JÚLIA CRISTINA CARDOSO CARRARO

BIOMARCADORES INFLAMATÓRIOS E EPIGENÉTICOS PRECOCES DE COMPONENTES DA SÍNDROME METABÓLICA E SUA ASSOCIAÇÃO COM A DIETA HABITUAL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Doctor Scientiae.*

VIÇOSA

MINAS GERAIS - BRASIL

2015

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T

C313b
2015

Carraro, Júlia Cristina Cardoso, 1987-
Biomarcadores inflamatórios e epigenéticos precoses de componentes da síndrome metabolica e sua associação com dieta habitual / Júlia Cristina Cardoso Carraro. - Viçosa, MG, 2015.
xv, 115f. : il. ; 29 cm.

Orientador : Josefina Bressan.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Síndrome Metabólica. 2. Inflamação. 3. Epigenética. I. Universidade Federal de Viçosa. Departamento de Nutrição e Saúde. Programa de Pós-graduação em Ciência da Nutrição. II. Título.

CDD 22. ed. 616.39

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APROVADA: 18 de dezembro de 2015.

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“Alguns homens veem as coisas como são, e dizem ‘Por quê?’ Eu sonho com as coisas que nunca foram e digo ‘Por que não?’” (George Bernard Shaw)

AGRADECIMENTOS

Gostaria de agradecer em primeiro lugar a Deus, que me concedeu, em todos estes anos, sabedoria e persistência para chegar até aqui.

Agradeço também a meus pais, Júlio e Conceição, que desde criança me incentivaram e, com todas as dificuldades, colocaram a minha educação como prioridade em suas vidas. Espero que esse seja apenas o começo dos resultados que devo a vocês!

Agradeço ao meu marido, Rodrigo, pelo suporte e paciência, por tentar, às vezes de maneiras avessas às minhas, me ver sempre seguindo em frente, ainda que isso significasse aceitar a distância e a saudade.

Agradeço à Universidade Federal de Viçosa e ao Departamento de Nutrição e Saúde, seus professores e funcionários, por terem feito parte de toda a minha história acadêmica e terem fornecido toda a infraestrutura necessária para uma educação de qualidade.

Agradeço à minha orientadora, professora Josefina Bressan, por ser metora deste trabalho, por seu apoio, orientação e financiamento nestes 4 anos e por permitir que este sonho se tornasse realidade.

Agradeço também à professora Helen Hermsdorff por toda ajuda e paciência, sendo parte fundamental na realização deste trabalho.

O meu obrigado aos amigos do LAMECC, pelas incontáveis horas compartilhadas, à professora Fernanda de Carvalho Vidigal e a toda equipe LATINMETS, por dividir comigo horas e horas de trabalho (e espero que também os frutos que virão!).

Obrigada à *Universidad de Navarra* e ao *Centro de Alimentación y Fisiología* por me receber com tanto carinho e disponibilidade em ajudar nesta etapa de minha formação. Meu agradecimento especial aos professores J. Alfredo Martínez e Fermín I. Milagro por todo apoio necessário e colaboração neste projeto.

Obrigada também aos amigos feitos na Espanha, sejam brasileiros ou espanhóis, por compartilhar momentos de alegrias e de incertezas e me ajudar a seguir adiante quando as coisas pareciam mais difíceis do que realmente eram. E aos amigos antigos e de sempre, bem como toda minha família, por acreditarem que eu seria capaz!

Enfim, os meus sinceros agradecimentos a todas as pessoas que direta ou indiretamente participaram desses longos anos de formação acadêmica. Que eu possa, no exercício das minhas (futuras) atividades profissionais, retribuir pelo menos em parte todo o apoio que recebi até aqui.

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LISTA DE ABREVIATURAS

BIA – Impedância bioelétrica

BMI – *Body Mass Index*

CRP – *C reactive protein*

DBP – *Diastolic Blood Pressure*

DEXA - Absorciometria por dupla emissão de Raio X

DNMT - *DNA methyltransferase*

HDL-c – *High Density Lipoprotein cholesterol*

FV – *Fruits and vegetables*

HEI – *Heathy Eating Index*

HOMA-IR - *Homeostasis Model Assessment - Insulin Resistance*

HRM – *High Resolution Melting*

ICAM – Molécula de adesão intercelular-1

IL-10 – Interleucina 10

IL-18 - Interleucina 18

IL-1r – Receptor de Interleucina 1

IL-1β - Interleucina 1β

IL-6 – Interleucina 6

IMC – Índice de Massa Corporal

IPAQ - Questionário Internacional de Atividade Física

LDL-c – *Low Density Lipoprotein cholesterol*

LINE-1 - *Long interspersed element-1*

MetS – *Metabolic Syndrome*

NCEP-ATPIII - *National Cholesterol Education Program/ Adult Treament Panel III*

NFκB – *Fator nuclear kappa B*

PAI-1 – Inibidor da ativação de plasminogênio-1

PBMC – Células mononucleares do sangue

PC – Perímetro da cintura

PCR – Proteína C reativa

RBP4 – Proteína ligadora de retinol 4

rRNA –RNA ribossômico

SAM - S-adenosilmetionina

SBP – *Sistolic Blood Pressure*

SM – Síndrome Metabólica

TC – *Total cholesterol*

TNF-α – Fator de necrose tumoral alfa

TyG – Índice Triglicerídeo - Glicose

WC – *Waist circumference*

WHO – *World Health Organization*

WHR – *Waist Hip Ratio*

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RESUMO

CARRARO, Júlia Cristina Cardoso, D.Sc., Universidade Federal de Viçosa, dezembro de 2015. **Biomarcadores inflamatórios e epigenéticos precoces de componentes da síndrome metabólica e sua associação com a dieta habitual**. Orientador: Josefina Bressan. Coorientadores: Fernanda de Carvalho Vidigal e Helen Hermana Miranda Hermsdorff.

A Síndrome Metabólica (SM) é caracterizada como um conjunto de alterações metabólicas relacionadas ao maior risco de doenças cardiovasculares, diabetes, e morte prematura. Seu desenvolvimento pode estar relacionado a vários fatores, como o estresse oxidativo, a inflamação, fatores ambientais (ex. dieta) e (epi)genéticos, sendo a obesidade e a resistência à insulina fatores centrais em sua fisiopatologia. Uma vez que a SM possui importantes repercussões na qualidade e expectativa de vida, torna-se importante o diagnóstico e intervenção precoce no intuito do aumento da sobrevida destes indivíduos. Assim, este estudo teve como objetivo determinar biomarcadores inflamatórios e epigenéticos precoces da SM, bem como avaliar a associação de componentes da dieta habitual com os componentes da SM, com as concentrações de marcadores inflamatórios e com modificações epigenéticas. Dois estudos transversais foram conduzidos, sendo um composto por profissionais de saúde aparentemente saudáveis do município de Viçosa – MG (n=226; 74% do sexo feminino; idade média de 28,9 ± 7,0 anos e média de IMC de 22,4 ± 3,4 kg/m²), e o segundo por estudantes das universidades de Navarra (UNAV) e Pública de Navarra (UPNA), da cidade de Pamplona, Espanha (n=153; 67% do sexo feminino; idade média de 21,0 ± 3,0 anos e média de IMC de 22,1 ± 2,5 kg/m²). Foram avaliados indicadores antropométricos, de composição corporal, marcadores metabólicos e inflamatórios e a metilação global do DNA e de promotores de genes codificadores de marcadores inflamatórios. Os marcadores epigenéticos foram avaliados por diferentes métodos (*High Resolution Melting* - HRM e MALDI-TOF-Sequenon). Em relação ao primeiro estudo, a hipermetilação global do DNA e a de promotores de genes inflamatórios (*IL-6, SERPINE1* e *PCR*) esteve associada a marcadores de adiposidade, como o perímetro da cintura - PC e índice de massa corporal - IMC. Indivíduos com maior metilação global também apresentaram aumento da pressão

arterial sistólica e de marcadores do metabolismo glicídico, maiores concentrações de TNF-α e menores de adiponectina, bem como pior qualidade da dieta. A hipermetilação dos genes inflamatórios, embora não relacionada a concentrações circulantes das respectivas citocinas, esteve relacionada a componentes específicos da dieta, como o maior consumo de calorias, de carboidratos ou de lipídios totais. No segundo estudo, as concentrações séricas de interleucina-6 (IL-6) estiveram associadas a indicadores de adiposidade (PC, razão cintura quadril - RCQ, IMC e percentual de gordura corporal), assim como ao maior risco de ocorrência da resistência à insulina e maiores concentrações séricas de PCR, mostrando-se melhor biomarcador precoce de desordens metabólicas que a interleucina-18 (IL-18). As concentrações destas interleucinas podem ser influenciadas pelo hábito de fumar, uma vez que o maior número de cigarros fumados ao dia esteve associado a maiores concentrações de IL-6 e IL-18. A melhor qualidade da dieta, bem como o maior consumo de frutas totais, laranja, maçã, vitamina C e fibra da fruta estiveram associadas à hipometilação do promotor de *TNF-α*. Em conjunto, os resultados dos dois estudos sugerem que as concentrações de citocinas inflamatórias e marcadores epigenéticos (metilação de DNA) podem ser bons preditores precoces de alterações metabólicas, podendo ser influenciados pela qualidade da dieta e ingestão de nutrientes e,ou grupos alimentares específicos, como o de frutas.

ABSTRACT

CARRARO, Júlia Cristina Cardoso, D.Sc., Universidade Federal de Viçosa, December, 2015. **Inflammatory and Epigenetic early biomarkers of Metabolic Syndrome components and its association with habitual diet.** Adviser: Josefina Bressan. Co-advisers: Fernanda de Carvalho Vidigal and Helen Hermana Miranda Hermsdorff.

The metabolic syndrome (MetS) is a cluster of metabolic abnormalities associated with increased risk of cardiovascular disease, diabetes, and premature death. The development of MetS may be related to many factors such as oxidative stress, inflammation, environmental (eg. diet) and (epi)genetic factors, being obesity and insulin resistance key factors in the pathophysiology. Since MetS has important repercussions on the quality of life and life expectancy, it is very important the early diagnosis and intervention in order to increase the survival of these subjects. Thus, this study aimed to determine early inflammatory and epigenetic biomarkers of MetS, and to evaluate the association of dietary components with MetS and with the biomarkers evaluated. Two cross-sectional studies were carried out, one composed by apparently healthy healthcare professionals from Viçosa - MG (n = 226, 74% of females, age average 28.9 ± 7.0 years old and BMI average 22.4 ± 3.4 kg/m²), and the second by students from the universities of Navarra (UNAV) and Public of Navarra (UPNA), from Pamplona , Spain (n = 153, 67% of females, age average 21.0 ± 3.0 years old and BMI average 22.1 ± 2.5 kg/m²).). Subjects from the both studies were evaluated by anthropometric, body composition, metabolic and inflammatory markers and, the global DNA methylation and methylation of promoters of genes encoding inflammatory markers. Epigenetic markers were evaluated by different methods (High Resolution Melting - HRM and MALDI-TOF-Sequenon). Regarding to the first study, the global DNA and inflammatory genes promoters hypermethylation (*IL-6, SERPINE1* and *PCR*) have been associated to adiposity markers such as waist circumference (WC) and body mass index (BMI). Individuals with higher global methylation also showed an increase in systolic blood pressure and glycemic metabolism markers, higher TNF-α concentrations and lower adiponectin, as well as poorer quality of the diet. The hypermethylation of inflammatory genes, though not related to circulating concentrations of the

cytokines, was related to specific components of the diet, such as higher caloric, carbohydrate or total fatty acids intake. About the second one, interleukin-6 (IL-6) concentrations were associated with adiposity traits (WC, waist hip ratio- WHR, BMI and body fat percentage), as well as the increased risk of resistance insulin and higher serum concentrations of CRP. IL-6 has been shown to be better early biomarker of metabolic disorders that interleukin-18 (IL-18). The concentrations of these interleukins can be influenced by smoking, once the largest number of cigarettes smoked per day was associated with higher concentrations of IL-6 and IL-18. The best quality of the diet and higher consumption of total fruit, orange, apple, vitamin C and fruit fiber were associated with hypomethylation of *TNF-α* promoter. Taken together, the results of both studies suggest that the inflammatory cytokines levels and epigenetic markers (DNA methylation) can be good predictors of early metabolic changes, which can be influenced by the quality of diet and intake of nutrients or specific

1. INTRODUÇÃO GERAL

A Síndrome Metabólica (SM) é caracterizada como um conjunto de alterações metabólicas relacionadas ao maior risco de doenças cardiovasculares, a saber, a pressão arterial aumentada, dislipidemia, obesidade central e glicemia de jejum elevada (ALBERTI et al., 2009).

A grande relevância dos estudos da SM se dá pelo aumento do risco de diversas doenças, como doenças coronárias, diabetes, e morte prematura (WILLIAMS et al., 2007), sendo o risco de infarto três vezes maior entre indivíduos sindrômicos (ISOMAA et al., 2001). A SM também aumenta o risco de desenvolvimento de esteatose hepática não-alcoólica (ERICKSON, 2009); síndrome do ovário policístico, aterosclerose (BRUCE; HANSON, 2010) e hiperuricemia (KUNITSKAYA; ARIEV, 2012).

Vários fatores podem influenciar no desenvolvimento da SM, como concentrações hormonais, estresse oxidativo, estado pró-inflamatório, distúrbios do sono, entre outros. No entanto, a resistência à insulina e a obesidade têm sido os principais fatores predisponentes (WILLIAMS et al., 2007, CAMPIÓN et al., 2009).

Por sua vez, os fatores genéticos são fortes contribuintes na gênese da SM, sendo a hereditariedade responsável por cerca de 10 a 30% das ocorrências (POLLEX; HEGELE, 2006). Dessa forma, cada vez mais se busca elucidar também os mecanismos epigenéticos de regulação metabólica.

Neste contexto, entende-se por epigenética, as modificações relativamente estáveis e hereditárias em nível cromossômico, sem mudanças na sequência de DNA (BERGER et al., 2009). Tais modificações podem ser influenciadas pelo ambiente (como dieta, atividade física, fumo, entre outros), mediante alterações na metilação, acetilação ou fosforilação do DNA e,ou de histonas (SPOTSWOOD; TURNER, 2002). Estas alterações levam à remodelação da cromatina, ocasionando em aumento ou diminuição da expressão de genes (WATERLAND; GARZA, 1999).

De fato, estudos de programação fetal em resposta a um ambiente materno adverso em relação à predisposição de doenças crônicas futuras têm demonstrado o papel da epigenética na gênese da SM (GALLOU-KABANI; JUNIEN, 2005).

Ainda no contexto da relação obesidade/SM, sabe-se que a inflamação exerce papel central entre as duas enfermidades, uma vez que a expansão do tecido adiposo promove o recrutamento de células imunes, aumentando assim a produção de citocinas pró-inflamatórias (VACHHARAJANI et al., 2009). Por sua vez, o aumento de citocinas inflamatórias favorece o desenvolvimento de desordens metabólicas tais como, resistência à insulina e doenças cardiovasculares (CHEN et al., 2012).

A dieta também possui influência importante na gênese das desordens metabólicas e aumento da inflamação (HERMSDORFF et al., 2009), tanto pelos mecanismos bem estabelecidos em relação ao aumento de fatores risco (SIMOPOULOS, 2008; VAN DIJK et al., 2009) quanto em termos de influência sobre marcadores epigenéticos (URIARTE et al., 2013; ZHANG et al., 2012).

Neste contexto, a busca por novas e apropriadas técnicas de diagnóstico precoce, como a determinação de biomarcadores, deve ser potencializada para a prevenção da SM (RYO et al., 2004). Esta busca pode ser de fundamental importância se tiver como alvo indivíduos jovens e saudáveis, de forma a predizer antecipadamente a ocorrência da enfermidade e otimizar seu tratamento (CARRARO et al., 2015). A determinação de biomarcadores precoces, bem como o efeito da dieta sobre os mecanismos epigenéticos poderão trazer novas perspectivas no âmbito do cuidado nutricional na prevenção de doenças crônicas não transmissíveis.

2. HIPÓTESE

A hipótese deste estudo é que a concentração circulante de citocinas inflamatórias e a metilação do DNA podem ser utilizados como biomarcadores precoces do desenvolvimento de desordens metabólicas.

3. OBJETIVOS

3.1. Objetivo Geral

Identificar biomarcadores inflamatórios e epigenéticos precoces do desenvolvimento de componentes da Síndrome Metabólica, bem como sua associação com componentes da dieta habitual.

3.2. Objetivos Específicos

Para a realização deste estudo foram desenvolvidos dois estudos transversais com indivíduos, aparentemente saudáveis, de diferentes populações:

a) *Estudo 1 – Profissionais de Saúde de Viçosa – MG (Brasil)*

- Avaliar indicadores antropométricos e do consumo alimentar na população estudada;

- Determinar as concentrações séricas de marcadores metabólicos: glicose, colesterol total e frações, triglicerídeos, insulina e ácido úrico séricos;

- Determinar as concentrações plasmáticas de biomarcadores inflamatórios: adiponectina, IL-10, IL-6, PAI-1, TNF-α, IL-1β, proteína C reativa (PCR) e complemento C3 dos indivíduos;

- Determinar as concentrações plasmáticas de malondialdeído;

- Determinar a metilação de genes codificadores de marcadores inflamatórios (IL-6, PAI-1 e PCR);

- Avaliar a associação entre indicadores antropométricos, componentes da dieta e de estilo de vida em relação ao estado inflamatório e oxidativo da população estudada;

- Avaliar a associação da metilação do DNA com a presença de SM e seus componentes;

- Avaliar a associação da dieta na relação entre metilação de DNA e SM.

b) Estudo 2 – Jovens universitários de Pamplona – Navarra (Espanha)

- Avaliar indicadores antropométricos e da dieta na população estudada;

- Determinar as concentrações de marcadores metabólicos: glicose, colesterol total e frações, triglicerídeos, insulina e ácidos graxos livres no plasma;

- Determinar as concentrações plasmáticas de biomarcadores inflamatórios: IL-6, IL-18 e TNF-α dos indivíduos;

- Avaliar a expressão e a metilação de TNF-α;

- Avaliar a associação entre indicadores antropométricos, dieta habitual e de estilo de vida em relação ao estado inflamatório;

- Avaliar a associação da metilação do DNA e expressão gênica com a presença de SM e seus componentes;

- Avaliar a associação da dieta nas relações entre marcadores (epi)genéticos, inflamatórios e componentes da SM.

4. REVISÃO BIBLIOGRÁFICA

Síndrome Metabólica

A Síndrome Metabólica (SM) está caracterizada como um conjunto de alterações metabólicas responsáveis pelo maior risco de eventos cardiovasculares. As principais alterações envolvidas são: pressão arterial aumentada, dislipidemias, obesidade central e glicemia de jejum elevada, relacionando-se intimamente à fisiopatologia da resistência à insulina (ALBERTI et al., 2009). Alguns autores relatam ainda como fatores relacionados à SM, o aumento das concentrações de apolipoproteína B (ALBERTI et al., 2005), aumento do estresse oxidativo e redução das defesas antioxidantes (WEST, 2000; PENCKOFER et al., 2002; STOCKER; KEANEY, 2004), aumento do ácido úrico (DA SILVA et al., 2015), microalbuminúria (GRUNDY, 2005) e metabolismo de carboidratos e lipídios prejudicados, bem como alterações nas funções dos tecidos adiposo, cardíaco, vascular e na função hemostática (GARAULET; MADRID, 2009).

De acordo com o *National Cholesterol Education Program* (NCEP) *Adult Treatment Panel III* (ATP III), os critérios de diagnóstico da SM são baseados na presença de 3 ou mais alterações dentre: perímetro da cintura, triglicerídeos séricos, pressão arterial e glicose de jejum aumentados, e redução dos níveis de lipoproteína de alta densidade (HDL-C). Esta classificação tem sido amplamente empregada na prática clínica e em estudos epidemiológicos (NCEP, 2001).

Segundo a Organização Mundial da Saúde, a SM pode ser definida pela presença de intolerância à glicose, diabetes ou resistência à insulina, associada a duas ou mais das seguintes alterações: pressão arterial e triglicerídeos séricos aumentados, redução de HDL-C, obesidade abdominal definida pelo perímetro da cintura aumentado e microalbuminúria (WHO, 2000).

Em 2009, uma definição unificada foi proposta por várias organizações, com os mesmos fatores diagnósticos propostos pelo NCEP-ATP IIII, exceto para a obesidade abdominal como fator obrigatório e, sendo a resistência à insulina o fator chave na fisiopatologia da doença (ALBERTI et al, 2009).

A prevalência de SM tem se apresentado elevada em países desenvolvidos, sendo observados valores entre 14 e 41% em estudo realizado em indivíduos não diabéticos de diversos países da Europa. O aumento da prevalência mostrou-se proporcional ao aumento da idade (BALKAU et al, 2002). Em países em desenvolvimento estes valores também são elevados. Estudos realizados com a população adulta da Índia e do Irã descreveram prevalências de SM de 41% e 33,7%, respectivamente (AZIZI et al, 2003; RAMANCHANDRAN et al, 2003). Em uma revisão sistemática Vidigal el al. (2013) estimaram uma prevalência de SM de 29,6% (14,9% a 65,3%) a partir de estudos pontuais realizados no Brasil. Estes valores podem chegar a 50%, sendo a SM considerada o maior problema de saúde pública da sociedade moderna (CAMERON et al., 2004). Entre caucasianos a prevalência é de cerca de 16%, mostrando a interferência de fatores étnicos no desenvolvimento da doença (ROSMOND, 2001; RAMPAL et al., 2012). Outro estudo demonstrou que a prevalência de SM é maior entre mulheres e entre indivíduos acima de 40 anos (RAMPAL et al., 2012). No entanto, a discrepância destes valores também pode ocorrer em função de diferentes critérios diagnósticos (TONG et al.,2007; JANGHORBANI; AMINI, 2012). Um aumento da ocorrência de SM é observado, principalmente, durante o período perimenopausal e nos primeiros anos pós-menopausa, o que pode ocorrer em função da deficiência de estrógeno, tanto sobre a atividade ovariana, quanto sobre o aumento do acúmulo de gordura central (JANSEN et al., 2008).

A etiologia da SM ainda é desconhecida, mas sugere-se que seu desenvolvimento poderia ser devido à complexa interação de fatores genéticos, idade, sedentarismo e estilo de vida, em especial, à dieta (RAMÍREZ -VARGAS et al, 2007). Vários fatores podem estar envolvidos na gênese ou manutenção de alterações metabólicas predisponentes da SM, sendo considerada uma doença multifatorial.

Existem cada vez mais evidências científicas de que a inflamação está diretamente associada à SM, assim, deriva-se a importância de se conhecer melhor os mecanismos implicados na etiologia de tais associações (SCHIFFRIN, 2008; WADSWORTH, 2008).

Já está bem elucidado que a obesidade, condição intimamente relacionada à presença da SM, pode ser caracterizada como uma resposta inflamatória subclínica, com uma produção anormal de adipocinas (substâncias com funções hormonais produzidas no tecido adiposo) e de fatores pró-inflamatórios, uma vez que os adipócitos apresentam algumas propriedades imunes e de ativação do complemento. Ademais, a obesidade promove a atração de monócitos para o tecido adiposo (HERMSDORFF et al., 2010).

Obesidade e o risco aumentado de SM

A SM tem recebido grande atenção nas últimas décadas, uma vez que sua prevalência tem aumentado de forma exacerbada, principalmente em países ocidentais. Este aumento tem ocorrido em função de dietas hipercalóricas, sedentarismo, e consequente excesso de adiposidade (CORNIER et al., 2008). Sua prevalência foi estimada em 20 a 30%, crescendo paralelamente ao aumento da obesidade (ALBERTI et al., 2005).

Cerca de 30% da população norte americana é obesa, sendo esta realidade já aplicável também às crianças (WU; KAER, 2013). A Organização Mundial da Saúde relatou que a obesidade crescerá de 1 bilhão de pessoas com sobrepeso e 350 milhões de obesos para cerca de 2,16 bilhões de indivíduos com sobrepeso e 1,12 bilhões de obesos em 2030, sendo a causa primária desta epidemia o desbalanço energético (JAMES, 2008).

O peso corporal tende a ser estável tanto em obesos quanto em magros, em função da existência de mecanismos regulatórios do gasto energético e, ou da ingestão alimentar (FRIEDMAN, HALAAS, 1998).

As adipocinas também estão relacionadas à regulação de vias metabólicas, integrando o tecido adiposo a receptores de hormônios nucleares e ao sistema nervoso central (LAZAR, 2006), e agindo sobre o metabolismo de lipídio e glicose (KERSHAW; FLIER, 2004). Uma dessa adipocinas, a adiponectina e seus receptores tem sido considerada um possível alvo terapêutico para a SM, em função de sua ação antidiabética e antiaterogênica (KADOWAKI; YAMAUCHI, 2005).

Os adipócitos são as células especializadas na função de estocagem de lipídios, como substrato energético, em situações de abundância de nutrientes (PELLEYMOUNTER et al., 1995).

A localização do tecido adiposo, se subcutâneo ou visceral, determina importantes diferenças metabólicas entre estes dois compartimentos corporais de acúmulo de lipídios, em função da capacidade de lipólise e lipogênese, sensibilidade à insulina, metabolismo energético e regulação endócrina (WAJCHENBERG, 2000). O acúmulo excessivo de triglicerídeos no tecido adiposo visceral tem sido descrito como mais deletério, uma vez que, por ser de mais fácil mobilização, promove um aumento de ácidos graxos livres na circulação portal, levando a um menor *clearance* de insulina no fígado, resistência à insulina, hiperinsulinemia, hipertensão, dislipidemias e, consequentemente, maior risco de doenças cardiovasculares (SHANKAR, 2012).

Além disso, as células adiposas possuem capacidade limitada de estocagem. Sendo assim, quando ocorre uma ingestão calórica elevada de maneira crônica, este armazenamento pode cessar, levando ao acúmulo ectópico de lipídios. Órgãos como coração, músculo esquelético, fígado e pâncreas podem apresentar concentrações anormais de triglicerídeos, podendo sofrer interferências negativas em nível celular e falência gradativa do órgão (UNGER et al., 2010). Este conceito de acúmulo de lipídios em células não adiposas e suas consequências metabólicas é chamado de lipotoxicidade, e pode incorrer em resistência à insulina, apoptose e inflamação (VIRTUE; VIDAL-PUIG, 2010).

Desta maneira, a obesidade causa acúmulo excessivo de gordura não somente no tecido adiposo, como em órgãos responsivos à insulina, principalmente o músculo esquelético e tecido hepático, podendo levar à resistência à insulina e SM, bem como ao diabetes tipo 2, esteatose hepática não alcoólica, certos tipos de câncer e redução da expectativa de vida (ZIMMET et al., 2001; GARNETT et al., 2007; AGIRBASLI et al., 2011).

Inflamação e SM

Sabe-se que a obesidade é uma condição inflamatória e está associada a maiores prevalências de SM. Sendo assim, vários marcadores inflamatórios (Proteína C Reativa – PCR, interleucina-6 – IL-6 e adiponectina) podem representar um elo entre a obesidade e suas complicações associadas, como a resistência à insulina e a SM, e serem assim considerados possíveis biomarcadores para a síndrome (DANDONA et al, 2004; CHEN et al., 2012; CARRARO et al., 2015).

Os mecanismos precisos que relacionam tais eventos permanecem, todavia, sem completa elucidação. As relações de causa-efeito entre os processos de inflamação com as doenças associadas ainda não estão claras, mas vários estudos têm associado a ocorrência destas ao aumento nas concentrações de alguns marcadores inflamatórios (DANDONA et al, 2004; DANDONA et al, 2005; MORENO-ALIAGA et al, 2005). Sendo assim, esforços têm sido realizados no sentido de identificar marcadores com o objetivo de sistematizar sua utilização no diagnóstico e controle de doenças relativas ao aumento do risco cardiovascular, como a SM.

O aumento das concentrações de diversas citocinas pró-inflamatórias tem sido associado às complicações metabólicas (resistência à insulina, doenças cardiovasculares, entre outras), como pode ser observado com o fator de necrose tumoral (TNF-α), a interleucina 6 (IL-6), interleucina 1β (IL-1β), PAI-1 (inibidor da ativação de plasminogênio 1), entre outros (FORD et al, 2003; HU et al., 2004; UM et al., 2004, VOLP et al., 2012, LOPEZ-LAGARREA et al., 2013).

A IL-6 e o TNF-α, no entanto, são as citocinas mais estudadas na obesidade e suas concentrações se encontram elevadas em indivíduos deste grupo, inclusive no plasma (HERMSDORFF et al, 2010; 2011). Estudos também têm sido conduzidos no intuito de estabelecer relações inversas entre concentrações circulantes de citocinas anti-inflamatórias e a SM. Todavia, estas relações estão mais bem elucidadas em relação à resistência à insulina e à obesidade, e não diretamente à SM. A adiponectina, por exemplo, uma adipocina anti-inflamatória, atenua a resistência à insulina por meio da redução do conteúdo

de triglicerídeos no fígado e no músculo esquelético (YAMAUCHI et al., 2001). Relações inversas também foram identificadas entre as concentrações de interleucina 10 (IL-10) e presença de SM em mulheres (ESPOSITO et al., 2003), provavelmente devido à sua ação inibitória sobre a produção de citocinas pró-inflamatórias (TEDGUI; MALLAT, 2001).

Fatores genéticos relacionados à SM

Várias evidências demonstram que fatores genéticos são fortes contribuintes no desenvolvimento da SM, sendo que a suscetibilidade genética e a hereditariedade respondem por cerca de 10 a 30% das ocorrências (POLLEX; HEGELE, 2006).

Vários estudos têm demonstrado genes candidatos à relação com o desenvolvimento da SM (CORELLA et al., 2010; POVEL et al., 2011), entre eles, o *FTO* (LIGUORI et al., 2014), genes relacionados ao metabolismo lipídico (KRISTIANSSON et al., 2012), à adipogênese (FARMER, 2006), ao controle circadiano (DASHTI et al., 2014), a fatores de crescimento (MINA et al., 2015) e à inflamação (FUENTES et al., 2013). A alteração na expressão destes genes, bem como a interação multigência pode interferir na ocorrência ou não da doença, no entanto seus mecanismos de ação ainda são pouco elucidados.

Nutriepigenética e a regulação da expressão gênica

O termo epigenética refere-se a modificações no cromossomo, sem alterações na sequência de DNA, que podem ser hereditárias (BERGER et al., 2009). Tais modificações são responsivas ao ambiente e compreendem diversos mecanismos, como alterações na metilação, acetilação ou fosforilação do DNA e,ou de histonas (SPOTSWOOD; TURNER, 2002). Estas alterações levam à remodelação da cromatina, ocasionando em aumento ou diminuição da expressão de genes (WATERLAND; GARZA, 1999).

A metilação é considerada uma das principais alterações epigenéticas, sendo caracterizada pela adição de um grupo metil à posição 5 do anel

pirimidínico de citosinas situadas próximas a uma guanina (dinucleotídeos CpGs) (JONES; TAKAI, 2001). Tais dinucleotídeos, quando localizados em regiões promotoras, regulam a transcrição gênica, de forma que, quanto mais metilado, mais condensada a cromatina, menor acesso dos fatores de transcrição e menor transcrição gênica (JIRTLE; SKINNER, 2007; ROBERTSON, 2005).

Estudos epigenéticos relacionados à obesidade têm revelado alterações na regulação da adipogênese e da inflamação, e que sítios CpGs específicos podem estar relacionados a alterações metabólicas (PIETILÄINEN et al., 2015).

Análises globais de metilação são feitas por meio de elementos repetitivos do genoma, tais como LINE-1 (long interspersed repeat sequences), cuja hipometilação está relacionada à instabilidade genômica e anormalidades cromossômicas (SHARMA et al., 2010; WILSON et al., 2007). Por outro lado, análises de genes específicos são geralmente realizadas em ilhas CpGs de promotores, os quais podem ser silenciados ou superexpressos conforme o nível de metilação (ESTELLER, 2002).

A metilação de ilhas CpGs pode ser alterada em função da dieta, como consumo habitual de doadores de metil (folato, betaína, vitamina B12), álcool, dietas hiperlipídicas, entre outros (AMARAL et al., 2014; BOEKE et al., 2012; VAN ENGELAND et al., 2003; MILAGRO et al., 2009, URIARTE et al., 2013). Padrões alimentares saudáveis, como a maior ingestão de frutas e verduras têm sido associados a modificações epigenéticas (DELGADO-CRUZATA et al., 2015) de maneira dose dependente (ZHANG et al., 2011) e, consequentemente, a alterações no padrão de expressão de genes alvo. Tais modificações epigenéticas e de expressão gênica estão relacionadas ao desenvolvimento de várias enfermidades (HANSEN et al., 2011; KIM et al., 2010; TOPEROFF et al., 2012), tanto de forma a aumentar como a diminuir o risco de ocorrência, e podem ser analisadas globalmente ou em relação a genes específicos de interesse.

Biomarcadores em saúde cardiovascular

O termo “biomarcadores”, proveniente de marcador biológico, se refere à subcategoria de sinais clínicos (indicações clínicas que podem ser observadas

externamente ao paciente), que podem ser medidos com acurácia e reprodutibilidade (STRIMBU; TAVEL, 2010). Foi definido pelo *National Institutes of Health Biomarkers Definitions Working Group* como "uma característica que pode ser medida objetivamente e avaliada como indicador de processos biológicos normais, patogênicos ou resposta farmacológica a intervenções terapêuticas" (NATIONAL INSTITUTES OF HEALTH, 2001). A Organização Mundial da Saúde, por sua vez, define biomarcadores como "qualquer substância, estrutura ou processo ou seus produtos, que possam ser medidos no corpo e predizer a incidência de resultados ou doenças" (WHO, 2001).

O estabelecimento de biomarcadores tem fundamental importância no desenvolvimento de novos medicamentos, e o entendimento das relações existentes entre estes e as respostas metabólicas pode otimizar a expansão de tratamentos para diversas patologias (STRIMBU; TAVEL, 2010).

A busca por novos biomarcadores, especialmente os não-invasivos, tem sido extensivamente realizada em relação ao diagnóstico de câncer (THOMAS; SWEEP, 2001; DUFFY, 2007). No entanto, também tem crescido a utilização e investigação de biomarcadores não-tradicionais em relação a doenças cardiovasculares, e sua relação com a obesidade, podendo auxiliar no entendimento de novos mecanismos que expliquem tal inter-relação e na sua prevenção (MORABIA; CONSTANZA, 2005).

Soro e plasma sanguíneos têm sido alvos de extensiva investigação nos últimos anos para estabelecimento de novos biomarcadores, sendo convencionalmente utilizadas as proteínas plasmáticas (ROULSTON, 1990; THOMAS; SWEEP, 2001).

A identificação de novos biomarcadores, todavia, requer a determinação de sua relevância e validade. Relevância se refere à capacidade de fornecer informações clinicamente importantes e de interesse público. Validade, por sua vez, se refere à necessidade de determinar a efetividade e utilidade do biomarcador. No entanto, este último parâmetro, longe de ser definitivo em "válido" ou "não válido", se comporta mais como um espectro. Neste sentido, o desenvolvimento de novos biomarcadores, geralmente, não é conclusivo. Ao

contrário, se busca progressivamente diminuir as incertezas e os erros associados à sua utilização em relação a um desfecho clínico (STRIMBU; TAVEL, 2010).

Diante do exposto, a busca de biomarcadores precoces (em níveis plasmáticos e moleculares) da SM se faz estritamente necessária, para que se possa intervir antes mesmo da instalação e manifestação clínica da doença, permitindo a atuação em níveis de prevenção e tratamento mais eficazes da mesma.

5. METODOLOGIA

5.1. Caracterização do estudo

Para o estudo de biomarcadores precoces de componentes da Síndrome Metabólica foram conduzidos dois estudos populacionais, de natureza transversal. O primeiro estudo foi composto por profissionais da saúde do município de Viçosa – MG (Brasil), e o segundo, por estudantes universitários da cidade de Pamplona - Navarra (Espanha). Ambos os estudos foram realizados em 2 encontros e visaram o estudo de pessoas aparentemente saudáveis, com o intuito de descrever alterações inflamatórias e epigenéticas em estágios subclínicos. A caracterização das amostras de ambos os estudos está descrita na Tabela 1:

Tabela 1: Caracterização da amostra dos estudos de Profissionais da Saúde de Viçosa – MG (estudo 1) e de Estudantes Universitários de Pamplona – Navarra (Estudo 2).

Variáveis	Estudo 1 (n=226)	Estudo 2 (n=153)
Sexo (% de sexo feminino)	74,3	67,3
Idade (anos)	28,90 ± 7,00	21,00 ± 3,00
Peso (kg)	63,90 ± 12,76	62,53 ± 11,04
Perímetro da Cintura (cm)	79,10 ± 9,91	72,75 ± 7,92
Razão Cintura Quadril	0,80 ± 0,07	0,74 ± 0,06
Índice de Massa Corporal (kg/m²)	22,40 ± 3,40	22,10 ± 2,50
Gordura corporal (%)	22,65 ± 6,50	20,0 ± 6,59
Pressão Arterial Sistólica (mmHg)	108,97 ± 12,35	114, 93 ± 11,43
Pressão Arterial Diastólica (mmHg)	67,19 ± 7,72	65,16 ± 7,96
Colesterol total (mg/dL)	183,96 ± 36,41	174,83 ± 27,69
HDL (mg/dL)	59,32 ± 15,26	59,77 ± 12,78
LDL (mg/dL)	105, 79 ± 30,86	101,46 ± 24,13
CT:HDL	3,26 ± 0,93	3,02 ± 0,67
Triglicerídeos (mg/dL)	95,02 ± 50,45	67,99 ± 26,43
Glicose de jejum (mg/dL)	87,39 ± 11,81	85,09 ± 7,69

Insulina de jejum (mg/dL)	7,28 ± 4,45	7,92 ± 3,31
HOMA-IR	1,64 ± 1,64	1,67 ± 0,73
PCR (mg/L)	1,82 ± 2,03	1,10 ± 0,81
IL-6 (pg/mL)	1,36 ± 0,92	1,04 ± 0,40
TNF-α (pg/mL)	6,68 ± 3,03	2,12 ± 2,19

Resultados apresentados em média ± desvio padrão.

5.2. Desenho experimental

Estudo 1: Projeto Profissionais de Saúde de Viçosa

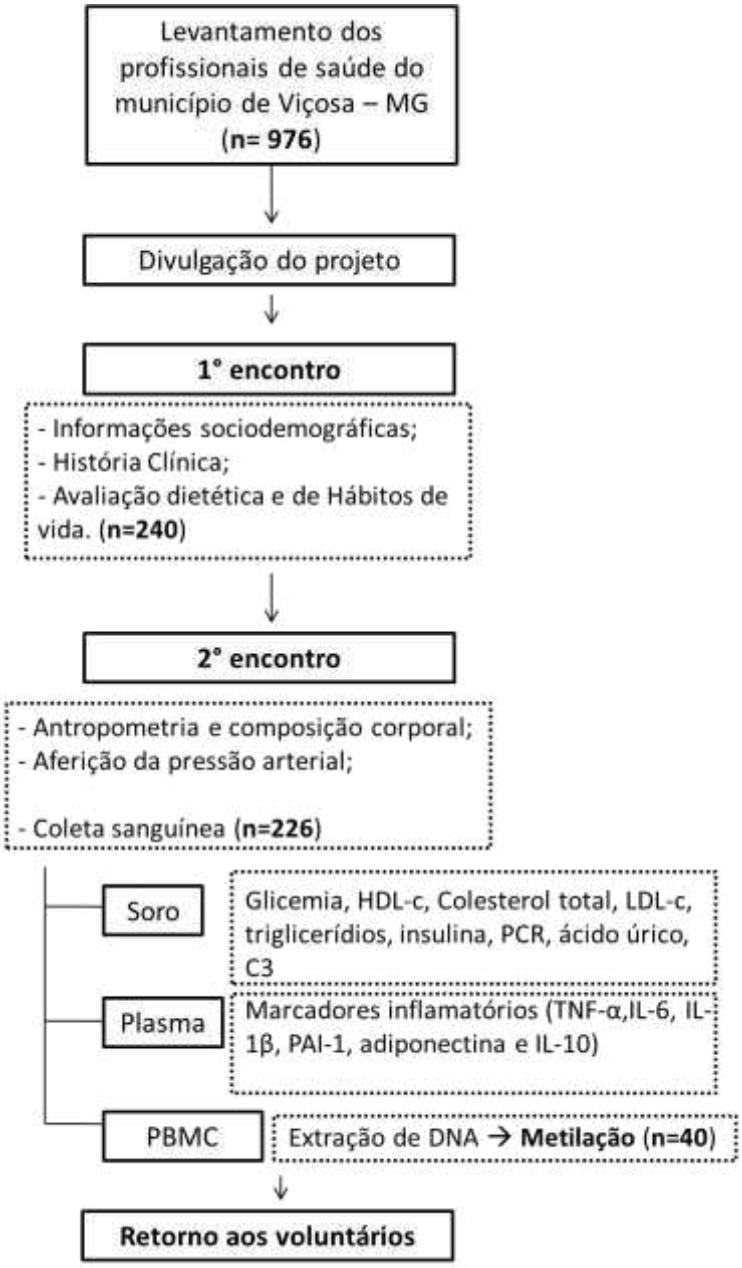

Figura 1: Desenho experimental do estudo com profissionais da saúde do município de Viçosa, MG (Brasil)

Estudo 2: Universitários de Pamplona - Espanha

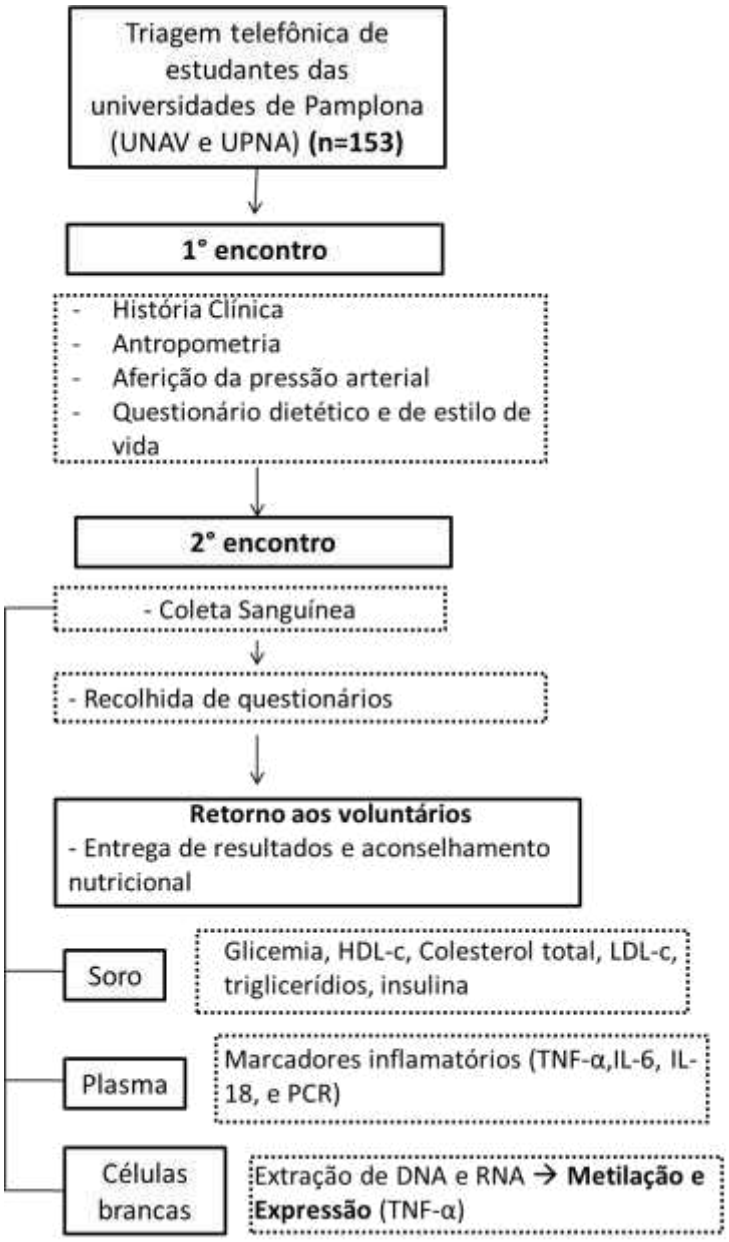

Figura 2: Desenho experimental do estudo com jovens universitários da cidade de Pamplona (Navarra, Espanha)

5.3. Instrumentos e técnicas de coleta de dados

Avaliação antropométrica e composição corporal

O peso foi determinado com roupa leve, sem sapatos, utilizando balança digital (Toledo®, Brasil e *Tanita Body Composition Analyzer* TBF 300, Japão), com precisão de 0,05 kg. A estatura foi determinada utilizando estadiômetro (Seca 206 ®, Brasil e Seca ® 713, Alemania) com o paciente em pé, sendo lida com precisão de 0,1 mm. O índice de massa corporal (IMC) foi calculado com o peso (kg) dividido pela estatura (m) ao quadrado e os participantes foram classificados de acordo com os pontos de corte da Organização Mundial da Saúde (WHO, 2000). O perímetro da cintura (PC) foi determinado com o indivíduo em pé, utilizando uma fita métrica no ponto médio entre a última costela e a crista ilíaca e foi expressa em centímetros. A avaliação da composição corporal foi realizada por meio da análise de bioimpedância elétrica tetrapolar horizontal (BIA), utilizando o aparelho Biodynamics modelo 310 ® no Estudo 1, e por meio de pregas cutâneas (bicipital, trcipital, suprailíaca e subescapular) aferidas com adipômetro Lange (Beta Technology, EUA) no Estudo 2.

Aferição da pressão arterial e coleta de sangue

A pressão arterial foi determinada em repouso, determinada em triplicata, utilizando-se esfigmomanômetro semi-automático (Omron HEM-705CP, Holanda), seguindo o procedimento recomendado pela *European Society of Hypertension* e pela *European Society of Cardiology* (MANCIA et al, 2007). As amostras de sangue foram obtidas por punção venosa, após um mínimo de 12 horas de jejum, por técnico de enfermagem treinado. As coletas foram feitas em tubos a vácuo sorogel para separação de soro, e a vácuo com EDTA para separação de plasma. O sangue foi centrifugado a 2500 rpm, em temperatura de 4ºC, por 10 minutos, para separação de plasma e soro. Aos tubos cujas alíquotas de plasma foram retiradas, adicionou-se solução fisiológica, homogeneizou-se adicionando lentamente a 13 mL de solução de separação (Polymorphprep ®). Esta solução foi centrifugada a 450 rpm, em temperatura ambiente, por 30 minutos para separação de células mononucleares. As células foram retiradas com pipetas pasteur

esterilizadas e adicionadas a 1mL de Dulbecco's PBS, procedendo duas posteriores lavagens neste tampão, ao centrifugar a 400 rpm, em temperatura ambiente, por 10 minutos cada. As células extraídas foram armazenadas em 1,5 mL de Trizol ® a -80ºC, até o momento da extração de ácidos nucléicos.

Determinação de marcadores metabólicos e inflamatórios

Foram dosadas no soro a glicemia de jejum pelo método de glicose oxidase; HDL-colesterol após precipitação; colesterol total e triglicerídeos por métodos enzimáticos, sendo calculados os valores de LDL-colesterol pelo método de Friedewald et al. (1972). A insulina plasmática foi determinada pelo método ELISA (Thermo Scientific Multiskan FC) com a utilização de kit para insulina humana (Human Insulin ELISA Kit - Linco Research®). A presença de resistência à insulina foi determinada por meio do índice HOMA-IR (Homeostasis Model Assessment - Insulin Resistance), calculado a partir da fórmula sugerida por Matthews et al (1985), bem como pelo método TyG, recentemente sugerido por Simental-Mendía et al (2008):

$$\text{HOMA-IR} = \frac{\text{IJ (}\mu\text{U/mL) x GJ (mmol/L)}}{22,5}$$

Onde IJ corresponde à insulinemia de jejum e GJ à glicemia de jejum.

TyG: Ln [(triglicerídeos de jejum (mg/dL)) x (glicemia de jejum (mg/dL))/2]

A concentração de PCR sérica foi determinada por ELISA (Thermo Scientific Multiskan FC), empregando-se kit comercial específico (DSL-10-42100 Proteína-C Reativa Ultra-Sensível/ Linco Research S.A.). Foi utilizado o protocolo fornecido pelo fabricante. A sensibilidade de detecção do kit foi de 1,6 ng/mL. A leitura dos dados foi realizada em leitor de ELISA (Thermo Scientific Multiskan FC). Os resultados foram expressos em ng/mL. O ácido úrico foi dosado por

método colorimétrico enzimático, no aparelho Cobas Mira Plus – Roche, de acordo com recomendações do fabricante.

As concentrações das citocinas plasmáticas foram determinadas por ELISA (Thermo Scientific Multiskan FC), empregando-se kits comerciais específicos para cada citocina (IL-6, IL-18, PAI-1, TNF-α, Il-1β e IL-10) (Biosource/Sellex/Europe S.A.). A concentração de adiponectina foi determinada por ELISA (Thermo Scientific Multiskan FC), empregando-se kit comercial específico (Linko Research S.A.). Foi utilizado o protocolo fornecido pelo fabricante. A sensibilidade de detecção do kit foi de 0,5 ng/mL. A leitura dos dados foi realizada em leitor de ELISA (Thermo Scientific Multiskan FC).

Análise de peroxidação de lipídios

Os níveis de MDA foram estimados no soro por meio do Teste de Substâncias Reativas ao Ácido Tiobarbitúrico – TBARS, de acordo com metodologia descrita por Buege e Aust (1978). Alíquotas de 400 µL de soro foram adicionadas a tubos de ensaio contendo a solução de TBARS (ácido tricloroacético a 15% e ácido tiobarbitúrico a 0,375%, dissolvidos em HCL 0,25 N), e então aquecidas em banho-maria a 90 °C por 15 minutos. Após a incubação, a solução foi resfriada e centrifugada a 1000 x g. O sobrenadante foi utilizado para a leitura de absorbância a 535 nm em espectrofotômetro (Shimadzu®, modelo UV – 1601) e os resultados foram expressos em nanomol de equivalentes de MDA por mL de plasma, utilizando-se o coeficiente de extinção molar de 1,56 x 105 $M^{-1}.cm^{-1}$.

Extração de ácidos nucléicos

A extração de ácidos nucléicos foi realizada por meio do MasterPure™ Complete DNA and RNA Purification Kit (Epicentre), sendo as células lisadas e os ácidos nucléicos precipitados de acordo com as determinações do fabricante. Após a extração de DNA e RNA, as concentrações e grau de pureza foram determinados em NanoDrop (NanoDrop 2000C, Thermo Scientific®, EUA), por meio de leitura de absorbância a 260nm e pela razão 260/280nm, respectivamente.

Retrotranscrição

Alíquotas de RNA foram padronizadas à concentração de 2 ng/µL. Foram adicionados 10 µL de amostra a 10 µL de master mix (2 µL de solução tampão, 0,8 µL de dNTP, 2 µL de primers randômicos, 1 µL de transcriptase reversa, 1 µL de inibidor de RNAse e 3,2 µL de água livre de nucleasse) e então incubados em termociclador a 25 ºC durante 10 minutos, 37 ºC durante 120 minutos, 85 ºC por 5 minutos, seguidos de resfriamento a 4 ºC. Após a síntese da dupla fita a partir do RNA total, as concentrações de cDNA de cada amostra foram determinadas em espectrômetro NanoDrop (NanoDrop 2000C, Thermo Scientific®, EUA), posteriormente ajustadas para uma mesma concentração, e estocados a -80°C.

Expressão gênica – Reação da polimerase em cadeia em tempo real (qPCR)

A reação foi realizada utilizando o cDNA sintetizado adicionado à solução master mix (Invitrogen®).

As reações foram feitas em triplicatas, pipetadas manualmente em capela com fluxo de ar. As reações foram feitas por Real-Time PCR (7900 HT Fast Real-Time PCR System – Applied Biosystem, Singapore) e analisado no programa SDS 2.4 (Apllied Biosystem software).

Os valores de C_T foram calculados por *software* fornecido pelo fabricante, sendo o C_T o ciclo de amplificação em que o sinal de fluorescência emitida está acima dos níveis de amplificação inespecífica e inversamente proporcional ao número de cópias inicial da amostra.

Para a normalização dos dados, as expressões de cada gene foi analisada em relação à expressão do gene constitutivo 18S rRNA.

Metilação de genes

O DNA extraído foi tratado com bissulfito de sódio (EpiTect ® Fast DNA Bisulfite Kit, Qiagen), conforme instruções do fabricante, para identificação de citosinas metiladas e não metiladas. Após este passo, foi quantificado em NanoDrop (NanoDrop 2000C, Thermo Scientific®, EUA) e diluído a 5 ng/mL para

análise de metilação global (Line-1) e a 10 ng/mL para metilação de genes específicos.

As análises de metilação foram realizadas pelo método High Resolution Melting (HRM) Real Time PCR (7900 HT Fast Real-Time PCR System – Applied Biosystem, Singapore), e analisado nos softwares SDS 2.4 e HRM v.2.0 (Apllied Biosystem software).

O DNA genômico tratado com bissulfito foi amplificado com as seguintes combinações de primers:

Tabela 2: Caracterização dos oligonucletídeos utilizados para a determinação de metilação de DNA, por *High Resolution Melting*, em células mononucleares.

Gene ou sítios específicos	**Sequência do Primer**	**Número de CpGs analisados/ Tamanho do amplicon (bp)**
LINE-1		
Foward	5’ GCGAGGTATTGTTTTATTTGGGA 3’	8 / 141
Reverse	5’ CGCCGTTTCTTAAACC 3’	
IL-6		
Foward	5’ TTATGTAGGAAAGAGAATTTGGTTTAG 3’	5 / 181
Reverse	5’ AAAAAATAAAATCATCCATTCTTCAC 3’	
IL-18		
Foward	5’ GAAAGAGGTATAGGTTTTGGAAGG 3’	
Reverse	5’ CAATTCCTTACTAACTATCCAAACAA 3’	
TNF-α		
Foward	5’ TTTTGGAAAGGATATTATGAGTATTGA 3’	4 / 99
Reverse	5’ CTAAAACCCTAAAACCCCCCTAT 3’	
CRP		
Foward	5’	4 /316

	GGTAATTTAGTAGTTATAGGAGTTTGTAATAAAT 3’	
Reverse	5’ AAAACACAACAACTTCTCCATAATCA 3’	
PAI-1		
Foward	5’ TGTGTTTGGTTGTAGGGTTAAGA 3’	7 / 151
Reverse	5’ TTACTTTTCTCCTACCTAAAATTCTCA 3’	

Análises de consumo alimentar e de estilo de vida

O consumo habitual foi estimado por meio de questionário de frequência alimentar de consumo semi-quantitativo adaptado do estudo PREDIMED, validado para a população espanhola (FERNÁNDEZ-BALLART et al, 2009). Escores de adesão ao padrão de dieta mediterrânea e de Índice de Qualidade da Dieta foram calculados para avaliar a qualidade da dieta. O padrão de dieta mediterrânea foi avaliado conforme sugerido por Trichopoulou et al. (2003), considerando-se o consumo abaixo ou acima da mediana (categorizada por sexo) de ingestão de alimentos típicos da dieta mediterrânea. O índice de qualidade da dieta foi calculado de acordo com a adaptação do Healthy Eating Index (GUENTHER et al., 2008) para a população brasileira (PREVIDELLI et al., 2011), sendo calculado de acordo com as recomendações de cada grupo alimentar. Dados sobre hábito de fumar e suplementação de vitaminas e minerais foram investigados em questionários estruturados. A prática de atividade física foi avaliada por meio do Questionário Internacional de Atividade Física (IPAQ) (MATSUDO et al., 2001), sendo classificados como ativos os indivíduos com prática de atividade física ≥150 minutos/semana.

5.4. Análises Estatísticas

Os dados registrados foram revisados com o objetivo de detectar informações ausentes e inconsistências nos dados e excluídos os *outliers*. As variáveis quantitativas com distribuição normal (segundo o teste de Kolmogorov-Smirnov e Shapiro-Wilk) foram expressas em média e desvio padrão (DP) e aquelas que não apresentaram a distribuição normal foram expressas em mediana e intervalo interquartílico. Para as variáveis qualitativas foi apresentada a distribuição de frequências.

Foi utilizado o teste de qui-quadrado de Pearson para comparação de proporções e, quando necessário, este foi substituído pelo teste exato de Fisher. Para a comparação de médias foram utilizados os testes t de Student (2 grupos) e a análise de variância (3 ou mais grupos). No caso de variáveis cuja distribuição não era normal foram utilizados os testes de Mann Whitney (para comparação de dois grupos independentes) e o teste de Kruskal-Wallis (3 ou mais), sendo que para esse último, uma vez detectada diferença entre os grupos, foi aplicado o teste *post hoc* de Tukey.

Para os testes de associações foi utilizado o coeficiente de correlação de Pearson para variáveis com distribuição normal ou Spearman nos demais casos, sendo corrigidos pelo método de *False Discovery Rate* de Benjamini-Hochberg.

Modelos de regressão linear múltipla e de Poison foram utilizados para identificar fatores preditivos da metilação de DNA e das concentrações séricas ou plasmáticas dos biomarcadores inflamatórios em relação aos determinantes da SM, após avaliação dos pressupostos de normalidade, homocedasticidade e colinearidade. Variáveis cujo $p<0,2$ foram mantidas nos modelos de regressão múltipla.

O nível de significância (α) adotado para todos os testes de hipóteses foi de 5% e os contrastes foram levantados em nível bilateral.

A análise estatística foi realizada utilizando-se os programas STATA 9.1 (StataCorp LP, College Station, TX) e SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

5.5. Aspectos éticos

Ambos os estudos foram submetidos à avaliação ética, sendo o primeiro aprovado (Ref. nº 005/2011), em 18/02/2011, pelo Comitê de Ética em Pesquisa com Seres Humanos da Universidade Federal de Viçosa, e o segundo pelo Comitê Ético de Investigação da Clínica da Universidade de Navarra (Ref. nº 79/2005), de acordo com os princípios da Declaração de Helsinki. Todos os voluntários assinaram o Termo de Consentimento Livre e Esclarecido e receberam resultados e orientações individualizadas.

6. RESULTADOS

Estudo 1

6.1. *LINE-1* and inflammatory gene methylation levels are early biomarkers of metabolic changes: association with adiposity

Estudo 2

6.2. Interleukin-6 is a better metabolic biomarker than interleukin-18 in young healthy adults

6.3. Higher fruit intake is related to *TNF-α* hypomethylation and better glucose tolerance in healthy subjects

LINE-1 and inflammatory gene methylation levels are early biomarkers of metabolic changes: association with adiposity

Submetido à revista Biomarkers

Running title: DNA methylation as biomarker of metabolic changes

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This work was supported by CNPq, Spanish Ministry of Economy and Competitiveness and CIBERobn (Carlos III Institute of Health), and author declares have no conflicts of interest.

Keywords: Global DNA methylation, metabolic syndrome, healthy eating index, IL-6, SERPINE1

Abstract

We analyzed whether global and inflammatory genes methylation can be early predictors of metabolic features and their associations with the diet, in a cross-sectional study (n=40). Higher global methylation was associated to adiposity, insulin resistance, and lower quality of the diet. Methylation of *IL-6, SERPINE1* and *CRP* genes was related to adiposity traits and macronutrients intake. *SERPINE1* higher methylation was also related to some metabolic alterations. *CRP* methylation was better predictor of insulin resistance than CRP plasma concentrations. Global and inflammatory gene promoter higher methylation can be good early biomarkers of adiposity and metabolic changes and are associated to the quality of the diet.

Keywords: Global DNA methylation, metabolic syndrome, healthy eating index, IL-6, SERPINE1

Introduction

Epigenetics has been proposed as a possible factor for understanding interindividual differences in the susceptibility to disease and response to therapy (Martínez et al., 2014). Epigenetics involves heritable changes in gene expression that cannot be explained by changes in DNA sequence, being the addition of a methyl group to the pyrimidine ring in position 5 of cytosine in CpG dinucleotides the most studied epigenetic mechanism (Jones and Takai, 2001). CpG islands methylation may be increased or decreased in response to environmental factors, such as dietary components (Uriarte et al., 2013, Amaral et al., 2014), and also to metabolic diseases, including diabetes (Toperoff et al., 2012) and cardiovascular diseases (Cash et al., 2011).

Studies about obesity epigenetics have reported that there is a paradoxical downregulation of lipo/adipogenesis and upregulation of inflammation pathways in subcutaneous adipose tissue, and that this CpG sites methylation is also correlated to metabolic disturbances (Pietiläinen et al., 2015). Moreover, differential methylation analyzes between obese subjects with or without Metabolic Syndrome (MetS) has showed that the most overrepresented genes were those related to structural components of the cell membrane, cell cycle regulation and inflammation (Guénard et al., 2014).

DNA methylation can be assessed globally, via repetitive elements of the genome, such as the *LINE-1* (long interspersed element-1) retrotransposon, which is associated with genomic instability and chromosomal abnormalities, or in the promoter regions of specific genes, which can be activated or silenced according to

the level of methylation (Martín-Núñez et al., 2014). Some investigations have linked changes in DNA methylation to obesity and components of the MetS, even before the onset of clinical symptoms (Lomba et al., 2010, Kulkarni et al., 2011, Zhao et al., 2012).

LINE-1 methylation have been associated to fasting glucose, blood lipids and a greater risk for MetS in the presence of obesity (Turcot et al., 2012), and may be considered a good biomarker of response to treatment for weight loss in obese (García-Lacarte et al., 2015).

Since pro-inflammatory cytokines, such as Interleukin-6 (IL-6), Plasminogen activator inhibitor 1 (PAI-1) and C reactive protein (CRP) are increased in obese subjects, mainly in those with higher central (Hermsdorff et al., 2011, McArdle et al., 2013, Lopez-Lagarrea et al., 2013, Paepegaey et al., 2014), it becames an interesting point of investigation about epigenetic control. In fact, *IL-6* promoter methylation was changed after weight loss by Roux-en Y gastric bypass in a study with obese subjects (Kirchner et al., 2013), and *SERPINE1* (gene enconding PAI-1) higher methylation was a good predictor of major reductions in body weight and total fat mass in obese subjects with MetS that received energy-restricted diets (Lopez-Lagarrea et al., 2013). However, a little is known about the CRP promoter methylation.

Search for new biomarkers is interesting in order to personalize and improve the treatment and prevention of diseases. This issue is particularly important if determined early, before the development of the clinical features of the disease (Carraro et al., 2015). Several early predictors of MetS had been investigated (Park

et al., 2013, Liao et al., 2013), including in peripheral blood mononuclear cells (PBMC) (Afman et al., 2014). In this sense, epigenetic marks can be used as biomarkers of disease or response (Goni et al., 2014) and it is an interesting approach, due to its reversible nature. Nutrition and lifestyle interventions could benefit from the understanding of the methylation marks and processes in order to improve the therapeutic management of chronic diseases. Our hypotheses is that subjects with lower global and inflammatory genes methylation could be more prone to develop metabolic changes and that this parameters can be used as early biomarkers these alterations.

This study aimed to analyze the association between the methylation levels of *LINE-1* and several inflammatory genes with MetS components and habitual diet in apparently healthy subjects, seeking the description of early epigenetic biomarkers of metabolic outcomes.

Subjects and methods

Subjects

This is a cross-sectional study aims to evaluate determinants of MetS in health professionals. Health professionals between 20 and 59 years old from Viçosa (Brazil) were invited to participate in the study. The study included all those who signed the consent, which were not in use of corticosteroids or antibiotics, without hospitalization or serious illness in recent months and that, in the case of females, were not pregnant or breastfeeding. A sample of 226 volunteers were selected, from which, a group of 40 individuals was randomly selected by age and gender, blinded to the group allocation, for the study of DNA methylation (Figure

1). The average age of the selected group was 28.9 ± 7.0 (22-53) years old, average body mass index (BMI) was 22.4 ± 3.4 kg/m^2, and 79% of them were women. This project was approved by the Ethics Committee on Human Research of the Universidade Federal de Viçosa (Ref. No. 005/2011), according to the principles of the Helsinki's Declaration.

Anthropometry and body fat composition

Anthropometric measurements were conducted according to routine standardized methods and body fat content was determined by bioelectric impedance (Biodynamics 310®, Biodynamics Corporation, Seattle, WA). The values of BMI (kg/m^2) and total body fat (%) were assessed as indicators of total adiposity, while waist circumference (WC), assessed in the midpoint between the last rib and the iliac crest, and waist-hip ratio (WHR) were measured as markers of central fat accumulation.

Blood pressure and biochemical assessments

Systolic (SBP) and diastolic (DBP) blood pressures were measured, following WHO criteria (Whitworth and Chalmers, 2004), using a digital HEM 142INT sphygmomanometer (OMROM Healthcare Co, Kyoto, Japan). Venous blood samples were drawn after a 12-h overnight fast. Plasma (EDTA) and serum were separated from whole blood by centrifugation at 3500 rpm, at 4º C for 10 min (Megafuge 11R, Thermo Scientific, Waltham, MA), and were immediately frozen at -80ºC until assay. Serum levels of triglycerides, total cholesterol (TC), high density lipoprotein–cholesterol (HDL), glucose and insulin were measured by standard methods as previously described (Hermsdorff et al., 2011). The plasma low density lipoprotein-cholesterol (LDL) data were calculated by the Friedewald equation

(Friedewald et al., 1972). Insulin resistance was estimated by the HOMA-IR, defined as fasting glucose (mmol/L) x fasting insulin (lU/ml)]/22.5 (Matthews et al., 1985), and the TyG index, calculated as Ln [(triglycerides (mg/dl)) x (glucose (mg/dl))/2] (Simental-Mendía et al., 2008). Serum CRP concentrations were determined by ELISA (Multiskan FC, Thermo Scientific) using the DSL-C-reactive protein ultra sensitive kit (Ref. 10-42100, Linco Research Inc., St Charles, MO), and subjects with CRP concentrations ≥ 10 mg/L were excluded to the sample to avoid acute inflammation interference. Uric acid was determined by an enzymatic colorimetric method in the Cobas Mira Plus equipment (Roche Diagnostics, Basel, Switzerland). Plasma concentrations of different cytokines, tumor necrosis factor-alpha (TNF-α) (Catalog number LHC3011), interleukins 1β, 6 and 10 (IL-6, IL-1β, IL-10) (Catalog number LHC0061, LHC0011 and LHC0101, respectively), and adiponectin (Catalog number LHP0041) were determined by multiplex ELISA, using a commercial kit (Biosource / Sellex). PAI-1 plasma concentrations were determined by ELISA, using a commercial kit (Invitrogen, Catalog number KHC3071).

Dietary intake assessment

Dietary intake was assessed with a validated semi-quantitative food frequency questionnaire with 136 food items (MARTIN-MORENO et al., 1993). Nutrient intake was estimated using an *ad hoc* computer software specifically developed for this aim, including the latest available information from Brazilian food composition tables. Healthy Eating Index (HEI), an indicator of diet quality developed according to current nutritional recommendations, was measured

according by Previdelli et al. (Previdelli et al., 2011) using a specific adaptation of HEI (Guenther et al., 2008) for the Brazilian population.

Peripheral blood mononuclear cell (PBMC) isolation

After separating the plasma by centrifugation, the remaining homogenate (red and white cells) was diluted in saline, Polymorphprep ® (AXIS-SHIELD PoC AS, Oslo, Norway) was added and then centrifuged at 450 rpm at room temperature for 30 minutes. Two subsequent washes were performed with dPBS, and the extracted cells were stored at -80 ° C until the time of analysis.

DNA isolation and methylation profile

DNA from PBMC was isolated by using the MasterPure kit (Epicenter, Madison, WI), and its quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA).

The quantitative analysis of 5-methylcitosine levels in LINE-1 and three gene promoters was determined after bisulfite treatment (EZ DNA methylation kit; Zymo Research, Irvine, CA) by using the high-resolution melting curve analysis (HRM) method in a 7900HT Fast Real-Time PCR System (Life Technologies Co., Carlsbad, CA), and analyzed by SDS 2.4 and HRM v.2.0 softwares (Life Technologies). The genes were selected because of their influence on obesity-related inflammation: *IL-6*, *SERPINE1* (encoding for the protein plasminogen activator inhibitor-1 or PAI-1) and *CRP*. Bisulfite-treated genomic DNA was amplified using the pairs of primers indicated in table 1:

Statistical analyses

Individuals were categorized according to gene methylation medians or global methylation medians for some analyses (Supplementary files). Results are reported as mean ± SEM or median (Interquartile range), according to distribution and tests were two-sized. Statistical comparisons between groups were performed by the parametric Student's t test or Mann–Whitney U test according to variable distribution, which was determined by the Shapiro–Wilk test. The variance was similar between the groups compared. Chi-square analyzes were performed to evaluate potential associations between lifestyle features (physical activity, smoking status, pharmacological agents (antidiabetic, antihypertensive and lipid-lowering drugs) and nutritional supplements) and global and gene methylation status. Pearson correlations were fitted to evaluate the potential correlations of LINE-1 or gene promoter methylation with adiposity indicators, metabolic features, and dietary factors. Benjamini-Hochberg method was performed to control the false discovery rate. Multiple linear regression models were performed to analyze the prediction of LINE-1 methylation (outcome) for selected variables ($p<0.20$). To analyze the prediction of inflammatory gene promoter methylation for selected variables, a Poisson regression was performed and adjusted by age and gender. Non-normally distributed variables were log-transformed prior to inclusion in linear regression analyses. Analyses of area under the curve (AUC) by ROC curve were performed to compare methylation and plasma cytokines as biomarkers by using the Hanley and McNeil method (JA and BJ., 1982).

Statistical analyses were performed with STATA 9.0 software (StataCorp LP, College Station, TX). A P-value <0.05 was considered statistically significant.

Results

Global methylation in *LINE-1* was positively associated with markers of adiposity, such as WC and BMI. These subjects evidenced higher circulating levels of glucose, insulin and TNF-α and lower adiponectin concentration, as well as higher HOMA-IR and lower quality of the diet (Supplementary file 1).

Global methylation levels were not influenced by gender and lifestyle features (data not shown). Futhermore, *LINE-1* methylation showed positive correlations with adiposity features (WC and BMI) (Figure 2).

According to a multiple linear regression analysis, *LINE-1* methylation was predicted by WC, TyG index, TNF-α concentrations and HEI, being the diet quality an independent predictor (Table 2).

Concerning methylation of the inflammatory genes, all the analyzed genes were associated with anthropometric traits. Subjects with higher methylation of *IL-6* had higher values of weight, WC, WHR and BMI. CRP was related to weight and SERPINE1 to WC, WHR and BMI. *SERPINE1* methylation also showed correlations with plasma lipid profile, uric acid, adiponectin, HEI and potassium intake, whereas *CRP* methylation was positively associated with the intake of total, saturated and monounsaturated fatty acids (Supplementary file 2). The methylation of inflammatory genes was not related to other lifestyle features and gender.

The methylation levels of *IL-6*, *SERPINE1* and *CRP* did not correlate with the concentrations of these inflammatory biomarkers in plasma (Table 3). However, these methylation levels were also associated with adiposity features. The mainly correlations, beyond these with anthropometric and metabolic features, are shown in Figure 3. Subjects with higher *IL-6* gene methylation were more prone to present higher WC ($p<0.001$), BMI ($p<0.001$), SBP ($p=0.003$) and WHR ($p=0.004$). Those with higher *SERPINE1* methylation had increased prevalence of higher WC ($p<0.001$), WHR ($p<0.001$) and uric acid ($p=0.004$). *CRP* gene promoter methylation, on the other hand, was not correlated to any variable after Benjamini-Hochberg correction. The methylation levels of the inflammatory genes were predictors of several MetS components, when analyzed by Poisson's regression, even after adjusting by age and gender. However, the prevalence ratios were not clinically relevant (data not shown). On the other hand, *CRP* methylation was a better biomarker of TyG index than CRP plasma concentrations (Figure 4).

Discussion

Epigenetic biomarkers have received attention due to new evidences about interactions between the environment, the epigenome and metabolic conditions. These biomarkers may contribute to personalize dietary treatments, allowing the early detection of individuals predisposed to certain diseases, and the identification of those patients who will respond better to an intervention (Campión et al., 2010, Milagro et al., 2013). These epigenectics changes had already been used like good biomarkers of cancers (Chen et al., 2014, Yang et al., 2013).

Although the methylation levels may vary according to the tissue, several studies have reported that blood cell samples are able to reflect epigenetic alterations in primary tissue (Vlassov et al., 2010), as well as being easier, less invasive and economic options (Al-Moundhri et al., 2010). In this context, LINE-1 methylation in PBMC has been studied in relation to the development of several diseases (Piyathilake et al., 2011, Kitkumthorn et al., 2012).

In this sense, there is evidence of the importance of epigenetic programming on obesity and MetS onset, and the relationships between global methylation and MetS have been studied in various conditions (Gallou-Kabani and Junien, 2005, Piyathilake et al., 2013). Animal studies described differences in gene promoter methylation level related to obesity, for example, leptin promoter hypermethylation in rats fed a high fat diet (Milagro et al., 2009). However, the relationship between global methylation and MetS seems somewhat controversial. Piyathilake et al. (Piyathilake et al., 2013) described associations between lower *LINE-1* methylation levels in PBMC and higher BMI, WC, body fat and HOMA-IR. Turcot et al. found inverse associations between *LINE-1* methylation in visceral adipose tissue of severely obese individuals and DBP and fasting glucose (Turcot et al., 2012), which is not in agreement with the relationships found in the current trial. On the other hand, other study found associations between higher global methylation and higher cardiovascular risk (Sharma et al., 2008).

Some investigations concerning the methylation patterns of specific inflammatory genes associated with obesity or adiposity have also been performed (Wang et al., 2010), since low-grade inflammation appears to be a link between obesity and associated metabolic disorders (McArdle et al., 2013). *DNMT* inhibitors

(Altucci and Stunnenberg, 2009) and anti-inflammatory effects of dietary compounds (vel Szic et al., 2010) can contribute to elucidate the interrelationship between inflammation, chronic disease and epigenetics, particularly DNA methylation.

Fluctuations in the methylation levels of specific gene promoters regulate the function of these regions to stimulate or inhibit the expression of genes by modifying the interaction of transcription factors and methyl-DNA binding proteins (Ross, 2003). Our hypothesis was that the promoters of the proinflammatory genes should be hypomethylated in order to upregulate the expression of these genes. However, the results have not confirmed this hypothesis.

The most plausible explanation for this unexpected finding would be that the higher concentrations of circulating pro-inflammatory cytokines related to metabolic disorders are, in many cases, caused by increased secretion from macrophages infiltrated in visceral adipose tissue (Brogren et al., 2008) and not from PBMC. Moreover, we found no correlation between promoter region methylation levels and cytokine circulating levels, which suggests that other regulatory factors, besides DNA methylation, may be involved. Furthermore, it is noteworthy that the inflammatory response is a complex mechanism that requires control of many factors, including cell type, tissue involved, type of inflammatory stimuli and the responsiveness of different signaling pathways (Medzhitov and Horng, 2009). In general, it has been reported that tissue-specific chronic inflammation is characterized by histone hypoacetylation and CpG hypermethylation, which contribute to persistent inflammatory response (Bäckdahl et al., 2009).

The binding of several transcription factors to the studied genes may help to explain part of the results. The analyzed CpGs in the promoter region of *IL-6* are in a binding site for C/EBP-β α and glucocorticoid receptor β (GR-β) isoform (Messeguer et al., 2002). The inhibition of binding of these transcription factors to DNA sequence due to increased methylation can lead, in the first case, to decreased modulation of leptin gene and lower regulation of energy homeostasis (Wang et al., 1995), and, in another case, to lower expression of anti-inflammatory proteins (Rhen and Cidlowski, 2005). In both cases, the hypermethylation of this region could be related to greater adiposity.

Regarding the promoter region of *SERPINE1*, the CpGs analyzed in this region are located in the binding regions of C/EBP-α and AhR:Arnt (Messeguer et al., 2002). Higher methylation of these sites may result in less control of energy homeostasis(Wang et al., 1995). Moreover, the AhR:Arnt complex is related to the regulation of circadian rhythms and is modulated by different food bioactive compounds. So, the methylation of the AhR:Arnt-related CpGs might influence the response to the beneficial actions of these compounds (Denison et al., 2002).

Finally, the CpG sites located in the *CRP* promoter are in the binding region of the GR-β and XBP1 (Messeguer et al., 2002). X-box binding protein 1 (XPB1) controls *IL-6* expression and endoplasmic reticulum stress, suggesting that changes in the functionality of this transcription factor might influence the inflammatory processes (Kaser et al., 2008).

Since the epigenome is flexible and can change in response to the diet (vel Szic et al., 2010), the restoration of the “healthy” epigenetic marks could be a

therapeutic aim in order to reduce the risk of chronic disease development. Several dietary factors have been reported to affect DNA methylation levels, such as methyl donors (Anderson et al., 2012) and protein or caloric restriction (Amaral et al., 2014). The intake of hypercaloric or isocaloric high fat diets are known to promote weight gain and changes in the methylation of leptin (Milagro et al., 2009) and fatty acid synthase promoter (Lomba et al., 2010). In addition, maternal intake of high fat diets can promote epigenetic modifications and alter the expression of nerohormones in the offspring, driving animals' choice for foods rich in sugar and fat (Vucetic et al., 2010). These epigenetic changes may be involved in the increased prevalence of obesity in many countries, showing that obesity and its comorbidities go beyond the simple result of energy balance (Milagro et al., 2013).

Although we can not assume a causal relationship, our results suggest that lower quality of the diet is associated with increased global methylation and with components of MetS. One of the nutrients that might be involved in this regulation is vitamin B12. Vitamin B12 is an important cofactor for the resynthesis of methionine (and, hence, of SAM) from homocysteine, and low quality diets rich in animal foods and poor in vegetables, fruits and legumes tend to be richer in this vitamin (Herrmann et al., 2001). Although this vitamin is important to erythropoiesis, amino acid and nucleic acid metabolism, and maintenance of the central nervous system, in excess can be related to higher DNA methylation. This inference seems to be supported by the lower DNA methylation levels found among vegetarians, individuals who are more prone to present low intakes of vitamin B12 (Geisel et al., 2005).

Despite the very clear relationships found between DNA methylation and the quality of the diet and metabolic risk, this study has some limitations, mainly the transversal nature of the (small) sample, which does not allow the establishment of cause-effect relationships. However, the strongest point of this work is that the results have been obtained from mostly healthy people, before the onset of obesity and MetS, highlighting the putative use of these epigenetic biomarkers as early predictors of metabolic complications.

The results reveal clear and negative relationships between diet quality and global and *SERPINE1* promoter methylation, and between the higher methylation of *LINE-1* and inflammatory gene promoters and features (mainly adiposity ones), even in apparently healthy subjects. It is an interesting starting point to follow up studies in order to verify if subjects with changes in DNA methylation levels will, in fact, develop metabolic syndrome or other non-communicable diseases.

Acknowledgements

The technical assistance of Ana Lorente is acknowledged.

Declaration of Interest: All the authors declare that there are any competing financial interests in relation to this work. This work was supported by Brazilian National Council for Scientific and Technological Development (CNPq - CNPq process 481518/2011-8; process 481019/2012-0 and process 444519/2014-9), Spanish Ministry of Economy and Competitiveness (ref. AGL2013-4554-R) and Carlos III Institute of Health (CIBERobn). We also thanks Brazilian Education

Personnel Improvement Coordination (Capes) for a PhD “sandwich” scholarship awarded to JCC Carraro (process number 99999.014962/2013-06). J Bressan is CNPq fellows.

References

Afman L, Milenkovic, D ,Roche, HM (2014). Nutritional aspects of metabolic inflammation in relation to health—insights from transcriptomic biomarkers in PBMC of fatty acids and polyphenols. ***Mol Nutr Food Res,*** **58: 1708-1720.**

Al-Moundhri MS, Al-Nabhani, M, Tarantini, L, Baccarelli, A ,Rusiecki, JA (2010). The Prognostic Significance of Whole Blood Global and Specific DNA Methylation Levels in Gastric Adenocarcinoma. ***PLoS ONE,*** **5: e15585.**

Altucci L ,Stunnenberg, HG (2009). Time for epigenetics. ***Journal of Biochemical Cell Biol,*** **41: 2-3.**

Amaral CL, Milagro, FI, Curi, R ,Martínez, JA (2014). DNA Methylation Pattern in Overweight Women under an Energy-Restricted Diet Supplemented with Fish Oil. ***BioMed Res Int,*** **2014: 10.**

Anderson OS, Sant, KE ,Dolinoy, DC (2012). Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. ***J Nutr Biochem,*** **23: 853-859.**

Bäckdahl L, Bushell, A ,Beck, S (2009). Inflammatory signalling as mediator of epigenetic modulation in tissue-specific chronic inflammation. ***Int J Biochem Cell Biol,*** **41: 176-184.**

Brogren H, Sihlbom, C, Wallmark, K, Lönn, M, Deinum, J, Karlsson, L ,Jern, S (2008). Heterogeneous glycosylation patterns of human PAI-1 may reveal its cellular origin. ***Thromb Res,*** **122: 271-281.**

Campión J, Milagro, F ,Martínez, JA (2010). Epigenetics and obesity. ***Progr Mol Biol Trans Sci,*** **94: 291-347.**

Carraro J, Hermsdorff, H, Puchau, B, Zulet, MÁ, Milagro, F, Bressan, J ,Martínez, JA (2015). Interleukin-6 is a better metabolic biomarker than interleukin-18 in young healthy adults. ***J Physiol Biochem*****: 1-9.**

Cash HL, Mcgarvey, ST, Houseman, EA, Marsit, CJ, Hawley, NL, Lambert-Messerlian, GM, Viali, S, Tuitele, J ,Kelsey, KT (2011). Cardiovascular disease risk factors and DNA methylation at the LINE-1 repeat region in peripheral blood from Samoan islanders. ***Epigenetics,*** **6: 1257-1264.**

Chen H, Yu, Y, Rong, S ,Wang, H (2014). Evaluation of diagnostic accuracy of DNA methylation biomarkers for bladder cancer: a systematic review and meta-analysis. ***Biomarkers,*** **19: 189-197.**

Denison MS, Pandini, A, Nagy, SR, Baldwin, EP ,Bonati, L (2002). Ligand binding and activation of the Ah receptor. ***Chemico-Biol Interact,*** **141: 3-24.**

Friedewald WT, Levy, RI ,Fredrickson, DS (1972). Estimation of the Concentration of Low-Density Lipoprotein Cholesterol in Plasma, Without Use of the Preparative Ultracentrifuge. ***Clin Chem,*** **18: 499-502.**

Gallou-Kabani C ,Junien, C (2005). Nutritional Epigenomics of Metabolic Syndrome: New Perspective Against the Epidemic. ***Diabetes,*** **54: 1899-1906.**

Garcia-Lacarte M, Milagro FI, Zulet MA, Martínez JA, Mansego ML (2015). LINE-1 methylation levels, a biomarker of weight loss in obese subjects, are influenced by dietary

antioxidant capacity. *Redox Report* [Online] Avaiable at: http://www.maneyonline.com/doi/abs/10.1179/1351000215Y.0000000029 Accessed on 10 November 2015.

Geisel J, Schorr, H, Bodis, M, Isber, S, Hübner, U, Knapp, J-P, Obeid, R ,Herrmann, W 2005. The vegetarian lifestyle and DNA methylation. *Clin Chem Lab Med.*

Guénard F, Tchernof A, Deshaies Y, Pérusse L, Biron S, Lescelleur O, Biertho L, Marceau S, Vohl, M. C. (2014). Differential methylation in visceral adipose tissue of obese men discordant for metabolic disturbances. *Physiological genomics,* 46(6): 216-222.

Goni L, Milagro, F, Cuervo, M ,Martínez, J (2014). Single-nucleotide polymorphisms and DNA methylation markers associated with central obesity and regulation of body weight. *Nutr Rev,* 72: 673-690.

Guenther PM, Reedy, J ,Krebs-Smith, SM (2008). Development of the Healthy Eating Index-2005. *J Am Diet Assoc,* 108: 1896-1901.

Hermsdorff H, Zulet, MÁ, Puchau, B ,Martínez, JA (2011). Central Adiposity Rather Than Total Adiposity Measurements Are Specifically Involved in the Inflammatory Status from Healthy Young Adults. *Inflammation,* 34: 161-170.

Herrmann W, Schorr, H, Purschwitz, K, Rassoul, F ,Richter, V (2001). Total Homocysteine, Vitamin B12, and Total Antioxidant Status in Vegetarians. *Clin Chem,* 47: 1094-1101.

Ja H ,Bj., M (1982). The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiol,* 143: 29-36.

Jones PA ,Takai, D (2001). The Role of DNA Methylation in Mammalian Epigenetics. *Science,* 293: 1068-1070.

Kaser A, Lee, A-H, Franke, A, Glickman, JN, Zeissig, S, Tilg, H, Nieuwenhuis, EES, Higgins, DE, Schreiber, S, Glimcher, LH, Blumberg, RS (2008). XBP1 Links ER Stress to Intestinal Inflammation and Confers Genetic Risk for Human Inflammatory Bowel Disease. *Cell,* 134: 743-756.

Kirchner H, Nylen C, Laber S, Barrès R, Yan J, Krook A, Zierath JR, Näslund, E. (2014). Altered promoter methylation of PDK4, IL1 B, IL6, and TNF after Roux-en Y gastric bypass. *Surgery for Obesity and Related Diseases*, 10(4): 671-678.

Kitkumthorn N, Tuangsintanakul, T, Rattanatanyong, P, Tiwawech, D ,Mutirangura, A (2012). LINE-1 methylation in the peripheral blood mononuclear cells of cancer patients. *Clin Chim Acta,* 413: 869-874.

Kulkarni A, Chavan-Gautam, P, Mehendale, S, Yadav, H ,Joshi, S (2011). Global DNA Methylation Patterns in Placenta and Its Association with Maternal Hypertension in Pre-Eclampsia. *DNA Cell Biol,* 30: 79-84.

Liao Y-C, Liang, K-W, Lee, W-J, Lee, W-L, Lee, I-T, Wang, J-S, Ting, C-T ,Sheu, WH-H (2013). Leptin to adiponectin ratio as a useful predictor for cardiac syndrome X. *Biomarkers,* 18: 44-50.

Lomba A, Martínez, JA, García-Díaz, DF, Paternain, L, Marti, A, Campión, J ,Milagro, FI (2010). Weight gain induced by an isocaloric pair-fed high fat diet: A nutriepigenetic study on FASN and NDUFB6 gene promoters. *Mol Gen Metabol,* 101: 273-278.

Lopez-Legarrea P, Mansego M L, Zulet MA, Martinez JA. (2013). Serpine1, PAI-1 protein coding gene, methylation levels and epigenetic relationships with adiposity changes in obese subjects with metabolic syndrome features under dietary restriction. *Journal of clinical biochemistry and nutrition*, 53(3): 139.

Martin-Moreno JM, Boyle, P, Gorgojo, L, Maisonneuve, P, Fernandez-Rodriguez, JC, Salvini, S ,Willett, WC (1993). Development and Validation of a Food Frequency Questionnaire in Spain. *Int J Epidemiol,* 22: 512-519.

Martín-Núñez G, Cabrera-Mulero, R, Rubio-Martín, E, Rojo-Martínez, G, Olveira, G, Valdés, S, Soriguer, F, Castaño, L ,Morcillo, S (2014). Methylation levels of the SCD1 gene promoter

and LINE-1 repeat region are associated with weight change: an intervention study. *Mol Nutr Food Res,* 58: 1528-1536.

Martínez J, Milagro, F, Claycombe, K ,Schalinske, K (2014). Epigenetics in adipose tissue, obesity, weight loss and type 2 diabetes. *Advances in Nutrition* 5: 71-81.

Matthews DR, Hosker, JP, Rudenski, AS, Naylor, BA, Treacher, DF ,Turner, RC (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia,* 28: 412-419.

Mcardle MA, Finucane, OM, Connaughton, RM, Mcmorrow, AM ,Roche, HM (2013). Mechanisms of Obesity-Induced Inflammation and Insulin Resistance: Insights into the Emerging Role of Nutritional Strategies. *Front Endocrinol,* 4: 52.

Medzhitov R ,Horng, T (2009). Transcriptional control of the inflammatory response. *Nat Rev Immunol,* 9: 692-703.

Messeguer X, Escudero, R, Farré, D, Nuñez, O, Martínez, J ,Albà, M (2002). PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics,* 18: 333-334.

Milagro FI, Campión, J, García-Díaz, DF, Goyenechea, E, Paternain, L ,Martínez, JA (2009). High fat diet-induced obesity modifies the methylation pattern of leptin promoter in rats. *J Physiol Biochem,* 65: 1-9.

Milagro FI, Mansego, ML, De Miguel, C ,Martínez, JA (2013). Dietary factors, epigenetic modifications and obesity outcomes: Progresses and perspectives. *Mol Aspect Med,* 34: 782-812.

Park KH, Zaichenko, L, Brinkoetter, M, Thakkar, B, Sahin-Efe, A, Joung, KE, Tsoukas, MA, Geladari, EV, Huh, JY, Dincer, F, Davis, CR, Crowell, JA ,Mantzoros, CS (2013). Circulating Irisin in Relation to Insulin Resistance and the Metabolic Syndrome. *J Clin Endocrinol Metab,* 98: 4899-4907.

Paepegaey AC, Genser L, Bouillot JL, Oppert JM, Clément K, Poitou C. (2015). High levels of CRP in morbid obesity: the central role of adipose tissue and lessons for clinical practice before and after bariatric surgery. *Surgery for Obesity and Related Diseases*, 11(1): 148-154.

Pietiläinen KH, Ismail K, Järvinen E, Heinonen S, Tummers M, Bollepalli S, Lyle R, Muniandy M, Moilanen E, Hakkarainen A, Lundbom J, Lundbom N, Rissanen A, Kaprio L, Ollikainen, M. (2015). DNA methylation and gene expression patterns in adipose tissue differ significantly within young adult monozygotic BMI-discordant twin pairs. *International journal of obesity* (2005) [Online] Avaiable at http://www.nature.com/ijo/journal/vaop/naam/abs/ijo2015221a.html Accessed on 10 November 2015.

Piyathilake CJ, Badiga, S, Alvarez, RD, Partridge, EE ,Johanning, GL (2013). A Lower Degree of PBMC L1 Methylation Is Associated with Excess Body Weight and Higher HOMA-IR in the Presence of Lower Concentrations of Plasma Folate. *PLoS ONE,* 8: e54544.

Piyathilake CJ, Macaluso, M, Alvarez, RD, Chen, M, Badiga, S, Siddiqui, NR, Edberg, JC, Partridge, EE ,Johanning, GL (2011). A higher degree of LINE-1 methylation in peripheral blood mononuclear cells, a one-carbon nutrient related epigenetic alteration, is associated with a lower risk of developing cervical intraepithelial neoplasia. *Nutrition,* 27: 513-519.

Previdelli A, Andrade, S, Pires, M, Ferreira, S, Fisberg, R ,Marchioni, D (2011). A revised version of the Healthy Eating Index for the Brazilian population. *Rrev Saúde Pub,* 45: 794-798.

Rhen T ,Cidlowski, JA (2005). Antiinflammatory Action of Glucocorticoids — New Mechanisms for Old Drugs. *New Engl J Med,* 353: 1711-1723.

Ross SA (2003). Diet and DNA Methylation Interactions in Cancer Prevention. *Annals N Y Academy Sci,* 983: 197-207.

Sharma P, Kumar, J, Garg, G, Kumar, A, Patowary, A, Karthikeyan, G, Ramakrishnan, L, Brahmachari, V ,Sengupta, S (2008). Detection of Altered Global DNA Methylation in Coronary Artery Disease Patients. *DNA Cell Biol,* 27: 357-365.

Simental-Mendía LE, Rodríguez-Morán, M ,Guerrero-Romero, F (2008). The Product of Fasting Glucose and Triglycerides As Surrogate for Identifying Insulin Resistance in Apparently Healthy Subjects. *Metab Synd Relat Dis,* 6: 299-304.

Toperoff G, Aran, D, Kark, JD, Rosenberg, M, Dubnikov, T, Nissan, B, Wainstein, J, Friedlander, Y, Levy-Lahad, E, Glaser, B ,Hellman, A (2012). Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. *Hum Mol Gen,* 21: 371-383.

Turcot V, Tchernof, A, Deshaies, Y, Pérusse, L, Bélisle, A, Marceau, S, Biron, S, Lescelleur, O, Biertho, L ,Vohl, M-C (2012). LINE-1methylation in visceral adipose tissue of severely obese individuals is associated with metabolic syndrome status and related phenotypes. *Clin Epigenetics,* 4: 10.

Uriarte G, Paternain, L, Milagro, FI, Martínez, JA ,Campion, J (2013). Shifting to a control diet after a high-fat, high-sucrose diet intake induces epigenetic changes in retroperitoneal adipocytes of Wistar rats. *J Physiol Biochem,* 69: 601-611.

Vel Szic KS, Matladi, NN, Guy, H ,Berghe, WV (2010). Nature or nurture: Let food be your epigenetic medicine in chronic inflammatory disorders. *Biochem Pharmacol,* 80: 1816-1832.

Vlassov VV, Laktionov, PP ,Rykova, EY (2010). Circulating Nucleic Acids as a Potential Source for Cancer Biomarkers. *Curr Mol Med,* 10: 142-165.

Vucetic Z, Kimmel, J, Totoki, K, Hollenbeck, E ,Reyes, TM (2010). Maternal High-Fat Diet Alters Methylation and Gene Expression of Dopamine and Opioid-Related Genes. *Endocrinol,* 151: 4756-4764.

Wang N, Finegold, M, Bradley, A, Ou, C, Abdelsayed, S, Wilde, M, Taylor, L, Wilson, D ,Darlington, G (1995). Impaired energy homeostasis in C/EBP alpha knockout mice. *Science,* 269: 1108-1112.

Wang X, Zhu, H, Snieder, H, Su, S, Munn, D, Harshfield, G, Maria, BL, Dong, Y, Treiber, F, Gutin, B ,Shi, H (2010). Obesity related methylation changes in DNA of peripheral blood leukocytes. *BMC Med,* 8: 87.

Whitworth JA ,Chalmers, J (2004). World Health Organisation-International Society of Hypertension (WHO/ISH) hypertension guidelines. . *Clin Exp Hypertens,* 26: 747-752.

Yang Q, Gao, J, Xu, L, Zeng, Z, Sung, JJY ,Yu, J (2013). Promoter hypermethylation of BCL6B gene is a potential plasma DNA biomarker for gastric cancer. *Biomarkers,* 18: 721-725.

Zhao J, Goldberg, J, Bremner, JD ,Vaccarino, V (2012). Global DNA Methylation Is Associated With Insulin Resistance: A Monozygotic Twin Study. *Diabetes,* 61: 542-546.

Supplementary file 1: Anthropometric, metabolic, inflammatory and dietetic factors of health professionals according by LINE-1 methylation median.

Variables	LINE-1 (≤75.8%) (n=19)	LINE-1 (>75.8%) (n=21)	p
Age† (y)	26.75 ± 1.30	29.67 ± 1.27	0.12
Anthropometric			
Weight† (kg)	57.30 (53.50-66.10)	61.70 (53.00-74.75)	0.20
WC (cm)	74.91 ± 1.46	80.39 ± 2.06	**0.04**
WHR†	0.76 (0.72-0.83)	0.81 (0.75-0.86)	0.12
BMI†	20.94 (18.90-22.40)	23.55 (20.49-25.88)	**0.02**
BF (%)	24.81 ± 1.01	23.99 ± 1.36	0.64
Metabolic			
SBP† (mmHg)	102.00 (96.00-106.30)	110.70.00 (99.65-117.60)	0.06
DBP (mmHg)	66.70 ± 1.40	68.64 ± 1.61	0.37
TC (mg/dL)	181.55 ± 5.77	180.67 ± 7.96	0.93
HDL† (mg/dL)	62.00 (55.00-67.00)	58.00 (49.00-64.00)	0.17
TG† (mg/dL)	79.00 (53.00-103.00)	80.00 (55.50-124.50)	0.59
CT:HDL†	3.03 (2.48-3.49)	3.07 (2.46-3.76)	0.69
Glucose† (mg/dL)	81.00 (80.00-85.00)	90.00 (79.00-94.50)	**0.04**
Insulin† (mU/L)	5.00 (3.10-7.50)	6.50 (4.60-11.95)	**0.04**
HOMA-IR†	1.06 (0.61-1.48)	1.51 (0.94-2.76)	**0.03**
TyG index	8.06 ± 0.08	8.21 ± 0.09	0.26
Uric acid† (mg/dL)	3.40 (3.10-4.00)	4.10 (3.20-4.40)	0.35

MDA (nmol/mL)	0.44 ± 0.05	0.42 ± 0.06	0.80
Inflmmatory			
C3 complement (g/L)	103.23 ± 3.42	106.68 ± 4.29	0.53
CRP† (mg/L)	1.20 (0.63-3.00)	0.90 (0.75-2.00)	0.87
IL-10† (pg/mL)	1.38 (0.97-1.93)	1.81 (1.43-3.01)	0.07
IL-1β† (pg/mL)	1.08 (0.94-1.30)	0.94 (0.94-1.08)	0.72
IL-6† (pg/mL)	1.29 (1.22-1.29)	1.29 (1.10-1.29)	0.64
TNF-α (pg/mL)	5.38 ± 0.53	7.48 ± 0.55	**0.01**
PAI-1 (pg/mL)	1286.49 ± 146.06	1033.51 ± 116.09	0.18
Adiponectin† (ng/mL)	17.38 (13.52-26.69)	11.21 (8.60-17.54)	**0.04**
Sleep hours	7.17 ± 0.26	7.12 ± 0.21	0.90
Diet			
Energy† (kcal)	2292.7 (1867.90- 2642.01)	2216.91 (1874.18-2887.13)	0.68
Carbohydrates† (g)	264.92 (205.26-339.98)	284.47 (227.42-358.08)	0.45
Lipids (g)	81.87 ± 6.87	80.21 ± 6.47	0.86
Protein† (g)	98.47 (76.91-141.39)	106.34 (83.33-152.95)	0.40
Fiber† (g)	29.96 (22.87-58.55)	34.82 (27.94-48.47)	0.51
Cholesterol† (mg)	350.76 (166.77-456.02)	327.16 (210.18-390.00)	1.00
Ca† (mg)	836.05 (494.47-1306.38)	686.45 (456.50-1189.70)	0.49
Mg† (mg)	265.91 (211.29-377.19)	315.19 (237.06-479.18)	0.15
P† (mg)	1422.83 (1080.70-1795.96)	1386.27 (1094.17-2341.02)	0.58

Fe† (mg)	10.53 (7.42-14.41)	10.69 (9.21-15.86)	0.45
Na† (mg)	1822.85 (1056.18-2922.99)	2205.01 (1000.51-3837.40)	0.36
K† (mg)	3074.34 (2285.03-3948.10)	3595.94 (2429.84-4987.17)	0.13
Cu† (µg)	1.27 (1.06-1.69)	1.31 (1.13-2.48)	0.15
Zn† (mg)	12.13 (9.08-14.86)	10.79 (8.52-15.21)	0.94
Retinol† (µg)	404.77 (212.00-1 373.05)	370.59 (198.42-559.21)	0.65
B1† (mg)	1.15 (0.93-1.69)	1.41 (0.89-1.84)	0.80
B2† (mg)	1.63 (0.99-2.26)	1.25 (0.73-2.04)	0.15
B6† (mg)	1.46 (0.87-1.88)	1.39 (0.74-2.01)	0.72
Niacin† (mg)	28.20 (18.61-41.16)	32.02 (21.28-41.54)	0.62
Vit C† (mg)	124.79 (67.28-230.10)	175.43 (66.42-290.18)	0.57
SFA (g)	31.09 ± 3.45	29.41 ± 3.28	0.73
MUFA (g)	27.16 ± 2.82	29.24 ± 3.37	0.64
PUFA† (g)	5.72 (4.21-6.93)	4.67 (3.39-7.61)	0.51
SFA (%)	12.35 ± 1.26	10.58 ± 0.84	0.24
MUFA† (%)	10.69 (6.64-12.97)	9.53 (7.39-12.45)	0.62
PUFA† (%)	2.15 (1.64-3.44)	1.81 (0.95-3.31)	0.34
Alcohol† (g)	9.69 (0.20-72.40)	9.48 (5.55-22.81)	0.60
Healthy Eating Index	60.62 ± 1.94	51.14 ± 2.34	**0.01**

Mean ± SEM – Student's t test/ † Median (Interquartile range) - Mann Whitney

WC: Waist circumference, WHR: Waist Hip Ratio, BMI: Body Mass Index, BF: Body Fat, SBP: Sistolic Blood Pressure, DBP: Diastolic Blood Pressure, TC: Total Cholesterol, TG: Triglycerides, TyG: Triglyceride-Glucose Index, MDA: Malondialdehyde, CRP: C Reactive Protein, TNF-α: Tumor Necrosis Factor α, PAI-1: Plasminogen Activation Inhibitor, SFA: Saturated Fatty Acid, MUFA: Monounsaturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids.

Supplementary file 2: Anthropometric, metabolic, inflammatory and dietetic factors of health professional according to inflammatory gene methylation medians.

Variables	IL-6 (≤43.37%) (n=18)	IL-6 (>43.37%) (n=21)	p	CRP (≤8.57%) (n=17)	CRP (>8.57%) (n=17)	p
Age† (y)	26.31 ± 1.02	30.14 ± 1.46	**0.04**	28.06 ± 1.62	28.39 ± 1.33	0.76
Anthropometric						
Weight† (kg)	55.80 (51.60-63.18)	61.70 (55.10-76.65)	**0.04**	59.00 (52.95-73.60)	59.30 (53.35-72.80)	**0.02**
WC (cm)	73.38 ± 1.43	81.83 ± 1.89	**0.00**	77.03 ± 1.72	78.47 ± 2.54	0.65
WHR†	0.75 (0.72-0.78)	0.82 (0.77-0.86)	**0.02**	0.79 (0.74-0.83)	0.77 (0.72-0.86)	0.83
BMI†	20.41 (18.90-22.69)	22.97 (20.98-25.88)	**0.01**	21.41 (19.65-26.03)	21.74 (20.20-24.15)	0.80
BF (%)	23.88 ± 1.03	24.81 ± 1.37	0.59	24.25 ± 1.04	24.74 ± 1.45	0.79
Metabolic						
SBP† (mmHg)	104.00 (96.16-	106.30 (101.75-	0.14	107.50 (100.45-	101.40 (96.65-	0.26

	107.50)	121.25)		117.85)	105.98)	
DBP (mmHg)	66.87 ± 1.28	69.00± 1.64	0.32	66.16 ± 1.33	68.95 ± 1.88	0.24
TC (mg/dL)	172.74 ± 6.83	188.90 ± 7.02	0.11	176.82 ± 7.79	191.05 ± 7.67	0.20
HDL† (mg/dL)	60.50 (55.50-66.25)	55.00 (48.00-66.50)	0.35	59.00 (50.50-68.50)	55.00 (51.00-65.75)	0.68
TG† (mg/dL)	79.00 (57.50-118.00)	80.00 (53.50-103.00)	0.72	93.00 (51.50-103.00)	71.50 (55.25-107.75)	0.09
CT:HDL†	2.99 (2.36-3.20)	3.29 (2.77-3.90)	0.09	2.98 (2.40-3.90)	3.05 (2.74-3.40)	0.19
Glucose† (mg/dL)	83.50 (78.50-91.25)	85.00 (79.50-91.50)	0.76	84.00 (78.00-88.50)	81.50 (79.00-91.25)	0.93
Insulin† (mU/L)	5.45 (3.48-11.30)	5.70 (4.60-8.95)	0.48	6.30 (3.95-8.95)	5.45 (3.83-7.93)	0.13
HOMA-IR†	1.08 (0.66-2.37)	1.20 (0.94-1.78)	0.44	1.36 (0.80-1.78)	1.10 (0.81-1.59)	0.19
TyG	8.12 ± 0.09	8.13 ± 0.09	0.96	8.10 ± 0.10	8.27 ± 0.09	0.22
Uric Acid† (mg/dL)	3.40 (2.95-4.00)	4.10 (3.40-4.47)	0.11	3.65 (3.40-4.38)	3.65 (3.13-4.18)	0.09
MDA (nmol/mL)	0.44 ± 0.06	0.42 ± 0.05	0.76	0.39 ± 0.05	0.44 ± 0.06	0.62
Inflmmatory						
C3 complement	99.28 ± 2.95	109.39 ± 4.27	0.06	105.29 ± 3.53	108.02 ± 4.03	0.62

(g/L)						
CRP† (mg/L)	0.85 (0.59-2.00)	2.00 (0.77-3.00)	0.13	1.02 (0.71-2.00)	1.50 (0.72-3.00)	0.82
IL-10† (pg/mL)	1.57 (1.21-2.04)	1.72 (1.13-2.48)	0.64	1.82 (1.25-2.38)	1.50 (1.15-1.79)	0.72
IL-1β† (pg/mL)	1.01 (0.94-1.30)	0.94 (0.94-1.08)	0.60	1.08 (1.00-1.30)	0.94 (0.94-1.08)	0.44
IL-6† (pg/mL)	1.29 (1.28-1.29)	1.29 (0.93-1.29)	0.56	1.28 (0.93-1.29)	1.29 (1.23-1.29)	0.93
TNF-α (pg/mL)	6.69 ± 0.66	6.25 ± 0.55	0.60	6.54 ± 0.76	6.17 ± 0.45	0.67
PAI-1 (pg/mL)	1085.49 ± 117.31	1165.72 ± 140.98	0.67	1354.88 ± 136.11	1186.67 ± 125.13	0.37
Adiponectin† (ng/mL)	17.42 (14.11-26.26)	11.21 (8.60-20.91)	0.11	16.20 (11.49-25.98)	14.68 (9.06-19.46)	0.55
Sleep hours	7.30 ± 0.24	7.13 ± 0.20	0.59	7.00 ± 0.22	7.28 ± 0.28	0.44
Diet						
Energy† (kcal)	2031.20 (1660.87-2433.61)	2555.93 (2044.38-2859.75)	**0.03**	2216.91 (1874.18-2675.63)	2086.43 (1859.79-2675.22)	0.13
Carbohydrates† (g)	228.09 (199.94-	325.83 (259.91-	**0.01**	293.49 (205.43-	255.97 (204.44-	0.97

	300.14)	350.19)		332.03)	369.41)	
Lipids (g)	74.95 ± 6.32	85.33 ± 6.93	0.28	69.49 ± 5.48	95.42 ± 7.69	**0.01**
Protein† (g)	96.43 (74.11-119.99)	106.36 (83.33-162.06)	0.23	106.34 (84.48-151.37)	99.30 (71.31-131.13)	0.25
Fiber† (g)	35.20 (19.91-64.11)	31.49 (27.93-40.03)	0.74	38.46 (28.24-52.34)	29.68 (21.59-37.82)	0.45
Cholesterol† (mg)	332.98 (210.96-409.58)	354.10 (172.12-535.96)	0.47	354.87 (161.08-429.22)	260.26 (194.60-460.00)	0.51
Ca† (mg)	657.75 (563.48-873.94)	1086.28 (445.19-1782.14)	0.16	656.42 (465.51-1421.72)	755.06 (572.28-988.70)	0.19
Mg† (mg)	283.57 (208.73-433.06)	315.17 (237.06-437.12)	0.29	308.80 (243.34-371.56)	309.02 (212.45-412.17)	0.70
P† (mg)	1256.94 (1078.16-1794.30)	1695.01 (1131.35-2231.25)	0.17	1539.68 (1050.40-2060.21)	1362.92 (1108.53-1723.20)	0.11
Fe† (mg)	10.38 (8.51-16.99)	10.60 (8.64-14.62)	0.92	10.56 (8.55-14.61)	9.53 (7.09-12.39)	0.77
Na† (mg)	1514.44 (816.70-2988.36)	2417.99 (1422.65-3617.56)	0.15	2018.70 (1080.37-3216.41)	2096.77 (978.98-3573.17)	0.29

K† (mg)	3085.86 (2168.02-3847.54)	3595.94 (2570.30-4653.79)	0.16	2992.83 (2407.10-3907.06)	3112.20 (2078.47-4068.97)	0.79
Cu† (µg)	1.42 (1.11-2.63)	1.27 (1.09-2.22)	0.79	1.19 (1.07-1.44)	1.27 (0.84-1.95)	0.59
Zn† (mg)	12.46 (9.58-15.04)	10.83 (7.99-15.91)	0.52	11.14 (8.50-15.22)	10.83 (7.95-15.01)	0.97
Retinoic acid† (µg)	312.05 (153.13-1124.39)	404.77 (232.47-900.95)	0.34	381.76 (216.28-782.31)	404.77 (149.64-1304.12)	0.72
Vitamin B1† (mg)	1.00 (0.84-1.44)	1.68 (1.13-2.30)	**0.01**	1.31 (0.89-1.68)	1.15 (0.96-2.07)	0.41
Vitamin B2† (mg)	1.16 (0.76-1.45)	1.71 (1.06-2.69)	**0.01**	1.21 (0.87-1.97)	1.52 (0.98-2.42)	0.42
Vitamin B6† (mg)	1.11 (0.71-1.53)	1.53 (0.91-3.07)	0.06	1.38 (0.77-1.81)	1.46 (0.89-2.51)	0.80
Niacin† (mg)	28.11 (21.16-33.74)	35.44 (19.63-62.21)	0.25	35.44 (22.42-42.11)	28.01 (19.59-48.04)	0.55
Vitamin C† (mg)	186.59 (71.91-290.87)	138.66 (64.68-234.68)	0.68	136.14 (67.07-178.73)	100.80 (56.75-303.26)	0.41
SFA (g)	25.95 ± 2.99	34.20 ± 3.56	0.09	24.44 ± 2.37	37.84 ± 3.96	**0.01**
MUFA (g)	28.18 ± 3.48	28.29 ± 2.97	0.98	23.84 ± 2.77	33.47 ± 3.69	**0.04**
PUFA† (g)	5.88 (4.10-7.48)	4.50 (3.26-6.96)	0.28	5.61 (3.79-7.38)	5.66 (3.41-8.04)	0.39
SFA (%)	11.05 ± 1.25	11.78 ± 0.97	0.64	9.99 ± 0.77	13.13 ± 1.24	**0.04**

MUFA† (%)		11.01 (9.13-13.66)	9.12 (6.78-11.61)	0.09	9.70 (6.48-14.15)	10.51 (6.97-13.27)	0.31
PUFA† (%)		2.50 (1.59-3.69)	1.65 (0.98-3.64)	**0.03**	1.99 (1.52-3.30)	2.58 (1.04-3.54)	0.22
Alcohol† (g)		7.78 (0.28-22.78)	11.49 (6.41-119.70)	0.21	8.04 (0.21-56.28)	22.22 (8.25-349.10)	0.97
Healthy Index	Eating	58.38 ± 3.67	53.92 ± 2.23	0.31	57.36 ± 2.34	56.28 ± 2.57	0.76

Continuation...

Variables	**SERPINE-1 (≤8.52%) (n=18)**	**SERPINE-1 (>8.52%) (n=19)**	**p**
Age† (y)	25.31 ± 0.69	30.68 ± 1.57	**0.01**
Anthropometric			
Weight† (kg)	57.60 (51.98-63.18)	64.80 (53.60-74.80)	0.08

WC (cm)	72.29 ± 1.42	82.96 ± 1.81	**0.00**
WHR†	0.73 (0.72-0.76)	0.82 (0.78-0.87)	**0.00**
BMI†	20.93 (19.10-22.48)	24.12 (20.94-25.85)	**0.02**
BF (%)	23.93 ± 0.96	25.18 ± 1.43	0.47
Metabolic			
PAS† (mmHg)	103.15 (97.55-106.60)	105.00 (98.30-120.70)	0.41
PAD (mmHg)	67.38 ± 1.29	68.97 ± 1.72	0.47
TC (mg/dL)	167.37 ± 6.37	191.63 ± 6.99	**0.02**
HDL† (mg/dL)	60.50 (53.30-67.50)	56.00 (51.00-65.00)	0.39
TG† (mg/dL)	86.00 (55.25-108.00)	80.00 (55.00-124.00)	0.86
CT:HDL†	2.80 (2.30-3.14)	3.29 (2.85-3.91)	**0.03**
Glucose† (mg/dL)	82.50 (78.50-91.25)	85.00 (79.00-93.00)	0.44
Insulin† (mU/L)	5.60 (3.58-9.73)	6.20 (4.40-9.70)	0.53
HOMA-IR†	1.12 (0.69-1.89)	1.32 (0.94-2.39)	0.46
TyG index	8.14 ± 0.09	8.15 ± 0.11	0.92

Uric acid† (mg/dL)	3.40 (3.03-3.65)	4.20 (3.60-4.50)	**0.03**
MDA (nmol/mL)	0.47 ± 0.06	0.41 ± 0.05	0.46
Inflmmatory			
C3 complement (g/L)	103.29 ± 3.41	106.52 ± 4.74	0.58
CRP† (mg/L)	1.20 (0.70-2.00)	1.42 (0.75-2.72)	0.82
IL-10† (pg/mL)	1.43 (1.07-2.05)	1.72 (1.38-2.15)	0.38
IL-1β† (pg/mL)	1.01 (0.94-1.30)	1.06 (0.94-1.08)	0.58
IL-6† (pg/mL)	1.29 (1.28-1.29)	1.29 (0.93-1.29)	0.33
TNF-α (pg/mL)	6.44 ± 0.68	6.59 ± 0.57	0.87
PAI-1 (pg/mL)	1178.99 ± 156.77	1105.13 ± 120.01	0.709
Adiponectin† (ng/mL)	17.70 (14.15-26.96)	11.21 (8.83-17.53)	**0.02**
Sleep hours	7.23 ± 0.23	7.20 ± 0.22	0.93
Diet			

Energy† (kcal)	2031.20 (1660.87-2721.12)	2292.75 (2020.42-2882.06)	0.09
CHO† (g)	243.34 (206.97-327.54)	322.42 (226.46-376.92)	0.11
LIP (g)	76.23 ± 7.77	84.64 ± 6.29	0.41
PTN† (g)	97.43 (65.12-129.36)	106.39 (84.40-161.84)	0.10
Fiber† (g)	35.19 (23.40-59.32)	31.51 (27.63-40.82)	0.79
Cholesterol† (mg)	243.34 (206.97-327.54)	338.80 (197.43-377.57)	0.67
Ca† (mg)	657.75 (482.89-886.70)	836.05 (517.09-1575.07)	0.14
Mg† (mg)	261.97 (209.26-390.32)	315.17 (240.53-465.39)	0.18
P† (mg)	1244.11 (968.51-1902.28)	1434.82 (1199.75-2193.59)	0.12
Fe† (mg)	11.16 (8.63-16.99)	10.12 (8.47-14.82)	0.85
Na† (mg)	2150.89 (1087.94-3697.07)	2043.99 (939.98-3423.96)	0.87
K† (mg)	2665.85 (1896.54-3841.45)	3595.94 (2619.78-4787.82)	**0.03**
Cu† (µg)	1.23 (1.08-1.97)	1.27 (1.11-2.35)	0.62
Zn† (mg)	12.46 (8.55-15.04)	10.47 (8.25-15.61)	0.80
Ret† (µg)	357.79 (171.91-663.12)	381.76 (214.48-1373.05)	0.50
Vitamin B1† (mg)	1.15 (0.84-1.65)	1.60 (0.96-2.34)	0.17

Vitamin B2† (mg)	1.25 (0.92-1.64)	1.52 (0.85-2.55)	0.35
Vitamin B6† (mg)	1.38 (0.83-1.53)	1.53 (0.75-2.28)	0.40
Niacin† (mg)	26.04 (18.59-38.94)	33.09 (20.78-45.72)	0.39
Vitamin C† (mg)	130.47 (71.91-235.88)	147.39 (62.50-282.86)	0.74
SFA (g)	27.35 ± 3.56	32.90 ± 3.56	0.28
MUFA (g)	25.31 ± 3.01	31.22 ± 3.59	0.22
PUFA† (g)	5.64 (3.43-7.15)	4.50 (3.14-7.36)	0.44
SFA (%)	11.21 ± 1.24	11.55 ± 1.07	0.84
MUFA† (%)	10.48 (6.85-13.06)	9.75 (7.20-12.51)	0.96
PUFA† (%)	2.13 (1.59-3.55)	1.65 (0.94-3.15)	0.09
Alcohol† (g)	8.21 (3.77-72.40)	12.41 (7.78-24.57)	0.29
Healthy Eating Index	62.11 ± 1.97	52.53 ± 2.07	**0.01**

Mean ±SEM – Student's t Test/ † Median (Interquartile range) - Mann Whitney

WC: Waist circumference, WHR: Waist Hip Ratio, BMI: Body Mass Index, BF: Body Fat, SBP: Sistolic Blood Pressure, DBP: Diastolic Blood Pressure, TC: Total Cholesterol, TG: Triglycerides, TyG: Triglyceride-Glucose Index, MDA: Malondialdehyde, CRP: C Reactive

Protein, TNF-α: Tumor Necrosis Factor α, PAI-1: Plasminogen Activation Inhibitor, SFA: Saturated Fatty Acid, MUFA: Monounsaturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids

Table 1: Primers used in the quantification of the methylation levels of the four genomic regions analyzed in PBMC.

Gene or specific sites		Primer sequence	Screened CpGs/ Amplicon size (bp)
LINE-1			
	Foward	5’ GCGAGGTATTGTTTTATTTGGGA 3’	8 / 141
	Reverse	5’ CGCCGTTTCTTAAACC 3’	
IL-6			
	Foward	5’ TTATGTAGGAAAGAGAATTTGGTTTAG 3’	5 / 181
	Reverse	5’ AAAAAATAAAATCATCCATTCTTCAC 3’	
CRP			
	Foward	5’ GGTAATTTAGTAGTTATAGGAGTTTGTAATAAAT 3’	4 /316
	Reverse	5’ AAAACACAACAACTTCTCCATAATCA 3’	
SERPINE-1			
	Foward	5’ TGTGTTTGGTTGTAGGGTTAAGA 3’	7 / 151
	Reverse	5’ TTACTTTTCTCCTACCTAAAATTCTCA 3’	

Table 2: Multiple linear regression model with LINE-1 methylation (global methylation) as a dependent variable

Variables	B coefficient	(CI 95%)	P-value
Global methylation as dependent			
WC (cm)	0.01	(0.00; 0.01)	0.57
TyG index	0.07	(-0.03; 0.17)	0.15
TNF-α (mg/L)	0.01	(-0.01; 0.03)	0.37
HEI	-0.01	(-0.01; 0.00)	**0.02**
			R^2=0.50 (p= **0.04**)

Table 3: Spearman's correlations between inflammatory genes promoter methylation and its plasma concentration.

Analysis performed	R^2	p
IL-6 (pg/mL) *versus* IL-6 methylation	-0.057	0.728
PAI-1 (pg/mL) *versus* SERPINE-1 methylation	-0.047	0.784
CRP (mg/L) *versus* CRP methylation	0.003	0.989

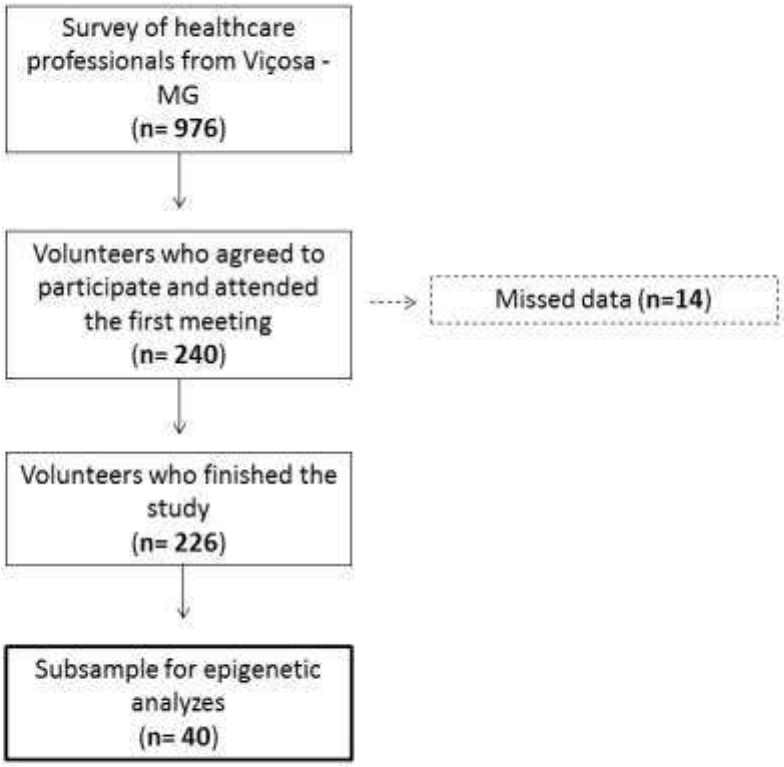


Figure 1: Diagram of study volunteers.

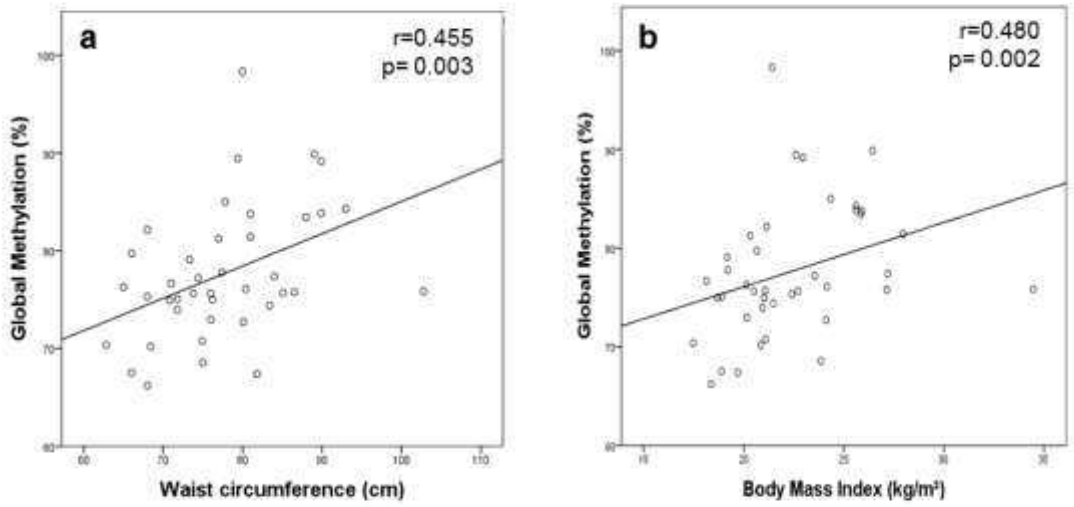


Figure 2: LINE-1 correlations with anthropometric features.

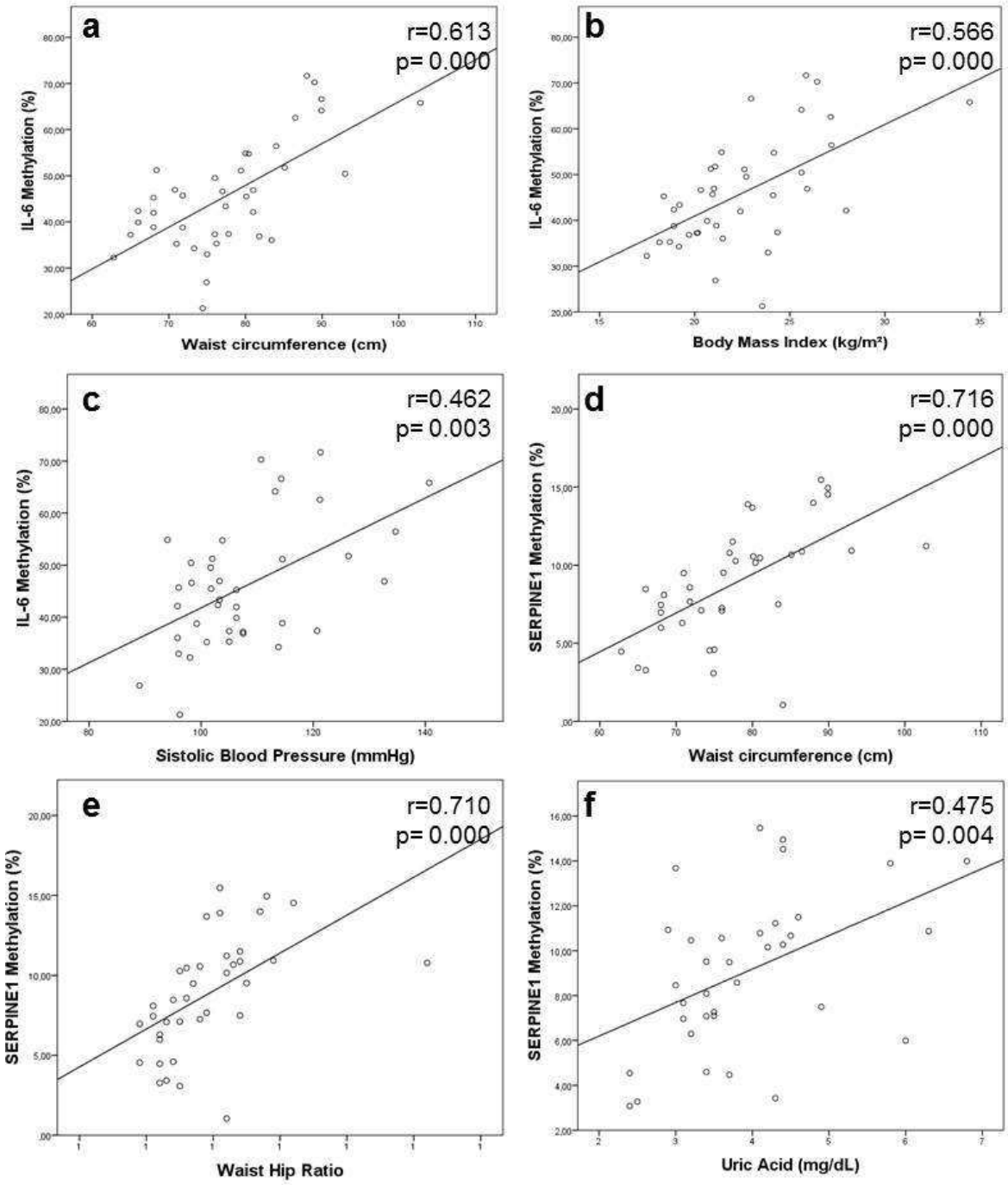
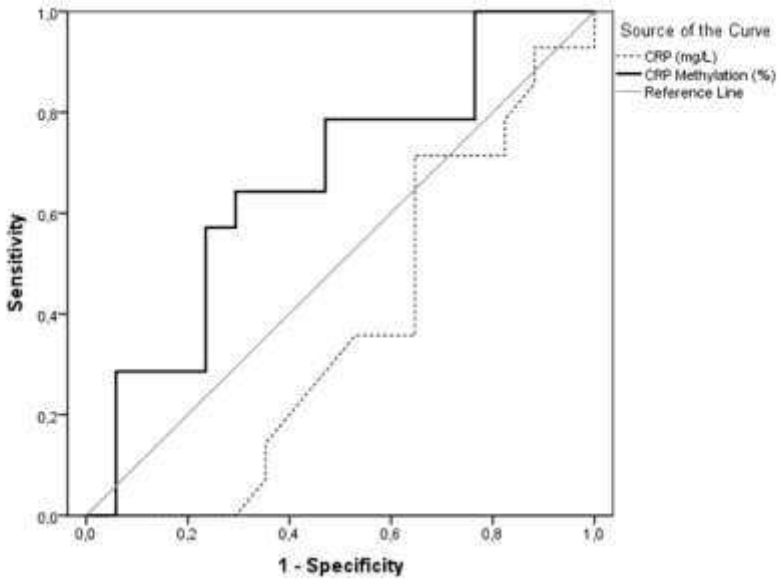


Figure 3: Correlations of the methylation levels of inflammatory genes and metabolic traits.



Test Result	AUC	SEM	CI (95%)	p
CRP (mg/L)	0.372	0.101	0.173-0.571	0.039
CRP methylation (%)	0.664	0.100	0.468-0.859	

Figure 4: Comparison between area under the curve (ROC curve) of CRP plasma concentrations and CRP gene promoter methylation in relation to insulin resistance, analyzed by TyG index.

J Physiol Biochem
DOI 10.1007/s13105-015-0391-2

ORIGINAL PAPER

Interleukin-6 is a better metabolic biomarker than interleukin-18 in young healthy adults

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Received: 22 October 2014 / Accepted: 5 February 2015

Introduction

Inflammation is a physiological defense response to injury and foreign substances, in order to maintain body homeostasis [27]. However, it can also be triggered by disorders, such as obesity or diabetes, in metabolically active tissues [4].

Obesity and excessive body fat content are associated with increased circulating levels of proinflammatory cytokines [47, 18], supporting a link between chronic low-grade inflammation and diseases such as diabetes and insulin resistance [41], dyslipidemia [18], and, consequently, metabolic syndrome (MetS) features [36]. In addition, previous studies have reported that weight loss leads to a reduction of these proinflammatory molecules [17], making the cytokines an interesting biomarker to follow changes associated to cardiometabolic risk and obesity management. On the other hand, it has been postulated that inflammation could trigger obesity [28]. Thaler and Schwartz (2010) proposed that inflammation in hypothalamic cells, caused by excess of nutrients (mainly fatty acids), can promote resistance to insulin or leptin actions, driving to excessive weight gain [38].

Therefore, the identification of early biomarkers to metabolic disturbances with diagnostic value, including inflammatory markers, in order to prevent future damages and facilitate early treatments is still a great challenge, mainly in young subjects.

In this context, interleukin 6 (IL-6) is a proinflammatory cytokine whose levels have been reported to increase in proportion to the degree of obesity, particularly central adiposity, and to insulin resistance [23]. The levels of IL-6 are often increased in obese subjects, both in adipose tissue and in the blood [11], decreasing in patients undergoing exercise and after caloric restriction [13]. IL-6 levels have been associated with the percentage of total body fat, waist circumference (WC), waist-hip ratio (WHR), and total cholesterol:HDL ratio (TC:HDL ratio), [18] as well as the development of chronic disorders, such as cardiovascular diseases [44].

Another cytokine associated with obesity is interleukin 18 (IL-18). IL-18 is produced by macrophages and Kupffer cells, and was first described as an interferon-γ-inducer factor (IGIF) in T and natural killer cells from mice infected with *Propionibacterium acnes* [10]. The secondary structure of IL-18 in the form of β-sheet folded makes it more closely related to IL-1β than any other cytokine [10]. Increased levels of this cytokine have been related to obesity, type 2 diabetes [45], insulin resistance [1], and atherogenesis [12].

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Published online: 27 February 2015

In turn, it is recognized that lifestyle factors, such as dietary pattern, physical activity, and smoking, can interfere with inflammatory marker concentrations [24, 16, 21, 14]. However, little is known about the relationships between inflammatory markers and metabolic traits in young healthy subjects.

The hypothesis of the present study is that subtle changes may occur in healthy people long time before the onset of the disorders, and may increase the risk to develop metabolic complications. Therefore, we aimed to assess the potential value of IL-6 and IL-18 as early biomarkers of metabolic disorders, as well as to identify the determinants of variation in the concentrations of these cytokines in young healthy adults, with emphasis in metabolic and lifestyle variables.

Materials and methods

Subjects

In this study, 153 apparently healthy young adults (50 males, 103 females), with a mean age of 21±3 (range: 18–34 years old) and a mean body mass index (BMI) of 22.1±2.5 (range 17.4–29.3 kg/m^2), were recruited through magazines, radio, web page, and intranet tools from the Universities of Navarra (UNAV) and Public of Navarra (UPNA). In the recruitment message, the age range (18–35 years old) was mentioned, as well as relevant clinical information for those interested in participating in this nutritional survey. The enrollment questionnaire was devised to assess the nutritional status and to provide appropriate dietary advises to the participants. In addition, a phone number and an e-mail address were provided for a continuous contact. Exclusion criteria included pregnancy, inflammatory, heart and respiratory diseases, hormonal treatments, or prescribed drugs that could affect glucose metabolism, alcohol and drug dependence, recent follow-up of diets designed to weight loss, or unstable weight in the last 3 months. These exclusion criteria were screened firstly by phone, and those that passed this step were further screened by a questionnaire fulfilled by a trained physician. Each volunteer signed a written informed consent, which was previously approved by the Investigation Ethics Committee of the Clínica Universidad de Navarra (ref number 79/2005), in accordance with the principles of the Helsinki Declaration.

Anthropometry and body fat distribution

Anthropometric determinations, such as weight, height, waist and hip circumferences, and skinfold thickness, as well as calculated indexes, were taken using standard measurement procedures [20]. BMI (kg/m^2) and total BF (%) were used as indicators of total adiposity, while measurements of WC (cm) and WHR were used as indicators of central fat accumulation [20].

Blood pressure and biochemical assessments

Systolic (SBP) and diastolic blood pressures (DBP) were measured following World Health Organization guidelines [42]. Venous blood samples were drawn after a 12 h overnight fast by venipuncture. The EDTA–plasma and serum samples were obtained by centrifugation (3500 rpm×15 min at 4 °C), and frozen immediately at −80 °C until assay. Serum concentrations of triglycerides, total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-c), Complement C3 (C3), and glucose and insulin were measured by standard methods as previously described [18]. Insulin resistance was calculated as homeostasis model assessment-estimated insulin (HOMA-IR) according to Matthews et al. [25]. Another index used to estimate insulin resistance was the triglyceride-glucose index (TyG), calculated according to Simental-Mendía et al. [37]. Plasma circulating concentrations of C-reactive protein (CRP, Immundiagnostik AG, Bensheim, Germany), high-sensitivity IL-6 (hs-IL-6; R&D Systems, Minneapolis, MN, USA), high-sensitivity tumor necrosis factor-alpha (hs-TNF-α; R&D Systems, Minneapolis, MN, USA), and IL-18 (Medical & Biological Laboratories Co., Naka-ku Nagoya, Japan) were evaluated by specific commercial enzyme-linked immunosorbent assay procedures in an automated analyzer system (Triturus, Grifols, Barcelona, Spain) as described by the manufacturer. In our laboratory, the inter- and intra-assay coefficients of variability were <10 %.

Lifestyle features

The participants were asked about smoking status (never, former, or current smokers), smoking time in life-course, and number of cigarettes per day. With respect to physical activity, the participants informed whether they performed regular physical activity (yes/no), and if so, the type and the physical activity (h/week) [16]. To

quantify the volume of activity, a metabolic equivalent index was also computed by assigning a multiple of resting metabolic rate to each activity [8].

Statistical analyses

Results are reported as mean±SEM and variable distribution was determined by the Shapiro–Wilk test. In order to analyze anthropometric, biochemical, inflammatory, and dietary pattern and lifestyle characteristics with respect to IL-6 and IL-18 concentrations, these cytokines were taken as suitable variables considering their medians as cutoff values (1.07 pg/mL and 180.0 ng/μL, respectively). Statistical comparisons between groups were performed by the parametric Student *t* test, Mann–Whitney *U* test, or χ^2 test, as appropriate. In order to analyze inflammatory marker concentrations with respect to anthropometrical and metabolic data of the study participants, BMI, WC, WHR, HOMA-IR, and TyG index were distributed into tertiles in order to assess trends. Finally, multiple linear regression models were performed to analyze the prediction of plasma IL-6 and IL-18 concentrations (outcome) for selected variables and using both cytokines as predictors of metabolic and inflammatory traits related to the MetS. Non-normally distributed variables were log-transformed prior to inclusion in linear regression analyses. Statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). A P value <0.05 was considered as statistically significant.

Results

Anthropometric, metabolic, inflammatory, and lifestyle characteristics are described according to the medians of IL-6 and IL-18 (Tables 1 and 2). Gender distribution (data not shown) and age did not differ between the lower and higher IL-6 or IL-18 groups. Individuals with higher concentrations of IL-6 had higher values of BMI, WC, WHR, body fat percentage, and plasma CRP concentration, as well as higher TyG index. Those subjects with higher concentrations of IL-18, in turn, showed an increased TC:HDL ratio (Table 1).

When evaluated by lifestyle, individuals with higher concentrations of both cytokines showed higher frequency of smoking or higher daily number of cigarettes (Table 2). No significant differences were found with regard to physical activity between groups (Table 2). IL-6, but not IL-18, showed a trend to increase with the rise of WC and TyG index (Fig. 1).

When using multiple linear regression models to analyze the factors that contribute to the variations in the studied interleukins (Table 3), age, BMI, glucose, triglycerides, C3, CRP, number of cigarettes smoked per day, and physical activity level contributed with approximately 24 % to IL-6 variation. Age, BMI, triglycerides, glucose, and CRP were independent predictors (P<0.005). In turn, BMI, total cholesterol, triglyceride, C3, HOMA-IR, and number of cigarettes smoked per day contributed to a variation of 19 % in plasma IL-18. BMI, C3, HOMA-IR and smoking habits were independent predictors (P<0.005).

Furthermore, IL-6 was able to predict the concentrations of TyG index, CRP, Total cholesterol and C3. However, once have been found that IL-6 levels are correlated to number of cigarettes smoked per day, the regressions were adjusted by number of cigarettes and others confounding variables. The relationship between IL-6 levels and TyG index and CRP remained significant even after adjustment for age and BMI and number of cigarettes smoked per day (p adjusted) as shown in Fig. 2. IL-18 predicted HOMA-IR and insulin and triglyceride concentrations, even after the adjustments (data not shown), but this influence was very poor in relation to the biological effects. All models were also further adjusted by sex, daily energy intake, and physical activity level (metabolic equivalents/day) without changing the associations (data not shown).

Discussion

Inflammation appears to be a defense metabolic process that links obesity onset and the development of MetS features [47]. Inflammatory biomarker concentrations, such as those of CRP, TNF-α, IL-6, and IL-18, are often elevated in obese individuals, and associated to higher cardiovascular risks and insulin resistance [1, 2].

The expansion of adipose tissue promotes the recruitment of immune cells, enhancing the production of proinflammatory cytokines, as well as the density of macrophages in visceral adipose tissue [39]. Furthermore, hypoxia, promoted by the expansion of adipose tissue, leads to increased production of these cytokines in order to trigger angiogenesis and improve the blood flow to the tissue [34]. However, the increase in the

Table 1 Anthropometric and clinical characteristics of the participants (n=153), according to the median of plasma IL-6 (1.07 pg/mL) and IL-18 (180.0 ng/μL) concentrations

Descriptive means	All (n=153)	Lower IL-6 (n=76)	Higher IL-6 (n=77)	P value	Lower IL-18 (n=76)	Higher IL-18 (n=77)	P value
Age (years)	20.8±0.2	20.7±0.2	20.9±0.3	0.572	21.0±0.3	21.0±0.3	0.614
BMI (kg/m^2)	22.0±0.2	21.4±0.2	22.7±0.3	*0.001*	21.9±0.3	22.2±0.3	0.622
WC (cm)	72.7±0.6	70.7±0.7	74.8±0.9	*0.001*	72.8±0.9	72.7±0.8	0.957
WHR	0.74±0.00	0.73±0.00	0.75±0.01	*0.024*	0.742±0.01	0.741±0.00	0.912
Body fat (%)	20.0±0.5	18.8±0.7	21.2±0.8	*0.002*	19.6±0.8	20.4±0.7	0.956
Systolic BP (mmHg)	114.9±0.9	114.3±1.4	115.6±1.2	0.481	116±1	114±1	0.439
Diastolic BP (mmHg)	65.2±0.6	64.5±0.9	65.6±0.9	0.335	66±1	64±1	0.204
Glucose (mg/dL)	85.1±0.6	84.3±0.8	85.9±0.9	0.181	84.2±0.8	86.0±0.9	0.138
Insulin (μU/L)	7.9±0.3	7.7±0.4	8.1±0.4	0.516	7.7±0.3	8.1±0.42	0.515
HOMA-IR	1.7±0.0	1.6±0.1	1.7±0.1	0.376	1.6±0.1	1.7±0.1	0.354
TyG index	8.0±0.0	7.99±0.2	8.06±0.2	*0.004*	8.02±0.2	8.04±0.2	0.438
Total cholesterol (mg/dL)	174.8±2.2	176.1±3.2	173.5±3.1	0.566	172.1±3.2	177.5±3.1	0.228
HDL-c (mg/dL)	59.8±1.0	60.3±1.5	59.2±1.4	0.605	61.4±1.4	58.1±13.0	0.111
TC: HDL-c	3.0±0.1	3.0±0.1	3.0±0.1	0.948	2.9±0.1	3.2±1.5	0.008
Triglycerides (mg/dL)	67.9±2.1	70.3±3.3	65.6±2.6	0.267	64.0±2.8	71.9±3.2	0.066
Complement C3 (g/L)	1.1±0.0	1.0±0.0	1.1±0.0	0.082	1.1±0.2	1.1±0.2	0.179
CRP (mg/L)	1.1±0.1	0.9±0.1	1.3±0.1	*<0.001*	1.1±0.1	1.1±0.1	0.621
TNFα (pg/mL)	2.1±0.2	1.9±0.2	2.3±0.3	0.190	2.0±0.3	2.2±0.2	0.657
IL-6 (pg/mL)	1.2±0.1	0.75±0.0	1.8±0.1	N/A	1.2±0.1	1.3±0.1	0.573
IL-18 (ng/μL)	484.1±65.2	563.6±99.5	403.6±83.7	0.220	130.2±3.8	833.4±116.8	N/A

P value from Student t test or Mann–Whitney U test (to non-normally distributed variables), when groups were compared

Data are mean±SEM

BP blood pressure, *CRP* C-reactive protein, *HOMA-IR* insulin resistance index, *HDL-c* high-density lipoprotein-cholesterol, *TC* total cholesterol, *N/A* not available

In italics are p values <0.05

circulating levels of these cytokines interferes with the insulin signaling pathway, leading to resistance to the action of this hormone in target tissues [47].

IL-6 is a cytokine produced by many cell types and elicit paracrine, autocrine, and endocrine effects, acting as a mediator of the acute response and determining the

Table 2 Lifestyle features of the participants (n=153), according to the medians of plasma IL-6 (1.07 pg/mL) and IL-18 (180.0 ng/μL) concentrations

Characteristics	All (n=153)	Lower IL-6 (n=77)	Higher IL-6 (n=76)	P value	Lower IL-18 (n=76)	Higher IL-18 (n=77)	P value
Self-reported physical active practice (yes)	82 (53.6)	38 (46.3)	44 (53.7)	0.289	41 (50)	41 (50)	0.930
Metabolic equivalents (h/week)	37.6±2.3	41.5±3.4	36.6±3.0	0.213	39.8±3.3	35.4±3.2	0.188
Smokers (yes)	50 (32.7)	23 (46)	27 (54)	0.556	19 (38)	31 (62)	*0.044*
Smoking time (years)	1.3±0.2	1.2±0.3	1.4±0.2	0.497	0.8±0.2	1.8±0.3	*0.019*
Cigarettes (n/day)	2.5±0.4	1.9±0.5	3.2±0.6	*0.003*	1.6±0.5	3.5±0.7	*0.021*

P values from Mann–Whitney U test to continuous variables and χ^2 test to dichotomy variables, when groups were compared

Data are mean±SEM or n (frequency in percents) to continuous or dichotomy variables

In italics are p values <0.05

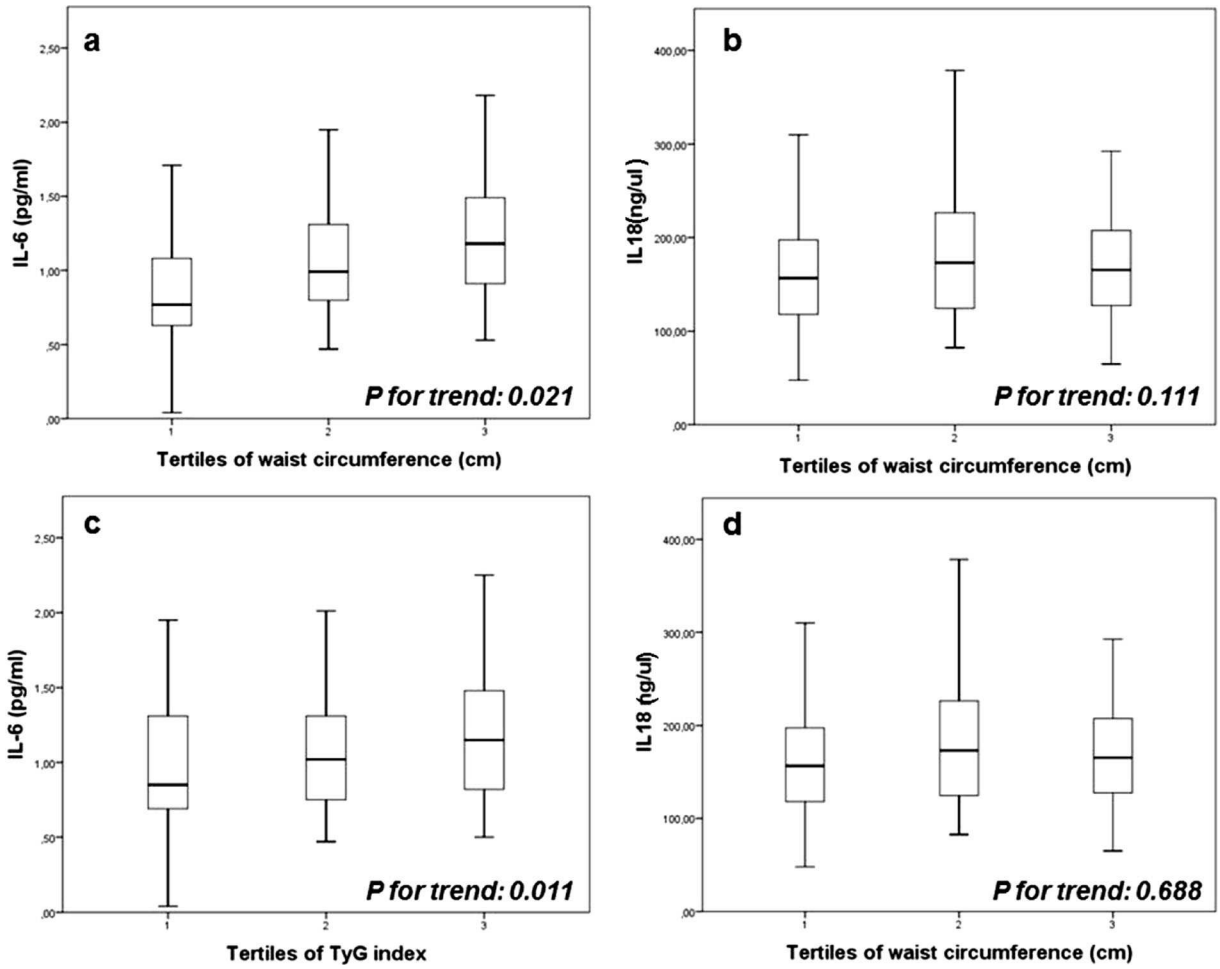

Fig. 1 Plasma IL-6 (**a**) and IL-18 (**b**) concentrations (n=153), according to the distribution of waist circumference (centimeters) into tertiles, and IL-6 (**c**) and IL-18 (**d**) according to the distribution of TyG index into tertiles. Data are expressed as median (interquartile range)

increase of plasma concentrations of acute phase proteins, such as CRP [44, 9]. As in this study, the correlation between IL-6 and abdominal adiposity has been previously documented [18, 21], and chronic elevations of IL-6 concentration have been associated with both obesity and insulin resistance [32]. IL-6, produced in large scale in adipose tissue macrophages, may have a pivotal role in obesity and insulin resistance, impairing insulin sensitivity by reducing tyrosine phosphorylation and increasing serine phosphorylation of key molecules in the insulin signaling pathway, leading to resistance to its action in target tissues, increasing lipolysis and decreasing glucose uptake in the adipose tissue [15]. Although in this study IL-6 levels were not associated with insulin resistance when measured as HOMA index, subjects with higher IL-6 concentration showed a higher TyG index. In this sense, Vasques et al. [40] suggested that the TyG index was a better predictor of insulin resistance than the HOMA-IR.

Regarding inflammatory markers, plasma concentration of IL-6 was a predictor of CRP, as has been reported in the scientific literature [43], even after adjustment for factors related to the lifestyle. Other studies have suggested that CRP concentrations were independently associated to insulin resistance [31], even in a healthy population [43], strengthening the relationship between IL-6 concentrations and insulin resistance. However, the predictive power of IL-6 in relation to C3 and total cholesterol was not significant after adjusting for the daily number of cigarettes smoked. In fact, it has been reported that smoking is associated with elevated levels of plasma IL-6 [16], and high concentrations of IL-6 may be a central mediator of the association between

Table 3 Multiple linear regression analyses showing the independent contributions of variables of studied domains to the variation of the plasma IL-6 and IL-18 concentrations

Variables	B coefficient	95 % Confidence interval	*P* value
Plasma IL-6 as dependent			
Age (years)	−0.015	(−0.030; 0.000)	*0.043*
BMI (kg/m^2)	0.025	(0.008; 0.042)	*0.003*
Triglycerides (mg/dL)	−0.002	(−0.003; 0.000)	*0.013*
Glucose (mg/dL)	0.006	(0.000; 0.011)	*0.040*
C3 complement (g/l)	0.215	(−0.019; 0.449)	0.072
CRP (mg/L)	0.118	(0.065; 0.170)	*<0.001*
Cigarette (*n*/day)	0.002	(−0.006; 0.011)	0.610
Physical activity (metabolic equivalents h/week)	−0.001	(−0.002; 0.000)	0.157
			R^2=0.242 (P<0.001)
Plasma IL-18 as dependent			
BMI (kg/m^2)	−0.032	(−0.059; −0.005)	*0.021*
Total cholesterol/HDL	0.008	(−0.101; 0.117)	0.879
Triglycerides (mg/dL)	0.002	(−0.001; 0.005)	0.113
C3 complement (g/l)	0.563	(0.188; 0.938)	*0.003*
HOMA-IR	0.119	(0.030; 0.208)	*0.009*
Smoking time (years)	0.038	(0.010; 0.066)	*0.008*
			R^2=0.193 (P<0.001)

Domains are adiposity indicator, lipid markers, glucose marker, inflammatory marker and lifestyle features

Adjusted R^2 and all independent variables included in each model are presented in the table. Non-normally distributed variables were log-transformed prior to inclusion in linear regression

In italics are p values <0.05

smoking and cardiovascular risk [26]. On the other hand, IL-6 elicited in exercise has been proposed to have an anti-inflammatory effect and to regulate the food intake by the sensitization of insulin and leptin central action [35]. In general, the increase in IL-6 production in skeletal muscle occurs concomitantly to a decrease in TNF-α secretion, resulting in an anti-inflammatory effect [33]. However, in this case, IL-6 levels were neither related to physical activity nor to TNF-α concentration, suggesting a classical proinflammatory role of IL-6 in relation to obesity.

In this study, although higher plasma concentrations of IL-18 were not associated with increased central adiposity, this cytokine was related with a higher TC:HDL ratio, considered a surrogate of cardiovascular risk. It was also observed that insulin and triglycerides levels, and insulin resistance assessed as HOMA-IR, were influenced by IL-18 levels, even after adjusting for age and lifestyle. These predictions, although statistically significant, seem to have little biologic importance, once IL-18 has a very small influence in the values of metabolic and inflammatory traits.

IL-18 has been previously related to obesity and type 2 diabetes [45], atherosclerosis [12], and MetS [22]. Zorrilla et al. [46] reported that IL-18 modulates food intake, metabolism, and adiposity, suggesting a protective effect of this interleukin in obesity. These data were confirmed by Netea et al. [29] who, using genetically modified mice, observed that the absence of IL-18 induced hyperphagia, hyperglycemia, obesity, and insulin resistance, whereas the administration of recombinant IL-18 improved such metabolic defects. Taken together, these outcomes suggest that elevated levels of IL-18 associated with adiposity may occur due to resistance to the action of this interleukin, such occurs with insulin or leptin [45]. Studies correlating the plasma concentration of IL-18 to individual components of MetS have been previously reported [6], as well as the progressive increase in the levels of IL-18 in relation to the number of MetS components, regardless adjusting for age, gender, BMI, and insulin levels [22]. However, in the present study, IL-18 did not appear to be a good biomarker of MetS components in young healthy subjects as it was not able to detect early changes in metabolic features.

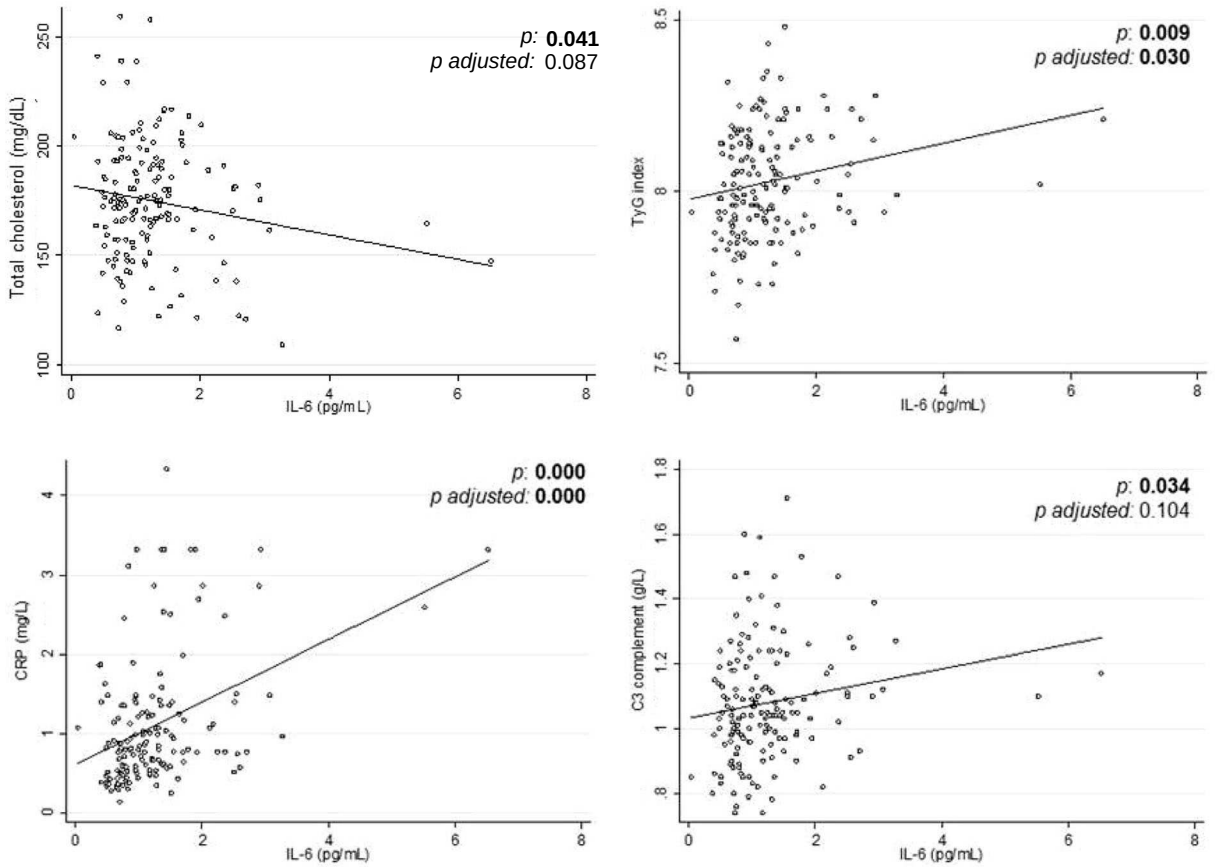

Fig. 2 Multiple linear regression analyses with IL-6 concentrations (pg/mL) as a predictor of total cholesterol, TyG index, CRP, and C3 complement adjusted by number of cigarettes smoked per day (n=153)

Regarding lipid metabolism, Blankenberg et al. [3] reported a positive correlation between IL-18 and serum triglycerides and a negative correlation with HDL. Although we have not observed a negative correlation between IL-18 and HDL, the higher TC:HDL ratio among individuals with higher circulating levels of IL-18 suggests a comparable cardiometabolic risk. As observed in our results, Blankenberg et al. [3] also found that smoking interfered in the concentrations of IL-6, but not in those of IL-18 [3].

In this study, differences in cytokine concentrations between genders were not observed. Men had a trend to higher levels of IL-6 but not significant. This result was similar to other results obtained in previous reports [5]. However, concerning IL-18, we have not observed differences between both genders. Although most of the studies have observed higher levels of IL-18 in men [30], Chen et al. [7] found similar levels of IL-18 between healthy men and women, but higher levels in men with MetS.

It is known that some components of the diet [19] and physical activity [24] can influence the inflammatory status. However, these relationships were not observed in the current study. The exception was the lowest phosphorus intake among individuals with higher levels of IL-6.

This study has some limitations, since relationships of cause and effect between the associations cannot be assumed due to the cross-sectional nature of the study. The sample composition (only young and apparently healthy volunteers, not including individuals with MetS diagnoses) does not allow the evaluation of potential associations of IL-6 and IL-18 with the disease. In fact, this experimental design was conducted in order to analyze these inflammatory mediators as early biomarkers of metabolic features/disorders even in young healthy subjects. A future research with this same population might help to clarify if these subjects with trend to insulin resistance really developed this disturbance.

In conclusion, the results of this study suggest that IL-6 concentrations may be linked to adiposity traits and

increased risk of insulin resistance, measured by TyG index, in young healthy subjects. Thus, IL-6 can be an early risk factor for the development of chronic diseases. In any case, such effects may be influenced by smoking habit, once the number of cigarette smoked per day was associated to higher concentrations of IL-6. Moreover, IL-6 seems to be a better early biomarker of metabolic traits in young healthy subjects than IL-18.

Acknowledgments We wish to thank the physician Blanca E. Martínez de Morentín, the nurse Salomé Pérez, and the technician Verónica Ciaurriz. This work was supported by *Línea Especial* about Nutrition, Obesity and Health (University of Navarra LE/97) and CIBERobn. We also thanks to Capes for a PhD scholarship awarded to JCC Carraro. J Bressan and HHM Hermsdorff are CNPq and FUNARBE-UFV fellows, respectively.

Conflict of interest The authors declare that they have no competing interests.

Author contributions JCCC analyzed and wrote the manuscript. HHMH contributed in the design, field work, data collection, and analysis. BP contributed in the design, field work, and data collection. MAZ, FIM, and JB contributed in the design and analysis. JAM contributed in the general coordination, design, and financial management. All authors assisted in editing the manuscript as well as reading and approving the final manuscript.

References

1. Ahmad R, Al-Mass A, Al-Ghawas D et al (2013) Interaction of osteopontin with IL-18 in obese individuals: implications for insulin resistance. PLoS ONE 8(5):e63944
2. Blackburn P, Després J-P, Lamarche B et al (2006) Postprandial variations of plasma inflammatory markers in abdominally obese men. Obesity 14(10):1747–1754
3. Blankenberg S, Luc G, Ducimetière P et al (2003) Interleukin-18 and the risk of coronary heart disease in European men: the prospective epidemiological study of myocardial infarction (PRIME). Circulation 108(20):2453–2459
4. Bondia-Pons I, Ryan L, Martinez JA (2012) Oxidative stress and inflammation interactions in human obesity. J Physiol Biochem 68(4):701–711
5. Breyer MK, Rutten EPA, Vernooy JHJ, Spruit MA, Dentener MA, van der Kallen C, vanGreevenbroek MMJ, Wouters EFM (2011) Gender differences in the adipose secretome system in chronic obstructive pulmonary disease (COPD): a pivotal role of leptin. Respir Med 105:1046–1053
6. Bruun J, Stallknecht B, Helge J, Richelsen B (2007) Interleukin-18 in plasma and adipose tissue: effects of obesity, insulin resistance, and weight loss. Eur J Endocrinol 157:465–471
7. Chen YC, Chen SD, Miao L, Liu ZG, Li W, Zhao ZX, Sun XJ, Jiang GX, Cheng Q (2012) Serum levels of interleukin (IL)-18, IL-23 and IL-17 in Chinese patients with multiple sclerosis. J Neuroimmunol 243(1–2):56–60
8. Ching PL, Willett WC, Rimm EB et al (1996) Activity level and risk of overweight in male health professionals. Am J Public Health 86(1):25–30
9. Diehl S, Rincón M (2002) The two faces of IL-6 on Th1/Th2 differentiation. Mol Immunol 39(9):531–536
10. Dinarello CA (1999) IL-18: A TH1 -inducing, proinflammatory cytokine and new member of the IL-1 family. J Allergy Clin Immunol 103(1):11–24
11. Fantuzzi G (2005) Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 115(5):911–919
12. Gerdes N, Sukhova GK, Libby P et al (2002) Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. J Exp Med 195:245–257
13. Goyenechea E, Parra D, Martínez JA (2007) Impact of interleukin 6–174G>C polymorphism on obesity-related metabolic disorders in people with excess in body weight. Metabolism 56(12):1643–1648
14. Gülçubuk A, Haktanir D, Cakiris A et al (2014) The effects of resveratrol on tissue injury, oxidative damage, and pro-inflammatory cytokines in an experimental model of acute pancreatitis. J Physiol Biochem 70(2):397–406
15. Gustafson B, Hammarstedt A, Andersson CX et al (2007) Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. Arterioscler Thromb Vasc Biol 27(11):2276–2283
16. Helmersson J, Larsson A, Vessby B, Basu S (2005) Active smoking and a history of smoking are associated with enhanced prostaglandin F2α, interleukin-6 and F2-isoprostane formation in elderly men. Atherosclerosis 181(1):201–207
17. Hermsdorff H, Zulet MA, Abete I, Martínez JA (2011) A legume-based hypocaloric diet reduces proinflammatory status and improves metabolic features in overweight/obese subjects. Eur J Nutr 50(1):61–69
18. Hermsdorff H, Zulet MA, Puchau B, Martínez JA (2011) Central adiposity rather than total adiposity measurements are specifically involved in the inflammatory status from healthy young adults. Inflammation 34(3):161–170
19. Hermsdorff HH, Zulet MA, Martínez JA (2011) The implication of unknown bioactive compounds and cooking techniques in relations between the variety in fruit and vegetable intake and inflammation. Am J Clin Nutr 93(6):1384
20. Hermsdorff HHM, Puchau B, Bressan J, Martínez JA (2009) Association of retinol-binding protein-4 with dietary selenium intake and other lifestyle features in young healthy women. Nutrition 25(4):392–399
21. Hermsdorff HHM, Puchau B, Bressan J, Martínez JA (2010) Association of body Fat distribution with proinflammatory gene expression in peripheral blood mononuclear cells from young adult subjects. OMICS: J Integr Biol 14(3):297–307
22. Hung J, McQuillan BM, Chapman CML (2005) Elevated interleukin-18 levels are associated with the metabolic syndrome independent of obesity and insulin resistance. Arterioscler Thromb Vasc Biol 25(6):1268–1273
23. Kim T, Choi S, Ha E et al (2013) IL-6 induction of TLR-4 gene expression via STAT3 has an effect on insulin resistance in human skeletal muscle. Acta Diabetol 50(2):189–200

24. Lavie CJ, Church TS, Milani RV, Earnest CP (2011) Impact of physical activity, cardiorespiratory fitness, and exercise training on markers of inflammation. J Cardiopulm Rehabil Prev 31(3):137–145
25. Matthews DR, Hosker JP, Rudenski AS, Nayloret BA et al (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28(7):412–9
26. McCarty MF (1999) Interleukin-6 as a central mediator of cardiovascular risk associated with chronic inflammation, smoking, diabetes, and visceral obesity: down-regulation with essential fatty acids, athanol and pentoxifylline. Med Hypotheses 52(5):465–477
27. Medzhitov R (2008) Origin and physiological roles of inflammation. Nature 454(7203):428–435
28. Moreno-Aliaga M, Campión J, Milagro FI et al (2005) Adiposity and proinflammatory state: the chicken or the egg. Adipocytes 1:1–16
29. Netea MG, Joosten LAB, Lewis E et al (2006) Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance. Nat Med 12(6):650–656
30. Opstad TB, Pettersen AA, Arnesen H, Seljeflot I (2011) Circulating levels of IL-18 are significantly influenced by the IL-18+183 A/g polymorphism in coronary artery disease patients with diabetes type 2 and the metabolic syndrome: an observational study. Cardiovasc Diabetol 10:110
31. Pannacciulli N, Cantatore FP, Minenna A et al (2001) C-reactive protein is independently associated with total body fat, central fat, and insulin resistance in adult women. International journal of obesity and related metabolic disorders. Int J Obes Relat Metab Disord 25(10):1416–1420
32. Pedersen M, Bruunsgaard H, Weis N et al (2003) Circulating levels of TNF-alpha and IL-6-relation to truncal fat mass and muscle mass in healthy elderly individuals and in patients with type-2 diabetes. Mech Ageing Dev 124(4):495–502
33. Petersen AMW, Pedersen BK (2006) The role of il-6 in mediating the anti-inflammatory effects of exercise. J Physiol Pharmacol 57(10):43–51
34. Quintero P, Milagro FI, Campion J, Martínez JA (2010) Impact of oxygen availability on body weight management. Med Hypotheses 74(5):901–907
35. Ropelle ER, Flores MB, Cintra DE et al (2010) IL-6 and IL-10 anti-inflammatory activity links exercise to hypothalamic insulin and leptin sensitivity through IKKβ and ER stress inhibition. PLoS Biol 8(8):e1000465
36. Safavi SM, Ziaei R, Marac MR (2012) Association of serum ceruloplasmin level with obesity: some components of metabolic syndrome and high-sensitive C-reactive protein in Iran. J Obes 2012:951093
37. Simental-Mendía L, Rodríguez-Morán M, Guerrero-Romero F (2008) The product of fasting glucose and triglycerides as surrogate for identifying insulin resistance in apparently healthy subjects. Metab Syndr Relat Disord 6:299–304
38. Thaler JP, Schwartz MW (2010) Minireview: inflammation and obesity pathogenesis: the hypothalamus heats up. Endocrinology 151(9):4109–4115
39. Vachharajani V, Granger DN (2009) Adipose tissue: a motor for the inflammation associated with obesity. IUBMB Life 61(4):424–430
40. Vasques ACJ, Novaes FS, de Oliveira MS et al (2011) TyG index performs better than HOMA in a Brazilian population: a hyperglycemic clamp validated study. Diabetes Res Clin Pract 93(3):e98–e100
41. Wang B, Li Q, Jiang Y et al (2011) Serum complement C3 has a stronger association with insulin resistance than high sensitive C-reactive protein in non-diabetic Chinese. Inflamm Res 60(1):63–68
42. Whitworth JA, Chalmers J (2004) World health organisation-international society of hypertension (WHO/ISH) hypertension guidelines. Clin Exp Hypertens 26(7–8):747–52
43. Yudkin JS, Stehouwer CDA, Emeis JJ, Coppack SW (1999) C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler Thromb Vasc Biol 19(4):972–978
44. Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V (2000) Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 148(2):209–214
45. Zilverschoon GRC, Tack CJ, Joosten LAB et al (2008) Interleukin-18 resistance in patients with obesity and type 2 diabetes mellitus. Int J Obes 32(9):1407–1414
46. Zorrilla EP, Sanchez-Alavez M, Sugama S et al (2007) Interleukin-18 controls energy homeostasis by suppressing appetite and feed efficiency. Proc Natl Acad Sci U S A 104(26):11097–11102
47. Zulet MA, Puchau B, Navarro C et al (2007) Biomarcadores del estado inflamatorio: nexo de unión con la obesidad y complicaciones asociadas. Nutr Hosp 22(5):511–527

Higher fruit intake is related to TNF-α hypomethylation and better glucose tolerance in healthy subjects

Submetido à revista Journal of Nutrigenetics and Nutrigenomics

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Running head: TNF-α promoter hypomethylation and fruit intake

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ABSTRACT

Background/Aim: This study hypothesized an association between healthy dietary patterns, hypermethylation of tumor necrosis factor-alpha (TNF-α) promotor and decreased risks of metabolic changes. Methods: 40 normal-weight young women encompassed this cross-sectional study. DNA was isolated from white blood cells and CpG site methylation in TNF-α were analyzed by Sequenom EpiTyper. Results: Contradicting our hypothesis, Healthy Eating Index score was negatively associated with CpG5 (r=-0.460, p=0.003) and TNF-α total methylation (r=-0.355, p=0.026). A higher intake of fruits was related to lower insulin, HOMA-IR, TNF-α total methylation and methylation at CpG5. No other dietary pattern was related to TNF-α methylation. TNF-α total methylation correlated positively with systolic blood pressure (r=0.323; p=0.042) and CpG5 methylation with body mass index (r=0.333, p=0.036). Furthermore, fiber intake was negatively associated with the methylation of CpG5 (r=-0.324, p=0.041) and the whole TNF-α sequence (r=-0.434, p=0.005), whereas vitamin C intake was negatively associated with TNF-α total methylation (r=-0.411, p=0.009). Intakes of apples and citrus fruits were negatively associated with TNF-α total methylation. Conclusion: A healthy dietary pattern and higher fruit intake (particularly apples and citrus fruits) were related to better glucose tolerance in healthy subjects, which could be mediated by lower TNF-α methylation.

Keywords: Biomarker; inflammation; epigenetic; DNA methylation; diet; fiber; vitamin C

1. INTRODUCTION

Healthy dietary patterns and diets rich in fruits and vegetables (FV) are widely recommended as a health promotion strategy due to high concentrations of vitamins, minerals, antioxidants, phytochemicals and dietary fiber [1]. The occurrence of these foods in the habitual diet contributes to explain the "fiber hypothesis," which states that increased fiber intake protects against Western diseases [2]. In this context, several studies have reported an association between FV intake and lower risk of chronic illnesses, such as cardiovascular diseases [3], oxidative stress [4] including lower DNA oxidation [5], and inflammation, being the expression of inflammatory genes inversely proportional to the consumption of fruits [6]. A dietary pattern rich in fruits and dairy products has also been related to decreased odds of impaired blood glucose, hypertrygliceridemia and metabolic syndrome (MetS) risks [7]. In addition, fruits and vegetables have low energy density, making them interesting for body weight management [8].

MetS has been defined as a cluster of medical disturbances related not only to increased cardiovascular risk and type 2 diabetes, but also to higher mortality [9]. MetS risk factors include diet [10], lifestyle [11], oxidative stress [12], genetics [13] and epigenetic mechanisms such as DNA methylation [14, 15].

Chronic inflammation has been proposed as a potential link between excessive weight and adiposity and metabolic complications of obesity [16]. Indeed, tumor necrosis factor α (TNF-α), one of the major mediators of inflammatory response [17], is usually overexpressed in obesity and with the number of MetS components [18], and is upregulated in white adipose tissue in obese individuals with insulin resistance [19].

On the other hand, several nutrients and bioactive compounds have been reported to affect epigenetic mechanisms involved in gene expression regulation, such as DNA methylation, contributing thus to the prevention of the development of metabolic disorders [20]. In fact, DNA methylation can be related to MetS phenotypes, and these relationships among epigenetics, diet and disease may be a cyclic interplay [21].

Overall, we hypothesized that healthy dietary patterns can change the relationship between epigenetic signature and metabolic traits, increasing the DNA methylation of TNF-α promotor, and hence, decreasing its expression. Thus, this study aimed to evaluate the effect of healthy dietary patterns and food intake on DNA methylation of TNF-α gene in white blood cells in normal-weight healthy subjects, and the interactions between diet, TNF-α methylation and the main features of MetS.

2. SUBJECTS AND METHODS

2.1.Subject

This cross-sectional study included 40 normal-weight healthy women, university students of Pamplona (Navarra, Spain), with a mean age of 21 ± 3 (range: 18–28 years) and a mean body mass index (BMI) of 21.0 ± 1.7 (range: 18.5–24.9 kg/m^2). Initial enrollment screening evaluations consisted of a medical history, physical examination, and fasting blood biochemistry, to exclude subjects with evidence of any chronic inflammatory, heart, or respiratory diseases, as detailed elsewhere [22]. Other exclusion criteria were hormonal treatment or drug prescription affecting glucose metabolism, alcohol and drug dependence, history of recent diet for weight loss, or unstable weight in the last 6 months. All followed procedures were in accordance with

98 the ethical requirements of the responsible committee on human experimentation (Investigation
99 Ethics Committee of the Clínica Universidad de Navarra, nº 79/2005), and with the Helsinki
100 Declaration of 1975, as revised in 2000 and later years. An informed consent was obtained from
101 all patients for being included in the study.

102 2.2.Anthropometry and body fat composition

103 Anthropometric measurements were conducted according to previously described
104 procedures [22]. BMI (kg/m^2) was calculated as the ratio between weight (kg) and the squared
105 height (m^2). Total body fat (%) was estimated by the equations of Durnin and Womersley [23],
106 using four skinfold thicknesses (biceps, triceps, subscapular, and suprailiac).

107 2.3.Dietary intake assessment

108 Dietary intake was assessed with a semi-quantitative food frequency questionnaire (136
109 food-items) validated for Spanish people [24]. Nutrient intake was estimated using an ad hoc
110 computer program specifically developed for this purpose, including the latest available
111 information included in the food composition tables for Spain [25, 26]. Daily energy and fat
112 intake were calculated as frequency nutrient composition of each portion size for each consumed
113 food item, where frequencies were measured in nine frequency categories (6+/d, 4–6/d, 2–3/d,
114 1/d, 5–6/wk, 2–4/wk, 1/wk, 1–3/mo, never or almost never) for each food item.

115 The participants were assigned into two groups (low and high fruit intake) according to
116 the median of fruit intake (293.4 g/day). The median cutoff criteria have been previously applied
117 [27] based on a valid and reliable method to assign two groups of risk in epidemiological studies
118 [28]. Mediterranean dietary pattern [29] and Healthy Eating Index (HEI) scores [30] were
119 calculated to evaluate the quality of diet. Healthy Eating Index is a measure of diet quality based

on the food-group recommendations, such as fruit, vegetables, grains, dairy foods, meat and beans, oils, saturated fat, sodium and calories of fats, alcohol and added sugar [30].

2.4.Blood pressure and biochemical assessments

Systolic and diastolic blood pressures were measured following WHO criteria [31]. Venous blood samples were drawn after a 12-h overnight fast. EDTA (ethylenediamine tetraacetic acid)–plasma and white blood cells (WBC) were separated from whole blood by centrifugation at 3500 rpm, at 5°C for 15 min (Model 5804R, Eppendorf, Germany), and immediately frozen at –80°C until assay (WBC in buffy-coat). Serum concentrations of triglycerides, total cholesterol (TC), high density lipoprotein–cholesterol (HDL-c), glucose and insulin were measured by standard methods as previously described [32]. The plasma low density lipoprotein–cholesterol (LDL-c) data were calculated by the Friedewald equation [33]. Insulin resistance was estimated by the HOMA-IR (Homeostatic model assessment – Insulin resistance), through calculations as follows: HOMA-IR = [fasting glucose (mmol/L) x fasting insulin (lU/ml)]/22.5 as described elsewhere [34]. Plasma concentrations of high-sensitive TNF-α (hs-TNF-α) were measured using enzyme immunoassay based kits (R&D Systems, Minneapolis, MN, USA) by means of an automated analyzer system (Triturus, Grifols, Barcelona, Spain).

2.5.DNA isolation and methylation assays

DNA from WBC was isolated by using the Master Pure kit (Epicenter, Madison, WI, USA). DNA quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA) and treated with sodium bisulfite (EZ DNA methylation kit; Zymo Research, Orange, CA) following the manufacturer's protocols.

The quantitative analysis of 5-methylcytosine levels of TNF-α gene promoter was performed with Sequenom EpiTyper (Sequenom, San Diego, CA, USA), which relies on base-specific cleavage followed by MALDI-TOF mass spectrometry, as described elsewhere [35]. Bisulfite-treated genomic DNA was amplified using two pairs of primers: 5'-GGGTATTTTTGATGTTTGTGTGTTT-3' (forward) and 5'-AAAAATCTCCCTTCTCCACTCACAA-3' (reverse), designed to amplify 20 CpG sites located between nucleotides -170 to +359 of the TNF-α gene [36].

2.6.RNA extraction and expression analysis

Total RNA from WBC was extracted with Trizol reagent (Invitrogen) and subsequently treated with DNase (DNA-free kit, Ambion/Applied Biosystems, Austin, TX, USA) as previously described [37]. Quantitative real-time PCR was performed in an ABI PRISM 7000 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Taqman probes for TNF-α were also supplied by Applied Biosystems. Gene expression levels were normalized by using 18s rRNA as internal control and calculated with the $2^{-\Delta\Delta Ct}$ method [38].

2.7.Statistical Analysis

Results are reported as mean ±SD and normality condition was determined by Shapiro-Wilk test. Statistical comparisons between groups were performed by Student's t-test or Mann–Whitney U test, as appropriate. Pearson and Spearman correlations were fitted to evaluate the potential correlations of TNF-α promoter methylation with anthropometric or metabolic features and dietary factors. Chi-square test was used to evaluate the association between low/high intake of fruits and categorical variables of interest (vitamin and mineral supplementation and physical activity). Subjects were also categorized according to tertiles of orange/tangerine intake. One way ANOVA was performed to determine means differences, and polynomial contrasts for trend

166 analyses. Statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL,
167 USA). A P-value < 0.05 was considered as statistically significant.

168

169 **3. RESULTS**

170 Subject's features below or above the median of fruit intake per day were similar in
171 relation to age, vitamin and mineral supplementation and physical activity practice. However,
172 those with higher FV intake showed lower fasting insulin concentration and HOMA-IR. There
173 was no difference in TNF-α expression in relation to fruit intake or another group of foods (Table
174 1).

175 Subjects with higher intake of fruit showed lower total methylation percentage of TNF-α
176 gene and CpG5 of the region analyzed (Figure 1). A trend towards significance ($p<0.1$) was also
177 observed in CpGs 14 and 19. TNF-α total methylation (TNF-α total) was related to higher
178 systolic blood pressure, and CpG5 methylation was associated with higher BMI values (Figure
179 1).

180 TNF-α total methylation was inversely correlated with fruit fiber and vitamin C ($p=0.005$
181 and 0.009, respectively), while the methylation levels of CpG5 were correlated with fiber (Figure
182 2).

183 Other food groups and nutrients did not correlate with CpG5 and TNF-α total
184 methylation, except for HEI, which was negatively associated with the methylation levels of both
185 regions, and whole cereals, which showed a trend towards significance (Table 2).
186 Orange/tangerine and apple intakes were particularly closely associated with TNF-α methylation

187 (Figure 3). In fact, orange/tangerine and apple were the most consumed fruits by these
188 participants (83.1 and 74.1 g/day, respectively).

189 **4. DISCUSSION**

190 The encouragement of higher consumption of fruits and vegetables is not a new strategy
191 for health promotion [39]. In this sense, our results reveal that the higher intake of fruits was
192 related to lower fasting insulin levels and HOMA-IR values, which means a better glucose
193 tolerance in these healthy subjects. FV intake has been previously related to lower insulin
194 resistance and MetS [40], and several biomarkers of FV intake, such as carotenoids and vitamin
195 C, have been negatively associated with glycemia, serum insulin concentrations and glycosylated
196 hemoglobin (HbA 1c) levels [41, 42].

197 As low-grade inflammation is involved in the development of insulin resistance, the
198 effect of changing dietary habits (particularly higher consumption of fruits) on chronic
199 inflammation may be one of the protective mechanisms regarding metabolic disorders [43].
200 Hotamisligil [44] reported that insulin receptor is an important target for TNF-α, and that this
201 cytokine may be involved in the switch of tyrosine to serine phosphorylation. In this context, a
202 beneficial effect of FV, in particular fruits, has been already described in relation to oxidative
203 stress [5, 45] and inflammation [6,46]. For example, it has been reported that a high FV intake
204 reduces interleukin-6 and TNF-α concentrations and is associated with higher antioxidant
205 capacity in plasma [46-48]. These effects are mainly attributed to antioxidants and bioactive
206 compounds found in fruits and vegetables, especially vitamin C and fiber [49], which
207 corroborates our results.

208 The beneficial effect of FV consumption on inflammation can be observed even at the
209 molecular level, for example with a reduced expression of inflammatory markers [6], and could
210 be regulated by epigenetic factors. For example, changes in dietary habits including fruit intake
211 have been positively associated with epigenetic modifications, such as changes in LINE-1 DNA
212 methylation [50] or a lower prevalence of DNA hypomethylation, an association that was dose-
213 dependent [51]. Changes in the methylation levels of specific gene promoters may regulate the
214 expression of genes by modifying the interaction of transcription factors and methyl-DNA
215 binding proteins [52]. In this sense, our results reveal that TNF-α promoter methylation is related
216 to healthy dietary patterns, in special, to higher fruit intake. Citrus fruits and apple were the main
217 fruits involved, being fiber and vitamin C particularly related. Dietary fiber content is considered
218 to have a beneficial effect on inflammation [53]. Krishnamurthy et al. [54] found that, for each
219 10-g/day increase in total fiber intake, the odds of elevated serum C-reactive protein (CRP)
220 levels were decreased by 11% and 38% in those subjects without and with chronic kidney
221 disease, respectively. Fiber intake was negatively associated with visceral adipose tissue, CRP
222 and fibrinogen, and positively associated with adiponectin in adolescents [55]. Fiber intake can
223 even reverse the side effects of a high-fat, high-carbohydrate meal on inflammatory markers,
224 endotoxemia and oxidative stress in normal-weight subjects [56]. However, no other food groups
225 also rich in dietary fiber, such as vegetables, legumes, nuts and whole cereals, were associated
226 with TNF-α methylation, showing a specific effect of fruit intake, and maybe, an interaction
227 between nutrients in the food matrix. Several dietary components found in vegetables, such as
228 sulforaphane and flavonoids, have been reported to influence DNA methylation levels [57, 58].
229 On the other hand, some polyphenols like curcumin, resveratrol and catechin can modulate NF-

κB action and chromatin remodeling and, hence, the inflammatory response through DNA methyltransferase (DNMT) action [59].

Our hypothesis was that the higher intake of "healthy food", like FV, could result in a hypermethylation of TNF-*α* promoter, and hence, in a lower expression of this cytokine. However, we found the opposite outcome, since the higher intake of fruit was related to positive metabolic effects but no changes in circulating TNF-α and TNF-*α* mRNA levels, and TNF-*α* promoter hypomethylation in WBC. It is not known whether these mechanisms are similar in patients with established metabolic disorders and healthy subjects. Moreover, some studies have also reported that, although DNA methylation of the cytosine in the CpG dinucleotide is typically associated with gene silencing, CpG promoters can be both methylated and transcriptionally active due to an increase in binding of transcription factors in a methylation-dependent manner [60, 61], and that promoter sequence and gene function are major predictors of promoter methylation states [62]. In this sense, a study that evaluated the effect of Roux-en Y gastric bypass on TNF-*α* methylation reported a decrease in TNF-*α* promoter methylation in whole blood that was accompanied by a reduction in TNF-α plasma levels, with no significant correlation between both features [63]. A bioinformatic analysis [64] revealed that the CpG5 site in the promoter of TNF-*α* comprises the binding sequences of STAT4, that regulates the differentiation of T $CD4^+$ cells; c-ETS, that controls the expression of cytokines and chemokines; and ELK, an ETS family member.

Confirming these findings, there was no difference in TNF-*α* mRNA and TNF-α plasma concentration between the groups, and neither fruit intake nor DNA methylation status were related to TNF-*α* expression, showing that TNF-*α* promoter hypomethylation was not followed by an increase in TNF-*α* expression in WBC, and that other regulatory mechanisms could be

involved, such as histone modifications [65], non-coding RNA, enhancer function [66], and others. In fact, several investigations reported that there is not a simple association between DNA methylation and gene expression [67, 68] and that dietary influence on DNA methylation, and hence on metabolic features, can result unexpected [69]. In fact, two possible DNA methylation regulatory mechanisms with opposite modes of gene expression regulation have been proposed [70]: 1) tissue-specific differentially methylated regions are negatively correlated with the expression of their associated genes, and 2) the occurrence of negative regulators, such as transcriptional repressors that exhibit specific binding activity to methylated DNA motifs, causes a positive correlation between gene methylation and gene expression.

Specific foods and nutrients found in FV can also influence DNA methylation [20]. In the current study, promising effects were found in relation to the consumption of apple and, specially, orange/tangerine/grapefruit group. In this context, it has been described that apple polyphenol dietary supplementation in rats inhibited adipocyte hypertrophy and enhanced lipolytic response through the regulation of genes involved in adipogenesis, lipolysis and fatty acid oxidation, which could be mediated, in part, by changes in DNA methylation [71]. The anti-inflammatory effects of orange juice were reviewed by Coelho and colleagues [72], who reported that the modulation of inflammatory markers by orange juice consumption can be due to bioactive compounds, such as the flavonoids hesperidin and naringenin. On the other hand, mice supplemented with grapefruit extract (rich in naringenin and kaempferol) showed a significant decrease in fasting glucose levels, which was accompanied by lower mRNA expression of some proinflammatory genes (monocyte chemoattractant protein-1 – MCP-1, TNF-*α, cyclooxygenase*-2, nuclear factor kappa B - NF-*κB*) in liver and epididymal adipose tissue [69]. In that study, the CpG3 site of TNF-*α* showed higher methylation in the grapefruit group compared with the non-

treated group, suggesting that DNA methylation changes in TNF-α in adipose tissue might contribute to reduce the inflammation associated with diabetes and obesity [69], which corroborates our findings.

The present study has some limitations, such as the sample size, the lack of data about WBC distribution (granulocytes, monocytes and lymphocytes) and the cross-sectional design, but is very conclusive in determining the positive effects of fruit intake even in apparently healthy normal-weight subjects and the occurrence of epigenetic changes in proinflammatory genes that could be related to these beneficial effects. However, this relationship between fruit intake and DNA methylation in inflammation-related genes should also be investigated in longitudinal and clinical studies.

In conclusion, a healthy dietary pattern and higher daily fruit intake are related to better glucose tolerance and lower methylation of TNF-α in white blood cells, being fiber and vitamin C from fruits putatively involved in this relationship.

5. ACKNOWLEDGEMENTS

We wish to thank the physician Blanca E. Martínez de Morentin, the nurse Salomé Pérez, and the technician Verónica Ciaurriz. We also thank to Capes for a PhD scholarship awarded to JCC Carraro. This work was supported by the Spanish Ministry of Economy and Competitiveness (ref. AGL2013-4554-R) and Carlos III Institute of Health (CIBERobn). Authors declare no conflict of interest.

6. REFERENCES

1. Slavin JL, Lloyd B: Health Benefits of Fruits and Vegetables. Adv Nutr 2012;3(4):506-16.
2. Casiglia E, Tikhonoff V, Caffi S, Boschetti G, Grasselli C, Saugo M, Giordano N, Rapisarda V, Spinella P, Palatini P: High dietary fiber intake prevents stroke at a population level. Clin Nutr 2013;32(5):811-8.

3. Radhika G, Sudha V, Mohan Sathya R, Ganesan A, Mohan V: Association of fruit and vegetable intake with cardiovascular risk factors in urban south Indians. Br J Nutr 2008;99(02):398-405.
4. Yuan L, Zhang L, Ma W, Zhou X, Ji J, Li N, Xiao R: Glutathione S-transferase M1 and T1 gene polymorphisms with consumption of high fruit-juice and vegetable diet affect antioxidant capacity in healthy adults. Nutrition 2013;29(7–8):965-71.
5. Cocate PG, Natali AJ, de Oliveira A, Longo GZ, Alfenas RdCG, Peluzio MCG, Santos EC, Buthers JM, Oliveira LL, Hermsdorff HHM: Fruit and vegetable intake and related nutrients are associated with oxidative stress markers in middle-aged men. Nutrition 2014;30(6):660-5.
6. Hermsdorff H, Zulet M, Puchau B, Alfredo Martínez J: Fruit and vegetable consumption and proinflammatory gene expression from peripheral blood mononuclear cells in young adults: a translational study. Nutr Metab 2010;7:42.
7. Hong S, Song Y, Lee KH, Lee HS, Lee M, Jee SH, Joung H: A fruit and dairy dietary pattern is associated with a reduced risk of metabolic syndrome Metabolism 2012;61(6):883-90.
8. Hornick BA, Weiss L: Comparative Nutrient Analysis of Commonly Consumed Vegetables: Support for Recommending A Nutrition Education Approach Emphasizing Specific Vegetables to Improve Nutrient Intakes. Nutr Today 2011;46(3):130-7.
9. Saito I, Iso H, Kokubo Y, Inoue M, Tsugane S: Metabolic Syndrome and All-Cause and Cardiovascular Disease Mortality - Japan Public Health Center-Based Prospective (JPHC) Study. Circ J 2009;73(5):878-84.

10. Unger RH, Clark GO, Scherer PE, Orci L: Lipid homeostasis, lipotoxicity and the metabolic syndrome. Biochimica et Biophysica Acta (BBA) - M Cell Biol Lipids 2010;1801(3):209-14.
11. Ramírez-Vargas E, Arnaud-Viñas MR, Delisle H: Prevalence of the metabolic syndrome and associated lifestyles in adult males from Oaxaca, Mexico. Salud Pub Mex. 2007;49:94-102.
12. Penckofer S, Schwertz D, Florczak K: Oxidative Stress and Cardiovascular Disease in Type 2 Diabetes: The Role of Antioxidants and Pro-Oxidants. J Cardiovasc Nurs 2002;16(2):68-85.
13. Pollex RL, Hegele RA: Genetic determinants of the metabolic syndrome. Nat Clin Pract Cardiovasc Med 2006;3(9):482-9.
14. Milagro FI, Gómez-Abellán P, Campión J, Martínez JA, Ordovás JM, Garaulet M: CLOCK, PER2 and BMAL1 DNA Methylation: Association with Obesity and Metabolic Syndrome Characteristics and Monounsaturated Fat Intake. Chronobiol Int 2012;29(9):1180-94.
15. Plagemann A, Harder T, Brunn M, Harder A, Roepke K, Wittrock-Staar M, Ziska T, Schellong K, Rodekamp E, Melchior K, Dudenhausen JW: Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome. J Physiol 2009;587(20):4963-76.
16. Zulet MA, Puchau B, Navarro C, Martí A, Martínez JA: Biomarcadores del estado inflamatorio: nexo de unión con la obesidad y complicaciones asociadas. Nutr Hosp 2007;22(5):511-27.
17. Fantuzzi G: Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 2005;115(5):911-9.
18. Xydakis AM, Case CC, Jones PH, Hoogeveen RC, Liu M-Y, Smith EOB, Nelson KW, Ballantyne CM: Adiponectin, Inflammation, and the Expression of the Metabolic Syndrome in Obese Individuals: The Impact of Rapid Weight Loss through Caloric Restriction. J Clin Endocrinol Metab. 2004;89(6):2697-703.
19. Wieser V, Moschen A, Tilg H: Inflammation, Cytokines and Insulin Resistance: A Clinical Perspective. Arch Immunol Ther Exp 2013;61(2):119-25.
20. Remely M, Lovrecic L, Garza A, Migliore L, Peterlin B, Milagro F, Martinez JA, Haslberger AG: Therapeutic perspectives of epigenetically active nutrients. Br J Pharmacol 2015;172(11):2756-68.

21. Turcot V, Tchernof A, Deshaies Y, Pérusse L, Bélisle A, Marceau S, Biron S, Lescelleur O, Biertho L, Vohl MC: LINE-1 methylation in visceral adipose tissue of severely obese individuals is associated with metabolic syndrome status and related phenotypes. Clin Epigenetics. 2012;4(10).
22. Hermsdorff HHM, Zulet MÁ, Puchau B, Bressan J, Martínez JA: Association of retinol-binding protein-4 with dietary selenium intake and other lifestyle features in young healthy women. Nutrition 2009;25(4):392-9.
23. Durnin JVGA, Womersley J: Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 Years. Br J Nutr 1974;32(01):77-97.
24. Fuente-Arrillaga C, Ruiz Z, Bes-Rastrollo M, Sampson L, Martinez-Gonzalez M: Reproducibility of an FFQ validated in Spain. Publ Health Nutr 2010;13:1364-72.
25. Mataix J: Tabla de composición de alimentos. 5th ed. Granada: Universidad de Granada; 2009.
26. Moreiras O, Carbajal A, Cabrera LCC: Tabla de composición de alimentos. 13th ed. Madrid: Ediciones Pirámide; 2010.
27. Hermsdorff H, Zulet MÁ, Abete I, Martínez JA: Discriminated benefits of a Mediterranean dietary pattern within a hypocaloric diet program on plasma RBP4 concentrations and other inflammatory markers in obese subjects. Endocrine 2009;36(3):445-51.
28. Willett WC: Nutritional Epidemiology. 2nd ed. New York: Oxford University Press; 1998.
29. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D: Adherence to a Mediterranean Diet and Survival in a Greek Population. NE J Med 2003;348(26):2599-608.
30. Guenther PM, Reedy J, Krebs-Smith SM: Development of the Healthy Eating Index-2005. J Am Diet Assoc 2008;108(11):1896-901.
31. Whitworth JA, Chalmers J: World Health Organisation-International Society of Hypertension (WHO/ISH) hypertension guidelines. . Clin Exp Hypertens 2004;26:747-52.
32. Hermsdorff H, Zulet MÁ, Puchau B, Martínez JA: Central Adiposity Rather Than Total Adiposity Measurements Are Specifically Involved in the Inflammatory Status from Healthy Young Adults. Inflammation 2011;34(3):161-70.
33. Friedewald WT, Levy RI, Fredrickson DS: Estimation of the Concentration of Low-Density Lipoprotein Cholesterol in Plasma, Without Use of the Preparative Ultracentrifuge. Clin Chem 1972;18(6):499-502.
34. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetol 1985;28(7):412-9.
35. Campión J, Milagro FI, Goyenechea E, Martínez JA: TNF-α Promoter Methylation as a Predictive Biomarker for Weight-loss Response. Obesity 2009;17(6):1293-7.
36. Hermsdorff HH, Mansego ML, Campión J, Milagro FI, Zulet MA, Martínez JA: TNF-alpha promoter methylation in peripheral white blood cells: Relationship with circulating TNFα, truncal fat and n-6 PUFA intake in young women. Cytokine 2013;64(1):265-71.
37. Puchau B, Hermsdorff HHM, Zulet M, Martínez JA: DDAH2 mRNA expression is inversely associated with some cardiovascular risk-related features in healthy young adults. Dis Markers 2009;27(1):37-44.
38. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 2001;25(4):402-8.
39. WHO. Diet, nutrition and the prevention of chronic diseases. . Organization WH, editor. Geneva, Switzerland: World Health Organization; 2003.
40. Salas-Salvadó J, Martinez-González MÁ, Bulló M, Ros E: The role of diet in the prevention of type 2 diabetes. Nutr Metab Cardiovasc Dis 2011; 21 (2):B32-B48.

41. Ylönen K, Alfthan G, Groop L, Saloranta C, Aro A, Virtanen SM: Dietary intakes and plasma concentrations of carotenoids and tocopherols in relation to glucose metabolism in subjects at high risk of type 2 diabetes: the Botnia Dietary Study. Am J Clin Nutr 2003;77(6):1434-41.
42. Carter P, Gray LJ, Talbot D, Morris DH, Khunti K, Davies MJ: Fruit and vegetable intake and the association with glucose parameters: a cross-sectional analysis of the Let/'s Prevent Diabetes Study. Eur J Clin Nutr 2013;67(1):12-7.
43. Martínez-González MA, García-Arellano A, Toledo E, Salas-Salvadó J, Buil-Cosiales P, Corella D, et al: A 14-Item Mediterranean Diet Assessment Tool and Obesity Indexes among High-Risk Subjects: The PREDIMED Trial. PLoS ONE 2012;7(8):e43134.
44. Hotamisligil GS: Mechanisms of TNF-alpha-induced insulin resistance. Exp Clin Endocrinol Diabetes 1999;107(2):119-25.
45. Cocate P, Natali A, Alfenas R, de Oliveira A, Dos Santos E, Hermsdorff H: Carotenoid consumption is related to lower lipid oxidation and DNA damage in middle-aged men. Br J Nutr 2015;114(2):257.
46. Holt EM, Steffen LM, Moran A, Basu S, Steinberger J, Ross JA, Hong CP, Sinaiko AR: Fruit and Vegetable Consumption and Its Relation to Markers of Inflammation and Oxidative Stress in Adolescents. J Am Diet Assoc 2009; 109(3):414-21.
47. Jiang Y, Wu S-H, Shu X-O, Xiang Y-B, Ji B-T, Milne GL, Cai Q, Zhang X, Gao YT, Zheng W, Yang G: Cruciferous Vegetable Intake Is Inversely Correlated with Circulating Levels of Proinflammatory Markers in Women. J Acad NutrDiet 2014;114(5):700-8.e2.
48. Root MM, McGinn MC, Nieman DC, Henson DA, Heinz SA, Shanely RA, Knab AM, Jim F: Combined Fruit and Vegetable Intake Is Correlated with Improved Inflammatory and Oxidant Status from a Cross-Sectional Study in a Community Setting. Nutrients 2012;4(1):29-41.
49. Hermsdorff HHM, Barbosa KBF, Volp ACP, Puchau B, Bressan J, Zulet MÁ, Martinez JA: Vitamin C and fibre consumption from fruits and vegetables improves oxidative stress markers in healthy young adults. Br J Nutr 2012;107(08):1119-27.
50. Delgado-Cruzata L, Zhang W, McDonald JA, Tsai WY, Valdovinos C, Falci L, Wang Q, Crew KD, Santella RM, Hershman DL, Greenlee H: Dietary Modifications, Weight Loss, and Changes in Metabolic Markers Affect Global DNA Methylation in Hispanic, African American, and Afro-Caribbean Breast Cancer Survivors. J Nutr 2015;145(4):783-90.
51. Zhang FF, Morabia A, Carroll J, Gonzalez K, Fulda K, Kaur M, Vishwanatha JK, Santella RM, Cardarelli R: Dietary Patterns Are Associated with Levels of Global Genomic DNA Methylation in a Cancer-Free Population. J Nutr 2011;141(6):1165-71.
52. Ross SA: Diet and DNA Methylation Interactions in Cancer Prevention. Annals NY Acad Sci 2003;983(1):197-207.
53. Wannamethee SG, Whincup PH, Thomas MC, Sattar N: Associations Between Dietary Fiber and Inflammation, Hepatic Function, and Risk of Type 2 Diabetes in Older Men: Potential mechanisms for the benefits of fiber on diabetes risk. Diabetes Care 2009;32(10):1823-5.
54. Krishnamurthy VMR, Wei G, Baird BC, Murtaugh M, Chonchol MB, Raphael KL, Greene T, Beddhu S: High dietary fiber intake is associated with decreased inflammation and all-cause mortality in patients with chronic kidney disease. Kidney Int 2012;81(3):300-6.
55. Parikh S, Pollock NK, Bhagatwala J, Guo D-H, Gutin B, Zhu H, Dong Y: Adolescent Fiber Consumption Is Associated with Visceral Fat and Inflammatory Markers. J Clin Endocrinol Metab 2012;97(8):E1451-E7.
56. Ghanim H, Dhindsa S, Batra M, Green K, Abuaysheh S, Chaudhuri A, Dandona P: The Intake of Fiber Suppresses the High-Fat High-Carbohydrate Meal-Induced Endotoxemia, Oxidative Stress and Inflammation. Endocrine Abst 2012; 29: P613.

57. Hsu A, Wong C, Yu Z, Williams D, Dashwood R, Ho E: Promoter de-methylation of cyclin D2 by sulforaphane in prostate cancer cells. Clin Epigenetics 2011;3:3.
58. Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W, Yang CS: Tea Polyphenol (−)-Epigallocatechin-3-Gallate Inhibits DNA Methyltransferase and Reactivates Methylation-Silenced Genes in Cancer Cell Lines. Cancer Res 2003;63(22):7563-70.
59. Rahman I, Chung S: Dietary polyphenols, deacetylases and chromatin remodeling in inflammation. J Nutrigenet Nutrigenomics. 2010;3:220–30.
60. Flower K, Thomas D, Heather J, Ramasubramanyan S, Jones S, Sinclair AJ: Epigenetic Control of Viral Life-Cycle by a DNA-Methylation Dependent Transcription Factor. PLoS ONE 2011;6(10):e25922.
61. Rishi V, Bhattacharya P, Chatterjee R, Rozenberg J, Zhao J, Glass K, Fitzgerald P, Vinson C: CpG methylation of half-CRE sequences creates C/EBPα binding sites that activate some tissue-specific genes. Proc Natl Acad Sci 2010;107(47):20311-6.
62. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schübeler D: Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet. 2007;39(4):457-66.
63. Kirchner H, Nylen C, Laber S, Barrès R, Yan J, Krook A, Zierath JR, Näslund E: Altered promoter methylation of PDK4, IL1 B, IL6, and TNF after Roux-en Y gastric bypass. Surg Obes Relat Dis 2014;10(4):671-8.
64. Messeguer X, Escudero R, Farré D, Nuñez O, Martínez J, Albà M: PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics. 2002;18(2):333-4.
65. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003; 33: 245-54.
66. Ong C-T, Corces VG: Enhancer function: new insights into the regulation of tissue-specific gene expression. Nat Rev Genet 2011; 12(4):283-93.
67. Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S: Expression of mRNA for DNA methyltransferases and methyl-CpG–binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. Hepatology 2001; 33(3):561-8.
68. Ehrlich M, Woods CB, Yu MC, Dubeau L, Yang F, Campan M, Weisenberger DJ, Long T, Youn B, Fiala ES, Laird PW: Quantitative analysis of associations between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. Oncogene 2006;25(18):2636-45.
69. La Garza AL, Etxeberria U, Palacios-Ortega S, Haslberger AG, Aumueller E, Milagro FI, Martinez JA: Modulation of hyperglycemia and TNF-alpha-mediated inflammation by helichrysum and grapefruit extracts in diabetic db/db mice. Food Funct 2014;5(9):2120-8.
70. Wan J, Oliver VF, Wang G, Zhu H, Zack DJ, Merbs SL, Quian J: Characterization of tissue-specific differential DNA methylation suggests distinct modes of positive and negative gene expression regulation. BMC Genomics 2015;16(1):49.
71. Boqué N, de la Iglesia R, de la Garza AL, Milagro FI, Olivares M, Bañuelos Ó, Soria AC, Rodríguez-Sanchez S, Martínez JA, Campión J: Prevention of diet-induced obesity by apple polyphenols in Wistar rats through regulation of adipocyte gene expression and DNA methylation patterns. Mol Nutr Food Res 2013;57(8):1473-8.
72. Coelho R, Hermsdorff H, Bressan J: Anti-inflammatory Properties of Orange Juice: Possible Favorable Molecular and Metabolic Effects. Plant Foods Hum Nutr 2013;68(1):1-10.

Table 1: Sample characterization according to median of fruit intake per day and associations between fruit intake and supplementation and physical activity.

Variables	Low intake (≤293.4 g/day)	High intake (>293.4 g/day)	P values
Age (y)	20.7 ± 2.5	20.7 ± 2.3	0.948
Body mass index (kg/m²)	21.2 ± 1.6	20.8 ± 1.9	0.433
Body fat (%)	22.2 ± 4.7	20.7 ± 5.1	0.344
Waist circumference (cm)	68.3 ± 4.6	67.8 ± 4.9	0.717
Glucose (mg/dL)	80.9 ± 7.3	78.4 ± 5.7	0.237
Insulin (µU/L)	8.36 ± 2.72	6.01 ± 3.39	**0.020***
HOMA-IR index	1.69 ± 0.60	1.16 ± 0.68	**0.014***
Total Cholesterol (mg/dL)	178 ± 25	183 ± 28	0.618
LDL-cholesterol (mg/dL)	102 ± 19	106 ± 25	0.536
HDL-cholesterol (mg/dL)	64 ± 13	63 ± 12	0.830
TC/HDL ratio	2.85 ± 0.54	2.96 ± 0.59	0.545
Triglycerides (mg/dL)	62.9 ± 23	65.9 ± 23.4	0.687
NEFA (mmol/L)	0.48 ± 0.25	0.42 ± 0.18	0.382
Systolic BP (mmHg)	113.2 ± 10.0	107.2 ± 9.8	0.063
Diastolic BP (mmHg)	63.5 ± 6.3	64.5 ± 8.7	0.680
TNF-α (pg/mL)	2.36 ± 3.90	2.37 ± 2.71	0.992
TNF-α mRNA (RE)	1.87 ± 0.53	1.81 ± 0.56	0.712
Vitamin/Mineral suppl (Yes)	5	11	0.053
Physical activity (Yes)	8	13	0.113

NEFA: non-esterified fatty acids; TC: Total cholesterol; BP: Blood pressure; TNF-α: Tumor necrosis factor α; RE: relative expression; suppl: Supplementation.

Results are shown as Mean ± SD or frequency (n) of occurrence. P values from Student's *t*-tests for means tests and from Chi-Square test for associations. * Indicates statistical significance ($p<0.05$).

Table 2: Correlations between healthy food groups and CpG5 and *TNF-α* total methylation.

Food Group Intake	CpG5 Methylation (%)		Total methylation (%)	
	R	p	R	p
Vegetables (g/day)	-0.143	0.379	-0.216	0.180
Legumes (g/day)	-0.245	0.128	-0.137	0.400
Nuts (g/day)	-0.104	0.523	-0.014	0.932
Natural juice (g/day)	-0.037	0.822	-0.030	0.855
Cereals (g/day)	-0.022	0.894	-0.116	0.477
Whole cereals (g/day)	-0.195	0.227	-0.276	0.084
Mediterranean Dietary Pattern	-0.162	0.318	-0.227	0.159
Healthy Eating Index	-0.460	**0.003***	-0.355	**0.026***

P values from Spearman or Pearson coefficient correlations, as appropriate. * Indicates statistical significance ($p<0.05$).

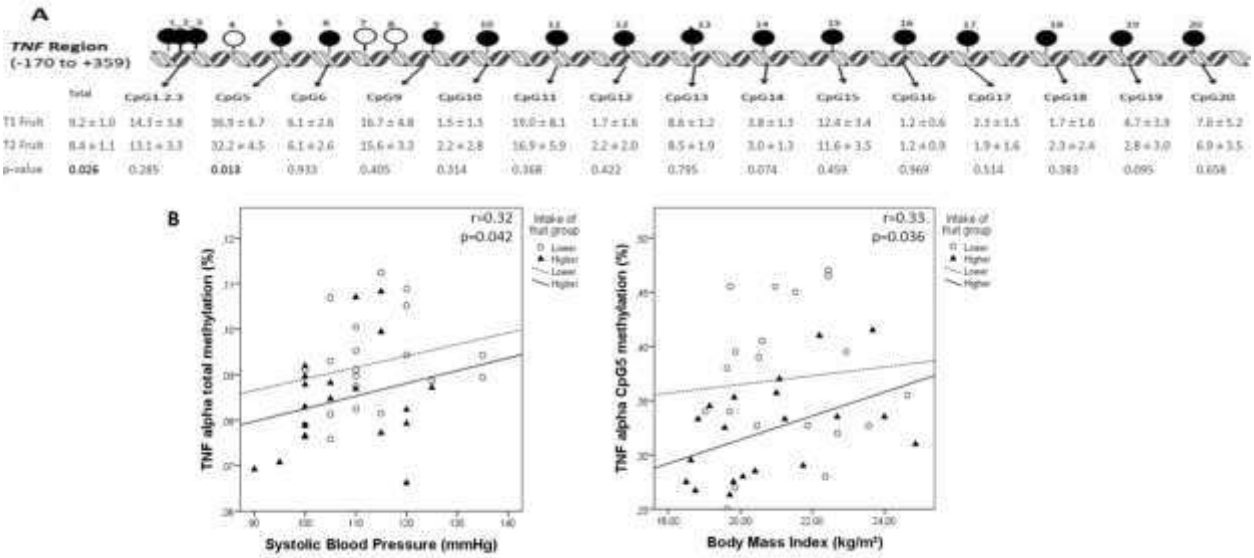
Figure Captions

Figure 1: Methylation (%) of 17 CpGs sites located in Tumor Necrosis Factor-α gene. (A) DNA methylation according to the median of fruit intake (293.4 g/day). (B) Correlation of the methylation levels of different CpGs and anthropometric/metabolic features.

Figure 2: Correlations between TNF-α total methylation (%) and some nutrients present in fruits.

Figure 3: CpG5 and TNF-α total methylation in relation to tertiles of orange/tangerine daily intake. Different letter indicates statistical differences between tertiles of intake. P for trend was determined by ANOVA with polynomial contrasts. Statistical significance <0.05.

509 **Figure 1**



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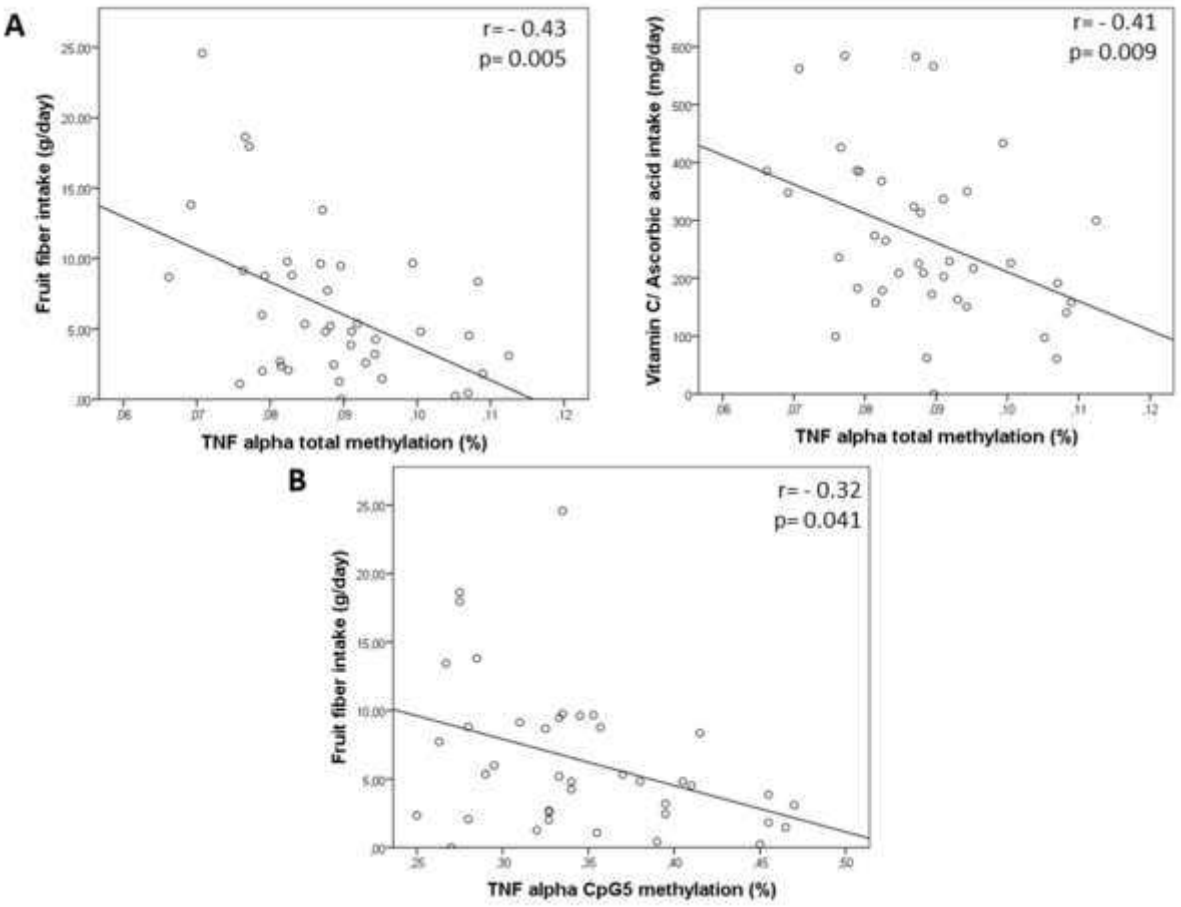
512 **Figure 2**



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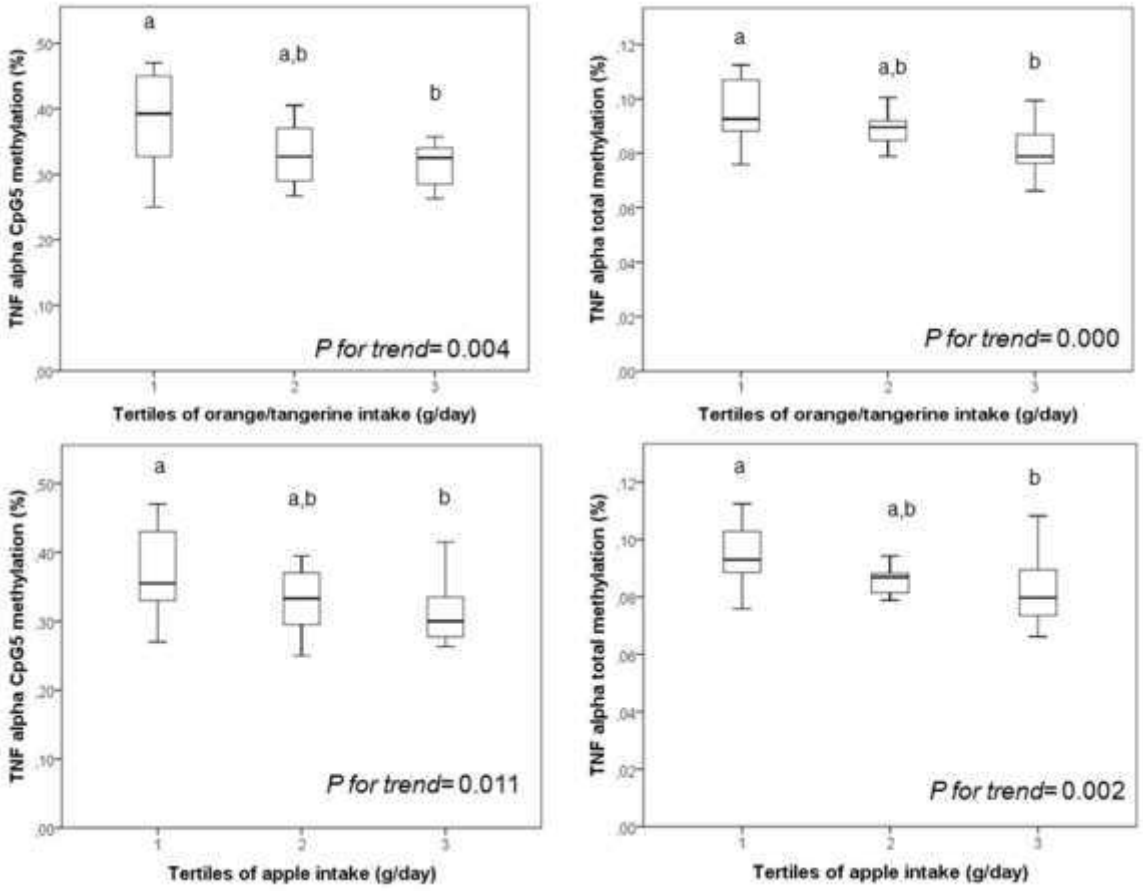
515 **Figure 3**



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7. CONCLUSÕES GERAIS

As concentrações de citocinas inflamatórias circulantes e marcadores epigenéticos (metilação de DNA) podem ser bons preditores precoces de alterações metabólicas, já que se encontram alterados antes mesmo do desenvolvimento da enfermidade. A IL-6, melhor que a IL-18, está associada à maior adiposidade e ao maior risco de resistência à insulina, podendo ser influenciada pelo hábito de fumar. O nível de metilação global (*LINE-1*) e dos promotores de genes inflamatórios específicos (*IL-6, SERPINE1, PCR e TNF-*α), estão associados a componentes de risco metabólico e podem ser influenciados pela qualidade da dieta e ingestão de nutrientes e,ou grupos alimentares específicos, como o de frutas.

8. REFERÊNCIAS BIBLIOGRÁFICAS

AGIRBASLI M, AGAOGLU NB, ERGONUL O, et al. Comparison of anthropometric indices in predicting metabolic syndrome components in children. *Metab Syndr Relat Disord* 2011; 9: 453–459

ALBERTI, K.G., ZIMMET P., SHAW J. The metabolic syndrome–a new worldwide definition. *Lancet*, 366(9491):1059–1062, 2005.

ALBERTI, K.G.; ECKEL, R.H.; GRUNDY, S.M.; ZIMMET, P.Z.; CLEEMAN, J.I.; et al. Harmonizing the metabolic syndrome: a joint interim statement of theInternational Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 120: 1640–1645, 2009.

AMARAL CL, MILAGRO FI, CURI R, MARTÍNEZ JA. DNA Methylation Pattern in Overweight Women under an Energy-Restricted Diet Supplemented with Fish Oil. BioMed Research International. 2014;2014:10.

AZIZI, F.; SALEHI, P.; ETEMADI, A.; ZAHEDI-ASL, S. Prevalence of metabolic syndrome in an urban population: Tehran lipid and glucosa study. *Diabetes Res Clin Pract* 61:29-37, 2003.

BALKAU, B.; CHARLES, M.A.; DRIVSHOLM, T.; BORCH-JONHSEN, K.; WAREHAM, N.; YUDKIN, J.S.; MORRIS, R.; ZAVARONI, I.; VAN DAM, R.; FESKINS, E.; GABRIEL, R. DIET, M.; NILSSON, P.; HEDBLAD, B.; European Group for the Study of Insulin Resistance (EGRI). Frequency of the WHO metabolic syndrome in European cohort, and an alternative definition of an insulin resistance syndrome. *Diabetes Metab* 28:364-376, 2002.

BERGER SL, KOUZARIDES T, SHIEKHATTAR R, SHILATIFARD A. An operational definition of epigenetics. Genes & Development. 2009;23(7):781-3.

BOEKE, C.E.; BACCARELLI, A.; KLEINMAN, K.P.; BURRIS, H.H.; LITONJUA, A.A.; RIFAS-SHIMAN, S.L.; TARANTINI, L.; GILLMAN, M.W. Gestational intake of methyl donors and global LINE-1 DNA methylation in maternal and cord blood. Prospective results from a folate-replete population. Epigenetics, 7(3): 253-260. 2012.

BRUCE, K.D.; HANSON, M.A. The Developmental Origins, Mechanisms, and Implications of Metabolic Syndrome. *J Nutr,* 140: 648–652, 2010.

BUEGE, J.A.; AUST, S.D. Microsomal lipid peroxidation. Methods in enzymology, 52: 302-310. 1978.

CAMERON AJ, *et al.* The metabolic syndrome: prevalence in worldwide populations. *Endocrinol Metab Clin N Am* 33: 351–376, 2004.

CAMPION, J., MILAGRO, F.I., MARTINEZ, JA. Individuality and epigenetics in obesity. *Obes Rev*, 10:383–92, 2009.

CARRARO, J.C.C., HERMSDORFF, H.H.M., PUCHAU, B. et al. Interleukin-6 is a better metabolic biomarker than interleukin-18 in young healthy adults. *Journal of Physiology and Biochemistry*, 1-9, 2015.

CHEN S-J, YEN C-H, HUANG Y-C, LEE B-J, HSIA S, et al. Relationships between Inflammation, Adiponectin, and Oxidative Stress in Metabolic Syndrome*. PLoS ONE,* 7(9): e45693, 2012.

CORELLA, D.; CARRASCO, P.; FITÓ, M.; MARTÍNEZ-GONZÁLEZ, M.A.; SALAS-SALVADÓ, J.; ARÓS, F.; LAPETRA, J.; GUILLÉN, M.; ORTEGA-AZORÍN, C.; WARNBERG, J.; FIOL, M.; RUIZ-GUTIERREZ, V.; SERRA-MAJEM, L.; MARTÍNEZ, J.A.; ROS, E.; ESTRUCH, R. Gene-environment interactions of CETP

gene variation in a high cardiovascular risk Mediterranean population. *J Lipid Res* 51(9):2798-807, 2010.

CORNIER, M. A. et al. The metabolic syndrome. *Endocr Rev*, 29, 777–822, 2008.

DANDONA, P.; ALJADA, A.; BANDYOPADHYAY, A. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 25:4-7, 2004.

DANDONA, P.; ALJADA, A.; CHAUDHURI, A.; MOHANTY, P.; GARG, R. Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circulation* 111: 1448-1454, 2005.

DASHTI, H.; SMITH, C.; TANAKA, T.; ORDOVÁS, J. Interactions between circadian gene variants and fatty acid intake for metabolic syndrome risk: a meta-analysis from the Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium (373.2). *The FASEB Journal*, 28 (1): 373-2. 2014.

DA SILVA, H.A.; CARRARO, J.C.C.; BRESSAN, J.; HERMSDORFF, H.H.M. Relação entre ácido úrico e síndrome metabólica em uma população com risco cardiometabólico. *Revista Einstein* [Online]. Disponível em http://www.scielo.br/pdf/eins/2015nahead/pt_1679-4508-eins-S1679-45082015AO3194.pdf Acesso em 13 de novembro de 2015.

DELGADO-CRUZATA L.; ZHANG, W.; MCDONALD, J.A.; TSAI, W.Y.; VALDOVINOS C.; FALCI, L. et al. Dietary Modifications, Weight Loss, and Changes in Metabolic Markers Affect Global DNA Methylation in Hispanic, African American, and Afro-Caribbean Breast Cancer Survivors. *The Journal of Nutrition* 145:783-790. 2015.

DUFFY, M.J. Role of tumor markers in patients with solid cancers: a critical review. *European Journal of Internal Medicine*, 18, 175–184, 2007.

ERICKSON, S.K. Nonalcoholic fatty liver disease. *J. Lipid Res.* 50: S412–S416, 2009.

ESPOSITO, K.; PONTILLO, A.; GIUGLIANO, F.; GIUGLIANO, G.; MARFELLA, R.; NICOLETTI, G.; GIUGLIANO, D. Association of Low Interleukin-10 Levels with the Metabolic Syndrome in Obese Women. *J Clin Endocrinol Metabol*, 88(3):1055–1058, 2003.

ESTELLER M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene. 2002;21:5427 – 40.

Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Experte Panel on Detection, Evaluation, and Treatment of high blood cholesterol in adults (Adult Treament Panel III). *JAMA* 285: 2486-2497, 2001.

FARMER, S.R. Transcriptional control of adipocyte formation. *Cell Metab*, 4: 263–273, 2006.

FERNANDEZ-BALLART, J.D.; PIÑOL, J.L.; ZAZPE, I.; CORELLA, D.; CARRASCO, P.; TOLEDO, E.; PEREZ-BAUER, M.; MARTÍNEZ-GONZÁLEZ, M.A.; SALAS-SALVADÓ, J.; MARTÍN-MORENO, J.M. Validation of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. In press 2009.

FORD, E.S.; MOKDAD, A.H.; GILES, W.H.; BROWN, D.W. The metabolic syndrome and antioxidant concentrations: findings from the Third National Health and Nutrition Examination Survey. *Diabetes* 52: 2346-2352, 2003.

FRIEDEWALD WT, LEVY RI, FREDRICKSON DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.

FRIEDMAN, J.M.; HALAAS, J.L. Leptin and the regulation of body weight in mammals. Nature 395: 763-770. 1998.

FUENTES, E.; FUENTES, F.; VILAHUR, G.; BADIMON, L.; PALOMO, I. Mechanisms of chronic state of inflammation as mediators that link obese adipose tissue and metabolic syndrome. *Mediators of inflammation*, 2013. [Online] Disponível em: http://www.hindawi.com/journals/mi/2013/136584/abs/ Acesso em 16 de novembro de 2015.

GALLOU-KABANI, C.; JUNIEN, C. New Perspective Against the Epidemic Nutritional Epigenomics of Metabolic Syndrome. Diabetes, 54(7): 1899-1906. 2005.

GARAULET, M.; MADRID, J.A. Chronobiology, genetics and metabolic syndrome. *Curr Opinion Lipidol*, 20:127–134, 2009.

GARNETT SP, BAUR LA, SRINIVASAN S, LEE JW, COWELL CT. Body mass index and waist circumference in midchildhood and adverse cardiovascular disease risk clustering in adolescence. *Am J Clin Nutr*, 86: 549–555, 2007.

GRUNDY, SM. Metabolic syndrome: therapeutic considerations. *Handb Exp Pharmacol*, 107–33. 2005.

GUENTHER, P.M.; REEDY, J.; KREBS-SMITH, S.M. Development of the Healthy Eating Index-2005. *Journal of American Dietary Association*, 108(11):1896-901. 2008

HERMSDORFF, H.H.M., PUCHAU, B., BRESSAN, J., MARTÍNEZ, J.A. Association of retinol-binding protein-4 with dietary selenium intake and other lifestyle features in young healthy women. *Nutrition*, 25(4), 392–399, 2009.

HERMSDORFF, H.H.M.; PUCHAU, B.; ZULET, M.Á.; MARTÍNEZ, J.A. Association of Body Fat Distribution with Proinflammatory Gene Expression in

Peripheral Blood Mononuclear Cells from Young Adult Subjects. *OMICS: A Journal of Integrative Biology* 14(3): 297-307, 2010.

HERMSDORFF, H.H.M.; ZULET, M.Á.; PUCHAU, B.; MARTÍNEZ, J.A. Central Adiposity Rather Than Total Adiposity Measurements Are Specifically Involved in the Inflammatory Status from Healthy Young Adults. *Inflammation* 34(3): 161-170, 2011.

HOTAMISLIGIL, G.S.; SHARGILL, N.S.; SPIEGELMAN, B.M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*, 259: 87, 1993.

HU FB, MEIGS JB, LI TY, RIFAI N, MANSON JE. Inflammatory markers and risk of developing type 2 diabetes in women. *Diabetes* 53:693–700, 2004.

ISOMAA, B., ALMGREN, P., TUOMI, T., FORSEN, B., LAHTI, K., NISSEN, M., TASKINEN, M.R., GROOP, L. Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care* 24, 683–689, 2001.

JAMES W: WHO recognition of the global obesity epidemic. *Inter J obes*, 32:S120–S126, 2008.

JANGHORBANI, M.; AMINI, M. Comparison of glycated hemoglobin with fasting plasma glucose in definition of glycemic component of the metabolic syndrome in an Iranian population Diabetes & Metabolic Syndrome. *Clin Res Rev*, xxx: xxx–xxx, 2012.

JANSSEN I, POWELL LH, CRAWFORD S, LASLEY B, SUTTON-TYRRELL K: Menopause and the metabolic syndrome: the Study of Women's Health Across the Nation. *Arch Intern Med*, 168(14):1568–1575, 2008.

JIRTLE RL, SKINNER MK. Environmental epigenomics and disease susceptibility. Nat Rev Genet. 2007;8(4):253-62.

JONES PA, TAKAI D. The Role of DNA Methylation in Mammalian Epigenetics. Science. 2001;293(5532):1068-70.

KADOWAKI, T.; YAMAUCHI, T. Adiponectin and adiponectin receptors. *Endocr Rev*, 26:439–451, 2005.

KERSHAW, E.E., FLIER, J.S. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab*, 89:2548–2556, 2004.

KIM M, LONG TI, ARAKAWA K, WANG R, YU MC, LAIRD PW. DNA Methylation as a Biomarker for Cardiovascular Disease Risk. PLoS ONE. 2010;5(3):e9692.

KRISTIANSSON, K.; PEROLA, M.; TIKKANEN, E.; KETTUNEN, J.; SURAKKA, I.; HAVULINNA, A.S. et al. Genome-wide screen for metabolic syndrome susceptibility Loci reveals strong lipid gene contribution but no evidence for common genetic basis for clustering of metabolic syndrome traits. *Circulation: Cardiovascular Genetics*, 5(2): 242-249. 2012.

KUNITSKAYA,N. A.; ARIEV, A.L. The Properties of Disorders of Carbohydrate Metabolism in Elderly Patients with Gout and Metabolic Syndrome. *Adv Gerontol*, 2 (4): 336–339, 2012.

LAZAR, M.A. The humoral side of insulin resistance. *Nat Med*, 12:43–44. 2006.

LIGUORI, R., LABRUNA, G., ALFIERI, A., MARTONE, D., FARINARO, E., CONTALDO, F., SACCHETTI, L.; PASANISI, F.; BUONO, P. The FTO gene polymorphism (rs9939609) is associated with metabolic syndrome in morbidly obese subjects from southern Italy. *Molecular and cellular probes*, 28(4): 195-199. 2014.

LOPEZ-LEGARREA, P.; MANSEGO, M.L.; ZULET, M.A.; MARTINEZ, J.A. Serpine1, PAI-1 protein coding gene, methylation levels and epigenetic relationships with adiposity changes in obese subjects with metabolic syndrome features under dietary restriction. *Journal of clinical biochemistry and nutrition*, 53(3): 139. 2013.

MANCIA, G.; DE BACKER, G.; DOMINICZAK, A.; CIFKOVA, R.; FAGARD, R. et al. Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Journal of Hypertension* 25(6):1105-87, 2007. Erratum in: *Journal of Hypertension* 25(8):1749, 2007.

MATSUDO, S.; ARAÚJO, T.; MATSUDO, V. et al. Questionário Internacional de Atividade Física (IPAQ): Estudo de validade e reprodutibilidade no Brasil. *Atividade Física e Saúde* 6(2):5-16, 2001.

MATTHEWS, D.R.; HOSKER, J.P.; RUDENSKI, A.S.; NAYLOR, B.A.; TREACHER, D.F.; TURNER, R.C. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28:412-9, 1985.

MILAGRO FI, CAMPIÓN J, GARCÍA-DÍAZ DF, GOYENECHEA E, PATERNAIN L, MARTÍNEZ JA. High fat diet-induced obesity modifies the methylation pattern of leptin promoter in rats. J Physiol Biochem. 2009;65(1):1-9.

MINA, T. H.; RÄIKKÖNEN, K.; RILEY, S. C.; NORMAN, J. E.; REYNOLDS, R. M. Maternal Distress Associates with Placental Genes Regulating Fetal Glucocorticoid Exposure and IGF2: Role of Obesity and Sex. *Psychoneuroendocrinology*, 59: 112-122. 2015.

MORENO-ALIAGA, M.J.; CAMPIÓN, J.; MILAGRO, F.I.; BERJÓN, A.; MARTINEZ, J.A. Adiposity and proinflammatory state: the chicken or the egg. *Adipocytes* 1:1-6, 2005.

MORABIA, A., COSTANZA, M,C. The obesity epidemic as harbinger of a metabolic disorder epidemic: trends in overweight, hypercholesterolemia, and diabetes treatment in Geneva, Switzerland, 1993–2003. *American Journal of Public Health*, 95, 632–635, 2005.

NATIONAL INSTITUTES OF HEALTH. Biomarkers Definition Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical and Pharmacological Therapeutics,* 69, 89–95, 2001.

PELLEYMOUNTER, M.A.; CULLEN, M.J.; BAKER, M.B.; HECHT, R.; WINTERS, D.; BOONE, T.; COLLINS, F. Effects of the obese gene product on body weight regulation in ob/ob mice, *Science*, 269 (1995) 540–543.

PENCKOFER S, SCHWERTZ D, FLORCZAK K. Oxidative stress cardiovascular disease in type 2 diabetes: the role of antioxidants and pro-oxidants. *J Cardiovasc Nurs,* 16: 68–85, 2002.

PIETILÄINEN KH, ISMAIL K, JÄRVINEN E, HEINONEN S, TUMMERS M, BOLLEPALLI S, LYLE R, MUNIANDY M, MOILANEN E, HAKKARAINEN A, LUNDBOM J, LUNDBOM N, RISSANEN A, KAPRIO L, OLLIKAINEN, M. DNA methylation and gene expression patterns in adipose tissue differ significantly within young adult monozygotic BMI-discordant twin pairs. International journal of obesity (2005). (2015). [Online] Avaiable at http://www.nature.com/ijo/journal/vaop/naam/abs/ijo2015221a.html Accessed on 10 November 2015.

POLLEX, R. L.; HEGELE, R. A. Genetic determinants of themetabolic syndrome. *Nat Clin Pract Cardiovasc Med*, 3, 482–489, 2006.

POVEL, C.M.; BOER, J.M.A.; REILING E.; FESKENS, E.J.M. Genetic variants and the metabolic syndrome: a systematic review. *Obesity rev*, 12, 952–967, 2011.

PREVIDELLI, A.; ANDRADE, S.; PIRES, M.; FERREIRA, S.; FISBERG, R.; MARCHIONI, D. A revised version of the Healthy Eating Index for the Brazilian population. Revista de Saúde Publica, 45: 794-798. 2011.

RAMANCHANDRAN, A.; SNEHALATHA, C.; SATYAVANI, K.; SIVASANKARI, S.; VIJAY, V. Metabolic syndrome in urban Asian Indian Adults: a population study using modified ATP criteria. *Diabetes Res Clin Pract* 61:29-37, 2003.

RAMÍREZ-VARGAS, E.; ARNAUD-VIÑAS; MDEL, R.; DELISLE, H. Prevalence of the metabolic syndrome and associated lifestyles in adult males from Oaxaca, Mexico. *Salud Publica Mex* 49(2):94-102, 2007.

RAMPAL, S., MAHADEVA, S. GUALLAR, E., BULGIBA, A., MOHAMED, R., et al. Ethnic Differences in the Prevalence of Metabolic Syndrome: Results from a MultiEthnic Population-Based Survey in Malaysia. *PLoS ONE* 7(9): e46365, 2012.

ROBERTSON KD. DNA methylation and human disease. Nat Rev Genet. 2005;6(8):597-610.

ROSEN, B.S.; COOK, K.S.; YAGLOM, J.; GROVES, D.L.; VOLANAKIS, J.E.; DAMM, D.; WHITE, T.; SPIEGELMAN, B.M. Adipsin and complement factor D activity: an immune-related defect in obesity. *Science,*244: 1483, 1989.

ROSMOND, R. Visceral obesity and the metabolic syndrome, in: Björntorp, P. (Ed.), International Textbook of Obesity. Wiley, Chichester, pp. 339–350, 2001.

ROULSTON, J.E. Limitations of tumour markers in screening. British Journal of Surgery, 77, 961–962, 1990.

RYO, M., NAKAMURA, T., KIHARA, S. et al. Adiponectin as a Biomarker of the Metabolic Syndrome. *Circulation Journal*, 68 (11), 975-981, 2004.

SCHIFFRIN, E.L. Oxidative Stress, Nitric Oxide Synthase, and Superoxide Dismutase: A Matter of Imbalance Underlies Endothelial Dysfunction in the Human Coronary Circulation. *Hypertension* 51:31-32, 2008

SHANKAR, G.. Role of Serotonin from Thought and Anxiety to Weight Gain and Metabolic Syndrome. *J Autacoids*, 1:3, editorial, 2012.

SHARMA S, KELLY TK, JONES PA. Epigenetics in cancer. Carcinogenesis. 2010;31(1):27-36.

SIMENTAL-MENDÍA LE, RODRÍGUEZ-MORÁN M, GUERRERO-ROMERO F. The Product of Fasting Glucose and Triglycerides As Surrogate for Identifying Insulin Resistance in Apparently Healthy Subjects. Metabolic Syndrome and Related Disorders. 2008;6(4):299-304.

SIMOPOULOS, A.P. The Importance of the Omega-6/Omega-3 Fatty Acid Ratio in Cardiovascular Disease and Other Chronic Diseases. Exp Biol Med, 233 (6): 674-688. 2008.

SPOTSWOOD, H.T, TURNER, B.M. An increasingly complex code. *Journal of Clinical Investigation*, 110 (5), 577-582, 2002.

STOCKER, R., KEANEY, J.F. Role of oxidative modification in atherosclerosis. *Physiol*, 84: 1381–1478, 2004.

STRIMBU, K., TAVEL, J.A. What are Biomarkers? *Current Opinion in HIV and AIDS,* 5 (6), 463-466, 2010.

TEDGUI, A.; MALLAT, Z. Anti-inflammatory mechanisms in the vascular wall. *Circ Res*, 88:877–887, 2001.

THOMAS, C.M., SWEEP, C.G. Serum tumor markers: past, state of the art, and future. *International Journal of Biological Markers,* 16, 73–86, 2001.

TONG, P.C., KONG, A.P., SO, W.Y., YANG, X., HO, C.S., MA, R.C., OZAKI, R., CHOW, C.C., LAM, C.W., CHAN, J.C., COCKRAM, C.S. The usefulness of the International Diabetes Federation and the National Cholesterol Education Program's Adult 10 Treatment Panel III definitions of the metabolic syndrome in predicting coronary heart disease in subjects with type 2 diabetes. *Diabetes Care*, 30, 1206-1211, 2007.

TOPEROFF G, ARAN D, KARK JD, ROSENBERG M, DUBNIKOV T, NISSAN B, et al. Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. Human Molecular Genetics. 2012;21(2):371-83.

TRICHOPOULOU, A.; COSTACOU, T.; BAMIA, C.; TRICHOPOULOS, D. Adherence to a Mediterranean Diet and Survival in a Greek Population. *New England Journal of Medicine,* 348(26):2599-608. 2003.

UM, J.Y.; CHUNG, H.S.; SONG, M.Y.; SHIN, H.D.; KIM, H.M. Association of interleukin-1beta gene polymorphism with body mass index in women. *Clin Chem*, 50:647–650, 2004.

UNGER, R.H.; CLARK, G.O.; SCHERER, P.E.; ORCI, L. Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1801(3), 209-214. 2010.

URIARTE, G.; PATERNAIN, L.; MILAGRO, F.I.; MARTÍNEZ, J.A.; CAMPION, J. Shifting to a control diet after a high-fat, high-sucrose diet intake induces epigenetic changes in retroperitoneal adipocytes of Wistar rats. J Physiol Biochem, 69:601–611. 2013.

VACHHARAJANI V, GRANGER DN. Adipose tissue: A motor for the inflammation associated with obesity. IUBMB Life. 2009;61(4):424-30.

VAN DIJK, S.J.; FESKENS, E.J.; BOS, M.B.; HOELEN, D.W.; HEIJLIGENBERG, R.; BROMHAAR, M.G.; DE GROOT, L.C.; DE VRIES, J.H.; MÜLLER, M.; AFMAN, L.A. A saturated fatty acid-rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome. *Am J Clin Nutr* 90(6):1656-64, 2009.

VAN ENGELAND, M.; WEIJENBERG, M.P.; ROEMEN, G.M.JM. et al. Cohort Study on Diet and Cancer Methylation in Sporadic Colorectal Cancer: The Netherlands Effects of Dietary Folate and Alcohol Intake on Promoter. Cancer Res, 63:3133-3137. 2003.

VIRTUE, S.; VIDAL-PUIG, A. Adipose tissue expandability, lipotoxicity and the metabolic syndrome—an allostatic perspective. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1801(3), 338-349. 2010.

VIDIGAL, F.C. BRESSAN, J.; BABIO, N.; SALAS-SALVADÓ, J. Prevalence of metabolic syndrome in Brazilian adults: a systematic review. BMC Public Health, 13:1198. 2013.

VOLP, A. C.P.; BARBOSA, K.B.F.; BRESSAN, J. Triacylglycerols and body fat mass are possible independent predictors of C3 in apparently healthy young Brazilian adults. *Nutrition* 28(5): 544–550, 2012.

WAJCHENBERG, B. L. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocrine reviews*, 21(6), 697-738. 2000.

WATERLAND, R.A.; GARZA, C. Potential mechanisms of metabolic imprinting thatlead to chronic disease. *Am J Clin Nutr*, 69:179–197, 1999.

WEST, I.C. Radicals and oxidative stress in diabetes. *Diabetic Med,* 17: 171–180, 2000.

WHO, 2000. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Technical Report Series 894:i–xii, pp. 1–253.

WILSON AS, POWER BE, MOLLOY PL. DNA hypomethylation and human diseases. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2007;1775(1):138-62.

WILLIAMS, E.S.; BAYLIN, A.; CAMPos, H. Adipose tissue arachidonic acid and the metabolic syndrome in Costa Rican adults. *Clin Nutr*, 26, 474–482, 2007.

WU L, KAER LV. Contribution of lipid-reactive natural killer T cells to obesity-associated inflammation and insulin resistance. Adipocyte. 2013;2(1):12-6.

YAMAUCHI T, KAMON J, WAKI H, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med,*7:941–6, 2001.

ZHANG, F.F.; SANTELLA, R.M.; WOLFF, M.; KAPPIL, M.A.; MARKOWITZ, S.B.; MORABIA, A. White blood cell global methylation and IL-6 promoter methylation in association with diet and lifestyle risk factors in a cancer-free population. Epigenetics 7 (6): 606-614. 2012.

ZHANG, F.F.; MORABIA, A.; CARROLL, J.; GONZALEZ, K.; FULDA, K.; KAUR, M.; VISHWANATHA, J.K.; SANTELLA, R.M.; CARDARELLI, R. Dietary Patterns Are Associated with Levels of Global Genomic DNA Methylation in a Cancer-Free Population. *The Journal of Nutrition*, 141:1165-1171. 2011.

ZIMMET, P.; ALBERTI, K.G.M.M.; SHAW, J. Global and societal implications of the diabetes epidemic. Nature 414, 782-787. 2001.