



# The Nitrate-Nitrite-Nitric Oxide Pathway: Findings from 20 Years of the Tehran Lipid and Glucose Study

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## Abstract

**Context:** We describe here the contributions of the Tehran lipid and glucose study (TLGS) to understanding different aspects of the nitrate (NO<sub>3</sub>)-nitrite (NO<sub>2</sub>)-nitric oxide (NO) pathway in health and disease.

**Evidence Acquisition:** All English-language documents from the TLGS, focused on NO pathway were searched using the PubMed, Scopus, and Embase databases.

**Results:** Reference values of serum concentrations of NO metabolites (nitrate+nitrite or NOx) were 11.5 - 76.4, 10.1 - 65.6, and 10.3 - 66.8 μmol/L in men, women, and the total population, respectively. Circulating NOx was affected by age, smoking habits, menopause status, thyroid hormones, and various pathologic conditions. Elevated serum NOx was related to increased incidence of metabolic syndrome (odds ratio (OR) = 1.75, 95% confidence interval (CI) = 1.19 - 2.59), hypertriglyceridemic-waist phenotype (OR = 1.39, 95% CI = 1.05 - 1.93), chronic kidney disease (OR = 1.86, 95% CI = 1.10 - 3.14) in women, and cardiovascular disease (hazard ratio (HR) = 1.35, 95% CI = 1.01 - 1.80] in the total population. In participants with low vitamin C intake, higher intakes of NO<sub>2</sub> (≥ 8.77 mg/d) were accompanied with increased risk of diabetes (HR = 2.43, 95% CI = 1.45 - 4.05). A decreased risk of hypertension (OR = 0.58, 95% CI = 0.33 - 0.98) and chronic kidney disease (OR = 0.50, 95% CI = 0.24 - 0.89) was observed in response to higher intakes of NO<sub>2</sub>.

**Conclusions:** Circulating NOx is associated with and could predict the risk of metabolic disorders in a general population. Moreover, dietary NO<sub>3</sub>/NO<sub>2</sub> exposure from usual diets seems to contribute to development of noncommunicable diseases.

**Keywords:** Nitric Oxide, Nitrate, Nitrite, Diabetes, Obesity, Metabolic Syndrome, Cardiovascular Disease

## 1. Context

Nitric oxide (NO), a simple ubiquitous signaling molecule, is suggested to be linked with several physiological processes including regulation of vascular homeostasis and blood pressure, inhibition of platelet activation, regulation of energy and lipid metabolism, mitochondrial biogenesis, and the modification of various physiological pathways (1-3). Interrupted NO metabolism, including either reduced or elevated production of NO, as well as its decreased bioavailability, has been reported as a risk factor and/or prognostic biomarker for development of chronic disorders especially cardiovascular disease (CVD), renal dysfunction, diabetes, hypertension (HTN), and different types of cancer (4-9).

In this review, we aim to describe contributions of the Tehran lipid and glucose study (TLGS) to understanding different aspects of the nitrate (NO<sub>3</sub>)-nitrite (NO<sub>2</sub>)-nitric oxide (NO) pathway in health and disease. All English-language documents from the TLGS focusing on serum concentrations of NO metabolites (nitrate+nitrite or NOx), or dietary intake of NO<sub>3</sub>/NO<sub>2</sub> were searched from PubMed, Scopus, and Embase databases. To summarize findings of these studies in the the first author's name, date of publication, number of participants, years of follow-up period in cohort studies, serum NOx concentrations (μmol/L), exposure levels of dietary NO<sub>3</sub> and NO<sub>2</sub>, definition of outcomes, and odds ratios (ORs) or hazard ratios (HRs) with their corresponding 95% confidence intervals (95% CIs) were ex-

tracted.

### 1.1. NOx Measurement in TLGS

TLGS is an ongoing community-based prospective study being conducted to investigate and prevent non-communicable diseases (NCDs), in a representative sample in the district 13 of Tehran, the capital city of Iran (10).

In the third phase of TLGS (2006 - 2008), in a sub-sample of participants (n = 4407), serum NOx levels were measured by a rapid and simple spectrophotometric method, validated in our laboratory (11,12). Inter- and intra-assay coefficients of variations of measurements were 5.2% and 4.4%, respectively; the sensitivity of the assay was 2.0  $\mu\text{mol/L}$  and its recovery was 93%  $\pm$  1.5% (13).

The mean ( $\pm$  SE) of serum NOx concentrations in normal subjects were 24.8  $\pm$  0.02 and 24.4  $\pm$  0.01  $\mu\text{mol/L}$  in the TLGS men and women, respectively (14). In our adults, the reference values of serum NOx concentration were 11.5 - 76.4, 10.1 - 65.6, and 10.3 - 66.8  $\mu\text{mol/L}$  in men, women, and total population, respectively (15). In the TLGS pediatric population (189 boys and 212 girls, aged 4 - 19 years), reference values for serum NOx concentrations were 13.6 - 69.2, 11.4 - 66.0, and 12.2 - 69.4  $\mu\text{mol/L}$  in boys, girls, and total population, respectively (16).

### 1.2. Factors Affecting Circulating NOx

In our population, a higher NOx levels was observed in men, compared to women aged 20 - 29 years (25.1  $\pm$  0.03 vs. 22.7  $\pm$  0.02  $\mu\text{mol/L}$ ), and serum NOx concentration showed a peak at 50 - 59 years in both genders; increased NOx levels between 50 and 59 years of age, declined after 60 years in men but not in women (14). Overall, there was also a significant increasing trend in circulating NOx across age groups, only in women (14). Our study conducted on 1209 middle-aged (40 - 60 years) TLGS participants indicated higher serum NOx values in postmenopausal women (median = 29, IQR = 21 - 43  $\mu\text{mol/L}$ ), compared to women with regular cycles (25.5  $\mu\text{mol/L}$ , IQR = 19 - 39) or men (26  $\mu\text{mol/L}$ , IQR = 20 - 37) (17). Serum NOx levels also show considerable elevation across pre-menopause to post-menopause transition (18); in a conditional fixed-effect logistic regression model, the chance of "transition to menopause" and "menopause" increased by 2.44 (95% CI = 1.17 - 5.08) and 2.27 (95% CI = 1.23 - 4.18) per 1 standard deviation increase in circulating NOx levels (18).

In a cross-sectional analysis, conducted on 1974 adult participants of the Tehran thyroid study, serum NOx levels were negatively associated with free thyroxin ( $T_4$ ) in men and anti-thyroperoxidase (TPO) levels in women (19). Insulin is another physiological factor that may affect serum

NOx concentration. In our population, a higher circulating NOx was observed in the highest compared to the lowest quartile of fasting serum insulin, an association that was statistically significant only in women (28.5 vs. 25.4  $\mu\text{mol/L}$ ) (20). NOx concentration was also weakly correlated with a homeostatic model assessment of insulin resistance and quantitative insulin sensitivity check index in women (20).

Effects of life style factors, like dietary habits and smoking, as potential moderators of circulating NOx, have been less documented. The possible adverse effects of passive cigarette and water pipe (qalyan) smoking on serum NOx concentration were documented following the first report in 2010 from the TLGS data (21). Serum NOx was significantly higher in the healthy active smokers (28.9 vs. 24.1  $\mu\text{mol/L}$ ), compared to nonsmokers; the number of cigarette smoked was also positively correlated with serum NOx concentrations. Qalyan smokers had higher serum NOx levels, compared to the non-smoker controls (34.3 vs. 22.5  $\mu\text{mol/L}$ ); serum NOx values were comparable between passive smokers and non-smokers (21).

Alterations of circulating NOx in relation to dietary factors or eating behaviors seem to be a neglected area in the field of NO metabolism. In a cross-sectional analysis in the TLGS population, we found a significant positive association between L-arginine intake from usual diet and serum NOx concentrations (22); this association was affected by sex, age, body mass index (BMI) and HTN, and a greater association was observed in women, middle-aged and older adults, overweight and obese subjects, as well as non-hypertensive compared to hypertensive subjects (22). Regular dietary intakes of  $\text{NO}_3/\text{NO}_2$  were correlated with their urinary excretion levels ( $r = 0.59$ , 95% CI = 0.49 - 0.67, and  $r = 0.29$ , 95% CI = 0.17 - 0.41, for  $\text{NO}_3$  and  $\text{NO}_2$ , respectively) in a sub-sample analysis of our population; fasting serum NOx was not related to  $\text{NO}_3/\text{NO}_2$  exposure ( $r = 0.19$ , 95% CI = 0.07, 0.32 and  $r = 0.09$ , 95% CI = -0.03, 0.23, for  $\text{NO}_3$  and  $\text{NO}_2$ , respectively) (23).

Other potential factors affecting circulating NOx, like physical activity, have not been assessed in the setting of the TLGS.

## 2. Association Between NCDs and Circulating NOx

### 2.1. Obesity

Reports from a cross-sectional analysis, using the TLGS data by our group showed positive associations between serum NOx concentrations and anthropometric measures including BMI, waist circumference (WC) and waist to hip ratio (WHR) in women (13), results that remained only for

BMI > 30 kg/m<sup>2</sup> ( $\beta = 5.4$ ,  $P = 0.001$ ) in multivariable adjusted model (13). A significant trend of increasing serum NOx concentrations was also observed across categories of BMI in women (13). In a prospective approach, we reported that serum NOx, beyond being a probable indicator, may also be a novel predictor of obesity phenotypes. Following a median of 6.3 years study of 2243 adults, we observed an association between development of hypertriglyceridemic waist phenotype (HTW) and elevated baseline serum NOx in women (HR=1.39, 95% CI=1.05-1.93)(24) (Table 1); compared to women in the first tertile of serum NOx, those in the third tertile ( $\geq 30.9$  vs.  $< 19.9$   $\mu\text{mol/L}$ ) had higher WC, both at baseline and follow-up examinations (24). We also showed that circulating NOx could predict changes of lipid accumulation product (LAP) index, a novel biomarker of central lipid accumulation related to risk of diabetes and CVD (25); serum NOx in the highest tertile was positively related to 6-years LAP changes ( $\beta = 5.23$ , 95% CI=1.69-7.78) (25).

## 2.2. Dysglycemia and Diabetes

In an earlier study in the TLGS population, we observed higher serum NOx values in diabetic subjects compared to their corresponding controls (34.6  $\mu\text{mol/L}$ , 95% CI = 31.3 - 38.2 vs. 30.2  $\mu\text{mol/L}$ , 95% CI = 27.9 - 32.6) (29).

The first investigation addressing potential association of serum NOx and diabetes was conducted on the TLGS population (15); a remarkable result to emerge from this cross-sectional analysis was that circulating NOx above reference values (65.6  $\mu\text{mol/L}$ ) increased chances of having type 2 diabetes in women (OR=1.67, 95% CI=1.10 - 2.55) (15).

## 2.3. Hypertension, Chronic Kidney Disease, and Cardiovascular Events

Limited number of studies have investigated NO-blood pressure relationship in the framework of a population-based setting. In the TLGS population, compared to controls, serum NOx levels were higher in both men and women with stage 1 HTN (14% and 23%, respectively); conversely, circulating NOx was significantly reduced in men with stage 2 HTN (30). In both stages, treated-hypertensive men had a higher serum NOx concentration, whereas in women, increased circulating NOx was observed just in stage 1 HTN (30). Considering serum NOx as an independent variable in the analysis, we observed that elevated serum NOx was not related to chances of having HTN (OR = 0.91, 95% CI = 0.49 - 1.70, and OR = 1.38, 95% CI = 0.95 - 2.01, in men and women, respectively) (15).

In a cross-sectional setting of 3462 TLGS participants, the odds of having chronic kidney disease (CKD) in both

men and women, in the highest compared to the lowest tertile of serum NOx ( $\geq 32$  vs.  $< 21$   $\mu\text{mol/L}$ ), were significantly higher (OR=1.61, 95% CI = 1.05 - 2.64 and OR = 2.64, 95% CI = 1.91 - 3.66); following adjustment for diabetes, CVD, HTN, medications and triglycerides (TG)-to-high density lipoprotein-cholesterol (HDL-C) ratio, elevated serum NOx was related to the likelihoods of CKD only in women (OR=2.48, 95% CI=1.76-3.49)(27) (Table 1). Our prospective design within the TLGS indicated that, in the presence of the well-known risk factors, serum NOx values  $\geq 32$   $\mu\text{mol/L}$  may predict 3-year risk of CKD in women (OR = 1.86, 95% CI = 1.10 - 3.14) (27). In a 3-year follow-up of adult (aged  $\geq 30$  years) men and women participated in the third phase of TLGS, results indicated that compared to the lowest quartiles of serum NOx, the incidence of CVD (10.1 vs. 4.4%,  $P = 0.002$ ) was higher in the highest quartile and the risk of CVD events increased by 35% (HR = 1.35, 95% CI = 1.01 - 1.80) for each 1 unit of increase in Ln-transformed serum NOx concentrations (28) (Table 1); in this analysis, incorporating circulating NOx into the traditional CVD risk model appropriately reclassified over 6% of individuals at risk (28).

## 2.4. Metabolic Syndrome

Only a few population-based studies have documented the importance of circulating NOx as a novel biomarker of MetS. In a study of 851 children and adolescents, aged 4 - 19 years, a higher prevalence of MetS (13.2% vs. 6.1%) was observed in the highest compared to the lowest quartile of serum NOx concentrations ( $\geq 33.0$  vs.  $< 19.0$   $\mu\text{mol/L}$ ) (31); age- and sex adjusted odds ratio of having MetS was significantly higher in the subjects with highest levels of NOx (OR = 2.2, 95% CI = 1.1 - 4.7) (31). Furthermore, co-clustering of NOx with other MetS components indicated that circulating NOx can be considered as a component of MetS (31).

In a short-term follow-up of the TLGS participants, risk of developing MetS was significantly higher (OR = 1.75, 95% CI = 1.19 - 2.59) in women who had higher basal serum NOx values ( $\geq 35$   $\mu\text{mol/L}$ ) (26) (Table 1); serum NOx-to-creatinine ratio, a marker of endogenous NO production, was also related to developing MetS in women (26).

## 3. Dietary Intake of NO<sub>3</sub> and NO<sub>2</sub> in Relation to Cardio-Metabolic-Renal Disease

Considering the importance of NO<sub>3</sub>/NO<sub>2</sub> exposure in NO homeostasis and its possible role in pathogenesis of NCDs, we recently developed a comprehensive database of the NO<sub>3</sub>/NO<sub>2</sub> content of commonly consumed food items (32). Following estimation of NO<sub>3</sub>/NO<sub>2</sub> exposure from usual diet in the TLGS population, we reported, for the first

**Table 1.** Associations Between Serum NOx Levels and the Incidence of Non-Communicable Diseases in the TLGS Population

Author	Study Population	Years of Follow-Up	Outcomes	Levels of Serum NOx ( $\mu\text{mol/L}$ )	Adjusted OR (95% CI) or HR (95% CI)
Ghasemi et al. (26)	Adult men (n = 644)	3.3	Metabolic syndrome	$\leq$ 75th percentile	1.00
				$>$ 75th percentile	0.93 (0.58 - 1.49) <sup>a</sup>
	Adult women (n = 1137)	3.3	Metabolic syndrome	$\leq$ 35.0	1.00
				$>$ 35.0	1.75 (1.19 - 2.59) <sup>a</sup>
Bahadoran et al. (27)	Adult men (n = 1063)	3	Chronic kidney disease	$<$ 21.0	1.00
				21.0 - 32.0	1.44 (0.67 - 3.11) <sup>a</sup>
				$\geq$ 32.0	0.98 (0.44 - 2.20) <sup>a</sup>
	Adult women (n = 1441)	3	Chronic kidney disease	$<$ 21.0	1.00
				21.0 - 32.0	1.53 (0.89 - 2.63) <sup>a</sup>
				$\geq$ 32.0	1.86 (1.10 - 3.14) <sup>a</sup>
Hadaegh et al. (28)	Adult men and women (n = 2443)	3.1	Cardiovascular events	Ln-transformed NOx as a continuous variable	1.35 (1.01 - 1.80) <sup>b</sup>
Bahadoran et al. (24)	Adult men (n = 762)	6	Hypertriglyceridemic-waist phenotype	$<$ 20.9	1.00
				20.9 - 29.9	1.41 (0.95 - 2.07) <sup>a</sup>
				$\geq$ 29.9	1.16 (0.78 - 1.72) <sup>a</sup>
	Adult women (n = 1172)	6	Hypertriglyceridemic-waist phenotype	$<$ 19.9	1.00
				19.9 - 30.9	1.19 (0.86 - 1.64) <sup>a</sup>
				$\geq$ 30.9	1.39 (1.05 - 1.93) <sup>a</sup>

Abbreviations: HR, hazard ratio; NOx, nitric oxide metabolites (nitrate+nitrite); OR, odds ratio.

<sup>a</sup> Indicates OR.

<sup>b</sup> Indicates HR.

time, the hazards of diabetes, HTN, CKD and CVD in response to different levels of dietary NO<sub>3</sub>/NO<sub>2</sub> (Table 2). Our findings indicate that incidence of diabetes was increased (HR = 2.43, 95% CI = 1.45 - 4.05, HR = 1.88, 95% CI = 1.12 - 3.15, respectively) in participants with low-vitamin C diets and higher intakes of total ( $\geq$  8.77 mg/d) and animal-based ( $\geq$  3.24 mg/d) NO<sub>2</sub>; we found no significant association between NO<sub>3</sub> in overall, and plant- or animal sources and the risk of diabetes (33). The highest compared to the lowest NO<sub>2</sub> intake ( $\geq$  10.7 vs.  $<$  7.6 mg/d) was accompanied with a significant reduced risk of HTN (OR = 0.58, 95% CI = 0.33 - 0.98) and CKD (OR = 0.50, 95% CI = 0.24 - 0.89) (34). We also observed that in subjects with lower dietary total antioxidant capacity (TAC), higher intakes of NO<sub>3</sub> ( $\geq$  430 mg/d) were accompanied with an increased risk of CVD (HR = 3.28, 95% CI = 1.54 - 6.99); no evidence was documented in relation to NO<sub>2</sub> and the occurrence of CVD events during 6.7 years of follow-up (35).

#### 4. Conclusions

Our observations imply that NO may play an independent role in predicting the CVD, CKD and obesity phenotypes, beyond the known classical indices. Higher NO<sub>2</sub> exposure alongside with a low-vitamin C intake may increase the risk of diabetes whereas high-NO<sub>3</sub> diet may decrease the risk of HTN and CKD. Furthermore, higher NO<sub>2</sub> intakes in the context of low-TAC diet contributes to development of CVD events. In our view, these findings make a major contribution towards enhancing current understanding of potential health outcomes in response to long-term exposure of NO<sub>3</sub>/NO<sub>2</sub>.

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**Table 2.** Association Between Dietary Intakes of NO<sub>3</sub> and NO<sub>2</sub> and the Incidence of Non-Communicable Diseases in the TLGS Population

Author	Study Population	Years of Follow-Up	Outcomes	Levels of Dietary Intakes of NO <sub>3</sub> or NO <sub>2</sub>	Adjusted OR (95% CI) or HR (95% CI)
Bahadoran et al. (33)	Adult men and women (n = 2139)	5.8	T2DM	> 410 mg/d NO <sub>3</sub>	1.38 (0.90 - 2.11) <sup>a</sup>
	Adult men and women (n = 2139)	5.8	T2DM	> 8.77 mg/d NO <sub>2</sub> alongside with a low-vitamin C diet	2.43 (1.45 - 4.05) <sup>a</sup>
	Adult men and women (n = 2139)	5.8	T2DM	> 8.77 mg/d NO <sub>2</sub> alongside with a high-vitamin C diet	0.91 (0.47 - 1.73) <sup>a</sup>
Bahadoran et al. (34)	Adult men and women (n = 1780)	5.8	CKD	< 365 mg/d NO <sub>3</sub>	1.00
				365 - 510 mg/d NO <sub>3</sub>	1.04 (0.68 - 1.57) <sup>b</sup>
				≥ 510 mg/d NO <sub>3</sub>	0.76 (0.43 - 1.24) <sup>b</sup>
	Adult men and women (n = 1780)	5.8	CKD	< 7.6 mg/d NO <sub>2</sub>	1.00
				7.6 - 10.7 mg/d NO <sub>2</sub>	0.76 (0.50 - 1.13) <sup>b</sup>
				≥ 10.7 mg/d NO <sub>2</sub>	0.50 (0.24 - 0.89) <sup>b</sup>
	Adult men and women (n = 1878)	5.8	HTN	< 359 mg/d NO <sub>3</sub>	1.00
				259 - 505 mg/d NO <sub>3</sub>	1.02 (0.68 - 1.51) <sup>b</sup>
				≥ 505 mg/d NO <sub>3</sub>	0.81 (0.48 - 1.38) <sup>b</sup>
Adult men and women (n = 1878)	5.8	HTN	< 7.5 mg/d NO <sub>2</sub>	1.00	
			7.5 - 10.6 mg/d NO <sub>2</sub>	0.66 (0.44 - 1.00) <sup>b</sup>	
			≥ 10.6 mg/d NO <sub>2</sub>	0.58 (0.33 - 0.98) <sup>b</sup>	
Bahadoran et al. (35)	Adult men and women (n = 2369)	6.7	CVD	≥ 430 mg/d NO <sub>3</sub> alongside with a high-TAC diet	1.10 (0.46 - 2.61) <sup>a</sup>
				≥ 8.9 mg/d NO <sub>2</sub> alongside with a high-TAC diet	1.10 (0.46 - 2.61) <sup>a</sup>
				Adult men and women (n = 2369)	6.7
≥ 8.9 mg/d NO <sub>2</sub> alongside with a low-TAC diet	2.14 (0.84 - 5.45) <sup>a</sup>				

Abbreviations: CKD, chronic kidney disease; CVD, cardiovascular disease; HR, hazard ratio; HTN, hypertension; NO<sub>2</sub>, nitrite, NO<sub>3</sub>, nitrate; OR, odds ratio; T2DM, type 2 diabetes; TAC, total antioxidant capacity.

<sup>a</sup> Indicates HR.

<sup>b</sup> Indicates OR.

## Footnotes

**Authors' Contribution:** The study was designed and implemented by Zahra Bahadoran and Asghar Ghasemi. Zahra Bahadoran, Sajad Jeddi, Parvin Mirmiran, Amir Abbas Momenan, Fereidoun Azizi and Asghar Ghasemi prepared the manuscript. Asghar Ghasemi revised and supervised overall project. All authors read and approved the final version of manuscript.

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