

Association of Oxidative Stress, Insulin Resistance, and Diabetes Risk Phenotypes: The Framingham Offspring Study

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Abstract

Objective: Systemic oxidative stress causes insulin resistance (IR) in rodents. We tested the hypothesis that oxidative stress and IR are associated in humans.

Research Design and Methods: We used cross-sectional data from 2002 non-diabetic subjects of the community-based Framingham Offspring Study. We measured IR with the homeostasis model and defined categorical IR as HOMA-IR >75thile. We measured oxidative stress using the ratio of urine 8-epi-PGF_{2a}/creatinine, and used age-sex-adjusted regression models to test the association of oxidative stress with IR in people without diabetes and among subgroups at elevated risk of diabetes.

Results: Across 8-epi-PGF_{2a}/creatinine tertile the prevalence of IR increased (18.0%, 27.5%, and 29.4%; $p < 0.0001$), as did mean levels of HOMA-IR (3.28, 3.83, 4.06 units; $p < 0.0001$). The IR-oxidative stress association was attenuated by additional adjustment for BMI ($p = 0.06$ across tertile for IR prevalence, $p = 0.004$ for mean HOMA-IR). Twenty-six percent of participants were obese (BMI ≥ 30 kg/m²), 39% had metabolic syndrome (ATP3 definition) and 37% had IFG (fasting glucose 5.6-6.9 mmol/l). Among 528 obese participants, IR prevalence was 41.3%, 60.6%, and 54.2% across 8-epi-PGF_{2a}/creatinine tertile ($p = 0.005$); among 781 with metabolic syndrome, IR prevalence was 41.3%, 56.7%, and 51.7% ($p = 0.0025$); and among 749 with IFG, IR prevalence was 39.6%, 47.2%, and 51.6% ($p = 0.04$).

Conclusions: Systemic oxidative stress is associated with IR in people at average or elevated risk of diabetes, even after accounting for BMI.

Introduction

Type 2 diabetes mellitus 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 about 50% of obese people 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-PGF_{2a} (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 IR 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 IR are associated in subgroups of people with high type 2 diabetes risk phenotypes, including obesity, impaired fasting glucose (IFG) and metabolic syndrome.

Subjects and Methods

Study Subjects

The Framingham Offspring Study is a community-based prospective observational study of cardiovascular disease (CVD) and its risk factors. (18) During the seventh exam cycle (1999-2001; n=3539), participants fasted overnight, provided blood and urine samples, and had a standardized medical examination. A total of 2002 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 who were missing urinary isoprostane measurements (n=456) because routine urine collection and storage did not commence until about 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 ($[\text{fasting glucose} \times \text{fasting insulin}] / 22.5$) as the dependent variable. We defined categorical insulin resistance as 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_{2a} concentrations, and plasma myeloperoxidase (MPO) concentrations. The primary analysis considered oxidative stress markers distributed by sex-specific tertiles: urine 8-epi-PGF_{2a}/creatinine had tertile thresholds of 93.2 ng/mmol and 146.9 ng/mmol in men, and 109.5 ng/mmol and 183.8 ng/mmol in women; MPO tertile thresholds were 33.0 ng/mL and 55.1 ng/mL in men, and 31.2 ng/mL 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 prediabetes phenotypes: 1) metabolic

syndrome using the 2005 updated Third Report of the National Cholesterol Education Program's Adult Treatment Panel (ATPIII) criteria as any three or more of: 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 (HDL-C) < 1.0 mmol/l (in men) or < 1.3 mmol/l (in women), or treatment for elevated cholesterol; and blood pressure $\geq 130/85$ mm Hg or treatment for hypertension; (21) or, alternatively, 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 (kg/m^2). We used blood pressure as the mean of the physician's two measurements after the subject had been seated for at least five minutes. We defined diabetes as a FPG concentration ≥ 7.0 mmol/l or current use of hypoglycemic drug therapy. Over 98% of individuals with diabetes among Framingham Offspring have type 2 diabetes. (23) We defined CVD by standard Framingham Heart Study criteria as any of the following: angina, coronary insufficiency, fatal and non-fatal myocardial infarction, or stroke, transient ischemic attack, heart failure, or intermittent claudication. (24)

Laboratory assay methods for glucose, insulin, lipids and urinary 8-epi-PGF_{2a} have been published previously. (4; 25) The Framingham laboratory participates in the Centers for Disease Control lipoprotein cholesterol laboratory standardization program. Fasting plasma glucose (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 Inc., St Louis, MO); intra-assay CVs were $< 6.1\%$. Urine 8-epi-PGF_{2a} was measured by ELISA (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_{2a} was indexed to creatinine as ng 8-epi-PGF_{2a}/mmol creatinine. Fasting serum MPO concentrations were measured with a commercially available (OXIS, Portland, OR) ELISA; the mean intra-assay CV was $3.2 \pm 2.7\%$.

Statistical Analysis

We used multivariable logistic regression or multivariable linear regression (ANOVA) to test associations of oxidative stress markers with IR prevalence or HOMA-IR levels. For the primary analyses we classified subjects by sex-specific tertiles of urine 8-epi-PGF_{2a}/creatinine, or MPO. Logistic regression and ANCOVA models testing proportions or levels of insulin resistance in these categories were adjusted for (i) age and sex, or (ii) age, sex and BMI, or (iii) age, sex, BMI, waist circumference, smoking, systolic blood pressure, hypertension treatment, hyperlipidemia treatment, and levels of the ratio of total to HDL cholesterol, and triglycerides. We used natural logarithmic transformation to approximately normalize the distributions of urine 8-epi-PGF_{2a}/creatinine, MPO and HOMA-IR for statistical testing, but for HOMA-IR in the results we report least square mean concentrations and their standard errors. The primary analysis was conducted on non-diabetic subjects overall, then repeated among prediabetes subgroups, including obesity ($\text{BMI} > 30 \text{ kg}/\text{m}^2$), IFG, or metabolic syndrome. We tested interactions by sex or prediabetes phenotype on associations of oxidative stress markers with IR. For sex-by-urine 8-epi-PGF_{2a}/creatinine and sex-by-MPO 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 were obese. Other high risk

phenotype prevalences were similar whether defined by metabolic syndrome or IFG (37-39%). Concentrations of urine 8-epi-PGF_{2a}/creatinine and MPO were uncorrelated (Spearman $r = 0.03$, $p = 0.21$). After adjusting for sex and age, concentration of log(urine 8-epi-PGF_{2a}/creatinine) was higher in subjects with metabolic syndrome (least square mean (LSM) 4.92, standard error (SE) 0.02 ng/mmol) compared with those without metabolic syndrome (LSM 4.84, SE 0.02 ng/mmol; $p = 0.003$) and in those with IFG (LSM 4.93, SE 0.02 ng/mmol) compared with those with normal fasting glucose (LSM 4.83, SE 0.02 ng/mmol; $p = 0.0005$). Sex and age adjusted concentrations of log(MPO) were similar in subjects with metabolic syndrome (LSM 3.70, SE 0.02 ng/mL) compared with those without metabolic syndrome (LSM 3.70, SE 0.02 ng/mL; $p = 0.83$), as well as in those with IFG (LSM 3.67, SE 0.02 ng/mL) compared with those with normal fasting glucose (LSM 3.72, SE 0.02 ng/mL; $p = 0.056$). Sex and age adjusted concentrations of HOMA-IR were higher in subjects with metabolic syndrome (LSM 5.09, SE 0.07) compared with those without metabolic syndrome (LSM 2.85, SE 0.06; $p < 0.0001$) and in those with IFG (LSM 4.92, SE 0.08) compared with those with normal fasting glucose (LSM 3.01, SE 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_{2a}/creatinine (Figure 1, upper panels). Figure 1, upper right panel shows a graded, dose-response relation between increasing tertiles of urine 8-epi-PGF_{2a}/creatinine and mean levels of HOMA-IR. The association of insulin resistance with urine 8-epi-PGF_{2a}/creatinine was weakened after adjustment for BMI (IR prevalence across tertiles $p = 0.06$; mean HOMA-IR across tertiles, $p = 0.004$;). Stratified by obesity (Figure 1, lower panels), prevalence of IR and adjusted mean levels of HOMA-IR increased significantly across tertiles of

urine 8-epi-PGF_{2a}/creatinine among those with BMI ≥ 30 kg/m² (IR prevalence, $p = 0.005$; HOMA-IR means, $p = 0.008$) but not strongly among those with BMI < 30 kg/m² (IR prevalence, $p = 0.22$; HOMA-IR means, $p = 0.02$); testing interactions of obesity-by-8-epi-PGF_{2a}/creatinine interaction gave $p = 0.16$ for IR prevalence and $p = 0.02$ for HOMA-IR level.

Stratified by prediabetes (Figure 2), the prevalence of IR and mean levels of HOMA-IR increased across tertiles of urine 8-epi-PGF_{2a}/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 IR prevalence, interactions of prediabetes-with-8-epi-PGF_{2a}/creatinine were not significant ($p = 0.09$ for MetS, $p = 0.31$ for IFG); in contrast, for HOMA-IR levels, interactions were significant or borderline ($p = 0.001$ for MetS, $p = 0.04$ for IFG).

Additional adjustment of models for age, sex, BMI, waist circumference, smoking, systolic blood pressure, hypertension treatment, hyperlipidemia treatment, and levels of the ratio of total to HDL cholesterol, and triglycerides. did not alter the primary association: in these models $p = 0.034$ for IR prevalence across tertiles of urine 8-epi-PGF_{2a}/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 MPO (age-sex-adjusted adjusted p -value = 0.26 for prevalence of IR, or $p = 0.48$ for concentrations of HOMA-IR).

Discussion

We observed that IR was positively associated with systemic oxidative stress, measured by increased concentrations of urine 8-epi-PGF_{2a}/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 IR, 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 IR leads to systemic oxidative stress. The association of oxidative stress with IR was not entirely explained by obesity, which we previously showed to be a major determinant of urine 8-epi-PGF_{2a}/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_{2a}/creatinine and IR were increased in people with these high diabetes-risk phenotypes; individuals with high-risk phenotypes and high concentrations of urine 8-epi-PGF_{2a}/creatinine had the highest levels of IR. Prospective analysis is needed to firmly establish that oxidative stress contributes to diabetes risk in the community. However, even people with normal glucose tolerance or without metabolic syndrome demonstrated a positive association of oxidative stress with IR. This observation weakens to some degree a counterargument that elevated oxidative stress makers are found in prediabetes as a result of residual confounding by the many correlated metabolic abnormalities or possible subclinical atherosclerosis known to occur in prediabetes 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 IR. From this perspective the data clearly show positive associations among oxidative stress, IR and prediabetes 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 IR or prediabetes phenotypes. In Japanese men, plasma concentrations of 8-epi-PGF_{2a} were

significantly correlated with glucose clamp-assessed IR. (8) Plasma concentrations of 8-epi-PGF_{2a} were higher in Indian Mauritians with impaired glucose tolerance compared to 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 IGT compared to similar subjects with normal glucose tolerance, (7) and in a study of 81 patients with nonalcoholic fatty liver disease and 30 healthy controls, 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_{2a}) 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 our large cross-sectional study indicates 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 IR 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 kappa B (NF- κ B) pathway (28; 29) and NF- κ B downstream signaling elements, especially the c-Jun amino-terminal kinase (JNK) pathway. (30) NF- κ B and JNK pathway activation decrease insulin signaling and insulin-mediated glucose uptake,

at least in rodents. (31; 32) Activation of NF- κ B stress signaling pathways also is associated with a generalized up-regulation of acute phase proteins, including TNF- α , IL6, and CRP, (33), themselves precursors of type 2 diabetes. (34; 35) NF- κ B activation also may induce IR 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 shows that antioxidant therapy with vitamin C significantly improves endothelial dysfunction associated with IR, (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 MPO, which by consuming nitric oxide limits its availability and function, are also a potent correlate of endothelial dysfunction. (45) However, in this study we did not find that elevated concentrations of MPO were associated with IR, although they have been associated in other studies with risk for coronary heart disease events. (46; 47) Taken together the data support the hypothesis that oxidative stress measured by urine 8-epi-PGF_{2a}/creatinine underlies IR, is associated with prediabetes, and could be a risk factor for type 2 diabetes in humans. The several complementary mechanistic pathways underlying IR point to multiple potential targets for the prevention and control of IR 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 adequate sample size to classify

subjects into phenotypic subgroups allowing examination of the joint effects of oxidative stress markers and prediabetes phenotypes. The study does have limitations. We used a spot analysis of urine 8-epi-PGF_{2a}/creatinine as an index of oxidative stress, rather than a 24-hour collection, and used a surrogate measure for IR. 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 prediabetes with IR. We only used one other measure of oxidative stress, MPO, which we did not find to be associated with IR. However, urine 8-epi-PGF_{2a}/creatinine and MPO were not correlated in our sample. MPO may reflect different aspects of oxidative stress, may not (in our study sample) be a valid oxidative stress marker, or its possible association with isoprostanes or IR masked by unmeasured confounding. It is possible that other markers of oxidative stress might provide additional information related to IR 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 ethnic and age groups.

In summary, we conclude that systemic oxidative stress is associated with IR among people without diabetes in the community. The association was statistically independent of BMI and was similar in obesity, metabolic syndrome, and IGT-defined prediabetes. 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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Table 1. Study Sample Characteristics

	Mean or %	SD
N	2002	
Age, years	60	9.3
Age minimum-maximum, years	33-87	
Women (%)	58.0	
BP \geq 130/85 mm Hg or treatment (%)	50.5	
HDL-C $<$ 1.3 mmol/l (W) or $<$ 1.0 (M) or treatment (%)	34.0	
Triglycerides \geq 1.7 mmol/l (%)	27.7	
Waist $>$ 88 cm (W) or $>$ 102 (M) (%)	60.1	
Body Mass Index (BMI), kg/m ²	27.7	5.0
BMI \geq 30 kg/m ² (%)	26.4	
Prediabetes		
Fasting plasma glucose 5.6-6.9 mmol/l (IFG) (%)	37.4	
ATP3 Metabolic syndrome (%)	39.0	
Log(Urine 8-epi-PGF2a/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	

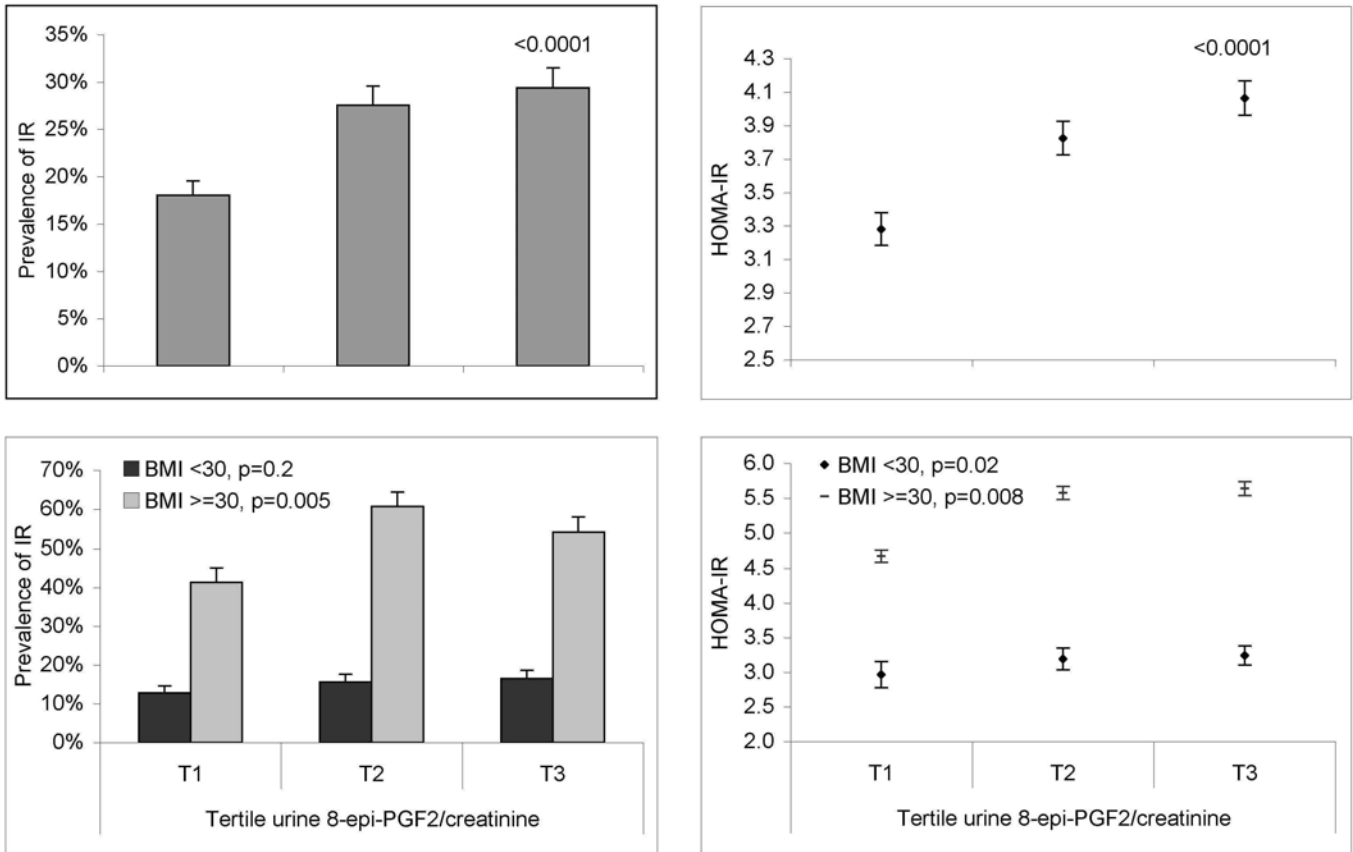


Figure 1. Top panels: The unadjusted prevalence of insulin resistance (IR, upper left) or age-sex-adjusted mean level of HOMA-IR (upper right) by tertile of creatinine-indexed 8-epi-PGF2α/creatinine. Bottom panels: The unadjusted prevalence of IR (lower left) or age-sex-adjusted mean level of HOMA-IR (lower right) by tertile of creatinine-indexed 8-epi-PGF2α/creatinine, stratified by body mass index (BMI, kg/m²). P-value, BMI-by-8-epi-PGF2α/creatinine interaction = 0.16 for IR prevalence and 0.02 for HOMA-IR level. P-values indicate significance of contrasts overall or within BMI category, and error bars are standard deviations for prevalence and standard errors for means.

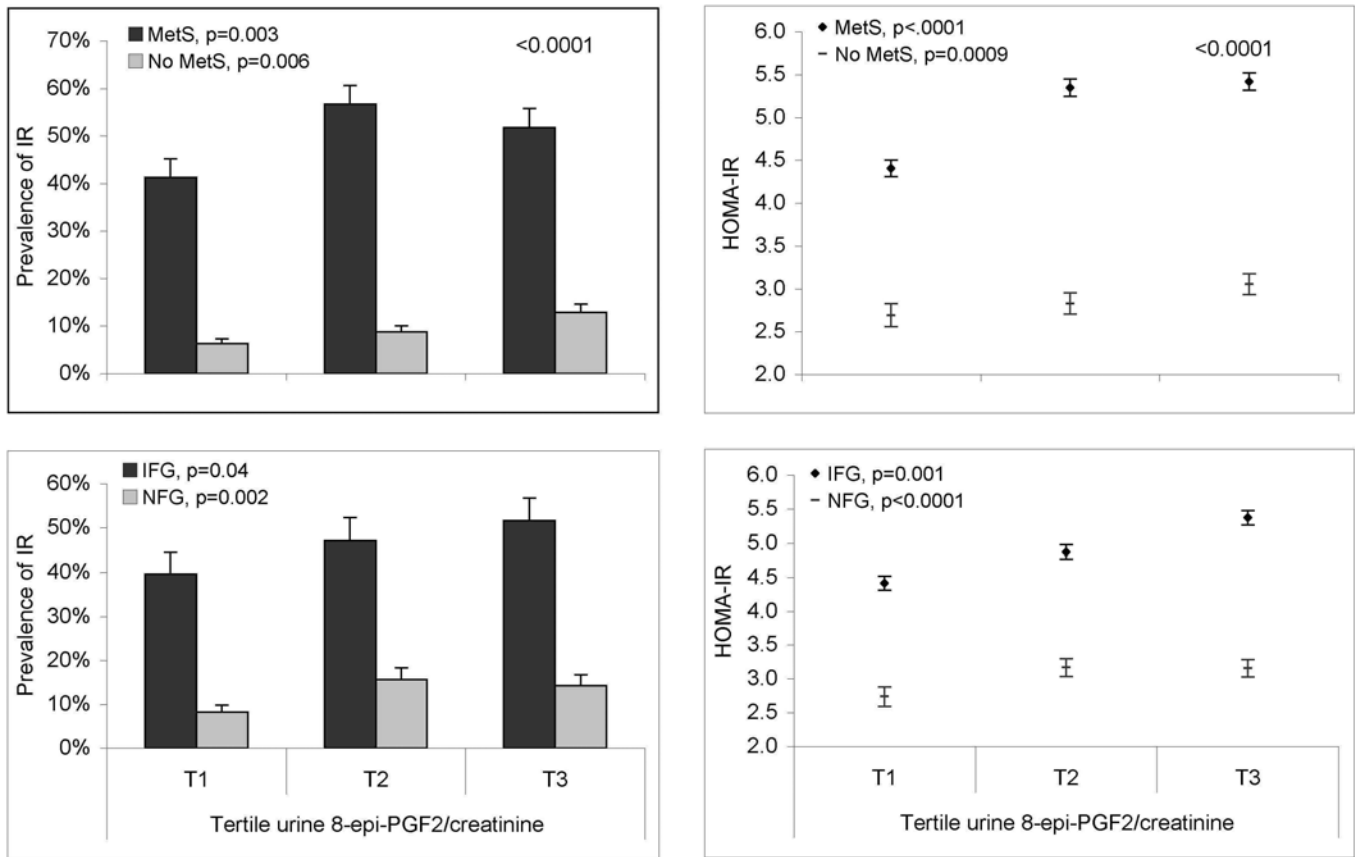


Figure 2. Top panels: The unadjusted prevalence of insulin resistance (IR, upper left) or age-sex-adjusted mean level of HOMA-IR (upper right) by tertile of urine 8-epi-PGF2a/creatinine, stratified by the presence or absence of metabolic syndrome (MetS). P-value, MetS-by-8-epi-PGF2a/creatinine interaction = 0.09 for IR prevalence and 0.001 for HOMA-IR level. Bottom panels: The unadjusted prevalence of insulin resistance (IR, upper left) or age-sex-adjusted mean level of HOMA-IR (lower right) by tertile of urine 8-epi-PGF2a/creatinine, stratified by normal (NFG) or impaired fasting glucose (IFG). P-value, NFG or IFG-by-8-epi-PGF2a/creatinine interaction = 0.31 for IR prevalence and 0.04 for HOMA-IR level. P-values indicate significance of contrasts within prediabetes category, and error bars are standard deviations for prevalence and standard errors for means.