A Prospective Study of Soluble Tumor Necrosis Factor-α Receptor II (sTNF-RII) and Risk of Coronary Heart Disease Among Women With Type 2 Diabetes

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OBJECTIVE — Tumor necrosis factor-α (TNF-α), a cytokine secreted by adipose tissue and other cells, might play a role in insulin resistance.

RESEARCH DESIGN AND METHODS — Of 32,826 women from the Nurses' Health Study who provided blood at baseline, we followed 929 women with type 2 diabetes. During 10 years of follow-up, we documented 124 incident cases of coronary heart disease (CHD).

RESULTS — After adjustment for age, smoking, BMI, and other cardiovascular risk factors, the relative risks (RRs) comparing extreme quartiles of soluble TNF-α receptor II (sTNF-RII) were 2.48 (95% CI 1.08–5.69; $P = 0.034$) for myocardial infarction (MI) and 2.02 (1.17–3.48; $P = 0.003$) for total CHD. The probability of developing CHD over 10 years was higher among diabetic subjects with substantially higher levels of both sTNF-RII (>75th percentile) and HbA_{1c} (>7%), compared with diabetic subjects with lower levels (25% vs. 7%, $P < 0.0001$). Diabetic subjects with only higher sTNF-RII or HbA_{1c} had similar (16–17%) risk. In a multivariate model, diabetic subjects with higher levels of both sTNF-RII and HbA_{1c} had an RR of 3.66 (1.85–7.22) for MI and 3.03 (1.82–5.05) for total CHD, compared with those with lower levels of both biomarkers.

CONCLUSIONS — Increased levels of sTNF-RII were strongly associated with risk of CHD among diabetic women, independent of hyperglycemia.

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Abbreviations: CABG, coronary bypass surgery; CHD, coronary heart disease; CRP, C-reactive protein; MI, myocardial infarction; NHS, Nurses' Health Study; PTCA, coronary angioplasty; sICAM; soluble intercellular adhesion molecule; sTNF-RII, soluble tumor necrosis factor-α receptor II; TNF-α, tumor necrosis factor-α.

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

Tumor necrosis factor-α (TNF-α) is a mediator of obesity-related insulin resistance (1). This multifunctional cytokine is produced primarily by several types of cells involved in the atherosclerotic process, including macrophages, endothelial cells, and smooth muscle cells (2), but is also derived from muscle cells (3) and adipose tissue (4). Its expression is increased by obesity (5) and decreased by weight reduction (6). Deletion of TNF-α or its receptors in obese knockout mice resulted in improved insulin sensitivity (7), and the actual defect induced by TNF-α is likely to be at or near the insulin receptor itself (8). TNF-α stimulates hepatic secretion of VLDL triglyceride (9,10).

Given the strong links among obesity, inflammation (11), type 2 diabetes, and vascular condition (12,13) and the potential role of TNF-α in insulin resistance, lipid metabolism, and inflammation, we hypothesize that TNF-α is associated with the development of coronary heart disease (CHD) in patients with type 2 diabetes. To test this hypothesis, we examined prospectively the association between plasma levels of soluble TNF-α receptor II (sTNF-RII, as a measure of TNF-α in plasma) and risk of CHD among women with type 2 diabetes in the Nurses' Health Study (NHS).

RESEARCH DESIGN AND METHODS

— The NHS was initiated in 1976 with the enrollment of 121,700 U.S. nurses aged 30–55 years. This prospective cohort study is followed through questionnaires related to lifestyle factors and health outcomes that are mailed biennially. Of 32,826 study participants who provided blood samples in 1989–1990, 1,194 had a confirmed diagnosis of type 2 diabetes. The present study included 929 women who did not report a diagnosis of myocardial infarction (MI), coronary bypass surgery (CABG), coronary angioplasty (PTCA), or stroke at the

time blood was drawn and had complete biomarker data.

Definition of diabetes

Cases of diabetes were reported by the respondents on the biennial questionnaires. We mailed a supplementary questionnaire to all women reporting a diagnosis of diabetes to obtain further information about the date of diagnosis, symptoms, diagnostic tests, and treatment for hypoglycemia. In accordance with the criteria of the National Diabetes Data Group (14), confirmation of diabetes required at least one of the following self-reports on the supplementary questionnaire: *1*) elevated plasma glucose concentration (fasting plasma glucose ≥7.8 mmol/l, random plasma glucose ≥11.1 mmol/l, and/or plasma glucose ≥11.1 mmol/l after ≥2 h during an oral glucose tolerance test) plus at least one classic symptom (excessive thirst, polyuria, weight loss, or hunger), 2) no symptoms but at least two elevated plasma glucose concentrations (by the above criteria) on different occasions, or 3) treatment with hypoglycemic medication (insulin or oral hypoglycemic agent). We used the National Diabetes Data Group criteria to define diabetes because in our participants diabetes was diagnosed before the American Diabetes Association released their criteria in 1997 (15). The validity of self-reported diagnosis of type 2 diabetes by the supplementary questionnaire has been established by a separate validation study through medical record reviews. Permission for medical record review was provided by 71 of 84 women, medical records were obtained for 62, and the diagnosis of type 2 diabetes was confirmed in 98.4% (16).

CHD end points

CHD outcomes consisted of fatal CHD, nonfatal MI, and CABG/PTCA. The end point did not include angina pectoris. Nonfatal MI was confirmed by reviewing medical records using the criteria of the World Health Organization of symptoms plus either typical electrocardiographic changes or elevated levels of cardiac enzymes (17). Physicians who reviewed the records were blind to the self-reported risk-factor status. Deaths were reported by next of kin, through the postal system, and through records of the National Death Index. With the use of all sources combined, the follow-up for deaths was >98% complete (18). Fatal CHD was confirmed by review of medical records or autopsy reports with the permission of the next of kin. In no instance was the cause on the death certificate accepted without corroboration. Sudden deaths (i.e., death within 1 h of symptom onset in a woman without known disease that could explain death) were included in the fatal CHD category.

Laboratory methods

Blood samples were centrifuged and aliquotted into cryotubes as plasma, buffy coat, and erythrocytes. Cryotubes were stored in liquid-nitrogen freezers at −130°C or lower.

Plasma was assayed for the presence of sTNF-RII using the Human sTNF-RII ELISA kit (R&D Systems, Minneapolis, MN). The coefficient of variation (CV) range was 2.6–4.8%. Plasma C-reactive protein (CRP) was measured by using the US CRP ELISA kit (DSL, Webster, TX) with a CV range of 2.8–5.1%. Because the CRP levels from the current assay consistently read higher CRP levels compared with an assay previously used in our studies (19), we performed a cross-validation study utilizing 204 samples obtained from the diabetic women cohort, in which the CRP levels were measured by both methods. The correlation coefficient between the two methods was 0.97, suggesting that the results reported using these assays should be comparable. Soluble intercellular adhesion molecules (sICAM-1s) were assayed in plasma using the Human sICAM-1 ELISA kit (R&D Systems) with a CV range of 3.3–4.8%. Plasma levels of sE-selectin were assayed by using the Human sE-selectin ELISA kit (R&D Systems) with a CV range of 5.7–8.8%. Concentrations of HbA_{1c} were based on turbidimetric immunoinhibition with hemolyzed whole blood or packed red cells with a CV of <3.0%. Fibrinogen was measured on a Hitachi 911 analyzer with reagents and calibrators from Kamiya Biomedical (Seattle, WA) with a CV of 1.16%. The concentrations of total cholesterol, HDL cholesterol, and triglycerides were measured simultaneously on the Hitachi 911 analyzer with reagents and calibrators from Roche Diagnostics (Indianapolis, IN); the CVs for these measurements were <1.8%. Concentrations of LDL cholesterol were measured by a homogenous direct method from Genzyme (Cambridge, MA) with a CV <3.1%. Concentrations of apolipoprotein B_{100} were measured in an immunonephelometric assay with reagents and calibrators from Wako Chemicals (Richmond, VA) with a CV <5%.

Assessment of lifestyle exposures

Participants provided information biennially on their lifestyle exposures. We calculated BMI as the ratio of weight (kilograms) to the square of height (meters). A history of high blood pressure was determined from self-reports preceding blood collection. A parental history of CHD (before age 60) was reported in 1976. Alcohol intake was assessed with validated (20) dietary questionnaires in 1990, 1994, and 1998.

Statistical analysis

The women were followed from June 1990 through June 2000. We used Cox proportional hazards analysis stratified on 5-year age categories and over each 2-year follow-up interval to estimate the relative risks (RRs) for each biomarker quartile compared with the lowest quartile. Accumulation of person-months of follow-up started in June 1990. Participants in whom CHD or stroke was diagnosed or who died during follow-up were censored at the date of diagnosis or death. All other participants were followed through June 2000. Tests of linear trend across increasing categories of sTNF-RII were conducted by treating the categories as a continuous variable and assigning the median intake for the category as its value. We performed Kaplan-Meier survival analysis to compare the CHD survival probabilities of persons with combinations of HbA_{1c} (≤/>7%) and sTNF-RII (≤/>75th percentile) levels. Finally, we evaluated the joint RR of sTNF-RII (≤/>75th percentile) and HbA_{1c} (≤/>7%) levels in a multivariate model. All statistical analyses were performed with SAS 8.0 statistical software (SAS Institute, Cary, NC).

RESULTS — During 10 years of follow-up (6,889 person-years), we documented 124 incident cases of CHD (46 nonfatal MI, 22 fatal CHD, and 56 CABG/PTCA) among 929 women with confirmed type 2 diabetes. Women with higher levels of sTNF-RII (Table 1) were older, heavier, and more sedentary and tended to consume less alcohol. Among women with higher levels of sTNF-RII,

Table 1—*Baseline characteristics across quartiles of sTNF-RII among 929 women with type 2 diabetes: the NHS*

	Quartiles of sTNF-RII			
	1	2	3	4
Median sTNF-RII (pg/ml)	1768.6	2205.1	2616.7	3322.7
Range sTNF-RII (pg/ml)	1,005.1–2,006.8	2,010.8–2,370.6	2,373.2–2,888.1	2,891.6–9,355.3
Age (years)	56.6	57.7	59.0	60.0
BMI (kg/m^2)	27.3	29.2	30.9	32.4
Current smokers	32 (14)	25 (11)	39 (17)	35 (14)
Physical activity (h/week)	3.0	2.8	2.8	2.3
Alcohol consumption (g/day)	4.5	2.7	2.2	1.9
Duration of type 2 diabetes (years)	7.4	7.9	7.1	9.1
Daily aspirin use	74 (33)	72 (32)	81 (35)	87 (35)
Postmenopausal hormone use	68 (30)	66 (29)	62 (27)	57 (23)
Insulin use	32 (14)	36 (16)	39 (17)	72 (29)
Oral diabetic medication use	38 (17)	52 (23)	42 (18)	54 (22)
Multivitamin supplement use	95 (42)	86 (38)	79 (34)	94 (38)
History of hypertension	115 (51)	120 (53)	143 (62)	170 (69)
History of hypercholesterolemia	126 (56)	118 (52)	120 (52)	131 (53)
History of angina pectoris	27 (12)	18 (8)	28 (12)	27 (11)
Parental history of MI	43 (19)	56 (25)	55 (24)	59 (24)
Biomarkers				
HbA_{1c} (%)	6.45	6.87	6.80	7.29
CRP (mg/l)*	4.7	7.2	8.8	11.7
sICAM-1 (ng/ml)	271.1	290.6	325.5	374.6
sE-selectin (ng/ml)	55.7	60.0	68.5	79.5
Fibrinogen (mg/dl)	349.4	367.5	386.2	397.4
Triglycerides (mg/dl) †	187.2	203.0	200.3	217.7
HDL cholesterol (mg/dl)	56.0	52.6	49.5	48.6
LDL cholesterol (mg/dl)	145.5	137.7	138.7	135.2
Apolipoprotein B_{100} (mg/dl)	103.4	101.9	101.7	102.1
Total cholesterol–to–HDL cholesterol ratio	4.6	4.7	4.7	4.9

Data are *n* (%) unless otherwise indicated. Means and percentages (except for age) are age adjusted for the age-groups: <49, 50–54, 55–59, 60–64, and ≥65 years. *According to a regression between two CRP assays among subgroup of 204 diabetic women ($r = 0.97$ corrected CRP = 0.4 + 0.67*x*). †Similar levels among 622 women who reported fasting >8 h before blood was drawn.

the proportion using postmenopausal hormones was lower, whereas the proportion using insulin and oral diabetic medications was higher. Women with higher levels of sTNF-RII were more likely to have had a longer duration of type 2 diabetes and to have a history of hypertension and parental history of MI. Increasing levels of sTNF-RII were associated with higher levels of HbA_{1c}, CRP, sICAM-1, sE-selectin, fibrinogen, and triglycerides and a higher total cholesterol–to–HDL cholesterol ratio.

Higher levels of sTNF-RII were strongly associated with a progressively higher risk of MI (Table 2). The age-adjusted RR for extreme quartiles was 3.01 (95% CI 1.37–6.64). The association was modestly attenuated in a multivariate model, adjusted for age, smoking, BMI, alcohol intake, physical activity, postmenopausal hormone use, aspirin use, parental history of MI, history of hypertension, history of angina pectoris, and total cholesterol–to–HDL cholesterol ratio (RR for the extreme quartiles 2.48 [95% CI 1.08–5.69], $P = 0.034$). The association was weaker for CABG/PTCA than for MI. The overall multivariate-adjusted RR of total CHD events between extreme quartiles of sTNF-RII was 2.02 (1.17–3.48, $P = 0.003$). This association was not appreciably changed after further adjustments for duration of type 2 diabetes, levels of HbA_{1c}, triglycerides, or CRP, as well as for levels of sICAM-1, fibrinogen, or sE-selectin (data not shown). After adjusting for age, smoking, BMI, alcohol intake, physical activity, postmenopausal hormone use, aspirin use, parental history of MI, history of hypertension, history of angina pectoris, and total cholesterol–to–HDL cholesterol ratio, addition of sTNF-RII further improved the prediction model beyond that provided by other risk factors ($P < 0.001$ based on the likelihood ratio test). In a secondary analysis, when excluding 248 participants in whom diabetes was diagnosed after blood was drawn ($n = 108$ remaining CHD cases) the multivariate adjusted RR for the extreme quartiles of sTNF-RII was 1.71(0.96–3.07, $P = 0.022$).

The association of sTNF-RII with CHD persisted in subgroup analysis according to categories of BMI, postmenopausal hormone use, duration of diabetes, alcohol intake, and levels of HbA_{1c} and CRP (data not shown). However, after adjusting for cardiovascular risk factors and sTNF-RII, CRP was not significantly associated with risk of CHD in diabetic women (RR comparing the highest with lowest quartile of CRP 1.03 [95% CI 0.59–1.81], $P = 0.904$). The other in-

Table 2—*Relative risks of MI, CABG/PTCA, and total CHD events across quartiles of sTNF-RII among 929 women with type 2 diabetes: the NHS*

	Quartiles of sTNF-RII				
	1	2	3	4	*P* value
Median (pg/ml)	1,768.6	2,205.1	2,616.7	3,322.7	
MI (fatal + nonfatal)					
n	8	16	16	28	
Age adjusted	1.00	1.89 (0.81–4.41)	1.80 (0.77–4.22)	3.01 (1.37–6.64)	0.005
MV*	1.00	1.86 (0.78–4.41)	1.62 (0.67–3.88)	2.48 (1.08–5.69)	0.034
CABG/PTCA					
n	13	6	14	23	
Age adjusted	1.00	0.46 (0.18–1.22)	1.04 (0.48–2.22)	1.64 (0.82–3.27)	0.031
MV*	1.00	0.53 (0.20–1.41)	1.07 (0.49–2.37)	1.77 (0.84–3.70)	0.041
CHD (MI + CABG/PTCA)					
n	21	22	30	51	
Age adjusted	1.00	1.02 (0.56–1.85)	1.33 (0.76–2.33)	2.16 (1.29–3.62)	<0.001
MV*	1.00	1.09 (0.60–2.01)	1.28 (0.72–2.29)	2.02 (1.17–3.48)	0.003
MV + duration of type 2 diabetes		1.02 (0.55–1.87)	1.25 (0.67–2.22)	1.87 (1.08–3.23)	0.007
MV + TGs	1.00	1.11 (0.61–2.04)	1.26 (0.70–2.24)	1.99 (1.16–3.45)	0.005
MV + HbA_{1c}	1.00	1.03 (0.56–1.89)	1.19 (0.67–2.13)	1.79 (1.04–3.10)	0.014
MV + CRP	1.00	1.08 (0.59–1.99)	1.27 (0.71–2.28)	2.04 (1.17–3.56)	0.004
MV + HbA_{1c} + CRP	1.00	1.03 (0.56–1.90)	1.21 (0.68–2.16)	1.86 (1.06–3.25)	0.011

Data are RR (95% CI) unless noted. *Multivariate model (MV), Cox regression, adjusted for age (<49, 50–54, 55–59, 60–64, ≥65 years), smoking (current, past, never), BMI (<23, 23–25, 25–28, 28–30, 30–34, ≥35 kg/m^2), alcohol intake (0, 0.1–4.9, ≥5 g/day), physical activity (0–1, 1–2, 2–4, ≥4 h/week), postmenopausal hormone use (premenopausal, current, past, never, missing), aspirin use (nondaily, daily), parental history of MI, history of hypertension, history of reported angina pectoris, and ratio of total cholesterol to HDL (quartiles).

flammation markers measured were not associated with the outcome.

The probabilities (Fig. 1) of developing CHD during 10 years of follow-up was significantly higher among diabetic women with higher levels of both sTNF-RII (>75th percentile) and HbA_{1c} (>7%) compared with women with lower levels (25% vs. 7%). Diabetic women with higher sTNF-RII or HbA_{1c} had intermediate (16–17%) risk ($P < 0.0001$). We examined the joint effect of sTNF-RII and glycemic control in a multivariate model (Fig. 2). Diabetic women with higher levels of both sTNF-RII and HbA_{1c} had RRs of 3.66 (95% CI 1.85–7.22) for MI and 3.03 (1.82–5.05) for total CHD events compared with diabetic women with lower levels of both biomarkers (*P* for interaction = 0.99 and 0.40, respectively).

CONCLUSIONS — In this prospective cohort study among women with type 2 diabetes, we found that higher levels of sTNF-RII were associated with an increased risk of developing CHD, independent of established cardiovascular risk factors. The increased CHD risk with higher sTNF-RII was independent of poor glycemic control.

To our knowledge, this is the first prospective study of the relationship between sTNF-RII levels and risk of CHD in diabetic patients. The strengths of the study include our well-established cohort with detailed and repeated measures of lifestyle exposures. Potential limitations also need to be considered. First, we did not directly measure plasma TNF-α in the frozen specimens. However, sTNF receptors derived by proteolytic cleavage of the TNF cell surface receptor have a high correlation with TNF-α, a longer half-life, and a higher ability of detection than that for TNF-α (21). Second, although a single measurement of biomarkers may be susceptible to short-term variation, we found that the intraclass correlation was high (0.78) for sTNF-RII for blood drawn 4 years apart in a subgroup of 82 blood samples from the Health Professional Follow-up Study. With respect to short-term stability, analysis of blood samples from the NHS showed that the levels of the inflammatory biomarkers sTNF-RII, CRP, and vascular cell adhesion molecule 1 were stable and statistically consistent for up to 36 h from collection to processing (22). Finally, we recognize that the NHS cohort does not represent a random sample of U.S. women. Nevertheless, the homogeneity of the cohort for educational attainment and socioeconomic status reduces the likelihood of residual and unmeasured confounding.

TNF-α, originally identified as a factor that promoted hemorrhagic necrosis in transplanted tumors (23), is associated with inhibition of insulin receptor signaling, a decrease in lipoprotein lipase activity, repression of the glucose transporter GLUT4, and induction of hyperlipidemia (24). TNF-α impairs insulin signaling by inhibiting the function of insulin receptor substrate 1 through serine phosphorylation (25) and may mediate insulin resistance through indirect effects, including increased free fatty acid oxidation, stimulation of insulin counter regulatory hormones or cytokines, and impairment of endothelial function (26). Adipose tissue is a major source of endogenous production of TNF-α; elevation in the level of TNF-α may be a critical mechanism by which fat cells induce peripheral insulin resistance (27).

Circulating TNF-α levels are elevated in obese insulin-resistant or diabetic patients, (28,29), but the role of TNF-α in the insulin resistance of human type 2 di-

Number of people at risk

year	0	1	2	3	4	5	6	7	8	9
Low sTNF-RII and low HbA_{1c}	456	454	446	443	441	436	424	417	412	400
low sTNF-RII and high HbA_{1c}	127	124	120	119	112	112	105	101	101	91
High sTNF-RII and low HbA_{1c}	226	224	221	215	204	204	197	188	185	174
High sTNF-RII and high HbA_{1c}	120	117	113	106	100	96	91	82	82	74

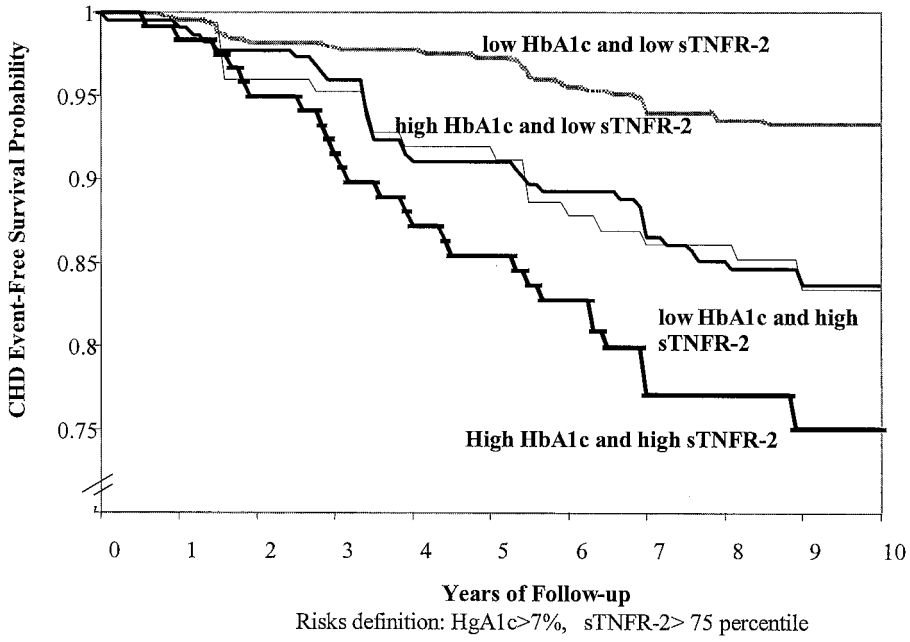

Figure 1—*CHD event–free survival among women with type 2 diabetes, stratified by risk combinations of HbA_{1c} and sTNF-RII: the NHS.*

abetes remains unclear (30). Although our cohort is based on women with confirmed type 2 diabetes rather than solely with insulin resistance, the results provide further support for an important role of TNF-α in the metabolic syndrome. Few previous prospective studies have assessed the association between TNF-α and CHD in nondiabetic populations. In a nested case-control analysis (31), the excess risk of recurrent coronary events 9 months after an initial MI among 272 individuals was predominantly seen among those with the highest (>95th percentile) levels of TNF-α (RR 2.7 [95% CI 1.4–5.2]). Among participants aged 70–79 years (32), TNF-α showed significantly positive associations with CHD risk (1.22

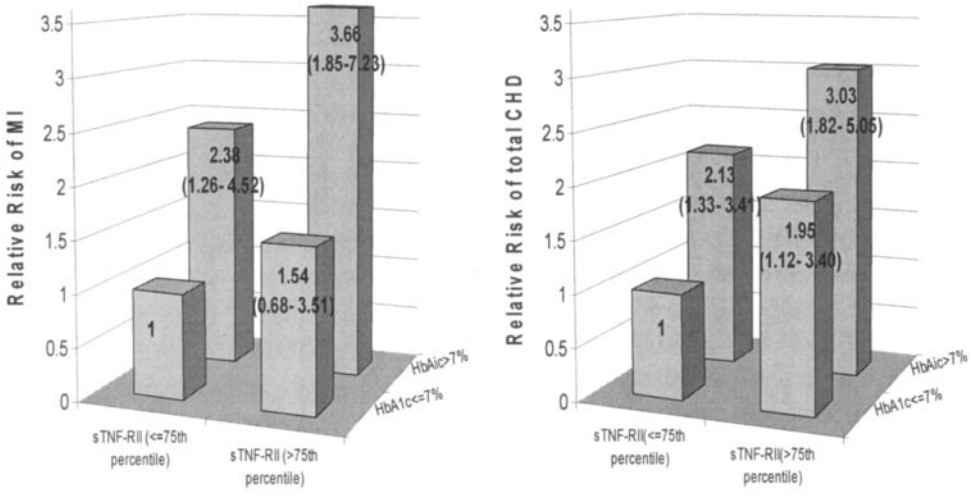

Figure 2—*Multivariate-adjusted RRs of MI and total CHD among women with type 2 diabetes according to baseline levels of sTNF-RII and HbA_{1c}: the NHS. The multivariate model, with Cox regression, was adjusted for age (<49, 50–54, 55–59, 60–64, ≥65 years), smoking (current, past, never), BMI (<23, 23–25, 25–28, 28–30, 30–34, ≥35 kg/m^2), alcohol intake (0, 0.1–4.9, ≥5 g/day), physical activity (0–1, 1–2, 2–4, ≥4 h/week), postmenopausal hormone use (premenopausal, current, past, never, missing), aspirin use (nondaily, daily), parental history of MI, history of hypertension, history of reported angina pectoris, and ratio of total cholesterol to HDL cholesterol (quartiles).*

[1.04–1.43] per 1 SD increase) during an average follow-up of 3.6 years.

Type 2 diabetes is characterized by progressive β-cell secretory dysfunction after insulin resistance, which is present many years before the onset of hyperglycemia in most patients. Both diabetes and CHD have been suggested to be vascular conditions with the shared pathophysiology of vascular endothelial dysfunction, promoted by inflammation (12,13). We recently showed an association between increased levels of sTNF-RII and a significant risk of developing type 2 diabetes among apparently healthy women in the NHS (19), although further adjustment for CRP substantially attenuated this association. In the current study, the role of sTNF-RII in predicting CHD remained robust and independent of its downstream inflammatory biomarkers including CRP, suggesting a unique role of sTNF-RII in atherosclerosis among diabetic women.

The combination of higher levels of both sTNF-RII and HbA_{1c} resulted in RRs of 3.6 for MI and 3.0 for total CHD in our study. In the U.K. Prospective Diabetes Study, each 1% reduction in HbA_{1c} was significantly associated with reductions in risk of 21% for any end point related to diabetes (33). In the Norfolk cohort of the European Prospective Investigation into Cancer and Nutrition, an increase of 1% in HbA_{1c} was independently associated with a 28% increase in risk of death (34), suggesting that HbA_{1c} is a strong predictor of mortality risk in diabetes. Hyperglycemia increased circulating TNF-α and IL-6 levels by an oxidative mechanism, suggesting a causal role for hyperglycemia in the immune activation of diabetes (35). However, these results should be interpreted with caution because the study did not provide a control group. At our study, levels of sTNF-RII were associated with CHD risk independent of $HbA_{1c.}$ The additive effects of poor glycemic control and inflammation suggest that glycemic control alone may account for most but not all of the CHD complications in diabetic women.

In conclusion, we found that high plasma levels of sTNF-RII were significantly associated with increased incidence of CHD, independent of established cardiovascular risk factors. The additive effects of poor glycemic control and inflammation suggest that both mechanisms may play a role in the development of vascular complications in diabetic women.

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