

Systemic nitric oxide metabolites and the chance of pre-diabetes regression to normoglycemia: A 9-year cohort study

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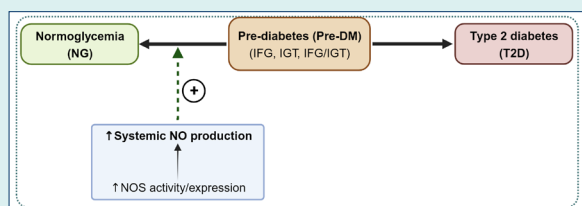
Abstract

Introduction: We aimed to track longitudinal changes of glycemic status in subjects with pre-diabetes (Pre-DM) in relation to their baseline levels of systemic nitric oxide (NO) production [i.e., measured as serum NO metabolites (NO_x), crude and body weight (BW)-adjusted NO_x to creatinine ratio (NO_x-to-Cr)] over 9 years.

Methods: This cohort study included 541 middle-aged Iranian men and women with Pre-DM, recruited in 2006-2008 and followed up to 2015-2017. The colorimetric Griess method was used to measure serum NO_x concentration. Multinomial logistic regression analyses estimated the odds ratios (OR) of Pre-DM regression and progression across tertiles (tertile 3 vs. tertile 1 and tertile 2) of serum NO_x, crude, and BW-adjusted NO_x-to-Cr ratio.

Results: Participants who regressed to normoglycemia (NG) had a higher BW-adjusted NO_x-to-Cr ratio than those who developed type 2 diabetes (T2D) or those who remained Pre-DM (0.52±0.34 vs. 0.43±0.25 and 0.48±0.29, $P=0.023$). Higher BW-adjusted NO_x-to-Cr increased chance of returning to NG (OR=2.05, 95% CI= 0.98-4.32, $P=0.058$) and decreased levels of 2h-serum glucose over time ($P_{\text{time} \times \text{group}}=0.025$), as well as the decreased overall mean of fasting (106, 95% CI=103-109 vs. 110, 95% CI=108-112 mg/dL, $P=0.008$) and 2h-serum glucose (153, 95% CI=146-159 vs. 163, 95% CI=158-168 mg/dL, $P=0.018$).

Conclusion: A higher endogenous NO production (i.e., indirectly measured by BW- and Cr-adjusted serum NO_x concentration) in Pre-DM subjects is associated with the chance of returning to NG.



Introduction

Nitric oxide (NO) is a signaling gasotransmitter,^{1,2} which regulates whole-body glucose and insulin metabolism.^{3,4} The involvement of NO in regulating glucose and insulin homeostasis in humans is supported by genetic studies introducing endothelial NO synthase (eNOS)-gene polymorphisms [e.g., 27bp-VNTR (27-base pair-variable number of tandem repeats),⁵ G894T (Glu298Asp or reference single nucleotide polymorphism (rs)1799983 in exon 7),⁶ E298D (polymorphism in Exon7 with substitution of glutamic acid (E) at codon 298 by aspartic acid (D)), and intervening sequence 18 (IVS18) + A27C

(Ala27Cys) polymorphism in intron 18⁷] as strong predictors of insulin resistance (IR) and type 2 diabetes (T2D). A predominant eNOS gene polymorphism, 4b4a VNTR polymorphism, is related to a lower circulating NO concentration⁸ and a higher risk of T2D.⁹

Disrupted NO metabolism seems to contribute to the pathophysiology of IR and T2D,¹⁰ however, the association of systemic NO production with pre-diabetes (Pre-DM) [i.e., a common intermediate dysglycemia characterized by isolated impaired fasting glucose (iIFG), isolated impaired glucose tolerance (iIGT), or combined IFG-IGT¹¹] has not yet been fully clarified. Epidemiologic evidence implies



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that serum NO metabolites (nitrate + nitrite=NO_x), a surrogate of NO production,¹² may improve predictive models of cardiometabolic outcomes.¹³⁻¹⁵ An increased NO production at early stages of dysglycemia has been reported¹⁶⁻¹⁸ that might be a compensatory mechanism for its function, a hypothesis needs to be elucidated within a longitudinal follow-up of Pre-DM subjects.

Here, we aimed to track changes in glycemic status [Pre-DM regression to normoglycemia (NG) or progression to T2D] in relation to baseline values of systemic NO production [measured as serum NO_x, as well as both crude and body weight (BW)-adjusted NO_x to creatinine ratio (NO_x-to-Cr)] over 9 years, among middle-aged Pre-DM adults. The use of Cr- and BW-adjusted rather than the crude NO_x concentration provides a more accurate estimation of whole-body NO production because serum nitrate is highly affected by renal function¹⁹ and has an extensive distribution volume (~28% of the BW).¹² We also determined longitudinal changes and cumulative average of glycemic parameters over time across different levels of systemic NO production in Pre-DM subjects.

Subjects and Methods

Study population

Here, we used participants' data from the Tehran Lipid and Glucose Study (TLGS), a community-based cohort study initiated in 1999 to investigate and prevent non-communicable diseases (NCDs).²⁰ Men and women (age ≥21 years) participated in the third TLGS examination (2006-2008) with Pre-DM (n=541), who were assessed for serum NO_x concentrations and had completed data, were recruited for the current study and followed up to 2015-2017. The median follow-up was 9.2 years (interquartile range= 7.8-10.2 years). Fig. 1 shows the flow of the study participants through the examinations. All study participants completed written informed consent.

Measurements of variables

Data collection has been reported elsewhere in detail.^{20,21} History of NCDs, medications (i.e., medications for dyslipidemia and hypertension, or use of NO-releasing drugs, including nitroglycerin, nitrocontin, and isosorbide dinitrate), lifestyle [smoking habits and physical activity (PA)] as well as measurements of BW, waist circumference (WC), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were performed by the trained interviewers at 3-y follow-up intervals. Details of PA measurements were described elsewhere.²² PA level was described as metabolic equivalent (MET) and categorized at three levels of light, medium, and high-PA (corresponding to MET<600, 600-1499, and ≥ 1500 minutes/week, respectively).²³

Biochemical measurements were described elsewhere in detail.²⁴ Blood samples were obtained following a 12-14 h overnight fasting. Both fasting (FSG) and 2hour-(2h-SG) serum glucose, triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) were measured at baseline and all subsequent examinations, using the enzymatic colorimetric methods (Pars Azmoon, Tehran, Iran).²⁴ The standard oral glucose tolerance test (OGTT) was performed after glucose ingestion (82.5 g glucose monohydrate solution equivalent to 75 g anhydrous glucose; Cerestar EP, Spain) in participants who did not receive hypoglycemic medications. The Jaffe kinetic alkaline picrate method was used for measuring serum Cr concentrations. Intra- and inter-assay coefficients of variation (CV) were <5.0% for all measurements. As described elsewhere in detail,²⁵ serum NO_x concentration was measured using the modified Griess method.^{26,27}

Definitions

Pre-DM was defined as IFG ($100 \leq \text{FSG} < 126$ mg/dL), IGT ($140 \leq \text{2h-SG} < 200$ mg/dL), or combined IFG-IGT.²⁸

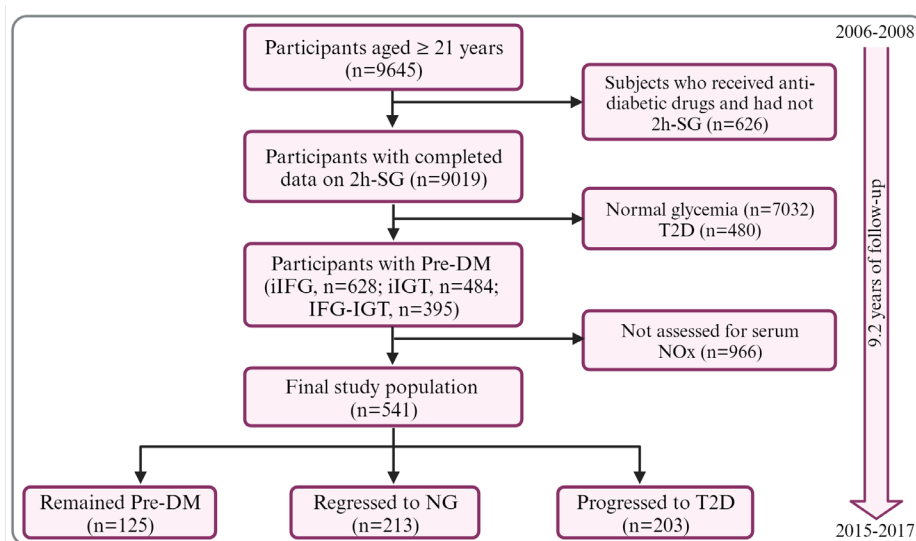


Fig. 1. Flowchart of study. 2h-SG, 2hour-serum glucose; Pre-DM, pre-diabetes; NG, normoglycemia; T2D, type 2 diabetes; iIFG, isolated impaired fasting glucose; iIGT, isolated impaired glucose tolerance; NO_x, nitric oxide metabolites (nitrate + nitrite). The figure was created with BioRender.

Incidence of NG was considered as the first occurrence of both normal fasting glucose (NFG: FSG <100 mg/dL) and normal glucose tolerance (NGT: 2h-SG <140 mg/dL). The incidence of T2D was considered as the first occurrence of FSG \geq 126 mg/dL, 2h-SG \geq 200 mg/dL, or the use of glucose-lowering drugs. Family history of T2D (FHD) was considered positive when having at least one parent/sibling with T2D.

Statistical methods

SPSS for Windows version 20 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. To compare the baseline characteristics of the study participants, analysis of variance and chi-square test were used for the continuous variables with normal distribution and categorical variables, respectively. Multinomial logistic regression analyses estimated the odds ratios (95% confidence intervals, CIs) for Pre-DM regression and progression across tertiles (tertile 3 vs. tertile 1 and tertile 2) of serum NOx (<35 and \geq 35 μ mol/L), serum NOx-to-Cr (<0.38 and \geq 0.38), and BW-adjusted NOx-to-Cr ratio (<0.50 and \geq 0.50). Potential covariates were chosen based on scientific²⁹ and statistical evidence.³⁰ A univariate analysis was performed to identify potential confounding variables, and P_E (P value for entry) <0.2 was considered inclusion criteria in the final multivariable models.³⁰ Table S1 indicates the potential covariates and their OR (95% CIs) in the univariate multinomial logistic regression. Finally, two logistic models were conducted: age, sex, FSG, and 2h-SG were adjusted in model 1, and model 2 was additionally adjusted for FHD, SBP, and WC. No evidence of interaction between serum NOx and sex was observed in relation to the outcomes ($P_{\text{sex} \times \text{NOx}} = 0.695$ and 0.711 , for NG and T2D, respectively); the analyses, therefore, were conducted overall.

To determine longitudinal changes of glycemic parameters (FSG and 2h-SG concentrations) and to estimate their cumulative average across different levels (tertile 3 vs. tertile 1 and tertile 2) of serum NOx, crude, and BW-adjusted NOx-to-Cr ratio, repeated-measures ANOVA was used.

Results

Over 9 years, Pre-DM regression and progression rates were 39.4% and 37.5%, respectively. Mean serum NOx and Cr concentrations were 33.1 ± 17.8 and 95.3 ± 14 μ mol/L at baseline.

Table 1 shows the baseline characteristics of the study participants. Participants who regressed to NG were significantly younger and had lower BW, WC, FSG, 2h-SG, and serum Cr than those who progressed to T2D. They also had a higher BW-adjusted NOx-to-Cr ratio than those who developed T2D or remained Pre-DM (0.52 ± 0.34 vs. 0.43 ± 0.25 and 0.48 ± 0.29 , $P = 0.023$). The frequency of using NO-releasing drugs was significantly different between groups (7.2%, 1.4%, and 3.4% in those

who remained Pre-DM, regressed to NG, and progressed to T2D, respectively).

Table 2 displays serum NOx concentrations and crude and BW-adjusted NOx-to-Cr ratio across Pre-DM phenotypes (iIFG, iIGT, and IFG-IGT) at baseline. No significant difference was found between Pre-DM phenotypes for serum NOx and other NOx-based values.

As indicated in Table 3, adjusted (age, sex, FSG, 2h-SG)-OR for regression to NG among the participants with serum NOx-to-Cr \geq 0.38 was 1.79 (95% CI=0.91-3.53), compared with people who had a ratio lower than 0.38. After further adjusting for FHD, SBP, WC, and PA, the association was strengthened but remained non-significant (OR=1.85, 95% CI=0.89-3.82). The Pre-DM subjects with BW-adjusted NOx-to-Cr \geq 0.5 had a higher chance of returning to NG, independent of the known potential confounders (OR=2.05, 95% CI=0.98-4.32, $P = 0.058$).

Using repeated measurement ANOVA (Table 4), we also found that higher levels of BW-adjusted NOx-to-Cr \geq 0.5 were associated with repeated lower levels of 2h-SG over time ($P_{\text{time} \times \text{group}} = 0.025$) and a lower overall mean of both FSG (106, 95% CI=103-109 vs. 110, 95% CI=108-112 mg/dL, $P = 0.008$) and 2h-SG (153, 95% CI=146-159 vs. 163, 95% CI=158-168 mg/dL, $P = 0.018$). Neither overall means nor trends of FSG and 2-SG changes over time differed between lower and higher levels of serum NOx and NOx-to-Cr ratio (data not shown).

Discussion

Following some preliminary observations about the potential compensatory overproduction of NO at the initial stages of dysglycemia, we followed longitudinal changes in the glycemic status of Pre-DM subjects based on their baseline systemic NO levels. A 9-year follow-up of Pre-DM subjects showed that the chance of returning to NG increased by 2-fold in those with higher baseline levels of whole-body NO production (indicated as adjusted NOx-to-Cr ratio \geq 0.5). The Pre-DM subjects with a higher systemic NO production also experienced a better longitudinal postprandial glucose tolerance (i.e., measured as repeated 2h-SG levels during the study with 3-year intervals). This glycemic parameter mainly denotes peripheral insulin sensitivity and is related to endothelial function.³¹

Pre-DM is now affecting about 30% of the middle-aged adults worldwide. Although over 70% of Pre-DM subjects will progress to T2D with an annual rate of 5-10% per year,³² the chance of NG is predicted to be 33-59% within 1-5 years.³³ Several biological (e.g., age, sex, FHD, obesity, hypertension, serum glucose, and lipid levels) and lifestyle (dietary factors, smoking, and PA) determinants may potentially predict the chance of Pre-DM regression and progression.^{29,34}

Change of systemic NO concentrations in T2D is stage-dependent since an increased NO production occurs

Table 1. Baseline characteristics of the study participants according to the outcomes (n=541)

	Remained Pre-DM (n=125)	Regressed to NG (n=213)	Progressed to T2D (n=203)
Age (y)	57.4±13.3	50.5±13.7 ^{a,b}	55.5±13.7
Men (%)	48.0	39.0	46.3
FHD (%)	18.4	22.0	29.4
Medications			
Lipid-lowering (%)	6.3	5.4	7.7
BP-lowering (%)	8.9	7.0	9.0
NO-releasing drugs (%)	7.2*	1.4	3.4
TNG (%)	4.8	1.4	0.2
Nitrocontin (%)	0.8	0	0
ISDN (%)	1.6	0	1.8
Smoking (%)	22.4	25.4	20.7
Physical activity (MET-h/week)	32.1±55.5	39.6±61.8	42.1±61.8
Light (%)	53.3	62.5	60.4
Moderate (%)	23.0	23.1	23.1
High (%)	23.7	14.0	16.5
BW (kg)	74.6±12.1 ^b	74.3±14.2 ^b	79.1±14.4
WC (cm)	98.3±11.1	95.2±12.5 ^b	101±11.0
SBP (mm Hg)	125±17.8	122±19.8	128±20.1
DBP (mm Hg)	75.6±9.6	76.4±9.2	76.9±11.6
FSG (mg/dL)	102±7.9 ^b	97.7±8.8 ^{a,b}	105±9.5
2h-SG (mg/dL)	135±30.0 ^b	130±29.3 ^b	150±31.0
TG to HDL-C ratio	4.8±3.4	4.6±3.9	5.2±3.8
Serum NOx (μmol/L)	33.1±18.2	33.7±18.6	32.4±16.8
Serum Cr (μmol/L)	96.2±11.3	93.0±13.6 ^{a,b}	97.1±15.5
NOx-to-Cr ratio	0.35±0.19	0.37±0.22	0.33±0.18
BW-adjusted NOx-to-Cr ratio	0.48±0.29	0.52±0.34 ^b	0.43±0.25

2h-SG, 2-hour serum glucose; BW, body weight; Cr, creatinine; DBP, diastolic blood pressure; FHD, family history of T2D; FSG, fasting serum glucose; HDL-C, high-density lipoprotein cholesterol; ISDN, isosorbide dinitrate; MET, metabolic equivalent; NG, normoglycemia; NOx, nitric oxide metabolites (nitrate+nitrite); Pre-DM, pre-diabetes; SBP, systolic blood pressure; T2D, type 2 diabetes; TG, serum triglyceride; TNG, nitroglycerin; WC, waist circumference.

Data are mean ± SD (unless stated otherwise).

Physical activity levels (Light <10, moderate 10-25, and high ≥25 MET-h/week).

^a Significant difference with Pre-DM ($P<0.05$); ^b Significant difference with T2D ($P<0.05$).

* A significant difference between groups (chi-square test was used, $P=0.020$).

Table 2. Nitric oxide (NO)-related parameters across Pre-DM phenotypes

	iIFG	iIGT	Combined IFG-IGT
Serum NOx (μmol/L)	32.9±17.4	32.9±18.7	33.5±17.6
Serum NOx-to-Cr ratio	0.34±0.19	0.36±0.22	0.35±0.19
BW-adjusted NOx-to-Cr ratio	0.46±0.28	0.52±0.34	0.48±0.29

Data are mean ±SD

BW, body weight; Cr, creatinine; iIFG, isolated impaired fasting glucose; iIGT, isolated impaired glucose tolerance; NOx, serum nitric oxide metabolites (nitrate+nitrite).

early, and a reduced production is observed in established T2D.^{35,36} Both human and animal studies reported an increased NO production in the pre-DM state,³⁷⁻⁴⁰ while in established T2D, a diminished NO production was observed.^{41,42} Both fractional and absolute rates of NO production from *L*-arginine are diminished in T2D compared to NG (19.3±3.9% vs. 22.9±4.5% per day, and 320 vs. 890 μmol per day, respectively).⁴¹ The rate of NO synthesis from plasma *L*-arginine turnover was reduced by 50%, resulting in a 16% diminished rate of whole-body

NO production in T2D compared to NG.⁴² The impaired NO production in T2D may be explained by decreased eNOS activity⁴³ and expression,⁴⁴ decreased eNOS sensitivity to its agonists,⁴⁵ elevated arginase activity,⁴⁶ and *L*-arginine deficiency.⁴⁶

A compensatory NO overproduction (reported up to 2-fold compared to controls³⁹) during the initial stages of impaired glucose and insulin homeostasis is mainly explained by hyperglycemia- and hyperinsulinemia-induced upregulation of NOS expression and activity.^{47,48}

Table 3. The odds ratio (95% CI) of pre-diabetes (Pre-DM) regression to normoglycemia (NG) and progression to type 2 diabetes (T2D) in relation to nitric oxide (NO)-related parameters

	Regressed to NG	Progressed to T2D
Serum NOx ($\geq 35 \mu\text{mol/L}$)		
Crude	1.07 (0.67-1.71)	0.95 (0.59-1.53)
Model 1	1.02 (0.62-1.66)	0.99 (0.60-1.63)
Model 2	1.45 (0.80-2.63)	1.33 (0.72-2.46)
Serum NOx-to-Cr (≥ 0.38)		
Crude	1.30 (0.82-2.08)	0.87 (0.54-1.41)
Model 1	1.79 (0.91-3.53)	1.22 (0.62-2.39)
Model 2	1.85 (0.89-3.82)	1.28 (0.63-2.56)
BW-adjusted NOx-to-Cr (≥ 0.50)		
Crude	1.46 (0.92-2.33)	0.84 (0.52-1.36)
Model 1	1.85 (0.93-3.66)	1.13 (0.57-2.22)
Model 2	2.05 (0.98-4.32)	1.30 (0.63-2.69)

Data are odds ratios (ORs) and 95% confidence intervals (95% CI) (odds values refer to T_3 vs. T_1 and T_2)

Multinomial logistic regression was used.

Model 1, adjusted for age, sex, fasting serum glucose (FSG), and 2-hour serum glucose (2h-SG).

Model 2, additionally adjusted for family history of T2D (FHD), systolic blood pressure (SBP), waist circumference (WC) and physical activity (PA).

BW, body weight; Cr, creatinine; NOx, nitric oxide metabolites (nitrate+nitrite).

These compensatory mechanisms may lead to adaptation and relative normalization of whole-body NO production to maintain its function in handling glucose and insulin homeostasis. NO regulates insulin secretion⁴⁹ and potentiates skeletal muscle insulin sensitivity and glucose uptake by increasing blood flow-dependent glucose delivery, increasing expression and membrane translocation of glucose transporter 4, and increasing transendothelial insulin transport.⁵⁰⁻⁵² Moreover, NO facilitates insulin-dependent and -independent glucose uptake in adipose tissue.^{53,54}

Some points should be considered when interpreting this study's findings. First, since two independent pathways (i.e., L-arginine-NOS and nitrate-nitrite pathways) are involved in endogenous NO production, the interpretation of serum NOx values might be complex. Serum nitrate is valid as a surrogate of NOS activity if

its non-NOS sources (i.e., ingested nitrate from food, water, and medications containing NO prodrugs, inhaled nitrogen oxides, originated nitrate from gut microbiota, and potential contamination of serum samples with nitrogen oxides during measurements) are excluded.⁵⁵ Second, because serum NOx concentration is affected by renal function, using its Cr-corrected values is considered a more accurate indicator of NOS activity than the crude NOx levels.⁵⁵ Third, due to its relatively large distribution volume (28% of BW) and low clearance rate ($30 \pm 2 \text{ mL/min/1.73 m}^2$), extensive, significant, and long-term changes in NO synthesis are required to affect circulating nitrate significantly¹²; serum NOx, therefore, may represent the long-lasting status of whole-body NO production. Fourth, serum NOx could not be considered equal to NO availability in the body because increased serum NOx may co-occur with a decreased biologically active form of NO and vice versa.⁵⁶

This investigation is the first attempt to address the potential association of whole-body NO production with the transition of Pre-DM to T2D and NG within a population-based setting. However, the study had some limitations. Our limited sample size may diminish the findings' generalizability. Because of the distinct pathophysiology of IGT and IFG,³¹ the observed association may differ for these subgroups; however, we did not address the hypothesis due to a relatively low sample size and insufficient power. Here, systemic NO production was estimated using the measurement of NO metabolites instead of direct evaluation. Due to the low levels and short half-life of circulating, measurement of its metabolites (nitrate+nitrite), rather than its direct measurement, is a common and practical approach in population-based studies.^{57,58} After overnight fasting, NOx measurement may reflect systemic NO production in subjects without renal dysfunction since the half-life of oral nitrate is about 5 hours.⁵⁹ In our study, blood sampling was taken after 12-14 hours of fasting, significantly reducing the contribution of ingested nitrate in the circulation.

Conclusion and perspective

In conclusion, our findings imply that a higher endogenous rate of NO production (i.e., indicated as BW adjusted

Table 4. Mean concentrations of glycemic parameters in subjects with lower and higher values of body weight (BW)-adjusted NOx-to-creatinine (Cr) ratio over 9 years of follow-up

	Baseline (2006-2008)	First follow-up (2009-2011)	Second follow-up (2012-2014)	Third follow-up (2015-2017)	Overall mean (95% CI)	P _{time×group}
FSG (mg/dL)						
<0.50	102±9.4	110±21.9	115±30.4	115±33.8	110 (108-112)	0.147
≥0.50	100±9.9	105±14.2	108±21.7	109±25.0	106 (103-109)*	
2h-SG (mg/dL)						
<0.50	138±31.9	153±54.7	173±69.1	188±77.0	163 (158-168)	0.025
≥0.50	139±30.3	142±45.6	155±59.9	175±64.5	153 (146-159)**	

Data are mean \pm SD. The repeated measures analysis of variance (ANOVA) was used. * $P=0.008$ ** $P=0.018$ for the overall mean difference between categories. 2h-SG, 2-hour serum glucose; FSG, fasting serum glucose; NOx, nitric oxide metabolites (nitrate+nitrite).

NO_x-to-Cr ratio ≥ 0.5) is associated with the chance of Pre-DM remission by 2-fold. This observation suggests that serum NO_x concentration (a rapid, inexpensive, and easily measured biomarker of NO production) may be used alongside other factors to predict the future state of Pre-DM subjects. However, our findings were derived from a relatively low-sample size population and needed to be approved by further larger sample-size prospective cohorts. The association of endogenous NO production and Pre-DM regression and progression must be distinguished across Pre-DM phenotypes (iIFG, iIGT, and combined IFG-IGT) to provide helpful evidence for designing individualized therapeutic strategies in Pre-DM. Future investigations must also address whether dietary approaches and therapeutic agents targeting NO production may affect Pre-DM regression and progression.

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Authors' Contribution

Conceptualization Zahra Bahadoran, Asghar Ghasemi.

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Competing Interests

The authors have no conflict of interest.

Ethical Statement

The study protocol was evaluated and approved by the ethics research council of the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics code: IR.SBMU.ENDOCRINE.REC.1401.112). Written informed consent was obtained from all TLGS participants.

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Supplementary file 1

Supplementary file 1 contains Table S1.

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Research Highlights

What is the current knowledge?

- ✓ Impaired nitric oxide (NO) metabolism contributes to the development of type 2 diabetes.
- ✓ Increased NO production was reported in the initial stages of dysglycemia and insulin resistance.
- ✓ The association of systemic NO production with changes in glycemic status in pre-diabetes (Pre-DM) subjects is unclear.

What is new here?

- ✓ Higher levels of NO production in Pre-DM subjects may increase the chance of returning to normoglycemia.

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