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ORIGINAL RESEARCH

Association of Omnivorous and Vegetarian Diets With Antioxidant Defense Mechanisms in Men

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BACKGROUND: Evidence that a vegetarian diet rich in antioxidants contributes to cardiovascular health are growing, however, the underlying molecular mechanisms remain unknown. HO-1 (heme-oxygenase-1), a marker of adaptive response, is protective against oxidative stress and has shown cardioprotective effects. Therefore, we evaluated circulating HO-1 levels and the effect of plasma from omnivorous and vegetarians in endothelial cells (human umbilical vein endothelial cells) on modulating NRF2 (nuclear factor erythroid 2-like 2)/HO-1 and nitric oxide production.

METHODS AND RESULTS: From 745 participants initially recruited, 44 omnivorous and 44 vegetarian men matched by age and absence of cardiovascular risk factors and diseases were included in this study. Circulating HO-1 was measured using ELISA and human umbilical vein endothelial cells were incubated with plasma from omnivorous and vegetarians. Higher circulating HO-1 concentrations were found in omnivorous compared with vegetarians. Plasma from omnivorous and not from vegetarians induced NRF2/HO-1 and nitric oxide production in human umbilical vein endothelial cells, and increased reactive oxygen species production and caspase activity after incubation with stressor stimulus.

CONCLUSIONS: We suggest that HO-1 induction in omnivorous may indicate a pro-oxidative status since HO-1 is activated under oxidative stress a state not seen in vegetarians.

Key Words: antioxidant ■ diet ■ vascular endothelium

Several lines of evidence indicate that a diet containing high consumption of fruits and vegetables is anti-inflammatory and antioxidative.[1] Epidemiologically, plant-based diets, such as vegetarian diet, can help humans in disease prevention.[2,3] Observational studies show that vegetarians have lower rates of hypertension, diabetes mellitus, obesity, several types of cancer, and total mortality.[3,4]

Failure to protect against oxidative stress and decreased NO bioavailability have been associated with endothelial dysfunction,[5] one of the main mechanisms that lead to cardiovascular diseases (CVDs).[6] Thus, enzymes with antioxidant properties may play beneficial effects in vascular system, minimizing oxidative stress.[7] NRF2 (nuclear factor erythroid 2-like 2) is considered a "master regulator" of the antioxidant response; under cellular stress, NRF2 is disassociated from its inhibitory protein KEAP (Kelch-like ECH-associated protein-1) and transported to the nucleus, where it binds to antioxidant response elements (ARE) regulating the expression of antioxidant proteins, including GSR (glutathione reductase) and HO-1 (heme-oxygenase-1).[8] HO-1 cleaves heme-producing bilirubin and CO_2, promoting cell protection through its antioxidative, anti-inflammatory, antiapoptotic properties.[7] HO-1 is highly expressed in the spleen, liver and bone marrow, the main organs responsible for metabolism of hemoglobin.[9] In

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CLINICAL PERSPECTIVE

What Is New?

- Circulating HO-1 (heme-oxygenase-1) level was higher in healthy omnivorous compared with vegetarians.
- The HO-1/NRF2 (nuclear factor erythroid 2-like 2) pathway and nitric oxide production were induced in endothelial cells incubated with plasma from omnivorous compared with the vegetarian, and an increase in reactive oxygen species and apoptosis was observed only in the omnivorous group in the stress condition.

What Are the Clinical Implications?

- Since HO-1 is activated under oxidative stress, an increase of circulating HO-1 in healthy omnivorous may indicate a pro-oxidative status.
- Circulatory factors present in omnivorous plasma (eg, inflammatory cytokines, oxidative stress, and growth factors) may induce an oxidative environment in endothelial cells and stimulate antioxidant defense via NRF2/HO-1 and nitric oxide production.

Nonstandard Abbreviations and Acronyms

ARE	antioxidant response elements
BMI	body mass index
CVDs	cardiovascular diseases
CARVOS	carotid atherosclerosis and arterial stiffness in vegetarians and omnivorous subjects
HbA1c	glycated hemoglobin
GSR	glutathione reductase
HO-1	heme-oxygenase-1
HDL-C	high-density lipoprotein cholesterol
hs-CRP	high-sensitivity C-reactive protein
HUVEC	human umbilical vein endothelial cells
IMT	intima-media thickness
LDH	lactate dehydrogenase
NRF2	nuclear factor erythroid 2-like 2
PWV	pulse wave velocity
ROS	reactive oxygen species
SBP	systolic blood pressure
tBHP	tert-butyl hydroperoxide solution
ZnPP	zinc protoporphyrin

the endothelium, basal expression of HO-1 is low, but can be rapidly induced by physiological and pathological stimuli, including heme group, oxidative stress, heavy metals, inflammatory cytokines, NO, hypoxia and growth factors.[10]

HO-1 may play an important role in CVDs development and has been seen as a potential treatment strategy.[11] HO-1 induction inhibited progression of atherosclerotic lesions and reversed plaque morphology and composition into a more stable phenotype in a rabbit model of atherosclerosis.[12] Pre-treatment of human umbilical vein endothelial cells (HUVECs) with Euxanthone (a compound extracted from *Polygala caudata*) increased NRF2 and HO-1 expression, and rescued the cells from ox-LDL (Oxidized Low-Density Lipoprotein)-induced cytotoxicity and apoptosis, suggesting its potential as a therapy for atherosclerosis.[13]

Incubation of cells with plasma/serum is a well-established in vitro model and has been used to evaluate the effects of caloric restriction diets in several types of cells,[14–16] and recently of vegetarian diet in cardiomyoblast cells.[17] These studies showed that circulating factors present in plasma/serum from humans or animals induce modification in cell culture,[14–17] including changes in NRF2 expression, on cell proliferation, apoptosis, and stress responsiveness.[14]

Despite intense research regarding NRF2/HO-1 in CVDs, there are few studies[14,18–20] that have evaluated the effect of diet on NRF2/HO-1 pathway. Therefore, our objectives were (1) to verify plasmatic concentration of HO-1 and its association with cardiovascular biomarkers and (2) investigate the effect of plasma from omnivorous and vegetarians in endothelial cells on modulating antioxidant defenses and vasodilator factors. Given that a healthy vegetarian diet is rich in fruits and vegetables, we hypothesized that a vegetarian diet could modulate NRF2/HO-1 pathway, differently from omnivorous diet.

MATERIALS AND METHODS

More details for Materials and Methods in Data S1.

Subjects and Biochemical Measurements

Schematic diagram of the study is shown in Figure 1. This study included 44 vegetarian and 44 matched by sex and gender individuals that participated in the CARVOS (Carotid Atherosclerosis and Arterial Stiffness in Vegetarians and Omnivorous Subjects) as previously described.[21] Apparently healthy individuals were classified according to their dietary patterns: omnivorous (consumed any type of meat at least ≥5 servings per week), vegetarians who excluded the consumption of meat, fish, and poultry for at least 4 years (n=14 vegetarians for 4–10 years, n=30 for >10 years) and these men could be lacto-ovo-vegetarians (consumed egg and milk), lacto-vegetarians (consumed milk), or strict

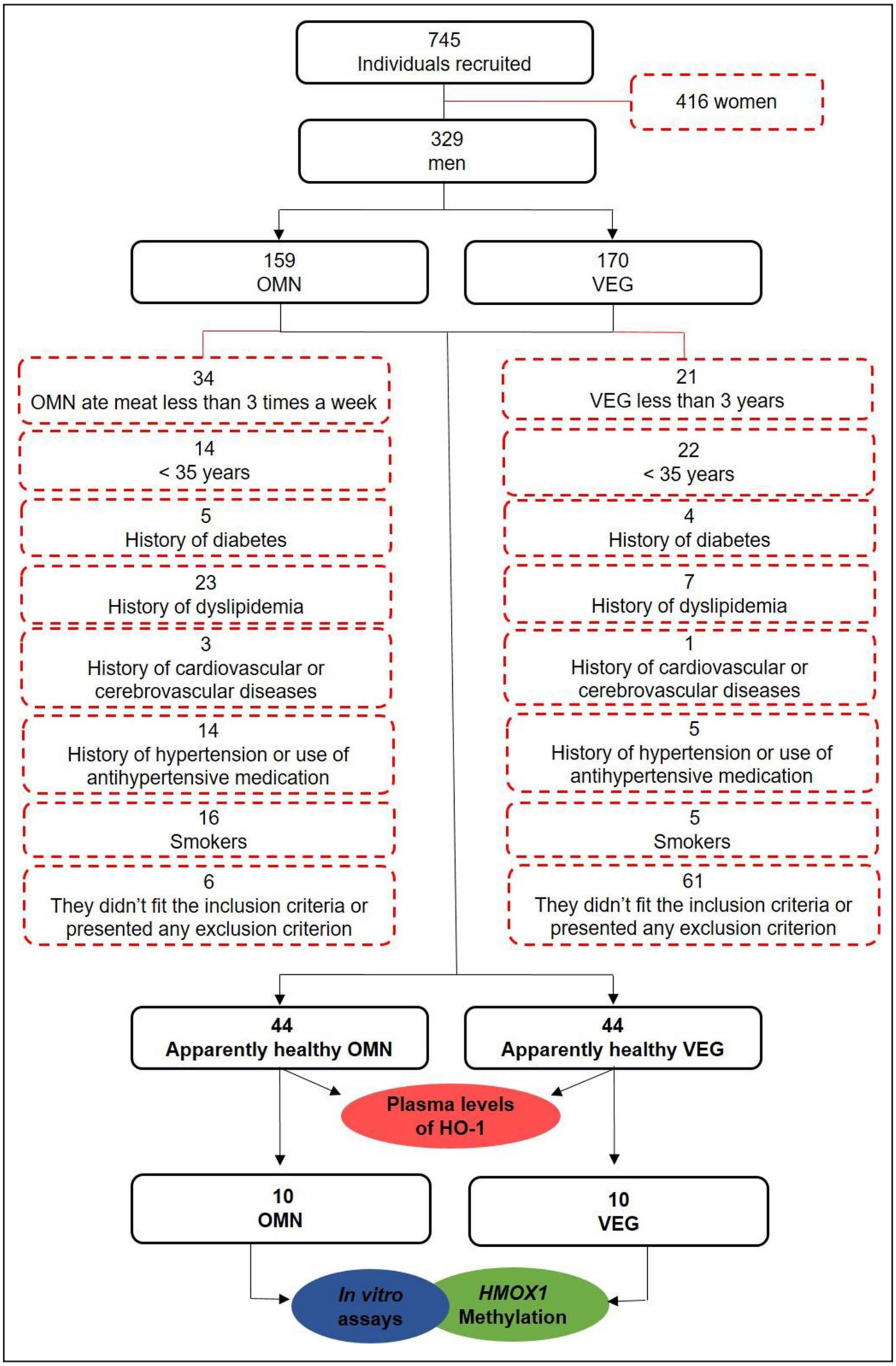

Figure 1. Diagram of the study workflow.

vegetarians (consumed no eggs or milk). Individuals with previous manifestations of CVD, history of diabetes mellitus, history of dyslipidemia, history of cardiovascular or cerebrovascular diseases, history of hypertension or intake of antihypertensive or lipid modifying medications, and smoking were excluded. For the in vitro study, a pool of plasma from omnivorous and vegetarians were used (n=10/group). Inclusion criteria for these assays were individuals aged ≥35 ≤52 years and body mass index (BMI) ≥18 <29 kg/m^2.

Blood samples were collected in tubes containing EDTA, after a 10 to 12 hours fasting. The tubes were immediately centrifuged and plasmas were stored at −80°C until its experimental use.

The study was approved by the Institutional Review Board at Heart Institute (InCor), Sao Paulo, Brazil (Protocol number: 3751/12/007), following the principles of the Declaration of Helsinki, and all participants provided written informed consent.

Evaluating Endothelial Function

The functional and anatomical properties of the right carotid artery, evaluated as carotid intima-media thickness (IMT) and relative carotid distensibility, were assessed using an ultrasound device consisting of a vessel wall echo-tracking system (Wall-Track System, PieMedical, Maastricht, The Netherlands), as previously described by Acosta-Navarro et al.[21] Pulse wave velocity (PWV) measurements were performed using a pressure sensitive transducer as previously described by Acosta-Navarro et al.[21]

Cell Culture and Plasma-Pool Incubation

HUVECs (CRL 2873, American Type Culture Collection, Manassas, Virginia, USA) were cultured in DMEM (Gibco, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco CA, USA), 50 μg/mL penicillin, 50 μg/mL streptomycin, and 0.5 μg/mL amphotericin B (Gibco CA, USA) at 37°C in an incubator with 5% CO_2 atmosphere. After reaching 90% to 100% confluence, HUVECs were incubated in the medium (without phenol red and fetal bovine serum) containing 10% (v/v) plasma-pool from omnivorous and vegetarians (n=10/group) for 24 hours. Cells were used until the eighth passage.

To investigate the effect of plasma in endothelial cells in an oxidative environment, HUVECs were seeded into 12 wells plates at a concentration of 1.5×10^5 cells in DMEM F12. After reaching 90% to 100% confluence, cells were incubated with plasma-pool for 24 hours. Next, cells were stimulated with tert-Butyl hydroperoxide solution (tBHP, Sigma Aldrich, Saint Louis, MO, USA) at 100 μmol/L. After 3 hours, cells were washed with PBS, incubated with 250 μL Cell Dissociation Buffer, enzyme-free, Hanks' Balanced Salt Solution (Thermo Scientific, California, USA) for 40 minutes, followed by the addition of 250-μL PBS. Then, 90 μL of suspended cells were transferred to a 96-well plate to perform cell viability, reactive oxygen species (ROS) and caspase assays.

Measurement of HO-1 Concentrations

HO-1 concentrations in plasma and cell supernatant were measured using the ELISA kit Human Total HO-1/*HMOX1* (heme-oxygenase-1 gene) ELISA (R&D Systems, MN, USA) according to the manufacturer's protocol. The plate was read at 450 nm in a spectrophotometer (Synergy 4, BioTek).

DNA Methylation Analysis of the *HMOX1*

DNA methylation analysis was based on methylation-sensitive restriction enzymes cleavage with Haemophilus parainfluenzae (HpaII) and Moraxella species (Msp) (New England BioLabs) followed by quantitative real-time polymerase chain reaction (PCR).[22] MspI digested DNA samples were used to identify possible errors caused by non-specific or incomplete digestion (Figure S1A through S1C). Undigested controls without the addition of any restriction enzyme were also subjected to quantitative real-time PCR amplification. PCR reactions were performed in triplicates on a StepOne Real-time PCR System (Applied Biosystems). Quantification of DNA methylation was based on ΔCt (cycle threshold) value by subtracting the mean Ct from the HpaII digested samples from the average Ct from the undigested samples.

Measurement of Total Glutathione

For this assay, 1×10^5 cells were inoculated in 12-well plate. Total glutathione levels were measured in cell lysates using a Glutathione Colorimetric Detection Kit (Thermo Fisher Scientific, California, USA according to the manufacturer's protocol. The plate was read at 405 nm in a spectrophotometer (Synergy 4, BioTek). The reaction was performed in triplicate.

mRNA and miRNA Expression

HUVECs were seeded at a concentration of 5×10^4 cells per well into a 48-well plate. HUVECs were lysed by QIAZOL reagent and total RNA was extracted using a miRNeasy Mini Kit (Qiagen, Leusden, Netherlands) according to the manufacturer's protocol. cDNA reaction was performed by miScript II RT Kit using HiFlex Buffer for mRNA and *HiSpec Buffer* for miRNAs. The primers of the mRNAs *NFE2L2, HMOX1, GSR,* and *HPRT1* (endogenous control) were obtained from Sigma.

miRNAs expression was quantified by miScript SYBR Green PCR Kit (Qiagen). The primers of miRNAs were obtained from Qiagen (Leusden, Netherlands): *miR-let-7a*, MS00031220; *miR-let-7b*, MS00003122, *miR-let-7c*, MS00003129 and *RNU6-2*, MS00033740. Relative quantification was calculated using the comparative 2(−Delta Delta C(T)).[23] Gene and miRNA expression data were analyzed using GeneGlobe Data Analysis Center (Qiagen) online platform. All PCR reactions were performed in duplicate for each sample. The primer sequences used are shown in Data S1.

NRF2 DNA Binding Assay

HUVECs were seeded into 25 cm^2 bottles at a concentration of 5×10^5 cells. Nuclear extract was obtained using Nuclear Extraction Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. NRF2 quantification was assessed by ELISA using NRF2 Transcription Factor Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance was measured at 450 nm in a spectrophotometer (Synergy 4, BioTek). The reaction was performed in triplicate.

Antioxidant Response Element Activation

ARE activation was analyzed by ARE Reporter Kit (BPS Bioscience, CA, USA). HUVECs were seeded at a concentration of 1×10^4 cells per well into a white 96-well plate. Before plasma incubation, cells were transfected using Lipofectamine 2000 (Thermo Scientific) with the ARE reporter or negative control reporter. The incubation with tBHP (50 μmol/L) for 3 hours was used as a positive control. The luminescence was measured in a microplate reader (Synergy 4, BioTek) using the Dual-Glo Luciferase Assay System (Promega, WI, USA). The reaction was performed in triplicate.

Measurement of Nitric Oxide Level

The production of intracellular NO was assessed using fluorescent dye 2-(3,6-diacetyloxy-4,5-diamino-9H-xanthen-9-yl)-benzoic acid (DAF-2 diacetate) (Cayman, Michigan, USA). The fluorescence intensity was measured in a microplate reader (Synergy 4, BioTek) at 485 to 520 nm. The reaction was performed in replicates of 5.

Measurement of Nitrite

Nitrite concentration was evaluated in the cell supernatants using Griess method.[24] For this assay, HUVECs were previously incubated for 1 hour with 1 μmol/L of zinc protoporphyrin (ZnPP), an inhibitor of HO-1 activity. Next, the cells were incubated with plasma in the presence of ZnPP or the vehicle DMSO (Sigma-Aldrich, Poole, UK) for 24 hours. The plate was read at 540 nm in a spectrophotometer (Synergy 4, BioTek). The reaction was performed in replicates of 5.

Cell Viability Assay

Cell viability was accessed using PrestoBlue Cell Viability Reagent, (Invitrogen, Thermo Fisher Scientific, California, USA). Cells were incubated with 10 μL of PrestoBlue Reagent for 1 hour. Fluorescence was read on a multi-functional plate reader (Synergy 4, BioTek) using excitation and emission wavelengths of 560 and 590 nm, respectively. The reaction was performed in replicates of 5.

Cellular Cytotoxicity Assay

Plasma membrane damage releases lactate dehydrogenase (LDH) into the cell culture media, which is indicative of cytotoxicity. LDH release was quantified using Pierce LDH Cytotoxicity Assay Kit (88954) (Thermo Fisher Scientific, California, USA) according to the manufacturer's protocol. The absorbance was read at 490 and 680 nm. The reaction was performed in replicates of 5.

ROS Production

The production of intracellular ROS was evaluated using 2,7-dichlorodihydrofluorescein diacetate (Cayman Chemical, Michigan, USA). HUVECs were loaded with 25 μmol/L of 2,7-Dichlorodihydrofluorescein diacetate (DCFH) in PBS for 45 minutes. The plate was read using excitation and emission wavelengths of 502 and 523 nm, respectively. The reaction was performed in replicates of 5.

Caspase Activity

To evaluate the caspase 3/7 activity, cells were incubated with 2.5 μmol/L Cell Event Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific, California, USA) in PBS containing 5% fetal bovine serum for 40 minutes. The fluorescence intensity was measured in a microplate reader (Synergy 4, BioTek) at excitation 502 and emission 569 nm. The reaction was performed in triplicate.

Statistical Analysis

ANOVA and the Bonferroni Multiple Comparison Test were used to compare study variables between 3 groups. Two-way ANOVA followed by Bonferroni post-test was used to assess differences within and between groups (diet and stress stimulus). For comparison between the 2 groups, t test was performed. Pearson (or Spearman) correlation coefficients were used to determine the relationship between HO-1 levels in plasma samples and the parameters clinical, and biochemical. For all experimental groups, data were expressed as mean±SEM. In the multivariable linear regression analysis, HO-1 was included as dependent variable to test association with type of diet (omnivorous and vegetarian), important biomarkers and risk factors for CVD (such as age, BMI, systolic blood pressure (SBP), glycated hemoglobin (HbA1c), total cholesterol, high-density lipoprotein cholesterol (HDL-C), hs-CRP (high-sensitivity C-reactive protein), IMT, PWV, and carotid distensibility) were included as independent variables too, to adjust the model. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and Stata version 10.0, respectively. Statistical significance was defined as $P<0.05$.

RESULTS

Circulating HO-1 Concentration is Higher in Omnivorous Versus Vegetarians

Main characteristics of the study subjects omnivorous (n=44) and vegetarians (n=44) are presented in Table 1. Significantly higher values of body weight, BMI, SBP, diastolic blood pressure, non-HDL-C, low-density lipoprotein cholesterol, triglycerides, apolipoprotein B, glucose, HbA1c, IMT, PWV were found in omnivorous compared with vegetarians (all $P<0.05$). We also evaluated HO-1 levels by obesity status. The number of obese individuals is almost 3.5 times higher in omnivorous than vegetarians group. There was no difference in HO-1 levels by obesity status: omnivorous ($P=0.287$, mean±SEM 795.7±122.3 pg/mL, n=12 normal weight versus 636.3±77.33 pg/mL, n=32 obese/overweight) and vegetarians ($P=0.854$, 417.9±51.76 pg/mL, n=35 normal weight versus 397.3±79.00 pg/mL obese/overweight, n=9).

First, HO-1 was measured in plasma from all subjects. HO-1 concentration was significantly higher in omnivorous compared with vegetarians (Figure 2) ($P=0.0007$). Furthermore, we correlated plasma HO-1 levels with clinical and laboratory biomarkers (Table 2). HO-1 was positively associated with HDL-C for all participants ($P=0.01$) and for the omnivorous group ($P=0.02$). Strong negative correlations were found between HO-1 and BMI ($P=0.03$) and hs-CRP ($P=0.009$) only in the omnivorous group.

Table 3 shows the normalized (per 1 SD) multivariate associations of HO-1 with dietary pattern omnivorous or vegetarian. In the multivariable linear regression models, a positive association between HO-1 and omnivore diet was encountered. This association was seen when the variables age and BMI were included in the models (model 1, $P<0.001$). In the model 2, the variables age, BMI, SBP, HbA1c, total cholesterol, HDL-C, hs-CRP were included for adjustment in the multivariable linear regression and association was maintained ($P<0.001$). In model 3, in addition to the previous variables, measures of subclinical atherosclerosis were included (IMT, PWV, and carotid distensibility) and the association was also maintained ($P=0.003$).

HO-1 Concentration is Not Associated With DNA Methylation Levels of the Gene *HMOX1*

To evaluate if HO-1 concentrations in plasma could be associated with differences in DNA methylation levels of

Table 1. Clinical and Laboratory Biomarkers of Omnivorous and Vegetarian Men

	Omnivorous (n=44)	Vegetarians (n=44)	t Test (*P* Value)
Age, y	46.80±1.44	45.45±1.17	0.47
Weight, kg	82.37±2.26	70.94±1.43	<0.0001***
BMI, kg/m^2	27.27±0.73	23.14±0.44	<0.0001***
SBP, mm Hg	129.20±2.28	119.5±1.57	0.0008***
DBP, mm Hg	83.98±1.57	75.73±1.29	0.0001***
Total cholesterol, mg/dL	202.80±5.32	180.10±6.09	0.006**
HDL-C, mg/dL	45.45±1.75	47.59±1.39	0.343
Non-HDL-C, mg/dL	157.30±5.52	132.50±6.52	0.004**
LDL-C, mg/dL	128.50±4.88	110.00±5.00	0.009**
Triglycerides, mg/dL	144.00±9.64	122.20±10.88	0.031*
Apolipoprotein B, g/L	1.01±0.03	0.87±0.04	0.018*
Fasting glucose, mg/dL	102.90±1.96	94.81±1.09	0.0006***
HbA1c, %	5.54±0.06	5.32±0.04	0.006**
Hemoglobin, g/dL	15.80±0.13	15.47±0.17	0.13
hs-CRP, mg/dL	1.20 (0.20–41.80)	1.01 (0.17–19.80)	0.08
IMT, mm	661.40±19.33	593.40±14.13	0.005**
Carotid distensibility	5.72±0.28	6.39±0.26	0.08
PWV, m/s	7.70±0.13	7.09±0.11	0.0008***

Data presented as mean±SEM (except for high-sensitivity C-reactive protein: median and interquartile range). Omnivorous: consumed any type of meat at least ≥5 servings per week. Vegetarians: excluded the consumption of meat, fish, and poultry for at least 4 years (lacto-ovo-vegetarians, lacto-vegetarians, or strict vegetarians). BMI indicates body mass index; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HDL-C high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; PWV, pulse wave velocity; and SBP, systolic blood pressure.

*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with omnivorous group.

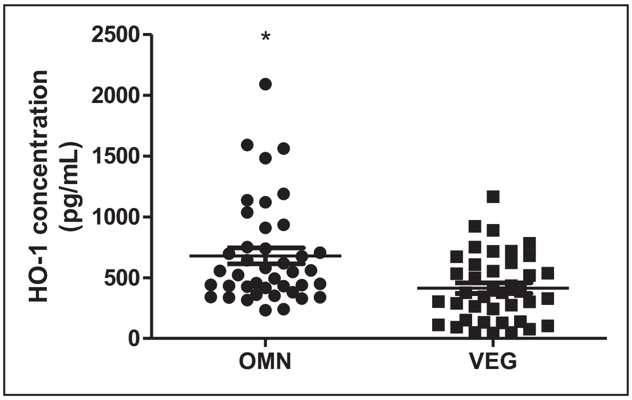

Figure 2. HO-1 (heme-oxygenase-1) level in plasma from omnivorous and vegetarians.
Data presented as mean±SEM. *$P<0.0001$ compared with vegetarians group (Unpaired t test, GraphPad Prism software). HO-1 indicates heme-oxygenase-1.

the cognate gene *HMOX1* in peripheral blood cells, we selected subjects of both omnivorous and vegetarian groups showing the higher and lower concentrations of HO-1 (omnivorous high/low HO-1 versus vegetarian high/low HO-1 concentration, 5 subjects of each). The Figure S1D shows that HO-1 concentrations were significantly different among the 4 groups, as follow: omnivorous high > vegetarian high and omnivorous low > vegetarian low ($P<0.0001$). The highest levels of HO-1 among vegetarians correspond to levels intermediate to the higher and lower extremes in omnivorous. No differences in DNA methylation were observed in these groups, since all subjects were classified as unmethylated at the *HMOX1* locus (methylation levels <10%) (Figure S1E).

Plasma From Omnivorous Induces HO-1/NRF2 Pathway and NO Production in Endothelial Cells

The characteristics of subjects included in vitro assays are shown in Table S1. No difference was observed in the clinical parameters evaluated between omnivorous and vegetarian group (all $P>0.05$).

HO-1 level was evaluated in the cell supernatant from HUVECs incubated with plasma from omnivorous and vegetarian groups. HO-1 concentration was significantly higher in omnivorous compared with the vegetarian group ($P=0.01$) (Figure 3A). Also, gene expression of *NFE2L2*, *HMOX1*, and *GSR* in HUVECs was verified (Figure 3B through 3D). HUVECs incubated with plasma from omnivorous increased *NFE2L2* ($P=0.03$), *HMOX1* ($P=0.0018$), *GSR* ($P=0.02$) expression compared with vegetarian group. The total glutathione was measured in cell lysates. No difference in

Table 2. Correlation Between Plasmatic HO-1 Concentrations With Clinical and Laboratory Biomarkers in Omnivorous and Vegetarian Men

HO-1 vs	All Participants (n=88)		Omnivorous (n=44)		Vegetarians (n=44)	
	r	*P* Value	*R*	*P* Value	*r*	*P* Value
Age, y	−0.02	0.79	0.01	0.94	0.09	0.54
Weight, kg	−0.00	0.93	−0.25	0.11	0.08	0.58
BMI, kg/m^2	0.00	0.95	−0.32	0.03*	−0.08	0.59
SBP, mm Hg	0.08	0.46	−0.26	0.09	0.03	0.85
DBP, mm Hg	0.05	0.59	−0.21	0.17	0.06	0.71
TC, mg/dL	0.03	0.75	0.08	0.60	0.00	0.95
HDL-C, mg/dL	0.25	0.01*	0.35	0.02*	0.25	0.10
Non-HDL-C, mg/dL	−0.03	0.73	−0.01	0.90	−0.05	0.75
LDL-C, mg/dL	−0.01	0.91	0.01	0.94	−0.00	0.98
Triglycerides, mg/dL	−0.07	0.52	−0.08	0.58	−0.15	0.34
Apolipoprotein B, g/L	−0.05	0.59	−0.04	0.79	−0.00	0.99
Fasting glucose, mg/dL	−0.00	0.94	−0.02	0.88	−0.26	0.09
HbA1c, %	−0.19	0.08	−0.20	0.20	−0.24	0.12
Hemoglobin, g/dL	−0.09	0.42	−0.00	0.99	−0.22	0.18
hs-CRP, mg/dL	−0.10	0.36	−0.40	0.00**	−0.03	0.84
Carotid distensibility	−0.00	0.97	0.07	0.68	−0.11	0.49
IMT, mm	−0.00	0.94	−0.12	0.44	0.15	0.33
PWV, m/s	0.18	0.09	0.05	0.74	0.15	0.33

BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HO-1, heme-oxygenase-1; hs-CRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; PWV, pulse wave velocity; and SBP, systolic blood pressure.

Significant at *$P<0.05$, **$P<0.01$.

Table 3. Multivariate Association Between Dietary Pattern and HO-1 Plasma Concentrations (Per 1 SD)

HO-1 vs	Model 1			Model 2			Model 3		
	β	CI 95%	*P* Value	β	CI 95%	*P* Value	β	CI 95%	*P* Value
Vegetarians	Reference			Reference			Reference		
Omnivorous	0.86	0.39–1.32	<0.001	0.86	0.39–1.34	<0.001	0.81	0.29–1.33	0.003

Model 1: Multiple linear model adjusted for age and body mass index. Model 2: Multiple linear models adjusted for age, body mass index, systolic blood pressure, glycated hemoglobin, total cholesterol, high density lipoprotein cholesterol and high-sensitivity C-reactive protein. Model 3: Multiple linear model adjusted for age, body mass index, systolic blood pressure, glycated hemoglobin, total cholesterol, high density lipoprotein cholesterol, high-sensitivity C-reactive protein, carotid intima-media thickness, pulse wave velocity and carotid distensibility; 95% CI obtained from multivariable linear regression; and β, standardized regression coefficients. HO-1 indicates heme-oxygenase-1.

the total glutathione (Figure S2A) among groups was found ($P>0.05$). In addition, we evaluated miRNAs expression of the let-7 family (let-7a, -b, and -c) (Figure S2B through S2D), which regulates genes associated with NRF2 pathway. There were no differences between the groups in miRNAs expression (all $P>0.05$).

NRF2 expression in the nucleus (Figure 4A) and ARE activity (Figure 4B) were also assessed. Plasma of omnivorous group increased NRF2 expression and activated ARE compared with the control group (without plasma incubation) ($P<0.05$ and <0.0001, respectively). Plasma of vegetarians resulted in ARE activating when compared with control ($P<0.05$) and this was significantly lower in relationship to the omnivorous group ($P<0.0001$).

Plasma from omnivorous increased intracellular NO ($P=0.02$) (Figure 5A) and nitrite levels ($P<0.05$) (Figure 5B) compared with the vegetarian group. To evaluate the relationship between HO-1 and NO, cells were incubated with ZnPP to inhibit HO-1 activity. We observed that nitrite concentration decreased after ZnPP treatment only in the omnivorous group ($P<0.05$).

ROS Production and Caspase Level Increased in Endothelial Cells Incubated With Plasma From Omnivorous Following tBHP-Induced Oxidative Damage

There was a significant interaction between stress stimulus and diet ($P=0.001$) for cell viability. As observed in

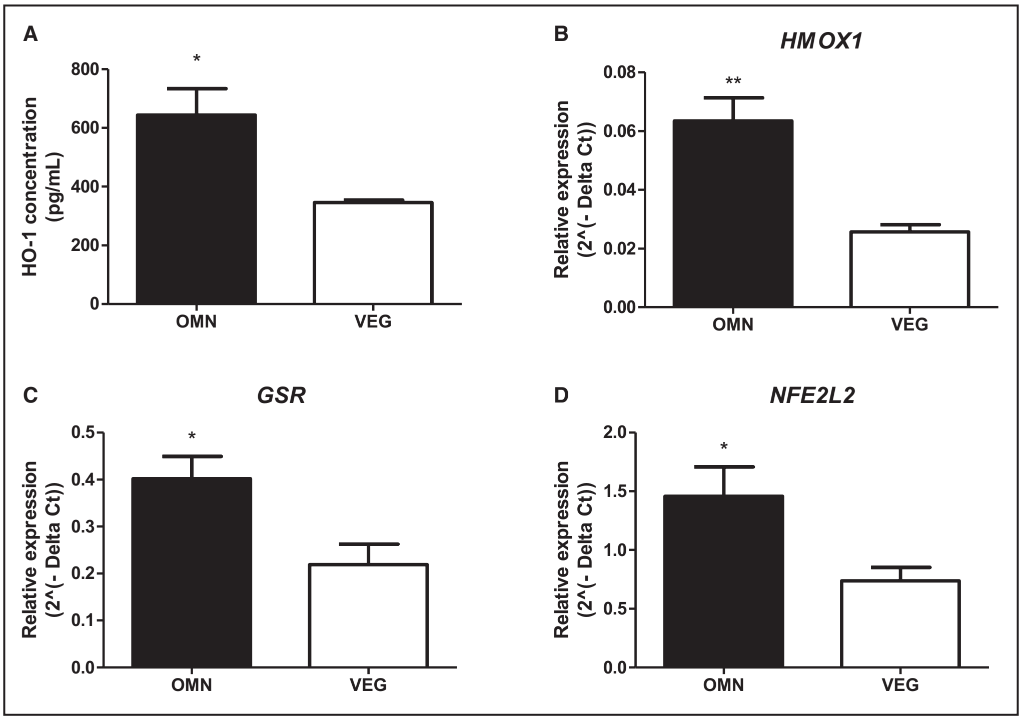

Figure 3. HO-1 (heme-oxygenase-1) concentration (A) and relative expression of the genes *HMOX1* (B), *GSR* (C), and *NFE2L2* (D) in human umbilical vein endothelial cells.
Endothelial cells were incubated with 10% (v/v) plasma samples from omnivorous and vegetarians for 24 hours. Data presented as mean±SEM. HO-1 indicates heme-oxygenase-1. *$P<0.05$ and **$P<0.01$ vs vegetarians group. Unpaired t test, GraphPad Prism software.

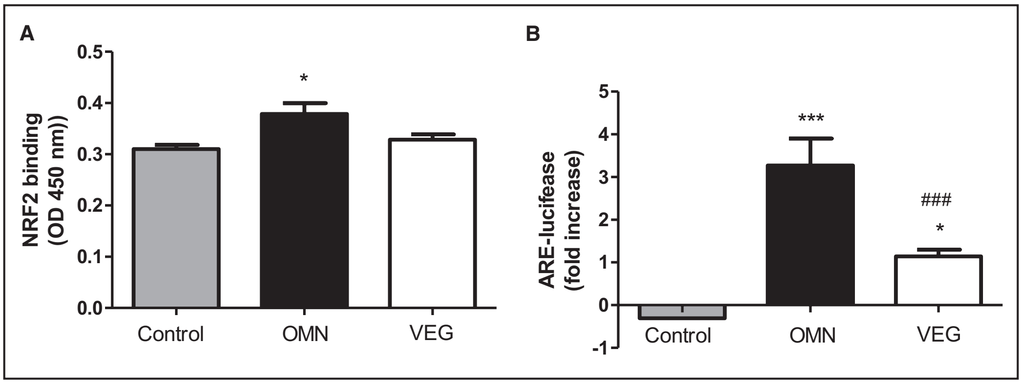

Figure 4. NRF2 (nuclear factor erythroid 2-like 2) expression in the nucleus (A) and antioxidant response elements activity (B).
Human umbilical vein endothelial cells were incubated with 10% (v/v) plasma from omnivorous and vegetarians for 24 hours. Data presented as mean±SEM. *P<0.05 and ***P<0.0001 vs control group. ###P<0.0001 vs omnivorous group. ANOVA, Bonferroni Multiple Comparison Test, GraphPad Prism software. ARE indicates antioxidant response elements; NRF2, nuclear factor erythroid 2-like 2; and OD, optical density.

Figure 6A, cell viability from omnivorous and vegetarians group decreased significantly when exposed to tBHP (all P<0.001). For LDH, ROS, and caspase levels, there was no interaction between the factors evaluated (all P>0.05). Cell incubation with plasma from the different groups and with tBHP did not change LDH release (P>0.05) (Figure 6B). Interestingly, tBHP-induced oxidative damage caused an increase in ROS production (P=0.009) and caspase activation (P=0.005), however, these effects were observed only in the omnivorous group, as shown in Figure 6C and 6D.

DISCUSSION

In the present study, we demonstrate higher circulating HO-1 production in healthy omnivorous compared with vegetarian men. Also, we show for the first time that plasma from omnivorous was able to increase the gene/protein *HO-1* expression, *GSR* and *NFE2L2* gene expression, as well as NO production in endothelial cells compared with a vegetarian group. NRF2 binding and ARE activity were significantly higher in the omnivorous group when compared with the vegetarians, indicating that HO-1 was activated by NRF2 through the binding to ARE. Moreover, cells incubated with plasma from the omnivorous group presented an increase in ROS production and apoptosis when exposed to stressor stimulus. HO-1 induction in omnivorous may indicate a pro-oxidative status since HO-1 is activated under oxidative stress.

Evidence indicates that a healthy vegetarian diet provides benefits in preventing, reversing atherosclerosis,

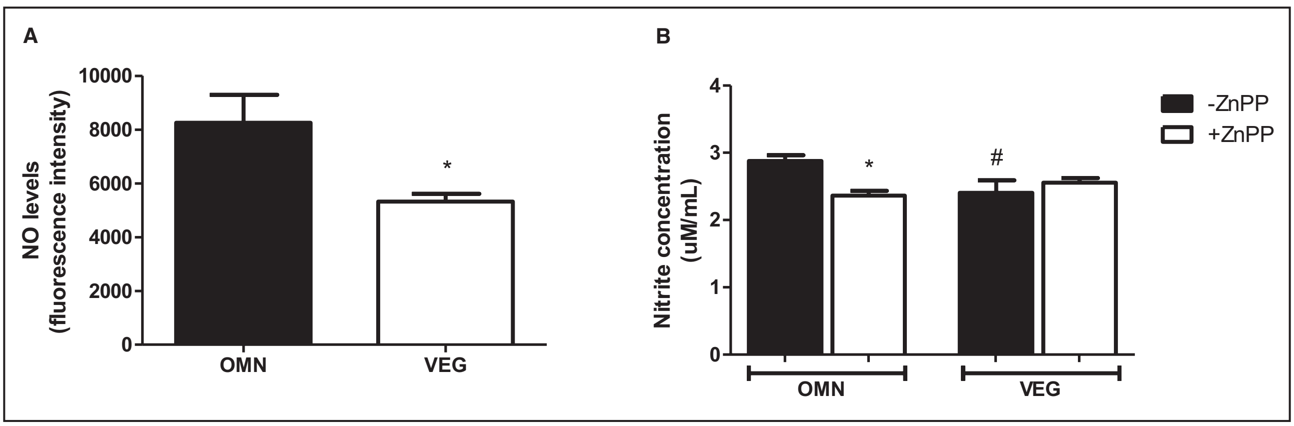

Figure 5. Intracellular nitric oxide levels (A) and nitrite concentration in the cell supernatant (B).
Human umbilical vein endothelial cells were incubated with 10% (v/v) plasma samples from omnivorous and vegetarians for 24 hours. Nitrite concentration was evaluated in the absence of zinc protoporphyrin (ZnPP) or presence of ZnPP (+ZnPP) at 1 μmol/L. DMSO was used as a vehicle for ZnPP. Data presented as mean±SEM. *P<0.05 vs omnivorous (**A**) and vs respective group—ZnPP (**B**). NO indicates nitric oxide; ZnPP, zinc protoporphyrin. #P<0.05 vs omnivorous–ZnPP group. (ANOVA, Bonferroni Multiple Comparison Test, GraphPad Prism software).

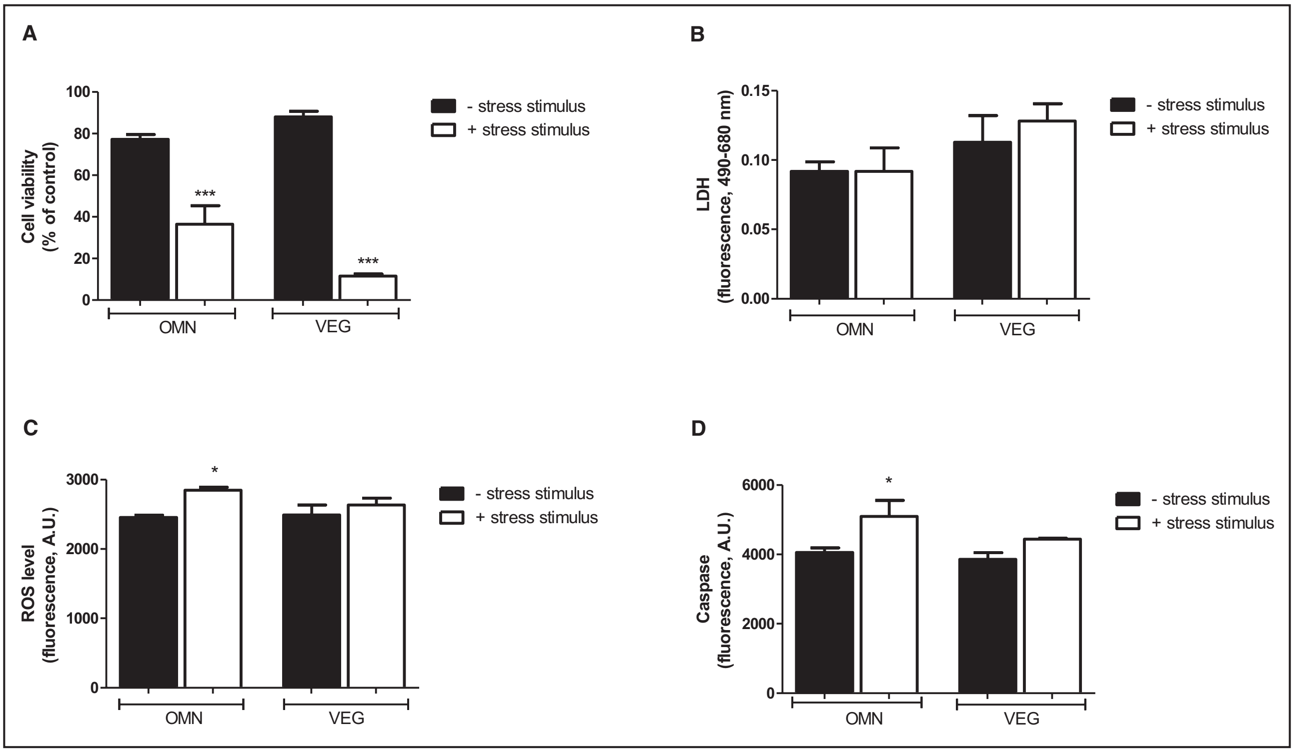

Figure 6. Cell viability (A), lactate dehydrogenase release (B), reactive oxygen species production (C), and caspase activation (D).

Human umbilical vein endothelial cells s were incubated with 5% (v/v) plasma samples from omnivorous and vegetarians for 24 hours in the absence (–) or presence (+) of stress stimulus induced by tert-butyl hydroperoxide solution at 100 μmol/L. Data presented as mean±SEM. AU indicates arbitrary units; LDH, lactate dehydrogenase; ROS, reactive oxygen species. *$P<0.05$ and ***$P<0.0001$ vs respective group—stress stimulus (2-way ANOVA followed by Bonferroni post-test, GraphPad Prism software).

and in decreasing CVD risk factors.[2,3,25] As previoulsy shown,[26–30] we found higher values of weight, BMI, SBP, diastolic blood pressure, non-HDL, low-density lipoprotein cholesterol, triglycerides, apolipoprotein B, glucose, HbA1c, IMT, PWV in omnivorous compared with vegetarians. In Brazil, a study concluded that vegetarians had a better nutritional status compared with omnivorous, with lower BMI and waist circumference, higher levels of plasma lipoprotein high-density, in addition to following a healthier lifestyle.[28] The benefits of vegetarian dietary patterns on cardiovascular health are probably the result of low consumption of animal foods that contain harmful substances, such as saturated fat, cholesterol, heme iron, and greater consumption of fruits and vegetables, which are rich in fibers, antioxidants, and phytonutrients.[25,31,32] Compared with omnivorous, people following a vegetarian/vegan diet have higher levels of antioxidants such as carotenoids, ascorbic acid, and beta-carotene.[33–35]

HO-1 catalyzes heme-producing carbon monoxide and biliverdin, which in turn is converted to bilirubin.[7] Physiologically, HO-1 induction may be a beneficial or adaptive response to several stimuli, presenting a protective role in several disorders.[7] HO-1 presence in plasma is unclear, although, it can be released into the plasma by smooth muscle cells, cardiomyocytes, leukocytes, macrophages, and endothelial cells, which were damaged by effects of oxidative stress, chronic inflammation, hypertension and/or other pathological states.[36] We showed that circulating HO-1 level was higher in plasma from omnivorous compared with vegetarians. Interestingly, these results are similar to those reported in disease conditions.[37,38] A previous study conducted by our research group showed that plasma HO-1 concentration was increased in pregnant women who subsequently developed severe preeclampsia.[37] Plasma HO-1 concentration was also elevated in individuals with newly-diagnosed type 2 diabetes mellitus compared with controls.[38] Importantly, the individuals included in this study are clinically healthy, without risk factors for CVD, and yet, the omnivorous showed higher levels of HO-1 than the vegetarian group.

Another possible explanation for this finding includes the dietary factors present in animal foods. At the transcriptional level, HO-1 is induced primarily by its substrate, free heme.[7] About 40% of the iron found in meat and fish is heme iron, which has high absorption and bioavailability.[39,40] On the other hand, a high intake of plant foods can result in high consumption of antioxidants, which in turn can reduce oxidative stress inhibiting ARE induction and HO-1 production.[41] Considering that HO-1 is responsible for the degradation of heme and release free iron,[7] dietary factors present in the omnivorous diet may be contributing to the increase of HO-1 in this group. The higher amounts of antioxidants observed in vegetarians[33,35,42] and the lower bioavailability of iron from plant foods,[43] may explain the low HO-1 expression observed in vegetarian group.

The relationship of HO-1 with clinical biomarkers of omnivorous and vegetarians was investigated. We found that HO-1 was positively associated with HDL-C both for all participants (omnivorous+vegetarians) and for omnivorous group. Also HO-1 was negatively associated with BMI and hs-CRP only in omnivorous. However, the mechanism underlying the association between HO-1 and HDL-C/BMI/hs-CRP is still unclear. In the multiple regression models, we showed that the HO-1 level was associated with omnivorous diet independently of age, BMI, hs-CRP, SBP, HbA1c, total cholesterol, HDL-C, and others factors of subclinical atherosclerosis such as IMT, PWV, and carotid distensibility.

We also evaluated if the HO-1 concentrations in plasma could be associated with differences in DNA methylation levels of the gene *HMOX1.* Despite dissimilarities in HO-1 protein levels, no differences in DNA methylation of the gene *HMOX1* were detected in omnivorous and vegetarian groups. HUVEC cells were unmethylated at this locus. This finding is in accordance with the expression of this gene at transcriptional and protein levels.

Besides that, we used as in vitro model the incubation of HUVECs with plasma from healthy subjects to explore the effects of omnivorous and vegetarian diet on HO-1 regulation and elucidate the underlying molecular mechanisms. Previous studies focused on the effects of caloric restriction diet (in different types of cells)[14–16] and the vegetarian diet (in cardiomyoblast cells),[17] have shown that circulatory factors present in the serum of animals and humans incubated in cell culture lead to changes in gene expression and significant antioxidant effect. Similarly, our results showed that omnivorous plasma was able to induce HO-1 gene/protein expression, to activate NRF2 gene/protein and ARE activity, indicating that HO-1 is regulated in part by NRF2. GSR gene expression was increased in HUVECs incubated with plasma from omnivorous compared with vegetarian group, however, total glutathione level did not differ between groups. The redox regulatory mechanism in omnivorous may be because of HO-1 induction and not by glutathione. Furthermore, we showed that the NO markers were increased in HUVECs incubated with plasma from omnivorous compared with vegetarian, while that inhibition of HO activity by ZnPP decreased nitrite concentration only in omnivorous group, suggesting that HO-1 may influence NO production. In fact, it was demonstrated in endothelial cells that HO-1 upregulation improves oxidative stress-induced senescence through the regulation of endothelial NO synthase, increasing endothelial NO synthase phosphorylation and NO production.[44] There are also data indicating that microRNAs can modulate cellular redox homeostasis, and are involved in several physiological and pathophysiological processes.[45] Ungvari et al[46] reported the presence of ARE consensus sequence in the 5′ flanking region of Dicer (ribonuclease III), a key enzyme of the microRNA biogenesis machinery[47]; the authors demonstrated that overexpression of Dicer1 in cerebromicrovascular endothelial cells isolated from aged rats improved angiogenic processes and partially restored miRNA expression, including let-7b.[46] However, we observed that there were no changes in miR-let-7 family expression (miR-let-7a, let-7b, and let-7c) in HUVECs incubated with plasma from omnivorous compared with the vegetarian group.

In the literature, it was reported the high consumption of red meat and processed meat results in increased oxidative stress,[48–50] while high consumption of fruits, vegetables, and whole grains is anti-inflammatory and antioxidative, and relieves oxidative stress.[51,52] Oxidative stress is involved in endothelial dysfunction and has been associated with the pathogenesis of CVD.[5] Therefore, we also investigated the effect of plasma from omnivorous and vegetarians in endothelial cells in an oxidative environment. We observed that cell viability, cytotoxicity, ROS level, and caspase level did not differ between omnivores and the vegetarian group. Nonetheless, we found different responses on endothelial cells incubated with plasma from different groups when exposed to stress stimulus: in the omnivorous group had in increase in ROS production, while in vegetarian group, ROS level was similar to HUVECs without stimulus.

In addition, the apoptotic rate was increased only in the HUVECs incubated with plasma from healthy omnivorous following tBHP exposure. Evidence demonstrates that endothelial cell apoptosis may compromise vasoregulation, increases low-density lipoprotein cholesterol storage, and stimulates monocyte migration into the vessel wall, resulting in atherosclerotic plaque formation.[53] Using the same in vitro model, Valgimigli et al[54] showed that serum of patients with acute coronary syndromes promoted increased rate of apoptosis in endothelial cell and that apoptosis levels were correlated with the number of complex coronary lesions.[54] Interestingly, coincubation of these cells with serum from these patients and water-soluble α-tocopherol (vitamin E, Trolox), decreased significantly apoptosis rate.[54] Another study demonstrated that exposure of endothelial cells to the serum of patients with advanced heart failure led to increased apoptosis and promoted angiogenic sprouting.[55] Circulatory factors (eg, inflammatory cytokines, oxidative stress, and growth factors) present in the plasma of omnivores may be contributing to these changes observed in endothelial cells. Taken together, our results suggest that vegetarian plasma better protected HUVECs against the oxidative damage induced by tBHP.

This study, however, has several limitations: (1) this cross-sectional non-interventional study does not prove causality between the effects of omnivorous/vegetarian diets versus HO-1 and neither HO-1 versus clinical biomarkers, however, the sample power calculations and the inclusion criteria strengthen the conclusions; (2) we have not assessed the mechanisms of the vegetarian diet involved in the biomarkers included in this study; (3) we used an immortalized cell line of HUVECs, which is basically adapted to 2-dimensional monolayer culture conditions and do not always accurately replicate the primary cells.

In conclusion, circulatory factors present in omnivorous plasma may induce an oxidative environment in endothelial cells and consequently stimulating NO production and antioxidant defense via NRF2/HO-1. Detailed mechanisms for protector effects of vegetarian diet in endothelial cell need to be further explored. This in vitro model may represent a potential tool for exploring the effects of diet on endothelial cells.

ARTICLE INFORMATION

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Cinegaglia analyzed data, interpreted results of experiments, and drafted the article. Acosta-Navarro acquired the data. Rainho analyzed, interpreted results, and drafted the methylation assay. Antoniazzi acquired and analyzed data. Mattioli contributed to the maintenance of cell culture and performed LDH assay. Carolina Vieira de Mello reviewed the article. D. Santos reviewed the article. Valeria Cristina Sandrim conceived and designed the study. All authors revised the article and approved its final version.

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Supplementary Materials

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SUPPLEMENTAL MATERIAL

Data S1.

SUPPLEMENTAL METHODS

Measurement of HO-1 concentration

HO-1 concentrations in plasma and cell supernatant were measured using the enzyme-linked immunosorbent assay kit Human Total HO-1/HMOX1 ELISA (R&D Systems, MN, USA) according to the manufacturer's protocol. In brief, the 96 well half-area microplate was prepared with capture antibody HO-1/HMOX1 and incubated overnight at room temperature. Next, samples, standards, detection antibody, and streptavidin-HRP were added, forming a sandwich complex. Then, final washes were performed, and the substrate solution was added and incubated. Finally, stop solution was added, and the plate was read at 450nm in a spectrophotometer (Synergy 4, BioTek®). A standard curve was generated by the incubation of HO-1 solutions (156.25–100,00pg/mL) with the previous reagents. The HO-1 concentration was expressed in pg/mL.

DNA Methylation analysis of the gene HMOX1

DNA methylation analysis was based on methylation-sensitive restriction enzymes (MSRE) cleavage with HpaII and MspI (isoschizomers with different sensitivities to DNA methylation at CCGG restriction sites), followed by quantitative real-time PCR [22]. Briefly, 150ng of genomic DNA from peripheral blood was digested with HpaII or MspI (New England BioLabs). Cleavage reactions of 30μL on 1X CutSmart® Buffer were performed at 37ºC overnight. Commercially available CpG methylated HeLa genomic DNA (New England Biolabs) and EpiTect unmethylated human control DNA (Qiagen) were used as fully methylated and fully non-menthylated references, respectively, during protocol optimization. MspI digested DNA samples were used to identify possible errors caused by non-specific or incomplete digestion (Figure S1 A-C). In addition, undigested controls without the addition of any restriction enzyme were also subjected to quantitative real-time PCR amplification using two locus-specific oligonucleotide pairs: forward 5'-TCATATGACTGCTCCTCTCCA3-’ and reverse 5'-GCGGCCGGTCACATTTA-3' to the target region and forward 5'-GTGATAGAAGAGGCCAAGACTG-3' and reverse 5'-CCCTGTGTTAACCATGACCTAT-3' to the control region. PCR reactions were performed in triplicates on a StepOne Real-time PCR System (Applied Biosystems) in a 20µL volume, containing 1X Power SYBR Green (Applied Biosystems), 25nM of each primer and 20ng of DNA as template. Quantification of DNA methylation was based on ∆CT value by subtracting the mean Ct (threshold cycle) from the HpaII digested samples from the average Ct from the undigested samples. The percentage of methylation was calculated by the formula 100 x (2-∆CT). O cut-off value of 10% was stablished for methylation.

Measurement of total glutathione (GSH)

Total GSH levels were measured in cell lysates using a Glutathione Colorimetric Detection Kit (Thermo Fisher Scientific, California, EUA). For this assay, $1x10^5$ cells were inoculated in 12-well plate. After plasma incubation, cells were suspended with iced PBS and cell scraper. Next, the cells was centrifuged, washed with PBS, and lysated in liquid nitrogen. Then, cell lysates and standard curve (0.78-25 µM) were added to a 96 well half-area microplate with Colorimetric Detection Reagent and Reaction Mixture. After 20 minutes of incubation, the plate was read at

405nm in a spectrophotometer (Synergy 4, BioTek®). The reaction was performed in triplicate.

mRNA and miRNA expression

HUVECs were seeded at a concentration of 5×10^4 cells per well into a 48-well plate. After plasma incubation, HUVECs were lysed by QIAZOL reagent and total RNA was extracted using a miRNeasy Mini Kit (Qiagen, Leusden, Netherlands) according to the manufacturer's protocol. Quantification and purity of isolated RNA from the samples were performed using a NanoDrop Spectrophotometer (Thermo Scientific, MA, USA) and was consistently found to be pure. cDNA reaction was performed by miScript II RT Kit using HiFlex Buffer for mRNA and *HiSpec Buffer* for miRNAs.

For mRNA expression, RT-QPCR was performed using PowerUP SYBR Green Master Mix (Thermo Fisher Scientific) containing 10 µL of SYBR green, 2 µL of each primer (200nM), 5 µL of nuclease-free water and 1µL of cDNA (5ng/µL). The *HPRT1* gene was chosen as an endogenous control because it was the gene most stable in our in vitro assay according to the previous study [56]. The primers of the mRNAs (*NFE2L2, HMOX1, GSR, HPRT1)* were obtained from Sigma®.

miRNAs expression was quantified by miScript SYBR® Green PCR Kit (Qiagen®). Each reaction contained 10 µL of QuantiTect SYBR Green PCR Master Mix, 2 µL of universal primer, 2 µL of each primer (300nM, forward and reverse), 2µL of cDNA (10ng/µL), and 4 µL of nuclease-free water, in a final volume of 20 µL. Normalization was performed to U6 snRNA. The primers of miRNAs (*miR-let-7a*, *miR-let-7b*, *miR-let-7c*, and *RNU6-2)* were obtained from Qiagen® (Leusden, Netherlands). Relative quantification was calculated using the comparative 2(-Delta

Delta C(T)) [23]. Gene and miRNA expression data were analysed using GeneGlobe Data Analysis Center (Qiagen®) online plataform. In all PCR reactions were performed in duplicate for each sample. The primer sequences used are shown in the table below:

NFE2L2	forward 5'CGTTTGTAGATGACAATGGG-3' reverse 5'AGAAGTTTCAGGTGACTGAG-3'
HMOX1	forward 5'CAACAAAGTGCAAGATTCTG-3' reverse 5'TGCATTCACATGGCATAAAG-3'
GSR	forward 5'GACCTATTCAACGAGCTTAC-3' reverse 5'CAACCACCTTTTCTTCCTTG-3'
HPRT1	forward 5' ATAAGCCAGACTTTGTTGG-3' reverse 5' ATAGGACTCCAGATGTTTCC-3'
miR-let-7a	MS00031220
miR-let-7b	MS00003122
miR-let-7c	MS00003129
RNU6-2	MS00033740

NRF2 DNA binding assay

HUVECs were seeded into 25cm^2 bottles at a concentration of 5×10^5 cells. After reaching 90–100% confluence and plasma incubation, the phosphate buffered saline (PBS) cold was added, and nuclear extract was obtained using Nuclear Extraction Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. NRF2 quantification was assessed by ELISA using NRF2 Transcription Factor Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). First, samples and controls were added to a 96-well plate for overnight incubation. The next day, the plate was washed with wash buffer and incubated with a primary antibody for 1h. Washes were repeated, and the second antibody was incubated for 1h. After, a developing solution was added for 45min under gentle agitation and protected from light. Then, stop solution was added, and the absorbance was measured at 450 nm in a spectrophotometer (Synergy 4, BioTek®). The reaction was performed in triplicate.

Antioxidant response element (ARE) activation

Antioxidant response element (ARE) activation was analyzed by ARE Reporter Kit (BPS Bioscience, CA, USA). In brief, HUVECs were seeded at a concentration of 1×10^4 cells per well into a white 96-well plate. After reaching confluence, cells were transfected using Lipofectamine® 2000 (Thermo Scientific) with the ARE reporter or negative control reporter for 12h. Next, the cells were incubated with a pool of plasma samples from the omnivorous or vegetarian group for 24 h. The incubation with tBHP (50 uM) for 3 h was used as a positive control. The luminescence was measured in a microplate reader (Synergy 4, BioTek®) using the Dual-Glo® Luciferase Assay System (Promega, WI, USA). The reaction was performed in triplicate.

Measurement of nitrite

NO production was also evaluated indirectly through the quantification of nitrite in the cell supernatants by Griess method [24]. For this assay, HUVECs were previously incubated for 1 hour with 1 µM of ZnPP, an inhibitor of HO-1 activity. Next, the cells were incubated with plasma in the presence of ZnPP or the vehicle DMSO (Sigma-Aldrich®, Poole, UK) for 24 hours, and the supernatant was collected. In brief, 50 µL of each sample was added to a 96-well plate with 50 µL of 1% sulfanilamide solution in 5% phosphoric acid for 10 min protected from light. Next, 50 µL of 0.1% N- (1-naphthyl)-ethylenediamine dihydrochloride solution was added and incubated for 10 min. The plate was read at 540 nm in a spectrophotometer (Synergy 4, BioTek®). The amount of nitrite in the cell supernatant was generated using nitrite solutions (0.39–50 μM) as a reference standard. The nitrite concentration was expressed in μM. The reaction was performed in replicates of 5.

Cellular cytotoxicity assay

Plasma membrane damage releases Lactate dehydrogenase (LDH) into the cell culture media, which is indicative of cytotoxicity. LDH release was quantified using Pierce LDH Cytotoxicity Assay Kit (88954) (Thermo Fisher Scientific, Califórnia, EUA). Briefly, 50μL of supernatant was transferred to a 96-well flat-bottom plate and 50 μL of Reaction Mixture was added. After 30 minutes of incubation, 50μL of Stop Solution was added and mixed by gentle tapping. The absorbance was read at 490nm and 680nm. To determine LDH activity, the 680nm absorbance value (background signal from instrument) was subtracted from the 490nm absorbance. The reaction was performed in replicates of 5.

Table S1. Clinical and laboratory biomarkers of omnivorous and vegetarian men included in the *in vitro* assay studies.

	Omnivorous (n=10)	Vegetarians (n=10)	*t*-test (*P*-value)
Age (Years)	42.20 ± 1.49	43.50 ± 2.03	0.61
Weight (kg)	78.18 ± 3.56	77.73 ± 3.34	0.92
BMI (kg/m^2)	25.99 ± 0.91	25.32 ± 1.18	0.66
SBP (mmHg)	121.40 ± 3.45	120.30 ± 3.60	0.82
DBP (mmHg)	81.00 ± 3.03	79.60 ± 3.85	0.77
Total cholesterol (mg/dl)	190.40 ± 11.92	187.40 ± 12.43	0.86
HDL-c (mg/dl)	47.60 ± 5.47	48.60 ± 3.43	0.87
Non-HDL-c (mg/dL)	142.80 ± 14.95	138.80 ± 13.47	0.84
LDL-c (mg/dl)	118.90 ± 11.53	114.10 ± 9.24	0.74
TG (mg/dl)	119.10 ± 18.87	122.70 ± 24.21	0.90
ApoB (g/l)	0.96 ± 0.11	0.85 ± 0.08	0.48
Fasting glucose (mg/dl)	96.60 ± 2.03	92.90 ± 2.21	0.23
HbA1c (%)	5.22 ± 0.13	5.34 ± 0.07	0.44
Hemoglobin (g/dl)	15.31 ± 0.26	15.78 ± 0.27	0.24
hsCRP (mg/dL)	1.00 (0.23-7.88)	0.58 (0.19-3.14)	0.20
IMT (mm)	623.50 ± 31.66	596.50 ± 24.01	0.50
Carotid distensibility	6.03 ± 0.43	5.79 ± 0.60	0.75
PWV (m/s)	7.45 ± 0.17	6.81 ± 0.25	0.05

Data presented as mean ± SEM (except for hsCRP: median and interquartile range). There was no statistical difference between groups (All data P>0.05).
Omnivorous: consumed any type of meat at least five or more servings per week. Vegetarians: excluded the consumption of meat, fish and poultry for at least 4 years (lacto-ovo-vegetarians, lacto-vegetarians or vegans).
BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; HDL-c – high density lipoprotein cholesterol ; LDL-c – low density lipoprotein cholesterol; non-HDL-c - non-high-density lipoprotein cholesterol; TG – triglycerides; ApoB – apolipoprotein; hsCRP – high sensitivity C-reactive protein; HbA1c - Glycated Hemoglobin; IMT - intima-media thickness; PWV – pulse wave velocity

Figure S1. Scheme of the *HMOX1* gene and its promoter-associated CpG island organized according to the physical location on chromosome 22q12.3. (A) This gene contains 5 exons (blue) spanning approximately 13,4 Kb of DNA (UCSC Genome Browser on Human, assembly GRCh38/hg38, http://genome.ucsc.edu) and a short promoter-associated CpG island (green) This island spans 259 bp, including the 5'UTR region. (B) and (C) MSRE-PCR (Methylation Sensitive Restriction Enzyme – Polymerase Chain Reaction) strategy based on cleavage with the methylation-sensitive *HpaII* restriction enzyme and its isoschizomer *MspI* used as cleavage control. Commercially human hypermethylated and unmethylated DNA, digested with the restriction enzymes were used as experimental references. (D) Five top and bottom concentrations of HO-1 in plasma in selected subjects of omnivorous and vegetarian groups (*P*<0,0001, Bonferroni's multiple comparisons test). (E) Matched DNA methylation analysis of the *HMOX1* gene in peripheral blood cells of the same subjects. No differences were detected among groups. All samples showed residual amplification of the target region, being classified as unmethylated at this locus.

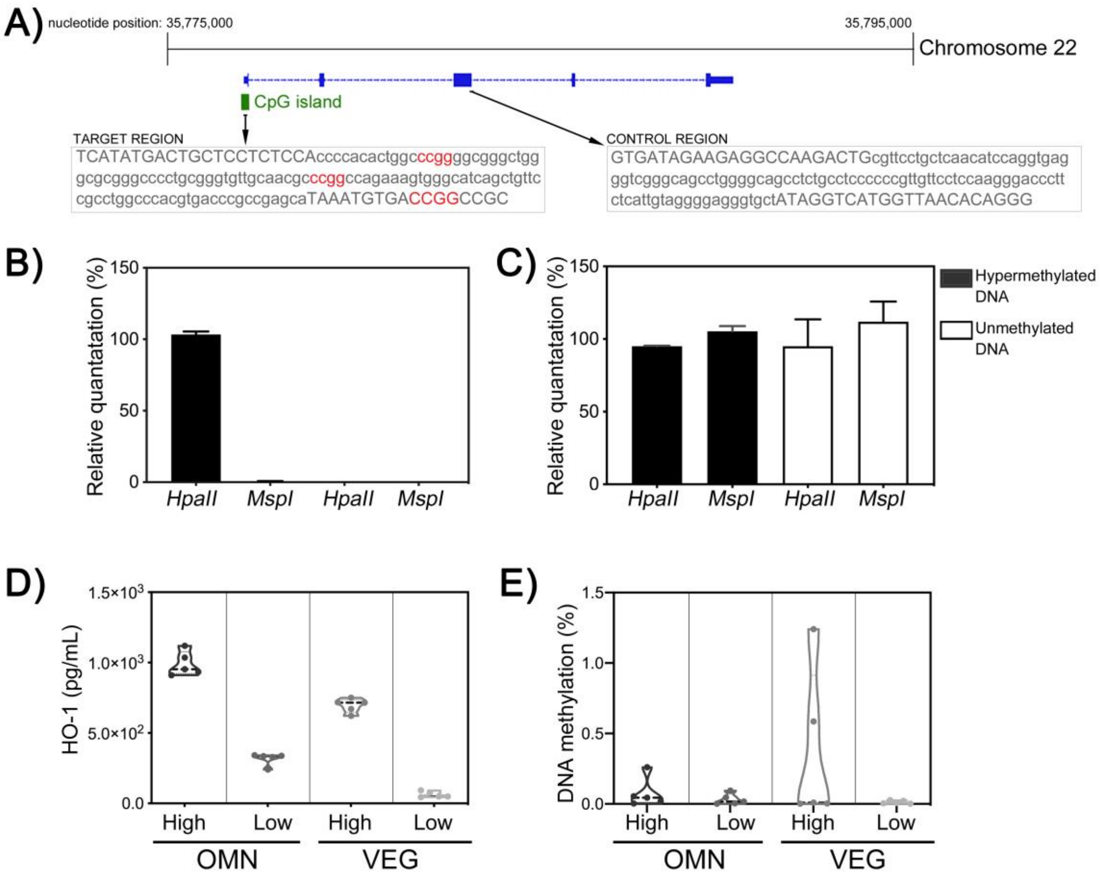

Figure S2. Total GSH (A) and relative expression of the miRNAs miR-let-7a (B), miR-let-7b (C), and miR-let-7c (D) in HUVECs. Endothelial cells were incubated with 10% (v/v) plasma samples from omnivorous (OMN) and vegetarians (VEG) for 24 hrs. Data presented as mean ± SEM. There was no statistical difference between the groups (All data *P*>0.05). (Unpaired t-test, GraphPad Prism software).