Sex Differences in Endothelial Function Markers Before Conversion to Pre-Diabetes: Does the Clock Start Ticking Earlier Among Women?

The Western New York Study

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OBJECTIVE — We examined whether biomarkers of endothelial function, fibrinolysis/thrombosis and adiponectin, predict the progression from normal to pre-diabetes more strongly among women than men over 6 years of follow-up from the Western New York Health Study.

RESEARCH DESIGN AND METHODS — In 2002–2004, 1,455 participants from the Western New York Health Study, who were free of type 2 diabetes and cardiovascular disease at baseline (1996–2001), were selected for reexamination. An incident case of pre-diabetes was defined as fasting glucose <100 mg/dl at the baseline examination and ≥100 and <126 mg/dl at the follow-up examination. Biomarkers of endothelial function (E-selectin and soluble intracellular adhesion molecule-1 [sICAM-1]), fibrinolysis/thrombosis (plasminogen activator inhibitor-1 [PAI-1]), and fasting insulin, adiponectin, and inflammation (high-sensitivity C-reactive protein) were measured in frozen (−190°C) baseline samples.

RESULTS — Multivariate analyses revealed higher adjusted mean values of biomarkers of endothelial dysfunction (E-selectin and sICAM-1) and fibrinolysis (PAI-1) and lower mean values of adiponectin only among women who developed pre-diabetes compared with control subjects. Formal tests for interaction between sex and case/control status were statistically significant for E-selectin ($P = 0.042$), PAI-1 ($P = 0.001$), sICAM-1 ($P = 0.011$), and frequency of hypertension ($P < 0.001$).

CONCLUSIONS — These results support the concept that women who progressed from normoglycemia to pre-diabetes have greater endothelial dysfunction than men as well as more hypertension and a greater degree of fibrinolysis/thrombosis. Whether this relates to the higher risk of heart disease among diabetic women awaits further study.

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Death rates from coronary heart disease (CHD) in the U.S. have fallen dramatically over the last several decades; however, the rate of decline has been greater among men than among women (1). The factors underlying this sex difference are unclear (2–6). It has also been suggested that women, especially those with type 2 diabetes, are more likely to have coronary microvascular disease than men (7,8). It is well known that diabetic women have a much higher risk for CHD than their male counterparts, a risk not completely explained by traditional biological and psychosocial factors (9,10).

Previous studies have shown that the frequency of nonfatal myocardial infarction is increased before the clinical diagnosis of type 2 diabetes (11) and that women with impaired glucose tolerance tend to have a more atherogenic risk profile than their male counterparts years before the diagnosis of clinical diabetes (12). This observation has led to the "ticking clock" hypothesis (13), wherein the elevated CHD risk among diabetic individuals may be due more to their long-standing atherogenic risk profile than to hyperglycemia per se, which is more strongly associated with the risk of microvascular events (14). Recently, attention has been focused on the role of emerging risk factors including markers of endothelial dysfunction, inflammation, and fibrinolysis/thrombosis as precursors of both CHD and type 2 diabetes (15–17). In particular, elevated concentrations of E-selectin and plasminogen activator inhibitor-1 (PAI-1) have been associated with a twofold risk of CHD and diabetes (18,19). Adiponectin concentrations, which may be involved in chronic low-grade inflammation (20), have also been seen in some populations to predict CHD risk (21,22). Thus, progression from normal to impaired fasting glucose (pre-diabetes) to diabetes would appear to be associated with a worsening of CVD risk factors, although longitudinal data on this hypothesis are scant. It is not known whether the clock starts ticking earlier among women and thus might contribute proportionately more to the risk for subsequent CHD in those with type 2 diabetes.

These analyses were designed to investigate the following questions: *1*) what traditional and emerging CHD risk factors

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Abbreviations: ACR, albumin-to-creatinine ratio; CHD, coronary heart disease; CVD, cardiovascular disease; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; PAI-1, plasminogen activator inhibitor-1; sICAM-1, soluble intracellular adhesion molecule-1.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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predict conversion from normoglycemia to pre-diabetes, 2) does sex modify the association between these emerging risk factors and conversion to pre-diabetes, and 3) are these sex differences independent of other covariates including adiposity and insulin resistance?

RESEARCH DESIGN AND METHODS

— The study design and methodology of this population-based investigation have been published previously (23,24). Briefly, participants in this study were originally enrolled as healthy control subjects in the Western New York Study, an epidemiologic case-control investigation of alcohol intake patterns and risk of CVD in Erie and Niagara Counties, New York, conducted from 1996 to 2001. The initial cohort of control participants was randomly selected from driver's license lists for those aged <65 years and from the Health Care Finance Administration rolls for those aged 65–79. In 2001–2004, we conducted the first follow-up of the apparently healthy myocardial infarction control group. Eligible participants for the follow-up study were men and women aged 39–79 years selected from the baseline examination cohort without known clinical CVD (self-reported myocardial infarction, angina, or revascularization surgery) or type 2 diabetes (measured fasting plasma glucose >125 mg/dl or self-report and taking medication) who were capable of completing the current study protocol (n = 2,652). Exclusion criteria also included self-report of a medical condition that would prohibit participation (e.g., all cancers except skin cancer, type 1 diabetes, or physical or mental impairment), a permanent change in residence to out of state, death, or inability to contact and determine eligibility. This left 2,139 individuals eligible for this examination of whom 1,455 completed the full clinic protocol (68.0% response rate). The mean ± SD follow-up time was 5.9 ± 0.8 years. The protocol was approved by the State University at Buffalo Health Science Institutional Review Board, and all participants provided written informed consent before participation.

At both the baseline and 6-year follow-up examinations, all participants received a clinical examination that included resting blood pressure and measures of height, weight, and waist girth. Resting seated blood pressure was obtained by trained and certified technicians according to a standardized protocol (25). Hypertension was defined as a systolic pressure ≥140 mmHg or a diastolic pressure ≥90 mmHg or use of antihypertensive medications regardless of blood pressure level. Height was recorded to the nearest one-quarter inch, and weight was measured to the nearest one-quarter pound. BMI (weight in kilograms divided by the square of height in meters) was calculated and served as a measure of relative weight. Waist girth (centimeters) was determined with participants standing erect with the abdomen relaxed, arms at the side, and feet together. A linen tape was placed horizontally between the bottom of the last rib and the top of the iliac crest around the smallest of these two reference points. The measurement was taken at end-expiration and recorded to the nearest 0.1 cm. Study subjects also provided a fasting (at least 10 h overnight) blood sample and were asked to refrain from smoking or vigorous physical activity for 24 h. Several standardized questionnaires were administered to determine cigarette use, physical activity (Stanford 7-Day Recall), alcohol use, general health and well-being, personal and family health history, medication use, and socioeconomic status. In this analysis, no one reported a positive history for gestational diabetes. Participants were instructed to bring all medications to the clinic visits to permit identification of oral medications as well as insulin use. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting glucose × fasting insulin/22.4 (26). A positive family history of type 2 diabetes was defined as a positive report in a first-degree relative. Data concerning family history of type 1 diabetes were not available.

Fasting glucose concentrations were determined by the glucose oxidase method (Beckman Instruments, Fullerton, CA). The interasssay coefficient of determination was <5%. Fasting triglycerides were measured with enzymatic techniques. Information on HDL cholesterol was not available. After identification of those who progressed (or not) to pre-diabetes, the baseline aliquots of serum or plasma were retrieved and sent by overnight courier to the Laboratory for Clinical Biochemistry Research, University of Vermont, Burlington, Vermont, or the Department of Endocrinology at the University of Pittsburgh, Pittsburgh, Pennsylvania, for assay. Interleukin-6, a major proinflammatory cytokine, was measured by an ultrasensitive enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Using this method, we have determined a routine coefficient of variation (CV) in the laboratory of 6.3%. Soluble E-selectin, also known as endothelial leukocyte adhesion molecule-1 and CD62E, was measured using a high-sensitivity quantitative sandwich enzyme (Parameter Human sE-Selectin Immunoassay; R&D Systems). Intra-assay and interassay CVs range from 4.7 to 5.0% and from 5.7 to 8.8%, respectively. Human soluble intercellular adhesion molecule-1 (sICAM-1) was measured by an enzyme-linked immunosorbent assay (Parameter Human sICAM-1 Immunoassay; R&D Systems). The laboratory CV was 5.0%. High-sensitivity C-reactive protein (hsCRP) was measured using the BNII nephelometer from Dade Behring with a particle-enhanced immunonepholometric assay. Intra-assay CVs range from 2.3 to 4.4%, and interassay CVs range from 2.1 to 5.7%. Adiponectin was assayed from a kit provided by Linco Research. This kit is a radioimmunoassay using a double antibody-polyethylene glycol separation. The assay has a detection limit of 1 ng/ml. The intra-assay CV ranges from 12.2 to 14.5%, and the interassay CV ranges from 3.7 to 6.1. Fasting insulin was assayed from a kit provided by Linco Research that has minimal cross-reactivity with human proinsulin. The assay has a lower detection limit of 2 μU/ml with an interassay CV ranging from 3.6 to 8.4% and an intra-assay CV ranging from 2.2 to 4.4%. A frozen spot urine sample was also retrieved and used for the assessment of the albumin-to-creatinine (ACR) ratio. Collections were done in the morning of the baseline visit and stored on liquid nitrogen. Albumin was assayed with a nephelometric immunoassay using a monospecific antiserum to human albumin. Creatinine concentration was determined using the modified Jaffé method. The urinary ACR (milligrams of albumin/grams of creatinine) was calculated and used as a surrogate of albumin excretion rate (27). Intraclass correlation coefficients among duplicate samples exceeded 0.97. A value exceeding 300 mg/g was considered to be evidence of overt nephropathy, and one study sample was omitted from these analyses. Of the values, >94% were <30 mg/g.

Statistical procedures

For these analyses, we identified a "case" of pre-diabetes as an individual whose

Table 1—*Age and age-adjusted mean levels of selected risk factors according to sex and case/control status*

	Women			Men			
Risk factor at baseline	Case subjects	Control subjects	*P* value	Case subjects	Control subjects	*P* value	*P* value for interaction*
n	52	156		39	117		
Age (years)	61.6 ± 10.8	56.5 ± 10.3	0.001	63.0 ± 11.3	60.9 ± 11.4	0.277	0.215
BMI (kg/m^2)	28.8 ± 5.4	27.5 ± 5.3	0.136	25.7 ± 4.9	24.7 ± 3.5	0.161	0.260
Waist (cm)	91.3 ± 12.9	86.2 ± 12.3	0.013	89.7 ± 12.8	86.9 ± 9.2	0.136	0.278
CRP (μg/ml)	4.7 ± 3.5	4.1 ± 2.9	0.236	2.5 ± 2.6	1.8 ± 1.8	0.047	0.951
Interleukin-6 (pg/ml)	3.0 ± 2.1	2.9 ± 1.6	0.734	1.8 ± 1.2	2.1 ± 1.8	0.236	0.337
E-selectin (ng/ml)	51.2 ± 17.9	45.5 ± 18.2	0.058	40.1 ± 16.9	42.0 ± 16.6	0.529	0.033
sICAM-1 (ng/ml)	274.7 ± 61.4	252.5 ± 52.8	0.012	252.2 ± 50.7	255.8 ± 57.7	0.733	0.016
PAI-1 (ng/ml)	37.3 ± 18.8	26.1 ± 16.3	<0.001	27.5 ± 15.6	28.4 ± 17.8	0.769	0.002
Adiponectin (ng/ml)	9.1 ± 5.2	11.4 ± 5.0	0.004	6.8 ± 4.4	7.2 ± 4.0	0.548	0.111
ln triglycerides (mg/dl)	4.7 ± 0.5	4.5 ± 0.5	0.024	4.7 ± 0.6	4.6 ± 0.5	0.172	0.227
Fasting glucose (mg/dl)	91.9 ± 4.8	88.3 ± 5.5	<0.001	94.0 ± 3.6	90.6 ± 5.1	<0.001	0.833
ln ACR (mg/g)	1.91 ± 0.8	1.79 ± 0.7	0.794	1.4 ± 0.9	1.4 ± 0.7	0.265	0.810
HOMA-IR	3.3 ± 1.6	2.8 ± 1.3	0.043	3.7 ± 1.9	3.1 ± 1.8	0.080	0.965
Hypertension (%)†	49.7	27.0	0.004	61.1	47.1	0.110	0.304

Data are means ± SD. *Interaction of sex by case-control status. †Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or receiving treatment.

fasting serum glucose was <100 mg/dl at baseline but was between 100 and 125 mg/dl (5.7 and 6.5 mmol/l) at follow-up. Each case patient was matched to three control subjects based upon sex, ethnicity (white or nonwhite), and year of study enrollment. Age was considered as a continuous covariate in all analyses. All control subjects had a fasting blood glucose concentration <100 mg/dl at both the baseline and follow-up examinations.

For this report, the data were analyzed using statistical techniques that incorporated the matching variables as covariates. The distributions of fasting triglycerides, ACR, and fasting insulin were log transformed (natural log) before analysis to approximate more normal distributions. General linear models (28) or logistic regression techniques (29) were used to compare the adjusted differences between case subjects and control subjects. Likelihood ratio tests were performed to determine statistical interactions between sex and case-control status by comparing the log likelihood between the two nested models, one with only the main effects and the other with both the main effects and the interaction terms in the model. All statistical tests were two-sided, and a *P* value <0.05 was considered statistically significant.

RESULTS — During a mean ± SD follow-up of 5.9 ± 0.8 years, 52 women, and 39 men progressed from normoglycemia to pre-diabetes (Table 1). Compared with the control subjects, the pre-diabetic women were older and had a higher mean waist circumference after adjustment for age, ethnicity, and year of study enrollment. They also displayed higher age-adjusted mean levels of E-selectin, sICAM-1, PAI-1, total triglycerides, and fasting glucose, a higher value for the HOMA-IR index, and a greater frequency of hypertension ($P < 0.05$). Mean concentrations of adiponectin were significantly lower among female case subjects compared with control subjects ($P = 0.004$). Among men, case subjects with pre-diabetes displayed significantly higher adjusted mean concentrations of hsCRP ($P = 0.047$) and a marginally higher mean value for the HOMA-IR index ($P = 0.08$). No significant differences were noted for any of the endothelial biomarkers or PAI-1. Statistically significant sex by case-control interactions were observed for E-selectin, sICAM-1, and PAI-1 (all $P < 0.05$). The results for adiponectin were suggestive but did not achieve a conventional level of statistical significance ($P = 0.111$), due perhaps to the limited sample size.

Table 2 presents the results adjusted further for BMI. Among women, differences in HOMA-IR and triglyceride concentrations diminished ($P = 0.149$ and 0.058, respectively). However, differences in E-selectin, sICAM-1, and adiponectin remained essentially unaltered. Among men, the results were also virtually unchanged, except for the HOMA-IR index, which was no longer significant ($P = 0.262$). The sex by case-control interactions noted in Table 1 remained significant. Additional adjustment for the HOMA-IR index (Table 3) resulted in nonsignificant differences in triglyceride concentrations among women. Formal tests for interaction between sex and case/control status were statistically significant for E-selectin ($P = 0.042$), PAI-1 ($P = 0.001$), sICAM-1 ($P = 0.011$), and the frequency of hypertension ($P < 0.001$) and were again suggestive but not statistically significant for adiponectin ($P = 0.131$). It was not possible to adjust the sex differences in risk factors for waist circumference because of the limited overlap in the distributions between men and women; i.e., adjustment for waist girth was equivalent to adjustment for sex. To examine whether differences in endothelial function were moderated by inflammation, we further adjusted for hsCRP (data not shown). The results remained virtually unchanged. Further consideration of weight change, family history of type 2 diabetes, physical inactivity, and use of lipid-lowering medications did not alter these findings.

CONCLUSIONS — It is clear from several studies that risk factors for CVD and diabetes are elevated long in advance of clinical diagnosis (9,11,13). Factors other than worsening hyperglycemia, however, are responsible for the origins of the increased risk of CHD in those with

Table 2—*Age- and BMI-adjusted mean levels of selected risk factors according to sex and case/control status*

	Women			Men			*P* value for
	Case subjects	Control subjects	*P* value	Case subjects	Control subjects	*P* value	interaction*
n	52	156		39	117		
Waist (cm)	87.9	85.6	0.023	92.9	92.4	0.627	0.219
CRP (μg/ml)	4.4	4.0	0.452	2.7	2.2	0.112	0.854
Interleukin-6 (pg/ml)	2.8	2.8	0.856	1.9	2.2	0.159	0.405
E-selectin (ng/ml)	51.0	45.5	0.073	41.1	43.6	0.424	0.036
sICAM-1 (ng/ml)	272.5	252.0	0.022	257.8	264.4	0.523	0.017
PAI-1 (ng/ml)	34.7	25.9	0.001	29.2	31.6	0.420	0.003
Adiponectin (ng/ml)	9.7	11.4	0.023	6.6	6.9	0.636	0.189
ln triglycerides (mg/dl)	4.7	4.5	0.058	4.7	4.6	0.213	0.257
Fasting glucose (mg/dl)	91.6	88.3	<0.001	94.2	90.7	<0.001	0.973
ln ACR (mg/g)	1.6	1.5	0.711	1.8	1.8	0.801	0.823
HOMA-IR	3.3	2.7	0.149	3.6	3.3	0.262	0.829
Hypertension (%)†	46.1	26.9	0.011	65.5	54.5	0.203	0.320

Data are means. *Interaction of sex by case-control status. †Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or receiving treatment.

diabetes. How long in advance of clinical diabetes this risk can be detected and whether or not sex differences may be observed are poorly understood.

Most extant studies have documented progression from impaired glucose tolerance to diabetes and have naturally focused on the traditional CVD risk factors that predict conversion to diabetes (13,30). Our report supports and extends previous work by noting the novel role that several emerging risk factors may play in progression from normoglycemia to "pre-diabetes," particularly among women.

Our results are clearly important as recent clinical trial evidence shows that delay in development or prevention of diabetes is possible, and preventive efforts should occur early in the pre-diabetic state (31,32). Whether certain prevention efforts or treatment should be sex specific awaits further study. However, this is among the first reports to show that women are more likely than men to manifest elevated levels of endothelial factors, fibrinolysis/thrombosis, and adiponectin during the transition from normoglycemia to pre-diabetes. Previous findings by Haffner et al. (12) demonstrated that those who progressed to type 2 diabetes were likely to have a more atherogenic cardiovascular risk profile than those who did not. Most of the study subjects had impaired glucose tolerance at baseline, however. The same pattern held true when the analyses were confined to normoglycemic individuals at the start of follow-up, consistent with our results.

Nondiabetic women are more likely to have a more favorable CVD risk profile than nondiabetic men and are less likely to develop and die from CVD at any age, findings that are not explained by sex differences in obesity or other metabolic or behavioral factors. One hypothesis to explain this "female advantage" is that women (especially younger women) are more insulin sensitive (less insulin resistant) than their male counterparts, an advantage that is diminished or abolished in the diabetic state (33). Surrogate indexes of insulin resistance including the HOMA-IR have been positively associated with E-selectin, PAI-1, and other endothelial markers (34,35). In this report, consideration of the HOMA-IR index failed to reduce or eliminate the sex differences in the risk factors of interest. Covariate adjustment for the HOMA-IR may

Table 3—*Age-, BMI-, and HOMA-IR–adjusted mean levels of selected risk factors according to sex and case/control status*

	Women			Men			*P* value for
	Case subjects	Control subjects	*P* value	Case subjects	Control subjects	*P* value	interaction*
n	52	156		39	117		
Waist (cm)	87.7	85.8	0.056	93.2	92.5	0.511	0.354
CRP (μg/ml)	4.5	4.1	0.457	2.8	2.2	0.110	0.845
Interleukin-6 (pg/ml)	2.8	2.9	0.762	1.9	2.2	0.225	0.458
E-selectin (ng/ml)	51.2	45.5	0.062	40.5	43.2	0.374	0.036
sICAM-1 (ng/ml)	273.0	254.5	0.036	254.8	264.0	0.374	0.010
PAI-1 (ng/ml)	34.6	27.0	0.003	26.8	31.7	0.097	0.001
Adiponectin (ng/ml)	9.6	11.1	0.055	6.8	6.9	0.903	0.131
ln triglycerides (mg/dl)	4.6	4.5	0.230	4.7	4.6	0.363	0.306
ln ACR (mg/g)	1.6	1.5	0.783	1.7	1.8	0.523	0.765
Hypertension (%)†	44.5	28.8	0.035	63.6	54.8	0.306	0.331

Data are means. *Interaction of sex by case-control status. †Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or receiving treatment.

not completely capture the effects of insulin resistance, however, as it is only modestly correlated with insulin resistance assessed with the hyperinsulinemic-euglycemic clamp method (36).

Several studies have shown that elevated concentrations of E-selectin and PAI-1 are significant independent predictors of both CHD and type 2 diabetes (18,19). Few studies have examined these novel risk factors with respect to the development of pre-diabetes in a population-based cohort. Caballero et al. (37) found abnormalities in both microvascular and macrovascular reactivity as well as abnormalities in markers of endothelial activation that were present in individuals at risk of developing type 2 diabetes, even at a stage of normoglycemia. Retinal microvascular abnormalities have been associated with blood pressure, inflammation, and endothelial dysfunction (38). Such microvascular abnormalities predicted ischemic heart disease in women but not men (39). Proinflammatory markers are also associated with endothelial dysfunction (40). However, covariate adjustment for hsCRP levels failed to remove the sex difference in these endothelial markers in our study.

Other risk factors for diabetes include impaired fibrinolysis characterized by elevated concentrations of PAI-1. Indeed, data from the Insulin Resistance Atherosclerosis Study (IRAS) indicated that PAI-1 was among the most predictive of all biomarkers examined (19). PAI-1 is associated with insulin resistance in many studies, although consideration of HOMA-IR did not eliminate the sex difference in this report. Because PAI-1 is secreted from hepatocytes and adipose tissue in addition to endothelium, it may function through a pathway different from that for the cellular adhesion molecules (41) to effect progression risk. Whether these emerging risk factors operate more strongly among women requires further investigation.

Abnormally elevated rates of albumin excretion have been suggested to reflect endothelial dysfunction or other vascular damage in several studies (42). In the current study, the ACR was not significantly related to progression to pre-diabetes in either sex. This could be due, at least in part, to the low prevalence of microalbuminuria in this cohort.

The strengths of our study include its population-based design, the measurement of several emerging risk factors including inflammation and endothelial dysfunction, and the detailed assessment of several covariates of interest. Limitations include the single determination of fasting glucose concentrations used to define pre-diabetes, although this definition is consistent with recent recommendations from the American Diabetes Association and should affect case subjects and control subjects similarly. Although fasting glucose levels show greater correlation over time than postchallenge levels (43), the use of an oral glucose tolerance test may have identified people with type 2 diabetes at follow-up who were classified as pre-diabetic. Finally, measures of endothelial markers were obtained only once, which could have led to some misclassification, although this also should tend to affect the case subjects and the comparison group similarly. It should be noted that the samples were not subjected to repeated freeze-thaw cycles. Lastly, although observational studies cannot prove causality, our data add compelling new evidence that the clock may indeed start ticking earlier among women than among men.

In summary, in this prospective study on the role of endothelial dysfunction and progression to pre-diabetes, we found that E-selection, sICAM-1, and PAI-1 concentrations were predictive of conversion among women but not among men. These results were independent of the effects of age, BMI, and HOMA-IR. These novel and important observations support a role for endothelial dysfunction in the progression to pre-diabetes.

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