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Original Article

Interplay between circulating nitric oxide and interleukin-17 in elderly outpatients with non-inflammatory conditions

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Abstract: Nitric oxide (NOx) availability in biological systems is associated with either favorable or unfavorable outcomes. In this sense, several studies bring about evidence that unbalanced NOx production may be underlying to the pathophysiology of vascular disorders. Our study investigated the possible association of clinical, biochemical and inflammatory variables with total circulating levels of NOx in elderly patients devoid of major inflammatory conditions. Clinical (demographics, lifestyle, anthropometry, pressoric traits) and biochemical characteristics (lipemic, glycemic and hormonal profiles) were assessed from 168 geriatrics outpatients eligible for primary care for age-related disorders. Furthermore, circulating levels of 10 inflammatory mediators and of NOx were measured. Correlation tests analyzed categorical or continuous traits according to serum NOx and found no association between NOx and any of the clinical or laboratory data but a negative correlation between plasma NOx concentrations and levels of the immune mediator IL17a ($r = -0.236$; $P = 0.004$). Evidence for a correlation between circulating NOx and IL17 is already present in the literature, mostly from studies conducted under inflammatory conditions. Our hypothesis is that such negative correlation can be attributed to an endogenous homeostatic system that IL17 production by the constitutively produced NOx from the vascular endothelium.

Keywords: Biomarker, cardiovascular event, vascular disorder, elderly, inflammation

Introduction

The phenomenon of demographic aging coupled with unhealthy living habits led to the worldwide plethora of cardiovascular diseases (CVD) [1]. Studies indicate that CVD-related deaths occur mostly due to coronary diseases, and that the largest contingent is affected in developing countries [1-3]. Data from the World Health Organization (WHO) shows that in the last decades, among the 50 million deaths, CVD accounted for 30% of this mortality, remaining the main cause of death in the world [4]. In this context, the main clinical outcome associated with the CVD is atherosclerosis [5], an inflammation-based phenotype involving focal activation of the immune cells to which, among avoidable and unavoidable contributors, accumulation of low density lipoproteins (LDL) on fibrous elements of the arteria intima stands as main pathophysiological causation factor [1, 6]. According to Galiuto and Locorotondo, severity and progress of an atherosclerotic process is also associated with its extent and intensity. In addition, fibrous plaques and fatty streaks may eventually be observed early in adulthood, characterizing the disease as no longer exclusive to the older age strata [2, 3, 5].

A determinant factor for the development of atherosclerotic processes corresponds to the integrity of the vascular endothelium, composed of a thin layer of specialized cells that lines the blood vessels internally. In healthy conditions, the endothelium promotes regulation of blood flow, vascular resistance, modulates immune responses, and releases the endothelium-derived relaxation factor, now known as nitric oxide (NOx) [7, 8]. A higher pro-

portion of circulating NOx is produced by the vascular endothelium through the catalytic action of the endothelial nitric oxide synthase (eNOS) enzyme which promotes the oxidation of L-arginine in presence of cofactors as oxygen and nicotinamide adenine dinucleotide phosphate [9]. This mediator promotes important vasodilation, and loss or deficiency of its expression favors the intensification of the atherosclerotic process and/or other vascular disorders [7-9].

However, NOx availability in biological systems can yield either favorable or unfavorable outcomes, depending on the type of immune-regulated process, mainly whether a pathogen-triggered or an autoimmune-driven inflammation [10]. In this sense, several studies bring about evidence that exacerbated NOx production may trigger tissue injuries, such as cerebral ischemia and Parkinson's disease [11-13]. Moreover, it is observed that higher expression or concentration of NOS isoforms are associated with increase in the frequencies of ischemic episodes and of neurotoxicity events in the brain [9, 14].

Some cell types that participate in inflammatory responses promote the release of NOx. This is mainly due to mediators with inflammatory properties that, by binding to their respective receptors, activate gene transcription intracellularly to promote synthesis and/or activation of mediators [7, 9]. The nuclear factor (NF) kB is an example of transcription factor that is widely studied in atherosclerotic processes, being able to stimulate the synthesis of pro-inflammatory cytokines. In addition to being associated with the regulation of inflammatory genes, NF-kB activates NOx production, among other reactive oxygen species [15, 16]. Considering the relationship between NOx and inflammatory mediators, and knowing that susceptibility to atherosclerotic events is associated with variations in NOx circulating levels [7, 14], this study investigated the possible association between classic risk factors for atherosclerosis and circulating concentrations of NOx and of pro- and anti-inflammatory cytokines in a sample of Brazilian older adults.

Material and methods

Clinical procedures

The clinical, biochemical, anthropometric, metabolic and inflammatory profiles, of 168 elderly participants of both gender from the prospective cohort study termed Prognosis and Therapeutics in Geriatrics (Proteger) was used. The study started in 2011 aiming to identify risk factors for primary or secondary prevention of vascular events in the Brazilian geriatric people. As inclusion criterion, we enrolled subjects aged ≥ 60 years who sought general geriatric consultations in any of our facilities. As an exclusion criterion, presence of autoimmune diseases, chronic or recurrent infections, active or previous neoplastic diseases, chronic kidney disease, and/or use an anti-inflammatory drug in the 30 days prior to clinical and biochemical exams. Participation was voluntary and upon signing a free and informed consent form, with the study being approved by the institutional ethics committee and conducted by principles of the Helsinki Declaration. Data was gathered from August 2011 to July 2014, during the visits at the Geriatrics Service of the Catholic University Hospital of Brasília (HUCB) and the Multidisciplinary Center for the Aged of the University Hospital of Brasília (HUB).

Through clinical and biochemical assessments, presence of blood pressure, glycaemic or lipid abnormalities were determined and comorbidities as well as the pharmacotherapy were assessed. To be considered physically active, individuals should have practiced 30 minutes or more of any exercise for at least four days a week [17], Smoking was defined as consuming more than 100 cigarettes over a lifetime [18]. Type 2 diabetes was defined according to the reference values established by the American Diabetes Association (fasting glycaemia ≥ 126 mg/dl) or current use of oral hypoglycemic drugs or insulin [19]. Systolic and diastolic blood pressures were measured as recommended by the VI Brazilian Guidelines for Hypertension. Patients with systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg were classified as hypertensive, as well as users of antihypertensive drugs [20]. As an anthropometric indicator of central obesity, the abdominal perimeter was measured using inelastic tape in the midpoint between the last rib and the iliac crest at the moment of the individual's expiration, according the cutoffs (94 cm for men and 80 cm for women).

Blood analysis

The biochemical profile was analyzed by means of total cholesterol (TC), high density lipopro-

Table 1. Description of the clinical, anthropometric and metabolic variables

Variables	(n = 168)
Male, %	41.6
Age, years	73.3 ± 8.9
Waist Circumference, cm	97.3 ± 11.6
Glucose level, mg.dl^{-1}	102.9 ± 28.1
Glycated Hemoglobin, %	5.9 ± 1.0
Insulin†, mUI/mL	6.7 (3.2, 10.8)
HOMA† index	1.6 (0.9, 3.0)
Type 2 Diabetes Mellitus§, %	22.0
Total cholesterol, mg.dl^{-1}	191.7 ± 32.2
Low density lipoprotein, mg.dl^{-1}	114.5 ± 32.2
Triglycerides, mg.dl^{-1}	142.0 ± 65.1
Hyperlipemia§, %	48.8
High density lipoprotein, mg.dl^{-1}	48.8 ± 10.9
Systolic blood pressure, mmHg	134.6 ± 19.3
Diastolic blood pressure, mmHg	80.4 ± 11.3
Systemic Hypertension§, %	76.2
C-protein reactive†, mg.dl^{-1}	1.1 (0.3, 4.4)
Thyroid stimulated Hormone†, mIU.l^{-1}	1.9 (1.3, 3.1)
Homocysteine†, µmol.l^{-1}	11.3 (9.0, 14.5)
Sedentary§, %	60.7
Smoker§, %	38.1
Interferon-γ†, pg/ml	6.2 (5.4, 7.0)
Interleukin-1β†, pg/ml	2.0 (0.1, 9.5)
Interleukin-2†, pg/ml	8.4 (7.4, 9.6)
Interleukin-4†, pg/ml	9.6 (8.4, 10.6)
Interleukin-6†, pg/ml	21.0 (12.7, 30.5)
Interleukin-8†, pg/ml	43.1 (27.2, 100.4)
Interleukin-10†, pg/ml	0.2 (0.0, 2.2)
Interleukin-12†, pg/ml	9.3 (7.1, 11.0)
Interleukin-17a†, pg/ml	38.1 (32.2, 42.8)
Tumor necrosis factor-α†, pg/ml	0.4 (0.0, 1.9)
Nitric Oxide, mM	57.9 ± 17.8

Data are expressed as average ± standard deviation for continuous parameters, as relative frequencies§ for categorical features, and as median and interquartile interval† for continuous traits with non-Gaussian distribution. HOMA = Homeostatic model assessment.

tein (HDL), triglycerides (TG), serum glucose, glycated hemoglobin, insulin, C-reactive protein (CRP), Thyroid stimulating hormone (TSH) and homocysteine. Blood samples were collected after a 12-hour fasting period and drawn in EDTA tube. Laboratory tests were performed based on routine clinical analysis, with reagents from Boehringer Mannheim (Germany) and processed on an automatic analyzer (Autohumalyzer, Human GMBH, Germany). Very low density lipoprotein (VLDL) was determined dividing the TG levels by 5, while the Friedewald equation was used to produce LDL readings, by subtraction of VLDL and HDL from total cholesterol [21]. For analytical steps, samples were categorized as positive or negative for selected metabolic traits. Lipemic categorization was according to the NCEP ATP III criteria, with each volunteer showing hyperlipidemia if having TC ≥ 200 mg/dL, LDL ≥ 130 mg/dL and/or TG ≥ 150 mg/dL [22]. Current use of antilipemic drugs accounted for the definition of hyperlipidemia. The HOMA index was determined based on the fasting insulin and glucose dosages, based on the product ratio between fasting insulinemia (mU/L) and fasting glycemia (mmol/L) by 22.5 [23].

Nitric oxide and total nitrate

To quantify the total serum nitric oxide (NOx), the enzymatic conversion tests of nitrate nitrite (NO^{-2}) by nitrate reductase were used, followed by colorimetric detection by the Griess diazotization reaction [24], according to specific set instructions (R&D Systems Inc, MN, USA). The readings of the colorimetric reactions were performed in a Biotek ELX 800 apparatus (DeMorellis, SP, Brazil) at a wavelength of 490 nm.

Citokines

For inflammatory measurements, serum samples were obtained and kept frozen at -80°C until evaluation of all cytokines. Concentrations were obtained by flow cytometry using two sets of multiplexed bead-based immunoassays, namely the Human Th1/Th2/TH17 kit and the Human Inflammatory kit (BD Biosciences, San Diego, CA, USA). Lab procedures followed the protocols provided by the manufacturer, which all together produced measurements for ten different circulating cytokines, as follows: Interleukin (IL) 1β, IL2, IL4, IL6, IL8, IL10, IL12p70, IL17a, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα).

Lyophilized cytokines provided in the kits were processed along with serum samples to generate a referential, standard curve for each cytokine, with readings obtained using the flow cytometer BD FACSCalibur, channel FL4. Three hundred events were acquired for each type of cytokine-bead used in the assay. Data were analyzed using software FCAP software, ver-

Table 2. Correlation between absolute serum inflammatory mediators and NOx scores according to the clinical and biochemical measures of sample

	Clinical measures; r; P									
	Age	Sex§	WC	DM2§	SAH§	SBP	DPB	Hyperlipemia§	Smoker§	Sedentary§
NOx	-.004; .960	-.006; .940	.062; .442	.048; .551	.165; .037	-.031; .700	-.052; .517	.075; .348	.003; .968	-.064; .424
	Biochemical; r; P									
	CRP	TSH	Homocysteine	TC	VLDL-c	HDL-c	TGL	Insulin	HOMA	HbA1c
NOx	-.075; .346	.020; .802	-.012; .877	.016; .845	.066; .414	.060; .455	.037; .639	-.039; .634	-.026; .748	.033; .686

The Spearman's§ or the Pearson's correlation test was used. Data are expressed in correlation index and significance level (*r*; *P*). CRP = C-reactive protein; DBP = diastolic blood pressure; DM2 = type 2 diabetes mellitus; HbA1c = glycated hemoglobin A1c; HDL-c = high density lipoprotein cholesterol; HOMA = Homeostatic model assessment; NOx = nitric oxide; SAH = systemic arterial hypertension; SBP = systolic blood pressure; TC = total cholesterol; TNF = tumor necrosis factor; TSH = thyroid-stimulating hormone; VLDL-c = very low density lipoprotein cholesterol; WC = waist circumference. Significance threshold set at $P < 0.0025$ according to the Bonferroni convention for multiple (k) correlation tests.

sion 3.0 (BD Biosciences®, San Diego, CA, USA). Concentrations were determined by interpolation from the corresponding standard curve.

Statistics

To measure the association of circulating levels of NOx, correlation coefficients were obtained between the cytokines and selected categorical and continuous variables of clinical relevance. For this, we evaluated the normal distribution of all continuous variables using the Kolmogorov-Smirnov test. The association was assessed by the Pearson correlation test, whereas the involvement of at least one categorical variable or a continuous trait with non-Gaussian distribution (skewness > 3) was done using the Spearman counterpart with men and women represented by 1 or 2, and absence or presence of a characteristic represented by 0 or 1, respectively. In addition, absolute NOx concentrations were tested across circulating levels of a panel of 10 different immunological mediators using the Spearman's correlation test. When the results showed a significant correlation, the differential levels of serum NOx were tested between the quartiles of the immune variables by means of analysis of variance with the Tukey post-hoc test. All analyzes were performed with the Statistical Package for the Social Sciences (SPSS) for Windows (version 17.0), with a significance level set at $P < 0.05$. Only for the exploratory correlation analysis, a two-tailed *p*-value was adopted with significance according to the Bonferroni principle for multiple comparisons (eg.: if $k = 10$ tests, then $\alpha \leq 0.005$).

Results

The sample consisted of 168 older adults, predominantly sedentary, female and aged 73.3 yrs in average. Mean results obtained by biochemical, clinical and anthropometric tests are in **Table 1**. It is noteworthy the high prevalence of classic vascular risk factors such as mean values of abdominal circumference, higher than those recommended for women (80 cm) and men (94 cm). Prevalence of patients with type 2 diabetes mellitus was 22%, with mean values of glycated hemoglobin close to the upper limit recommended by Brazilian Diabetics Society (6.5% as borderline index for good glycaemic control in aged patients). We also found 76.2% of the sample with systemic hypertension, with mean systolic blood pressure was above normal (> 130 mmHg). Analyzes have also been performed regarding the hormonal profile since TSH, for example, influences cellular metabolism and, consequently, interferes with cardiac functioning. It was found that the mean values found were within the recommended values (between 0.4 and 4 mIU/L).

Regarding the inflammatory profile, mean CRP (predictor of cardiovascular risk) was found above referential range (0.1-0.25 mg/dL). Altogether, clinical, anthropometric and metabolic variables (**Table 1**) indicated that one or more chronic disorders were abundant phenotypes in the sample. However, due to the exclusion factors, conditions were not compatible with active, systemic inflammation or with diseases of accelerated prognosis, thus revealing basal inflammatory parameters for all other immunomediators assessed. In this scenario, we observed no significant correlation between absolute levels of serum inflammatory variables and NOx scores (**Table 2**).

Analyzes of absolute circulating cytokines across NOx concentrations (**Table 3**) indicated a negative correlation with cytokine IL17a ($r = -0.236$; $P = 0.004$). This way, serum nitric oxide

Table 3. Correlation between serum inflamatory mediators and nitric oxide (NOx) levels

	NOx, mM	
	r	P
Interferon-γ, pg/ml	-.160	.052
Interleukin-1β, pg/ml	-.030	.712
Interleukin-2, pg/ml	-.115	.164
Interleukin-4, pg/ml	-.187	.023
Interleukin-6, pg/ml	-.112	.166
Interleukin-8, pg/ml	.053	.510
Interleukin-10, pg/ml	-.057	.484
Interleukin-12, pg/ml	.028	.729
Interleukin-17a, pg/ml	-.236	.004
Tumoral Necrosis Factor-α, pg/ml	-.008	.926

The Spearman's correlation test was used. Data are expressed in correlation index and significance level (*r*; *P*). IFN = interferon; IL = interleukin; TNF = tumor necrosis factor; NOx = nitric oxide. Significance threshold set at $P < 0.005$ according to the Bonferroni convention for multiple (k) correlation tests.

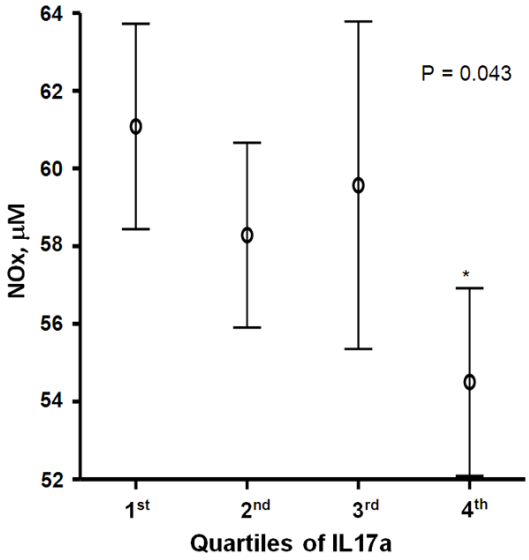

Figure 1. Circulating NOx and quartiles of IL17a. The significance was verified through analysis of variance with the Tukey post-hoc test. The vertical bars represent the standard error.

levels were tested throughout the quartiles of the immune mediator (**Figure 1**), confirming from another perspective the negative correlation between NOx and IL17, unparalleled for any of the other cytokine when tested under same analysis.

Discussion

The present study evidenced that circulating levels of nitric oxide was inversely correlated with the proinflammatory cytokine IL17. Abundant information regarding IL17 has arisen in the last decade due to its ability to act as a novel proinflammatory rout of specialization of the CD4 T cell line, namely the anew characterized Th17 [25, 26]. Among these reports, there are those that demonstrate how IL17 is pivotally expressed in diseases as psoriasis, rheumatic diseases, multiple sclerosis and bone disorders [25-27]. On the other hand, physiological statuses compatible with metabolic syndrome (hypertension, hypercholesterolemia, and diabetes mellitus) are high prevalent in the elderly population, and increase the risk to atherosclerosis, possibly because of impaired production and lower bioactive concentration of NOx by the vascular endothelium [28].

In this biochemically intricate scenario, it does not surprise that the literature already points out to a relationship between these two apparently unrelated mediators, with clear evidence developed elsewhere demonstrating NOx as a negative regulator of IL17-producing cells. It is now clear that the potent inhibition exerted by NOx on cellular function and proliferation of $CD4^+$ T-cells is mediated by downregulation of aryl hydrocarbon receptors (AhR - the main trigger of Th17 activation).

Niedbala, et al. [29] observed that mice deficient in inducible nitric oxide synthase (iNOS, or NOS-2) had higher levels of Th17 cells in systemic circulation as well as of AhR-expressing cells in the spinal fluid, probably as part of an unbalance. Although diligently studied in the last years and found to act as a binding-dependent transcription factor, the physiological role of AhR is still not well understood [30]. Biobinders such as dioxin and 6-formolindole [3,2-b] carbazole (FICZ) [29] are contributors to the development of autoimmune diseases, many of them with onset dependent on Th17 activity as newly disclosed [30]. It is noteworthy that lymphocytes are not reprogramed since proliferation of Th17 cells is resumed in the absence of NOx [29, 31].

Ghasemi, et al. [32] sought 3505 healthy adult to propose a reference, physiological range for serum NOx levels (10.3-66.8 µM). Considering that the mean value presented by our analysis

lays within this range and that subjects do not exhibit inflammatory or other complicated health conditions (see exclusion criteria), we ponder their endothelial beds to be regularly preserved, allowing physiological levels of NOx despite the high prevalence of comorbidities. In this context, we hypothesize that the negative correlation found between total NOx and IL17 expresses an endogenous hemostasis systems in which production of IL17 is limited by the NOx constitutively released by healthy vascular endothelium, so to avoid exacerbation or perpetuation of inflammatory responses and, consequently, the appearance of autoimmunities or hypersensitivities [25].

Regarding IL17, although there are no reference values of risk for disease prediction, this cytokine has demonstrated a significant role in the immune system, not just for its ability to activate and/or suppress the expression of other cells, but also because its function interplays with molecules with major physiological importance [33, 34]. Our results supports evidence for a novel form of regulation of the immune system exerted by a rather ubiquitous, multifunctional molecule (NOx) to which a relationship with IL17 is already imputed by the literature, mostly derived from studies with autoimmune conditions but observed herein on non-inflammatory grounds. All in all, we hypothesize that such negative correlation is attributable to an endogenous homeostatic system that limits IL17 production by the constitutively produced NOx from the healthy vascular endothelium. Future studies should estimate the magnitude of the preventive and/or therapeutic contribution(s) of NOx on the Th17-dependent atherosclerotic phenotype. In addition, one should investigate the contribution of the different isoforms of NOS to the regulation of the Th17 profile, which still generates much scientific and academicals debate.

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Disclosure of conflict of interest

None.

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