) of  $>0.8$  with gas chromatography–mass spectrometry procedures. Because there are no published data on the potential effects of prolonged storage on the measurement of IsoP, we simultaneously performed the assay on stored (20- and 10-year) samples and fresh samples from 6 controls and 11 individuals with type 1 diabetes without renal failure. IsoP levels were similar and within the normal range at all time points (controls: 0.96, 1.6, and 1.01 ng/mg creatinine, respectively; individuals with type 1 diabetes: 1.3, 1.2, and 0.98 ng/mg creatinine, respectively), suggesting stability of the assay.

### Statistical analyses

All statistical analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC). Given the dependence of plasma tocopherol concentration on lipid levels,  $\alpha$ - and  $\gamma$ -tocopherol were reported per unit cholesterol (16). Urinary IsoP (in nanograms per milliliter) were normalized for urinary creatinine concentration and expressed as nanograms per milligram of creatinine. Univariate associations between

measurements at the first time point (baseline) and subsequent CAD status were determined using the Student  $t$  test for normally distributed continuous variables or the Wilcoxon two-sample rank sum test for nonnormally distributed continuous variables. The  $\chi^2$  or Fisher exact test were used as appropriate for univariate analysis of categorical variables. Since  $\alpha$ -tocopherol has been shown to act as a pro-oxidant under conditions of low reactive oxidant fluxes (17), Spearman rank correlations were used at each time point for the entire cohort, and separately for incident cases and noncases, to investigate the association between  $\alpha$ -tocopherol and urinary IsoP according to high versus low/normal HbA<sub>1c</sub>. Mixed models were used to graph trajectories of the main independent variables over time by subsequent CAD status. Trajectories were plotted as a function of time: time before CAD incidence in cases and time before the end of follow-up in noncases; cubic splines with continuous second derivatives were used to smooth lines (18). The GENMOD procedure in SAS was used to assess the association between the repeated measurements of the main independent variables and CAD incidence after adjustment for duration of diabetes and significant univariate risk factors. Nonnormally distributed variables were logarithmically transformed to more closely satisfy the assumption of normality for entry into multivariable models (as noted in Tables 1–3 and Fig. 1).

**RESULTS**—The concentrations of measured biomarkers of interest (i.e., urinary IsoP and  $\alpha$ - and  $\gamma$ -tocopherol) over time in the entire study group are given in Supplementary Table 1. The concentration of  $\alpha$ -tocopherol increased over time, paralleling the increase in the use of vitamin supplements in the entire EDC population (data not shown). No statistically significant changes, however, were observed overall in urinary IsoP or  $\gamma$ -tocopherol. Among individuals free of CAD at study entry and with stored samples available at three times during the 20-year follow-up ( $n = 356$ ), 88 (24.7%) developed a CAD event. Participant characteristics at the first of three time points (baseline) by subsequent CAD status are shown in Table 1. As expected, individuals who subsequently developed CAD were more likely to be older, with a longer duration of diabetes and higher blood pressure, lipid levels, albumin excretion rate, and white blood cell count compared with those who did not develop an event. Incident cases were also more

likely to have smoked, have micro- or macroalbuminuria, and lower estimated glomerular filtration rate. Although no significant differences in urinary IsoP,  $\alpha$ -tocopherol per unit cholesterol, or the ratio of  $\alpha$ -tocopherol to urinary IsoP were observed between subsequent cases and noncases, it is surprising that  $\alpha$ -tocopherol (not adjusting for cholesterol) was elevated among subsequent cases, whereas  $\gamma$ -tocopherol levels per unit cholesterol were elevated in individuals without CAD compared with subsequent cases.

In investigating the strength and direction of a potential association between

urinary IsoP and  $\alpha$ -tocopherol concentrations, generally inverse associations were observed regardless of the level of glycemic control (<7.5 or >7.5%) or subsequent CAD status, although the strength of the correlation differed by time point (data not shown).

Separate mixed models were constructed for each main independent variable [i.e., urinary IsoPs (per milligram of urinary creatinine), plasma  $\alpha$ - and  $\gamma$ -tocopherol (per unit cholesterol), and the  $\alpha$ -tocopherol-to-IsoP ratio] to assess the independent association between repeated measurements of each variable and the subsequent

development of CAD (Table 2). In multivariable mixed models, a borderline significant direct association was observed between urinary IsoP concentrations and CAD ( $P = 0.06$ ), whereas a significant inverse association was observed between plasma  $\alpha$ -tocopherol concentration and CAD ( $P = 0.02$ ). Repeated measurements of  $\gamma$ -tocopherol concentration did not seem to independently relate to subsequent CAD status ( $P = 0.54$ ). Interestingly, the  $\alpha$ -tocopherol-to-urinary IsoP ratio was strongly inversely associated with CAD ( $P = 0.003$ ). Similar results of an inverse association between the  $\alpha$ -tocopherol-to-urinary IsoP ratio and CAD incidence ( $P = 0.04$ ) were observed when including in the data from analyses of an additional 93 individuals with IsoP measured only in morning 4-hour clinic urine samples.

Figure 1 presents the trajectories of the  $\alpha$ -tocopherol, urinary IsoP (Fig. 1A), and their ratio (Fig. 1B) from the fully adjusted models (Table 2) before the occurrence of a CAD event or the end of follow-up (Table 3 gives the number of cases and noncases at each point during the follow-up period). Although differences did not appear to be of a great magnitude throughout the follow-up period,  $\alpha$ -tocopherol and the  $\alpha$ -tocopherol-to-urinary IsoP ratio were higher, especially during the 7 years before a CAD event or the end of follow-up, whereas urinary IsoP concentrations were lower among individuals who remained free of a CAD event.

**CONCLUSIONS**—These data suggest that among individuals with type 1 diabetes, the profile of OxS (i.e., urinary isoprostanes) and its potential antioxidant defense (i.e., plasma  $\alpha$ -tocopherol) differ over time in those who develop incident CAD compared with those who do not. This difference is particularly apparent in the 7 years before the incident event and is well characterized by the ratio of  $\alpha$ -tocopherol to urinary IsoP. No association was observed between repeated measures of plasma  $\gamma$ -tocopherol and subsequent development of CAD. Thus, a direct association of urinary IsoP concentrations after adjustment for other covariates was observed, whereas plasma  $\alpha$ -tocopherol and the  $\alpha$ -tocopherol-to-urinary IsoP ratio were inversely related to subsequent CAD status.

Clinical trials of the effect of the use of antioxidant vitamin supplements in both the general population as well as individuals with diabetes have generally suggested no benefit of supplementation for the primary prevention of cardiovascular

**Table 1—Participant characteristics at the first time point (baseline) by subsequent CAD status**

Participant characteristics	No CAD (n = 268)	CAD (n = 88)	P value
Years to CAD/censorship	18.8 (15.9–20.3)	13.0 (6.7–17.2)	<0.0001
Race/ethnicity, % (n)			
Non-Hispanic white	96.6 (259)	98.9 (87)	
Black	3.4 (9)	1.1 (1)	0.46
Age (years)	27.1 (8.0)	33.1 (7.0)	<0.0001
Duration (years)	18.8 (7.5)	24.7 (7.4)	<0.0001
Female sex, % (n)	51.9 (139)	53.4 (47)	0.80
BMI (kg/m <sup>2</sup> )	23.7 (3.3)	24.2 (3.1)	0.17
Ever smoked, % (n)	29.6 (79)	50.0 (44)	0.0005
HbA <sub>1c</sub> (%)	8.7 (1.4)	8.9 (1.5)	0.21
Systolic blood pressure (mmHg)	112.3 (13.9)	118.9 (15.6)	0.0002
Diastolic blood pressure (mmHg)	71.4 (9.7)	76.1 (11.1)	0.0002
Hypertension, % (n)	10.4 (28)	32.9 (29)	<0.0001
Cholesterol (mg/dL)	181.2 (36.4)	211.3 (46.1)	<0.0001
Triglycerides (mg/dL)	71.5 (56.0–102.5)	96.0 (72.0–131.0)	0.0003
Microalbuminuria, % (n)	40.7 (109)	63.6 (56)	0.0002
Macroalbuminuria, % (n)	19.8 (53)	43.2 (38)	<0.0001
Albumin excretion rate ( $\mu$ g/min)	10.9 (5.9–58.4)	47.3 (10.5–475.4)	<0.0001
eGFR (mL/min/1.73 m <sup>2</sup> )	114 (90–127)	104 (70–119)	0.0003
White blood cell count $\times 10^3$ /mm <sup>2</sup>	6.3 (1.8)	7.1 (1.8)	0.0005
Urinary 15-isoprostane F <sub>2t</sub> (ng/mg creatinine)	8.0 (5.8–11.2)	8.7 (6.7–11.7)	0.11
$\alpha$ -Tocopherol (mg/L)	8.0 (6.6–9.6)	8.8 (6.8–11.1)	0.02
$\alpha$ -Tocopherol per unit cholesterol	0.0046 (0.0038–0.0052)	0.0044 (0.0037–0.0053)	0.30
$\gamma$ -Tocopherol (mg/L)	2.1 (1.6–2.7)	2.1 (1.6–2.9)	0.63
$\gamma$ -Tocopherol per unit cholesterol	0.0012 (0.0009–0.0015)	0.0011 (0.0007–0.0013)	0.04
Ratio of $\alpha$ -tocopherol per unit cholesterol to urinary 15-isoprostane F <sub>2t</sub> per milligram creatinine	0.0006 (0.0004–0.0008)	0.0005 (0.0003–0.0007)	0.09

Data are mean (SD) or median (interquartile range) unless otherwise indicated. eGFR, estimated glomerular filtration rate (per the Chronic Kidney Disease Epidemiology Collaboration).

Table 2—Multivariable mixed models for the subsequent development of CAD

Main independent variables	Estimate	SE	95% CI	P value
Model 1: Urinary isoprostanes per mg creatinine				
IsoP*	0.0008	0.0005	−0.0000, 0.0017	0.0631
QIC	1,068.6899			
Model 2: $\alpha$ -Tocopherol per unit cholesterol				
$\alpha$ -Tocopherol*	−0.2697	0.1126	−0.4904, −0.0490	0.0166
QIC	1,059.6421			
Model 3: $\gamma$ -Tocopherol per unit cholesterol				
$\gamma$ -Tocopherol*	−0.2207	0.3599	−0.9262, 0.4848	0.5398
QIC	1,062.5363			
Model 4: $\alpha$ -Tocopherol per unit cholesterol and urinary isoprostanes per mg creatinine				
$\alpha$ -Tocopherol*	−0.2857	0.1154	−0.5120, −0.0595	0.0133
IsoP*	0.0008	0.0005	−0.0001, 0.0017	0.0730
QIC	1,052.6160			
Model 5: $\alpha$ -Tocopherol per unit cholesterol to urinary isoprostanes per mg creatinine ratio				
$\alpha$ -Tocopherol/IsoP*	−0.7244	0.2449	−1.2045, −0.2444	0.0031
QIC	1,052.6151			

\*Logarithmically transformed; estimates are per 10-unit increase. Models also were adjusted for duration of diabetes, sex, race/ethnicity, BMI, ever smoked, HbA<sub>1c</sub>, hypertension, HDL and non-HDL cholesterol, albumin excretion rate (log), glomerular filtration rate as estimated using the Chronic Kidney Disease Epidemiology Collaboration study equation, white blood cell count, type of urine sample used, and years until CAD or censorship. QIC, quasi-likelihood under the independence model information criterion (an index of a model's goodness of fit; the lower the value, the better the fit).

disease and even an increased mortality risk (5). Indeed, a meta-analysis of six randomized clinical trials comparing cardiovascular death risk with vitamin E versus placebo reported no benefit of supplementation, whereas similar meta-analyses for the effect of  $\beta$ -carotene supplementation suggested an increased risk of both all-cause and cardiovascular disease mortality (19). Interestingly, however, secondary prevention trials have provided somewhat more encouraging results for vitamin E supplementation, especially for myocardial infarction (20–22), although not all studies agree (23). We also have previously shown that a protective effect of  $\alpha$ -tocopherol against CAD seemed to be confined to persons with type 1 diabetes and kidney disease in a small case-control study (6). Thus, an issue raised is whether antioxidants may provide protection against cardiovascular disease only in susceptible population subgroups, such as those with a prior diagnosis (secondary prevention) or who are genetically susceptible. Indeed, in three clinical trials a reduced risk of cardiovascular disease with vitamin E therapy was apparent only among individuals with both type 2 diabetes and the haptoglobin 2–2 genotype (24–26).

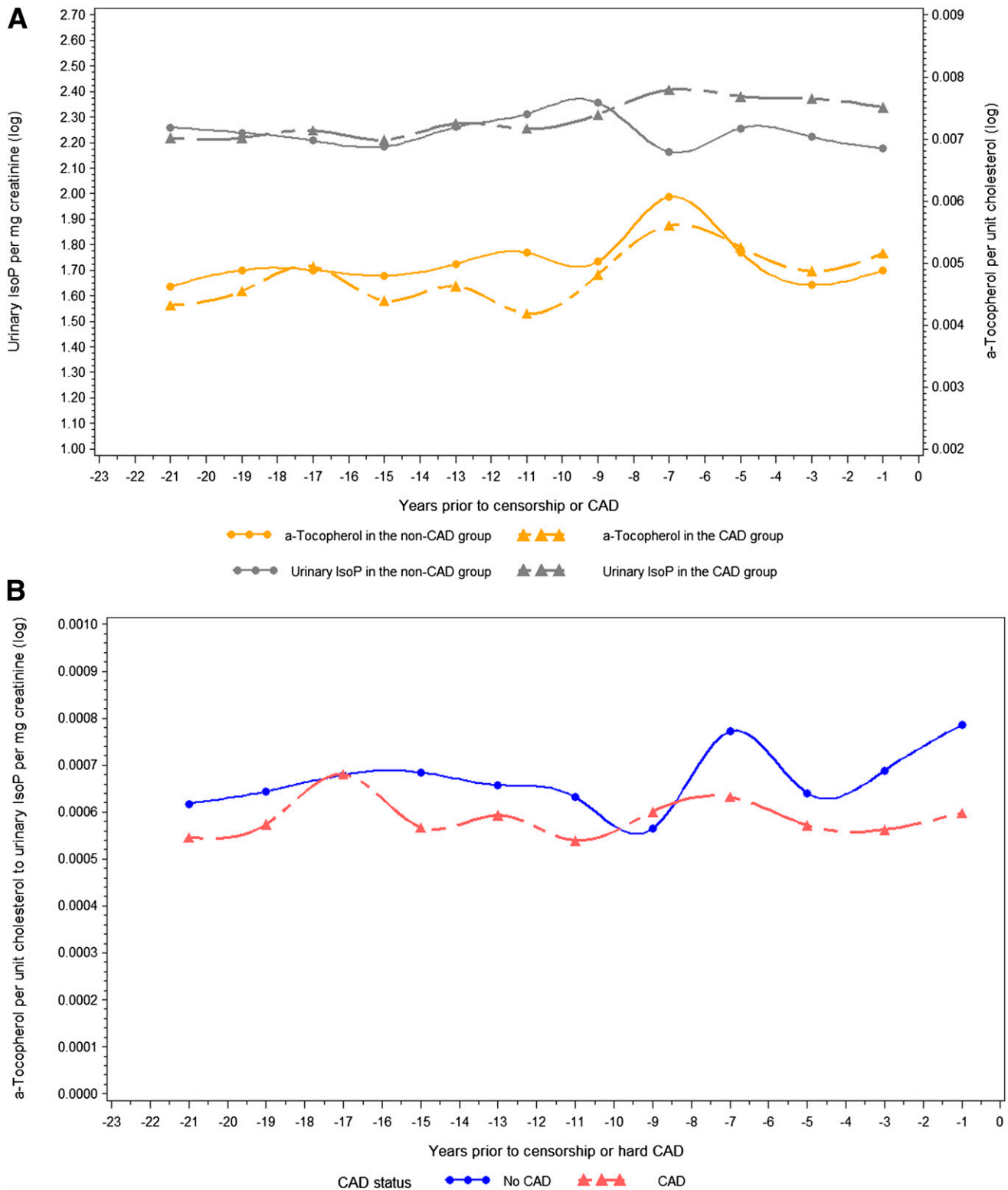
We also have previously raised the premise that an adequate response (e.g., antioxidant intake) to a specific insult (e.g., OxS) may reduce or delay the development of pathologic conditions associated with the insult (27). Thus, despite difficulties in recognizing markers representing protection or resistance in response to a specific stress, the concurrent evaluation of markers representing insult against those representing protection from or resistance to insults has great implications. In the current study, we aimed to assess whether an adequate antioxidative response to OxS could delay or prevent the development of CAD in a cohort of individuals with long-standing type 1 diabetes. Given that the majority of clinical trials of antioxidant supplementation in relation to cardiovascular disease focused on vitamin E, we measured the plasma concentration of  $\alpha$ - and  $\gamma$ -tocopherol. The former has been reported to have greater biologic activity and to be the only form maintained in human plasma (28), whereas the latter is the form most commonly found in the American diet (4).

We further assessed levels of urinary IsoPs, prostaglandin-like compounds derived primarily from nonenzymatic, free radical-induced peroxidation of arachidonic acid (29). Contrary to reactions catalyzed

by enzymes, it is reasonable to assume that the attack by reactive oxygen species on arachidonic acid yields equimolar levels of all F-series isoprostanes. F<sub>2</sub>-isoprostanes, the first isoprostane class discovered, are detectable in their esterified form in all normal biological tissues and in their free form in all normal biological fluids, including plasma and urine (3,30). Importantly, their concentration in urine is stable and not affected by arachidonic acid auto-oxidation or daily variation (29,31), contributing to their establishment as the gold standard biomarker of oxidant stress.

The Framingham Heart Study reported that urinary isoprostane concentrations were significantly associated with smoking, diabetes, and BMI, suggesting a role for systemic OxS in cardiovascular disease (32). A small case-control study from Germany also noted that urinary isoprostane excretion increased with an increasing number of traditional coronary heart disease risk factors (33). Indeed, several case-control studies of humans suggested the presence of elevated isoprostane concentrations in either plasma or urine among individuals with cardiovascular disease (33–37). Thus, plasma isoprostane levels were shown to be higher in individuals with atherosclerosis of the carotid or iliofemoral arteries (34), as well as among those with angiographic evidence of CAD (35), compared with controls. Urinary isoprostane levels also were reported to be increased with both subclinical atherosclerosis (36) and manifest CAD (33,36,37).

An important question that remained unanswered, however, was, Is OxS the initiator of processes leading to the development of CAD? Similarly to previous reports in the general population, we also observed a direct association between urinary concentrations of isoprostane and subsequent CAD in individuals with type 1 diabetes, although the association was not very strong, perhaps suggesting that the level of OxS per se is not the sole critical factor in disease pathogenesis, at least in this population. However, simultaneous evaluation of urinary IsoP and plasma  $\alpha$ -tocopherol resulted in significant effects for both biomarkers. The  $\alpha$ -tocopherol-to-urinary IsoP ratio over time was even more significant for subsequent CAD, consistent with the hypothesis that an adequate response to an insult may reduce risk associated with that insult. Thus, although the EDC study is not a clinical trial, our findings point to the value of concurrent evaluation of



**Figure 1**— $\alpha$ -Tocopherol and urinary IsoP trajectories (A) and  $\alpha$ -tocopherol-to-urinary IsoP ratio trajectories (B) (adjusted for duration of diabetes, race, sex, BMI, having ever smoked, HbA<sub>1c</sub>, hypertension, HDL and non-HDL cholesterol, albumin excretion rate (log), estimated glomerular filtration rate, and white blood cell count) before a CAD event or the end of follow-up.

insult and response to that insult to identify risk and potentially lead to novel approaches to preventing the progression of insults to full disease (e.g., by selective

administration of antioxidant therapy to those with a low  $\alpha$ -tocopherol-to-IsoP ratio), an approach that may merit further evaluation. Nonetheless, whether findings

from the current study in type 1 diabetes could be directly applied to the general population is unclear. Indeed, it has been shown that urinary isoprostane levels are

Table 3—Number of cases and noncases at each time point

	Years before CAD or censorship										
	-21	-19	-17	-15	-13	-11	-9	-7	-5	-3	-1
Cases (n)	8	11	18	27	27	24	21	27	31	22	24
Noncases (n)	94	109	62	96	87	113	49	24	38	17	5

increased when type 1 diabetes is diagnosed, and although they were shown to decline after 16 weeks of insulin treatment, isoprostane concentrations seem to remain elevated in persons with type 1 diabetes compared with controls (38). It is, then, possible that a relationship with CAD may be more difficult to observe in this compared with the general population if increased levels of OxS are present in type 1 diabetes before the initiation of atherosclerosis.

Pro-oxidant properties of  $\alpha$ -tocopherol have previously been described, especially at low oxidant fluxes and in the absence of co-antioxidants (17). Thus, a direct association between IsoP and  $\alpha$ -tocopherol may have been expected at lower levels of glycemic control ( $\text{HbA}_{1c} < 7.5\%$ ), contrary to our observations in this study. These findings, however, may suggest that the distribution of  $\text{HbA}_{1c}$ , even at lower levels, did not achieve the level of radical flux, leading to tocopherol-mediated peroxidation.

Strengths of the current study comprise the large cohort of individuals with type 1 diabetes, the long (20-year) prospective follow-up, and the biennial assessment of both risk factors and the outcome. In addition, the measurement of plasma levels of the antioxidants  $\alpha$ - and  $\gamma$ -tocopherol at three time points is also a strength because, contrary to dietary intake or supplement use data, plasma concentrations reflect biologically relevant levels of the vitamins. Finally, the measurement of urinary IsoP concentration, currently considered the “gold standard” biomarker of OxS, at three time points during the follow-up period is a further asset of this study.

As with any research study, conclusions based on this investigation are limited by several weaknesses, imposed by our choice of study design and the assumptions we made. Thus, although measuring biomarkers and assessing risk factors at multiple time points would have improved the precision of measurements, individuals with fewer than three stored samples had to be excluded from these analyses. This practice would have affected study participants with inadequate stored samples

(whether due to failure to collect samples, inadequate sample collection, or use of samples for previous analyses), especially those exhibiting cardiovascular and/or fatal events earlier during the follow-up period. Moreover, despite improved precision, biomarker assessment at three time points over a 20-year follow-up may not be an adequate representation of an individual's OxS status. We further restricted the assessment of OxS and antioxidative response to measurements of urinary IsoP and plasma tocopherol concentrations, respectively, among an array of currently known oxidative/antioxidative biomarkers. Whether other combinations of markers of stress and response or whether the total antioxidative potential/reserve of an individual may be more relevant to the subsequent risk for CAD cannot be derived from these data. Nevertheless, previous published reports of an association between total antioxidant status and cardiovascular outcomes have produced conflicting results (6,39–42). Finally, we cannot exclude the presence of subclinical CAD at study entry, a common limitation of both observational studies and clinical trials.

In conclusion, lower  $\alpha$ -tocopherol and the  $\alpha$ -tocopherol-to-urinary IsoP ratio were inversely related to CAD; to a lesser extent, higher urinary IsoP concentrations were directly related to CAD. These data thus provide some support for the hypothesis that a greater capability ( $\alpha$ -tocopherol) to respond to OxS (isoprostanes) relates to CAD incidence.

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T.C. researched and analyzed data and wrote the manuscript. R.W.E. performed and analyzed assays. G.L.S. analyzed assays. T.J.O. researched data, contributed to the discussion, and reviewed and edited the manuscript. T.C. 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.

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