Plasma NOx Concentrations in Glucose Intolerance and Type 2 Diabetes
— A Case-control Study in a Vietnamese Population

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Aim: The Vietnamese develop type 2 diabetes (T2D) and metabolic syndrome (MS) at a lower BMI than other ethnicities. Thus, biomarkers that identify subjects at an increased risk of T2D independently of obesity are being sought. Recent studies show that circulating NO metabolites (NOx) are increased in T2D. We investigated whether plasma NOx levels predict insulin resistance and glucose intolerance before the development of T2D, independently of obesity.

Methods: The current study was derived from a population-based study in HCMC, Vietnam, which was designed to investigate the prevalence of MS and T2D in a population aged 30-69 years. Four hundred and twenty-two subjects were recruited from the study and were stratified into 4 age- and gender-matched groups according to a glucose tolerance test {normal glucose tolerance (NGT), impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and T2D}.

Results: Plasma NOx concentrations were significantly increased in T2D but not in IFG or IGT compared with NGT. Multiregression analysis showed that plasma NOx levels were inversely correlated with BMI in T2D whereas no association was found between plasma NOx levels and BMI in non-diabetic subjects. Moreover, there was no correlation between plasma NOx levels and homeostasis model assessment-insulin resistance (HOMA-IR) in both diabetic and non-diabetic subjects.

Conclusion: Plasma NOx levels did not predict glucose intolerance or insulin resistance before the development of T2D and the increase in plasma NOx levels in T2D was not caused by adiposity. Thus, plasma NOx is not a useful marker for the prediction of high-risk subjects for T2D among Vietnamese.

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Key words: Case-control study, Insulin resistance, Adiposity

Introduction

Obesity is a major predictor of type 2 diabetes (T2D); however, similar to other Asians, Vietnamese are known to develop metabolic syndrome and T2D at a lower body mass index (BMI) than Caucasians1-3. Moreover, abdominal obesity, dyslipidemia and hypertension despite having a normal BMI are more common among Vietnamese (unpublished observation), suggesting the widespread presence of insulin resistance without being overweight or obese. Therefore, it is hoped to identify circulating biomarkers that can predict high-risk subjects for developing T2D independently of obesity. Thus far, C-reactive protein...
(CRP) in association with TNF-α receptor 2 and IL-6 has been shown to predict T2D independently of BMI and other indices of obesity in some studies; however, others have shown that the association between CRP and T2D is largely due to obesity.

Recently, several studies indicated that circulating levels of the metabolites of nitric oxide (NOx) are associated with T2D. A population-based study conducted by Zahedi et al. demonstrated that serum NOx concentrations were significantly elevated in subjects with metabolic syndrome and T2D compared to their corresponding controls; however, there is a contradicting report showing that increased plasma NOx levels were observed in T2D but not in non-diabetic subjects with the presence of insulin resistance. Thus, although these studies unequivocally demonstrated that circulating NOx levels are increased in T2D subjects, it is still controversial whether circulating NOx levels can predict high-risk subjects before the development of T2D.

This study was undertaken to investigate whether plasma NOx levels predict high-risk subjects for T2D independently of obesity using a population in which obesity is rare and yet the incidence of T2D is markedly increasing.

**Methods**

**Study design and measurements**

The present study is derived from a population-based study in HCMC, Vietnam. Subjects aged 30-69 years attended a health check up and underwent anthropometric and clinical measurements (weight, height, waist circumference and blood pressure), sampling of venous blood at 5:00-7:00 a.m. after overnight fasting and 75-g oral glucose tolerance test (OGTT). Blood pressure was measured in the sitting position after 5 min of rest. BMI was determined as weight in kilograms divided by the square of the height in meters (kg/m²). Fasting serum was separated from coagulated whole blood and insulin, high sensitive C-reactive protein (hsCRP), total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured by Diag Center International (Lab Group International-Division Vietnam) on the same day as blood sampling. The TG/HDL-C ratio was calculated as a surrogate marker for insulin resistance. The homeostasis model of assessment of the insulin resistance (HOMA-IR) score was calculated as fasting insulin (μU/mL) multiplied by fasting glucose (mmol/L) divided by 22.5. Plasma specimens were separated from non-coagulated whole blood and were frozen at -70°C until NOx measurements.

**75-g OGTT**

Fasting and 2-h postload glucose levels of 75-g OGTT were measured. The criteria of the 1999 World Health Organization were used to stratify subjects into 4 groups: normal glucose tolerance (NGT); impaired fasting glucose (IFG); impaired glucose tolerance (IGT) and type 2 diabetes (T2D); for T2D, fasting ≥ 7.0 mmol/L (126 mg/dL), 2-h ≥ 11.1 mmol/L (200 mg/dL); for IGT, fasting < 7.0 mmol/L (126 mg/dL) and 2-h ≥ 7.8 mmol/L (140 mg/dL) and < 11.1 mmol/L (200 mg/dL); and for IFG ≥ 6.1 mmol/L (110 mg/dL) and < 7.0 mmol/L (< 126 mg/dL) and if measured, 2-h < 7.8 mmol/L (140 mg/dL). A total of 422 subjects were recruited from the list of the main study in such a way that each OGTT-stratified group was matched for gender and age group.

The study was approved by the Institutional Review Board of the Health Services of Ho Chi Minh City and all participants signed informed consent.

**Measurements of plasma NOx**

The concentrations of plasma NO2⁻ (nitrite) and NO3⁻ (nitrate), stable metabolites of NO, were measured by the Griess reaction using an HPLC-Griess system (NOx Analyzer ENO-10; EiCom Instrument, Kyoto, Japan) as previously described. Briefly, 30 μL plasma is mixed with a equal volume of methanol and centrifuged at 15,000 rpm for 15 min to precipitate protein. Ten microliters each of supernatants are injected into the NOx Analyzer using an automatic injector (Gilson, Middleton, WN, USA). In the analyzer, nitrite and nitrate are separated on a reverse-phase separation column. Nitrite is then mixed with the Griess reagent to form a purple azo dye in the reaction coil, whose absorbance is measured at 540 nm by a flow-through spectrophotometer. The absorbance reaches a peak with a retention time of approximately 4.5 min. Nitrate is reduced to nitrite with a cadmium reduced copper column, which subsequently reacts with the Griess reagent. This peak arrives with a retention time of approximately 8 min. The area under the absorption curve is compared with that of a standard solution containing sodium nitrite and sodium nitrate (Wako Pure Chemical Industries Inc.) to determine plasma nitrite and nitrate concentrations. Plasma NOx concentration was obtained by summing nitrite and nitrate concentrations.

**Statistical analysis**

All statistical analyses were performed using SPSS for Windows, version 14.0 (SPSS, Chicago, IL). Cate-
Table 1. Characteristics of 4 OGTT-stratified groups

|                     | NGT (n = 120) | IFG (n = 111) | IGT (n = 101) | T2D (n = 90) | All (n = 422) |
|---------------------|---------------|---------------|---------------|--------------|--------------|
| **Gender, n (%)**   |               |               |               |              |              |
| Men                 | 57 (13.5)     | 51 (12.1)     | 47 (11.1)     | 47 (11.1)    | 201 (47.9)   |
| Women               | 63 (14.9)     | 60 (14.2)     | 54 (12.8)     | 43 (10.2)    | 220 (52.1)   |
| **Age (yrs)**       | 50.9 ± 11.2   | 50.1 ± 10.8   | 53.1 ± 10.0   | 57.7 ± 8.0***| 52.7 ± 10.6  |
| **BMI (kg/m²)**     | 22.7 ± 2.9    | 23.4 ± 3.5    | 24.2 ± 4.1**  | 23.8 ± 3.8*  | 23.5 ± 3.6   |
| **Waist circumference (cm)** | 78.4 ± 9.4   | 80.4 ± 9.5    | 82.9 ± 10.2** | 83.1 ± 9.2***| 81.0 ± 9.7   |
| **Systolic BP (mmHg)** | 118 ± 18      | 123 ± 22      | 127 ± 20**    | 132 ± 20***  | 125 ± 20     |
| **Diastolic BP (mmHg)** | 73 ± 10       | 75 ± 12       | 78 ± 11**     | 76 ± 10*     | 75 ± 11      |
| **Fasting glucose (mmol/L)** | 5.1 (0.4)    | 5.7 (0.3)     | 5.5 (0.7)     | 7.0 (2.7)    | 5.6 (0.8)    |
| **Fasting insulin (μU/mL)** | 6.4 (6.0)    | 7.6 (6.3)*    | 9.0 (8.4)***  | 10.2 (10.2)***| 8.1 (7.3)    |
| **HOMA-IR**         | 1.5 (1.4)     | 1.9 (1.6)*    | 2.3 (2.0)***  | 3.1 (2.8)*** | 2.0 (2.0)    |
| **Total cholesterol (mg/dL)** | 210 ± 40      | 203 ± 39      | 210 ± 47      | 212 ± 55     | 208 ± 45     |
| **HDL-C (mg/dL)**   | 55 ± 14       | 54 ± 15       | 52 ± 12       | 49 ± 13**    | 53 ± 14      |
| **Non HDL-C (mg/dL)** | 155 ± 41      | 149 ± 40      | 157 ± 46      | 163 ± 54     | 156 ± 45     |
| **Triglyceride (mg/dL)** | 137 (101)     | 138 (119)     | 168 (114)     | 204 (160)*** | 156 (128)    |
| **TG: HDL-C ratio** | 2.6 (2.8)     | 2.6 (3.8)     | 3.2 (3.3)     | 4.4 (4.1)*** | 3.1 (3.5)    |
| **hsCRP (mg/L)**    | 1.0 (2.0)     | 1.0 (2.0)     | 2.0 (2.5)     | 2.0 (3.0)*** | 1.0 (2.0)    |
| **NOx (μmol/L)**    | 26.4 (17.6)   | 26.4 (16.4)   | 23.5 (21.0)   | 35.7 (31.3)**| 26.5 (21.5)  |

BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model of assessment of insulin resistance; hsCRP, high sensitive C-reactive protein; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; NOx, nitrite and nitrate combined; OGTT: oral glucose tolerance test; T2D, type 2 diabetes; TG: triglyceride.

Average age, BMI, waist circumference, total cholesterol, HDL-C and non HDL-C are expressed as the mean ± SD and differences among groups were examined by one-way ANOVA and t-test. Triglyceride, TG: HDL-C ratio, fasting glucose, fasting insulin, HOMA-IR, hsCRP and NOx are presented as the median followed by interquartile range in parentheses and differences among groups were examined by the non-parametric Mann-Whitney test. P values refer to differences as determined by t tests or the Mann-Whitney test. *P<0.05, **P<0.01, ***P<0.001 compared with NGT group.

Results

Characteristics of 4 OGTT-stratified groups

Characteristics of 4 OGTT-stratified groups are shown in Table 1. Four groups were similar in gender. Subjects with T2D were older than those with NGT and IFG (P<0.001). Both IGT and T2D groups had significantly greater BMI (P<0.01 and P<0.05, respectively) and waist circumference (P<0.01 and P<0.001, respectively) than NGT group. Both systolic and diastolic blood pressure were significantly higher in both IGT (P<0.01 for both systolic and diastolic blood pressure) and T2D (P<0.001 and P<0.05, respectively) groups than NGT group.

While no significant difference was found in the concentration of serum total cholesterol or non HDL-C among the 4 groups, T2D group had lower HDL-C (P<0.01) and higher serum triglyceride (P<0.001), resulting in a higher TG/HDL-C ratio (P<0.001) than NGT. This higher TG/HDL-C ratio was not observed in IFG or IGT group compared with NGT group.
Fasting serum insulin levels were higher in IFG, IGT and T2D groups ($P<0.05$, $P<0.001$ and $P<0.001$, respectively) compared with NGT group. HOMA-IR was significantly greater in IFG, IGT and T2D groups ($P<0.05$, $P<0.001$ and $P<0.001$, respectively) compared with NGT group.

Serum hsCRP and plasma NOx concentrations were higher in T2D ($P<0.01$) but not in IFG or IGT group compared with NGT group.

### Table 2. Spearman univariate correlations (r) between plasma NOx concentrations and demographic, anthropometric and biochemical variables in 4 OGTT-stratified groups

| Variable                  | NGT ($n=120$) | IFG ($n=111$) | IGT ($n=101$) | T2D ($n=90$) |
|---------------------------|---------------|---------------|---------------|--------------|
| Gender                    | $-0.218^*$    | $-0.240^*$    | $-0.149$      | $-0.176$     |
| Age                       | 0.024         | 0.050         | 0.097         | 0.002        |
| BMI                       | 0.079         | $-0.038$      | $-0.079$      | $-0.247^*$   |
| Waist circumference       | 0.088         | 0.008         | 0.077         | -0.183       |
| Systolic blood pressure   | 0.079         | 0.141         | 0.018         | -0.099       |
| Diastolic blood pressure  | 0.018         | 0.150         | 0.008         | -0.251*      |
| Fasting glucose           | 0.172         | 0.215*        | 0.125         | 0.191        |
| Fasting insulin           | $-0.076$      | $-0.113$      | $-0.050$      | $-0.174$     |
| HOMA-IR                   | $-0.057$      | $-0.098$      | $-0.019$      | $-0.099$     |
| Total cholesterol         | 0.090         | $-0.101$      | $-0.080$      | $-0.071$     |
| Triglyceride              | 0.265**       | 0.124         | 0.133         | 0.144        |
| HDL-C                     | $-0.141$      | $-0.158$      | $-0.239^*$    | $-0.208^*$   |
| Non HDL-C                 | 0.104         | $-0.056$      | $-0.017$      | $-0.018$     |
| TG: HDL-C ratio           | 0.258**       | 0.133         | 0.159         | 0.198        |
| hsCRP                     | $-0.027$      | $-0.155$      | $-0.001$      | $-0.016$     |

BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model of assessment of insulin resistance; hsCRP, high sensitive C-reactive protein; NOx, nitrite and nitrate combined; TG, triglyceride.

r indicates Spearman correlation coefficient between plasma NOx concentration and each variable. *$P<0.05$, **$P<0.01$

### Univariate correlation analysis to determine factors associated with plasma NOx concentrations in 4 OGTT-stratified groups

To identify variables that are associated with plasma NOx concentrations, univariate correlation analysis was performed separately in each OGTT-stratified group. The Spearman correlation coefficients of variables in the association with plasma NOx concentration are shown in Table 2. Among subjects with T2D, BMI ($r = -0.247$, $P<0.05$), HDL-C ($r = -0.208$, $P<0.05$) and diastolic blood pressure ($r = -0.251$, $P<0.05$) were inversely correlated with plasma NOx concentrations whereas no significant association was found between plasma NOx concentrations and HOMA-IR.

Although plasma NOx levels were similar among 3 non-diabetic groups (NGT, IFG and IGT), factors associated with plasma NOx were different. Among NGT subjects, plasma NOx levels were positively correlated with male sex ($r = -0.218$, $P<0.05$), serum TG levels ($r = 0.265$, $P<0.01$) and TG: HDL-C ratios ($r = 0.258$, $P<0.01$). Among IFG subjects, plasma NOx levels were positively correlated with male sex ($r = -0.240$, $P<0.05$) and fasting glucose levels ($r = 0.215$, $P<0.05$). Among IGT subjects, plasma NOx levels were inversely correlated with serum HDL-C levels ($r = -0.239$, $P<0.05$); however, no significant association was found between plasma NOx concentrations and HOMA-IR or fasting insulin when the analysis was performed on any OGTT-stratified group as well as on 3 non-diabetic groups combined (data not shown).

### Multiple regression analysis to determine independent predictors of higher plasma NOx concentrations in 4 OGTT-stratified groups

Forward stepwise multiple linear regression analysis was performed to determine independent determinants of plasma NOx concentrations among 4 OGTT-stratified groups (Table 3). Gender, age and all other covariates that were found to positively correlate with plasma NOx concentrations by univariate correlation analysis were taken into multiple regression models. In T2D subjects, independent predictors of plasma NOx levels were diastolic blood pressure (B-coefficient = $-0.239$, $P=0.022$) and BMI (B-coefficient = $-0.251$, $P=0.001$).
The independent predictors of plasma NOx levels were different among 3 non-diabetic groups despite their similar NOx levels. In each group, a single determinant of plasma NOx concentrations was found with a quite low coefficient of determination of the regression (adjusted $R^2$ values varied from $0.044$ to $0.093$). Serum triglyceride levels were the determinant ($B$-coefficient $0.317$, $P<0.001$) that accounted for $9.3\%$ variations of plasma NOx concentrations in NGT subjects. Male gender was the determinant ($B$-coefficient $-0.230$, $P=0.015$) that accounted for only $4.4\%$ variations of plasma NOx levels in IFG subjects while serum HDL-C levels were the determinant ($B$-coefficient $-0.241$, $P=0.015$) that accounted for only $4.9\%$ variations of plasma NOx levels in IGT subjects.

### Discussion

The current study clearly showed that plasma NOx levels were elevated in subjects with T2D compared with NGT among Vietnamese. This finding was in accordance with previous reports by others on Iranian, Taiwanese and Japanese populations\(^{13, 14, 20}\), however, plasma NOx levels in IFG and IGT were neither significantly increased nor decreased compared with NGT. Furthermore, no correlation was found between plasma NOx concentrations and HOMA-IR in both diabetic and non-diabetic subjects. Thus, plasma NOx levels predict neither insulin resistance nor glucose intolerance before the development of T2D.

Several experimental studies using mouse models have shown that adipose tissue is a potential source of NO\(^{21-22}\) and the overproduction of NO by inducible NO synthase (iNOS) is involved in the development of insulin resistance in both genetic and diet-induced obesity\(^{23-26}\). However, the current study revealed that plasma NOx concentrations were not positively associated with obesity in T2D subjects but were instead inversely correlated with BMI, suggesting that the increase in plasma NOx levels in T2D was not caused by adiposity. Moreover, we found no association between plasma NOx levels and BMI or waist circumference in non-diabetic subjects where plasma NOx levels were not increased.

In the current study, we could not pinpoint why plasma NOx levels were increased in T2D, since the only variables that were significantly associated with higher plasma NOx levels in multiple regression analysis were lower BMI and lower diastolic blood pressure. The inverse association of diastolic blood pressure with plasma NOx levels was most likely due to the consequence of the effect of NO on blood pressure, since excess NO has been shown to decrease blood pressure in T2D\(^{27}\). There was a borderline pos-
itive correlation between fasting glucose and NOx concentrations \((r = 0.191, P = 0.071)\) in T2D subjects (see Table 2); therefore, the inverse association between plasma NOx levels and BMI might indicate that higher plasma NOx levels in T2D are associated with more severe diabetes. In an animal model, the upregulation of iNOS mRNA was found in the pancreatic islets of Zucker diabetic rats, which further led to β cell destruction and impaired insulin secretion. Both nicotinamide and aminoguanidine, which lower NO production, ameliorated β cell destruction and hyperglycemia in this model\(^{28}\). The finding suggests that increased NO could exacerbate T2D by further decreasing insulin secretion. Thus, although plasma NOx did not predict high-risk subjects for T2D, it might predict the outcome of T2D.

Although plasma NOx levels in IFG and IGT were neither significantly increased nor decreased compared with those in NGT, predictors of plasma NOx levels were different among 3 non-diabetic groups. While the serum TG level was an independent predictor of the plasma NOx level in NGT, it was not in IGT; instead, the serum HDL-C level was an independent predictor in this group. We do not have enough evidence to explain exactly why serum TG and HDL-C levels were correlated with plasma NOx levels in NGT and IGT, respectively; however, it is clear that the association of plasma NOx levels with serum TG or HDL-C levels was not due to insulin resistance, since no correlation of plasma NOx levels with HOMA-IR was found in any of 3 non-diabetic groups. There was an association between plasma NOx levels and the TG: HDL-C ratio, which is considered to be a surrogate marker for insulin resistance, in NGT. However, the fact that plasma NOx levels did not correlate with HOMA-IR in NGT indicates that the association between plasma NOx levels and the TG: HDL-C ratio in this case was not due to insulin resistance. Although the exact mechanism regulating plasma NOx levels has not been well understood, plasma NOx levels can be affected by the expression levels and activity of eNOS and iNOS. The divergence in the predictors of plasma NOx levels in 3 non-diabetic groups suggests that the expression levels and activity of eNOS and iNOS may be different in the 3 non-diabetic groups even though plasma NOx levels were similar.

**Conclusion**

Plasma NOx concentrations were significantly increased in T2D but not in subjects with glucose intolerance before the development of T2D. Plasma NOx levels were not associated with adiposity or insulin resistance in both diabetic and non-diabetic subjects. Thus, plasma NOx is not a useful marker to predict high-risk subjects for T2D in the Vietnamese population.

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