# Association of Oxidative Stress, Insulin Resistance, and Diabetes Risk Phenotypes

## The Framingham Offspring Study

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**OBJECTIVE** — Systemic oxidative stress causes insulin resistance in rodents. We tested the hypothesis that oxidative stress and insulin resistance are associated in humans.

**RESEARCH DESIGN AND METHODS** — We used cross-sectional data from 2,002 nondiabetic subjects of the community-based Framingham Offspring Study. We measured insulin resistance with the homeostasis model and defined categorical insulin resistance as homeostasis model assessment of insulin resistance (HOMA-IR) >75th percentile. We measured oxidative stress using the ratio of urine 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) to creatinine and used age- and sex-adjusted regression models to test the association of oxidative stress with insulin resistance in individuals without diabetes and among subgroups at elevated risk of diabetes.

**RESULTS** — Across 8-epi-$PGF_{2\alpha}$/creatinine tertiles, the prevalence of insulin resistance increased (18.0, 27.5, and 29.4% for the first, second, and third tertiles, respectively; $P < 0.0001$), as did mean levels of HOMA-IR (3.28, 3.83, and 4.06 units; $P < 0.0001$). The insulin resistance–oxidative stress association was attenuated by additional adjustment for BMI ($P = 0.06$ across tertiles for insulin resistance prevalence; $P = 0.004$ for mean HOMA-IR). Twenty-six percent of participants were obese (BMI ≥30 kg/m$^2$), 39% had metabolic syndrome (according to the Adult Treatment Panel III definition), and 37% had impaired fasting glucose (IFG) (fasting glucose 5.6–6.9 mmol/l). Among 528 obese participants, respectively, insulin resistance prevalence was 41.3, 60.6, and 54.2% across 8-epi-$PGF_{2\alpha}$/creatinine tertiles ($P = 0.005$); among 781 subjects with metabolic syndrome, insulin resistance prevalence was 41.3, 56.7, and 51.7% ($P = 0.0025$); and among 749 subjects with IFG, insulin resistance prevalence was 39.6, 47.2, and 51.6% ($P = 0.04$).

**CONCLUSIONS** — Systemic oxidative stress is associated with insulin resistance in individuals at average or elevated risk of diabetes even after accounting for BMI.

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**Abbreviations:** 8-epi-$PGF_{2\alpha}$, 8-epi-prostaglandin $F_{2\alpha}$; CVD, cardiovascular disease; FPG, fasting plasma glucose; HOMA-IR, homeostasis model assessment of insulin resistance; IFG, impaired fasting glucose; NF-κB, nuclear factor-κB; NOS, nitric oxide synthase.

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



Type 2 diabetes is extremely common and increasing rapidly worldwide. The diabetes epidemic is driven, in part, by a parallel epidemic of obesity (1). Whereas obesity is a major risk factor for type 2 diabetes, the mechanisms whereby excess body fat leads to diabetes remain uncertain. Insulin resistance and obesity-associated traits comprising the metabolic syndrome account for some of the risk (2). However, only ~50% of obese individuals at risk for diabetes are insulin resistant, suggesting that other factors are involved in obesity-related diabetes risk (3). Recent evidence demonstrates that obesity is a key determinant of systemic oxidative stress in humans (4). Oxidative stress, in turn, is a determinant of insulin resistance, at least in rodents (5). Oxidative stress may be one pathway whereby obesity, insulin resistance, and the metabolic syndrome lead to type 2 diabetes in humans.

Markers of systemic oxidative stress are elevated in clinical type 2 diabetes (6), but there are only limited data relating the degree of oxidative stress to insulin resistance in pre-diabetic states (7–15). Investigations have been impeded by limited availability of reliable biomarkers of oxidative stress for use in epidemiological studies. We measured two such markers, urinary concentrations of creatinine-indexed 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) (16) and plasma concentrations of myeloperoxidase (17), in subjects of the Framingham Offspring Study. We used these data to test the hypothesis that elevated levels of oxidative stress markers are associated with insulin resistance in individuals without diabetes and that these relations are present after accounting for variation in BMI. We also tested the hypothesis that oxidative stress and insulin resistance are associated in subgroups of individuals with high type 2 diabetes risk phenotypes, including obesity, impaired fasting glucose (IFG), and metabolic syndrome.

## RESEARCH DESIGN AND METHODS

— The Framingham Offspring Study is a community-based

**Table 1—*Study sample characteristics***

| Characteristics | Measures |
|---|---|
| *n* | 2,002 |
| Age (years) | 60 ± 9.3 |
| Range | 33–87 |
| Women | 58.0 |
| Blood pressure ≥130/85 mmHg or treatment | 50.5 |
| HDL cholesterol <1.3 (women) or <1.0 (men) mmol/l or treatment | 34.0 |
| Triglycerides ≥1.7 mmol/l | 27.7 |
| Waist >88 (women) or >102 (men) cm | 60.1 |
| BMI (kg/m$^2$) | 27.7 ± 5.0 |
| BMI >30 | 26.4 |
| Pre-diabetes | |
| FPG 5.6–6.9 mmol/l (IFG) | 37.4 |
| Metabolic syndrome* | 39.0 |
| Log(urine 8-epi-PGF$_{2\alpha}$/creatinine) (ng/mmol) | 4.87 ± 0.59 |
| Log(plasma myeloperoxidase) (ng/ml) | 3.70 ± 0.54 |
| HOMA-IR | 3.72 ± 2.3 |
| Insulin resistance | 25.0 |

Data are means ± SD or percent unless otherwise indicated. *According to Adult Treatment Panel III criteria.

prospective observational study of cardiovascular disease (CVD) and its risk factors (18). During the seventh exam cycle (1999–2001; $n = 3,539$), participants fasted overnight, provided blood and urine samples, and had a standardized medical examination. A total of 2,002 subjects provided data for the present analysis after exclusion of those examined at home or in a nursing home ($n = 206$ incomplete exams), those with prevalent diabetes ($n = 449$) or CVD ($n = 305$) or missing covariate information ($n = 121$), and those missing urinary isoprostane measurements ($n = 456$) because routine urine collection and storage did not commence until ~3 months into examination 7. The institutional review board of Boston University Medical Center approved the study protocol, and all subjects gave written informed consent.

## Exposure and outcome measures

The primary analysis examined insulin resistance, measured using the homeostasis model ([fasting glucose × fasting insulin]/22.5) as the dependent variable. We defined categorical insulin resistance as homeostasis model assessment of insulin resistance (HOMA-IR) level in the top quartile of the distribution among subjects without diabetes (19,20).

The primary independent exposure variables were systemic concentrations of oxidative stress markers, measured by urine creatinine-indexed 8-epi-PGF$_{2\alpha}$ concentrations, and plasma myeloperoxidase concentrations. The primary analysis considered oxidative stress markers distributed by sex-specific tertiles: urine 8-epi-PGF$_{2\alpha}$/creatinine had tertile thresholds of 93.2 and 146.9 ng/mmol in men and 109.5 and 183.8 ng/mmol in women; myeloperoxidase tertile thresholds were 33.0 and 55.1 ng/ml in men and 31.2 and 48.4 ng/ml in women. We assessed associations of insulin resistance with oxidative stress overall by BMI and as a function of two other pre-diabetes phenotypes: *1*) metabolic syndrome using the 2005 updated Third Report of the National Cholesterol Education Program's Adult Treatment Panel criteria as any three or more of the following: fasting plasma glucose (FPG) 5.6–6.9 mmol/l, waist circumference ≥102 cm (in men) or ≥88 cm (in women), fasting triglycerides ≥1.7 mmol/l, HDL cholesterol <1.0 mmol/l (in men) or <1.3 mmol/l (in women) or treatment for elevated cholesterol, and blood pressure ≥130/85 mmHg or treatment for hypertension (21); or *2*) IFG (FPG 5.6–6.9 mmol/l) (22).

We measured height, weight, and waist circumference with the subject standing. We calculated BMI as weight in kilograms divided by the square of height in meters. We used blood pressure as the mean of the physician's two measurements after the subject had been seated for at least 5 min. We defined diabetes as an FPG concentration ≥7.0 mmol/l or current use of hypoglycemic drug therapy. Over 98% of individuals with diabetes among Framingham Offspring Study subjects have type 2 diabetes (23). We defined CVD by standard Framingham Heart Study criteria as any of the following: angina, coronary insufficiency, fatal and nonfatal myocardial infarction, stroke, transient ischemic attack, heart failure, or intermittent claudication (24).

Laboratory assay methods for glucose, insulin, lipids, and urinary 8-epi-PGF$_{2\alpha}$ have previously been published (4,25). The Framingham laboratory participates in the Centers for Disease Control lipoprotein cholesterol laboratory standardization program. FPG was measured with a hexokinase reagent kit (A-gent Glucose Test; Abbott, South Pasadena, CA). Glucose assays were run in duplicate; intra-assay coefficients of variation (CV) were <3%. Fasting plasma insulin was measured with a human-specific insulin assay having essentially no cross-reactivity to insulin split products (Linco, St. Louis, MO); intra-assay CVs were <6.1%. Urine 8-epi-PGF$_{2\alpha}$ was measured by enzyme-linked immunosorbent assay (Cayman, Ann Arbor, MI); intra-assay CVs were <9.7%. Urine creatinine measured by reaction of creatinine and alkaline picrate (Abbott Spectrum CCX) assay CVs were <4%. Urinary content of 8-epi-PGF$_{2\alpha}$ was indexed to creatinine as nanograms 8-epi-PGF$_{2\alpha}$ per millimole creatinine. Fasting serum myeloperoxidase concentrations were measured with a commercially available (OXIS, Portland, OR) enzyme-linked immunosorbent assay; the mean intra-assay CV was 3.2 ± 2.7%.

## Statistical analysis

We used multivariable logistic regression or multivariable linear regression (ANOVA) to test associations of oxidative stress markers with insulin resistance prevalence or HOMA-IR levels. For the primary analyses, we classified subjects by sex-specific tertiles of urine 8-epi-PGF$_{2\alpha}$/creatinine or myeloperoxidase. Logistic regression and ANCOVA models testing proportions or levels of insulin resistance in these categories were adjusted for *1*) age and sex; *2*) age, sex, and BMI; or *3*) age, sex, BMI, waist circumference, smoking, systolic blood pressure, hypertension treatment, hyperlipidemia treatment, triglycerides, and levels of the ratio of total to HDL cholesterol. We used natural logarithmic transformation to approximately normalize the distributions of urine 8-epi-PGF$_{2\alpha}$/creatinine, myeloperoxidase, and HOMA-IR for statistical testing, but for HOMA-IR in RESULTS we report least-squares mean (LSM) concen-

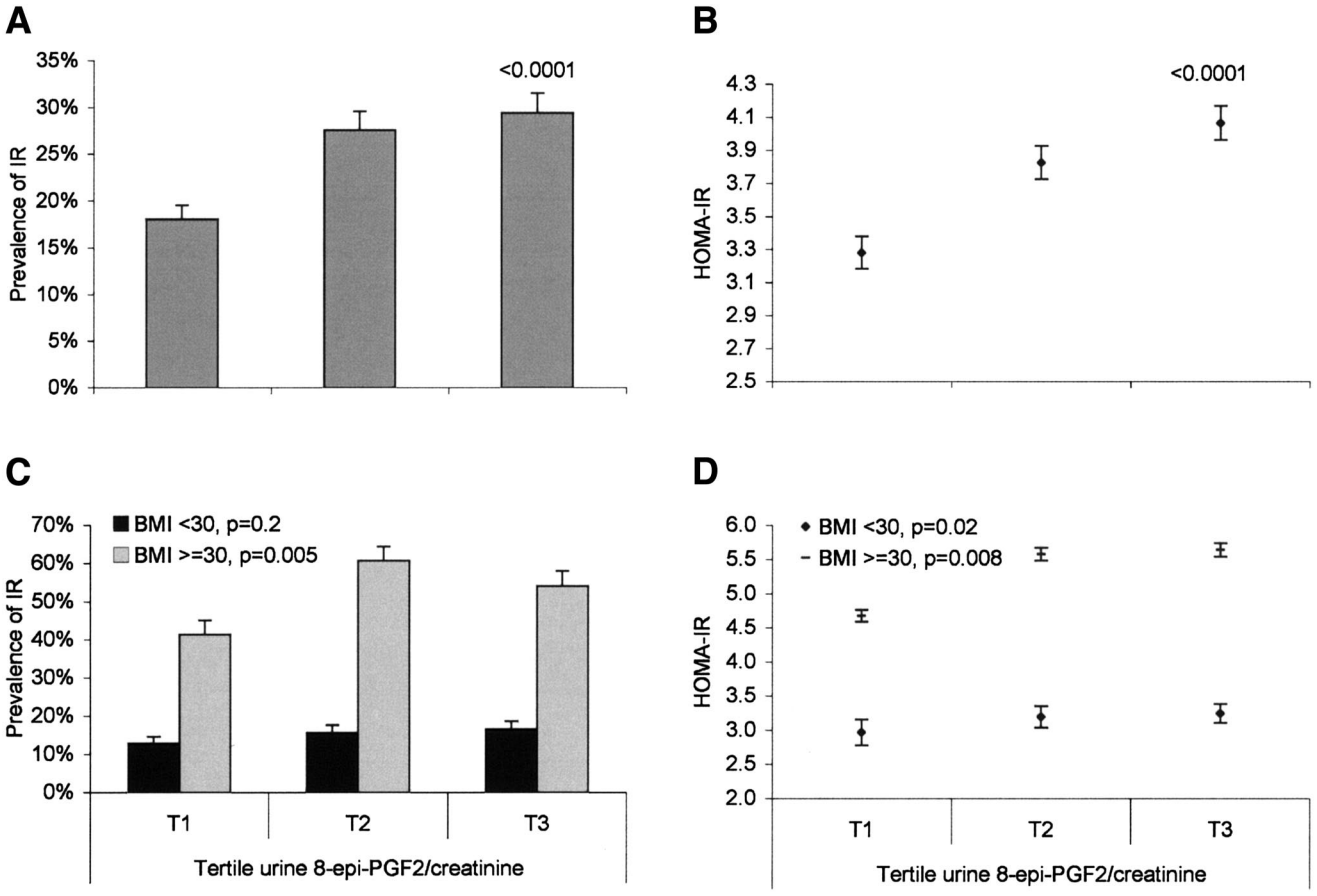


**Figure 1—**A *and* B*: Unadjusted prevalence of insulin resistance (IR)* (A) *or age and sex-adjusted mean level of HOMA-IR* (B) *by tertile of creatinine-indexed 8-epi-PGF*$_{2\alpha}$*.* C *and* D*: Unadjusted prevalence of insulin resistance (C) or age- and sex-adjusted mean level of HOMA-IR* (D) *by tertile of creatinine-indexed 8-epi-PGF*$_{2\alpha}$*, stratified by BMI. For BMI–by–8-epi-PGF*$_{2\alpha}$*/creatinine interaction,* P *= 0.16 for insulin resistance and* P *= 0.02 for HOMA-IR level.* P *values indicate significance of contrasts overall or within BMI category, and error bars are SDs for prevalences and SEs for means.*

trations ± SE. The primary analysis was conducted on nondiabetic subjects overall and then repeated among pre-diabetic subgroups, including obesity (BMI >30 kg/m$^2$), IFG, or metabolic syndrome. We tested interactions by sex or pre-diabetes phenotype on associations of oxidative stress markers with insulin resistance. For sex–by–urine 8-epi-PGF$_{2\alpha}$/creatinine and sex-by-myeloperoxidase interactions, we obtained $P > 0.05$, so we present analyses with men and women combined. We performed all analyses using SAS, version 8.1 (26).

**RESULTS** — Characteristics of study subjects are displayed in Table 1. By definition, 25% of subjects had insulin resistance, and a similar proportion was obese. Other high-risk phenotype prevalences were similar whether defined by metabolic syndrome or IFG (37–39%). Concentrations of urine 8-epi-PGF$_{2\alpha}$/creatinine and myeloperoxidase were uncorrelated (Spearman $r = 0.03$; $P = 0.21$). After adjusting for sex and age, concentration of log(urine 8-epi-PGF$_{2\alpha}$/creatinine) was higher in subjects with metabolic syndrome (LSM ± SE 4.92 ± 0.02 ng/mmol) compared with those without metabolic syndrome (4.84 ± 0.02 ng/mmol; $P = 0.003$) and in those with IFG (4.93 ± 0.02 ng/mmol) compared with those with normal fasting glucose (4.83 ± 0.02 ng/mmol; $P = 0.0005$). Sex- and age-adjusted concentrations of log(myeloperoxidase) were similar in subjects with (3.70 ± 0.02 ng/ml) and without (3.70 ± 0.02 ng/ml; $P = 0.83$) metabolic syndrome, as well as in those with IFG (3.67 ± 0.02 ng/ml) compared with those with normal fasting glucose (3.72 ± 0.02 ng/ml; $P = 0.056$). Sex- and age-adjusted concentrations of HOMA-IR were higher in subjects with metabolic syndrome (5.09 ± 0.07) compared with those without metabolic syndrome (2.85 ± 0.06; $P < 0.0001$) and in those with IFG (4.92 ± 0.08) compared with those with normal fasting glucose (3.01 ± 0.06; $P < 0.0001$).

The prevalence of insulin resistance and mean concentrations of HOMA-IR increased significantly with increasing concentrations of urine 8-epi-PGF$_{2\alpha}$/creatinine (Fig. 1*A* and *B*). Fig. 1*B* shows a graded, dose-response relation between increasing tertiles of urine 8-epi-PGF$_{2\alpha}$/creatinine and mean levels of HOMA-IR. The association of insulin resistance with urine 8-epi-PGF$_{2\alpha}$/creatinine was weakened after adjustment for BMI (insulin resistance prevalence across tertiles, $P = 0.06$; mean HOMA-IR across tertiles, $P = 0.004$). Stratified by obesity (Fig. 1*C* and *D*), prevalence of insulin resistance and adjusted mean levels of HOMA-IR increased significantly across tertiles of urine 8-epi-PGF$_{2\alpha}$/creatinine among those with BMI ≥30 kg/m$^2$ (insulin resistance prevalence, $P = 0.005$; HOMA-IR means, $P = 0.008$) but did not increase strongly among those with BMI <30 kg/m$^2$ (insulin resistance prevalence, $P =$

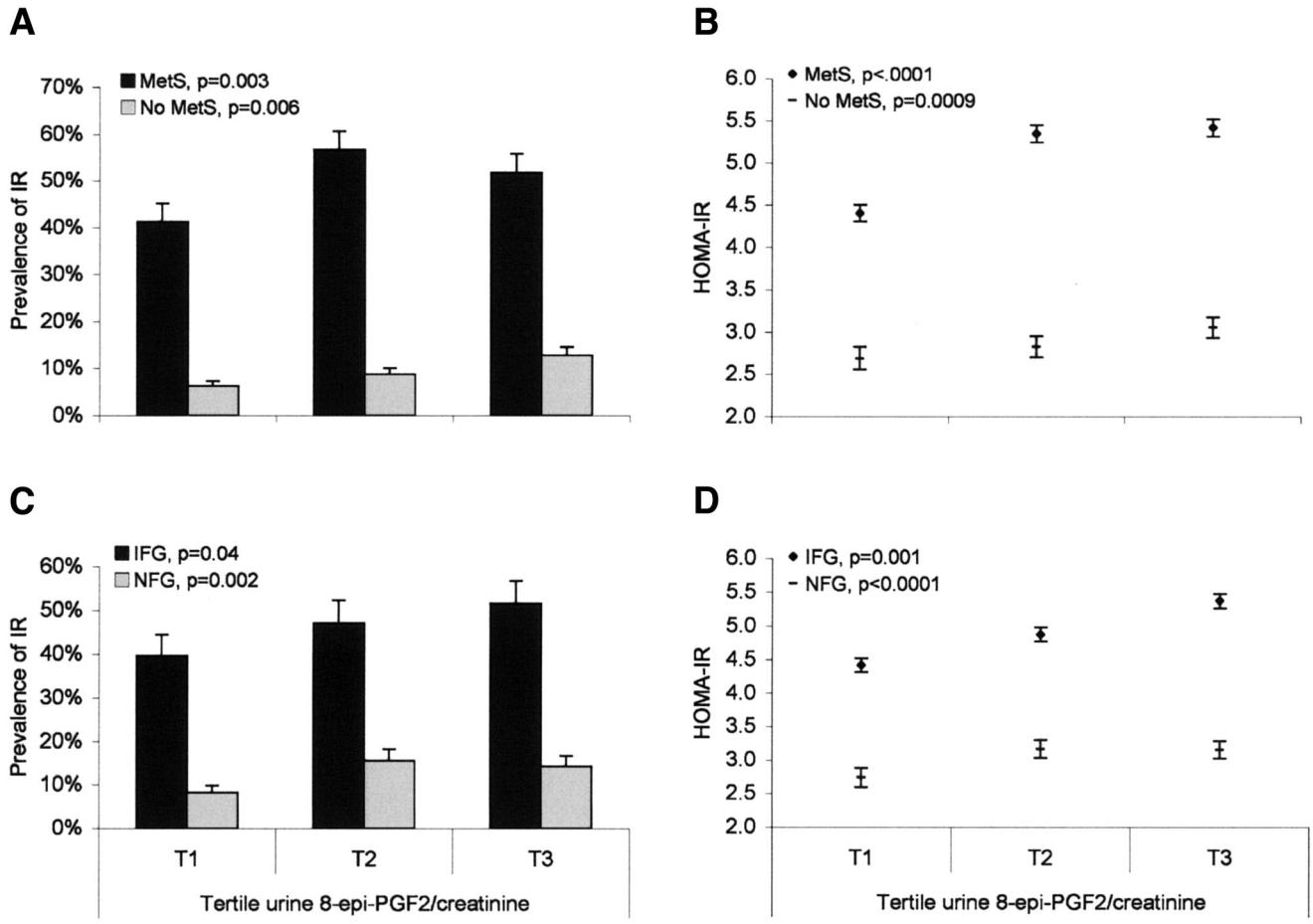


**Figure 2—**A *and* B*: Unadjusted prevalence of insulin resistance (IR)* (A) *or age- and sex-adjusted mean level of HOMA-IR* (B) *by tertile of urine 8-epi-PGF$_{2\alpha}$/creatinine, stratified by the presence or absence of metabolic syndrome (MetS). For metabolic syndrome–by–8-epi-PGF$_{2\alpha}$/creatinine interaction,* P *= 0.09 for insulin resistance prevalence and 0.001 for HOMA-IR level.* C *and* D*: Unadjusted prevalence of insulin resistance* (C) *or age- and sex-adjusted mean level of HOMA-IR* (D) *by tertile of urine 8-epi-PGF$_{2\alpha}$/creatinine, stratified by normal fasting glucose (NFG) or impaired fasting glucose (IFG). For normal fasting glucose– or impaired fasting glucose–by–urine 8-epi-PGF$_{2\alpha}$/creatinine interaction,* P *= 0.31 for insulin resistance prevalence and 0.04 for HOMA-IR level.* P *values indicate significance of contrasts within pre-diabetes category, and error bars are SDs for prevalences and SEs for means.*

0.22; HOMA-IR means, $P = 0.02$); testing interactions of obesity–by–8-epi-PGF$_{2\alpha}$/creatinine interaction gave $P = 0.16$ for insulin resistance prevalence and $P = 0.02$ for HOMA-IR level.

Stratified by pre-diabetes (Fig. 2), the prevalence of insulin resistance and mean levels of HOMA-IR increased across tertiles of urine 8-epi-PGF$_{2\alpha}$/creatinine among those with ($P = 0.003$) or without ($P = 0.006$) metabolic syndrome and with ($P = 0.04$) or without ($P = 0.002$) IFG. For insulin resistance prevalence, interactions of pre-diabetes with 8-epi-PGF$_{2\alpha}$/creatinine were not significant ($P = 0.09$ for metabolic syndrome; $P = 0.31$ for IFG); in contrast, for HOMA-IR levels, interactions were significant or borderline ($P = 0.001$ for metabolic syndrome; $P = 0.04$ for IFG).

Additional adjustment of models for age, sex, BMI, waist circumference, smoking, systolic blood pressure, hypertension treatment, hyperlipidemia treatment, triglycerides, and levels of the ratio of total to HDL cholesterol did not alter the primary association. In these models, $P = 0.034$ for insulin resistance prevalence across tertiles of urine 8-epi-PGF$_{2\alpha}$/creatinine and $P = 0.021$ for mean HOMA-IR across tertiles.

The prevalence of insulin resistance and mean levels of HOMA-IR were not different across tertiles of myeloperoxidase (age- and sex-adjusted $P = 0.26$ for prevalence of insulin resistance or $P = 0.48$ for concentrations of HOMA-IR).

**CONCLUSIONS** — We observed that insulin resistance was positively associated with systemic oxidative stress, measured by increased concentrations of urine 8-epi-PGF$_{2\alpha}$/creatinine, among individuals without diabetes in the community. These data from a large community-based cohort are consistent with other in vitro and rodent model evidence demonstrating that oxidative stress is a key pathway leading to insulin resistance and support the hypothesis that oxidative stress may be a risk factor for type 2 diabetes in humans (5). However, our cross-sectional study design cannot exclude the alternative explanation that insulin resistance leads to systemic oxidative stress. The association of oxidative stress with insulin resistance was not entirely explained by obesity, which we previously showed to be a major determinant of urine 8-epi-PGF$_{2\alpha}$/creatinine concentrations in the Framingham cohort (4). We have also shown in this cohort that obesity, metabolic syndrome, and IFG are potent determinants of incident type 2 diabetes (27). Here we report that concentrations of urine 8-epi-PGF$_{2\alpha}$/creatinine

and insulin resistance were increased in individuals with these high diabetes-risk phenotypes; those with high-risk phenotypes and high concentrations of urine 8-epi-$PGF_{2\alpha}$/creatinine had the highest levels of insulin resistance. Prospective analysis is needed to firmly establish that oxidative stress contributes to diabetes risk in the community. However, even individuals with normal glucose tolerance or without metabolic syndrome demonstrated a positive association of oxidative stress with insulin resistance. This observation to some degree weakens a counterargument that elevated oxidative stress makers are found in pre-diabetes as a result of residual confounding by the many correlated metabolic abnormalities or possible subclinical atherosclerosis known to occur in pre-diabetes that were not adjusted for here. Our goal was to assess oxidative stress as a main effect, adjusted only for age, sex, and BMI. However, even after additional adjustment for standard CVD risk factors, oxidative stress had a significant marginal association with insulin resistance. From this perspective, the data clearly show positive associations among oxidative stress, insulin resistance, and pre-diabetes in humans.

The present study substantially extends the relatively sparse human data in this field. Three cross-sectional studies with a few dozen subjects each have previously reported positive correlations of oxidative stress (by a variety of measures) with insulin resistance or pre-diabetes phenotypes. In Japanese men, plasma concentrations of 8-epi-$PGF_{2\alpha}$ were significantly correlated with glucose clamp–assessed insulin resistance (8). Plasma concentrations of 8-epi-$PGF_{2\alpha}$ were higher in Indian Mauritians with impaired glucose tolerance compared with similar subjects with normal glucose tolerance (9). In another Indian population, total antioxidant capacity (measured by levels of red cell superoxide dismutase and catalase or plasma reduced glutathione and ascorbic acid) was lower in impaired glucose tolerant subjects than in similar subjects with normal glucose tolerance (7), and in a study of 81 patients with nonalcoholic fatty liver disease and 30 healthy control subjects, oxidative stress (measured by copper-zinc superoxide dismutase activity) was positively correlated with HOMA-IR (10). However, other studies have found no association of oxidative stress (measured by levels of oxidized LDL or urine 8-epi-$PGF_{2\alpha}$) with metabolic syndrome or HOMA-IR (11–13) after adjustment for BMI, and two small prospective studies of oxidative stress found elevated concentrations of urinary isoprostanes to be protective (14) or have no association with the development of new cases of type 2 diabetes (15). These conflicting results from small (26 and 52 cases of diabetes, respectively) longitudinal studies and from our large cross-sectional study indicate that a large prospective analysis is needed to confirm or refute the hypothesis that oxidative stress is a type 2 diabetes precursor.

The mechanisms whereby oxidative stress is associated with insulin resistance and diabetes risk cannot be elucidated from our observational data. Other data reveal several potential mechanisms to suggest implications of our findings. Oxidative stress can be defined as an imbalance between the production of highly reactive molecular species (primarily oxygen and nitrogen) and antioxidant defenses against their production and action. Mechanisms influencing this balance include activation of stress-signaling pathways, specifically the transcription factor nuclear factor-κB (NF-κB) pathway (28,29) and NF-κB downstream signaling elements, especially the Jun $NH_2$-terminal kinase pathway (30). NF-κB and Jun $NH_2$-terminal kinase pathway activation decrease insulin signaling and insulin-mediated glucose uptake, at least in rodents (31,32). Activation of NF-κB stress signaling pathways also is associated with a generalized upregulation of acute phase proteins, including tumor necrosis factor-α, interleukin-6, and C-reactive protein (33), themselves precursors of type 2 diabetes (34,35). NF-κB activation also may induce insulin resistance via endothelial dysfunction that arises from altered fatty acid flux, elevated concentrations of asymmetric dimethylarginine, and impaired nitric oxide synthase (NOS) regulation (36–38). Treatment studies in humans show that antioxidant therapy with vitamin C significantly improves endothelial dysfunction associated with insulin resistance (39) and that NOS-mediated endothelial dysfunction in skeletal muscle is associated with impaired nutritive flow redistribution and diminished insulin-mediated and insulin-independent glucose uptake (40,41). We and others have recently shown that biomarkers of endothelial dysfunction are precursors of incident diabetes independent of obesity, inflammation, and other diabetes risk factors (42–44). Elevated concentrations of myeloperoxidase, whose availability and function are limited by consuming NO, are also a potent correlate of endothelial dysfunction (45). However, in this study we did not find that elevated concentrations of myeloperoxidase were associated with insulin resistance, although they have been associated with risk for coronary heart disease events in other studies (46,47). Taken together, the data support the hypothesis that oxidative stress measured by urine 8-epi-$PGF_{2\alpha}$/creatinine underlies insulin resistance, is associated with pre-diabetes, and could be a risk factor for type 2 diabetes in humans. The several complementary mechanistic pathways underlying insulin resistance point to multiple potential targets for the prevention and control of insulin resistance and its consequences.

Strengths of this study include a large community-based sample assessed using standardized clinical measures and biomarker assays with good precision. We had an adequate sample size to classify subjects into phenotypic subgroups, allowing examination of the joint effects of oxidative stress markers and pre-diabetes phenotypes. The study does have limitations. We used a spot analysis of urine 8-epi-$PGF_{2\alpha}$/creatinine as an index of oxidative stress, rather than a 24-h collection, and used a surrogate measure for insulin resistance. Use of spot urine samples and surrogate measures like HOMA-IR will cause misclassification that may diminish the true magnitude of associations of oxidative stress with pre-diabetes with insulin resistance. We only used one other measure of oxidative stress, myeloperoxidase, which we did not find to be associated with insulin resistance. However, urine 8-epi-$PGF_{2\alpha}$/creatinine and myeloperoxidase were not correlated in our sample. Myeloperoxidase may reflect different aspects of oxidative stress, may not (in our study sample) be a valid oxidative stress marker, and its possible association with isoprostanes or insulin resistance may be masked by unmeasured confounding. It is possible that other markers of oxidative stress might provide additional information related to insulin resistance that was not detected in this study. Finally, the Framingham cohort is largely white and middle-aged to elderly, so findings may have limited generalizability to other ethnicities and age-groups.

In summary, we conclude that systemic oxidative stress is associated with insulin resistance among individuals without diabetes in the community. The association was statistically independent of BMI and was similar in obesity, metabolic syndrome, and impaired glucose tolerance–defined pre-diabetes. Our data raise the hypothesis that oxidative stress is associated with risk of type 2 diabetes and could be a target for insulin sensitization to prevent diabetes.

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