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"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Universidade Estadual Paulista "Júlio de Mesquita Filho"
Instituto de Biociências
Programa de Pós-Graduação em Biotecnologia

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COMPARAÇÃO DE PADRÕES DE DIETA VEGETARIANA VERSUS ONÍVORA SOBRE O EFEITO DE ATIVAÇÃO DA VIA NRF2 EM CÉLULAS ENDOTELIAIS

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Orientadora: Profa. Dra. Valéria Cristina Sandrim

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para a obtenção do título de Doutora no Programa de Pós-graduação em Biotecnologia.

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e ajuda-nos o ideal na estrada imensa da vida.
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*Aos meus pais **Carlos** e **Silvia**,*
Meus exemplos de força, coragem e determinação,
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No pensar, clareza
No sentir, cordialidade,
No querer, prudência:
Almejando-as
Posso então esperar,
Que eu corretamente
Possa encontrar-me
Nas trilhas da vida
Diante de corações humanos
No âmbito do dever.
Pois clareza
Provém da luz da alma,
E cordialidade
Contém o calor espiritual,
Prudência
Intensifica a força da vida.
E tudo isso,
Em confiança em Deus anseia,
Em caminhos humanos conduz
A passos bons e seguros na vida.

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RESUMO

Evidências apontam que a dieta vegetariana diminui a probabilidade de desenvolver doenças cardiovasculares (DCVs). Um dos principais mecanismos que levam às doenças cardiovasculares é a disfunção do endotélio, associada com a diminuição da biodisponibilidade de óxido nítrico (NO) e a produção excessiva de espécies reativas de oxigênio (ROS). Do ponto de vista de biomarcadores de estresse oxidativo/antioxidante e de envelhecimento biológico, enzimas com propriedades antioxidantes e o comprimento do telômero podem apresentar efeitos na modulação do sistema vascular. O presente estudo inclui dois manuscritos, sendo o primeiro relacionado a via de regulação NRF2/HO-1 e o segundo ao comprimento dos telômero em onívoros (ONI) e vegetarianos (VEG). No primeiro manuscrito, o objetivo foi verificar a concentração de HO-1 circulante, bem como investigar o efeito da incubação do plasma de ONI e VEG em células endoteliais sob a modulação da via NRF2/HO-1 e a produção de NO. Dos 745 indivíduos inicialmente recrutados, 44 ONI e 44 VEG do sexo masculino aparentemente saudáveis foram incluídos no estudo. A concentração de HO-1 circulante foi mensurado usando o ensaio de ELISA. As células endoteliais foram incubadas com amostras de plasma de ONI e VEG. Nós observamos que a concentração de HO-1 circulante foi maior nos ONI em relação aos VEG. A incubação das células endoteliais com o plasma de ONI induziu o aumento da expressão gênica/proteica do NRF2 e HO-1, bem como a atividade do ARE e a produção de NO, quando comparado com o grupo VEG. Estes achados indicam que a produção de HO-1 circulante nos ONI pode estar relacionada a um maior status pro-oxidativo. A ativação da via HO-1/NRF2 e a produção de NO em cultura de células endoteliais incubadas com o plasma de ONI, parece refletir um mecanismo adaptativo de proteção destas células contra possíveis danos. No segundo manuscrito, nosso objetivo foi avaliar o comprimento do telômero em leucócitos (LTL, do inglês *leukocyte telomere length*) nos ONI e VEG e sua associação com biomarcadores clássicos de risco cardiovascular. O LTL foi mensurado em 39 amostras de ONI e 41 de VEG através da reação em cadeia da polimerase (PCR) quantitativa em tempo real (RT-QPCR). Apesar de não haver diferença no comprimento do telômero entre ONI e VEG, uma forte correlação negativa entre LTL e a espessura intima-média da carótida (IMT, do inglês *intima-media thickness*) foi observada nos ONI, mas não no grupo VEG. Além disso, os ONI que foram classificados com o LTL curto apresentaram maior IMT comparado com VEG. Nossos achados sugerem que o comprimento do telomero pode ser utilizado como um marcador de aterosclerose subclinico no grupo de onívoros.

Palavras-chave: células endoteliais, dieta vegetariana, heme-oxigenase, risco cardiovascular, comprimento do telômero.

ABSTRACT

Several studies report that a vegetarian diet lowers the probability of developing cardiovascular diseases (CVDs). The endothelial dysfunction is one of the main mechanism that leads to CVDs, associated with decreased nitric oxide (NO) bioavailability and excessive production of reactive oxygen species (ROS). Regarding oxidative/antioxidant stress and biological aging biomarkers, enzymes with antioxidant properties and telomere length may have effects on vascular system modulation. The present study includes two manuscripts related to: 1) regulation of NRF2/HO-1 pathway and 2) telomere length in omnivorous (OMN) and vegetarians (VEG). In the first manuscript, our objectives were to verify circulating HO-1 levels and the effect of plasma incubation from omnivorous and vegetarians in endothelial cells on modulating of NRF2/HO-1 pathway and NO production. From 745 participants initially recruited, 44 omnivorous and 44 vegetarian men apparently healthy were included in this study and circulating HO-1 was measured using ELISA assay. Endothelial cells were incubated plasma samples from OMN and VEG. We found higher circulating HO-1 production in omnivorous compared to vegetarian. Moreover, the plasma collected from omnivorous was able to increase the gene/protein NRF2/HO-1 expression, ARE activity, and NO production in endothelial cells culture compared to vegetarian group. We suggest that HO-1 induction in omnivorous may indicate a pro-oxidative. Activation of the HO-1 / NRF2 pathway and NO production in endothelial cell culture incubated with ONI plasma seems to reflect an adaptive mechanism of protection of these cells against possible damage. In the second manuscript, our objectives were to evaluate leukocyte telomere length (LTL) in VEG and OMN subjects and its association with classical cardiovascular risk biomarkers. LTL was measured in 39 omnivorous and 41 vegetarians by Real-Time Quantitative PCR reaction. Although telomere length was not different between omnivorous and vegetarians, we found a strong negative correlation between LTL and intima-media thickness (IMT) in omnivorous, but not in vegetarian group. In addition, omnivorous who were classified with short telomere length had higher carotid IMT compared to vegetarians. Our data suggest that telomere length can be a marker of subclinical atherosclerosis in the omnivorous group.

Key words: endothelial cells, vegetarian diet, heme-oxygenase, cardiovascular risk, telomere length.

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1. INTRODUÇÃO

1.1. Risco cardiovascular

Segundo dados da Organização Mundial de Saúde, as doenças cardiovasculares (DCVs) são a principal causa de mortes, responsáveis por ~31% de óbitos mundialmente. As DCVs envolvem um grupo de distúrbios do coração e dos vasos sanguíneos, entre elas podemos citar as doenças causadas pela aterosclerose como, doença coronariana, doença cerebrovascular, doenças vascular periférica e hipertensão [1], sendo que 85% destas mortes, são por ataque cardíaco e acidente vascular cerebral (AVC) [2]. Os fatores de risco cardiovascular incluem: dieta inadequada, sedentarismo, consumo excessivo de álcool, tabagismo, bem como a idade e fatores genéticos [1]; logo, os efeitos destes fatores de risco podem se manifestar nos indivíduos através do aumento da pressão arterial, diabetes, hiperlipidemia, sobrepeso e obesidade (Organização Pan-Americana da Saúde, OPAS, 2017) [3].

Várias evidências apontam que o comprometimento funcional do endotélio é um dos primeiros sinais do processo de aterosclerose, mas também pode ser um importante fator na progressão e no desenvolvimento da doença cardiovascular aterosclerótica [4]. O endotélio vascular é uma monocamada ativa de células que cobre o lúmen dos vasos sanguíneos, separando a parede vascular do sangue circulante [4]. Estas células endoteliais mantém a homeostase interna dos vasos sanguíneos através da produção substâncias vasoconstritoras e vasodilatadoras, desempenhando importante papel na regulação da resistência vascular e pressão sanguínea [4,5]. Em condições patológicas, a produção desses fatores vasoativos é desregulada, podendo levar à diminuição de substâncias vasodilatadoras (como o óxido nítrico) e ao aumento simultâneo de vasoconstritores [4,6].

O óxido nítrico (NO), um poderoso fator vasodilatador, é sintetizado pelo aminoácido L-arginina através da eNOS (NOS endotelial); rapidamente, o NO se desloca do endotélio para as células do músculo liso vascular para ativar a guanilato ciclase solúvel (GCs), que por sua vez, induz um aumento na concentração de monofosfato cíclico de guanosina (GMPc), levando assim a um relaxamento das células musculares lisas com consequente vasodilatação [5]. Porém, sugere-se que um defeito na atividade da eNOS, leva a redução da biodisponibilidade de NO e ao aumento de radicais livres, incluindo peroxinitrito ($ONOO^-$) e óxido nitroso (N_2O_3) na vasculatura, causando danos no DNA e envelhecimento endotelial [7,8].

Logo, a diminuição do NO, o aumento de estresse oxidativo resultante da produção excessiva de espécie reativa de oxigênio (ROS) e/ou a falha nos mecanismos de defesa dos antioxidantes, têm sido associados com a disfunção do endotélio [4,9]. Consequentemente, o sistema vascular está predisposto à vasoconstrição, agregação de plaquetas, aderência leucocitária e inflamação [4,5]. Tendo isso em vista, o foco do presente estudo foi a célula endotelial.

Estudos *in vitro* e *in vivo* já demonstraram os efeitos benéficos do maior consumo de frutas e vegetais associado com a diminuição nos danos celulares, no DNA e no estresse oxidativo [10]. Evidências epidemiológicas confirmam estes achados, mostrando que a dieta vegetariana [11,12], DASH (da sigla em inglês *Dietary Approaches to Stop Hypertension*) [13], mediterrânea [14] e de restrição calórica [15], podem modular positivamente as doenças cardiovasculares. Em contraste, o consumo excessivo de carne vermelha ou processadas leva ao aumento de estresse oxidativo [16–18]. Sendo assim, o presente estudo deu ênfase à dieta vegetariana.

1.2. Padrão alimentar vegetariano

O número de vegetarianos e adeptos ao vegetarianismo tem aumentado nos últimos anos. Estudos epidemiológicos apontam que em 2009, ~3% da população americana seguiu uma dieta vegetariana; em 2012, esse número passou para ~5%, e 43% dos americanos revelaram que consomem pelo menos uma refeição vegetariana na semana [19]. No Brasil, uma pesquisa realizada recentemente em 142 municípios, mostrou que essa proporção passou de 8% em 2012 para 14% em 2018, representando 30 milhões de brasileiros (IBOPE Inteligência, 2018) [20]. Baseado no modelo de outros países como Estados Unidos e Reino Unido, foi lançado no Brasil pela Sociedade Vegetariana Brasileira (SVB) uma campanha em 2009 nomeada "Segunda Sem Carne", convidando as pessoas a tirar a carne do cardápio pelo menos uma vez na semana, estimulando hábitos mais saudáveis e a consumirem mais verduras e frutas [21]. Grandes organizações, como a Organização Mundial de Saúde, reconhecem os benefícios de uma alimentação vegetariana para a saúde, se pronunciando sobre os riscos do consumo elevado de carne [20]. A Associação Dietética Americana (ADA), declara: "*as dietas vegetarianas apropriadamente planejadas são saudáveis, adequadas em termos nutricionais e apresentam benefícios para a saúde na prevenção e no tratamento de*

determinadas doenças, e pode ser adotada em qualquer ciclo da vida" [22]. O Conselho Regional de Nutricionistas – 3ª Região, também publicou em 2012 o parecer sobre as dietas vegetarianas com recomendações aos nutricionistas [23].

O padrão alimentar vegetariano exclui o consumo de todos os tipos de carnes, sendo classificado de acordo com o grau de restrição de alimentos de origem animal: lactovegetariano (permite laticínios) e ovolactovegetariano (permite ovos e laticínios) e vegetariano estrito (exclui qualquer tipo de alimento de origem animal) [24]. Outra definição conhecida como veganismo refere-se a uma filosofia ou um modo de viver, que busca excluir todo e qualquer tipo de exploração e sofrimento dos animais, seja no consumo de alimentos/produtos de origem animal, bem como nos produtos testados em animais [25]. No âmbito da alimentação, vegano e vegetariano estrito são sinônimos [25].

Em geral, a dieta vegetariana é rica em fibras, vitaminas C e E, ácido fólico, potássio, magnésio, carboidratos complexos), fitoquímicos e antioxidantes, e baixa em gorduras saturadas, colesterol e gordura total [26]. Estes compostos podem ser obtidos através de alimentos como cereais, legumes, verduras, nozes, sementes e frutas; produtos animais como laticínios e ovos, podem ser ou não incluídos nesse tipo de dieta [27]. Entretanto, seguir uma alimentação vegetariana desequilibrada e/ou excluir todos os alimentos de origem animal, pode resultar em desvantagens à saúde para estes indivíduos, como falta de vitamina D, vitamina B12, ferro heme e zinco [28]. A deficiência de vitamina B12 ocorre principalmente entre os indivíduos veganos e foi associada com o aumento no nível de homocisteína no plasma [29,30].

Evidências relatam que o padrão alimentar vegetariano diminui o risco de desenvolver doenças cardiovasculares [31,32]. Um estudo transversal (*Adventist Health Study 2*), que incluiu 592 participantes, mostrou que a probabilidade de indivíduos veganos/vegetarianos desenvolverem hipertensão foi 44% menor em relação aos onívoros; a prevalência de diabetes foi de 8,9% em veganos/vegetarianos, 18,8% em psicovegetarianos e 15,6% em onívoros [33]. Um dos maiores estudos de coorte envolvendo 89.000 adventistas californianos que consumiam dietas vegetarianas (psicovegetarianos, ovolactovegetarianos, veganos) comparado aos semivegetarianos e onívoros, mostrou que a taxa de hipertensão diminuiu gradualmente a medida que as pessoas se alimentavam a base de planta; e o mesmo foi observado para diabetes e obesidade [34]. Os efeitos da dieta na pressão sanguínea foram comprovados em estudos de coorte com intervenção de dietética. Foi observado que

indivíduos vegetarianos que incluíram a carne na dieta por 11 dias, apresentaram aumento na pressão arterial [35]. Foi observado que a retirada da carne da dieta dos indivíduos que seguia uma dieta onívora, levou a redução da pressão arterial em apenas 7 dias, considerando que antes da intervenção dietética, os medicamentos anti-hipertensivos ou antiglicêmicos foram reduzidos ou eliminados [36].

Estes achados estão de acordo com os aspectos tipos encontrados nos vegetarianos em relação aos onívoros, que incluem: menor pressão sanguínea, índice de massa corpórea (IMC), glicose, hemoglobina glicada, colesterol da lipoproteína de baixa densidade (c-LDL), colesterol da lipoproteína de alta densidade (c-HDL) colesterol total (CT) e gordura total (GT) [12,37]. De modo interessante, um estudo transversal envolvendo indivíduos com 50 anos ou mais, avaliou a resposta de dilatação vascular acessada por dilatação mediada por fluxo e dilatação independente do endotélio (após a administração de nitroglicerina, NTG); foi observado que a função vascular nos vegetarianos foi melhor em relação aos onívoros, e que estes efeitos foram dependentes da dieta e independentes de outros fatores relacionados à aterosclerose (tabagismo, envelhecimento, diabetes, hipertensão, hiperlipidemia) [38]. Os autores ainda relataram que a melhor resposta a dilatação vascular nos vegetarianos, foi diretamente relacionada ao tempo que estes indivíduos adotaram a dieta, sugerindo que a dieta vegetariana pode ter efeito direto na função endotelial [38]. *Navarro* e colaboradores confirmaram estes achados, mostrando que outros marcadores de função vascular, como velocidade de onda de pulso (VOP), espessura intima-média da carótida (c-IMT, do inglês *carotid intima-media thickness*), também estavam diminuídos nos vegetarianos em relação aos onívoros [39].

Estudos sugerem que ROS apresenta papel importante no controle da função vascular a manutenção do tônus vascular [40]. Em condições fisiológicas, o equilíbrio entre substâncias pró-oxidantes e antioxidantes é mantido ligeiramente em favor dos pró-oxidantes, favorecendo um estresse oxidativo moderado [41]. Entretanto, o excesso de ROS e/ou a diminuição da defesa antioxidante foram associados com a disfunção endotelial e ao aumento da pressão sanguínea [9]. Isso justifica o fato de que grande parte dos efeitos benéficos da dieta vegetariana para a saúde cardiovascular, tenham sido atribuídos a alta quantidade de antioxidantes endógenos e exógenos (provenientes dos alimentos baseados em plantas) presente neste indivíduos [28]. Alguns estudos observaram menores níveis de estresse oxidativo (peroxidação lipídica) [42,43], e maiores níveis de antioxidantes como (glutationa peroxidase,

carotenoides, Vitamina E e ácido ascórbico) nos vegetarianos em relação aos onívoros [11,42,44]. De modo interessante, biomarcadores antioxidantes e de estresse oxidativo foram mensurados no plasma de onívoros e vegetarianos jovens (20-30 anos de idade) e idosos (60-70 anos) [11]. Os níveis de Vitamina C e β-caroteno estavam diminuídos nos onívoros idosos, sendo que nos vegetarianos, os níveis plasmáticos foram similares entre os indivíduos jovens e idosos [11]. Da mesma forma, os níveis de biomarcadores de estresse oxidativo (quebra e oxidação do DNA, proteínas carboniladas e ácidos graxos dienos conjugados) não diferiu significativamente entre os onívoros e vegetarianos, porém os vegetarianos idosos apresentaram menores níveis de estresse oxidativo quando comparado aos onívoros da mesma idade [11].

Vale ressaltar que parte destes antioxidantes são fitoquímicos (como vitamina C e carotenoides), os quais apresentam atividade pró-oxidantes *in vivo*, e ainda assim desempenham importantes funções regulatórias e de sinalização celular, incluindo o balanço redox e controle da ativação do elemento de resposta antioxidante (ARE) [28]. O ARE é uma região promotora do gene sensível-redox que regula enzimas antioxidantes, incluindo a heme oxigenase (HO) [28].

1.3. Heme-oxigenase

A heme-oxigenase é uma enzima envolvida na degradação do grupo heme. Resumidamente, a HO cliva o heme em 3 produtos biológicos ativos: monóxido de carbono (CO), íons ferro (Fe2+) e biliverdina, que por sua vez é convertido em bilirrubina pela biliverdina redutase [45]. *Tenhuen* e colaboradores, identificaram três isoformas da HO [46]. A primeira, HO-1 é uma proteína induzível por estímulos, como heme (o principal indutor), metais pesados, hipóxia e oxidantes (como peróxido de hidrogênio e NO [47]. A HO-2 é uma proteína sintetizada constitutivamente, e no geral não responde a nenhum indutor da HO-1 [48]. Por fim, a HO-3 também participa da degradação heme, mas sua função ainda é indefinida [48].

A HO-1 em particular, é altamente expressa no baço, fígado e medula óssea, enquanto que nos demais tecidos, como no endotélio, sua expressão ocorre em níveis basais [49]. Sua indução fisiológica pode ser uma resposta benéfica ou adaptativa aos estímulos, apresentando papel fundamental na manutenção da homeostase vascular e, exercendo efeitos antioxidativo, anti-inflamatório, antiapoptótico, antiproliferativo e vasodilatador [45]. Em nível molecular, a

expressão HO-1 é ativada principalmente pelo fator de transcrição NRF2 (*nuclear factor, erythroid 2-like 2*), através da ligação com o elemento de resposta antioxidante (ARE). Em condições normais, o NRF2 se liga a proteína inibidora Keap-1 (*Kelch-like ECH-associated protein-1*) rica em cisteína, tornando-se alvo de ubiquitinação e degradação proteossomal, mantendo seu nível baixo no citoplasma [50]. Porém, o NRF2 é oxidado quando exposto a oxidantes endógenos, pró-oxidantes derivado de fitoquímicos ou eletrófilos (incluindo produtos de oxidação lipídica eletrolítica 4-hidroxinonenal, HNE), resultando na dissociação do NRF2 e Keap-1 [50]. Agora livre, o NRF2 é transportado para o núcleo, onde encontra outros fatores de transcrição como Maf (*v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog*), liga-se na sequência ARE, promovendo a transcrição da HO-1 e de outras enzimas antioxidantes [50]. Além disso, HO-1 também pode ser induzido por outros fatores de transcrição como *activator protein-1* (AP-1) e *signal transducer* e *activator of transcription 3* (STAT3) [49].

A contribuição da atividade da HO-1 e de seus produtos finais, tem sido extensamente investigada no contexto das doenças cardiovasculares. A degradação do heme livre parece ser a primeira contribuição da atividade da HO-1 na proteção do tecido [45]. A bilirrubina é um potente antioxidante endógeno, desempenhando importante papel anti-inflamatório através da inibição da NADPH oxidase [48]. CO atua como um segundo mensageiro para aumentar a resistência celular ao estresse oxidativo, através da ativação da guanilato ciclase solúvel (GCs) e consequente aumento de monofosfato cíclico de guanosina (GMPc) [51]. Estudos sugerem que CO interage com NO, os quais concomitantemente promovem a vasodilatação, ativam antioxidantes, além de inibir a apoptose e ações inflamatórias [51].

Diante de todos estes mecanismos citoprotetores mediados pela HO, a literatura tem mostrado que alguns antioxidantes sintéticos (como *dihydromyricetin*, *willow bark*, resveratrol) são capazes de induzir a HO-1 através do NRF2 nas células endoteliais, e tem sido proposto como potencial alvo terapêutico para a prevenção das doenças cardiovasculares [52–54]. Entretanto, o uso de suplementos antioxidantes pode alterar o sistema de defesa e de síntese dos antioxidantes endógenos nas células, além de modificar os níveis de morte celular [10]. Portanto, uma alimentação baseada em plantas como a dieta vegetariana parece ser a melhor opção para a saúde cardiovascular.

Além disso, é importante considerar as dificuldades em avaliar os efeitos desses antioxidantes sintéticos em níveis fisiológicos, pois a adição de extratos ou alimentos

integrais podem não representar a real dose ou a biotransformação destes em situações *in vivo* [55]. Por exemplo, em células de hepatócitos de animais, 50 e 75mmol/L de resveratrol ativou o NRF2 e induziu a produção de enzimas antioxidantes (ex. catalase, superóxido dismutase, glutationa peroxidase) [56], entretanto estas doses são muito mais elevadas do que as alcançadas na circulação em condições fisiológicas. Para resolver isso, alguns estudos têm incluído os indivíduos como 'biorreatores', utilizando amostras de plasma/soro destes sujeitos após a ingestão de alimentos ou extratos de interesse [55,57]. Estudos evidenciando os efeitos benéficos da dieta de restrição calórica, e mais recentemente da dieta vegetariana, mostraram que fatores circulatórios presentes no soro de animais e humanos, incubados em cultura de células, conferem modificações na expressão gênica e significante efeito antioxidante, antiproliferativo e anti-apoptótico [43,58–60]. Dessa forma, nós sugerimos que o plasma dos vegetarianos module a HO-1 de maneira diferente dos onívoros nas células endoteliais.

Considerando que indivíduos vegetarianos apresentam risco cardiovascular reduzido em relação aos onívoros, e que isso pode estar relacionado a alta quantidade de antioxidantes presentes nessa dieta, é importante avaliar quais são os mecanismos moleculares de proteção da dieta vegetariana ao risco cardiovascular. É possível que o plasma de vegetarianos module a HO-1 de maneira diferente dos onívoros, tornando este um possível alvo farmacológico. Além disso, não há dados na literatura mostrando o efeito do plasma de vegetarianos em cultura de células endoteliais, portanto este estudo pode contribuir para o conhecimento sobre os parâmetros celulares e bioquímicos deste grupo que vem crescendo mundialmente.

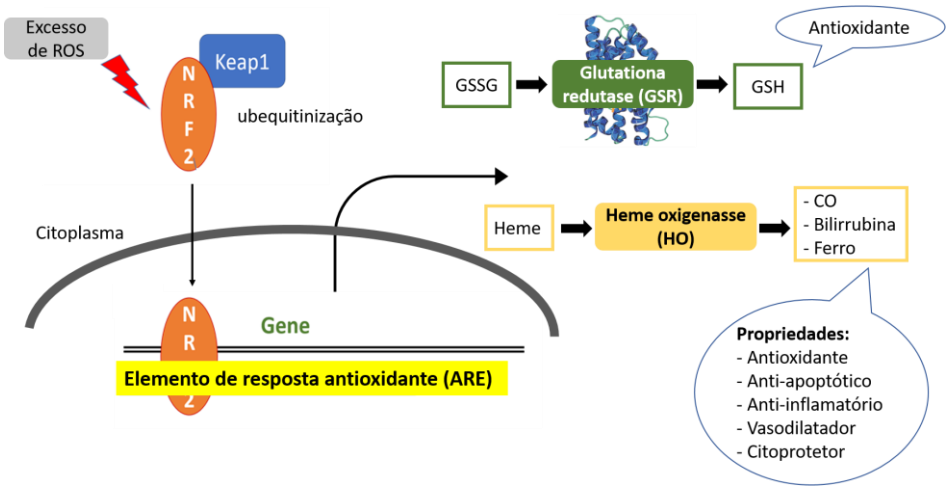

Figura 1. Via de regulação do NRF2/HO. Em condições fisiológicas normais, o NRF2 se encontra no citoplasma associado a proteína Keap-1, tornando-se alvo de ubequitinização e degradação. Em condições de estresse como quando induzido pelo excesso de ROS, o NRF2 se dissocia da Keap-1, sendo posteriormente transportado para o núcleo onde reconhece o ARE, determinando a síntese de moléculas antioxidantes como a heme oxigenasse e a glutationa.

1.4. Telômeros

Os telômeros são estruturas de nucleoproteínas repetidas (TTAGGG) localizadas na região 3' das extremidades dos cromossomos [61]. Tais nucleoproteínas representam uma capa protetora (da sigla em inglês *cap*), apresentando duas principais funções: 1) proteger as extremidades dos cromossomos de mecanismos inapropriados de reparo ao DNA, que de outra forma, poderiam ser reconhecidos como quebras na cadeia dupla do DNA e resultar na fusão de um cromossomo com outros; 2) impedir a degradação de genes próximos ao final dos braços dos cromossomos, preservando o material genético [62]. Devido a replicação do DNA incompleta, a sequência dos telômeros não são completamente replicadas, ocorrendo uma perda dos pares de base progressivamente, tornando os telômeros mais curtos a cada replicação das células somáticas [63]. Eventualmente, as células podem entrar em senescência e apoptose, resultando na perda dos telômeros [64]. Em adição, a ação de uma enzima especializada chamada telomerase, promove a manutenção e o alongamento dos telômeros [64]. Por ser uma transcriptase reversa, a telomerase adiciona nucleotídeos nas extremidades do DNA recém sintetizado, mantendo o comprimento do telômero [65]. Muitos tipos de células como, as células germinativas, células-tronco e em alguns tipos de células cancerígenas apresentam um alto nível de atividade da telomerase, enquanto que nas células somáticas, o seu nível de atividade é baixo ou indetectável [64]. Logo, o comprimento do telômero é considerado um "relógio mitótico" e tem sido proposto como um potencial biomarcador de envelhecimento [66–70].

Além do problema de replicação, outros fatores podem contribuir com o encurtamento do telômero, como inflamação, estresse oxidativo, fatores ambientais incluindo o alto consumo de álcool e a ingestão calórica em excesso [71].

Diante deste cenário, estudos têm mostrado que o encurtamento do telômero está envolvido na patogênese de doenças relacionadas ao envelhecimento, como as DCVs [66–70]. Um estudo longitudinal observou que o encurtamento do telômero foi associado com a incidência e progressão da aterosclerose, mostrando potencial utilidade clínica na predição da aterosclerose [66]. Outros estudos demonstraram maior encurtamento do telômero em indivíduos com aterosclerose [67], hipertensão [68,69] e doença arterial coronariana [70].

Evidências apontam que, além do envelhecimento, o aumento da inflamação e o estresse oxidativo aceleram o atrito do telômero, possivelmente explicando a associação

observada entre o comprimento do telômero e DCVs [64,72]. De modo interessante, tanto o comprimento do telômero como o risco cardiovascular podem ser modificados pela dieta [14,39,73,74]. Uma dieta equilibrada rica em frutas, vegetais e grãos integrais é anti-inflamatória e antioxidativa, que por sua vez, diminui a produção de ROS e danos ao DNA [10,75]. *Lian* e colaboradores [73] mostraram que os telômeros mais longos foram associados com menor risco de desenvolver hipertensão somente no grupo de indivíduos que consumiam mais vegetais [73]. Em outro estudo, a maior adesão à dieta mediterrânea foi associada com telômeros mais longos, entretanto, não houve correlação entre o comprimento dos telômeros e os componentes dietéticos da dieta mediterrânea, sugerindo que a preservação do comprimento dos telômeros resultou do efeito global deste padrão de dieta. Em contrapartida, o alto consumo de carne vermelha e processada pode resultar no aumento do estresse oxidativo [16–18], o qual pode induzir danos ao DNA e afetar o comprimento do telômero [76,77]. Diante deste contexto, uma dieta vegetariana balanceada pode proteger os cromossomos.

Estudos epidemiológicos têm investigado o comprimento do telômero nas doenças cardiovasculares, bem como sua relação com a dieta [70,73,74], entretanto, nenhum estudo incluindo indivíduos vegetarianos aparentemente saudáveis foi realizado até o momento. Considerando que a dieta vegetariana é rica em frutas e vegetais, nossa hipótese é que a dieta vegetariana pode prevenir ou reduzir o encurtamento do telômero.

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3. MANUSCRITO I

HO-1 levels is increased in omnivorous men healthy compared to vegetarians.

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ABSTRACT

Several evidence report that a healthy vegetarian diet is rich in antioxidants and contributes to cardiovascular health. Endothelial dysfunction is one of the main mechanisms that lead to cardiovascular diseases (CVDs). Failure to protect against oxidative stress and decreased nitric oxide (NO) bioavailability has been associated with endothelial dysfunction. Increased heme-oxygenase-1 (HO-1) is a marker of adaptive response and is protective against oxidative stress. Thus, it is important to understand the effects of vegetarian and omnivorous diet on the modulation of HO-1. Our objectives were to verify circulating HO-1 levels and the effect of plasma incubation from omnivorous and vegetarians in endothelial cells on modulating of NRF2/HO-1 pathway and NO production. From 745 participants initially recruited, 44 omnivorous and 44 vegetarian men apparently healthy were included in this study and circulating HO-1 was measured using ELISA assay. Endothelial cells were incubated plasma samples from omnivorous and vegetarians. We found higher circulating HO-1 production in omnivorous compared to vegetarian. Moreover, the plasma collected from omnivorous was able to increase the gene/protein NRF2/HO-1 expression, ARE activity, and NO production in endothelial cells culture compared to vegetarian group. We suggest that HO-1 induction in omnivorous may indicate a higher pro-oxidative status compared to vegetarian group since HO-1 is activated under oxidative stress.

INTRODUCTION

Several evidence report that a healthy diet containing high consumption of fruits, vegetables, and whole grains is anti-inflammatory and antioxidative, besides lower oxidative stress and DNA damage [1,2]. Epidemiologically, plant-based diets, such as vegetarian diet, can help humans in diseases prevention [3,4]. As reviewed by Le and Sabaté [4], the vegetarian diet lower rates hypertension, prevalence of diabetes, obesity, several types of cancer, and total mortality. These findings are in agreement with the typical aspects observed in vegetarian subjects including lower blood pressure, body mass index (BMI), glucose, total cholesterol, low-density lipoprotein cholesterol (LDL), and non–high-density lipoprotein cholesterol (non-HDL) [5,6]. Moreover, was observed that vascular function was better in vegetarians compared with omnivorous [6,7]. Researchers attribute these benefits of a vegetarian diet to less cholesterol, total and saturated fat, and higher intake of fiber, vitamin C, vitamin E, magnesium, carbohydrates (mainly the most complex), phytochemicals and antioxidants, compared with omnivorous diet [8,9].

Endothelial dysfunction is one of the main mechanisms that lead to cardiovascular diseases (CVDs) and represents the initial step in the pathogenesis of atherosclerosis [10]. Failure to protect against oxidative stress and decreased nitric oxide (NO) bioavailability (an endothelium-derived vasodilator) has been associated with endothelial dysfunction [11]. Therefore, enzymes with antioxidant properties may play beneficial effects in the modulation of vascular systems, minimizing oxidative stress [12]. NRF2 is considered a ‘‘master regulator’’ of the antioxidant response; under cellular stress, NRF2 is disassociated from its inhibitory protein Kelch-like ECH-associated protein-1 (KEAP-1) and transported to the nucleus, where it binds to antioxidant response elements (ARE) determining the synthesis of antioxidant molecules, such as glutathione reductase (GSR) and heme-oxygenase-1 (HO-1) [13]. GSR enzyme reduces oxidized glutathione (GSSG) to generate reduced glutathione (GSH) [14]. HO-1 cleaves heme-producing bilirubin and carbon monoxide (CO) and has an important role in the maintenance of vascular homeostasis, exerting antioxidative, anti-inflammatory, antiapoptotic, antiproliferative and vasodilator effects [12]. HO-1 is highly expressed in the spleen, liver and bone marrow, that is the main organs responsible for the metabolism of hemoglobina [15]. In other tissues, as in the endothelium, basal expression of HO-1 is very low, but can be rapidly induced by several physiological and pathological stimuli, including heme group, oxidative stress, heavy metals, inflammatory cytokines, NO, hypoxia and growth

factors [16]. All of these characteristics explain the growing interest in investigating the cardiovascular protective effects of HO-1.

HO-1 induction inhibited the progression of atherosclerotic lesions and reversed the plaque morphology and composition into a more stable phenotype in a rabbit model of atherosclerosis [17]. Interestingly, HO-1 upregulation improved H_2O_2-induced senescence in human umbilical vein endothelial cells (HUVECs) and enhanced the interaction between HO-1 and endothelial nitric oxide synthase (eNOS), resulting in of NO production [18].

There is also evidence that miRNAs can modulate NRF2/HO-1 pathway [19,20]. Ungvari and contributors [21] reported the presence of a ARE consensus sequence in the 5′ flanking region of Dicer (ribonuclease III), a key enzyme of the miRNAs biogenesis machinery [22]; a downregulation of Dicer1 expression in primary cerebromicrovascular endothelial cells (CMVECs) isolated from aged rats was associated with oxidative stress and with alterations in miRNA expression profile, including miRNA-let-7 family, while overexpression of Dicer1 restored miRNA expression and improved angiogenic processes [21].

Despite intense research regarding HO-1 in cardiovascular diseases, until now, no studies showed the effect of vegetarian diet on the modulation of NRF2/HO-1 plasmatic and neither in endothelial cells. Incubation of cells with plasma/serum is a well-established *in vitro* model and has been used to evaluate the effects of caloric restriction diet in several types of cells [23–25], and recently of vegetarian diet in cardiomyoblast cell [26].

In this regard, our objectives were to verify plasmatic concentration of HO-1 and investigate the effect of plasma incubation from omnivorous and vegetarians in endothelial cells on modulating of antioxidant defenses and vasodilator factors, including NRF2, HO-1, GSR, glutathione (GSH), and NO. 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

Subjects and biochemical measurements

Schematic diagram of the study is shown in **Figure 1**. In this observational cross-sectional CARVOS (Carotid atherosclerosis and arterial stiffness in vegetarians and omnivorous subjects) Study**,** a total of 745 subjects were recruited in São Paulo, Brazil between June 2013 and January 2014 through social activities and internet. We selected two groups of subjects according to the dietary patterns: vegetarians and omnivorous. The vegetarians subjects were defined as who practiced a diet including food of plant origin, egg, milk, and dairy, but exclude all types of meat for at least 4 years (lacto-ovo-vegetarians, lacto-vegetarians or vegans). Omnivorous were considered as consuming any type of meat at least five or more servings per week.

From 745 recruited, 416 volunteers were women, which were excluded because we would like to exclude the hormonal effects of clinical and biochemical parameters. Exclusion criteria were: history of diabetes, history of dyslipidemia, history of cardiovascular or cerebrovascular diseases, history of hypertension or intake of antihypertensive medication, and smoking. Next, it excluded vegetarian less than 3 years and omnivorous who eat meat less than four times per week. After applying inclusion and exclusion criteria, 241 men were excluded and 44 omnivorous and 44 vegetarian apparently healthy men were recruited in the current study. We included only apparently healthy individuals to reduce any interference with biomarkers.

For *in vitro* study, a pool of plasma from omnivorous and vegetarians were used (N= 10/group). Inclusion criteria for these assays were individuals ≥ 35 ≤ 52 years old and BMI ≥18 <29 kg/m^2.

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), after a 10–12 hours fasting, immediately centrifuged and 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 subjects gave written informed consent.

Carotid intima-media thickness (IMT) assessment

To measure carotid IMT, the functional and anatomical properties of the right carotid artery, evaluated as carotid intima-media thickness (IMT), were assessed using an ultrasound device consisting of a vessel wall echo-tracking system (Wall-Track System, PieMedical, Maastricht, The Netherlands), as described by Acosta-Navarro [6]. All subjects were submitted to B-mode conventional vascular ultrasound of the extracranial carotid artery. The right common carotid artery was obtained 2cm below the carotid bifurcation, and the following measurements were taken: IMT and diameter; beat-to-beat carotid systolic-diastolic variation; and percentage of that systolic-diastolic variation considered the relative distensibility [27].

Pulse wave velocity (PWV) measurements

PWV measurements was performed as described by Acosta-Navarro [6]. In brief, described by common carotid artery and femoral artery PWV were noninvasively recorded by using a pressure sensitive transducer. The distance between the recording sites (D) was measured, and PWV was automatically calculated as PWV = D/t, whereas (t) means pulse transit time. Measurements were repeated during 10 different cardiac cycles, and the mean was used for the final analysis. All procedures were performed according to the expert consensus document on arterial stiffness [28].

Cell culture and plasma-pool incubation

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

Measurement of HO-1 concentrations

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.

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®).

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 [29]. 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 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)) [30]. 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.

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®).

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 2 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).

Measurement of nitric oxide (NO) level

The production of intracellular NO was assessed using fluorescent dye DAF-2 diacetate (Cayman, Michigan, EUA). After plasma incubation, the cells were loaded with 5 µM of DAF-2 diacetate for 30 minutes and rinsed with PBS. Then, 200 uL of PBS was added and incubated for 15 minutes. The fluorescence intensity was measured in a microplate reader (Synergy 4, BioTek®) at 485-520 nm.

Measurement of nitrite

NO production was also evaluated indirectly through the quantification of nitrite in the cell supernatants by Griess method [31]. 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.

Statistical Analyses

Analysis of variance (ANOVA) and the Bonferroni's Multiple Comparison Test were used to compare study variables between 3 or more groups. For comparison between the two groups, *t*-tests were performed. Pearson's (or Spearman´s) 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. Important variables in cardiovascular disease conditions (such as age, BMI, SBP, HbA1c, total cholesterol, HDL, and hsPCR were included as independent variables in the multiple linear regression analysis. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and MedCalc Statistical Software version 19.0.5 (MedCalc Software bvba, Ostend, Belgium), respectively. Statistical significance was defined as $P<0.05$.

RESULTS

Main characteristics of the study subjects omnivorous (N= 44) and vegetarians (N= 44) are presented in **Table 1.** Higher values of weight, BMI, SBP, DBP, non-HDL, LDL-c, triglycerides, ApoB, glucose, HbA1c, IMT, PWV was found in omnivorous compared to vegetarians, with statistical significance differences.

First, HO-1 was measured in plasma from subjects. HO-1 concentration was significantly higher in omnivorous compared to vegetarians (**Figure 2**). Furthermore, we correlated plasma HO-1 levels with clinical parameters **(Table 2)**. HO-1 was positively associated with HDL for all participants and for omnivorous group. Strong negative correlations were found between HO-1 and BMI/hsCRP/ only in the omnivorous group.

Next, we performed multivariate analyses with combined data (**Table 3**). For all participants (omnivorous + vegetarians), only the association between HO-1 and HDL remain significantly different. While for omnivorous group, there was no longer an association between HO-1 and BMI/HDL/hsCRP after including age, BMI, SBP, HbA1c, total cholesterol, HDL, and hsPCR as independent variables in multivariate analysis.

The characteristics of subjects included *in vitro* assays are shown in **Table S1**. No difference was observed in the parameters evaluated between omnivorous and vegetarian group included *in vitro* assays. HO-1 level was evaluated in the cell supernatant from HUVECs incubated of plasma from omnivorous and vegetarian group. HO-1 concentration was significantly higher in omnivorous compared to vegetarian group (**Figure 3A**). In addition, the total GSH was measured in cell lysates. No significant differences in the total GSH (**Figure 3B**) among groups were found. Also, gene expression of *NFE2L2*, *HMOX1*, and *GSR* in HUVECs was verified (**Figure 4**). HUVECs incubated with plasma from omnivorous increased *NFE2L2*, *HMOX1*, *GSR* expression compared to vegetarian group. Additionally, we evaluated miRNAs expression of the let-7 family (let-7a, -b, and –c) (**Figure S1 A-C**), which regulates genes associated with NRF2 pathway. There was no significant differences between the groups in miRNAs expression.

To examine the NRF2 pathway, NRF2 expression in the nucleus (**Figure 5A**) and ARE activity (**Figure 5B**) were assessed. Plasma of omnivorous group increased NRF2 expression and activated ARE compared to control group (without plasma incubation).

Plasma of vegetarians resulted in the ARE activating when compared to control and this activating was significantly lower in relation to omnivorous group.

Moreover, the NO and nitrite production were evaluated. Plasma from omnivorous increased intracellular NO (**Figure 6A**) and nitrite levels (**Figure 6B**) compared to vegetarian group. To evaluate the relationship between HO-1 and NO, HO-1 activity was inhibited using ZnPP. Interestingly, nitrite concentration was decreased by ZnPP treatment only in omnivorous group.

DISCUSSION

In the present study, we demonstrate higher circulating HO-1 production in healthy omnivorous compared to vegetarian men. Also, we show for the first time that plasma collected from omnivorous was able to increase the gene/protein *HO-1* expression, *GSR* and *NFE2L2* gene expression, as well as NO in endothelial cells culture compared to vegetarian group. Moreover, we found an increase of NRF2 binding in the omnivorous group, and ARE activity was significantly higher when compared with vegetarian group, indicating that HO-1 was activated by NRF2 through the binding to ARE. Since HO-1 can be induced by several physiological and pathological stimuli [16], we suggest that HO-1 induction in omnivorous may indicate a higher pro-oxidative status compared to vegetarian group.

Evidence indicates that a healthy vegetarian diet provides benefits in preventing and reversing atherosclerosis and in decreasing CVD risk factors [3,4,32]. As expected, we found higher values of weight, BMI, SBP, DBP, non-HDL, LDL-c, triglycerides, ApoB, glucose, HbA1c, IMT, PWV in omnivorous compared to vegetarians, corroborating with literature findings [5,33]. The benefits of vegetarian dietary patterns on cardiovascular health are the result of lower exposure to harmful substances contained in animal products, such as saturated fat, cholesterol, heme iron, and greater consumption of fruits and vegetables, which is rich in fiber, antioxidant content and phytonutrients [8,32]. Compared with omnivorous, people following a vegetarian/vegan diet have higher levels of antioxidants such as carotenoids, ascorbic acid, and beta-carotene [26,34,35].

To the best of our knowledge about vegetarian diet, this is the first study to show circulating HO-1 levels in healthy vegetarian individuals. HO-1 catalyzes heme-producing carbon monoxide (CO) and biliverdin, the latter being subsequently converted in bilirubin [12]. Physiologically, HO-1 induction may be a beneficial or adaptive response to several stimuli, presenting a protective role in several disorders [12]. HO-1 presence in plasma is unclear, although, it may be released into the plasma from smooth muscle cells, cardiomyocytes, leukocytes, monocytes/macrophages and/or endothelial cells, which were damaged by the effect of hypertension, oxidative stress and chronic inflammation [36]. We showed that circulating HO-1 level was higher in plasma from apparently healthy omnivorous compared to vegetarians. Interestingly, these results are similar to those reported in disease conditions [37,38]. Previous study conducted by our research group showed that plasma HO-1 concentration was increased in pregnant women who subsequently develop severe preeclampsia [37]. Plasma

HO-1 concentration was also elevated in individuals with newly-diagnosed type 2 diabetes mellitus compared to controls, and HO-1 was positively associated with plasma glucose concentrations [38]. Importantly, the individuals included in this study are clinically healthy, and yet, the omnivorous showed higher levels of HO-1 than vegetarian group.

Another possible explanation for this finding includes the dietary factors present in animal foods. At transcriptional level, HO-1 is induced primarily by its substrate, free heme [12]. About 40% of the iron from meat and fish is heme iron, which has high absorption and bioavailability [39]. Considering that HO-1 is responsible for the degradation of heme and release free iron [12], dietary factors present in the omnivorous diet may be contributing to the increase of HO-1 in this group. On the other hand, higher amounts of antioxidants observed in vegetarians [34,40] and the lower bioavailability of iron from plant foods [41], may explain the low HO-1 expression observed in vegetarian group.

The relationship of HO-1 with clinical parameters of omnivorous and vegetarians was also investigated. We found that HO-1 was positively associated with HDL both for all participants (omnivorous + vegetarians) and for omnivorous group. However, this relationship remains significant only for all participants after adjustments in the multiple regression analysis, including age, BMI, SBP, HbA1c, total cholesterol, HDL, and hsCRP as independent variables. The mechanism underlying the association between HO-1 and HDL in omnivorous is still unclear. However, studies have been performed with proteins associated with HDL particles, such as apolipoprotein A1 and with D-4F (an A-1 mimetic peptide) [42,43]. Kruger and contributors showed that D-4F induced HO-1 expression and HO activity, inhibited superoxide formation, preventing endothelial fragmentation and restoring the vascular reactivity in diabetic rats [42]. Further studies are needed to explore this relationship between HDL and HO-1.

Besides that, we investigated the effect of plasma incubation from omnivorous and vegetarians on NRF2/HO-1 pathway in endothelial cells. Previous studies focused on the effects of caloric restriction diet (in different types of cells) [23–25] and the vegetarian diet (in cardiomyoblast cells) [26], 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, to activate NRF2 gene/protein and ARE activity, indicating that HO-1 is regulated in part by NRF2. In addition, GSR gene expression was increased in HUVECs

incubated with plasma from omnivorous compared to vegetarian group, however total GSH level did not differ between groups. The redox regulatory mechanism in omnivorous may be due to HO-1 induction and not by glutathione. As observed by Poljsak and contributors[2], the low amount of antioxidants is not problematic, since ROS levels remain low. Under normal physiological conditions, the balance between pro-oxidant and antioxidant substances is kept slightly in favor of pro-oxidant, favoring a mild oxidative stress[44]. In this circumstance, ROS upregulates antioxidant defenses to maintain redox balance [45], promoting the control of vascular function and maintenance of vascular tone [46]. Thus, it is possible that circulatory factors present in omnivorous plasma may be inducing an oxidative environment in endothelial cells, and consequently stimulating antioxidant defense via NRF2/HO-1.

Furthermore, we showed that the NO markers were increased in HUVECs incubated with plasma from omnivorous compared to 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 cell that HO-1 upregulation ameliorates oxidative stress-induced senescence via regulating endothelial nitric oxide synthase (eNOS), resulting in an enhancement of eNOS phosphorylation and NO production [18].

There is also data indicating that microRNAs can modulate cellular redox homeostasis, and plays a central role in several physiological and pathophysiological processes [47]. Ungvari and contributors [21] reported the presence of ARE consensus sequence in the 5′ flanking region of Dicer (ribonuclease III), a key enzyme of the microRNA biogenesis machinery [22]; the authors demonstrated that overexpression of Dicer1 cerebromicrovascular endothelial cells (CMVECs) isolated from aged rats improved angiogenic processes and partially restored miRNA expression, including let-7b [21]. 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 to vegetarians group.

The limitations of this study include: 1) this cross-sectional study does not prove causality between the effects of different dietary types and HO-1 and neither of HO-1 and clinic parameters, however, the sample power calculations and the inclusion criteria strengthen our conclusions 2) relative restricted number of individuals included in each group; 3) we have not evaluated the mechanisms of the vegetarian diet involved in the biomarkers

studied; 4) 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, HO-1 induction in omnivorous may indicate a pro-oxidative status since HO-1 is activated under oxidative stress. Moreover, plasma from omnivorous appears to contain metabolic factors that lead to modifications in endothelia cells involving NRF2/HO-1 pathway and NO production. This *In vitro* model may represent a potential tool for exploring the effects of vegetarian diet on endothelial cells.

ABBREVIATIONS

ApoB: apolipoprotein; ARE: antioxidant response elements; BMI: body mass index; CAD: coronary artery disease; carbon monoxide (CO); CVDs: cardiovascular diseases; CRP: c-reactive protein; DBP: diastolic blood pressure; eNOS: endothelial nitric oxide synthase; GSH: reduced glutathione; GSR: glutathione reductase; GSSG: oxidized glutathione; HbA1c: Glycated Hemoglobin; HDL: high density lipoproteins; HMOX1: heme-oxygenase-1 gene; HO-1: heme-oxygenase-1; HUVECs: in human umbilical vein endothelial cells; IMT: intima-media thickness; KEAP-1: Kelch-like ECH-associated protein-1; LDL: low density lipoproteins; MicroRNAs: miRNAs; NFE2L2: Nuclear factor, erythroid 2-like 2 gene; NO: nitric oxide; non-HDL: non-high-density lipoprotein cholesterol; OMN: omnivorous; PWV – pulse wave velocity; SBP: systolic blood pressure; TC: total cholesterol; TG – triglycerids. VEG: vegetarian.

AUTHOR CONTRIBUTIONS

N.C. performed assays, analyzed data, interpreted results and drafted L.A. and J.A-C. acquired the data. V.S. conceived and designed the study. All authors revised the article and approved its final version.

COMPETING FINANCIAL INTERESTS:

The authors declare no competing financial interests.

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TABLES

Table 1. Clinical parameters of omnivorous and vegetarians subjects.

	Omnivorous (N=44)	**Vegetarians (N=44)**	***t*-test (*P*-value)**
Age (Years)	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 (mmHg)	129.20 ± 2.28	119.5 ± 1.57	0.0008***
DBP (mmHg)	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 (mg/dl)	45.45 ± 1.75	47.59 ± 1.39	0.343
Non-HDL (mg/dL)	157.30 ± 5.52	132.50 ± 6.52	0.004**
LDL (mg/dl)	128.50 ± 4.88	110.00 ± 5.00	0.009**
TG (mg/dl)	144.00 ± 9.64	122.20 ± 10.88	0.031*
ApoB (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
hsCRP (mg/dL)	12.89	1.73	0.08
IMT (mm)	661.40 ± 19.33	593.40 ± 14.13	0.005**
PWV (m/s)	7.70 ± 0.13	7.09 ± 0.11	0.0008***

Data presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to omnivorous group.
Abbreviations: BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; HDL-c – high density lipoproteins; LDL – low density lipoproteins; non-HDL - 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.

Table 2. Correlation between plasmatic HO-1 concentrations *versus* clinical parameters.

HO-1 *vs.*	**All participants**		**Omnivorous**		**Vegetarians**	
	r	*P*-value	*r*	*P*-value	*r*	*P*-value
Age (Years)	-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 (mmHg)	0.08	0.46	-0.26	0.09	0.03	0.85
DBP (mmHg)	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 (mg/dl)	0.25	0.01*	0.35	0.02*	0.25	0.10
Non-HDL (mg/dL)	-0.03	0.73	-0.01	0.90	-0.05	0.75
LDL (mg/dl)	-0.01	0.91	0.01	0.94	-0.00	0.98
TG (mg/dl)	-0.07	0.52	-0.08	0.58	-0.15	0.34
ApoB (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
hsCRP (mg/dL)	-0.10	0.36	-0.40	0.00**	-0.03	0.84
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

Significant at *$P<0.05$, **$P<0.01$.
Abbreviations: BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; TC - total cholesterol**;** HDL – high density lipoproteins; LDL – low density lipoproteins; non-HDL - 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.

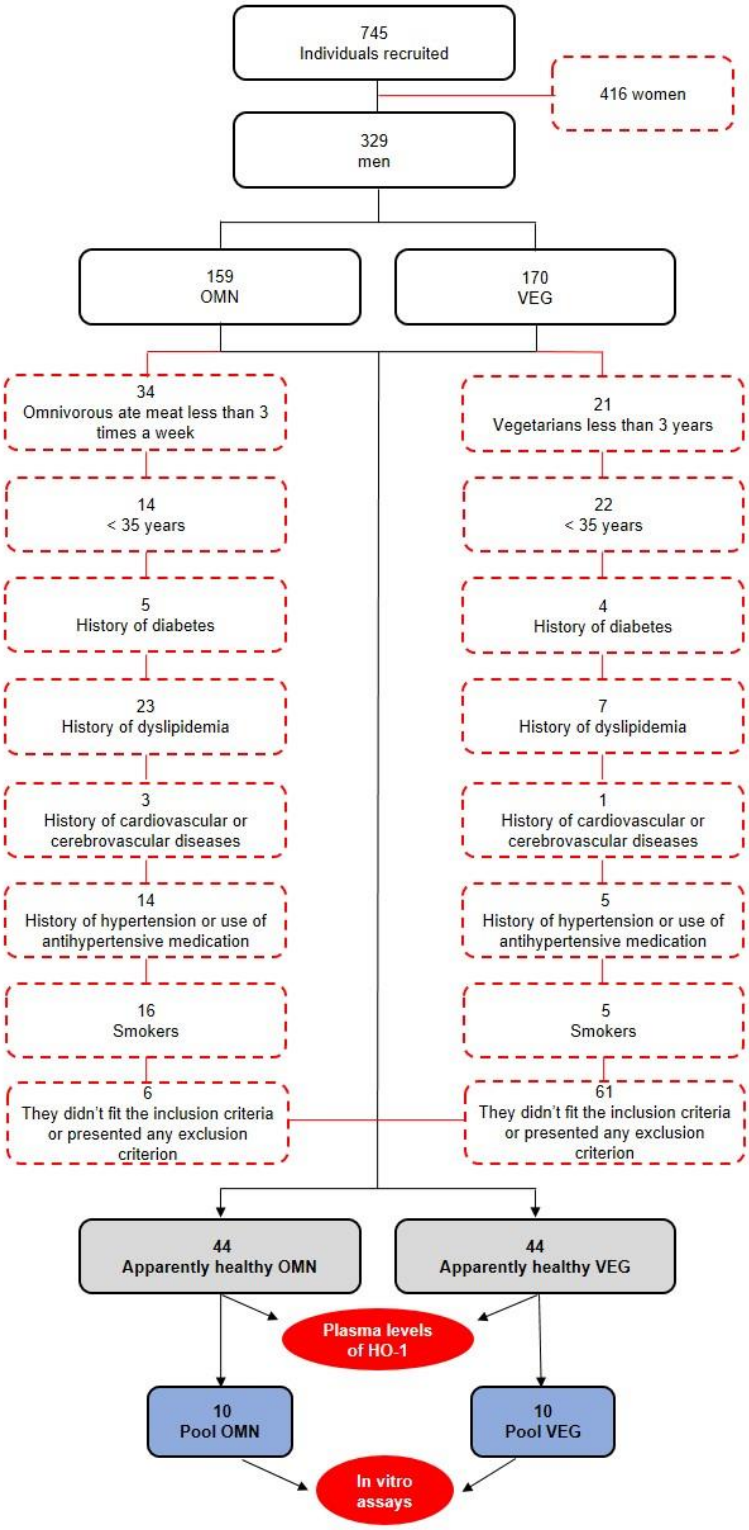
Table 3. **Multivariate relationships.**

HO-1 *vs.*	**All participants**		**Omnivorous**		**Vegetarians**	
	β	*p*-value	β	*p*-value	β	*p*-value
Age (Years)	0.89	0.89	6.94	0.39	4.75	0.43
BMI (kg/m^2)	19.52	0.23	3.88	0.83	2.49	0.89
SBP (mmHG)	2.92	0.51	-4.80	0.35	5.28	0.25
HbA1c (%)	-202.41	0.21	132.11	0.51	-221.29	0.22
TC (mg/dl)	-0.34	0.82	1.55	0.47	-0.40	0.77
HDL (mg/dl)	14.43	0.00**	7.35	0.26	9.15	0.07
hsPCR (mg/dL)	-0.80	0.94	-62.71	0.07	9.07	0.76

Dependent variable: Plasmatic HO-1 concentration. Significant at ***P*<0.01.
Abbreviations: BMI – body mass index; SBP – systolic blood pressure; HbA1c - Glycated Hemoglobin; TC - total cholesterol; HDL – high density lipoproteins; hsCRP – high sensitivity c-reactive protein. β- standardized regression coefficients, obtained from multiple linear regression analysis regression models.

FIGURES

Figure 1. Diagram of the study workflow



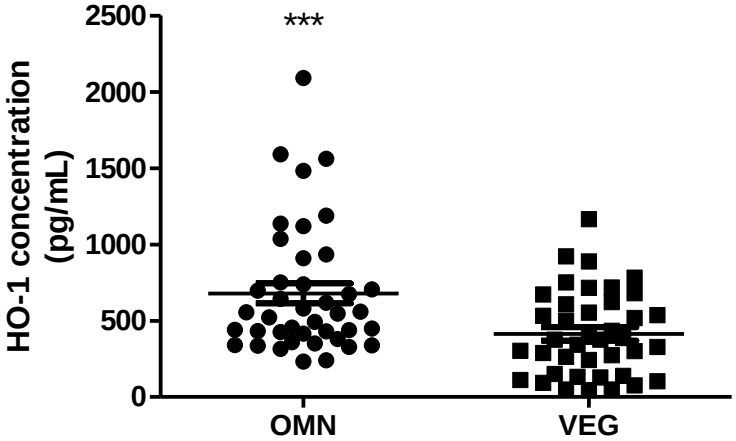


Figure 2. HO-1 level in plasma from omnivorous and vegetarians (A). Data presented as mean ± SEM. ****P*<0.0001 compared to VEG group (Mann Whitney test, GraphPad Prism software).

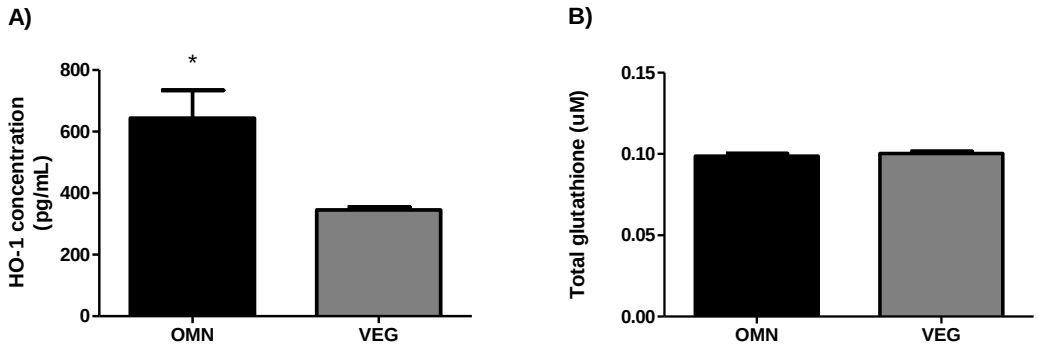


Figure 3. HO-1 concentration (A) and total GSH (B). HUVECs were incubated with 10% (v/v) plasma samples from omnivorous (OMN) and vegetarians (VEG) for 24 hrs. HO-1 level was measured in the cell supernatant. GSH level was evaluated in cell lysates Data presented as mean ± SEM. **P*<0.05 vs. VEG group (Unpaired *t*-test, GraphPad Prism software).

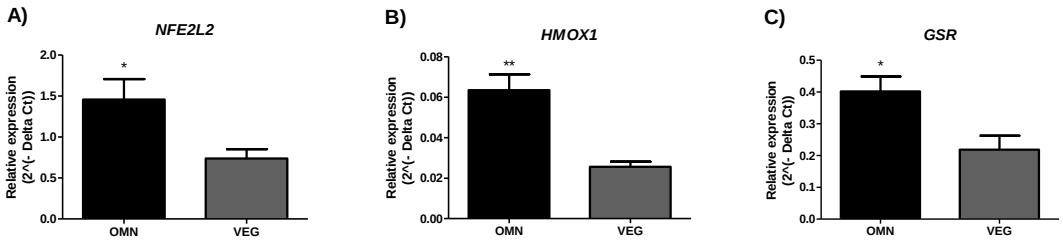


Figure 4. Relative expression of the genes *NFE2L2* (A), *HMOX1* (B), and *GSR* (C) in HUVECs. Endothelial cells were incubated with 10% (v/v) plasma samples from omnivorous (OMN) and vegetarians (VEG) for 24 hrs. Data presented as mean ± SEM. **P<0.05* and ***P<0.01* vs. VEG group. (Unpaired *t*-test, GraphPad Prism software).

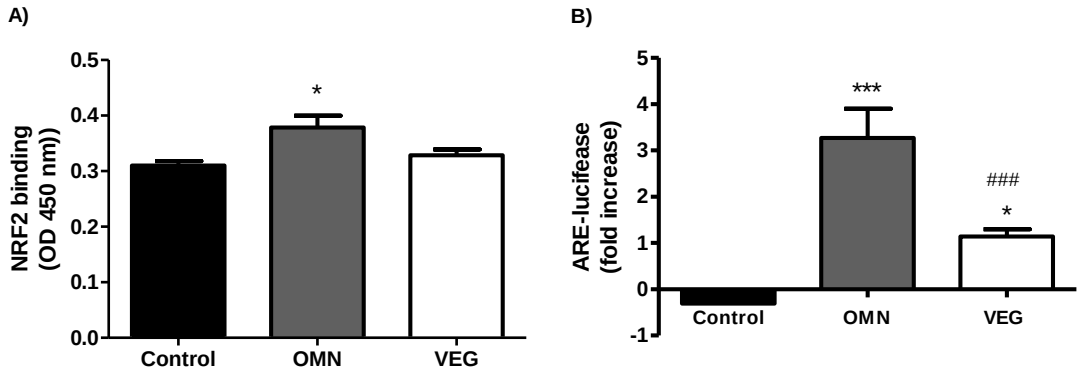


Figure 5. NRF2 expression in the nucleus (A) and ARE activity (B). HUVECs were incubated with 10% (v/v) plasma from omnivorous (OMN) and vegetarians (VEG) for 24 hrs. Data presented as mean ± SEM. *P<0.05 and ***P<0.0001 vs. control group. $^{###}P$<0.0001 vs. OMN group (ANOVA, Bonferroni's Multiple Comparison Test, GraphPad Prism software).

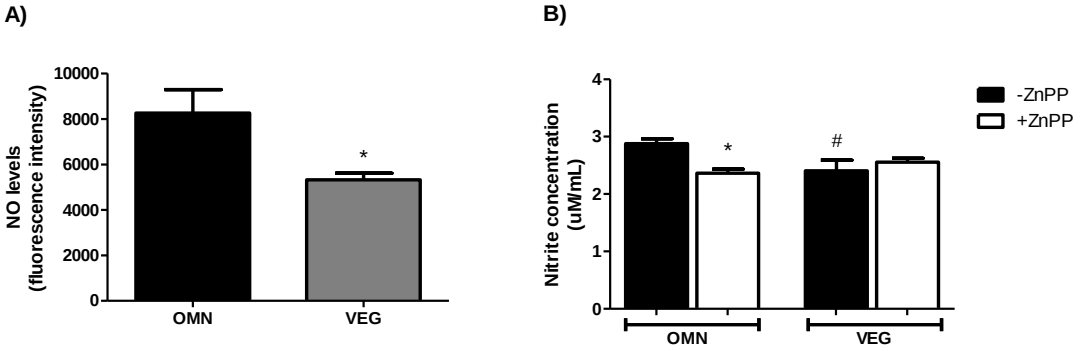


Figure 6. Intracellular NO levels (A) and nitrite concentration in the cell supernatant (B). HUVECs were incubated with 10% (v/v) plasma samples from omnivorous (OMN) and vegetarians (VEG) for 24 hrs. Nitrite concentration was evaluated in the absence (-ZnPP) or presence of ZnPP (+ZnPP) at 1 μM. DMSO was used as a vehicle for ZnPP. Data presented as mean ± SEM. *P<0.05 OMN –ZnPP vs. OMN +ZnPP. $^{\#}P$<0.05 vs. OMN –ZnPP group (ANOVA, Bonferroni's Multiple Comparison Test, GraphPad Prism software).

SUPPLEMENTARY TABLE

Table S1. Clinical parameters of omnivorous and vegetarians subjects included in vitro assays.

	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 (mg/dl)	47.60 ± 5.47	48.60 ± 3.43	0.87
Non-HDL (mg/dL)	142.80 ± 14.95	138.80 ± 13.47	0.84
LDL (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)	1.96 ± 0,75	1.44 ± 0.60	0.38
IMT (mm)	623.50 ± 31.66	596.50 ± 24.01	0.50
PWV (m/s)	7.45 ± 0.17	6.81 ± 0.25	0.05

Data presented as mean ± SEM. There was no statistical difference between groups (All data $P>0.05$).
Abbreviations: BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; HDL-c – high density lipoproteins; LDL – low density lipoproteins; non-HDL - non-high-density lipoprotein cholesterol; TG – triglycerides; ApoB – apolipoprotein; CRP – high sensitivity c-reactive protein; HbA1c - Glycated Hemoglobin; IMT - intima-media thickness; PWV – pulse wave velocity.

SUPPLEMENTARY FIGURE

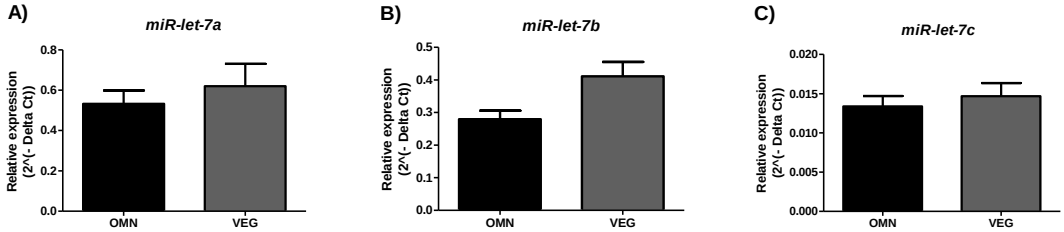


Figure S2. Relative expression of the miRNAs *miR-let-7a* (A), *miR-let-7b* (B), and *miR-let-7c* (C) in HUVECs. Endothelial cells were incubated with 10% (v/v) plasma samples from omnivorous (OMN) and vegetarians (VEG) for 24 hrs. Data presented as mean ± SEM. There was no statistical difference between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

4. MANUSCRITO II

Shortening telomere is associated with subclinical atherosclerosis biomarker in omnivorous but not in vegetarian healthy men

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Keywords: vegetarian, telomere length, carotid intima-media thickness, cardiovascular biomarkers.

ABSTRACT

Telomere length is considered to be a biomarker of biological aging and age-related disease. There are few studies that have evaluated the association between telomere length and diet, and none of them have evaluated the impact of a vegetarian diet on telomere length and its correlation with cardiovascular biomarkers in apparently healthy subjects. Therefore, our objectives were to evaluate leukocyte telomere length (LTL) in vegetarians and omnivorous subjects and its association with classical cardiovascular risk biomarkers. From the total of 745 participants initially recruited, 44 omnivorous and 44 vegetarian men apparently healthy were selected for this study and LTL was measured in 39 omnivorous and 41 vegetarians by Real-Time Quantitative PCR reaction. Although telomere length was not different between omnivorous and vegetarians, we found a strong negative correlation between LTL and IMT (intima-media thickness) in omnivorous, but not in vegetarian group. In addition, omnivorous who were classified with short telomere length had higher carotid IMT compared to vegetarians. Our data suggest that telomere length can be a marker of subclinical atherosclerosis in the omnivorous group.

INTRODUCTION

Telomeres are structures of TTAGGG nucleotides repeats at the ends of chromosomes; they prevent the ends of chromosomes from fusing with other chromosomes and from being recognized by proteins of DNA repair as a double-strand break, providing genomic stability and preservation [1].

Because telomere sequences do not fully replicate during DNA replication, they become progressively shorter with each replication of somatic cells [2]. Thus, telomere length is considered a biomarker of biological aging [3] with growing interest in cardiovascular diseases (CVDs) [4–8]. A prospective longitudinal study (average 5.5-yr follow-up) showed that the shortening of telomere length appears to have a predictive value in the incidence and progression of carotid atherosclerosis [4]. Further studies have shown shorter telomere length in subjects with CVDs like atherosclerosis [5], hypertension [6,7], and coronary heart disease [8]. Evidence indicates that, beyond aging, increase of inflammation and oxidative stress accelerates telomere attrition, possibly explaining the association observed between telomere length and CVDs [9,10].

Interestingly, both telomere length and cardiovascular risk can be modified by diet [11–14]. A healthy diet containing high consumption of fruits, vegetables, and whole grains is anti-inflammatory and antioxidative, besides lower severe oxidative stress and DNA damage [15,16]. Lian and collaborators [12] have shown that longer LTL was associated with lower risk of hypertension only in those who consume higher levels of vegetables. In addition, greater adherence to Mediterranean diet was associated with longer telomeres, however, none of the individual dietary components showed an association with telomere length, suggesting that this association may be a consequence of the global effect of this diet.

On the other hand, the high consumption of red/processed meat leads to increased oxidative stress [17–19], which can induce DNA damage and may affect telomere length [20,21]. In this context, a balanced vegetarian diet (which exclude all kinds of meat) would help protect the chromosome ends.

The vegetarian diet is characterized by a high intake of legumes, vegetables, fruits, grains, nuts, seeds, and may or may not include eggs and dairy (milk and milk products) [22]. In general, the vegetarian diet is rich in fibers, vitamin C and E, folic acid, magnesium, phytochemicals, and antioxidants but low in total fat, saturated fatty acid, cholesterol,

compared to omnivorous diet [23]. These factors are known to reduce the risk of CVDs [22], and several studies report that vegetarians have a lower cardiovascular risk in comparison with omnivorous subjects [11,24,25]. These findings are in agreement with the typical aspects observed in vegetarian subjects, including lower systolic and diastolic blood pressure, better insulin sensitivity and blood lipids profile [26,27].

Epidemiologic studies have been investigated the telomere length in CVDs, as well as their relationship with diet [8,12,14], but until now, no studies in healthy vegetarian subjects have been done. Given that healthy vegetarian diet is rich in fruits and vegetables, we hypothesized that vegetarian diet can prevent or delay telomere shortening and it can be correlated with lower probability of developing age-related disease, such as CVDs. Therefore, our objectives were to evaluate telomere length in vegetarians and omnivorous subjects and its association with classical cardiovascular risk biomarkers.

RESULTS

Main characteristics of the study subjects omnivorous (n= 39) and vegetarians (n= 41) are given in Table 1. Higher values of BMI, SBP, DBP, TC, LDL, non-HDL, ApoB, HbA1c, fasting glucose, and IMT were found in omnivorous compared to vegetarians. However, no difference was observed in telomere length between omnivorous and vegetarians (Table 1).

Table 2 shows the correlation of telomere length versus clinical and biochemical parameters. For all the participants, LTL was inversely correlated with SBP, DBP, TC, LDL, and Non-HDL. When we divided the subjects by diet pattern, we found a strong negative correlation between LTL and IMT only in the omnivorous group. In addition, LTL was negatively correlated with TC and non-HDL in vegetarian subjects.

Multivariate relationships between LTL and important variables in CVDs are shown in Table 3. For all participants (omnivorous + vegetarians), only the association between LTL and TC remain significantly different. In omnivorous group, the inverse relationship between LTL and IMT was kept. While for vegetarians, there was no longer an association between LTL and the parameters TC, and non-HDL, after including age, BMI, SBP, TC, HbA1c and IMT as independent variables in multivariate analysis.

Based on these findings, we compared the carotid IMT measurements in subjects with short and long LTL (Figure 1). Interestingly, omnivorous who were classified with short telomere length had higher carotid IMT compared to vegetarians (581.1 mm ± 23.02 *vs.* 693.3 ± 31.04 mm, P= 0.019).

DISCUSSION

This is the first study that investigated leukocyte telomere length and its association with cardiovascular risk in healthy vegetarian men. There was no significant difference in telomere length between apparently healthy omnivorous and vegetarian men. The main finding of this observational cross-sectional study was the negative association between IMT and telomere length in omnivorous, but not in the vegetarian group. Interestingly, among individuals with short telomere length, a greater IMT rate was observed in omnivorous compared to vegetarians. In vegetarians, lower TC and HbA1c was first correlated with short LTL, however, after adjustments in the multiple regression analysis, this relationship did not remain significant.

Only one study in the literature reported information about LTL in vegetarians [8]. However, this study was addressed to verify LTL in Indian individuals with coronary artery disease (CAD) and the authors only reported that there was a trend towards longer telomeres in vegetarian subjects compared to those who had a mixed diet, but this difference was not statistically significant [8].

Previous studies have shown that shortened LTL can be a powerful marker of increased carotid artery intima medial thickness (IMT) [28,29]. IMT is a non-invasive image method for evaluating modification of functional and structure of the vascular wall, which is important in the development of cardiovascular diseases, such as atherosclerosis [30]. The Framingham Heart Study including 1062 individuals, observed an inverse association of LTL with IMT in obese men, but not in non-obese [28]. In agreement with our finding in omnivorous group, a prospective longitudinal study (over 6 year of follow-up), showed that the shortening of telomere length was associated with increased incidence of subclinical carotid vascular damage (assessed by IMT) in the general population, specifically in men; moreover, subjects with LTL shortened over time had a higher risk of incident cardiovascular events, compared to those in whom LTL lengthened [29]. Another study also suggests that besides being an aging marker and of atherosclerosis, LTL may be a determining factor in arterial aging, perhaps because short telomeres might decrease replicative potential and compromise vascular repair, contributing to the atherosclerotic process [2].

Previously, a cross-section study including healthy individuals over the age of 50 years showed that vascular dilatory functions in vegetarians were better than those

omnivorous, and that these effects were dependent of diet and independent from others factors of atherosclerosis, such as smoking, diabetes, hypertension, hyperlipidemia and aging [31]. Also, the authors reported that degree vasodilatation responses were correlated with the years of a vegetarian diet, suggesting that vegetarian diet have a direct effect on vascular endothelial [31]. The mechanism underlying the association between LTL and IMT in omnivorous subjects is still unclear. However, we founded increased BMI, SBP, DBP, TC, LDL, non-HDL, ApoB, HbA1c, fasting glucose, and IMT in omnivorous compared to vegetarians. Consistent with our findings, previous studies have shown that omnivorous present higher BMI, SBP, DBP and worse insulin sensitivity and blood lipids profile, compared to vegetarian subjects [26,32]. Besides that, emerging evidence associates shorter LTL with disease risk factors such as altered lipidic profile [33], development of insulin resistance [34], increased blood pressure [35] and carotid IMT [28,29], suggesting a potential role of telomeres in the development of age-related diseases, including CVDs. Thus, it is possible that metabolic consequences identified in omnivorous compared to vegetarian cause changes in the process related to atherosclerosis and telomere shortening. Another possible explanation for this finding includes increased oxidative stress, resulting from the excessive production of reactive oxygen stress (ROS) and/or the failure of the antioxidant defense mechanisms [36,37]. Due to their high content of guanines, telomeric DNA is sensitive to damage by oxidation, and ROS, including hydroxyl radicals, produce single-strand breaks [37]. In contrast, some dietary factors such as antioxidants, vitamin intake and healthy lifestyle were reported to decrease rates of LTL shortening [10]. These factors can improve inflammation, lower ROS, reduce DNA damage, and increase telomerase activity [15,16,38]. On the other hand, the high consumption of red meat and processed meat lead to increased oxidative stress [17–19], which can induce DNA damage and may have an impact on the telomere length [20,21]. Given that vegetarian subjects have higher plasma levels of antioxidants (ex. carotenoids, ascorbic acid, and beta-carotene) [39,40], a balanced vegetarian diet could be protective against vascular aging.

The limitations of this study include: 1) this cross-sectional study does not prove causality between the effects of different dietary types and LTL and neither of LTL and IMT, however, the sample power calculation and the inclusion criteria strengthen our conclusions 2) relative restricted number of individuals included in each group; 3) we have not evaluated the mechanisms of the vegetarian diet involved in the biomarkers studied.

CONCLUSION

Despite there is no significant difference in telomere length, the contribution of LTL in relationship carotid IMT was observed in omnivorous but not in vegetarians, even in apparently healthy individuals. Our data suggest that telomere length can be a marker of subclinical atherosclerosis in the omnivorous group. Further studies are warranted to confirm the impact of the vegetarian diet on modifications of telomere length and its protective role of vascular aging.

MATERIALS AND METHODS

Subjects and biochemical measurements

Experimental design and samples are shown in Figure S1. In this observational study, we included only apparently healthy individuals, who were vegetarians at least 4 years (lacto-ovo-vegetarians, lacto-vegetarians or vegans) and omnivorous who consumed any type of meat at least five or more servings per week. From 745 recruited individuals, 416 volunteers were women and were excluded because some parameters could be influenced by differences related to phases of the menstrual cycle. After applying inclusion and exclusion criterions, 241 men were excluded and 44 omnivorous and 44 vegetarian apparently healthy men who participated of the final phase of CARVOS Study (Carotid Atherosclerosis, Stiffness Aortic and Risk Factors in Vegetarians and Omnivorous Subjects) were recruited to the study. To telomere assay, DNA samples from eight subjects were not available. Then the analysis was performed to 39 omnivorous and 41 vegetarians. The volunteer men filled out questionnaires regarding past medical history, dietary preferences, family history, physical activity, educational level, and personal data.

We excluded subjects with a history of diabetes, history of dyslipidemia, history of cardiovascular or cerebrovascular diseases, history of hypertension or intake of antihypertensive medication, smoking and age < 35 years. It also excluded vegetarian less than 3 years and omnivorous who eat meat less than four times per week or didn't fit the inclusion criteria.

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 subjects gave written informed consent.

The characteristics recorded for each participant included age, body mass index (BMI), height, systolic blood pressure (SBP), and diastolic blood pressure (DBP).

Blood samples were collected in tube containing ethylenediaminetetraacetic acid (EDTA), after a 10–12 hours fasting, immediately centrifuged and stored at −80 °C until used to measure laboratory analysis, which included total cholesterol (TC); high density lipoproteins (HDL), low density lipoproteins (LDL); non-high-density lipoprotein cholesterol

(non-HDL); triglycerides (TG); apolipoprotein (ApoB); C-reactive protein (CRP), Glycated Hemoglobin (HbA1c), and fasting glucose.

Carotid IMT assessment

As described by Acosta-Navarro [11], in brief, the functional and anatomical properties of the right carotid artery, evaluated as carotid intima-media thickness (IMT), were assessed using an ultrasound device consisting of a vessel wall echo-tracking system (Wall-Track System, PieMedical, Maastricht, The Netherlands). IMT method was performed in the Heart Institute (InCor), a reference hospital from Sao Paulo, being evaluated by a trained professional doing registered calculations. This method provides a reproducible and reliable measurement suitable for routine practice [41] and has been validated and used in other clinical studies [42–44].

All subjects were submitted to B-mode conventional vascular ultrasound of the extracranial carotid artery. The right common carotid artery was obtained 2cm below the carotid bifurcation, and the following measurements were taken: IMT and diameter; beat-to-beat carotid systolic-diastolic variation; and percentage of that systolic-diastolic variation considered the relative distensibility [42].

Relative telomere Length

Peripheral blood samples were collected in tubes containing EDTA, followed by DNA extraction with a high salt method (Lahiri and Schnabel, 1993). DNA was quantified using a NanoDrop Spectrophotometer Thermo Scientific, Nanodrop 200 model, and diluted to 75 ng in 96 well plates. Relative quantification method, described by Cawthon, was used to measure telomere length (Cawthon, 2002). The telomere reaction proceed for one cycle at 95°C for 10 min, followed by 18 cycles at 95°C for 15 s and 54°C for 2 min and primers used were Tel-1 primer (GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T) and Tel-2 primer (TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA). The 36B4 reaction proceeded for one cycle at 95°C for 10 min, followed by 30 cycles at 95°C for 15 s and 58°C for 1 min 10 s and primers used were 36B4u (CAG CAA GTG GGA AGG TGT AAT CC), 36B4d (CCC ATT CTA TCA TCA ACG GGT ACA A). Each reaction was

performed in triplicate for each sample and results were averaged in further calculations. For PCR reactions, PlatinumTaq (Invitrogen) was used and amplicon formation was monitored using SYBR-Green fluorescent dye (Invitrogen). All PCR reactions and fluorescence measures were carried out in an ABI-7500 real-time PCR machine (ABI). For telomere length quantification, the cycle threshold (Ct) for each telomere and control gene 36B4 PCR reaction were calculated using the ABI software algorithm. The telomere/control gene 36B4 (T/C) ratio reflects the relative size of telomere for each sample. Considering the exponential kinetics of the PCR reaction, this ratio may be expressed as the following equation: $2^{-\Delta Ct}$, where $-\Delta Ct$=-(Ct telomere - Ct control gene 36B4) of sample n. For group comparisons, $2^{-\Delta Ct}$ values for each sample were grouped and analyzed together.

Statistical analysis

Unpaired Student's t-test was used to compare telomere length between vegetarians and omnivorous. Pearson's (or Spearman´s) correlation coefficients were used to determine the relationship between telomere length and the parameters clinical, and biochemical. Important variables in cardiovascular disease conditions (such as age, BMI, SBP, HbA1c, TC, and IMT) were included as independent variables in the multiple linear regression analysis. Another analysis was performed between the groups, comparing IMT according to telomere length. Subjects telomere length was categorized by long and short telomeres presence, considering median (103.6) as a cut point. Subjects with telomere length measure lower than cut point were included in the short telomere subgroup and those equal or higher than cut point were included in the long telomere subgroup. ANOVA, Kruskal-Wallis test was used to evaluate IMT level in relation to short and long telomere length between the groups. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and Stata version 10.0. P values <0.05 were considered statistically significant.

Abbreviations

ApoB: apolipoprotein BMI: body mass index, CAD: coronary artery disease, CVDs: cardiovascular diseases, CRP: c-reactive protein, DBP: diastolic blood pressure, HbA1c: Glycated Hemoglobin, HDL: high density lipoproteins, IMT: intima-media thickness, LDL: low density lipoproteins, LTL: leukocyte telomere length, non-HDL: non-high-density lipoprotein cholesterol, SBP: systolic blood pressure, TC: total cholesterol, TG – triglycerides.

AUTHOR CONTRIBUTIONS

N.C. drafted manuscript. N.C. and L.A. analyzed data and interpreted results of experiments, D.R. performed telomere assay. D.M. conceived the study, genotyping and data analysis. J.A-C. acquired the data. V.S. conceived and designed the study. All authors revised the article and approved its final version.

COMPETING FINANCIAL INTERESTS:

The authors declare no competing financial interests.

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FIGURE 1

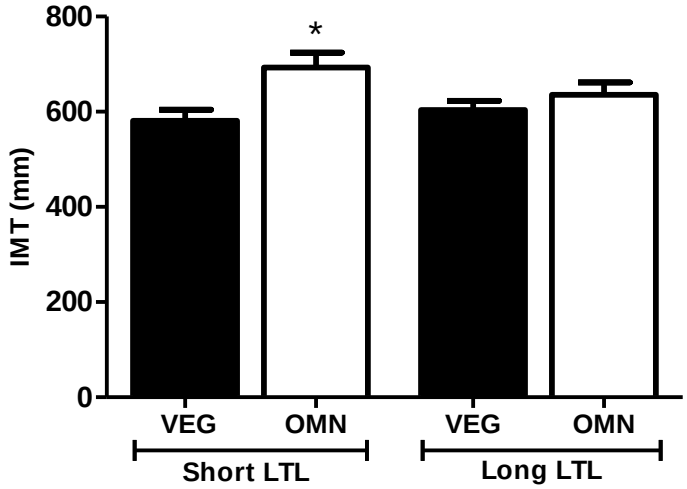

Figure 1. Carotid IMT level in relation to short and long telomere length. Data are presented as median and standard error. Omnivorous with short telomere length had higher carotid IMT compared to vegetarians. Significant at *$P<0.05$ compared to omnivorous with short LTL (*ANOVA, Kruskal-Wallis test, GraphPad Prism software*).

TABLES

Table 1. Clinical parameters of omnivorous and vegetarians subjects.

	Omnivorous (n=39) Mean ± SEM	**Vegetarian** (n=41) Mean ± SEM	***P*-value**
Age (Years)	47.00 ± 1.50	45.15 ± 1.23	0.341
BMI (kg/m^2)	27.67 ± 0.76	23.31 ± 0.46	< 0.00***
SBP (mmHg)	128.80 ± 2.44	119.80 ± 1.67	0.00**
DBP (mmHg)	83.51 ± 1.62	76.32 ± 1.31	0.00**
TC (mg/dl)	200.70 ± 5.58	181.60 ± 6.43	0.02*
HDL (mg/dl)	45.28 ± 1.80	47.88 ± 1.47	0.26
LDL (mg/dl)	127.10 ± 5.28	111.00 ± 5.29	0.03*
Non-HDL (mg/dl)	155.50 ± 5.82	133.90 ± 6.91	0.02*
TG (mg/dl)	141.80 ± 10.16	114.20 ± 11.62	0.07
ApoB (g/l)	1.00 ± 0.04	0.88 ± 0.04	0.04*
CRP (mg/L)	3.12 ± 1.06	1.80 ± 0.52	0.06
HbA1c (%)	5.56 ± 0.07	5.29 ± 0.04	0.00**
Fasting Glucose (mg/dl)	103.30 ± 2.16	94.88 ± 1.17	0.00***
IMT (mm)	668.20 ± 21.12	593.20 ± 14.81	0.00**
LTL (T/L ratio)	101.00 ± 5.63	111.20 ± 7.25	0.27

Abbreviations: BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; TC - total cholesterol; HDL – high density lipoproteins; LDL – low density lipoproteins; non-HDL - non-high-density lipoprotein cholesterol; TG – triglycerides; ApoB – apolipoprotein; CRP – c-reactive protein; HbA1c - Glycated Hemoglobin; IMT - intima-media thickness; LTL – Leukocyte telomere length.

*P<0.05, **P<0.01, ***P<0.001 compared to omnivorous group.

Table 2. Correlation between telomere length *versus* clinical and biochemical parameters.

LTL *vs.*	**All participants**		**Omnivorous**		**Vegetarians**	
	r	*P*-value	*r*	*P*-value	*r*	*P*-value
Age (Years)	-0.13	0.21	-0.26	0.10	-0.06	0.70
BMI (kg/m2)	-0.12	0.27	-0.09	0.57	-0.19	0.23
SBP (mmHG)	-0.24	0.02**	-0.28	0.08	-0.17	0.27
DBP (mmHG)	-0.25	0.02**	-0.31	0.05	-0.17	0.27
TC (mg/dl)	-0.31	0.00**	-0.26	0.10	-0.31	0.04*
HDL (mg/dl)	0.17	0.11	0.08	0.61	0.24	0.12
LDL (mg/dl)	-0.30	0.00**	-0.23	0.14	-0.26	0.09
Non-HDL (mg/dl)	-0.32	0.00**	-0.25	0.12	-0.34	0.02*
TG (mg/dl)	-0.19	0.08	-0.09	0.54	-0.20	0.19
ApoB (g/l)	-0.24	0.03*	-0.3	0.05	-0.16	0.29
CRP (mg/L)	0.00	0.94	-0.15	0.34	0.18	0.26
HbA1c (%)	-0.08	0.43	-0.13	0.42	0.05	0.75
Fasting Glucose (mg/dl)	-0.15	0.16	-0.09	0.58	-0.04	0.79
IMT (mm)	-0.27	0.01*	-0.38	0.01*	-0.02	0.85

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

Abbreviations: BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; TC - total cholesterol; HDL – high density lipoproteins; LDL – low density lipoproteins; non-HDL - non-high-density lipoprotein cholesterol; TG – triglycerides; ApoB – apolipoprotein; CRP – c-reactive protein; HbA1c - Glycated Hemoglobin; IMT - intima-media thickness.

Table 3. **Multivariate relationships.**

LTL *vs.*	**All participants**		**Omnivorous**		**Vegetarians**	
	β	*p*-value	β	*p*-value	β	*p*-value
IMT (mm)	-0.06	0.17	-0.16	0.01*	-0.01	0.87
Age (Years)	-0.10	0.86	0.20	0.78	-0.39	0.70
BMI (kg/m^2)	-0.58	0.68	2.59	0.15	0.31	0.91
SBP (mmHG)	-0.27	0.49	-0.13	0.77	-0.13	0.87
HbA1c (%)	7.0	0.64	1.48	0.93	19.10	0.53
TC (mg/dl)	-0.26	0.04*	-0.13	0.42	-0.35	0.10

Dependent variable: telomere length (TL). Significant at *P<0.05.

Abbreviations: IMT - intima-media thickness; BMI – body mass index; SBP – systolic blood pressure; HbA1c - Glycated Hemoglobin; TC - total cholesterol; β- standardized regression coefficients, obtained from multiple linear regression analysis regression models.

SUPPLEMENTAL FIGURE
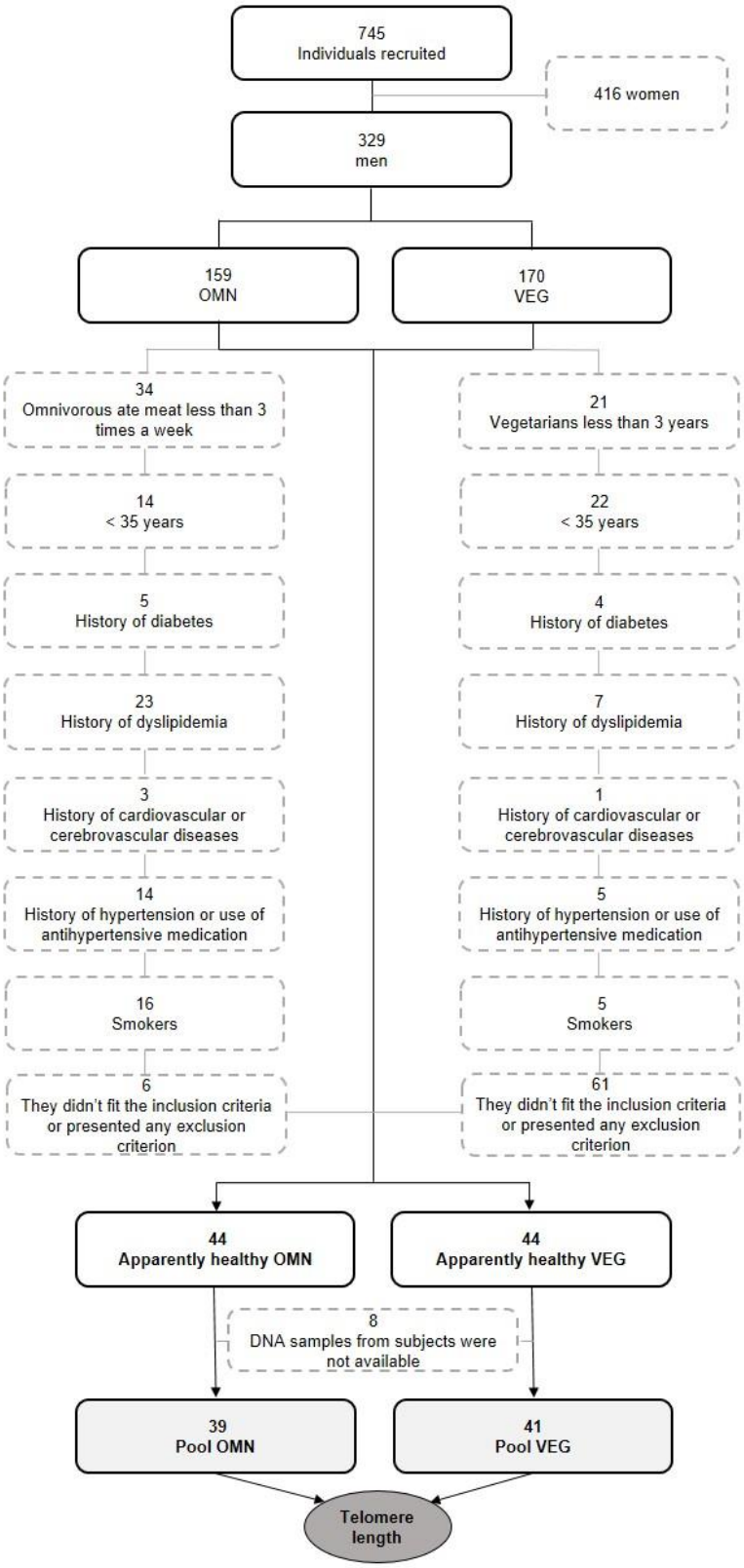
Figure S1. Experimental design and samples.