JOSÉ LUIZ MARQUES ROCHA

EPIGENETIC MECHANISMS INVOLVED IN THE INTERACTION BETWEEN DIET AND THE EXPRESSION OF INFLAMMATION-RELATED GENES

Thesis submitted to the Universidade Federal de Viçosa, as part of the requirements of Program in Science of Nutrition for obtaining the title of *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2016

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APPROVED: February 19, 2016.

Helen Hermana M. Hermsdorff	Maria do Carmo Gouveia Pelúzio
Júlia Cristina Cardoso Carraro	Solange Silveira Pereira

Josefina Bressan
(Advisor)

"A mind that is stretched by a new experience
can never go back to its old dimensions."

Oliver Wendell Holmes

ACKNOWLEDGEMENTS

I would like to say a huge thank you:

To my family and friends for understanding my absence and for supporting my needs and dreams during these years; without you I would not have the strength to complete this important step.

To Prof. Josefina Bressan my advisor in science and personal life. Thank you for giving me invaluable opportunities that were of great importance in my training as a scientist and as a human. Thank you for your unconditional support.

To my co-advisors Prof. J. Alfredo Martínez and Prof. Fermin I. Milagro who always ensure that my work would be of high quality. Thank you for believing in my work. I will never forget the support, care and attention.

To colleagues from Department of Nutrition and Health, Federal University of Viçosa and also from Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra for the companionship, availability and the experiences we shared.

To Education Ministry of Brazil and its institutions for funding this project with research grants; And also to the Brazilian people, taxpayers, for funding my education all these years.

To all people who in some way contributed to carrying out this life project; Accept my warmest Thanks!

SUMMARY

LIST OF ABBREVIATIONS

AcM: LPS-activated macrophage

DNMTs: DNA methyltransferases

FAs: Fatty acids

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

ICAM-1: Intercellular adhesion molecule 1

IRAK: IL-1 receptor–associated kinase

lincRNA, long intergenic noncoding RNA

LINE-1: Long interspersed nucleotide element-1

lncRNA: long noncoding RNA

MCP-1: monocyte chemotactic protein 1

MET: Metabolic equivalent

MetS: Metabolic syndrome

miRNA: micro ribonucleic acid

mRNA: messenger ribonucleic acid

MS-HRM: Methylation-sensitive high resolution melting

MyD88: myleloid differentiation primary response protein 88

OS: Oxidative stress

ox-LDL: Oxidized Low-density lipoprotein

PAI-1: plasminogen activator inhibitor 1

PBMC: Peripheral blood mononuclear cells

pri-miRNAs: primary transcripts

RESMENA-S: Reduction of the Metabolic Syndrome in Navarra – Spain

RISC: RNA-induced silencing complex

SERPINE1: Serpin peptidase inhibitor

SHIP1: SH2 domain containing inositol5-phosphatase 1

SOCS1: suppressor of cytokine signaling 1

TAC: Total antioxidant capacity

TLR4: Toll-like receptor 4

THP-1: Human monocytic cell line

TNF: Tumor necrosis factor

UTR: Untranslated region

VCAM-1: Vascular cell adhesion molecule 1

WBC: White blood cell

ABSTRACT

ROCHA, José Luiz Marques, D.Sc., Universidade Federal de Viçosa, February, 2016. **Epigenetic mechanisms involved in the interaction between diet and the expression of inflammation-related genes.** Advisor: Josefina Bressan.

Epigenetic mechanisms are involved in the regulation of gene expression including microRNAs (miRNA) and DNA methylation; and play a crucial role in the development and maintenance of pathophysiological complications. Therefore, the aims of this work were: 1) To evaluate the effect of a weight loss strategy based on the Mediterranean dietary pattern (RESMENA diet) on expression of some selected inflammation-related genes and miRNAs in white blood cells (WBC) of individuals with metabolic syndrome (MetS); 2) To clarify *in vitro* the roles of selected miRNAs on the expression of inflammation-related genes in human acute monocytic leukemia cells (THP-1). Moreover, we investigated the regulatory role of fatty acids (palmitic acid, oleic acid, eicosapentaenoic acid and docosahesaenoic acid) on the expression of these miRNAs in monocytes, macrophages and LPS-activated macrophages (AcM); 3) To explore in young and apparently healthy adults, the relation between DNA methylation levels of LINE-1, *TNF* and *IL6* in periferal blood monoclear cells (PBMC) and anthropometric, biochemical, clinical, dietary, inflammatory and oxidative stress parameters. To the first aim, the clinical, anthropometric, and biochemical characteristics of 40 individuals with MetS (20 men, 20 women; age: 48.8±10.02y; BMI: 35.41±4.42 kg/m^2) were evaluated before and after an 8 weeks hypocaloric (-30% of energy requeriments) diet based on the Mediterranean dietary pattern. Food consumption and nutrient intake were assessed with a food frequency questionnaire and 48-h weighed food records. Total RNA was isolated from WBC and the expression of some inflammation-related miRNAs and mRNAs (IL-6, TNF, ICAM-1, IL-18, SERPINE1, VCAM-1) was assessed by qRT-PCR. To achieve the second aim, THP-1 were differentiated into macrophages and activated with LPS for 24 hours. The three cell types were transfected with miR-Let-7b-5p and miR-155-3p mimics or negative control. qRT-PCR analyses were performed to evaluate selected genes with potential role in inflammatory pathways related to these miRNAs. To the third aim, one hundred fifty-six individuals (91 women, 65 men; age: 23.1±3.5 years; BMI: 22.0±2.9 kg/m^2) were evaluated for anthropometric, biochemical and clinical markers, including some components of the antioxidant defense system and inflammatory response. Moreover, DNA methylation of LINE-1, *TNF* and *IL6* and the expression of some inflammation-related genes were analyzed in PBMC. The

RESMENA nutritional intervention improved mostly anthropometric and biochemical features. The expression of miR-155-3p was decreased in PBMC, whereas let-7b was strongly upregulated as a consequence of the diet treatment. However, they were not correlated with the expression of the pro inflammatory genes in the same cells. The changes in the expression of let-7b, miR-125b, miR-130a, miR-132-3p, and miR-422b were associated with changes in diet quality when assessed by the Healthy Eating Index. Moreover, low consumption of lipids and saturated fat were associated with higher expression of let-7b after the nutritional intervention. The *in vitro* transfection of miR-155-3p mimic led to upregulation of IL6 in the three cell types analyzed. In the same way, SERPINE1 was upregulated in monocytes and macrophages. However, TLR-4 was downregulated in transfected monocytes and macrophages. After transfection with let-7b mimic, TNF/IL6 and SERPINE1 expression were downregulated in monocytes and AcM, respectively; however, TNF, IL6 and SERPINE1 were upregulated in macrophages. In addition, oleic acid was able to increase the expression of miR-155 in monocytes when compared with the DHA treatment but not in relation with non-treated cells. On the other side, oleic acid increased the expression of Let-7b in macrophages and AcM. Finally, the results from DNA methylation in young adults showed that adiposity was lower among individuals with higher LINE-1 methylation. On the contrary, body fat-free mass was higher among those with higher LINE-1 methylation. Individuals with higher LINE-1 methylation had higher daily intakes of calories, iron and riboflavin. However, those individuals who presented lower percentages of LINE-1 methylation reported higher intakes of copper, niacin and thiamin. Interestingly, the group with higher LINE-1 methylation had a lower percentage of current smokers and more individuals practicing sport. On the other hand, *TNF* methylation percentage was negatively associated with waist circumference, waist-to-hip ratio and waist-to-height ratio. Our data suggest that the modulation of the expression of miRNAs and DNA methylation through a nutrition approach might be a future alternative or adjunct to current pharmacologic therapy targeting endogenous miRNAs or target genes. Furthermore, this study also expands the understanding of the role of fatty acids in the epigenetic regulation in immune cells.

RESUMO

ROCHA, José Luiz Marques, D.Sc., Universidade Federal de Viçosa, Fevereiro, 2016. **Mecanismos epigenéticos envolvidos na interação entre a dieta e a expressão de genes relacionados com a inflamação.** Orientadora: Josefina Bressan.

Mecanismos epigenéticos estão envolvidos na regulação da expressão gênica, incluindo metilação do DNA e microRNA (miRNA) que desempenham um papel crucial no desenvolvimento e manutenção de complicações metabólicas. Portanto, os objetivos deste estudo foram: 1) Avaliar o efeito de uma estratégia para perda de peso com base no padrão mediterrâneo (dieta RESMENA) na expressão de alguns genes e miRNAs relacionados com inflamação em células brancas do sangue (WBC) de indivíduos com síndrome metabólica (MetS); 2) Estudar *in vitro* a ação de alguns miRNAs selecionados na expressão de genes relacionados com a inflamação em células humanas de leucemia monocítica aguda (THP-1). Além disso, investigar o papel regulador de ácidos graxos (ácido palmítico, ácido oleico, ácido eicosapentaenoico e ácido docosahexaenoico) na expressão destes miRNAs em monócitos, macrófagos e macrófagos activados por LPS (AcM); 3) Explorar em células mononucleares de sangue periféricos (PBMC) de jovens adultos aparentemente saudáveis, a relação entre os níveis de metilação do DNA (*LINE1*, *TNF* e *IL6*) e os parâmetros antropométricos, bioquímicos, clínicos, nutricionais e marcadores do estado inflamatório e do estresse oxidativo. Para isso, 2 estudos foram conduzidos em duas populações distintas, além de um estudo *in vitro*. No primeiro, características clínicas, antropométricas e bioquímicas de 40 indivíduos com MetS (20 homens e 20 mulheres, idade: 48.8 ± 10 anos; IMC: 35.4 ± 4.4 kg/m^2) foram avaliadas antes e depois de 8 semanas de uma dieta hipocalórica (-30% das necessidades energéticas) baseada no padrão alimentar mediterrânico. A ingestão de nutrientes foi avaliada com um questionário de frequência alimentar. O RNA total foi isolado a partir de WBC e a expressão de alguns miRNAs e mRNAs (IL6, TNF, ICAM-1, IL-18, SERPINE1, VCAM-1) foram avaliados por meio de qRT-PCR. Para melhor compreensão dos dados, um estudo *in vitro* foi desenvolvido. THP-1 foram diferenciadas em macrófagos e ativados com LPS durante 24 horas. Os três tipos celulares foram transfectados com miR-Let7b-5p, miR-155-3p ou controle negativo. E a expressão dos miRNAs e genes foi realizada por qRT-PCR. Para o terceiro objetivo, foram utilizados dados de um estudo transversal com 156 indivíduos (91 mulheres, 65 homens com idade média: 23.1 ± 3.5 anos; IMC: 22.0±2.9 kg/m^2). Foram avaliados parâmetros antropométricos, bioquímicos clínicos, e alguns componentes dos sistemas

de defesa antioxidante e resposta inflamatória. Além disso, a metilação do DNA e a expressão de alguns genes foram analisadas em PBMC. A intervenção nutricional RESMENA melhorou características antropométricas e bioquímicas dos participantes. A expressão de miR-155-3p foi diminuída enquanto Let-7b foi fortemente aumentada após 8 semanas de intervenção nutricional. As alterações na expressão de let-7b, miR-125b, miR-130a, miR-132-3p e miR-422b foram associadas com mudanças na qualidade da dieta, quando avaliada pelo índice de alimentação saudável. Além disso, o baixo consumo de lipídios e de gordura saturada foram associados com maior expressão de let-7b após o tratamento dietético. A transfecção *in vitro* do miR-155-3p levou ao aumento da expressão de IL6 nos três tipos celulares analisados. Da mesma forma, SERPINE1 foi regulado positivamente em monócitos e macrófagos. No entanto, TLR-4 foi regulado negativamente em monócitos e macrófagos transfectados com esse miRNA. Após transfecção com let-7b, TNF/IL6 e SERPINE1 foram reprimidos em monócitos e AcM, respectivamente. Além disso, o tratamento com ácido oleico foi capaz de aumentar a expressão de miR-155 em monócitos quando comparado com o tratamento de DHA, mas não em relação com as células controle. Por outro lado, o ácido oleico, aumentou a expressão de let-7b em macrófagos e AcM. Finalmente, os resultados de metilação do DNA mostraram que a adiposidade foi menor entre os indivíduos com maior metilação global do DNA (LINE-1). Indivíduos com maior metilação do DNA apresentaram maior ingestão diária de calorias, ferro e riboflavina. No entanto, os indivíduos que apresentaram menor porcentagem de metilação do DNA relataram maior consumo de cobre, niacina e tiamina. Curiosamente, o grupo com maior metilação de LINE-1 teve menor porcentagem de fumantes e mais pessoas que praticavam atividade física. Além disso, a porcentagem de metilação de TNF foi negativamente associada com o perímetro da cintura, relação cintura-quadril e relação cintura-estatura. Os nossos dados sugerem que a modulação da expressão de miRNAs e metilação de DNA por meio de uma abordagem nutricional podem ser uma alternativa futura ou adjunta à terapia farmacológica atual. Além disso, este estudo amplia o entendimento de como os ácidos graxos podem atuar nas células imunológicas relacionadas com a inflamação.

1. GENERAL INTRODUCTION

Economic growth accompanied by changes in the dietary and physical behavior of individuals can dramatically increase the risk of developing metabolic disorders, including obesity with its associated complications (XIAO, J. *et al.*, 2016). Genetics factors are believed to be essential for the development of the chronic diseases (MILAGRO, F.I. *et al.*, 2013; MURPHY, R. *et al.*, 2013). However, these genetic factors alone cannot explain the development of some the components of the metabolic syndrome (MetS) (THAMAN, R.G. *et al.*, 2013; SCHUBELER, D., 2015), which suggests the involvement of epigenetic mechanisms in these processes (VILLENEUVE, L.M. *et al.*, 2010; MARTINEZ, J.A. *et al.*, 2014). Moreover, it has been demonstrated that complex interactions between various genes and the environment are implicated in some changes in gene expression and in the occurrence of chronic complications (KEATING, S.T. *et al.*, 2013; MILAGRO, F.I. *et al.*, 2013). Therefore, the epigenetic mechanisms involved in the regulation of gene expression including microRNA (miRNA) and DNA methylation appear to play a crucial role in the development and maintenance of pathophysiological complications (ABENTE, E.J. *et al.*, 2016; EK, W.E. *et al.*, 2016).

The term “epigenetic” was originally defined by C. H. Waddington in 1938 as ‘‘the causal interactions between genes and their products, which bring the phenotype into being’’ (HOLLIDAY, R., 1987). Currently, “epigenetic” refers to the study of heritable traits of gene expression and subsequent phenotypic changes that do not include alterations of DNA sequences. More recently the terms “nutrigenetics” and “nutrigenomics” were defined as the science of the effect of genetic variation on dietary response and the role of nutrients and bioactive food compounds in gene expression, respectively (SIMOPOULOS, A.P., 2010; FENECH, M., 2015). Exploitation of this

genomic information along with high-throughout “omic” technologies allows the acquisition of new knowledge aimed at obtaining a better understanding of nutrient-gene interactions depending on the genotype with the ultimate goal of developing personalized nutrition strategies for optimal health and disease prevention (FENECH, M., 2015).

In the cell cytosol, epigenetic mechanisms include the action of single-strand RNA molecules (19–24 nucleotides in length) called miRNA, which control the translation of various mRNAs (WANG, J. *et al.*, 2016). The miRNA binds to the specific sites on the 3´ untranslated region (3´UTR) of the target mRNA leading to inhibition of mRNA translation by several mechanisms: complete degradation of messenger RNA (mRNA), destabilization of mRNA through cleavage and deadenylation, binding of miRNA to 3’UTR and direct repression of mRNA translation (EL GAZZAR, M. *et al.*, 2012; REBANE, A. *et al.*, 2013). Dietary factors have been shown to modify miRNA expression profiling, notably in inflammatory-related conditions (MILAGRO, F.I. *et al.*, 2013; REMELY, M. *et al.*, 2015).

Other heritable epigenetic mark is DNA methylation. This process involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs) (SCHUBELER, D., 2015). In mammals, DNA methylation occurs at cytosines in any context of the genome (LISTER, R. *et al.*, 2009; MENG, H. *et al.*, 2015). The regions of DNA with an increased numbers of CpG clusters are designated ‘‘CpG islands’’ and are usually methylated in a tissue-specific manner reflecting DNA stability and transcriptional gene status (MENG, H. *et al.*, 2015). Generally, a low methylation status of CpG islands situated in the promoter is associated with gene expression, while transcription is repressed under

a high methylation status of DNA (NICOLETTI, C.F. *et al.*, 2015; MARQUES-ROCHA, J.L. *et al.*, 2016).

Although research in this area has exponentially grown in the last 10 years, much remains to be learned about the relationship among diet, inflammation, MetS and the mechanisms underlying its deregulation. Thus, by an epigenetic approach, this work searched better understanding of this new frontier in nutrition research and its potential application to nutrition practice.

2. LITERATURE REVIEW

2.1 Metabolic Syndrome and Inflammation

The MetS refers to the clustering of cardiovascular risk factors that include central obesity, hyperglycemia, dyslipidemia, and hypertension (IDF, 2014). The ultimate importance of this cluster is to identify individuals at high risk of both T2DM and cardiovascular disease (KAUR, J., 2014). Growing evidence implicates obesity-induced inflammation as an important mechanism linking obesity to the MetS in metabolically active organs (ABELLA, V. *et al.*, 2014). Assessment of gene expression networks in obese adipose (AT) tissue has identified a robust pattern of overexpressed inflammatory genes associated with metabolic disease (CHEN, Y. *et al.*, 2008). Multiple inflammatory mediators abnormally secreted by AT and the crosstalk between immune and metabolic cells can impair insulin signaling and induce oxidative stress and endothelial dysfunction, leading to systemic insulin resistance and cardiovascular disease (MAURY, E. *et al.*, 2010). Deregulated macrophage-myocyte and macrophage-hepatocyte signaling can impair insulin sensitivity as well (HUANG, W. *et al.*, 2010). Hypothalamic inflammation, which is induced very rapidly by a high-fat diet (THALER, J.P. *et al.*, 2012) may provoke hyperphagia and has been documented to impair insulin release from β cells, peripheral insulin action, and potentiate hypertension

(PURKAYASTHA, S. *et al.*, 2011). Thus, chronic excess of nutrients, such as lipids and glucose may simultaneously trigger inflammatory responses, which further disrupt metabolic function, enhancing stress, and inflammation (GE, Q. *et al.*, 2014). Such a vicious cycle is identified as a mechanism leading to further metabolic deterioration. Therefore, breaking this vicious circle by resetting the immunological balance in obesity is a crucial approach for the management of MetS.

2.2 MicroRNA biogenesis and mechanism of action

miRNA genes are usually transcribed by RNA polymerase II (Pol II) to form a capped and polyadenylated transcript (see Figure 1). The miRNA precursor, termed primary miRNA (pri-miRNA), forms a hairpin-shaped loop structure that is cleaved by the RNase III Drosha and DiGeorge syndrome critical region 8 (DGCR8), yielding a hairpin-shaped precursor miRNA (pre-miRNA) that is ~70 nucleotides in length. The pre-miRNA is exported from the nucleus into the cytoplasm, where it is further cleaved by the RNase III enzyme Dicer, yielding an imperfect miRNA–miRNA* duplex that is about 22 nucleotides in length. Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC). miRNAs incorporated in the RISC often recognize their targets — nucleotides 2–7 of miRNA (known as the 'seed region') (KRUTZFELDT, J. *et al.*, 2006; WANG, Z., 2010; VAN ROOIJ, E. *et al.*, 2012).

Association of a miRNA with its mRNA target results in degradation of the mRNA as well as translational inhibition (Figure 1). Recently, pseudogenes have also been implicated in regulating miRNA activity. Pseudogene transcripts are often conserved across species and many contain conserved miRNA binding sites, referred to as competing endogenous RNAs (ceRNAs), which act as decoys or sponges by sequestering miRNAs and preventing them from binding to their mRNA targets. Stress

conditions can influence miRNA biogenesis at multiple levels (indicated on the figure 1 by lightening bolts) (KRUTZFELDT, J. *et al.*, 2006; WANG, Z., 2010; VAN ROOIJ, E. *et al.*, 2012). More specific information about miRNA biogenesis and mechanisms of action may be accessed elsewhere (KRUTZFELDT, J. *et al.*, 2006; REBANE, A. *et al.*, 2013).

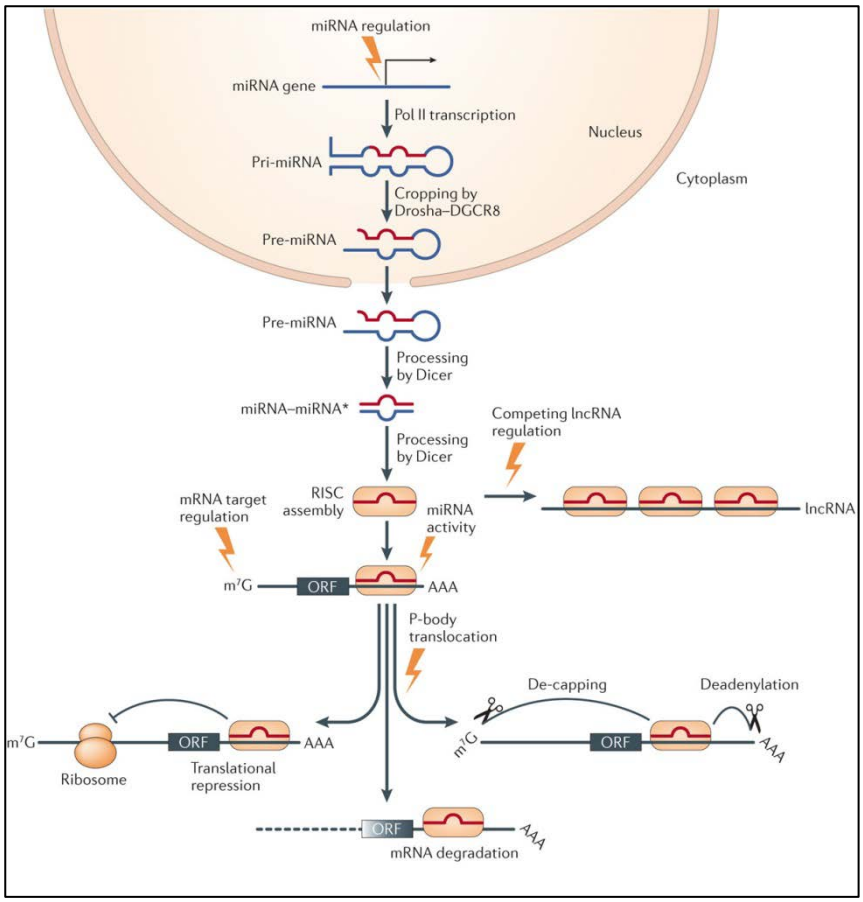

Figure 1. General biogenesis and mechanism of action of miRNAs. lncRNA, long non-coding RNA; m7G, 7-methylguanosine (a modified form of guanosine attached to the 5′ ends of mRNAs); ORF, open reading frame. Figure adapted from Van-Rooij and collaborators (2012).

2.3 Noncoding RNAs, cytokines, and inflammation-related diseases

Published Review • The FASEB Journal

Noncoding RNAs, cytokines, and inflammation-related diseases

José Luiz Marques-Rocha,* Mirian Samblas,[†,‡] Fermin I. Milagro,[†,‡,§] Josefina Bressan,* J. Alfredo Martínez,[†,‡,§,¶] and Amelia Marti[†,‡,§,¶,1]

*Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil; [†]Department of Nutrition, Food Science, and Physiology, [‡]Center for Nutrition Research, University of Navarra, Pamplona, Spain; [§]Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición, Physiopathology of Obesity, Carlos III Institute, Madrid, Spain; and [¶]Instituto de Investigación Sanitaria de Navarra, Pamplona, Spain

ABSTRACT **Chronic inflammation is involved in the onset and development of many diseases, including obesity, atherosclerosis, type 2 diabetes, osteoarthritis, autoimmune and degenerative diseases, asthma, periodontitis, and cirrhosis. The inflammation process is mediated by chemokines, cytokines, and different inflammatory cells. Although the molecules and mechanisms that regulate this primary defense mechanism are not fully understood, recent findings offer a putative role of noncoding RNAs, especially microRNAs (miRNAs), in the progression and management of the inflammatory response. These noncoding RNAs are crucial for the stability and maintenance of gene expression patterns that characterize some cell types, tissues, and biologic responses. Several miRNAs, such as miR-126, miR-132, miR-146, miR-155, and miR-221, have emerged as important transcriptional regulators of some inflammation-related mediators. Additionally, little is known about the involvement of long noncoding RNAs, long intergenic noncoding RNAs, and circular RNAs in inflammation-mediated processes and the homeostatic imbalance associated with metabolic disorders. These noncoding RNAs are emerging as biomarkers with diagnosis value, in prognosis protocols, or in the personalized treatment of inflammation-related alterations. In this context, this review summarizes findings in the field, highlighting those noncoding RNAs that regulate inflammation, with emphasis on recognized mediators such as TNF-α, IL-1, IL-6, IL-18, intercellular adhesion molecule 1, VCAM-1, and plasminogen activator inhibitor 1. The down-regulation or antagonism of the noncoding RNAs and the administration of exogenous miRNAs could be, in the near future, a promising therapeutic strategy in the treatment of inflammation-related diseases.—Marques-Rocha, J. L., Samblas, M., Milagro, F. I., Bressan, J., Martínez, J. A., Marti, A. Noncoding RNAs, cytokines, and inflammation-related diseases. *FASEB J.* 29, 3595–3611 (2015). www.fasebj.org**

Key Words: circular RNA · lincRNA · microRNA · lncRNA · obesity

Inflammation is a complex protective process that requires a cross-talk between different types of immune cells to remove or neutralize harmful stimuli (1). In the classic view, the inflammatory process is induced by an invasion of foreign pathogens of biologic origin or by tissue damage. Neutrophils, dendritic cells, and macrophages express almost all types of TLRs participating in the transmission of a signal from the plasma membrane through a multistep cascade to responsive transcription factors. Members of the TLR family have emerged as the primary evolutionarily conserved sensors of pathogen-associated molecular patterns (1). Binding of the TLRs to their respective ligands initiates a wide spectrum of responses, from phagocytosis to production of a variety of cytokines, which in turn shape and enhance the inflammatory and adaptive immune responses. Typical transcription factors that activate inflammatory mediators are NF-κB (2), activator protein 1 (3), signal transducer and activator of transcription (STAT) (4), CCAAT enhancer binding protein (C/EBP) (5), and Ets-like gene 1 (6). The interactions between transcription factors that compete for binding sites in the promoter regions of specific target genes are highly complex. Usually the multistep signaling leads to a prompt transcription of genes resulting in accumulation of specific mRNAs coding for TNF-α, IL-1, IL-6, IL-8, monocyte chemotactic protein 1 (MCP-1), and other cytokines involved in inflammation (7).

Some cytokines may elicit a broad inflammatory response, while others act on specific cell types. The activation, proliferation, and recruitment phenomena of specific differentiated immune cells are involved in resolving the nonhomeostatic state [for a review, see Shi (8)]. Thus, macrophages stimulate the inflammatory responses of

Abbreviations: ADAM17, ADAM (A disintegrin and metalloproteinase) metallopeptidase domain 17; AKT/GSK, protein kinase B/glycogen synthase kinase; BMI, body mass index; C/EBP, CCAAT enhancer binding protein; circRNA, circular RNA; COX-2, cyclooxygenase 2; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule 1; IL-1R,

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[1] Correspondence: Department of Nutrition, Food Science, and Physiology, University of Navarra, c/ Irunlarrea 1, 31008, Pamplona, Navarra, Spain. E-mail: amarti@unav.es

doi: 10.1096/fj.14-260323

neutrophils, fibroblasts, and endothelial cells (2). Other sentinel cells may present antigens to the T-helper cells, which play a central role in coordinating immune responses, such as clonal expansion of T cells and B cell responses (9).

Acute inflammation is an important part of the immune response, but chronic inappropriate inflammation can cause metabolic disorders (10). For example, chronic low-grade inflammation has been repeatedly associated with the onset and prevalence of metabolic syndrome (11, 12). The International Diabetes Federation estimates that one quarter of the world's adult population has metabolic syndrome (13). This phenomenon is defined by a combination of interconnected cardiometabolic alterations that include the presence of arterial hypertension, insulin resistance, dyslipidemia, cardiovascular disease, and abdominal obesity (11). With the pathologic enlargement of the adipose tissue in obesity, the blood supply to adipocytes may be reduced, with subsequent hypoxia leading to a an elevated production of proinflammatory mediators [TNF-α and IL-6, plasminogen activator inhibitor-1 (PAI-1), and C-reactive protein (CRP)] and increased infiltration of immune cells, particularly adipose tissue macrophages (14, 15). These altered signals mediate multiple processes, including insulin sensitivity (16), oxidative stress (17), energy metabolism, blood coagulation, and inflammatory responses (12). These pathologic conditions predispose to diabetes mellitus, hepatic steatosis, atherosclerosis, plaque rupture, and atherothrombosis (11). However, to date, the available information is controversial and does not necessarily imply an unequivocal causal role. The data obtained by functional genomics techniques indicate that several hundreds of genes participate in the inflammatory response and that their coordinated expression is tightly regulated [reviewed in Jura and Koj (18)]. Nevertheless, the involved pathways and the regulatory mechanisms are not completely understood.

In the last few years, there has been a growing interest in the role of microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) in the development of several inflammation-related diseases (**Table 1**). These noncoding RNAs have emerged as important transcriptional regulators in both physiologic and pathophysiological conditions (19–21). In physiologic homeostasis, these nucleic acids may participate in cell differentiation, proliferation, apoptosis, hematopoiesis, limb morphogenesis, and important metabolic pathways, such as insulin secretion, triglyceride and cholesterol biosynthesis, and oxidative stress (20, 22, 23). Given their fundamental biologic roles, it is not surprising that miRNAs expression is tightly controlled and that its dysregulation can lead to disease onset.

Advances in basic biology combined with recent whole-genome and transcriptome studies demonstrate that the control of protein expression levels is far more dynamic and complex than previously presumed (24). Gene transcription is regulated at different levels, but only some of them have been elucidated. Although miRNAs constitute only 3% of the human genome, it is estimated that they regulate approximately 90% of genes (23, 25). The mechanisms are not fully understood, but it is known that miRNAs are crucial for the stability and maintenance of the gene expression patterns that characterize cell types, tissues, and biologic processes and responses (23). miRNAs may act directly on the target genes or indirectly, by first regulating transcription factors that in turn control gene expression. Generally miRNAs bind to the target sequence located in the 3′ UTR of their target mRNA through at least 6 to 8 nt long complementary sequences. They cause gene silencing mainly through degradation of target mRNA or inhibition of protein translation [for reviews, see Rebane and Akdis (24), Djuranovic *et al.* (26), and Magenta *et al.* (27)]. On the other hand, the lncRNAs are non-protein-coding RNAs longer than 200 nt. It has been reported that alterations in the levels of lncRNAs affect gene expression and thereby cell homeostasis (28). In addition, miRNAs and lncRNAs do not abolish the expression of their target genes but only reduce the amount of mRNA and/or protein (22).

Recent advances with the omics technologies have produced progress in the understanding of the role of noncoding RNAs on gene expression and cell metabolism in several processes, including inflammation and oxidative stress (20, 22, 29). Moreover, miRNAs might provide a link between immune signaling pathways and might also play a role in controlling the switch from a strong early proinflammatory response to the resolution phase of the inflammatory process [for reviews, see O'Neill *et al.* (30) and Olivieri *et al.* (31)]. *In vitro* and *in vivo* studies have demonstrated the essential participation of some of these RNAs in the production and regulation of several proinflammatory cytokines (21, 24, 32). Collectively, these findings indicate a participation of miRNAs and lncRNAs in the onset, progression, and maintenance of several pathologies. Additionally, noncoding RNAs could have a role as new biomarkers with diagnostic value in prognosis protocols or in the personalized treatment of diseases related to metabolism (33–35). In this context, this review summarizes available findings in the field, highlighting those noncoding RNAs that regulate the expression of important inflammation mediators, such as TNF-α, IL-1, IL-6, IL-18, PAI-1, intercellular adhesion molecule 1 (ICAM-1), and VCAM-1.

MIRNAS AND REGULATION OF INFLAMMATION

The molecular networks that control the onset, peak magnitude, and resolution of inflammation must be properly tuned for optimal health maintenance (36). Both innate and adaptive immune responses are highly controlled, and recent studies have shed light on the epigenetic regulation in this intricate defense system (18, 37). This type of regulation is termed epigenetic because it causes heritable changes in gene expression, but they are

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IL-1 receptor; IRAK, IL-1 receptor–associated kinase; lincRNA, long intergenic noncoding RNA; lncRNA, long noncoding RNA; MCP-1, monocyte chemotactic protein 1; miRNA, microRNA; MyD88, myeloid differentiation primary response protein 88; NLR, NOD-like receptor family; PAI-1, plasminogen activator inhibitor 1; Ppargc1b, peroxisome proliferator-activated receptor γ, coactivator 1β; SHIP1, SH2 domain containing inositol-5-phosphatase 1; SOCS1, suppressor of cytokine signaling 1; STAT, signal transducer and activator of transcription; THP-1, human monocytic cell line; THRIL, TNF- and HNRNPL-related immunoregulatory lincRNA

TABLE 1. *Some inflammation-associated diseases and related noncoding RNAs*

Disease	Noncoding RNAs	Effect	Reference
Cardiovascular disease	miR-155	It modulates atherogenesis *in vivo*.	194
	miR-302a	It modulates cholesterol efflux and atherosclerosis.	195
	miR-712	It induces endothelial inflammation and atherosclerosis.	196
Diabetes mellitus	miR-126, miR-15a, miR-29b, miR-223, miR-28-3p	Reduced miR-15a, miR-29b, miR-126, miR-223, and elevated miR-28-3p levels in plasma predated the manifestation of the disease.	197
	miR-375	It is required for normal glucose homeostasis.	198
	let-7	Overexpression leads to insulin resistance and impaired glucose tolerance.	199
Adipogenesis and obesity	miR-103, miR-107, miR-143	They may accelerate fat cell development.	54
	miR-935, miR-4772, miR-223, miR-376b	The basal expression can differentiate between those who responders and those who do not responder to diet-induced weight loss.	58
	miR-378, miR-378	Mice genetically lacking this miRNA are resistant to high-fat diet-induced obesity and exhibit enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity of insulin target tissues.	59
	miR-221	It is down-regulated in the human subcutaneous adipose tissue of obese patients compared to nonobese individuals.	130
	miR-221, miR-28, miR-486, miR-486, miR-142, miR-130, miR-423-5p	These circulating miRNAs are associated with BMI, percentage fat mass, waist, and regional fat distribution.	52
Osteoarthritis	miR-149	It is down-regulated in osteoarthritic chondrocytes and is correlated to increased expression of TNF-α, IL-1β, and IL-6.	200
Asthma	miR-19	It promotes type 2 helper T cytokine production and amplifies inflammatory signaling.	201
Periodontitis	miR-181b, miR-19b, miR-23a, miR-30a, miR-let7a, miR-301a	The expression of these miRNAs is much higher in periodontitis gingivae than in healthy ones.	202

not accompanied by changes in DNA sequence (38). In this context, the participation of several miRNAs in the regulation of inflammatory processes has been reported (24, 29, 39, 40).

Each miRNA can target hundreds of mRNAs within a given cell type, and a single mRNA is often the target of multiple miRNAs (41). Mechanistically, miRNAs have been implicated as negative regulators of inflammatory processes at the posttranscriptional level (30). Additionally, miRNAs can regulate the transcription of other miRNAs (39). Among many other miRNAs researched in this field, miR-146, miR-155, and miR-223 are probably the most intensively studied. They have been identified as inflammatory response miRNAs that are up-regulated by NF-κB (21, 39, 42). Several studies indicate that miR-146 and miR-155 together play a key role in regulating several critical pathways that orchestrate innate immune responses and chronic inflammatory processes that are conserved across many different human tissue systems. This may be a consequence of their related ribonucleotide sequence (43–45). The participation of the most relevant miRNAs in the inflammatory process is summarized in **Table 2**.

Numerous miRNAs seem to function mainly in the regulation of inflammatory pathways, especially NF-κB signaling (46). When inflammation is initiated, rapid transcriptional up-regulation of several proinflammatory cytokines occurs (24). Simultaneously, the expression of numerous miRNAs is initiated by the same transcription factors. These miRNAs either silence the expression of the positive signaling proteins or the inhibitors of the same pathway (23, 41). On the other hand, numerous miRNAs are also down-regulated during the activation of immune cells. When anti-inflammatory miRNAs are expressed at lower levels or proinflammatory miRNAs are expressed at higher levels, the immune system is overactivated and *vice versa* (24). Thus, the dysregulation of miRNA expression can lead to immune deficiency, autoimmunity, or chronic infection (25, 31, 47, 48).

Several reports have examined the involvement of the miRNAs in low-intensity inflammation related to obesity and associated diseases [for reviews, see Hulsmans *et al.* (20), Aranda *et al.* (22), and Schroen and Heymans (49)]. In addition, obesity is associated with increased levels of blood monocytes (50), which are already activated in the circulation and are characterized by an increase in NF-κB activity, in the transcription of proinflammatory genes (*e.g.,* TNF-α and IL-6), and in the production of reactive oxygen species, as described elsewhere (14). Recent computational and experimental studies have shown that miRNAs appear to play regulatory roles in many biologic processes associated with obesity, including adipocyte differentiation and lipid metabolism (51). In addition, many

TABLE 2. *Most relevant miRNAs in the inflammatory process and their target genes*

miRNA	Target gene	Main results/properties	Cell type or tissue	Reference
miR-125b	TNF-α	miR-125b targets the 3′ UTR of TNF-α transcripts, being down-regulated in response to LPS. It is implicated in the response to endotoxin shock.	Mouse RAW264.7 macrophages stimulated with LPS	72
miR-126	VCAM-1	miR-126 regulates VCAM-1 expression and plays a pivotal role in the regulation of primary erythropoiesis.	Mouse embryonic stem cell (*in vitro*) and mouse embryo yolk sac (*in vivo*)	139
		miR-126 inhibits VCAM-1 expression and limitation of leukocyte adherence to endothelial cells.	HUVEC	86
		miR-126 expression increases in response to antiatherogenic triglyceride-rich lipoproteins or polyunsaturated fatty acids treatment. VCAM-1 expression decreases as a result of decreased IRF-1.	Human aortic endothelial cells	138
		After TNF-α induction, VCAM-1 expression is induced in both arteriolar and glomerular endothelium. miR-126 is up-regulated and inhibits VCAM-1 translation.	C57BL/6 mice and immortalized human glomerular endothelial cell line	140
		VCAM-1 expression, increased by TNF-α and decreased by mir-126, may play a critical role in early juvenile dermatomyositis.	Muscle biopsy samples and serum from humans	83
miR-132	NF-κB	miR-132 overexpression is sufficient to induce NF-κB translocation, acetylation of p65, and production of IL-8 and MCP-1.	Primary human adipose–derived stem cells and *in vitro* differentiated adipocytes	203
	IL-6	miR-132 is negatively related to macrophage infiltration and to IL-6 levels in patients with nonalcoholic fatty liver disease.	Human adipose tissue	100
miR-145	TNF-α	miR-145 enhances the production/release of TNF-α and increases the amount of membrane bound TNF-α *via* inhibition of ADAM17.	Human *in vitro* differentiated adipocytes	75
miR-146	IL-6	miR-146 decreases the expression of IL-6, but not TNF-α.	Mouse RAW264.7 macrophages stimulated with LPS	109
	IL-1β	IRAK inhibits mir-146 leading to up-regulation of IL-1 and inhibits inflammatory response in periodontal inflammation.	Primary human gingival fibroblasts in culture	91
	TNF-α	miR-146 is NF-κB dependent and acts as an inhibitor targeted to signaling proteins of innate immune responses as TNF-α.	Human monocytic cell line THP-1	73, 81
		miR-146 negatively regulates NF-κB activity and inflammation.	Breast cancer cells	204
		miR-146 controls monocyte responses during inflammatory challenge through targeting v-rel avian reticuloendotheliosis viral oncogene homolog B, a member of the noncanonical NF-κB/Rel family.	Peripheral blood Ly-6Chigh monocytes in mice and CD141 monocytes in humans	205
miR-155		miR-155 increases TNF-α levels by augmenting transcript stability through binding to its 3′ UTR.	Mouse RAW264.7 macrophages stimulated with LPS	72
miR-155	NF-κB	miR-155 is substantially up-regulated by polyriboinosinic:polyribocytidylic acid and IFN-β.	Primary murine macrophages	44
		miR-155 represses the expression of B cell lymphoma 6, a transcription	B cell lymphoma of mice	206

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TABLE 2. (*continued*)

miRNA	Target gene	Main results/properties	Cell type or tissue	Reference
		factor that attenuates proinflammatory NF-κB signaling.		
	IL-1	miR-155 regulates the TLR/IL-1 pathway during dendritic cell maturation.	Human monocyte–derived dendritic cells	207
miR-181	IL-1β, TNF-α and IL-6	TNF-α decreases miR-181 expression. miR-181 participates in cell stress response and cell survival, and inhibits the expression of TNF-α, IL-1β, and IL-6.	Mouse primary astrocyte culture and C57BL/6J mice	93
	NF-κB/VCAM-1/ E-selectin	Overexpression of miR-181b inhibits importin α3 expression and an enriched set of NF-κB-responsive genes, such as adhesion molecules VCAM-1 and E-selectin *in vitro* and *in vivo*.	HUVECs, THP-1 cells, male C57BL/6 mice, and human plasma	208
miR-187	TNF-α, IL-6 and IL-12	miR-187 negatively regulates cytokine production.	TLR4-stimulated monocytes	209
miR-221	ICAM-1	HIV-1 viral protein TAT modulates ICAM-1 expression *via* mir-221/-222.	HUVECs and HIV transgenic rats	129
		miR-221 is up-regulated in obese subjects, associated with BMI in adipose tissue, and down-regulated during preadipocyte differentiation and adipocyte maturation.	Subcutaneous human preadipocytes and adipose tissue biopsy samples	130
		miR-221 suppresses ICAM-1 translation and regulates IFN-γ-induced ICAM-1 expression.	Human cholangiocytes	131
	TNF-α	miR-221 is down-regulated by TNF-α treatment.	Human preadipocytes	77
miR-222	ICAM-1	miR-222 reduces ICAM-1 expression and prevents the association of cytotoxic T lymphocyte cells to tumor cells.	Primary human glioblastoma multiforme tissue and human colorectal cancer cells	132
miR-223	PAI-1	miR-223 prevents accumulation of NLR family, pyrin domain containing 3 (NLRP3) protein and inhibits IL-1β production from the inflammasome.	Human primary monocytes	210
miR-30c		miR-30c inhibits both PAI-1 mRNA and protein levels.	HUVEC/plasma samples from humans	151
		miR-30c cosilences PAI-1 and activin receptor-like kinase 2 (ALK2) and up-regulates adipogenic key transcription factors, such as PPARγ and C/EBP-α.	Human multipotent adipose–derived stem cells	152
miR-421		miR-421 inhibits both PAI-1 mRNA and protein levels. miR-421 plasma levels are increased in patients with high PAI-1 plasma levels.	HUVEC/plasma samples from humans	151
miR-449a/b		Hypoxia and reactive oxygen species decrease miR-449a/b and increases PAI-1 expression.	Hypoxic fibroblasts	148
Let-7	IL-6	Let-7 decreases expression of IL-6.	Transfection in bone marrow–derived mesenchymal stem cells	118

miRNAs are dysregulated in metabolic tissues from obese animals and humans, which potentially contributes to the pathogenesis of obesity-associated complications (20, 51). For example, a cross-sectional validation study reported that 15 specific circulating miRNAs were significantly deregulated in prepubertal obesity, including a decrease of miR-221 and miR-28-3p and an increase of miR-486-5p, miR-486-3p, miR-142-3p, miR-130b, and miR-423-5p in plasma (52). The circulating concentrations of these miRNAs were significantly associated with body mass index (BMI) and other measures of obesity, such as percentage fat mass, waist circumference, and regional fat distribution, and with laboratory parameters, including homeostasis model assessment of insulin resistance, high-molecular-weight

adiponectin, CRP, and circulating lipids in concordance with anthropometric associations (52). Thus, the very early detection of an abnormal circulating miRNA profile may be a promising strategy to identify obese children who may experience metabolic abnormalities accompanied by inflammation. The main miRNAs involved in the obesity pathophysiology are summarized in **Fig. 1**.

miRNAs modulate not only fat mass size but also the metabolic consequences of obesity and adipose tissue metabolism (53). A set of miRNAs (including miR-103, miR-107, and miR-143) are induced during adipogenesis (which may accelerate fat cell development) but are down-regulated in the obese state (54). In fact, there are many miRNAs involved in the regulation of adipogenesis, although it is not the main focus of this review. Adipocyte differentiation is not directly implicated in local inflammation, but chronic low-grade inflammation in the adipose tissue is usually associated with a reduction of the adipogenic capacity and an inability to increase adipose tissue mass by adipocyte hyperplasia. As a consequence, it leads to adipocyte hypertrophy and increased hypoxia, endoplasmic reticulum stress, adipocyte senescence, autophagy, apoptosis, necrosis, and death, which in turn aggravate lipotoxicity, macrophage infiltration, insulin resistance, and local and systemic inflammation (55). On the other hand, mouse models with an adipose tissue–specific reduction in proinflammatory potential display a reduced adipogenic capacity, whereas an impaired local proinflammatory response in the adipocyte leads to increased ectopic lipid accumulation, glucose intolerance, and systemic inflammation (56). These observations have important implications for understanding adipose tissue deregulation in obese mice and humans as well as the link between insulin and chronic inflammation and obesity (54). In addition to altered adipocyte miRNA expression during obesity, there is an altered expression of miRNAs in the circulation (57) and in peripheral blood mononuclear cells (58). Moreover, the basal expression of certain miRNAs in blood cells can differentiate between those who will respond and not respond (miR-935, miR-4772, miR-223, and miR-376b) to diet-induced weight loss (58). Another important miRNA is miR-378. Mice genetically lacking miR-378 and miR-378*, while leaving the host gene peroxisome proliferator–activated receptor γ, coactivator 1β (*Ppargc1b*), intact, are resistant to high-fat diet-induced obesity and exhibit enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity of insulin target tissues (59). In this context, the role of miRNAs in the regulation of the expression of some of the main molecules involved in the inflammatory processes will be discussed.

MIRNAS AND INFLAMMATORY CYTOKINES

Cytokines are a broad and loose category of small proteins (~5–20 kDa) that are important in cell signaling (9). These mediators are released by cells and affect the behavior of other cells, and sometimes the releasing cell itself (60). The cytokines play a central role in whole body homeostasis by influencing a variety of biologic and physiologic processes (61). Some of them control food intake, energy balance, insulin action, lipid and glucose metabolism, angiogenesis and vascular remodeling, blood

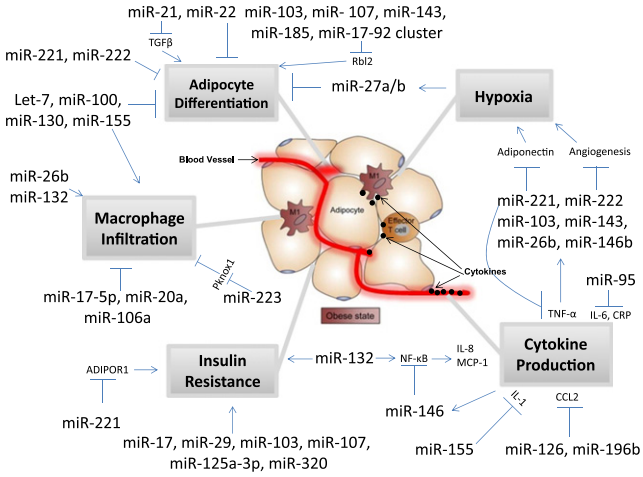

Figure 1. Summary of some miRNAs involved in obesity pathophysiology and related inflammation. Obesity-associated inflammation is characterized by macrophage infiltration into adipose tissue, cytokine production, decrease of triglyceride storage, stimulation of lipolysis, and impairment of insulin signaling (185). Adipose tissue hypoxia, increased hypertrophy, and adipocyte death are associated with chronic inflammation and insulin resistance in obesity. Both inflammation and hypoxia inhibit new adipocyte differentiation from preadipocytes (185) and lead to endoplasmic reticulum stress, adipocyte senescence, autophagy, and apoptosis, which in turn aggravate insulin resistance and local and systemic inflammation (55). In obesity, changes in miRNA expression profiles may induce adipocyte dysfunction. miR-103, miR-107, miR-143, and miR-185 are up-regulated in lean subjects and down-regulated in obese subjects, whereas miR-27a/b and miR-100 are down-regulated in lean subjects and up-regulated in obese subjects (54, 130). Regarding adipocyte differentiation, in human stem cells derived from subcutaneous adipose tissue, miR-21 antagonizes TGF-β signaling and increases adipogenesis (186). Mesenchymal stem cells overexpressing miR-221 and miR-222 display reduced ability to differentiate to adipocytes compared to control cells. miR-17-92 cluster accelerates adipogenesis by negatively regulating retinoblastoma-like protein 2 (Rbl2) transcripts (187). Adiponectin receptor 1 (ADIPOR1) is direct target of miR-221/222. miR-221 and miR-222 are inversely related to adiponectin levels and inhibit angiogenesis, thereby increasing hypoxia. Expression pattern of miR-27a/b in obesity is controlled by hypoxia. Number of macrophages infiltrating fat depot is associated with expression of miR-26b and miR-155 in subcutaneous and omental adipose tissue (100). miR-17-5p, miR-20a, and miR-106a are reported to suppress monocytic differentiation (188). miR-223 plays crucial role in modulating macrophage polarization, suppressing infiltration of proinflammatory M1 macrophages by targeting PBX/knotted 1 homeobox 1 (Pknox1) *in vitro* (189). Concerning cytokine secretion, miR-132 activates NF-κB pathway and stimulates chemokine production, including IL-8 and chemokine C-C motif ligand 2 (CCL-2). miR-126 and miR-196b reduce production of CCL-2. miR-155 down-modulates IL-1 production. miR-95 expression is negatively correlated with levels of CRP and IL-6. expression of miR-26b, miR-103, miR-143, miR-146b, miR-221, and miR-222 is regulated by TNF-α (100). miR-17, miR-29, miR-103, miR-107, miR-125a-3p, miR-132, and miR-320 are involved in obesity-related insulin resistance (190, 191). Mechanisms by which miRNAs regulate gene expression in obesity have been reviewed elsewhere (20, 51, 192, 193). Arrows indicate activation; blunted arrows, inhibition.

pressure, and coagulation, as well as inflammatory and immune responses (9, 18). The complex network of cytokines balances proinflammatory and anti-inflammatory effects. Disequilibrium between pro- and anti-inflammatory cytokines or the uncontrolled production of cytokines can result in inflammatory phenomena (61–63).

The expression of cytokines and proinflammatory factors, including IL-1, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, VEGF, prostaglandin E2, cyclooxygenase 2 (COX-2), matrix metalloproteinases, and basic fibroblast growth factor, is highly regulated at many levels, including gene transcription, mRNA translation, and mRNA degradation (64). Although so far virtually overlooked, the regulatory relationship between cytokines and miRNA seems to be reciprocal: not only do miRNAs target cytokine mRNA and thereby regulate cytokine expression, but also the cytokine signaling likewise has an impact on miRNA expression (65). For instance, IL-1β and TNF-α are potent stimulators for miR-146a and miR-155 induction in a variety of cell types (21). Importantly, miR-23b is down-regulated by IL-17, a cytokine that plays a profound role in the regulation of the innate immune activation (66). The role of the miRNAs as regulators of the main proinflammatory mediators is reported next.

TNF-α

TNF-α is a proinflammatory cytokine predominantly synthesized by macrophages (67). The regulation of production and secretion of this protein is multileveled and involves an extensive cross-talk at the intra- and extracellular level, including a self-regulatory loop (68). TNF-α is synthesized as a 26 kDa transmembrane protein, which is cleaved by ADAM (A disintegrin and metalloproteinase) metallopeptidase domain 17 (ADAM17), a member of the metalloproteinase family (69). This cleavage results in the release of the secreted 17 kDa form of TNF-α (70). Interestingly, several miRNAs have been shown to control TNF-α production, but at the same time, TNF-α affects the expression of several miRNAs, although some of these processes are still unclear. In this context, it has been hypothesized that miR-155 could directly increase TNF-α levels by augmenting transcript stability through binding to its 3′ UTR region (71). Alternatively, miR-155 could target gene transcripts coding for proteins that are known to act as repressors of TNF-α translation. Tili *et al.* (72) demonstrated that the up-regulation of miR-155 in LPS-activated macrophages resulted in an enhanced translation of TNF-α mRNA. Additionally, mice overexpressing miR-155 produce more TNF-α when challenged with LPS (73). Moreover, *in vivo* studies showed that transgenic mice overexpressing miR-155 in the B cell lineage produce more TNF-α when challenged with LPS and are hypersensitive to LPS/D-galactosamine-induced septic shock with respect to their normal counterparts (72). Some results also demonstrated that miR-155 and miR-146a serve as a mediator of the glucose-induced TNF-α/TGF-β1/NF-κB pathway (21). Finally, one study showed that human monocyte exposure to cold (32°C) potentiated LPS-induced production of TNF and IL-6 while blunting IL-10 production (74). This dysregulation was associated with increased expression of miR-155, which potentiates TLR signaling by negatively regulating SH2 domain–containing inositol-5-phosphatase 1 (SHIP1) and suppressor of cytokine signaling 1 (SOCS1). Indeed, SHIP1 and SOCS1 were suppressed at 32°C, and miR-155 antagomirs increased SHIP1 and SOCS1 and reversed the alterations in cytokine production in cold-exposed monocytes. In contrast, miR-155 mimics phenocopied the effects of cold exposure, reducing SHIP1 and SOCS1 and altering TNF and IL-10 production. Importantly, miR-155 deficiency reduced hypothermia and improved survival (78 *vs.* 32%), which was associated with increased SHIP1, SOCS1, and IL-10.

TNF-α is a strong inducer of NF-κB signaling. Thus, Lorente-Cebrian *et al.* (75) demonstrated that miR-145 overexpression regulated adipocyte lipolysis *via* multiple mechanisms involving increased production and processing of TNF-α in human adipocytes. The increase in TNF-α production by miR-145 was mediated *via* activation of p65, a member of the NF-κB complex. In addition, miR-145 also down-regulates the expression of the protease ADAM17, resulting in an increased fraction of membrane bound TNF-α, which is the more biologically active form of TNF-α (75). Moreover, it was recently shown in human non-adipose cells that miR-145 decreases ADAM17 expression by direct binding to the 3′ UTR of the gene (76). On the other hand, the treatment of preadipocytes with TNF-α for 24 h decreased miR-221 expression at all the concentrations tested (77). miR-221 also is down-regulated by TNF-α in cultured human preadipocytes. In a study with human adipocytes, the treatment with TNF-α up-regulated miR-378 expression on the d 15 after the induction of preadipocyte differentiation (78). The overexpression of miR-181a repressed TNF-α protein expression, while the inhibition had the opposite effect (79). Additionally, miR-181a suppression decreased the expression of fatty acid synthesis–associated genes (phosphodiesterase 3B and lipoprotein lipase, among others), revealing a new therapeutic target for antiobesity drugs.

The transfection of miR-346 in an LPS-activated human monocytic cell line (THP-1) has been shown to inhibit TNF-α secretion. miR-346 controls the release of TNF-α protein and the stability of its mRNA by regulating the stabilization of tristetraprolin, a RNA binding protein (80). Moreover, it has been found that miR-146b-5p decreases the expression of TNF-α in THP-1 monocytes by a targeted repression of IL-1 receptor–associated kinase (IRAK) and TNF receptor–associated factor 6, two key adapter molecules in the IRAK/NF-κB pathway (30, 81). Other miRNAs, such as miR-19a and miR-181, also regulate adipogenesis by affecting the expression of TNF-α as well as some genes involved in adipogenesis (82). Furthermore, Schroen *et al.* (49) summarized the inverse regulation of miRNAs during adipogenesis and adiposity by TNF-α, including the up-regulation of miR-221 and miR-222 and the inhibition of miR-103 and miR-104. By contrast, miR-125 may be implicated in the posttranscriptional repression of TNF-α mRNA—and hence the need for its down-regulation concerning TNF-α production (83).

Of interest, TNF-α induces the down-regulation of miR-103 and miR-143 (54) and the up-regulation of miR-221 and miR-222 in adipocytes (54, 84). Suarez *et al.* (85) reported that TNF-α increases the expression of miR-155, miR-31, miR-17, and miR-191 without changing miR-20a,

miR-222, and miR-126 in human endothelial cells. Such investigation also evidenced that E-selectin and ICAM-1 are targets of TNF-α-induced miR-31 and miR-17-3p, respectively, regulating neutrophil binding to endothelial cells (85). A constitutively expressed, endothelial cell–restricted miRNA (miR-126) modulated TNF-α-induced VCAM-1 expression (86). Taken together, the recent results show that inducible miRNAs may make up a feedback loop to control TNF-α production in an inflammation context.

IL-1

IL-1 is a cytokine that plays a key role in inflammatory, immunity, infectious, and degenerative diseases (87, 88). Although 2 different genes encode for IL-1α and IL-1β, both molecules bind to the same IL-1 receptor (IL-1R). However, they have diverse functions: whereas IL-1α is constitutively expressed in epithelial membranes of gastrointestinal tract, lung, liver, kidney, endothelial cells, and astrocytes, IL-1β is produced by monocytes and macrophages and in response to other cytokines (89, 90). Thus, their expression levels are under strict control to ensure activity under the appropriate conditions.

As occurs with other cytokines, the expression of IL-1α and IL-1β is regulated by miRNAs. For example, in human gingival fibroblasts, IL-1β levels increased after miR-146 inhibition (91). In cortical neuronal cells overexpressing miR-34a, miR-451, and miR-874 and subjected to stretch injury, mRNA levels of IL-1β and TNF-α were dramatically up-regulated (92). Furthermore, miR-181 inhibition resulted in the up-regulation of inflammatory cytokines, including IL-1β (93). IL-1 is produced by active leukocytes; it binds to its receptor and forms the TLR/IL-1 complex. Myeloid differentiation primary response protein 88 (MyD88) is the first adapter molecule recruited to the complex and activates the signaling pathway (94). In this context, it has been described that miR-155 regulates MyD88 protein expression by translational repression (95) and leads to the suppression of IL-1 function.

On the other hand, IL-1β is predicted as a target of miR-21 and miR-132. Recent studies have shown that miR-21 is implicated in several processes in human and mouse tissues, including an anti-inflammatory effect in different inflammation-related diseases. Thus, LPS induces miR-21 expression, and miR-21 limits the LPS-induced inflammatory response (96). Moreover, IL-1β is implicated in changing wound-associated macrophages to an anti-inflammatory mode and also induces the expression of the anti-inflammatory cytokine IL-10 (96). In addition, miR-21 protects against inflammatory and injury responses in fatal colitis (97). miR-132 may act as a homeostatic process counteracting inflammation both in experimental autoimmune encephalomyelitis and in inflammatory bowel disease (98, 99). Furthermore, miR-132 is correlated with adipose tissue morphology, metabolic markers, leptin, and adiponectin (100).

IL-6

IL-6 is a proinflammatory cytokine that regulates energy homeostasis, cell proliferation, and inflammatory responses (101). Increased IL-6 production is a hallmark of many human chronic inflammatory states, including sepsis, rheumatoid arthritis, and inflammatory bowel disease (102). The diverse roles of IL-6 during the inflammatory response might be explained by the ability to initiate 2 modes of signaling: classic signaling *via* the interaction of IL-6 with its membrane-bound IL-6Ra subunit (103), and trans signaling *via* a naturally occurring soluble IL-6Ra receptor that is proteolytically cleaved from the cell surface (104). In both scenarios, IL-6 exerts specific actions *via* the signal transducers, leading to the activation of the Janus family kinases/STAT and MAPK cascades (103). IL-6 expression is also regulated by NF-κB and is influenced by a variety of external stimuli. Various elements in the 3′ UTR of IL-6 mRNA contribute to IL-6 mRNA degradation and regulate IL-6 expression (105, 106).

The IL-6/STAT3 signaling pathway exerts complex actions in regulating the innate immune response (107). Activation of STAT3 may promote IL-6 production, and IL-6 itself can lead to the phosphorylation of STAT3 (108). Chen *et al.* (107) demonstrated that the expression of IL-6 in response to LPS initiates a positive feedback loop in which secreted IL-6 down-regulated miR-223 expression, leading secondarily to an increase in STAT3, which then drove the expression of IL-6 and IL-1β in a positive regulatory loop. The change in miR-223 expression was also accompanied by a substantial increase in STAT3 protein expression. Furthermore, the overexpression of miR-223 was associated with a significant decrease in STAT3 levels and a reduction in the production of IL-6 and IL-1β, while miR-223 antagomirs (antisense oligonucleotides) increased the production of IL-6 and IL-1β. Interestingly, IL-6 was found to be a main factor in inducing the decrease in miR-223 expression after LPS stimulation, which formed a positive feedback loop to regulate IL-6 and IL-1β (107).

On the other hand, miR-146a/b up-regulation provides a negative feedback on the innate immune system, being another important regulator of IL-6 metabolism (109). Bhaumik *et al.* (110) showed in human fibroblasts that the overexpression of miR-146a/b suppressed IL-6 and IL-8 secretion and down-regulated IRAK, a crucial component of the IL-1 receptor signal transduction pathway. The authors hypothesized that the increased expression of miR-146a/b served to restrain the excessive secretion of the inflammatory cytokines IL-6 and IL-8, thereby limiting inflammation in this context. It has been reported that miR-146a mimics decreased, while miR-146a inhibitor increased, the expression of IL-6 but did not affect TNF-α expression in LPS-stimulated RAW264.7 cells (109). The authors proposed that miR-146a might function as a negative feedback in the Notch1 signaling pathway during the LPS-induced production of inflammatory cytokines. Notch signaling is a highly conserved pathway that plays an important role in inflammatory disorders (111). It can act cooperatively with TLR pathways to activate Notch target genes and increase the production of TLR-induced cytokines in macrophages. Such study confirmed that miR-146a may play a critical role in the inflammatory response.

An assay with monocyte-derived cells demonstrated that IL-6-mediated suppression of miR-200c directs the constitutive activation of an inflammatory signaling circuit (112). The autoregulatory loop is established and maintained by the IL-6-directed suppression of miR-200c expression.

Furthermore, signals downstream of this regulatory RNA bifurcate to regulate p65 activity *vs.* that of a Jun NH2-terminal kinase to heat shock factor 1 pathway, which are required to demethylate the IL-6 promoter to facilitate p65/c-Jun-directed transcription of IL-6 (112). Dou *et al.* (113), in a study with hepatocytes of db/db mice (C57BL/KsJ), found that the expression of miR-200s was decreased and was accompanied by elevated levels of IL-6. Moreover, the treatment with IL-6 (10 ng/ml for 24 h) suppressed the expression of miR-200s. The authors proposed than IL-6 impairs activation of the PI3K/protein kinase B (AKT)/glycogen synthase kinase pathway *via* down-regulation of miR-200s, and up-regulation of the transcriptional regulator friend of Gata 2, a multitype zinc finger protein that influences gene transcription by specifically interacting with other transcription factors (114).

Further, it has been demonstrated that miR142-3p directly targets the 3′ UTR of IL-6 in dendritic cells (115) and that IL-6 expression decreases in hematopoietic cells in a miR142-3p-dependent manner (116). An IL-6/miR142-3p feedback loop–dependent regulation process has been described. Thus, IL-6 inhibits miR142-3p expression by inducing DNA methyltransferase 1–mediated hypermethylation of the miR142-3p promoter (117). Interestingly, miR142-3p also suppresses IL-6 secretion by targeting the 3′ UTR of IL-6. Also, the overexpression of let-7 *via* the transfection of let-7 precursors decreases IL-6 expression, which is consistent with the inhibition of IL-6 3′ UTR luciferase activity in bone marrow–derived mesenchymal stem cells (118). However, even if IL-6 contains a potential binding site for let-7a in its 3′ UTR, another study reported different data. Overexpression of let-7a *in vitro* increased IL-6 production in stimulated mesangial cells compared to nontransfected controls (119). These data open the door to studies examining the role of let-family expression in inflammation.

Of interest, miR-329 plays a pivotal role in the inhibition of IL-6 mRNA expression by targeting the NF-κB subunit, p65 (120). Moreover, the same study showed than miR-23a and let-7c directly regulate the inhibition of IL-6 mRNA. In the same way, miR-132 expression is inversely related with IL-6 levels in patients with nonalcoholic fatty liver disease (100). Together, these findings suggest that multiple miRNAs are involved in the regulation of IL-6 expression.

IL-18

IL-18 is a cytokine of the IL-1 family that is expressed as an inactive form and subsequently activated by proteolytic cleavage performed by caspase 1, which belongs to the NOD-like receptor (NLR) complex (121). IL-18 is produced in a variety of tissues and has been associated with inflammatory function, autoimmune diseases, and cancer (122, 123). For example, IL-18 has a predictive role in the development of type 1 diabetes (124), whereas its serum levels are correlated with hyperglycemia and atherogenesis in patients with type 1 diabetes (125). Only miR-197 has been studied in relation to IL-18. This miRNA interacts with the mRNA sequence of IL-18 and inhibits the expression of the cytokine (126). Moreover, the expression of miR-197 is inversely correlated with the levels of IL-18 in peripheral blood mononuclear cells from patients with hepatitis B (126).

ICAM-1

ICAMs are glycoproteins belonging to the immunoglobulin superfamily that bind to β2 integrins present in leukocytes (127). ICAM-1 is considered the most important adhesion molecule for leukocyte recruitment to inflamed areas (128). Recent studies have demonstrated that ICAM-1 expression is under critical control of a number of miRNA species, such as miR-221, miR-222, and miR-339. These miRNAs are complementary to ICAM-1 3′ UTR region and modulate ICAM-1 expression at the postranscriptional level by binding to the untranslated region. Thus, miR-221 and miR-222 down-regulate ICAM-1 expression in HUVEC and lead to significant reduction of monocyte adhesion to the cells (129). miR-221 is down-regulated in the human subcutaneous adipose tissue of obese patients compared to nonobese individuals (130) and can be related to chronic inflammatory state in obesity. In addition, miR-221 and miR-222 are down-regulated during adipogenesis and associated with BMI in human adipose samples (130). Different studies have examined the modulation of ICAM-1 molecule by miRNAs in diseases with inflammatory responses. Indeed, in an important group of biliary disorders called cholangiopathies, IFN-γ suppresses miR-221, resulting in increases of ICAM-1 expression in cholangiocyte cells and further regulation of inflammatory responses in cholangiopathies (131). In glioblastoma multiforme cells, the suppression of ICAM-1 by miR-222 and miR-339 is related to tumor escape from the host immune response and therefore to poor prognosis (132). Finally, Kim *et al.* (133) have reported that ICAM-1 is up-regulated by miR-130 during the inflammatory response.

VCAM-1

VCAM-1 is a sialoglycoprotein that belongs to the immunoglobulin superfamily (134). In response to metabolic and inflammatory activation, this intercellular adhesion molecule is up-regulated in endothelial cells (135) and mediates leukocyte adhesion by interacting with its integrin ligand, which is expressed in the membrane of leukocytes and activated neutrophils (136). An increase in VCAM-1 expression in endothelial cells is associated with the promotion of inflammation and tissue damage (134). miR-126 (also referred as miR-126-3p), along with its analogous strand (antisense) miR-126* (referring to the 5′ part of the transcript, also called miR-126-5p), is the most studied miRNA in relation with the regulation of VCAM-1.

In mammals, miR-126 is expressed from intron 7 of epidermal growth factor–like domain 7, an endothelial cell–derived secreted protein essential for proper vascular development (137). This miRNA can bind to the 3′ UTR of the transcript for human VCAM-1 (86), inhibiting mRNA translation and suppressing *de novo* protein synthesis (138), thereby blocking adhesion and infiltration of leukocytes into the vasculature wall. Transfection of endothelial cells with an antisense construct to target endogenous miR-126 permits an increase in TNF-α-stimulated VCAM-1 expression (86). Classically, miR-126 participates in biologic processes like angiogenesis and primitive erythropoiesis (139), and it has a potential role in the development of

renal microvascular inflammation and dermatomyositis (83, 140). However, a major emerging function of miR-126 is to regulate key molecules in PI3K/AKT and MAPK signaling pathways. It does change the expression levels of both negative regulators like sprouty-related EVH1 domain–containing protein 1 and phosphoinositide kinase 3 regulatory subunit 2 (PIK3R2 or p85β), and activators like insulin receptor substrate 1 (141). Agudo *et al.* (142) demonstrated that miR-126 is specifically expressed in plasmacytoid dendritic cells and controls the function of these cells. It regulates the expression of genes encoding molecules involved in the innate response, such as VEGF receptor 2, suggesting new opportunities for therapeutic purposes. In this sense, miR-126 up-regulation could promote chronic inflammation and autoimmunity, and a balanced expression of miR-126 should be sought to avoid the development of several pathologies. As a consequence, miR-126 could be considered a new modulator of innate immunity [for review, see (143)].

PAI-1

PAI-1 has been described as a member of the serine protease inhibitor superfamily (144) that inhibits the activation of both plasminogen activator and urokinase-type plasminogen activator, which act in fibrinolysis (145). PAI-1 is produced by fibroblasts, endothelial cells, and platelets (146, 147), and its expression increases with hypoxia and reactive oxygen species (148). This peptide has a variety of biologic functions and is involved in different diseases, such as systemic inflammation, atherosclerosis, metabolic syndrome, fibrosis, and cancer, as has been reviewed by Iwaki *et al.* (149). Inflammatory response regulators, such as monocytes, TNF-α, IL-1β, and LPS, are known to stimulate PAI-1 production in endothelial cells (150). Recent studies have described some miRNAs that bind to the 3′ UTR of PAI-1 and inhibit its production. For example, under hypoxic conditions, PAI-1 expression is augmented in fibroblasts by decreasing miR-449a/b expression (148). Other miRNAs that bind to the 3′ UTR of PAI-1 and down-regulate its expression are miR-30c, miR-421, miR-486, and miR-449a/b. Both miR-30c and miR-421 inhibit PAI-1 expression in endothelial cells, lowering PAI-1 plasma levels (151). Moreover, miR-30c induces human adipocyte differentiation while repressing PAI-1 expression in adipose tissue (152). It has been also shown that miR-486 is repressed and leads to the up-regulation of PAI-1 in liposarcoma or adipose tissue–derived tumors (153). The stress conditions also modify PAI-1 levels through miRNAs regulation.

OTHER NONCODING RNAS AND REGULATION OF INFLAMMATION

lncRNAs

Although only a handful of lncRNAs have been fully characterized—the result of the emergence of microarray and high-throughput sequencing techniques—the interest in these regulatory molecules is increasingly growing. They are noncoding RNAs longer than 200 nt with important roles in diverse biologic functions, including inflammation (154). Cui *et al.* (155) identified a number of lncRNAs with altered expression in response to LPS stimulation. One of the most up-regulated transcripts was lnc-IL7R, which overlaps with the 3′ UTR of the human IL-7 receptor α-subunit gene (*IL7R*). When transfected, it was able to diminish the LPS-induced inflammatory response. Another lncRNA, Ptprj-as1, is highly expressed in macrophage-enriched tissues and is transiently induced by proinflammatory factors, such as LPS (156). Rapicavoli *et al.* (157) studied the lncRNAs induced by inflammatory signaling *via* TNF-α. Among the hundreds of lncRNAs whose expression was modified, including 54 pseudogene lncRNAs, Lethe (a pseudogene lncRNA) was selectively induced when TNF-α activated NF-κB, and it functioned in negative feedback signaling to NF-κB by binding to this transcription factor and preventing it from interacting with DNA (157). Also, microbial recognition receptors, such as the TLRs, are able to induce the expression of numerous lncRNAs. One of them is lncRNA-COX-2, which mediates both the activation and repression of distinct classes of immune genes (158). Another lncRNA, nuclear enriched abundant transcript 1, induces the expression of antiviral genes, including IL-8 (159). Finally, in osteoarthritis, lncRNA H19, known for influencing IGF-2 expression, was found to be significantly up-regulated. However, proinflammatory cytokines, such as IL-1β and TNF-α, down-regulated the expression of lncRNA H19 as well as that of miR-675 (160). With this background, more research is needed to identify lncRNAs implicated in the regulation of the inflammatory processes.

Long intergenic noncoding RNAs

The long intergenic noncoding RNAs (lincRNAs) are a newly described subtype of noncoding RNAs that can act as regulators for both transcription and posttranscriptional/ translation (161). Some of them were recently identified in blood and adipose tissue during low-dose experimental endotoxemia with LPS in humans, and 2 of them, linc-TP53I13 and linc-DMRT2, were suppressed in adipose tissue of obese humans (162). Other lincRNA, such as lincRNA-DYNLRB2-2, inhibited inflammation through G protein–coupled receptor 119 in THP-1 macrophage-derived foam cells by promoting ABCA1-mediated cholesterol efflux (163). Finally, a total of 159 lincRNAs were found to be differentially expressed following innate activation of THP1 macrophages (164). Among them, linc1992, called TNF and HNRNPL-related immunoregulatory lincRNA (THRIL), was required for the expression of many cytokines and immune response genes, particularly TNF-α, by binding to its promoter.

Circular RNAs

There are thousands of endogenous circular RNAs (circRNAs) in mammalian cells, some of which are highly abundant and evolutionarily conserved. Evidence is emerging that some circRNAs might regulate miRNA function, and roles in transcriptional control have also been suggested. A recent review has described methods for

the identification and characterization of endogenous circRNAs, including molecular methods and genome-wide approaches, with a focus on the advantages and disadvantages of various techniques (165). Therefore, the study of this class of noncoding RNAs has potential implications for therapeutic and research applications in inflammation-related diseases. To understand the regulation and function of these unusual molecules is a future challenge within this field.

NONCODING RNA CHANGES IN RESPONSE TO ANTI-INFLAMMATORY DRUGS

Although it is not the main topic of this review, it has been described that several drugs can exert their beneficial effects at least in part through the regulation of the expression of some miRNAs. Despite their secondary effects, steroidal and nonsteroidal anti-inflammatory drugs are being used in the control and management of several inflammation-related diseases, including autoimmune diseases (166). Several studies, both *in silico* (167) and in animals (168), have reported that these drugs can affect the expression of many miRNAs involved in the inflammatory response. For example, celecoxib, a nonsteroidal anti-inflammatory drug, up-regulated miR-222 while deterring aromatase-expressing breast tumor growth in mice (168). Budenoside, a steroid that reduces inflammation, has been reported to protect the lung from cigarette smoke–induced miRNA alterations and changed the smoking-related dysregulation of miRNA expression in liver (169). Resolvins are compounds that are made by the human body from the ω-3 fatty acids (170). It has been described that resolvin D1 anti-inflammatory actions are mediated by the up-regulation of specific miRNAs, such as miR-208a and miR-219, and their target anti-inflammatory genes, including IL-10 (171). In summary, although the development of anti-inflammatory drugs targeting miRNAs and modulating their expression represents a promising therapeutic approach, there is much work to be done in the coming years.

CONCLUSIONS AND FUTURE DIRECTIONS

The role of noncoding RNA in the coordination of diverse cellular functions, such as stem cell maintenance, differentiation, and apoptosis, underscores their emerging importance as regulators of cellular homeostasis, especially of the inflammatory process, as reviewed here. Gene expression is a complex, dynamic process influenced by the environment (*i.e.,* diet, stress, and toxins) in which the noncoding RNAs are involved in the regulation of pathways associated with the development of inflammation-related diseases. In this way, the noncoding RNAs are related to predisposition and development of inflammation-related diseases, including obesity, atherosclerosis, type 2 diabetes, osteoarthritis, autoimmune diseases (*e.g.,* lupus erythematosus, multiple sclerosis, type 1 diabetes, inflammatory bowel disease, celiac disease), asthma, periodontitis, and cirrhosis.

There are 2 promising applications of noncoding RNAs in the prognosis and treatment of these diseases. First, noncoding RNAs can be used as susceptibility biomarkers, early prognostic biomarkers, confirmatory biomarkers of disease, or biomarkers of treatment response (58, 172). The levels of cell-free miRNAs in serum are stable and reproducible, and they are emerging as new noninvasive biomarkers for the diagnosis of different types of cancer (173) and other diseases, including inflammation-related diseases (174). These circulating miRNAs are protected by microvesicles and protein/lipoprotein complexes (high-density lipoprotein, low-density lipoprotein complexes) in order to avoid RNase-mediated degradation, and they might contribute to intercellular signaling (172).

Second, noncoding RNAs could be used as a therapeutic strategy in inflammation-related diseases. In this case, 2 approaches are being investigated: the down-regulation or antagonization of the miRNAs related with the disease, and the administration of exogenous miRNAs that mimic the action of the endogenous beneficial ones (175). A growing body of evidence suggests that antagonizing miRNA activity through the use of miRNA inhibitors or synthetic anti-miRNA oligonucleotides, perfectly antisense to naturally occurring miRNAs, may be a useful approach to quench the pathogenic effects of up-regulated miRNAs (176). These specifically designed anti-miRNAs may have their half-life extended through the use of various strategies, including locked nucleic acid probes (177). Some studies have been shown to be highly efficacious in human cells in primary culture, but the implementation of these technologies in human clinical trials awaits further development (178). On the other hand, the administration of miRNA inducers that increase the expression of tissue-specific miRNAs could be an alternative approach for the management of inflammation-related diseases (30). Similarly, the administration of systemic miRNAs (miRNA mimics) or introducing genes coding for miRNAs into viral constructs or liposomes could be used as a future targeted therapy for many diseases (179). In this way, the development of nanotechnologies for increasing the bioavailability and the delivery of the exogenous miRNAs in order to specifically target the desired cells or tissues (*i.e.,* small nanoparticles that effectively complex miRNA and facilitate the uptake of miRNA into the cells) is rapidly progressing (180). Although the immune responses related to the carriers remain to be addressed, liposome-, metal- and polymer-mediated miRNA delivery (181), or even intravenous delivery of virus-mediated miRNAs (182), have been achieved in the treatment of cancer and liver disease.

The development of miRNA-based therapies for inflammation-related diseases is warranted because of the severe problem that these diseases represent to society. However, huge challenges remain to be overcome before the clinical use of miRNA-targeting therapeutics, especially in relation to the development of safe and effective delivery of targeting miRNAs in specific tissues *in vivo* (183). Among these remaining questions, we can highlight that one individual miRNA has multiple potential targets, which may coordinate or antagonize each other's functions. In addition, the interactions between miRNAs and mRNAs depend on the cell type and tissue, which explains the difficulties in predicting the side effects that may be associated with miRNA-based therapies. In order to affect only the intended organ, a solution is the conjugation of the anti-miR oligonucleotides with ligands for target organ

specific cell surface receptors (184). In spite of these difficulties, the next few years will see the development of miRNA-based therapies that, after being tested for safety and efficacy in animal models, could be used in the treatment of metabolic, autoimmune, and degenerative diseases, cancer, and many other conditions that are characterized or associated with chronic inflammation. FJ

The authors thank the government of Spain (Ministerio de Economía y Competitividad, ref. AGL2013-45554-R), Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERobn), and the CNPq Foundation [Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil)] for financial support, and the CAPES Foundation (Ministry of Education of Brazil, Brasília, DF 70040-020) for providing J.L.M.-R. with a research grant (process 6409-13-0). M.S. has a predoctoral fellowship from the Center for Nutrition Research of the University of Navarra.

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Received for publication July 4, 2014.
Accepted for publication June 2, 2015.

2.4 DNA Methylation and Inflammation

The methylation of DNA is regulated by DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) in the presence of S-adenosyl-methionine (SAM); this is the methyl donor for methylation of cytosine residues at the C-5 position to yield 5-methylcytosine (EHRLICH, M., 2009) (See Figure 2). This happens at 5' cytosine of C-G dinucleotides, referred to as CpGs. These nucleotide pairs are relatively sparse in the genome, and areas of comparatively high CpG density are referred to as CpG islands, identified as regions > 200 bp with a > 50% G+C content and 0.6 observed/expected ratio of CpGs (ILLINGWORTH, R.S. *et al.*, 2009). Approximately 60–70% of genes have a CpG island associated with their promoters, and promoters can be classified according to their CpG density (WEBER, M. *et al.*, 2007). Levels of DNA methylation at a promoter-associated CpG island are generally negatively associated with gene expression, although some specific genes show the opposite effect (GUTIERREZ-ARCELUS, M. *et al.*, 2013). DNA methylation induces transcriptional silencing by preventing the binding of transcription factors to the DNA or by recruiting methyl CpG-binding protein 2 to the DNA (BIRD, A., 2001), which in turn, recruits histone-modifying complexes (FUKS, F. *et al.*, 2003).

DNA methylation also functions to repress repetitive elements, such as long interspersed nuclear elements-1 (LINE-1) (ALVES, G. et al., 1996). LINE-1, considered the most common repetitive elements of interspersed DNA repeats (WEISENBERGER, D.J. et al., 2005), are moderately CpG rich and most heavily methylated (SHENG, W. et al., 2012). In addition, because of their high genome dissemination, LINE-1 methylation status has been proposed as a surrogate marker for estimating global DNA methylation level (WEISENBERGER, D.J. et al., 2005; BARCHITTA, M. et al., 2014).

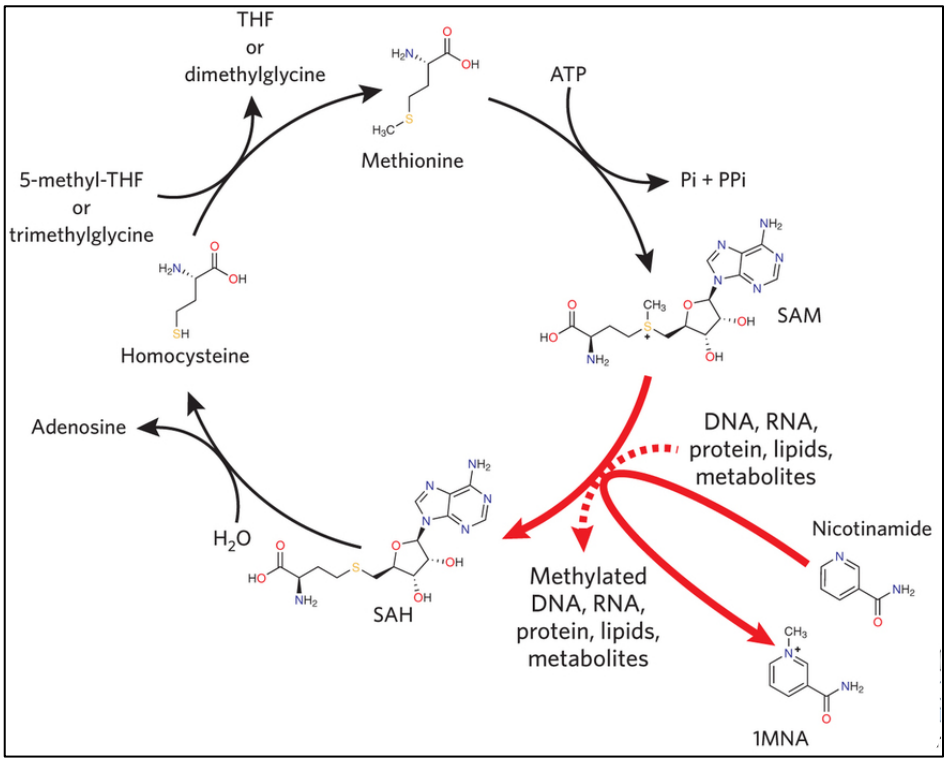

Figure 2. S-adenosyl-methionine (SAM) cycle. Methionine reacts with ATP to form S-adenosyl methionine (SAM), which is the methyl (-CH3) donor for DNA methylation. SAM-derived cytosine methylation of DNA is not a random process but occurs at the 5' carbon of the pyrimidine nucleus of cytosine within short cytosine–guanine dinucleotide (CpG) sequences. 1MNA, 1-Methylnicotinamide; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate. Figure adapted from Shlomi & Rabilowitz (2013).

While DNA methylation is an essential component of normal development and transcriptional regulation, aberrant patterns of DNA methylation are associated with a number of inflammatory diseases and conditions (EHRLICH, M., 2009; VOELTER-MAHLKNECHT, S., 2016). Obesity is well-known to contribute to a chronic low-grade inflammation via increased secretion of pro-inflammatory cytokines such as TNF, IL6 and IL-1β from macrophages infiltrating adipose tissue (OLEFSKY, J.M. et al., 2010). In this context, high-fat diet feeding in two subsequent generations of mice has recently been reported to result in DNA hypomethylation of inflammation-associated genes in adipose tissue of third generation mice (DING, Y. et al., 2014). In a study of the methylation status of the IL6 promoter (-1,200 to +30) in subjects with

rheumatoid arthritis compared to healthy controls, a single CpG site at -1,181 was significantly less methylated in subjects with rheumatoid arthritis, and the methylation of this dinucleotide resulted in reduced affinity with a nuclear protein in an *in vitro* assay (NILE, C.J. et al., 2008). The available evidence appears to indicate that aberrant DNA methylation contributes to inflammation through hypomethylation and subsequent upregulation of inflammatory gene expression.

Lower level of LINE-1 methylation in leukocytes has been associated with an increased risk of several cancers (HOU, L. et al., 2010; BARCHITTA, M. et al., 2014). Moreover, an inverse correlation between DNA methylation of TLR4 and the inflammatory response to LPS stimulation in intestinal epithelial cells has been reported (TAKAHASHI, K. et al., 2009), while DNA methylation of TLR4 in circulation was also inversely associated with the C-reactive protein, ICAM-1 and VCAM-1 responses to air pollution (BIND, M.A. et al., 2012). However, in terms of epigenetic modification and inflammation, the causal directionality remains questionable.

Studies in humans have shown that certain dietary compounds can modulate the status of DNA methylation (SCHIANO, C. *et al.*, 2015). Different nutrients such as folate, vitamin B6, vitamin B12, choline and methionine, play an important role in DNA methylation through their influence on SAM levels and methyltransferase inhibitor S-adenosylhomocysteine (SAH) levels (LEE, H.S., 2015) (see Figure 2). Moreover, dietary components including green tea, red wine, and cocoa also may act by epigenetic mechanisms (FANG, M. *et al.*, 2007; CRESCENTI, A. *et al.*, 2013), A study supplementating high-fat sucrose diet with apple polyphenols (APs) showed different methylation patterns of aquaporin 7 (Aqp7), leptin, peroxisome proliferator-activated receptor gamma co-activator 1 alpha (Ppargc1a), and sterol regulatory element binding

transcription factor 1 (Srebf1) promoters in adipocytes from apple-supplemented rats compared to high-fat sucrose fed rats (BOQUE, N. *et al.*, 2013). The authors proposed that AP exerts the antiobesity effects through the regulation of genes involved in adipogenesis, lipolysis, and fatty acid oxidation, in a process that could be mediated in part by epigenetic mechanisms.

3. HYPOTESIS

We hypothesized that a hypocaloric dietary pattern, as well as the improvement of the overall diet quality components designed to reduce MetS features, could have a modulatory effect on the expression of genes and miRNAs related to the inflammatory state. In addition, we suggested that the *in vitro* overexpression of selected miRNAs could clarify the results found *in vivo*. Finally, we believe that a DNA methylation study in healthy individuals could explore new biomarkers to early detection of risks factors related to MetS.

4. GENERAL OBJECTIVE

The general aim of this work was to search for a better understanding the potential epigenetic underlying mechanisms and the putative interactions with the dietary intake through of the study of miRNAs and DNA methylation in distinct populations and its potential application to dietetic practice.

4.1 Specific objectives

4.1.1 To evaluate the effect of a weight loss strategy based on the Mediterranean dietary pattern on anthropometric measurements, biochemical markers, and the expression of some selected inflammation-related genes and miRNAs in WBC of individuals with MetS.

4.1.2 To clarify the potential regulatory roles of miR-155-3p and let-7b on inflammation-related genes (*IL6*, *TNF*, *SERPINE1* and *TLR4*) in human acute monocytic leukemia cells (THP-1).

4.1.3 To investigate the influence of different fatty acids (palmitic acid, oleic acid, eicosapentaenoic acid and docosahesaenoic acid) on the expression of miR-155-3p and let-7b in monocytes, macrophages and LPS-activated macrophage.

4.1.4 To explore the relationship of DNA methylation levels of LINE-1, TNF and IL6 in PBMC with anthropometric, biochemical, clinical, dietary, inflammatory and oxidative stress parameters in young and apparently healthy adults.

5. GENERAL METHODOLOGY

The methodology used is described in each chapter of the results section; however, the main features of the two projects involved are highlighted in this session.

5.1 Study of the miRNAs

This study was conducted through analysis of blood samples collected in RESMENA-S study (Reduction of the Metabolic Syndrome in Navarra-Spain), Department of Food Science and Physiology at the University of Navarra, Spain, from 2009 to 2010 (ZULET, M.A. *et al.*, 2011). Samples were analyzed at baseline and after 8 weeks of nutritional intervention.

5.1.1 The RESMENA-S study

Subjects and methods

Inclusion and exclusion criteria

The inclusion and exclusion criteria for the study are shown in Table I. The criteria for MetS were based on those established by the International Diabetes Federation (IDF, 2014). The study 065/2009 was approved by the local ethical committee (Research

Ethics Committee of the University of Navarra). All study participants signed an informed consent document after verbal and written instructions and according to local legislation. This trial is registered at www.clinicaltrials.gov as NCT01087086.

Table I
Inclusion and exclusion criteria

Inclusion		*Exclusion*
Age: 35-65 years old		Subjects with difficulty for changing dietary habits
Central obesity (WC[1] > 94 cm males and > 80 cm females)		Subjects with psychiatric or psychological disorders
plus any two of the following four factors:		Subjects with eating disorders (bulimia; test of Edinburgh)
Raised triglycerides	≥ 150 mg/dL or specific treatment for this lipid abnormality	Subjects with weight instable for 3 months before the beginning of the study
Reduced HDL cholesterol	< 40 mg/dL in males < 50 mg/dL in females or specific treatment for this lipid abnormality	Subjects under any pharmacological treatment
Raised blood pressure	Systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension	Subjects with chronic diseases related to the metabolism of energy and nutrients (gastric ulcer, disorders of the digestive system, hyperthyroidism, hypothyroidism)
		Peri- and postmenopausal women
Raised fasting plasma glucose	≥ 100 mg/dL or previously diagnosed type 2 diabetes	Subjects on special diets
	If above 100 mg/dL , OGTT is strongly recommended but is not necessary to define presence of the syndrome	Subjects with food allergies or intolerances

[1]WC: waist circumference; BP: blood pressure; OGTT: oral glucose tolerance test.

Reproduced from Zulet, M.A. (2011)

Recruitment of participants

The recruitment of the participants is being carried out with the help of the Department of Endocrinology of the Health Department of Navarra and the Department of General Medicine of the University Hospital of Navarra. Advertisements (poster approved by the Ethical Committee), internet, interviews to local press and to the University of Navarra information office, and databases from previous studies in the Department were used for recruitment.

Study design

The study was designed as a 6-month weight-loss caloric restriction trial divided in 2 consecutive phases. The first phase consisted of an 8-week controlled parallel intervention period, which was followed by a 16- week self-control second phase. At the beginning of the study, subjects were randomized either to a moderately high

protein weight-loss diet (group A, n = 50) or to a weight-loss diet based on the American Heart Association recommendations (Group B, n = 50) (KRAUSS, R.M. *et al.*, 2000). Diets in both groups were designed (see Table II) on a daily caloric restriction of 30% of the subjects total energy baseline needs.

Table II
Examples of 1-day diet for each dietary group (1,300 kcal diet)

	Group A	*Group B*
Breakfast	Orange (175 g) 2 low-fat yogurts (2 x 125 g)	Orange (175 g) 2 low-fat yogurts (2 x 125 g) 1 slice of refined white bread (15 g)
Morning snack I	1 low-fat yogurt (125 g)	Apple (125 g) 1 low-fat yogurt with sugar (125 g)
Morning snack II	2 thin slices of ham (45 g) 2 slices of whole-grain bread (20 g)	
Lunch	Vegetables (cooked; 250 g) Whole-grain pasta (cooked; 45 g) Lean fish (cooked; 140 g) Apple (125 g)	Vegetables (cooked; 240g) Pasta (cooked; 90 g), 1 slice of refined white bread (15 g) Lean fish (40 g) Melon (250 g) 1 low-fat yogurt (125 g)
Afternoon snack I	1 low-fat yogurt (125 g)	Banana (75 g)
Afternoon snack II	Walnuts (10 g) Low-fat cheese (60 g)	
Dinner	Salad (200 g) 1 slice of whole-grain bread (30 g) Lean meat (cooked, 80 g) Pear (150 g)	Salad (200 g) 1 slice of refined white bread (30 g) 2 slices of ham (60 g) Pear (150 g)

Reproduced from Zulet,M.A. (2011)

Group A diets were characterized by a macronutrient distribution of 40/30/30 (carbohydrate/lipid/protein), a high adherence to the Mediterranean dietary pattern, an intake of low glycemic index carbohydrates, a higher supply of energy from protein at the end of the day, a high total antioxidant capacity, and a meal frequency of 7 meals/day. A weekly intake of at least 3 portions of wholegrain pasta, 3-4 portions of legumes, 3 portions of fatty fish and 6 fruits/vegetables portions was mandatory. Group B diets were characterized by a macronutrient content of 55/30/15 (carbohydrate/lipid/ protein) distributed in 3-5 meals/day. During the first phase of the study, subjects

received dietary counseling by qualified nutritionists every 15 day. Figure 3 shows the experimental design of the study.

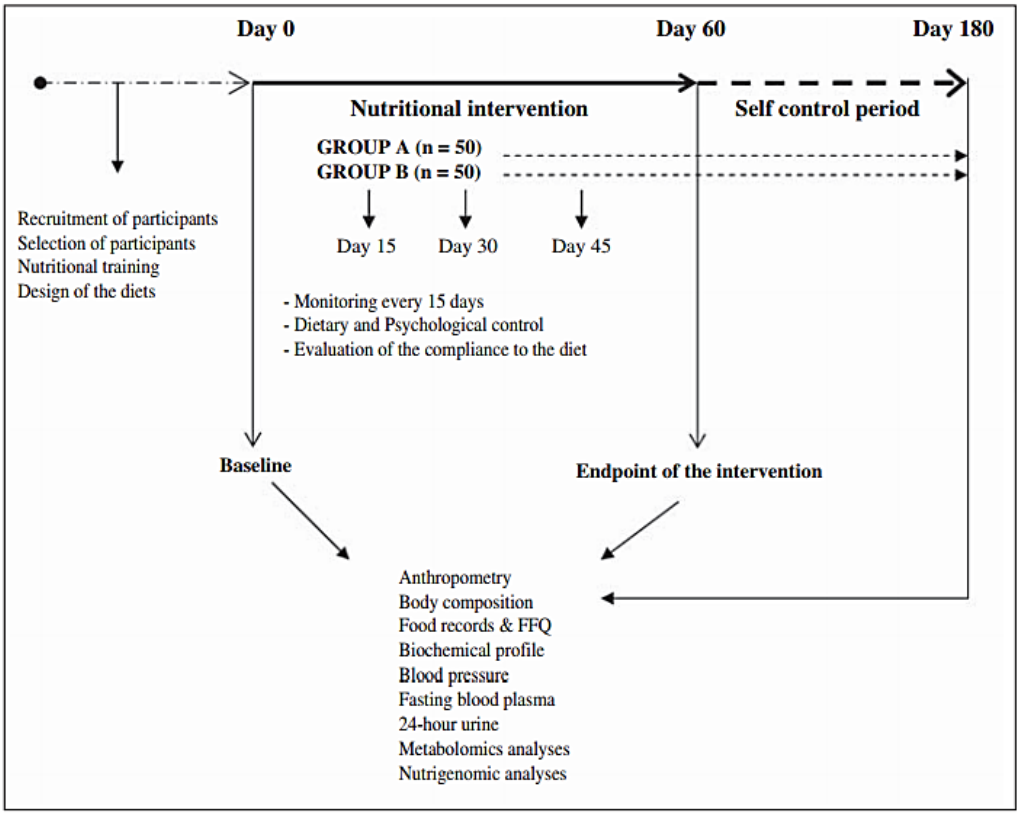

Figure 3. Experimental design of the RESMENA-S study. Reproduced from Zulet, M.A. (2011)

Screening visit

Recruited participants will attend a screening visit in which they received a written document with information about the study together with the informed consent to be signed. Both documents were approved by the Research Ethics Committee of the University of Navarra. During this visit, any doubt concerning the participation of the subject in the study was solved by qualified staff. After a medical examination by a physician, anthropometric parameters and blood pressure will be measured and a fasting blood sample was drawn by a nurse for the biochemical determination of metabolic syndrome clinical features. The subject will be asked to fill in a validated questionnaire concerning food frequency and dietary habits (SUN questionnaire) and a 72-hour food

record, data that together with the calculation of the basal metabolic rate of the subject was used by the dietitian to design their personalized diets (TOLEDO, E. *et al.*, 2010).

Study visits

Visits were planned for each subject in the course of the study at days 0, 15, 30, 45, and 60. Details of the determinations and measurements for each of them are given in table III. In addition to the intervention visits, participants from group A were requested to attend an informative group session to reinforce psychological attitude at the beginning of the study. In this session, two qualified nutritionists explained the benefits of the dietary pattern to follow in the study, the options to create their own personalized menus with the established personalized diets, as well as emphasized the importance of eating habits and compliance during both the controlled and self-control phases of the study. At the end of the study, participants received a report about the evaluation of their nutritional status in a last dietary counseling visit with the dietitian.

Table III
Determinations and measurements to be performed during the study visits

Visit day	*0*	*15*	*30*	*45*	*60*	*180*
Body weight	x	x	x	x	x	x
Blood pressure (SBP, DBP)	x				x	x
Waist and hip circumference	x				x	x
Skin-folds[1]	x				x	x
Body composition by bioimpedance	x	x	x	x	x	x
Body composition by DEXA	x				x	x
Collection of fasting blood[2]	x				x	x
24-h urine collection	x				x	x
Fasting plasma glucose	x				x	x
Fasting plasma insulin	x				x	x
Free fatty acids	x				x	x
Cholesterol, HDL-Chol, LDL-Chol	x				x	x
Total proteins	x				x	x
Inflammatory markers[3]	x				x	x
Oxidative-stress markers[4]	x				x	x
Metabolomics analyses[5]	x				x	x
Gene expression analyses[6]	x				x	x
Dietary counseling	x	x	x	x	x	x
72 hour-weight food record	x	x	x	x	x	x
VAS questionnaire					x	
State-Trait Anxiety Inventory (STAI)	x				x	x
Beck depression Inventory	x				x	x
Anxiety thermometer	x	x	x	x	x	x

Reproduced from Zulet, M.A. (2011)

5.2 Study of DNA methylation in apparently healthy adults

This study was conducted by analyzing data and blood samples previously collected as part of the project entitled "Evaluation of oxidative and inflammatory status of a university population: possible association with the development of metabolic syndrome" registered (No.50703155347) in 09/05/2007, at the Universidade Federal de Viçosa.

Subjects and methods

Recruitment, Inclusion and exclusion criteria

This was a cross-sectional study where one hundred sixty-one volunteers were recruited from the city of Viçosa, Minas Gerais, Brazil. Initial screening excluded subjects with evidence of any diagnosed organic underlying disease (gastrointestinal, kidney, liver, respiratory or heart disease), cancer, infectious and inflammatory disorders, diabetes (fasting glucose level > 126 mg/dl), hypertension (systolic and diastolic blood pressure values ≥ 140 and 90 mmHg, respectively), pregnancy, disorders affecting body composition (e.g. lipodystrophy and Cushing syndrome) or lipid-lowering treatment. Other exclusion criteria were contraceptive use up to 2 months before participation in this study, recent follow up of diets designed for weight loss or unstable weight (change > 10% in habitual weight) in the past 6 months. In accordance with the principals of the Helsinki Declaration and after a clear explanation of the study protocol, each participant gave a written informed consent to participate. The study was approved by the Committee of Ethics in Research with Human Beings of the Universidade Federal de Viçosa (Of. Ref. n° 009/2006 and 019/2011).

Anthropometric and body composition assessments

Height and Body weight were measured according validated protocols and body mass index (BMI) was calculated (BARBOSA, K.B. *et al.*, 2014). Waist and hip

circumferences were measured with an inelastic and flexible tape. Triceps, biceps, subscapular and suprailiac skinfold thicknesses were measured by using a skinfold caliper. The sum of skinfold thickness was calculated. Total body fat percentage was measured to the nearest 0.1% using a body composition analyzer (Biodynamics 310 model, Washington, USA). Body fat mass and body fat-free mass were estimated using the same body composition analyzer. Truncal fat percentage was computed as the sum of subscapular and suprailiac skinfold thicknesses divided by the sum of four skinfold measurement (WARNBERG, J. *et al.*, 2006). Finally, truncal adiposity index was calculated by the ratio of subscapular to triceps skinfold thickness (MORENO, L.A. *et al.*, 2001).

Blood pressure assessment

Systolic and diastolic blood pressures were measured with a mercury sphygmomanometer (BIC, SP, Brazil) following World Health Organization criteria (WHITWORTH, J.A. *et al.*, 2004).

Dietary intake and lifestyle assessments

A seventy-two-hour food record was used to collect information about energy and nutrient intake (BARBOSA, K.B. *et al.*, 2014). A booklet was given to the participants to record everything they ate or drank over a period of three non-consecutive days, including a weekend day. Dietary intake was computed using specific software (DietPro®, version 5.0, AS Systems). Covariates about diet and lifestyle, such as vitamin supplementation, smoking status (smokers or non-smokers), number of cigarettes per day, regular physical activity (yes or no) and volume of physical activity, were also collected. To quantify the volume of physical activity, an activity metabolic equivalent (MET) was used (AINSWORTH, B.E. *et al.*, 2000). This index represents

the ratio between energy expenditure during each specific activity and resting metabolic rate.

Analyses of biological samples

Blood samples were drawn by vein puncture after a 12-hour overnight fast. Ethylenediaminetetraacetic acid (EDTA) plasma, heparin plasma, serum, erythrocytes and, PBMC samples were separated from whole blood by centrifugation (ULMER, A.J. *et al.*, 1984). All samples were immediately stored at -80°C until assay.

6. RESULTS

6.1 CHAPTER 1 - Published Original Paper • Nutrition

Expression of inflammation-related miRNAs in white blood cells from subjects with metabolic syndrome after 8 wk of following a Mediterranean diet–based weight loss program.

Nutrition 32 (2016) 48–55

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Applied nutritional investigation

Expression of inflammation-related miRNAs in white blood cells from subjects with metabolic syndrome after 8 wk of following a Mediterranean diet–based weight loss program

José Luiz Marques-Rocha B.Sc. [a,b,c], Fermin I. Milagro Ph.D. [b,c,d], Maria Luisa Mansego Ph.D. [b,c], Maria Angeles Zulet Ph.D. [b,c,d], Josefina Bressan Ph.D. [a], J. Alfredo Martínez M.D., Ph.D. [b,c,d,*]

[a] Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil
[b] Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain
[c] Center for Nutrition Research, University of Navarra, Pamplona, Spain
[d] CIBERobn, Physiopathology of Obesity, Carlos III Institute, Madrid, Spain

ARTICLE INFO

Article history:
Received 15 May 2015
Accepted 18 June 2015

Keywords:
microRNA
miR-130a
Inflammation
miR-132-3p
Let-7b
miR-155

ABSTRACT

Objectives: The aim of this study was to evaluate the influence of a dietary strategy for weight loss (the RESMENA [reduction of metabolic syndrome in Navarra, Spain] diet) on the expression of inflammation-related microRNAS (miRNAs) and genes in white blood cells (WBC) from individuals with metabolic syndrome (MetS).

Methods: The clinical, anthropometric, and biochemical characteristics of 40 individuals with MetS (20 men and 20 women; age: 48.84 ± 10.02 y; body mass index: 35.41 ± 4.42 kg/m^2) were evaluated before and after an 8-wk hypocaloric diet based on the Mediterranean dietary pattern. Nutrient intake was assessed with a food frequency questionnaire and 48-h weighed food records. Total RNA was isolated from WBC and the expression of some inflammation-related miRNAs and mRNAs (*IL-6, TNF-α, ICAM-1, IL-18, SERPINE1, VCAM-1, GAPDH*) was assessed by quantitative polymerase chain reaction.

Results: The RESMENA nutritional intervention improved most anthropometric and biochemical features. The expression of miR-155-3p was decreased in WBC, whereas Let-7b was strongly upregulated as a consequence of the dietary treatment. However, they were not correlated with the expression of the proinflammatory genes in the same cells. The changes in the expression of let-7b, miR-125b, miR-130a, miR-132-3p, and miR-422b were significantly associated with changes in diet quality when assessed by the Healthy Eating Index. Moreover, low consumption of lipids and saturated fat (g/d) were associated with higher expression of let-7b after the nutritional intervention.

Conclusions: The Mediterranean-based nutritional intervention was able to induce changes in the expression of let-7b and miR-155-3p in WBC from patients with MetS after 8 wk. Moreover, the quality of the diet has an important effect on the miRNAs expression changes. These results should be highlighted because these miRNAs have been associated with inflammatory gene regulation and important human diseases.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. JLM-R and MLM contributed to the analysis and the writing of the manuscript. JB was involved in the fieldwork as well as in the critical reading of the manuscript. MAZ, JAM, and FIM were responsible for general coordination, follow-up, design, financial management, and the editing of the manuscript. All of the authors actively participated in manuscript preparation, and they all read and approved the final manuscript. The authors have declared that no competing interests exist.

* Corresponding author. Tel.: +34 94 842 5600; fax: +34 94 842 5619.
E-mail address: jalfmtz@unav.es (J. A. Martínez).

http://dx.doi.org/10.1016/j.nut.2015.06.008
0899-9007/

Introduction

Metabolic syndrome (MetS) is a complex disorder defined by a cluster of interconnected cardiometabolic alterations, including hypertension, insulin resistance, dyslipidemia, and abdominal obesity [1]. Individuals exhibiting combinations of these metabolic disturbances have a substantial synergistic cardiovascular risk greater than the sum associated with each abnormality [2]. Furthermore, the pathologic enlargement of the adipose tissue in obesity leads to an elevated production of proinflammatory mediators [3]. These altered signals mediate multiple processes, which predispose to diabetes mellitus, hepatic steatosis, atherosclerosis, plaque rupture, and atherothrombosis [1]. However, to date, the available information is controversial and does not necessarily imply a causal role neither putatively involve epigenetic mechanisms.

Data obtained from functional genomic methodologies indicate that several hundred genes participate in the inflammatory response and that their coordinated expression is tightly controlled [4]. Hence, microRNAs (miRNAs) are small noncoding RNAs that have the ability to control multiple genes, establishing an orchestrated network governing remote processes through intertwined pathways [5]. Several studies have highlighted the significance of miRNAs in maintaining metabolic homeostasis, and the regulation of these miRNAs could serve as a potential therapeutic target for metabolic disorders [6]. For example, the inflammatory toll-like receptor/nuclear factor-κB–related miR-181a is downregulated in monocytes of obese patients, which is associated with a higher number of MetS components and with coronary artery disease [7]. In this sense, miRNA expression contributes to regulate body weight, sometimes in relation to inflammation regulation [8]. Thus, some of these transcripts could be used as prognostic biomarkers of response to hypocaloric diets.

The aim of this study was to evaluate the effect of a weight loss strategy based on the Mediterranean dietary pattern (the RESMENA [reduction of metabolic syndrome in Navarra, Spain] diet) on anthropometric measurements, biochemical markers, and the expression of some selected inflammation-related genes and miRNAs in white blood cells (WBC) of individuals with MetS. We hypothesized that a hypocaloric pattern, as well as the improvement of the overall diet quality components designed to reduce MetS features, could have a positive effect on the expression of genes and miRNAs related to the inflammatory state. We sought to further explore the possible epigenetic underlying mechanisms and the putative interactions with the diet.

Methods

Study population

Forty white individuals (20 men and 20 women) with a body mass index (BMI) of 35.41 ± 4.42 kg/m^2, aged 48 ± 10 y, and diagnosed with MetS according to the International Diabetes Federation cutoffs [9] were enrolled in this study. The inclusion and exclusion criteria have been previously reported [10], but it should be pointed out that individuals with presence of psychiatric disturbances, eating disorders, chronic diseases related to the metabolism of nutrients, major body weight changes in the previous 3 mo, and difficulties in changing food habits were excluded. Volunteers were recruited through local newspaper advertisements and the database of Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain. Calculations were based on findings of previous studies [10,11]. The study protocol was performed in accordance with the ethical guidelines of the Declaration of Helsinki, and was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009). All individuals provided written informed consent also approved by the same research ethics committee.

Study protocol

The research is based on a subsample of the RESMENA-S study [10], a controlled intervention study that aimed to reduce MetS features based on energy restriction over 6 mo [10,11] and with components of the Mediterranean diet to specifically combat MetS features. The complete project is registered at www.clinicaltrials.gov (NCT01087086) and can be accessed elsewhere [10]. The diet of the RESMENA study prescribed an energy restriction of 30% applied to the total energy requirements of each patient. Resting metabolic rate was calculated using the Harris–Benedict equation, in which the Wilkens-adjusted weight was applied [12]. The physical activity factor was considered to calculate the total energy requirements according to the Food and Nutrition Board, National Research Council: Recommended Dietary Allowances [13]. Fasting blood samples, habitual dietary intake, and body composition were measured at baseline and at the end point of the 8-wk intervention period following standardized protocols, as published elsewhere [10,12].

Diet and dietary assessments

Participants were provided a 7-d menu plan as previously described [14]. The plan was composed of seven meals per day, including breakfast, lunch, dinner, two snacks in the morning, and two more snacks in the afternoon. Some characteristics of the RESMENA diet were a moderately high protein intake (24.6% ± 2.8%), higher daily average intake (seven meals daily), and increased total antioxidant capacity (TAC) than the usual recommendations. Moreover, the dietary advice included a cholesterol content <300 mg and focused on low glycemic index and glycemic load (GL) carbohydrate meals [10,12].

Dietary intake was assessed with a semiquantitative 136-item food frequency questionnaire previously validated in Spain for energy and nutrient intake [14]. A 48-h weighed food record was required at the beginning and at the end of the study. Diet composition was analyzed using the DIAL software (Alce Ingenieria, Madrid, Spain). The amount of eicosapentaenoic fatty acid and docosahexaenoic fatty acid was obtained through the DIAL software to estimate ω-3 fatty acid intake. The Healthy Eating Index (HEI) was calculated using the DIAL software as described elsewhere [15]. The program gives different values between 0 and 100 considering the servings per day of cereals, vegetables, fruits, dairy products, and meat. Also, this score takes into account the percentage of energy provided by total and saturated fats, the amount of cholesterol and sodium daily, and the variety of the diet. The final value was classified into five categories: >80 points indicates “excellent diet”; 71 to 80 points, a “very good diet”; 61 to 70 points, a “good diet”; 51 to 60, an “acceptable diet”; and 0 to 50 points, an “inadequate diet.” Dietary TAC was calculated using a list that takes into account raw or cooked food preparations. The dial software provides a list of the total antioxidant content (mmol/100 g) of >3100 foods, beverages, spices, herbs, and supplements used worldwide. The TAC value corresponding to the different scheduled/ingested servings per day was calculated [16]. Finally, GL was obtained from the updated international database published online by the Human Nutrition Unit, School of Molecular Biosciences, University of Sydney [17].

Anthropometric, clinical, and biochemical assessments

Anthropometric measurements (body weight, height, waist and hip circumferences) were carried out with the individuals in their underwear using validated processes [10]. Body fat was measured by dual energy x-ray absorptiometry (Lunar iDXA, software version 6.0, Madison, WI, USA) as described elsewhere [10]. Blood pressure was recorded with a standard mercury sphygmomanometer (Minimus II, Riester, Junginger, Germany). Measurements were taken three times after a 5-min resting period, following World Health Organization criteria [18].

Venous blood samples were drawn by venipuncture after a 12-h overnight fast. The ethylenediamine tetraacetic acid plasma and serum samples and WBCs were separated from whole blood by centrifugation at 3500 g at 5°C for 15 min (Model 5804 R, Eppendorf, Germany) and were frozen immediately at −80°C until assay (WBC in buffy coat). Glucose, total cholesterol (TC), high-density lipoprotein cholesterol, triacylglycerols, and acid uric serum concentrations were measured in an autoanalyzer Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits. Insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). Insulin resistance was estimated by the homeostasis model assessment (HOMA) index {HOMA-IR = [glucose (mmol/L) × insulin (μU/mL)]/22.5} [19]. Low-density lipoprotein cholesterol (LDL-C) levels were calculated with the Friedewald formula: LDL-C = TC−high-density lipoprotein cholesterol−very-low-density lipoprotein [20]. Plasma malondialdehyde (MDA) was calorimetrically determined with a commercial kit (BIOXYTECH LPO-586, Oxis Research, Portland, OR, USA). Plasma-oxidized LDL-C levels were measured using a capture-ELISA kit from Mercodia (Uppsala, Sweden). Plasma concentrations of plasminogen activator inhibitor (PAI)-1 (BioVendor, Germany), interleukin (IL)-6 (R&D Systems, Minneapolis, MN), tumor

necrosis factor (TNF)-α (R&D Systems) and high-sensitivity C-reactive protein (Demeditec, Germany) were measured using ELISA kits and an automated analyzer system (Triturus, Grifols, Barcelona, Spain). In our laboratory, the inter- and intra-assay variability were <10% for all analytical determinations.

RNA extraction, miRNA, and mRNA quantitative PCR

At baseline and at the end of the study, total RNA was extracted from WBC using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined at 260/280 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

For the miRNA analysis, 20 ng of total RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies, Foster City, CA, USA) according to the manufacturer's protocol and the miRNA-specific reverse-transcription primers provided with the TaqMan MicroRNA Assay (Life Technologies), as shown in Table 1. The miRNA-specific cDNA was amplified in triplicate with the TaqMan Universal polymerase chain reaction (PCR) master mix and the respective specific probe provided in the TaqMan MicroRNA Assay (Life Technologies).

To perform the mRNAs analyses, 2 μg of total RNA was reverse-transcribed using the high-capacity complementary DNA (cDNA) reverse transcription (RT) kit (Life Technologies) according to the manufacturer's protocol. cDNA was amplified in triplicate with the TaqMan Universal PCR master mix and the respective specific probe provided in the TaqMan Gene Expression Assays (Life Technologies; Table 1). The analyzed miRNAs and genes were selected on the basis of previous studies strongly supporting their possible involvement in inflammatory pathways related to MetS features.

miRNA and mRNA levels were normalized to the endogenous controls, *RNU48* and *GAPDH* (glycerol-3-phosphate dehydrogenase), respectively. The ΔΔCT method was used for quantification and the fold changes are reported as $2^{-\Delta\Delta CT}$ [21]. All quantitative real-time RT-PCR measurements were performed using a 7900 HT Fast Real-Time PCR system (Life Technologies).

Statistical analyses

A group size of 40 was estimated to be necessary to obtain a significant ($P < 0.05$) difference in the reduction of waist circumference of 4.3 ± 6.8 cm with a power of 80% [10]. Only those individuals who completed the study were analyzed. The results are expressed as mean ± SD. Normality distributions of the measured variables were determined according to the Shapiro–Wilk test. Differences between the beginning and the end of the intervention period were analyzed by a paired *t* test. Spearman correlation and linear regression analyses were applied to assess the potential relationships and associations among diet, biochemical, and anthropometrical components with miRNA and mRNA variation. Analyses were carried out using SPSS 15.1 software for Windows (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Table 1
References of the specific PCR primers/probes used for the measurement of the miRNAs and mRNAs

miRNAs	TaqMan MicroRNA Assay reference
Let-7b	002619
miR-125b	000449
miR-130a	000454
miR-132-3p	000457
miR-146a	000468
miR-155-3p	002287
miR-223-5p	002098
miR-422b	001314
miR-4772- p	464414
RUN18	001006
Genes (mRNAs)	**TaqMan assay reference**
IL-6	Hs00985639_m1
TNF-α	Hs01113624_g1
ICAM-1	Hs00164932_m1
IL-18	Hs01038788_m1
SERPINE1	Hs01126606_m1
VCAM-1	Hs01003372_m1
GAPDH	Hs02758991_g1

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; IL, interleukin; miR, microRNA; PCR, polymerase chain reaction; SERPINE, serine protease inhibitor, member E; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule

Results

All clinical, laboratory, and dietary characteristics of study participants at the beginning and at the end of the dietary intervention are shown in Table 2. After 8 wk, the RESMENA diet improved most anthropometric and biochemical markers included in this study (Table 2). There was a significant reduction of all variables related to body weight, such as BMI, waist circumference, and fat mass ($P < 0.05$). There was also a significant reduction of TC, triacylglycerols, glucose, and insulin levels and, consequently, of the HOMA index ($P < 0.05$). Moreover, the circulating levels of MDA and PAI-1 were lower when compared with the levels at baseline ($P < 0.05$).

Table 2
Anthropometric, biochemical, and dietary characteristics of the participants at baseline and after 8 wk on the RESMENA Diet

Variables	Baseline	8 wk	*P* value*
	Mean ± SD	Mean ± SD	
Anthropometric measurements (N = 40)			
Body weight (kg)	99.7 ± 16.5	92.6 ± 15.8	**<0.01**
BMI (kg/m^2)	35.4 ± 4.4	32.8 ± 4.2	**<0.01**
Waist circumference (cm)	110.9 ± 12.1	103.2 ± 10.1	**<0.01**
Waist/hip ratio	0.96 ± 0.10	0.92 ± 0.09	**<0.01**
DXA total fat mass (%)	42.6 ± 6.0	40.1 ± 6.6	**<0.01**
DXA gynoid fat mass (kg)	7.96 ± 1.74	7.33 ± 1.66	**<0.01**
DXA android fat mass (kg)	3.93 ± 0.94	3.72 ± 0.87	**0.02**
DXA truncal fat mass (kg)	26.73 ± 5.18	25.92 ± 5.03	**<0.01**
Blood pressure (N = 40)			
SBP (mm Hg)	147.39 ± 20.87	134.10 ± 16.07	**<0.01**
DBP (mm Hg)	84.43 ± 9.01	76.69 ± 9.07	**<0.01**
Biochemical parameters (N = 40)			
Total cholesterol (mg/dL)	219 ± 45	202 ± 45	**<0.01**
HDL-C (mg/dL)	43 ± 10	41 ± 9	0.06
LDL-C (mg/dL)	137 ± 39	131 ± 39	0.29
Ox-LDL (U/L)	44.3 ± 18.5	40.2 ± 13.9	0.11
TG (mg/dL)	196 ± 124	150 ± 100	**<0.01**
Glucose (mg/dL)	124 ± 38	109 ± 25	**<0.01**
Insulin (μU/mL)	14.18 ± 8.12	8.95 ± 5.97	**<0.01**
HOMA index	4.46 ± 3.01	2.49 ± 1.82	**<0.01**
Uric acid (mg/dL)	5.82 ± 1.38	5.94 ± 1.06	0.49
Inflammatory markers (N = 40)			
MDA (μM)	0.84 ± 0.36	0.74 ± 0.28	**<0.01**
CRP (mg/L)	3.30 ± 3.45	3.53 ± 5.75	0.71
IL-6 (pg/mL)	2.63 ± 1.73	2.76 ± 1.58	0.53
PAI-1 (pg/mL)	157 ± 127	144 ± 152	**<0.01**
TNF-α (pg/mL)	0.69 ± 0.50	0.88 ± 0.94	0.99
Dietary characteristics (n = 35)			
Energy intake (kcal/d)	2255 ± 287	1353 ± 559	**<0.01**
Meal frequency (meals/d)	5.35 ± 1.30	6.47 ± 1.08	**<0.01**
Proteins (% TCV/d)	17.1 ± 2.8	24.5 ± 2.8	**<0.01**
Lipids (% TCV/d)	44.4 ± 6.0	37.8 ± 7.0	**<0.01**
CHO (% TCV/d)	37.9 ± 5.9	37.4 ± 6.4	0.64
Fiber (g/d)	18.3 ± 10.2	20.2 ± 8.5	0.07
GI (U/d)	663 ± 217	332 ± 124	**<0.01**
GL (U/d)	112 ± 44	53 ± 26	**<0.01**
Saturated fatty acids (g/d)	34.3 ± 13.2	16.1 ± 5.7	**<0.01**
ω-3 fatty acids (g/d)	0.33 ± 0.16	0.34 ± 0.04	0.06
TAC (mmol/d)	8.32 ± 4.74	13.89 ± 5.21	**<0.01**
Healthy Eating Index (U)	55.9 ± 11.8	71.4 ± 10.2	**<0.01**

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; DXA, dual-energy x-ray absorptiometry; GL, glycemic load; HDL-C, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; GI, glycemic index; LDL-C, low-density lipoprotein cholesterol; MDA: malondialdehyde; ox-LDL, oxidized low-density lipoprotein cholesterol; RESMENA, reduction of metabolic syndrome in Navarra, Spain; SBP, systolic blood pressure; TG, tricylglycerols; TCV, total caloric value

Bolded *P* values indicate statistically significant (less than 0.05).

* *P* value: comparison by paired samples statistics from baseline (Student's *t* test).

The dietary characteristics of the participants changed in accordance with the recommendations provided by the RESMENA diet. There was a lower intake of energy, lipids, and saturated fatty acids ($P < 0.05$). On the other hand, meal frequency (meals/day), protein intake, and TAC of the diet were higher ($P < 0.05$) at the end of the nutritional intervention (Table 2). These changes subsequently improved ($P < 0.05$) the HEI.

The analysis of the expression of proinflammatory genes in WBC showed no relevant changes ($P > 0.05$) as a result of the 8-wk nutritional intervention (Table 3). However, the expression of miR-155-3p decreased ($P = 0.007$), whereas let-7b was strongly upregulated ($P = 0.002$) as a consequence of the dietary intervention (Table 3). However, they were not correlated with the expression of the proinflammatory genes analyzed. Interestingly, the expression of let-7b was highly correlated ($P < 0.05$) with the other miRNAs analyzed, both at baseline (miR-130a, miR-132-3p, miR-146a, miR-155-3p, miR-223-5p, miR-422b, and miR-4772-3p) or after the RESMENA diet (miR-125b, miR-130a, miR-146a, miR-223-5p, miR-422b, and miR-4772-3p; Table 4).

The changes (8 wk versus baseline) in the miRNA expression levels were statistically associated with the changes in the diet quality, when assessed by the HEI. The improvement of HEI positively correlated with the changes in the expression of some miRNAs (Table 5) such as let-7b ($r^2 = 0.478$; $P = 0.008$), miR-125b ($r^2 = 0.359$; $P = 0.047$), miR-130a ($r^2 = 0.546$; $P = 0.001$), miR-132-3p ($r^2 = 0.551$; $P = 0.001$), and miR-422b ($r^2 = 0.374$; $P = 0.042$). Some of these significant correlations were found even after adjustment for energy restriction, sex, and age: let-7b ($\beta = 0.425$; $P = 0.035$), miR-130a ($\beta = 0.469$; $P = 0.009$), and miR-132-3p ($\beta = 0.543$; $P = 0.003$; Table 6). Furthermore, the miR-155 expression was associated with changes in the HEI and weight loss in a regression model adjusted for sex and age ($\beta = 0.577$; $P = 0.014$ and $\beta = 0.740$; $P = 0.003$, respectively; Table 6).

Table 3
Relative expression (fold change) of some miRNAs and genes (mRNAs) at baseline and after 8 wk on the RESMENA diet

Relative expression	Baseline	8-wk	*P* value*
(N = 40)	Mean ± SD	Mean ± SD	
miRNAs†			
Let-7b	1.04 ± 0.63	1.51 ± 0.90	**<0.01**‡
miR-125b	1.22 ± 0.84	1.33 ± 0.72	0.70
miR-130a	1.29 ± 0.75	1.52 ± 0.86	0.76
miR-132-3p	0.54 ± 0.48	0.51 ± 0.25	0.20
miR-146a	0.57 ± 0.23	0.58 ± 0.25	0.83
miR-155-3p	0.89 ± 0.49	0.59 ± 0.27	**<0.01**‡
miR-223-5p	0.82 ± 0.50	0.89 ± 0.47	0.26
miR-422b	0.70 ± 0.43	0.74 ± 0.36	0.13
miR-4772-3p	2.60 ± 0.59	2.78 ± 0.27	0.56
mRNA†			
IL-6	0.62 ± 0.47	1.05 ± 0.86	0.20
TNF-α	1.01 ± 0.86	1.53 ± 1.25	0.08
ICAM-1	2.21 ± 1.79	1.92 ± 1.64	0.66
IL-18	2.30 ± 2.16	1.54 ± 1.32	0.21
SERPINE1	5.59 ± 4.15	4.74 ± 2.96	0.91
VCAM-1	5.21 ± 4.62	3.02 ± 2.32	0.30

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor; ICAM, intercellular adhesion molecule; PCR, polymerase chain reaction; RESMENA, reduction of metabolic syndrome in Navarra, Spain; SERPINE, serine protease inhibitor, member E; VCAM, vascular cell adhesion molecule
Bolded *P* values indicate statistically significant (less than 0.05).
* *P* value: comparison by paired samples after log transformation (Student's *t* test).
† miRNA and mRNA expression were measured by quantitative real-time PCR and normalized by *RNU48* and *GAPDH*, respectively.
‡ Different from baseline.

Table 4
Spearman correlations between the relative expression of Let-7b and other miRNAs* analyzed at baseline and after 8 wk on the RESMENA diet

Let-7b (N = 40)*	Baseline		8 wk	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
miR-125b	0.290	0.09	0.360	**0.02**†
miR-130a	0.702	**<0.01**†	0.636	**<0.01**†
miR-132-3p	0.002	0.99	0.025	0.88
miR-146a	0.049	0.77	0.425	**<0.01**†
miR-155-3p	−0.435	**0.03**†	−0.159	0.41
miR-223-5p	0.605	**<0.01**†	0.674	**<0.01**†
miR-422b	0.441	**<0.01**†	0.404	**<0.01**†
miR-4772-3p	0.380	**0.02**†	0.632	**<0.01**†

PCR, polymerase chain reaction; RESMENA, reduction of metabolic syndrome in Navarra, Spain
Bolded *P* values indicate statistically significant (less than 0.05).
* miRNAs were measured by quantitative real-time PCR and normalized by *RNU48*.
† $P < 0.05$.

Individually analyzing the components of HEI, it was evident that intakes of lipids and saturated fatty acids (g/d) were negatively associated with the expression of let-7b ($r^2 = -0.443$; $P = 0.014$ and $r^2 = -0.432$; $P = 0.017$, respectively; Fig. 1), suggesting that the improvement in HEI, in part as consequence of the low consumption of lipids and saturated fat intake (g/d), could be implicated in the increased expression of let-7 b in WBC.

Discussion

In the present study, the effects of a novel dietary strategy on the expression levels of miRNAs and genes related to the inflammatory process in WBC were reported. To our knowledge, this is a pioneer study in patients with MetS evaluating the effects of an energy-restricted intervention based on Mediterranean diet principles, including a modified macronutrient distribution and increased meal frequency, as well as the

Table 5
Spearman correlations between changes in the healthy eating index and changes in the relative expression of the miRNAs and genes analyzed*

Δ healthy eating Index (*n* = 35)	Correlation coefficient	*P* value
Δ Let-7b	0.478	**<0.01**†
Δ miR-125b	0.359	**0.04**†
Δ miR-130a	0.546	**<0.01**†
Δ miR-132-3p	0.551	**<0.01**†
Δ miR-146a	−0.026	0.88
Δ miR-155- p	0.414	0.12
Δ miR-223-5p	0.023	0.90
Δ miR-422b	0.374	**0.04**†
Δ miR-4772-3p	−0.238	0.20
Δ IL-6	−0.258	0.16
Δ TNF-α	−0.059	0.75
Δ ICAM-1	0.058	0.74
Δ IL-18	0.153	0.41
Δ SERPINE1	0.020	0.91
Δ VCAM-1	−0.049	0.81

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; IL, interleukin; PCR, polymerase chain reaction; SERPINE, serine protease inhibitor member; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule
Bolded *P* values indicate statistically significant (less than 0.05).
* Changes (8 wk vs baseline) in miRNA and gene expression were measured by quantitative real-time PCR and normalized by *RNU48* and *GAPDH*, respectively.
† $P < 0.05$.

Table 6
Regression coefficients between changes in the healthy eating index and changes in the relative expression of some miRNAs analyzed (N = 35)*

Linear regression models	ANOVA test	β-coefficient	*P* value
Δ miR-130a			
Δ HEI	0.024†	0.469	**0.009**†
Energy restriction		0.039	0.858
Age		−0.028	0.866
Sex		0.337	0.053
Δ miR-132-3p			
Δ HEI	0.025†	0.543	**0.003**†
Energy restriction		0.234	0.170
Age		−0.038	0.817
Sex		0.133	0.432
Δ Let-7b			
Δ HEI	0.025†	0.410	**0.024**†
Energy restriction		−0.191	0.276
Δ miR-155			
Δ HEI	0.007†	0.577	**0.014**†
Weight loss		0.740	**0.003**†
Age		−0.313	0.094
Sex		−0.020	0.922

ANOVA, analysis of variance; HEI, Healthy Eating Index; PCR, polymerase chain reaction
Bolded *P* values indicate statistically significant (less than 0.05).
* Changes (8 wk vs baseline) in miRNA expression were measured by quantitative real-time PCR and normalized by *RNU48*.
† $P < 0.05$.

presence of bioactive ingredients, such as fiber and beneficial fatty acids, and controlling glycemic index and GL, dietary TAC, and HEI score.

Metabolic diseases are characterized by the failure of regulatory genes or enzymes to effectively orchestrate specific pathways involved in the control of many biological processes [2]. In addition to the classical regulators of metabolic homeostasis, recent discoveries have evidenced the putative remarkable role of miRNAs in the post-transcriptional regulation of a number of genes, and their involvement in many pathologic states, such as the MetS features [22]. However, results from human studies are still unclear and more research is needed to find links between miRNAs and the pathways involved.

The main results of the current study were the expression changes of miRNA-155 and let-7b, especially the latter. Let-7 family was, after lin-4, the second miRNAs found and has nine members, namely, let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, and miR-98. All members are believed to exert similar functions because they share a common seed region (nucleotides 2–8), which mediates miRNA interaction with target mRNAs [23]. Previous studies have identified let-7 as a tumor suppressor, which is downregulated or even lost in many human cancers [24], regulating multiple cellular processes including cell division and DNA repair pathways. More recently, the role of let-7 in the physiopathology of some diseases has received significant attention. Furthermore, aberrant let-7 expression has been associated with a variety of human diseases including cardiovascular events, liver fibrosis, and lung disorders. Moreover, it has been demonstrated that let-7b regulates both atherogenic and adipogenic phenomena. Biostatistical tests and network analyses suggest that let-7b could act as a global synergistic key mediator controlling hundreds of protein-coding genes [25]. Interestingly, let-7b expression had a high correlation with all of the miRNAs analyzed at baseline or at the end of the period studied. A recent work suggested that let-7b could be a strong candidate as a master regulator of products on coding and non-coding genes related with ovarian carcinoma [25]. Other publications have provided new insights into the protective properties of let-7b in preventing the endothelial dysfunction associated with cardiovascular disease [26]. Circulating let-7b levels were lower in patients with acute myocardial infarction than in healthy adults [27]. Additionally, overexpression of let-7b promoted nitric oxide production [26], which is considered a key factor in vascular protective actions including vasodilatation. Although this study did not imply a direct action, we found that blood pressure was lower at the end of the dietary intervention period.

Global knockdown of the let-7 family with an antimiR was sufficient to prevent and treat impaired glucose tolerance in mice with diet-induced obesity, at least in part by improving insulin sensitivity in liver and muscle [23]. In the same study, antimiR treatment of mice on a high-fat diet also resulted in increased lean and muscle mass, but not increased fat mass, and prevented ectopic fat deposition in the liver. These findings demonstrate that let-7 regulates multiple aspects of glucose metabolism and suggests antimiR-induced let-7 knockdown as a potential treatment for type 2 diabetes.

In this trial, the expression of miR-155 was lower at the end of the nutritional intervention and was associated with the quality of the diet and with the weight loss by regression analysis. These results suggest that miR-155 could be a prognostic marker of obesity, as advanced by previous studies. In this context, some authors have shown that the expression of miR-155 was higher in diet-induced obese rats than in control animals [28]. In the same study, a 30% calorie restriction led to significant reduction in the expression of miR-155. Recent findings identified miR-155 as a central apigenin-regulated miRNA in inflammation and provided evidence of the underlying mechanism by which apigenin and diets rich in apigenin, a flavonoid abundant in parsley and celery, contribute to restore homeostasis [29]. Additionally, quercetin decreased lipopolysaccharide (LPS)-induced expression of miR-155 in macrophages [30], highlighting the benefits of dietary interventions as a strategy to restore proper immune function in vivo.

Overexpression of miR-155 has been reported in lung, breast, and colon tumors [31] and has been positively associated with intestinal inflammation and ulcerative colitis [32]. Furthermore, in vivo studies have found that transgenic mice overexpressing miR-155 in B-cell lineage (Eμ-miR-155) produced more TNF-α when challenged with LPS and were hypersensitive to LPS/D-galactosamine–induced septic shock with respect to their normal counterparts [33]. Interestingly, miR-155 targets the lipid phosphatase SHIP1 [34], an important signal for macrophage activation. The exposure of cultured macrophages to LPS leads to upregulation of miR-155, which targets the CCAAT/enhancer binding protein β (*C/EBPβ*) mRNA, implicated in the regulation of proinflammatory cytokines during macrophage activation and the acute-phase response [35].

To evaluate the quality of the diet, some indices or scores have been developed, such as the HEI, the Alternate HEI, or the Diet Quality Index and derivatives [16]. Most of these tools take into consideration the Mediterranean diet guidelines, widely recognized as a healthy pattern [36]. They consist of a single score that results from computing different components, such as foods, food groups, or a combination of foods and nutrients. In this context, the HEI score was selected because it takes into account macro- and micronutrient intake, as well as food variety. The improvement of HEI score due to the 8-wk RESMENA intervention was related to changes in the expression of let-7b, miR-125, miR-130, miR-132, and miR-422. Additionally, the consumption of total and saturated fat inversely correlated with the HEI and

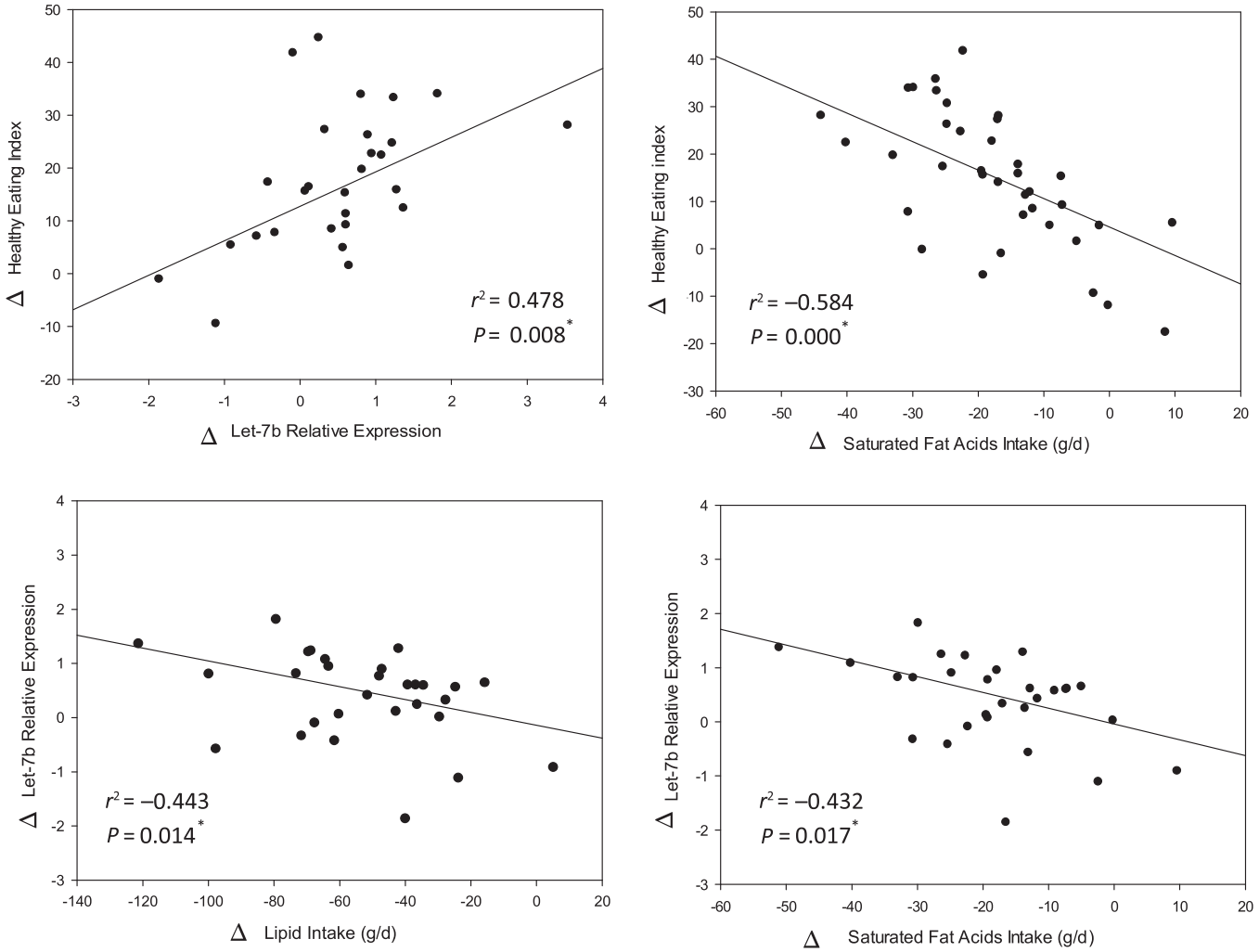

Fig. 1. Correlations between the changes in the Healthy Eating Index (HEI) and the relative expression of let-7b and changes in the intake of lipids and saturated fat (g/d). Spearman correlations; $^{*}P < 0.05$; Changes (8 wk vs baseline); n = 39 to HEI and n = 30 to let-7b relative expression analysis.

let-7b expression. Our regression analyses also indicated that the expression of these miRNAs was more dependent on the quality of the diet than on the weight loss achieved or the energy restriction. To our knowledge, this was the first time that these associations were described in the scientific literature. It has been previously mentioned that the diet may influence the expression of some miRNAs, which has been considered useful in the assessment of nutritional status in dietary intervention studies. For example, miR-125b has been reported to regulate in a negative manner the expression of the vitamin D receptor (*VDR*) [37]. This functional link between *VDR* expression and miR-125b might explain why *VDR* expression is upregulated, whereas miR-125b is downregulated in distinct types of cancer as the genomic locus that contains miR-125b is deleted in several cancers [37]. On the other hand, miR-125b has been reported to post-transcriptionally regulate vitamin D_3 hydroxylase (*CYP24*), an enzyme that catalyzes the inactivation of vitamin D; the low expression of miR-125b in cancer tissues may be a possible mechanism for explaining the high *CYP24* expression in cancer tissues [38]. The influence of nutrients on the expression of other miRNAs is still unclear, being a new promising area of nutrigenomic research.

In this study, MDA and PAI-1 levels decreased significantly after the intervention. High levels of plasma MDA, a biomarker of lipid peroxidation, have been associated with type 2 diabetes [39], whereas calorie-restricted dietary strategies have been previously associated with lower MDA levels [40]. In this sense, a recent publication revealed that Let-7b overexpression inhibited reactive oxygen species production [26]. In this context, it can be hypothesized that the increased expression of let-7 at the end of the intervention may contribute to decrease reactive oxygen species production and lipid peroxidation.

PAI-1, encoded by the *SERPINE1* gene, is the principal inhibitor of tissue plasminogen activator and urokinase, and therefore is an inhibitor of fibrinolysis [41]. This serine protease is produced by vascular endothelium, liver, monocytes/macrophages, platelets, and adipose tissue [42]. High plasma levels of PAI-1 have been associated with higher risk for cardiovascular diseases [43]. Furthermore, PAI-1–dependent mechanisms are also implicated in the pathogenesis of obesity, insulin resistance, and type 2 diabetes [44]. In fact, increased PAI-1 levels can be considered a component of MetS. Clinical studies have demonstrated a strong correlation between PAI-1 and BMI and fibrinolysis. As elevated levels of inflammatory cytokines could increase PAI-1

expression, the link between obesity and elevated PAI-1 levels could be the low-grade inflammatory state [45]. Although the RESMENA dietary strategy was successful for weight loss and the improvement of most of the anthropometric and biochemical parameters, including PAI-1, the circulating levels of other inflammatory biomarkers, such as CRP, TNF-α, and IL-6, were not reduced.

The study has some limitations. First, gene expression varies depending on the tissue or cell type [22], and it is possible that specific miRNAs are not widely expressed in human WBC. Second, this study specifically aimed to evaluate the association between the variables included and conclusions on causality cannot be measured. Also, the number of participants in this study is not very high, but it may be proposed that type 2 errors were overcome because important statistical differences were found. In the analysis of before and after responses in a single group, confounding by measured or unmeasured factors that are potentially time varying across the time periods compared remains a critical threat. However, we used statistical methods adjusting for measured confounding factors and quantifying the potential effect of unmeasured factors on effect estimates. Finally, despite limitations of structured questionnaires of this type, the advantage of food frequency questionnaire is its feasibility for establishing long-term habitual dietary intake [46].

Conclusion

The results of this study provide evidence that the quality of a Mediterranean-based nutritional intervention was related to changes in the expression of let-7b and miR-155-3p in WBC from patients with MetS after 8 wk. These results should be highlighted because these miRNAs have been associated with important human diseases linked to MetS, such as cancer, atherogenic and adipogenic processes, and other inflammatory conditions.

Acknowledgments

The authors acknowledge the volunteers of this study and the excellent technical assistance of Ana Lorente and the dietitians Rocio de la Iglesia, Aurora Perez-Cornago, and Patricia Lopez-Legarrea. They also acknowledge the Spanish Ministry of Economy and Competitiveness (AGL2013-45554-R project), the CAPES Foundation (Ministry of Education of Brazil, Brasília–DF 70040-020, Brazil) for providing JLM-R with a research grant (process n° 6409-13-0), Instituto de Salud Carlos III (CIBERobn) and CNPq Foundation (Brazil) for financial support.

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6.2 CHAPTER 2

Overexpression of miR-155-3p upregulates IL-6 but reduces TLR-4 expression in THP-1 cells.

José Luiz Marques-Rocha B.Sc.[a], **Marcos Garcia-Lacarte** B.Sc.[b], **Mírian Samblas** B.Sc.[b], **Josefina Bressan** Ph.D.[a], **J. Alfredo Martínez** M.D. Ph.D[b,c,d,*], **Fermin I. Milagro** Ph.D[b,c].

Author details:

[a] Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil
[b] Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain
[c] CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Health Institute, Madrid, Spain
[d] IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors have declared that no competing interests exist.

Authors' contributions:

JLM-R, ML, and MS were involved in the design of the study, performance of the experiments, data analysis, and manuscript writing. JB participated in the experimental design and data analysis. JAM and FIM conceived the study, and were involved in financial support, experimental design, data analysis, and final approval of the manuscript. All the authors read and approved the manuscript.

***Corresponding author:**
J. Alfredo Martínez
Address: c / Irunlarrea 1, 31008, Pamplona (Navarra, Spain)
Department of Nutrition, Food Science and Physiology; Center for Nutrition Research
University of Navarra
E-mail: jalfmtz@unav.es
Tel: 948425600 (ext: 806424)

ABSTRACT

Objectives: The main goal of the current study was to clarify the roles of miR-155-3p and let-7b on the expression of inflammation-related genes in white blood cells. A second goal was to investigate the regulatory role of pro and anti-inflammatory fatty acids (FAs) on the expression of these miRNAs in monocytes, macrophages and LPS-activated macrophages (AcM).

Methods: Human acute monocytic leukemia cells (THP-1) were differentiated into macrophages and activated with LPS for 24 hours. The three cell types were transfected with miR-Let-7b-5p and miR-155-3p mimics or mirVana® miRNA mimic negative control. Quantitative real time PCR was performed to analyze selected genes with possible involvement in inflammatory pathways related to miR-155 and Let-7b.

Results: The transfection of miR-155-3p mimic led to upregulation of IL6 in the three cell types. In the same way, SERPINE1 was upregulated in monocytes and macrophages. However, TLR-4 was downregulated in transfected monocytes and macrophages. After transfection with let-7b mimic, TNF/IL6 and SERPINE1 expression were downregulated in monocytes and AcM, respectively; however, TNF, IL6 and SERPINE1 were upregulated in macrophages. Oleic acid was able to increase the expression of miR-155 in monocytes when compared with the DHA treatment but not in relation with non-treated cells. On the other side, oleic acid increased the expression of Let-7b in macrophages and AcM .

Conclusions: Overall, these findings indicate that miR-155-3p and Let-7b play pro- and anti-inflammatory roles in THP-1 cells, respectively. However, these effects depend on the cell type. Moreover, oleic acid has beneficial properties in non-activated and LPS-activated macrophages by increasing Let-7b expression.

Keywords: Let-7b, inflammation, epigenetics, SERPINE1, monocyte, macrophage+

Institution where study was performed:

Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, C/ Irunlarrea, 31008, Pamplona, Navarra, Spain

ABBREVIATIONS

AMPK: AMP-activated protein kinase

FAs: Fatty acids

g: gram

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

IL: Interleukin

IκB: Inhibitor of κB

IRAK: IL-1 receptor-associated kinase

LPS: Lipopolysaccharide

miRNA: microribonucleic acid

MUFAs: Monounsaturated Fatty Acid

NF-K\κB: Nuclear factor κB

PAI = Plasminogen activator inhibitor

PUFAs: Polyunsaturated fatty acids

qRT-PCR: Quantitative real-time PCR

SFAs: Saturated fatty acids

SERPINE1: Serine protease inhibitor gene

SIRT: sirtuin

SMAD2 = SMAD family member 2

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor alpha

TRAF6: TNF receptor-associated factor 6

UFV: Federal University of Viçosa

UTR: Untranslated region

Introduction

MicroRNAs (miRNAs) are short non-coding RNAs that modulate physiological and pathological processes by inhibiting target gene expression through the blockade of protein translation or by inducing mRNA degradation (Marques-Rocha, Samblas et al. 2015). Recent findings have revealed critical functions for miRNAs in several cellular and biological processes, including proliferation, differentiation, and development, as well as in the regulation of genes relevant to many inflammation-related diseases (Marques-Rocha, Samblas et al. 2015). The first report linking miRNA with immune responses came from miRNA expression profiling performed in a monocytic cell line treated with the lipopolysaccharide (LPS), a toll-like receptor (TLR) 4 ligand (Taganov, Boldin et al. 2006). Currently, more than 100 miRNAs are known to be expressed by cells of the immune system, and they have the potential to broadly influence the molecular pathways that control the development and function of immune responses (O'Connell, Rao et al. 2010).

Specific miRNAs, including miR-155 and Let-7b, were initially linked with the inflammatory response by virtue of their potent up-regulation in multiple immune cell lineages by TLRs ligands, inflammatory cytokines (e.g. TNF, IL-6), and specific antigens (O'Connell, Taganov et al. 2007). miR-155 has been implicated in the differentiation and activation of cells of both the adaptive and the innate immune systems (O'Connell, Rao et al. 2010). There is extensive evidence supporting miR-155 as a negative regulator of innate immune or inflammatory response (Gottwein, Mukherjee et al. 2007; Ceppi, Pereira et al. 2009). On the contrary, some studies have also shown that miR-155 may also have a pro inflammatory role (Jin, Kim et al. 2014; Woodbury, Freilich et al. 2015). In the same way, let-7b, a regulator of developmental timing and cell proliferation, is able to mediate immune responses and adjust inflammation (Bao, Feng et al. 2013). Actually, we screened the targets of let-7b by

using Targetscan (version 6.0, www.targetscan.org) and found that *TLR4*, serine proteinase inhibitors (*SERPINE1*) and *TNF* were putative target genes of this miRNA. However, the influence of let-7b on the downstream gene expression in monocytes and macrophages has not been understood. In addition, the regulatory role of these miRNAs on the expression of some inflammation-related genes remains unknown.

In a recent publication of our group, a Mediterranean-based nutritional intervention was able to induce changes in the expression of let-7b and miR-155-3p in white blood cells (WBC) from patients with metabolic syndrome after 8 weeks (Marques-Rocha, Milagro et al. 2016). Moreover, a low consumption of lipids and saturated fat were associated with higher expression of let-7b after the nutritional intervention (Marques-Rocha, Milagro et al. 2016). In this sense, recent studies have analysed the role of different miRNAs on the pathogenesis of diseases associated with high-fat diets and different types of fatty acids (Garcia-Segura, Perez-Andrade et al. 2013). Interestingly, it has been reported that the adult offspring of mothers fed a high-fat diet before conception presented reduced levels of some miRNAs (e.g. let-7 family) during pregnancy and lactation, suggesting that an epigenetic mechanism is involved in explaining how dietary induced changes in gene expression is maintained until adulthood (Zhang, Zhang et al. 2009). However, to our knowledge there are no record about recognized anti and proinflammatory fatty acids (FAs) in the expression of the miRNA-155-3p and Let-7b.

In the present study, therefore, we aimed to clarify the potential regulatory roles of miR-155 and let-7b on inflammation-related genes (*IL6*, *TNF*, *SERPINE1* and *TLR4*) in human acute monocytic leukemia cells (THP-1). Furthermore, we investigated the influence of different FAs on the expression of these miRNAs in monocytes, macrophages and LPS-activated macrophages (AcM).

Material and methods

Cell culture

Human acute monocytic leukemia cells (THP-1) were obtained from the American Type Culture Collection (ATCC, TIB-202, USA) and maintained in culture in RPMI-1640 (ATCC, 30-2001, USA) supplemented with 2 mM of L-glutamine, 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4500 mg/L of glucose, 1 mM of sodium pyruvate, 1500mg/L of sodium bicarbonate, 100 U/ml penicillin-streptomycin (GIBCO, Life Technologies, CA, USA) and 10% fetal bovine serum (GIBCO, Life Technologies, CA, USA) at 37ºC in a 5% CO_2 humidified atmosphere. Cells were grown at a density of 4 x 10^5 cells/ml as recommended by the ATCC. Then, for the differentiation into macrophages, monocytic THP-1 cells were incubated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA) for 48 hours at 37^{o}C in a 5% CO2 incubator. Thereafter, macrophages were activated with 200 ng of LPS for 24 hours. Finally, the cells were examined and photographed with a confocal imaging system (Olympus FV1200).

mirVana® miRNA mimic transfection

Cells in the three differentiation states were transfected with either mirVana® miR-Let-7b-5p mimic, mirVana® miR-155-3p mimic or mirVana® miRNA mimic negative control (Applied Biosystems, CA, USA) using Lipofectamine® RNAiMAX Transfection Reagent (Catalog Number 4464070, Applied Biosystems, CA, USA) according to manufacturer's protocol. The BLOCK-iT Alexa Fluor Red Fluorescent Oligo control was transfected into THP-1 cells using Lipofectamine® RNAiMAX Transfection Reagent to assess transfection efficiency (See Figure S1 at supplementary file).

Treatment with fatty acids

THP-1 monocytes, macrophages and LPS-activated macrophages were incubated with a concentration of 100 µM of palmitic acid, oleic acid, eicosapentaenoic acid (EPA) and docosahesaenoic acid (DHA). The palmitic acid, EPA and DHA were dissolved in ethanol and oleic acid was dissolved in endotoxin free water, and then mixed with Bovine Serum Albumin in a 1:2 (BSA: free fatty acid) molar ratio such was previously described (Gonzalez-Muniesa, P et al., 2011). Then, the fatty acids were added to RPMI-1640 medium supplemented with a 10% of Fetal Bovine Serum and incubated during 30 hours at 37^{o}C in a 5% CO_2 humidified atmosphere. After the incubation, the cell pellet was collected for the analysis.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from THP-1 cells using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined at 260/280nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). cDNA was synthesized from 1 µg of total RNA using random primers and MultiScribeTM MMLV reverse transcriptase (Applied Biosystems, CA, USA). For mature miRNAs, 20 ng of RNA were retrotranscribed by using the Taqman MicroRNA RT kit (Applied Biosystems, CA, USA) and miRNA-specific primer sets supplied by the manufacturer (See Table S1 at supplementary file). Quantitative real time PCR was performed with the ABI prism 7900HT Sequence Detection System (Applied Biosystems, CA, USA). The analyzed genes were selected on the basis of previous studies supporting their possible involvement in inflammatory pathways related to miR-155 and Let-7b. β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were used as an internal reference for mRNAs analysis and U6 and U48 expression were used as control miRNAs. All assays were performed in triplicate. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method to determine the relative quantitative level and was expressed as a

fold-difference to the relevant control (Vandesompele, De Preter et al. 2002). Thus, the amount of target, normalized to endogenous gene and relative to the control is given by $2^{-\Delta\Delta Ct}$ [ΔCt = Ct (target gene) – Ct (endogenous gene); ΔΔCt = ΔCt for any sample - ΔCt for the control].

Statistical analysis

Results were expressed as mean values plus or minus SEM or SD and interpreted by repeated-measure ANOVA. Analyses were carried out using REST 2009 (Relative Expression Software Tool), SPSS 15.1 software for Windows (SPSS Inc, Chicago, IL, USA) and GraphPad Prism® version 5.0 (La Jolla, CA, USA) was used to show graphically the results. Differences were considered to be statistically significant when the p value was less than 0.05.

Results

Considering the basal expression, activated macrophages (AcM) presented higher values of TNF, IL6 and SERPINE1 than the other cell types ($p<0.05$ for all) after normalization by the respective negative control (See at supplementary file, Figure S2). In the same way, macrophages expressed more TLR4 than monocytes and AcM ($p<0.05$ for all). Moreover, macrophages presented higher expression of SERPINE1 but lower values of TNF than the monocytes ($p<0.05$ for all) (Figure S2).

When compared with the respective negative control groups, the relative levels of let-7b were increased after overexpression with Let-7b mimic 23.5 times in monocytes, 121.7 times in macrophages and 421.4 times in AcM (Figure 1A). In the same way, the transfection with miRNA-155 mimic increased the expression of this miRNA 30.1 times in monocytes, 193.7 times in macrophages and 508.9 times in AcM (Figure 1B).

After transfection with let-7b, TNF and IL6 expression were downregulated in monocytes (in comparison to negative control group) by a mean factor of 0.505 (S.E.

range=0.257 – 0.724; p=0.014) and 0.327 (S.E. range is 0.207 – 0.503; p<0.001), respectively (Figure 2). Differently, the transfection with the same miRNA increased the expression of TNF, IL6 and SERPINE1 in macrophages (p<0.05). These results were not replicated in AcM. In addition, SERPINE1 was downregulated in AcM by a mean factor of 0.666 (S.E. range is 0.459 – 0.950; p=0.046) (Figure 2).

The transfection of miR-155 mimic led to the upregulation (in comparison to control group) of IL-6 in monocytes (mean factor= 1.439; S.E. range= 1.198 – 1.988; p=0.024), macrophages (mean factor= 2.122; S.E. range= 1.238 – 4.104; p=0.010) and AcM (mean factor= 2.175; S.E. range= 1.081 – 4.374; p=0.030) (Figure 3). In the same way, SERPINE1 was upregulated in monocytes and macrophages by a mean factor of 1.339 (S.E. range= 1.103 – 1.988; p=0.041) and 1.283 (S.E. range is 1.001 – 1.756; p=0.016), respectively. However, TLR-4 was downregulated in transfected monocytes and macrophages by a mean factor of 0.712 (S.E. range= 0.567 – 0.922; p=0.005) and 0.707 (S.E. range=0.506 – 0.988; p=0.002) (Figure 3).

The relative expression of Let-7b was inhibited in monocytes as a result of the treatment with DHA (mean factor= 0.671 ± 0.106; p=0.030) when compared with the negative control (Figure 4). However, Let-7b was upregulated when monocytes were treated with EPA and compared with the DHA treatment (p=0.030). In macrophages and AcM, the expression of Let-7b was higher as a result of the treatment with oleic acid (7.610 ± 0.633 and 5.266 ±1.283, respectively; p<0.05 for both). Moreover, palmitic acid increased the expression of this miRNA in AcM when compared with the non-treated and PUFA (DHA or EPA)-treated cells (p<0.05). Concerning miR-155, oleic acid was able to increase the expression of this miRNA in monocytes when compared with the DHA treatment (p<0.05) but not in relation with non-treated cells (Figure 5). Moreover, the treatment with EPA increased the expression of miR-155 in macrophages (3.379 ± 0.432; p<0.001).

Discussion

miRNAs play an important role in the gene regulation network. It has been established that miRNAs participate in regulating 30–100% of proteins by modulating the expression levels of specific genes (Garzon, Calin et al. 2009). Accumulating studies have focused on the effect of miRNAs in immune system and found that noncoding RNAs contribute to the tight regulation of the inflammatory process (Marques-Rocha, Samblas et al. 2015). In this sense, FAs may act as either pro- or anti-inflammatory agents depending on the dose and chemical structure (Volpe and Nogueira-Machado 2013) and have been found to alter the expression of specific miRNAs (Garcia-Segura, Perez-Andrade et al. 2013). Thus, we have analysed the regulatory role of two important miRNAs in the context of the inflammatory process and the effects of different FFAs in the expression of these miRNAs.

In mammalian immune system cells, TLRs recognize pathogen-associated molecules or pathogen-associated molecular patterns (PAMPs) and hence play a pivotal role in the innate immunity (Ozinsky, Underhill et al. 2000). TLR4 is one of the most intensely studied. It is expressed in a variety of cell types including blood cells, especially in professional antigen presenting cell such as dendritic cells, monocytes and macrophages (Zeuner, Bieback et al. 2015). In our study, overexpression of miR-155 was able to reduce significantly the expression of TLR4 in monocytes and macrophages. To the best of our knowledge, this is the first work that shows this negative relationship. It has been previously reported that let-7b mediates TLR4 expression via post-transcriptional regulation and inhibits NF-kB activity through MyD88 dependent pathway (Teng, Wang et al. 2013). However, surprisingly, we did not find differences in TLR4 expression in Let-7b transfected cells. A similar result was reported by Teng et al. in gastric epithelial cell lines; the treatment with let-7b mimics or let-7b inhibitors caused reciprocal modification of TLR4 protein expression with no significant alteration of

TLR4 mRNA expression (Teng, Wang et al. 2013). The inconsistent results suggest that, in different cell types, let-7 family may regulate TLRs in a different manner despite their high degree of homology. Moreover, contrary to their traditional roles as repressors of gene activation, recent evidence suggests that, in certain cases, some miRNAs can switch from repression to activation of protein translation during the cell cycle (Vasudevan, Tong et al. 2007). These findings emphasize the complexity of miRNA-mediated regulation of gene expression.

miR-155 is conserved across vertebrate species and is involved in the inflammatory response (Woodbury, Freilich et al. 2015). For example, it has been shown that miR-155 enhanced production of TNF and IL6 in peripheral blood-derived macrophages in humans (Kurowska-Stolarska, Alivernini et al. 2011). On the other hand, the suppression of TNF with an inhibitor results in a significant decline of miR-155 (Omran, Ashhab et al. 2013). Other reports have also shown a direct relationship between TNF and miR-155 in different diseases, such as epilepsy (Ashhab, Omran et al. 2013), B cell lymphomas (Pedersen, Otero et al. 2009), and septic shock (Tili, Michaille et al. 2007). However, we did not find significant relation between miR-155 and mRNA of TNF in THP-1 cells. Differences in the kinetics of expression between miR-155 and some of its targets have been previously reported, showing delays of 8–12 h (Zhou, Huang et al. 2010; Thounaojam, Kundu et al. 2014). These differences have been attributed to a combination of the rate of transcription, the rate of miR loading into the RISC complex, and the rate of mRNA decay (Hausser and Zavolan 2014).

In this context, several studies have observed that miR-155 is induced by LPS, cytokine IFN-β and various TLR ligands in murine macrophages (O'Connell, Taganov et al. 2007; Tili, Michaille et al. 2007). miR-155, once induced, is involved in the activation of TNF and IL6 by targeting the Fas-associated death domain protein, I B kinase ε, and receptor (TNF receptor superfamily)-interacting serine-threonine kinase 1 (Tili,

Michaille et al. 2007). It has been reported that monocytes and macrophages overexpressing miR-155 exhibit decreased SHIP-1 expression that may lead to increased production of pro-inflammatory cytokines (Pauley, Satoh et al. 2008). MiR-155 also plays a role in the innate immune response by regulating suppressor of cytokine signaling (SOCS)-1, a negative regulator of dendritic cell antigen-presenting capacity (Rodriguez, Vigorito et al. 2007; Tili, Michaille et al. 2007; Lu, Thai et al. 2009). All these findings are corroborated in this present work, either by miR-155 upregulation in LPS-activated macrophages or by the upregulation of proinflammatory genes in all transfected cell types. For example, miR-155 mimic also upregulated IL6 in monocytes, macrophages and AcM. In a recent study, a significant reduction in IL6 mRNA expression was found after LPS stimulation by knock-out (□15,820-fold reduction) or silencing (□15,968-fold reduction) of miR-155 compared with control microglial cells (Woodbury, Freilich et al. 2015). This result demonstrates that miR-155 is essential for robust IL6 expression in response to inflammatory challenges. However, how miR-155 increases IL6 expression is unknown. There are no predicted miR-155-binding sites in the 3-UTR of the IL6 mRNA transcript (Griffiths-Jones, Saini et al. 2008; Kozomara and Griffiths-Jones 2014). Meanwhile, SOCS1, which normally inhibits IL6/IL6ST (gp130) JAK/STAT3 signaling, is a validated murine and human miR-155 target (Betel, Wilson et al. 2008). In human microglia and macrophages, miR-155 also elicits IL6 induction, putatively via SOCS1 repression (Moore, Rao et al. 2013). SOCS1 repression enhances the IL6/IL6ST (gp130) JAK/STAT3 axis; STAT3 activation in turn activates transcription of IL6 and progliogenic genes including GFAP, skewing neural stem cells differentiation to the glial lineage (Peng, Sun et al. 2011).

Plasminogen activator inhibitor-1 (PAI-1), encoded by the *SERPINE1* gene, is well recognized as a proinflammatory and cardiovascular risk marker (Kohler and Grant 2000). In our study, SERPINE1 expression showed an increase in monocytes and

macrophages transfected with miR-155 mimic. These findings might be probably explained by a parallel effect of this miRNA on factors that control SERPINE1 expression. For example, SMAD2, known to regulate SERPINE1 (Gaspar, Li et al. 2007), is directly targeted by miR-155 (Martinez-Nunez, Louafi et al. 2009; Louafi, Martinez-Nunez et al. 2010). On the other hand, Let-7b was able to reduce the expression of SERPINE1 in LPS-activated macrophages but, controversially, it upregulated SERPINE1 in non-activated macrophages. According to bioinformatics tools such as Targetscan and microSniper, *SERPINE1* gene has predicted target binding sites for several miRNAs including Let-7 family. In a recent work, Let-7g improved multiple endothelial functions by inhibiting *in vitro* and *in vivo* SERPINE1 expression (Liao, Wang et al. 2014). In this same study, stroke patients with higher PAI-1 levels were associated with lower serum levels of Let-7g. The authors proposed the regulation of PAI-1 by SMAD2, who is regulated also by Let-7g, and reinforced the importance of other miRNAs such Let-7b on this process. miRNAs generally negatively control gene function; however, when an internal environmental factor changes, they may switch to positively regulating gene function. Thus, the contrasted expression of let-7b suggests that other mechanisms may be involved aside from microRNA/target gene. One possible explanation is that the treatment with phorbol 12-myristate 13-acetate/ionomycin (PMA) increases the phagocytic properties of the cells and induces a constitutive production of pro-inflammatory cytokines (Martinon, Petrilli et al. 2006). The action of PMA may have been influenced more than the inhibition by Let-7b, resulting in higher expression of other pro-inflammatory genes (*TNF* and *IL6*) as observed in macrophages (Figure 2). It is important to highlight that these properties also increased the efficiency of miRNAs transfection as shown in Figure 1. Moreover, pro-inflammatory miRNAs and genes are dramatically upregulated (approximately 80-fold) by LPS (Arango, Diosa-Toro et al. 2015) in agreement with other reports

(O'Connell, Taganov et al. 2007; Tili, Michaille et al. 2007; Ruggiero, Trabucchi et al. 2009) and our data (Figure S2 - Supplementary file). These events led to higher expression of Let-7b in LPS-activated macrophages than in non-activated macrophages. Therefore, we suggest than this elevated expression of Let-7b was powerful enough to reduce the expression of SERPINE1 in AcM when compared with the respective control group.

The inhibitory role of Let-7b (reducing TNF and IL6 expression in monocytes) is confirmed by other reports. Guo et al. reported that let-7b had differential expression patterns in inflamed tissues compared with healthy controls (Guo, Liu et al. 2015). The let-7b/TLR4 pathway serves as a potential contributor to cell polarization and inflammatory ablation. Further investigation of the mechanisms that control let-7b expression demonstrated that a TLR4/NF-κB/STAT3/AKT regulatory signaling pathway plays a critical role in the regulation of plasticity of the inflammatory system (Ti, Hao et al. 2015). Moreover, Let-7b was involved in the inflammation and immune response via regulating the activation of NF-kB and mediating the downregulation of IL6 (Li, Jia et al. 2012; Teng, Wang et al. 2013).

The ω-3 long-chain PUFAs DHA (22:6n–3) and EPA (20:5n–3) are the FAs most frequently associated with diverse health-promoting activities. Besides their anti-hypertriglyceridemic effects, they are usually considered as anti-inflammatory mediators (Volpe and Nogueira-Machado 2013). Indeed, it has been demonstrated that ω-3 PUFAs, including DHA, can modify the expression of miRNAs in cancer (Davidson, Wang et al. 2009; Mandal, Ghosh-Choudhury et al. 2012). In the present study, DHA treatment was able to decrease the expression of let-7b in monocytes. Moreover, EPA increased the expression of miR-155-3p in macrophages. One report also showed down-regulation of let-7 in rats after treatment with dietary fish oil (Davidson, Wang et al. 2009). However, we do not know whether the effects of omega

3 fatty acids on miRNAs expression are the result of a direct effect or are mediated by other metabolites arising from essential FAs. Examples of the latter are lipid mediators such as resolvins, lipoxins, protectins, and maresins, which are synthesized endogenously from essential FA precursors during inflammation (Serhan 2009). It has been reported that some of these proresolving lipid mediators can regulate the expression of genes involved in inflammation resolution by modulating specific miRNAs (Fredman and Serhan 2011; Recchiuti, Krishnamoorthy et al. 2011). On the other hand, conclusive evidence indicates that DHA (from fish oil) synergizes to enhance mitochondrial Ca^{2+} accumulation, thereby inducing apoptosis (Kolar, Barhoumi et al. 2007). Thus, elevated concentration of this FA may induce processes related with cellular death and influence indirectly the contradictory expression of let-7b and miR-155-3p.

Similarly to PUFAs, MUFAs, especially oleic acid, have been associated with anti-proliferative effects in cancer and decreased risk of CVD (Perez-Martinez, Garcia-Rios et al. 2011; Moon, Batirel et al. 2014). Moreover, high MUFA diet or replacement of SFAs by MUFAs improve abdominal fat distribution, insulin sensitivity (Summers, Fielding et al. 2002; Finucane, Lyons et al. 2015) and postprandial oxidative stress in patients with metabolic syndrome (Perez-Martinez, Garcia-Quintana et al. 2010). One mechanism involved might be the prevention by oleate of JNK-1/2 or NF-κB activation in response to TNF-α or palmitate (Perdomo, Beneit et al. 2015). In endothelial cells, oleate reduced MCP-1 and ICAM-1 and increased eNOS expression induced by proinflammatory cytokines. Furthermore, oleate impaired the proliferation induced by TNF, angiotensin II or palmitate and the apoptosis induced by TNF (Perdomo, Beneit et al. 2015). However, the molecular mechanism by which oleate exerts its protective role is not fully understood. It was observed that let-7f was induced by oleic acid in Caco-2 cells (Gil-Zamorano, Martin et al. 2014). This result was similar to that found for let-7b

in the present work: Let-7b levels were higher in AcM and macrophages treated with oleic acid. To our knowledge, this is the first time that these results are observed in monocytes and macrophages, providing evidence to better understanding the actions of oleic acid in the regulation of the expression of miRNAs.

On the other side, SFAs, such as palmitic acid, have been associated with adverse cardiovascular effects (Harvey, Walker et al. 2010; Shen, Eguchi et al. 2013). One of the mechanisms that may be involved in the deleterious effects of SFAs is that they can act as ligands for TLR and activate different signaling pathways involved in the inflammatory response (Lee, Zhao et al. 2004; Rocha, Caldas et al. 2016). Thus, it has been described that palmitate may induce insulin resistance in myotubes through TLR2, inducing activation of NF-κB, JNK and p38 (Senn 2006) and decreasing the levels of IκB-α (Perkins 2007). Palmitate also induces insulin resistance in muscle cells (Coll, Eyre et al. 2008) and other non-neuronal cell types (Yang, Qian et al. 2010). Nevertheless, oleate has superior protective effects against palmitate damage to neuronal cells, even compared to other PUFAs such as DHA (ω-3) and linoleate (ω-6) that have received wider clinical study (Kwon, Lee et al. 2014). However, other authors have described that both oleate and palmitate induced insulin resistance in primary hepatocytes, associated with the accumulation of dyacilglycerols and/or ceramide (Chabowski, Zendzian-Piotrowska et al. 2013). In our study, palmitic acid treatment increased the expression of let-7b only in LPS-activated macrophages.

This study presents some limitations that should be considered. Firstly, it remains unclear whether transfected miRNAs behave similarly to endogenous miRNAs. We cannot exclude the possibility that some of the observed changes in gene expression are indirect. Secondly, the supraphysiological levels of mature miRNAs and these artifactual RNA species may lead to non-specific changes in gene expression as speculated in a recent publication (Jin, Gonzalez-Martin et al. 2015). However, to

minimize the potential bias, a negative control with the same concentration of the miRNA mimic was used in all transfection experiments. Thirdly, we have not measured the secreted protein to the medium. Thus, is not conclusive that changes on mRNA expression influenced at the protein level.

Conclusions

To sum up, these findings indicate the pro- and anti- inflammatory roles of miR-155-3p and Let-7b on THP-1 cells, respectively. However, the effects depend on the cell type. Moreover, our data suggest that the modulation of the expression of miRNAs through a nutritional approach might be a future alternative or adjunct to current pharmacologic therapy targeting endogenous miRNAs. In addition, this study contributes to the understanding of how the fatty acids may act in inflammation-related immune cells.

Acknowledgments

This study was supported by research grant to JLM-R (CAPES Foundation, Ministry of Education of Brazil, PDSA process nº 6409-13-0), and CIBERobn and MINECO (ref. AGL2013-45554-R).

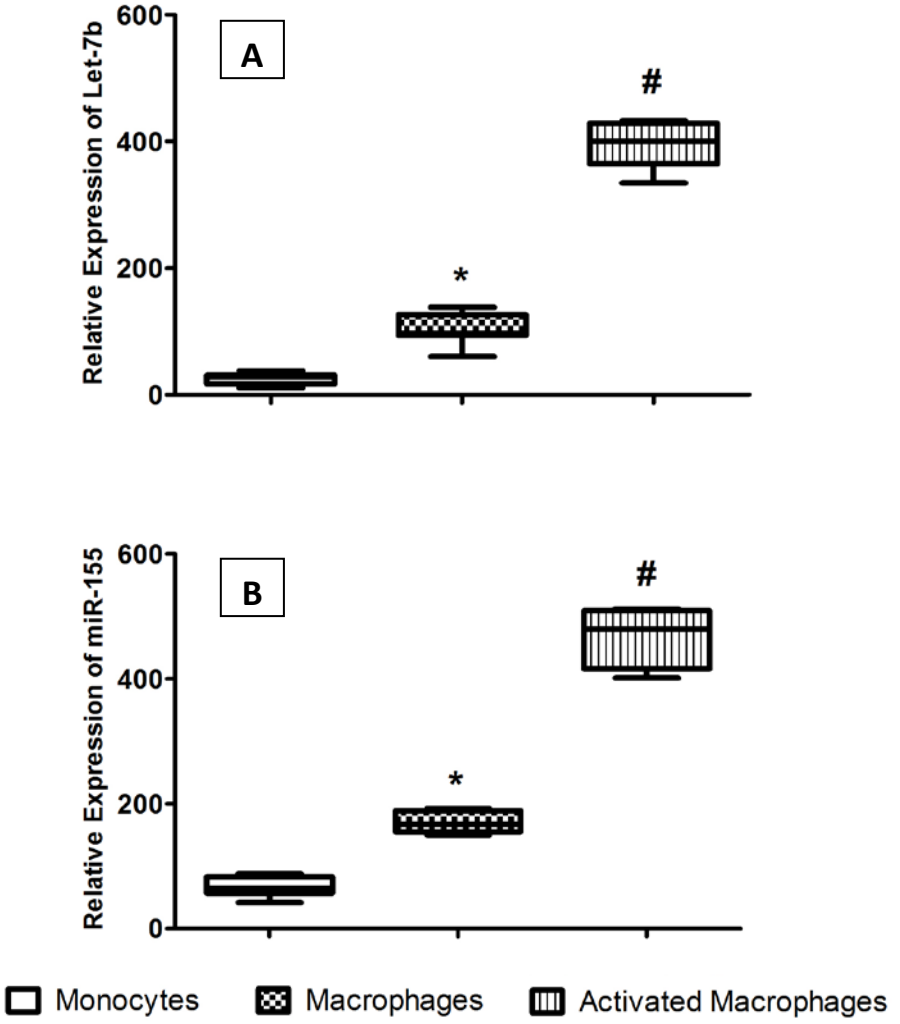


Figure 1. Expression of Let-7b (A) and miR-155-3p (B) in monocytes, macrophages and activated macrophages transfected with the respective miRNA (n=10) when compared with the respective negative control. Data are shown as the means ± standard error range. *P<0.05 when compared with monocytes; #P<0.05 when compared with other cell groups.

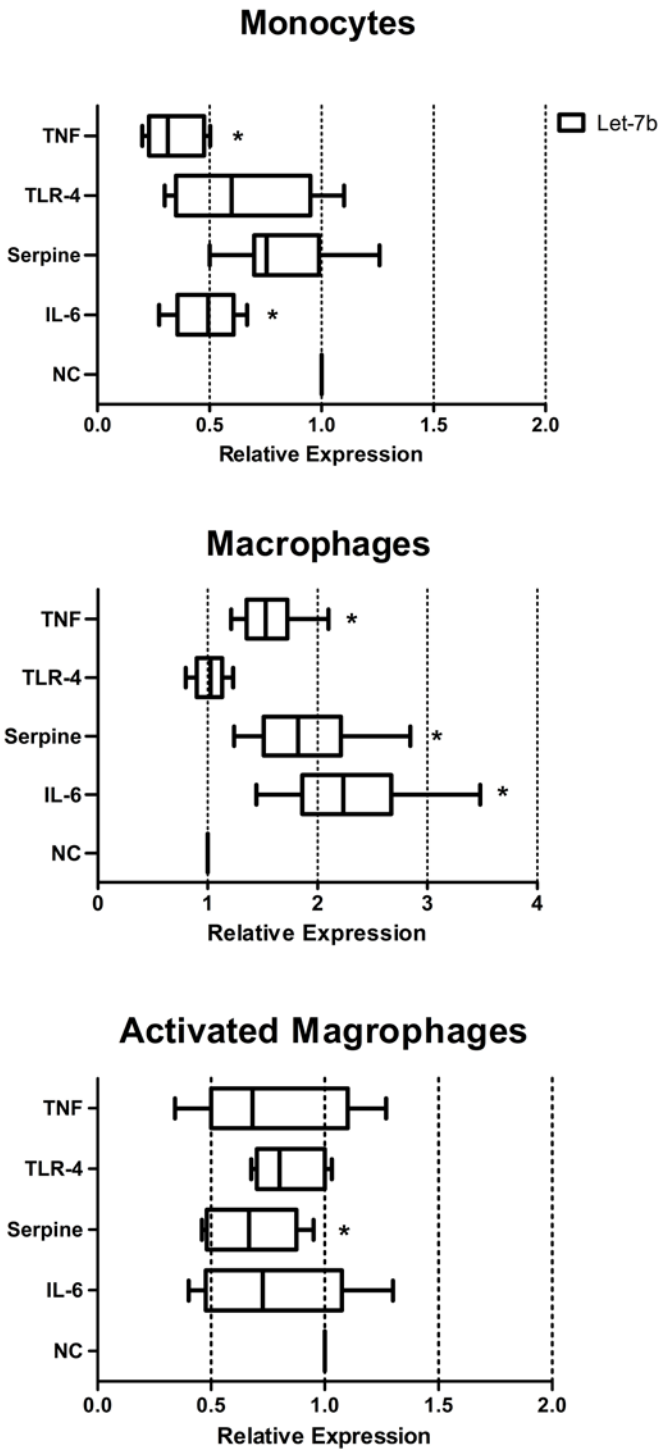


Figure 2. Relative expression of inflammation-related genes after transfection with Let-7b mimic in monocytes, macrophages and activated macrophages (n=8) compared with the respective negative control group (NC). Data are shown as the means ± standard error range.*P<0.05 when compared with the respective NC.

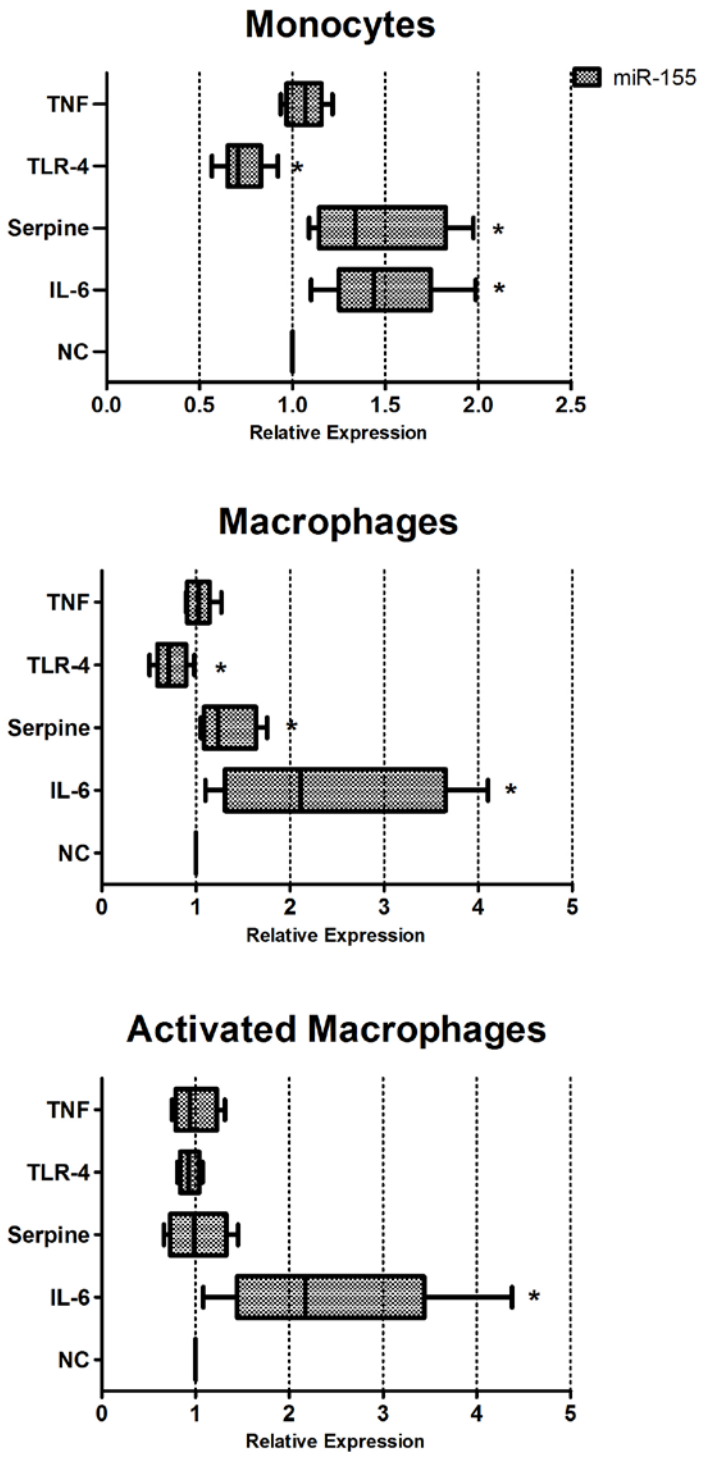


Figure 3. Relative expression of inflammation-related genes after transfection with miR-155 mimic in monocytes, macrophages and activated macrophages (n=8) compared with the respective negative control group (NC). Data are shown as the means ± standard error range.*P<0.05 when compared with the respective NC.

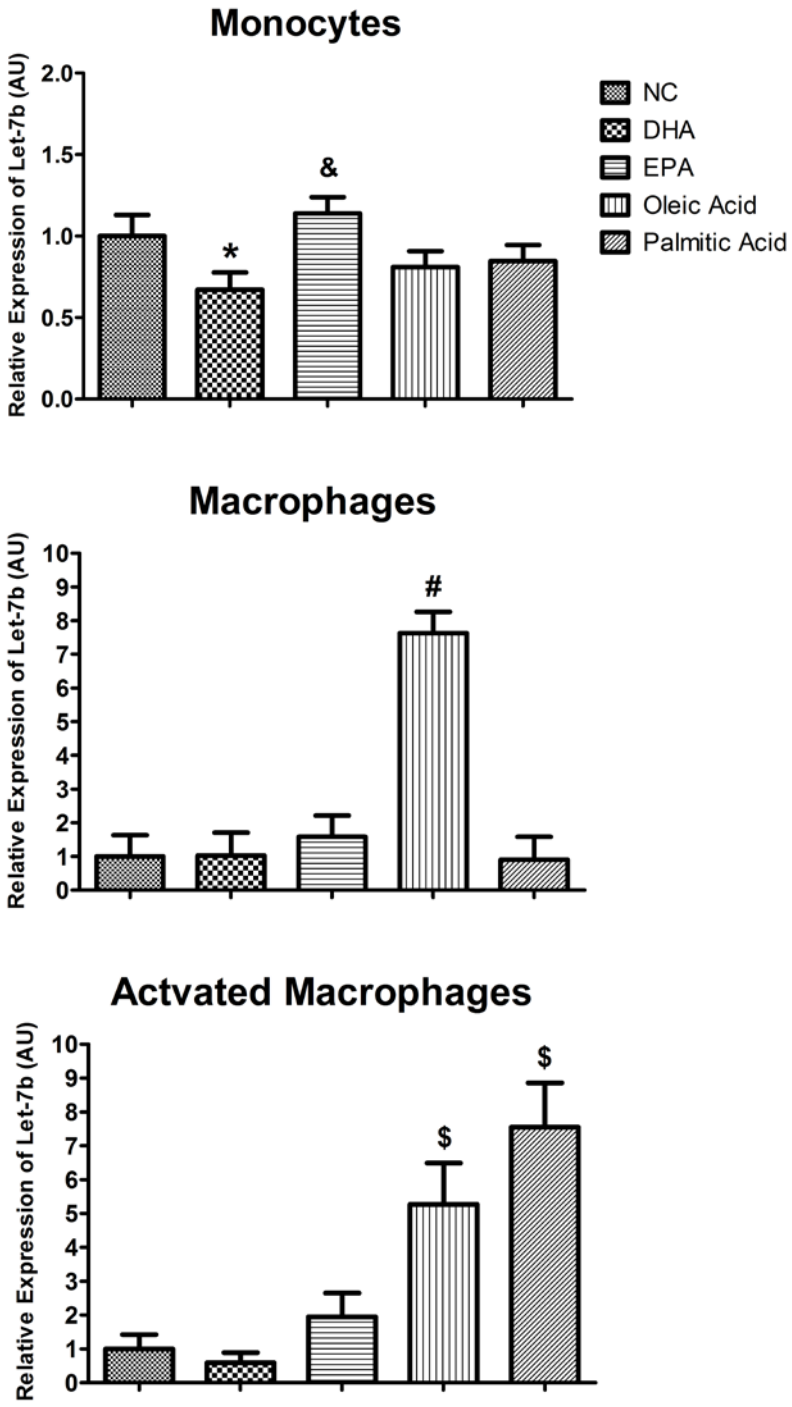


Figure 4. Relative expression of let-7b in monocytes, macrophages and activated macrophages after treatment with different types of fatty acids. DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid. Data are shown as the means ± standard deviation. * $P<0.05$ when compared with the negative control (NC). $^{\#}$ $P<0.05$ when compared with all other treatment groups. $^{\$}$ $P<0.05$ when compared with NC, DHA and EPA treatment groups.

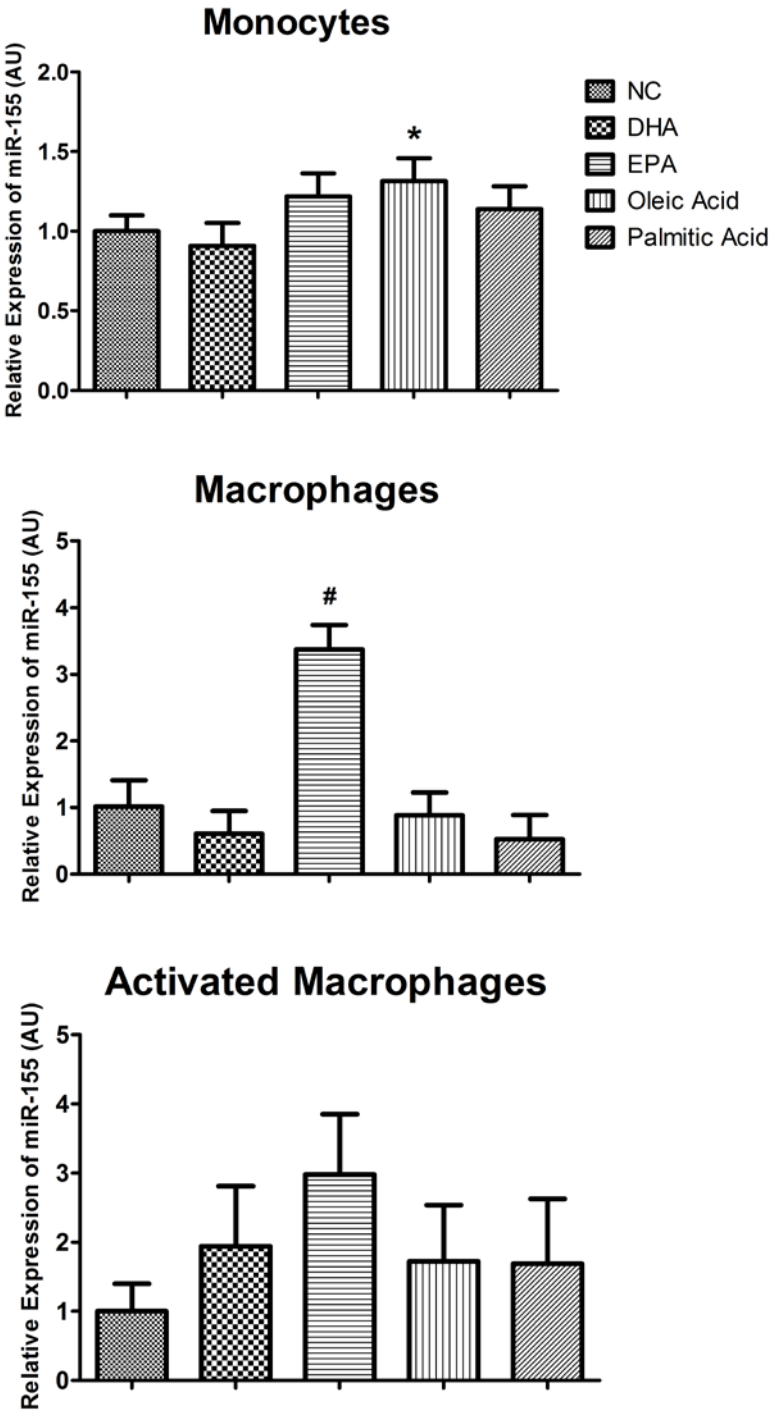
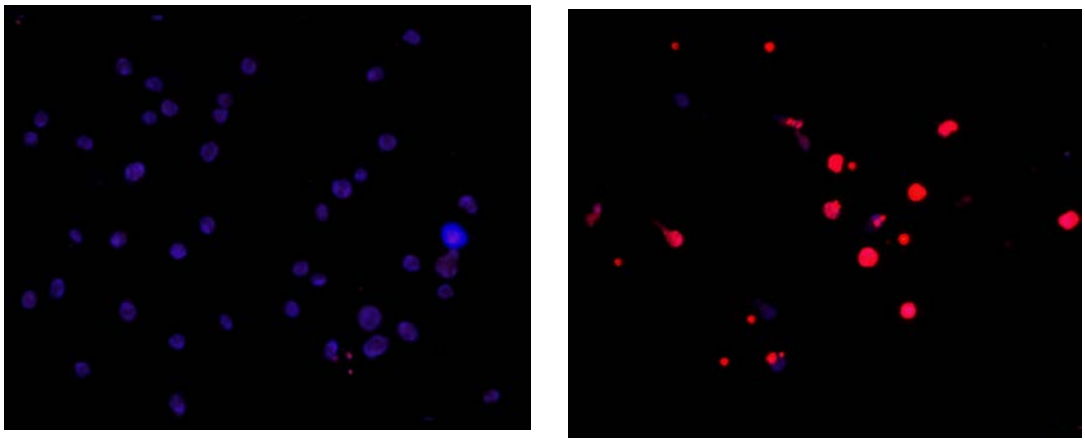


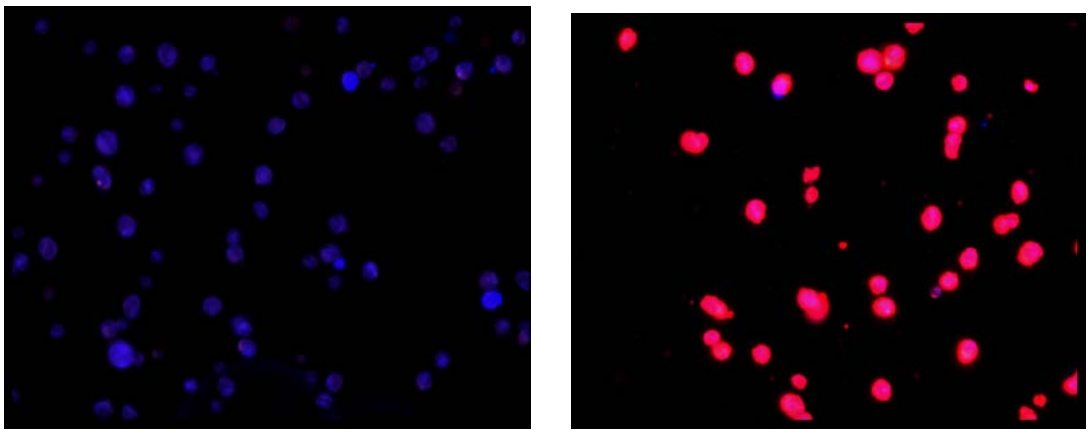
Figure 5. Relative expression of miR-155 in monocytes, macrophages and activated macrophages after treatment with different types of fatty acids. DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid. Data are shown as the means ± standard deviation. * $P<0.05$ when compared with the DHA treatment. $^{\#}$ $P<0.05$ when compared with all other treatment groups.

SUPPLEMENTARY DATA

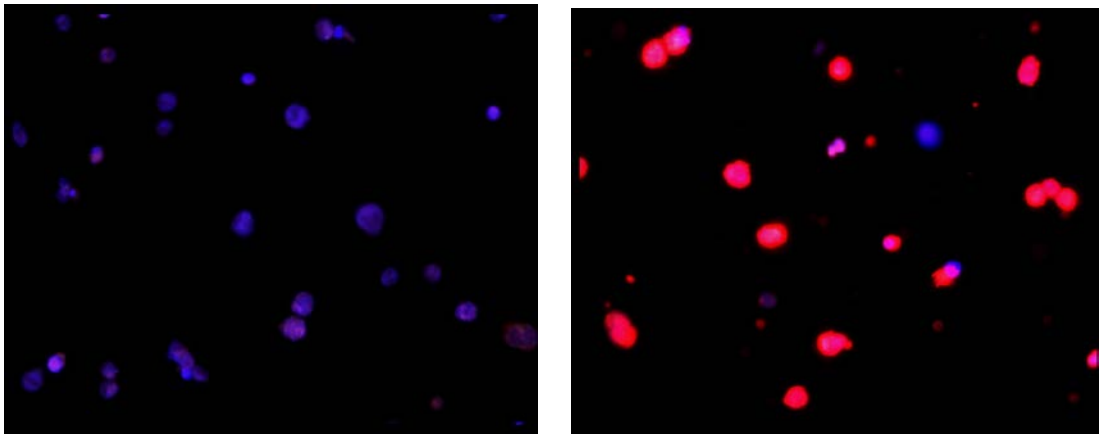
MONOCYTES



MACROPHAGES



ACTIVATED MACROPHAGES



Negative Control **Lipofectamine + Alexa Fluor Red Fluorescent Oligo**

Figure S1. Efficiency of transfection with lipofectamine in THP-1 cells after 48 h.

SUPPLEMENTARY DATA

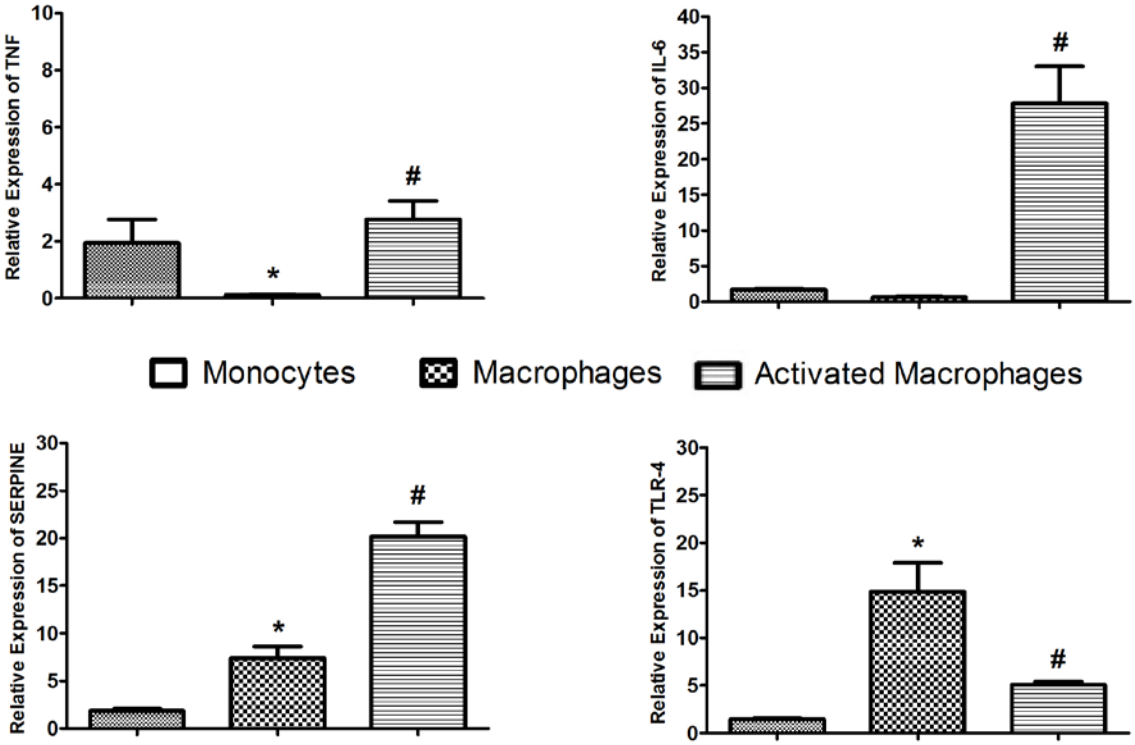


Figure S2. **Basal expression of genes related with inflammatory response in monocytes, macrophages and activated macrophages (n=14 in each group).** Data are shown as the means ± standard deviation. * P<0.05 when compared with monocytes. #P<0.05 when compared with other cell groups.

SUPPLEMENTARY DATA

Table S1. References of the specific PCR primers/probes used for the measurement of the miRNAs and mRNAs.

miRNAs	TaqMan® MicroRNA Assay reference
Let-7b	002619
miR-155-3p	002287
U6	001973
U48	001006
Genes (mRNAs)	**TaqMan® Assay reference**
IL6	Hs00985639_m1
TNF	Hs01113624_g1
SERPINE1	Hs01126606_m1
TLR4	Hs00152939_m1
GAPDH	Hs02758991_g1
B-ACTIN	Hs00159357_m1

Abbreviations: GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IL-6: interleukin-6; miR: microRNA; SERPINE1: serine protease inhibitor, member E1; TNF: tumor necrosis factor; TLR4: Toll-like receptor 4; U6 and U48: TaqMan® microRNA Control Assays

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6.3 CHAPTER 3 - Published Original Paper • Epigenetcs

LINE-1 methylation is positively associated with healthier lifestyle but inversely related to body fat mass in healthy young individuals.

Authors:

Jose Luiz Marques-Rocha[a], Fermin I. Milagro[b,c], Maria Luisa Mansego[b], Denise Machado Mourão[a], J. Alfredo Martínez[b,c,d], and Josefina Bressan[a]

[a]Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil; [b]Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain; [c]CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Health Institute, Madrid, Spain; [d]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

EPIGENETICS
2016, VOL. 0, NO. 0, 1–12
http://dx.doi.org/10.1080/15592294.2015.1135286

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RESEARCH PAPER

LINE-1 methylation is positively associated with healthier lifestyle but inversely related to body fat mass in healthy young individuals

José Luiz Marques-Rocha[a], Fermin I. Milagro[b,c], Maria Luisa Mansego[b], Denise Machado Mourão[a], J. Alfredo Martínez[b,c,d], and Josefina Bressan[a]

[a]Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil; [b]Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain; [c]CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Health Institute, Madrid, Spain; [d]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

ABSTRACT

Objectives: To investigate if epigenetic biomarkers from white blood cells (WBC) are associated with dietary, anthropometric, metabolic, inflammatory and oxidative stress parameters in young and apparently healthy individuals. ***Methods***: One hundred 5six individuals (91 women, 65 men; age: 23.1±3.5 years; body mass index: 22.0±2.9 kg/m^2) were evaluated for anthropometric, biochemical and clinical markers, including some components of the antioxidant defense system and inflammatory response. DNA methylation of *LINE-1, TNF-α* and *IL-6* and the expression of some genes related to the inflammatory process were analyzed in WBC. ***Results***: Adiposity was lower among individuals with higher *LINE-1* methylation. On the contrary, body fat-free mass was higher among those with higher *LINE-1* methylation. Individuals with higher *LINE-1* methylation had higher daily intakes of calories, iron and riboflavin. However, those individuals who presented lower percentages of *LINE-1* methylation reported higher intakes of copper, niacin and thiamin. Interestingly, the group with higher *LINE-1* methylation had a lower percentage of current smokers and more individuals practicing sport. On the other hand, *TNF-α* methylation percentage was negatively associated with waist girth, waist-to-hip ratio and waist-to-stature ratio. Plasma *TNF-α* levels were lower in those individuals with higher *TNF-α* methylation. ***Conclusions***: This study suggests that higher *LINE-1* and *TNF-α* methylation are associated with better indicators of adiposity status in healthy young individuals. In addition, energy and micronutrient intake, as well as a healthy lifestyle, may have a role in the regulation of DNA methylation in WBC and the subsequent metabolic changes may affect epigenetic biomarkers.

Abbreviations: BMI, Body mass index; CI, Confidence interval; g, gram; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL-c, High-density lipoprotein; kcal, Kilocalories; kg, Kilogram; kg/m2, Kilogram/square meter; IL, Interleukin; LDL-c, Low-density lipoprotein; LINE-1, Long interspersed nucleotide element-1; MET, Metabolic equivalent; μg/g, Microgram per gram; mM, Millimolar; MS-HRM, Methylation-sensitive high resolution melting; ng/g, Nanogram per gram; OS, Oxidative stress; OR, Odds ratio; ox-LDL, Oxidized Low-density lipoprotein; PBMC, Peripheral blood mononuclear cells; ROS, Oxygen-reactive species; TAC, Total antioxidant capacity; TC/HDL-c, Castelli index; TC, Total cholesterol; TNF-α, Tumor necrosis factor α; UFV, Federal University of Viçosa; UTR, Untranslated region; VLDL, Very Low-density lipoprotein; WBC, White blood cell

ARTICLE HISTORY
Received 12 October 2015
Revised 10 December 2015
Accepted 17 December 2015

KEYWORDS
adiposity; Biomarker; diet; epigenetics; IL-6; inflammation; TNF-α

Introduction

Lifestyle and nutrients may induce transient or permanent alterations in the epigenetic marks that regulate the expression of genes involved in metabolic processes and networks, which could be one of the factors leading to chronic diseases.[1] The most common epigenetic modification is DNA methylation. Approximately 50% of the human genome is composed of repetitive sequences such as LINE (Long Interspersed Nuclear Elements), which is the most common repetitive element of interspersed DNA repeats.[2] These elements are usually methylated, and their transcription and retrotransposition are suppressed by a variety of control mechanisms including methylation or non-coding RNA.[3] Because of its high

CONTACT J. Alfredo Martínez jalfmtz@unav.es
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Institutions where study was performed:
1) Laboratory of Energy Metabolism and Body Composition - Department of Nutrition and Health, Federal University of Viçosa (Viçosa, Brazil). Address: Departamento de Nutrição e Saúde. Universidade Federal de Viçosa - Av. PH Rolfs, s/n, Centro. Viçosa, Brazil. 36.570-000.
2) Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, C/ Irunlarrea, 31008, Pamplona, Spain
Supplemental material data for this article can be accessed on thepublisher's website.

genome dissemination, *LINE-1* methylation status has been proposed as a surrogate marker for estimating global DNA methylation level.[2]

A global decrease in the methylation of peripheral blood DNA was found to be an independent risk factor for many cancers[4] and with developmental, autoimmune, and other chronic diseases.[5] LINE-1 methylation levels in peripheral blood mononuclear cells (PBMCs) have been reported to predict response to a dietary weight-loss intervention[6] and have been associated with serum glucose levels.[7] The methylation levels of individual genes, such as those of tumor necrosis factor α (*TNF-α*), have been also proposed as biomarkers of response to a hypocaloric diet.[8] Methylation status may, therefore, serve as a biomarker for early diagnostics, prediction of prognosis, and response to treatments. Moreover, some dietary factors are able to alter the percentage of methylation, both at *LINE-1* and in proinflammatory genes. For example, energy restriction has been reported to decrease *IL-6* methylation levels in buffy coat DNA[7] and *TNF-α* methylation has been associated with n-6 polyunsaturated fatty acids (PUFAs) intake.[9] However, the ethiopathogenic mechanisms remain poorly understood. Thus, the objective of this study was to explore the relation between DNA methylation levels of *LINE-1, TNF-α* and *IL-6* in white blood cells (WBC) and anthropometric, biochemical, clinical, dietary, inflammatory and oxidative stress parameters in young and apparently healthy adults.

Material and methods

Subjects

One hundred 5six healthy subjects were recruited to participate in the study (91 women and 65 men; age: 23.1 ± 3.5 y and BMI: 22.0 ± 2.9 kg/m^2). Most of the study population self-reported to be white (n = 131) followed by black (n = 16) and others (n = 9). Initial screening excluded subjects with evidence of any metabolic disease; chronic inflammation, hydric balance disorders, changes in body composition and problems in nutrient absorption or metabolism. Other exclusion criteria were as follows: drug or nutritional treatment that affects energy balance, dietary intake, lipid profile, insulin levels or glucose metabolism, contraceptive use up to 2 months before participation in the study and weight loss diet follow-up or unstable weight in the past 6 months. In agreement with the principles of the Helsinki Declaration and following a clear explanation of the study protocol, each participant signed a written informed consent form. The study was approved by the Human Research Ethics Committee of the Federal University of Viçosa, Brazil (protocol number 019/2011).

Anthropometric and body composition assessments

Height was measured with a stadiometer (Seca 206 model, Hamburg, Germany) to the nearest 0.1 cm. Body weight was measured to the nearest 0.1 kg by using an electronic microdigital scale (Tanita TBF-300 A model, Tokyo, Japan). Body mass index (BMI) was calculated by the quotient between body weight and square height (kg/m^2). Waist and hip perimeters were measured with an inelastic and flexible tape to the nearest 0.1 m. Triceps, biceps, subscapular and suprailiac skinfold thicknesses were measured to the nearest 1 mm by using a skinfold caliper (Lange caliper, Cambridge Scientific Industries Inc., Maryland, USA). The sum of skinfold thickness was calculated. Total body fat percentage was measured to the nearest 0.1 % using a body composition analyzer (Biodynamics 310 model, Washington, USA). Body fat mass and body fat-free mass were estimated using the same body composition analyzer. Truncal fat percentage was computed as the sum of subscapular and suprailiac skinfold thicknesses divided by the sum of 4 skinfold measurements.[10] Finally, truncal adiposity index was calculated by the ratio of subscapular to triceps skinfold thickness.[11]

Blood pressure assessment

Systolic and diastolic blood pressures were measured with a mercury sphygmomanometer (BIC, SP, Brazil) following World Health Organization criteria.[12]

Dietary intake and lifestyle assessments

A seventy-2-hour food record was used to collect information about energy and nutrient intake. A booklet was given to the participants to record everything they ate or drank over a period of 3 non-consecutive days, including a weekend day. Dietary intake was computed using specific software (DietPro®, version 5.0, AS Systems).

Covariates about diet and lifestyle, such as vitamin supplementation, smoking status (smokers or non-smokers), number of cigarettes per day, regular physical activity (yes or no) and volume of physical activity, were also collected. To quantify the volume of physical activity, an activity metabolic equivalent (MET) was used.[13] This index represents the ratio between energy expenditure during each specific activity and resting metabolic rate. METs were computed by a multiple of resting metabolic rate (MET score) to each activity. The MET scores were provided by Compendium of Physical Activities, a

coding scheme that classifies specific physical activity by rate of energy expenditure.[14] METs were calculated by multiplying time spent on each activity by a specific MET score to that activity. The scores were then summed over all activities to obtain a mean value of overall week, expressed in hours per day.

Analyses of biological samples

Blood samples were drawn by vein puncture after a 12-hour overnight fast. Ethylenediaminetetraacetic acid (EDTA) plasma, heparin plasma and serum samples were separated from whole blood by centrifugation at 3500 rpm at 5°C for 15 min (Eppendorf AG, 5804 R model, Hamburg, Germany) while erythrocytes were separated from whole blood by centrifugation at 3000 rpm at 5°C for 10 min. All samples were immediately stored at −80°C until assay.

Lipid and glucose profile

Serum glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-c), and triacylglycerol concentrations were assessed in an automated biochemical analyzer (BS-200, Shenzhen Mindray Bio-medical Electronics Co., Nanshan, China) using specific colorimetric kits (Bioclin, Quibasa, Minas Gerais, Brazil). Low-density lipoprotein cholesterol (LDL-c) data were calculated by the Friedewald equation as previously validated.[15] The total cholesterol-to-HDL-c ratio was also assessed.[16] Plasma insulin concentrations (sensitivity 2 μU/mL) were measured by an enzyme-linked immunosorbent assay (ELISA) kit as described by the supplier (Linco Research, St. Charles, USA). The homeostasis model assessment of insulin resistance (HOMA-IR), calculated as fasting glucose (nmol/L) x fasting insulin (μU/mL)/22.5,[17] was used to estimate insulin resistance.

Antioxidant markers

Plasma total antioxidant capacity (TAC) was assessed by a colorimetric assay, which relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metmyoglobin (Cayman Chemical, Ann Arbor, MI, USA). Plasma oxidized-LDL (ox-LDL) concentrations were determined by ELISA (Mercodia, Uppsala, Sweden). Glutathione peroxidase (GPx) activity (nmol/[mL/min]) was measured in erythrocytes using a commercially available kit as described by the supplier (Cayman Chemical, Cat. 703102). Uric acid and ceruloplasmin concentrations were assessed with an automated biochemical analyzer (BS-200, Shenzhen Mindray Bio-medical Electronics Co., China) using specific commercially available kits (Bioclin).

Inflammatory markers

Plasma IL-6, TNF-α and C-reactive protein (CRP) levels were determined by using commercial ELISA kits from Cayman Chemical. Adiponectin levels were also assessed by ELISA (SPIBIO, Montigny le Bretonneux, France). Serum complement factor-3 (C3) was quantified with an automated biochemical analyzer (model BS-200) using a specific colorimetric kit (Bioclin).

Trace elements in nails

Nail samples were collected at the time of interview and stored at room temperature in clean polypropylene bags. Fingernail and toenail samples were treated with sub boiling nitric acid in a high-pressure Teflon digestion vessel using a microwave digestion system (Ethos Plus, Millestone, Sorisole, Italy). A Perkin Elmer Analyst 800 atomic absorption spectrometer (Norwalk, CT, USA), equipped with transverse-heated graphite atomizer, Zeeman background corrector and AS-800 autosampler, was used for measuring selenium at 196.0 nm with a spectral band width of 2.0 nm.[18] An electrodeless discharge lamp (Perkin Elmer) was used as a light source operated at 280 mA. Pyrolytic coated graphite tubes with end caps supplied by Perkin Elmer were used. Zinc and copper concentrations in digested acid solutions were analyzed by flame atomic absorption spectrophotometry. Zinc and copper hollow cathode lamps provided resonance lines of 213.9 and 324.8 and were operated both at 15 mA with a slit width seat at 0.7 nm.

RNA extraction and real time quantitative PCR

Total RNA was extracted from WBC using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined at 260/280 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Two μg of total RNA was reverse-transcribed using the SYBR® Green RT-PCR Reagents Kit (Cat. 4306736, Life Technologies, Waltham, MA, USA) according to the manufacturer's protocol. cDNA was amplified in triplicate with SYBR® Green PCR Master Mix (Cat. No. 4309155) and the respective specifics primers (see Supplementary 1). The analyzed genes were selected on the basis of previous studies supporting their possible involvement in inflammatory pathways related to metabolic syndrome. mRNA levels were normalized to the endogenous control *GAPDH* (glycerol-3-phosphate dehydrogenase). The ΔΔCt (crossing threshold) method was used for quantification (ABI) and the fold changes reported as $2^{-\Delta\Delta Ct}$.[9] All quantitative real-time RT-PCR measurements were performed using a 7900 HT Fast Real-Time PCR system (Life Technologies).

DNA isolation and methylated DNA standards

DNA was isolated from the WBC using the Master Pure DNA Purification Kit for Blood Version II (Epicenter, Madison, WI, USA) according to the instructions provided by the manufacturer. Purified DNA was stored at −20°C until use. Purified DNA was quantified by Pico-Green dsDNA Quantitation Reagent (Invitrogen). Cells-to-CpG™ Methylated gDNA Control Kit (Life Technologies) and DNA from placenta cells (D3160, Sigma Aldrich, St. Louis, MO, USA) were used as methylated and non-methylated DNA standards, respectively. To generate a range of methylated and unmethylated DNA standards, the 2 standard DNA controls were mixed in 0, 20, 40, 60, 80, and 100% methylated to unmethylated template ratios. One microgram of each standard and sample DNA was bisulfite-converted (BSC) by using the Epitect Fast Bisulfite Conversion Kit (Qiagen, Venlo, Limburg, The Netherlands) according to the manufacturer's instructions, thus converting non-methylated cytosines into uracil. All bisulfite-converted DNAs were diluted to 5 ng/μL for use in PCR.

Methylation-sensitive high resolution melting (MS-HRM) analysis

Specific primers against the completely methylated sense strand sequence were designed according to the recommendations of Wojdacz and Dobrovic (2007)[19] in order to minimize PCR bias. The promoter region of the consensus *LINE-1* sequence (GenBank: X58075) was used to design primer sets with the Primer3 website (http://frodo.wi.mit.edu/primer3/). Thus, the primers for analyzing *LINE-1* methylation were: forward, 5′-GCGAGGTATTGTTTTATTTGGGA-3′; reverse, 5′-CGCCGTTTCTTAAACC-3′. They screened 8 CpGs in an amplicon length of 141 bp and were first employed by Tse et al.[20] The primers for analyzing *IL-6* methylation were: forward, 5′-TTATGTAGGAAAGAGAATTTGGTTTAG-3′ and reverse, 5′-AAAAAATAA AATCATCCATTCTTCAC-3′. They covered 5 CpGs in an amplicon length of 181 bp as mentioned elsewhere.[7] The primers for *TNF-α* were: forward, 5′-TTTTGGAAAGGATATTATGAGTATTGA-3′ and reverse, 5′-CTAAAACCCTA AAACCCCCCTAT-3′. They covered 4 CpGs in an amplicon length of 99 bp as mentioned elsewhere.[9] Genomic sequence and CpGs sites covered by the MS-HRM primers studying *TNF-α* and *IL-6* methylation are shown in Figures S1 and S2. PCR amplification of the DNA was carried out using a 7900 HT Fast Real-Time PCR System (Life Technologies) equipped with the SDS Software (Version 2.4.1, Life Technologies). PCR was performed in a 10-μL reaction volume, and 5 ng of bisulfite-converted DNA templates for *LINE-1* assay were added to each well, which contained 1×MeltDoctor™ HRM Master Mix (HRM) (Life Technologies) and 0.2 μM each primer. The cycling protocol conditions included a single enzyme activation step of 10 minutes at 95°C followed by 40 cycles of the following steps: denaturation 95°C, 15 seconds, and annealing 60°C, 1 minute. The MS-HRM step was performed after 40 cycles of amplification and the MS-HRM analysis was initiated by denaturing all products at 95°C for 1 minute, followed by annealing at 55°C for 1 minute. Samples were slowly warmed to 95°C at 0.1°C/second. The High ReSolution Melt Software v2.0 (Life Technologies) was employed for end-product analysis. This algorithm allowed the raw melt curves to be normalized for fluorescence intensity, and a temperature shift was applied to align the normalized melt curves, which facilitated the analysis of samples with varying Ct values. A difference curve was then derived from the first derivative of the melt curves. Data for the difference melt curves were exported to Excel (Office 2007; Microsoft Corp., Redmond, WA) for further analyses. Graphs were plotted and inverted vertically. Both peak-height and area-under-the-curve from the normalized, temperature-shifted, difference curves were used to generate a standard curve and determine the degree of methylation of each DNA sample. All participant DNA samples were analyzed on a 384-well plate, which included a no-template control (NTC) and a set of reference methylation standards. Reference methylation standard curves and experimental samples were tested in triplicate.

Statistical analysis

The Kolmogorov-Smirnov normality test was used to determine variable distribution. Accordingly, the parametric Student *t* test or nonparametric Mann-Whitney *U* test was performed to detect differences between subjects with higher and lower DNA methylation percentage than the median value. Dichotomous variables were analyzed by chi-squared X^2 test. Contrasts and Tukey's post-hoc tests for One-Way ANOVA were performed to analyze differences among tertiles and quartiles. P for trend was calculated. The Spearman correlation coefficients were used to screen the statistical associations between DNA methylation and interest variables. Linear regression model, used to identify the predictors of DNA methylation, was adjusted for covariates such as calories, sex, age, smoking status and regular physical activity, when they showed significant effect. Nutrients from the diet were adjusted by total energy intake by using the residual method.[21] Results are presented as mean ± SD (standard deviation). Confidence intervals (95% CIs) were used to describe linear regression coefficients (β). $p < 0.05$ was considered statistically

significant. Statistical analyses were performed by using SAS software system version 8.0 for Windows (SAS Institute Inc., Cary, NC 27513, USA). GraphPad Prism® version 6.0 C (La Jolla, CA, USA) was used to show graphically the results.

Results

Baseline anthropometric, clinical and biochemical measurements from participants are presented in Supplementary **Table 2** (S2). The study population was considered healthy and some expected differences in anthropometric variables (i.e., body weight, BMI, waist circumference, truncal fat percentage, etc.) were found between genders.

LINE-1 methylation levels were significantly lower in women (about 7.3% less) ($p < 0.01$). In addition, *LINE-1* methylation were positively associated with body weight ($r = 0.296$; $p = 0.032$; $n = 120$). These results were confirmed when the subjects were divided according to the median of body weight (61.0 kg), even when analyses were made separately by gender ($p < 0.05$ for both). More detailed analyses showed that indicators of adiposity, such as skinfolds and total body fat, were lower among individuals with higher *LINE-1* methylation ($p < 0.05$ for all) (**Table 1**). On the contrary, body fat-free mass was higher (p for trend = 0.016) among those with higher *LINE-1* methylation. These individuals also exhibited lower plasma levels of ceruloplasmin (p for trend = 0.002) and higher IL-6 in plasma (p for trend = 0.006), as shown in **Table 1**.

Calorie (kcal) intake was higher (p for trend = 0.04) among individuals with higher *LINE-1* methylation even after adjusted for body weight (**Table 2**). A similar result was observed for daily iron intake (mg) even after adjusting by calories and body weight ($p < 0.05$). It is worth emphasizing that iron intake explained, through the r values, about 8.4% of *LINE-1* methylation, even after adjusting by energy, gender and smoking (**Fig. 1**). In the same way, higher daily riboflavin (B2 vitamin) intake was found in those individuals with bigger values to LINE-1 methylation ($p < 0.05$ inter quartiles). However, those individuals who presented lowers percentages of LINE-1 methylation reported eating higher amounts of copper, niacin (B3 vitamin) and thiamin (B1 vitamin) (p for trend <0.05 for all). Interestingly, the group with higher *LINE-1* methylation (<83.02%) had a lower percentage of current smokers ($p = 0.012$) and more individuals practicing sport ($p < 0.05$) (**Table 3**). These results are confirmed with the lower number of cigarettes smoked per day ($p = 0.041$) and higher physical activity per day ($p = 0.047$) in those subjects with bigger values to LINE-1 methylation. These results suggest that high *LINE-1* methylation could be associated to a healthier lifestyle.

Not only *LINE-1* methylation was associated positively with *TNF-α* and *IL-6* methylation percentage when linear regression was adjusted by gender, smoking and age ($r = 0.204$; $p = 0.048$ and $r = 0.332$; $p = 0.003$) (**Fig. 2**), but also when the subjects were divided according to the median of *LINE-1* methylation (83.02%; $p<0.05$ for both). Results from logistic regression analysis (odds ratio-OR) confirmed that individuals with higher *LINE-1* methylation levels (third tertile, >83.1 %) were more likely to have higher percentage of *IL-6* methylation (3.08 times) than those with lower methylation levels (first tertile, <77.10 %; IC: 1.13−8.36; $p = 0.013$).

Individuals whose *TNF-α* methylation percentage was above the tertile 3 (T3) showed lowers values of waist girth, waist-to-hip ratio, waist-to-stature ratio and selenium in nails ($p < 0.05$) (**Table 3**). Moreover, they presented lower *TNF-α* expression in WBC (p for trend = 0.041), suggesting that the hypermethylation of this gene was associated with inhibition of its transcription (**Table 5** and **Fig. 3A**). It should be noted that the plasma levels of *TNF-α* were also lower in those individuals with greater methylation for this gene (p for trend = 0.048). On the other hand, these individuals presented higher expression of *IL-18* and plasma values of IL-6 ($p < 0.05$ for all) (**Table 3**). As expected, the individuals with higher *TNF-α* methylation showed also higher methylation of *IL-6* and *LINE-1* (**Table 5**).

No differences were found between the groups whose *IL-6* methylation levels were above and below the median (≤ or >56.57 %). However, when only men were taken into account, the levels of selenium in nails and mRNA levels of *IL-6* and *TNF-α* were lower in those individuals with higher *IL-6* methylation values ($p < 0.05$ for all). On the other hand, a negative association was found between *IL-6* methylation in white blood cells and relative expression (mRNA) of *IL-6* in the same cells (**Fig. 3B**).

Discussion

There is growing evidence on the involvement of epigenetic mechanisms in disease onset, including obesity and type 2 diabetes.[1] A decrease in global DNA methylation is associated with increased genomic instability and chromosomal rearrangements,[22] a common biological mechanism in several diseases including cancer. Reduced DNA methylation in WBCs may be an indicator of systemic hypomethylation and of cumulative environmental impacts. Conversely, an increase in DNA methylation levels of repetitive elements may have a protective effect

Table 1. Anthropometric, clinical and biochemical data (mean ± SD) of **all individuals (N=120)** categorized by quartiles of ***LINE-1*** **methylation (%)**.

Variables	*Q1* < 77.01%	*Q2* 77.02 to 83.01%	*Q3* 83.02 to 89.05%	*Q4* >89.06%	P-for linear trend
Age (y)	23.2 (3.2)	24.5 (4.1)	23.0 (3.8)	22.8 (3.0)	0.280
BMI (kg/m^2)	21.9 (2.0)	22.6 (3.3)	22.0 (2.7)	22.4 (3.1)	0.732
Waist perimeter (cm)	77.7 (8.2)	80.5 (8.9)	78.0 (7.5)	78.9 (9.4)	0.532
Hip perimeter (cm)	98.0 (4.9)	**97.5 (6.5)**[a]	**95.8 (5.6)**[a]	**93.1 (7.5)**	**0.013**
Waist-to-hip ratio	0.79 (0.06)	0.82 (0.06)	0.81 (0.06)	**0.84 (0.06)**[a]	**0.018**
Total body fat - BIA (%)[a]	23.6 (6.0)	**22.6 (7.0)**[b]	**20.5 (6.7)**[b]	**18.2 (7.8)**	**<0.001**
Body fat mass - BIA(kg)	18.6 (5.8)	16.9 (7.0)	14.6 (4.3)	14.0 (5.2)	0.080
Truncal fat (%)	56.7 (5.7)	59.6 (7.3)	59.0 (7.2)	60.9 (6.4)	**0**.052
Sum of 4 STs (mm)[a]	68.9 (17.7)	67.6 (24.8)	**60.0 (20.4)**[a]	**56.1 (30.3)**	**0.050**
Body free fat mass - BIA(kg)	43.8 (8.4)	**47.0 (11.7)**[a]	**48.3 (9.9)**[a]	**51.3 (10.1)**	**0.016**
Systolic blood pressure (mmHg)	10.7 (0.8)	11.0 (0.6)	10.9 (1.0)	**11.4 (1.0)**[a]	**0.038**
Diastolic blood pressure (mmHg)	7.3 (0.6)	7.3 (0.5)	7.3 (0.7)	7.5 (0.8)	0.521
Glucose (mg/dL)[a]	91.0 (8.0)	91.8 (7.0)	90.7 (6.8)	90.5 (6.9)	0.362
Insulin (μU/mL)	15.8 (2.8)	17.2 (4.7)	16.2 (3.9)	13.4 (2.4)	0.423
HOMA-IR[a]	3.63 (0.67)	4.06 (1.09)	3.87 (1.09)	3.14 (0.48)	0.215
Total cholesterol (mg/dL)	162.4 (29.6)	162.7 (21.6)	158.7 (35.8)	151.1 (32.8)	0.426
HDL-c (mg/dL)[a]	48.8 (7.6)	45.6 (11.2)	43.7 (10.1)	42.4 (10.3)	0.087
LDL-c (mg/dL)[a]	91.8 (23.5)	91.7 (18.0)	96.2 (30.1)	91.6 (26.5)	0.890
VLDL-c (mg/dL)	20.5 (7.8)	20.7 (9.1)	20.1 (7.3)	17.0 (5.8)	0.218
Triacylglycerol (mg/dL)	102.7 (39.4)	108.6 (52.3)	100.9 (36.5)	85.4 (29.1)	0.063
Total cholesterol-to-HDL-c ratio[a]	3.31 (0.54)	3.60 (0.80)	3.74 (0.86)	3.56 (0.69)	0.971
CRP-hs	2.00 (2.21)	1.81 (1.58)	1.74 (2.64)	0.99 (1.37)	0.224
IL-6 (pg/mL)	15.8 (14.8)	21.6 (22.5)[a]	**19.8 (15.5)**[a]	**35.5 (21.0)**	**0.006**
TNF-α (pg/mL)[a]	4.13 (1.96)	4.45 (1.58)	4.21 (2.01)	5.05 (2.75)	0.331
C3 complement[a]	110.3 (24.6)	110.8 (25.3)	114.3 (25.6)	110.3 (21.0)	0.904
Ceruloplasmin (mg/dL)[a]	40.37 (8.40)	**36.87 (7.91)**[a]	**36.50 (7.82)**[a]	**32.51 (5.98)**	**0.002**
Adiponectin	32.7 (10.3)	32.4 (22.9)	22.1 (9.0)	23.1 (8.8)	0.294
GPx activity (nmol/[ml/min])[a]	576 (310)	540 (267)	542 (279)	643 (251)	0.580
Total antioxidant capacity (mM)[a]	1.50 (0.80)	1.76 (0.94)	1.46 (0.72)	1.89 (1.07)	0.196
Ox-LDL (U/L)[a]	62.7 (28.0)	67.7 (22.1)	80.7 (31.8)	76.2 (32.4)	0.081
Selenium (ng/g of nail)	0.41 (0.07)	0.40 (0.08)	0.39 (0.09)	0.35 (0.06)	0.109
Copper (ng/g of nail)	6.97 (4.10)	6.42 (2.90)	7.39 (5.70)	7.04 (4.50)	0.894
Zinc (ng/g of nail)	124.8 (25.0)	114.8 (16.8)	146.0 (99.0)	128.4 (71.7)	0.376

BIA: Electrical bioimpedance; HOMA-IR: homeostatic model assessment; CRP-hs: high-sensitivity C-reactive protein; GPx: Glutathione peroxidase; Ox-LDL; Oxidized low-density lipoprotein; ST skinfold thickness. One-way ANOVA test with linear contrast analysis were performed.
[a]different from Q1.
[b]different from all quartiles.

against genomic instability and unwanted chromosomal rearrangements.[23] However, there are many gaps about the determinants of DNA methylation levels in healthy people prior to disease onset, and there is great interest in finding new, early biomarkers for disease risk reduction and health promotion.

Our findings support previous results showing that women have significantly lower levels of *LINE-1* methylation.[24-26] Lower levels of global methylation in women may be due to different levels of dietary folate or other one-carbon nutrients in men and women.[27] Women may also have a higher folate requirement than men

Table 2. Daily nutrient intake (mean ± SD) for **all individuals** categorized according to the quartiles of ***LINE-1*** **methylation (%) (n=115)**.

Variables	*Q1* < 77.01%	*Q2* 77.02 to 83.01%	*Q3* 83.02 to 89.05%	*Q4* >89.06%	P-for linear trend
Energy (kcal/BW)	39.26 (9.09)	38.52 (8.80)	**44.62 (9.81)**[a,b]	**47.69 (13.28)**[a,b]	**0.004**
Carbohydrate*	339.7 (54.3)	344.1 (36.8)	350.0 (55.2)	348.1 (46.6)	0.880
Protein (g)*	106.7 (21.6)	98.6 (14.9)	103.3 (19.6)	102.5 (17.3)	0.671
Lipid (g)*	99.2 (15.6)	94.9 (12.3)	100.1 (20.8)	96.0 (14.9)	0.492
Alcohol (g)*	59.67 (86.27)	44.32 (77.34)	**5.34 (40.32)**[a,b]	**1.29 (53.01)**[a,b]	**0.050**
Iron (mg)*	64.32 (13.27)	64.47 (9.84)	**74.31 (19.69)**[a,b]	**83.88 (25.03)**[a,b]	**0.022**
Cupper (mg)*	4.47 (5.53)	3.90 (5.73)	**0.93 (5.84)**[a,b]	**0.23 (7.64)**[a,b]	**0.001**
Niacin (mg)*	32.50 (27.12)	32.19 (26.54)	**14.70 (27.47)**[a,b]	**4.08 (28.04)**[a,b]	**0.001**
Riboflavin (mg)*	1.36 (0.57)	**1.50 (0.51)**[a]	**1.56 (0.52)**[a]	**1.80 (0.72)**[a,b]	**0.050**
Thiamin (mg)*	4.95 (5.50)	**4.61 (5.52)**[a,b]	**1.29 (5.14)**[a,b]	**0.46 (6.45)**[a,b]	**<0.001**

BW, body weight.
One-way ANOVA test with linear contrast analysis were performed.
[a]different from Q1.
[b]different from all quartiles.
*Arbitrary values after adjustment for the calories using the residual method. Twenty-one

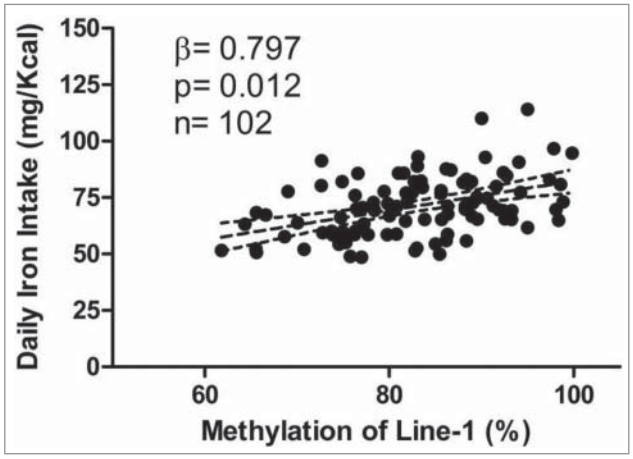

Figure 1. Linear regression model showing association between daily iron intake (refined by calories - residue method)[21] and *LINE-1* methylation (%) after adjustment for smoking and gender. The dotted lines represent the confidence rating limit (IC-95%).

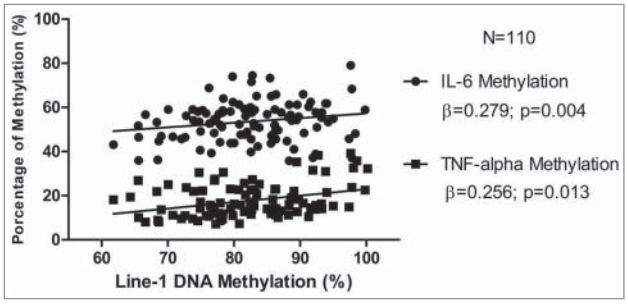

Figure 2. Linear regression model (r) showing association among *LINE-1* methylation (in %) and *IL-6* (r=0.204; p=0.048) and *TNF-α* (r=0.332; p=0.003) methylation percentage after adjusting by gender and smoking.

because of regular loss of red blood cells through menstruation, but it can not explain the gender-specific difference in global methylation in postmenopausal women.[27,28] There is still discussion about hormonal factors and the importance of DNA methylation for X-chromosome inactivation in women.[24,29] Further studies are needed to decipher the relationship between gender and *LINE-1* methylation.

The results from this research are also in line with other studies that reported association between *LINE-1* methylation and smoking status.[25] Cigarette smoke is considered one of the most powerful environmental modifiers of DNA methylation.[30,31] For example, an experimental study showed that cigarette smoke can induce DNA demethylation in repeat elements such as *LINE-1*.[32] The specific mechanisms of how cigarette smoke may alter DNA methylation are becoming better understood and may be reviewed elsewhere.[33] Our findings suggest that the toxic effects of tobacco could be at least partly mediated by modulating the epigenetic landscape. On the other hand, higher *LINE-1* methylation has been previously described in individuals with higher exercise levels,[34,35] suggesting that an increase in *LINE-1* methylation might be associated with healthy lifestyle habits. As epigenetic marks are potentially reversible, this result may have public health implications and merits further investigation.

Methylation of DNA is a biochemical process in which a methyl group is added to DNA nucleotides. Several nutrients act as key enzyme cofactors and play essential roles in methyl group metabolism and DNA methylation in particular, being riboflavin, vitamin B12 and folate the major determinants of one-carbon metabolism.[36] In this sense, our results confirm the importance of riboflavin in DNA methylation process.

Iron intake was positively associated with *LINE-1* methylation level even after adjustment for gender, calories and body weight. A study with 892 individuals also showed similar association between iron intake and *LINE-1* methylation in leukocytes.[25] Iron, together with 2-oxoglutarate and oxygen, is an essential cofactor for the 10–11 translocation (TET) family of proteins that hydroxylates 5-methylcytosine to 5-hydroxy methylcytosine and further oxidizes to 5-carboxylcytosine and 5-formylcytosine, which have all been suggested to be precursors for both active and passive DNA demethylation.[37] The mechanisms are not well understood, but these data reinforce the role of dietary iron on the methylation status.

A recent trial explored the associations between changes in lifestyle modifications, such as diet, and global epigenetic biomarkers in blood of overweight female breast cancer survivors. After a weight loss intervention consisting on dietary and physical activity changes, *LINE-1* methylation levels were significantly elevated as compared to baseline.[38] Particularly, a 10 % increase in the frequency of fruit consumption was associated with an increase in *LINE-1* methylation levels of 0.42 %. In a study with one 177 young healthy women, those who

Table 3. Lifestyle features (mean ± SD) for **all individuals** categorized according to the median of ***LINE-1* methylation (%)**.

Lifestyle features	*LINE-1*≤83.03% (n=54)	*LINE-1*> 83.03% (n=53)	P Value
Vitamin supplementation users (%)[a]	6.66	5.10	0.569
Current smokers (%)[a]	20.75	5.50	**0.012**
Smoking (cigarettes/day)[b]	3.6 ± 4.8	0.9 ± 1.7	**0.041**
Regular practice of sport (%)[a]	61.96	79.62	0.050
MET (h/day)[b]	30.3 ± 9.0	39.2 ± 10.0	**0.047**

MET, activity metabolic equivalent;
[a]Chi-Square Test for dichotomous variables was performed.
[b]Not normal distribution. Analyzed by Mann-Whitney U test.

Table 4. Anthropometric, clinical and biochemical data (mean ± SD) of **all individuals (N=107)** categorized by tertiles of *TNF-α* methylation (%).

Variables	T1 <13.41%	T2 13.41 to 20.45 %	T3 >20.45%	P-for linear trend
Age (y)	22.8 (3.5)	23.9 (3.6)	23.5 (3.0)	0.845
BMI (kg/m^2)	21.8 (2.4)	22.2 (2.7)	21.8 (2.9)	0.806
Waist perimeter (cm)	79.7 (7.7)	79.5 (8.3)	**74.9 (7.9)**[a,b]	**0.012**
Hip perimeter (cm)	95.2 (6.0)	95.7 (6.6)	94.5 (7.1)	0.344
Waist-to-hip ratio	0.84 (0.06)	0.83 (0.05)	**0.79 (0.05)**[a,b]	**0.007**
Waist-to-stature ratio	0.47 (0.04)	0.46 (0.04)	**0.44 (0.05)**[a]	**0.034**
Total body fat - BIA (%)[a]	23.5 (6.17)	23.8 (6.16)	24.0 (6.39)	0.578
Body fat mass - BIA(kg)	14.3 (4.2)	15.0 (4.3)	15.0 (5.8)	0.716
Truncal fat (%)	57.1 (6.1)	59.1 (7.0)	58.2 (6.6)	0.966
Sum of 4 STs (mm)[a]	59.3 (20.4)	63.3 (24.8)	60.4 (25.2)	0.567
Body free fat mass - BIA(kg)	47.5 (10.1)	48.2 (10.6)	45.7 (10.0)	0.772
Systolic blood pressure (mmHg)	10.9 (0.91)	10.7 (0.93)	11.1 (0.89)	0.346
Diastolic blood pressure (mmHg)	7.2 (0.67)	7.3 (0.65)	7.4 (0.66)	0.953
Glucose (mg/dL)[a]	90.6 (7.0)	90.5 (6.7)	91.0 (6.2)	0.709
Insulin (μU/mL)	16.0 (3.9)	14.7 (2.6)	15.5 (4.1)	0.621
HOMA-IR [a]	3.71 (1.05)	3.43 (0.62)	3.62 (0.88)	0.845
Total cholesterol (mg/dL)	165.6 (31.9)	159.9 (24.5)	152.7 (29.6)	0.673
HDL-c (mg/dL)[a]	47.8 (10.6)	44.7 (10.0)	45.9 (10.16)	0.667
LDL-c (mg/dL)[a]	97.8 (24.8)	94.3 (22.1)	**85.8 (19.9)**[a]	**0.050**
VLDL-c (mg/dL)	20.3 (8.5)	20.1 (7.1)	19.6 (8.3)	0.498
Triacylglycerol (mg/dL)	101.7 (42.6)	100.5 (35.8)	98.3 (41.8)	0.671
Total cholesterol-to-HDL-c ratio[a]	3.51 (0.53)	3.62 (0.88)	3.34 (0.65)	0.217
CRP-hs	2.02 (2.67)	1.20 (1.39)	1.30 (1.10)	0.186
IL-6 (pg/mL)	16.39 (14.14)	15.86 (14.17)	**28.5 (22.4)**[a,b]	**0.002**
TNF-α (pg/mL)[a]	5.97 (2.10)	4.60 (1.79)	**4.09 (2.43)**[a]	**0.048**
C3 complement[a]	111.6 (28.5)	113.6 (20.5)	112.2 (22.3)	0.416
Ceruloplasmin (mg/dL)[a]	37.7 (8.2)	35.7 (6.8)	37.3 (9.2)	0.927
Adiponectin	28.5 (11.9)	31.5 (21.8)	32.1 (16.2)	0.845
GPx activity (nmol/[ml/min])[a]	492 (213)	560 (318)	636 (269)	0.667
Total antioxidant capacity (mM)[a]	1.58 (0.79)	1.64 (0.73)	1.75 (0.99)	0.671
Ox-LDL (U/L)[a]	66.26 (27.56)	69.70 (35.45)	73.92 (21.37)	0.498
Selenium (ng/g of nail)	0.40 (0.08)	0.41 (0.09)	**0.36 (0.06)**[a]	**0.017**
Copper (ng/g of nail)	8.02 (6.00)	6.69 (3.69)	6.20 (4.14)	0.416
Zinc (ng/g of nail)	118.8 (25.2)	133.9 (77.1)	122.0 (25.5)	0.268

BIA: Electrical bioimpedance; HOMA-IR: homeostatic model assessment; CRP-hs: high-sensitivity C-reactive protein; GPx: Glutathione peroxidase; Ox-LDL; Oxidized low-density lipoprotein; ST skinfold thickness. One-way ANOVA test with linear contrast analysis were performed.
[a]different from T1.
[b]different from all tertiles.

had consumption of fruit below the median value (i.e., <201 gr/day) were 3.7 times more likely to display *LINE-1* hypomethylation than women whose consumption was above the median (OR 3.7; 95 % CI 1.4–9.5).[39]

Several studies have examined the association between BMI and *LINE-1* methylation levels with conflicting results.[40] Elevated BMI has been shown to be associated with lower *LINE-1* methylation in some investigations,[41,42] but, in other study, participants with BMI ≥40 kg/m^2 had higher *LINE-1* methylation levels than those with BMI ≤25.[43] On the other hand, some studies found no associations between BMI and *LINE-1* methylation.[44,45] Although the current research did not find an association between BMI and *LINE-1* status, body weight

Table 5. Relative expression and percentage of methylation data (mean ± SD) for **all participants (n=105)** categorized by median of *TNF-α* methylation (%).

Variables	T1 <13.41%	T2 13.41 to 20.45 %	T3 >20.45%	P-for linear trend
Relative expression				
IL-6[*]	1.09 (0.67)	1.01 (0.65)	0.98 (0.59)	0.731
IL-18[*]	1.42 (0.60)	**1.95 (0.86)**[a]	**1.97 (0.98)**[a,b]	**0.005**
ICAM-1[*]	0.66 (0.34)	0.71 (0.30)	0.76 (0.38)	0.483
RIL-1[*]	0.61 (0.35)	0.70 (0.36)	0.59 (0.37)	0.381
TNF-α[*]	1.18 (0.44)	1.10 (0.46)	**0.93 (0.39)**[a]	**0.041**
Percentage of methylation				
IL-6 (%)	51.19 (7.44)	54.31 (10.07)	**57.69 (10.78)**[a]	**0.015**
LINE-1 (%)	81.07 (7.81)	84.61 (9.02)	**86.05 (8.59)**[a]	0.053

[*]AU: arbitrary units. One-way ANOVA test with linear contrast analysis were performed.
[a]different from T1.
[b]different from all tertiles.

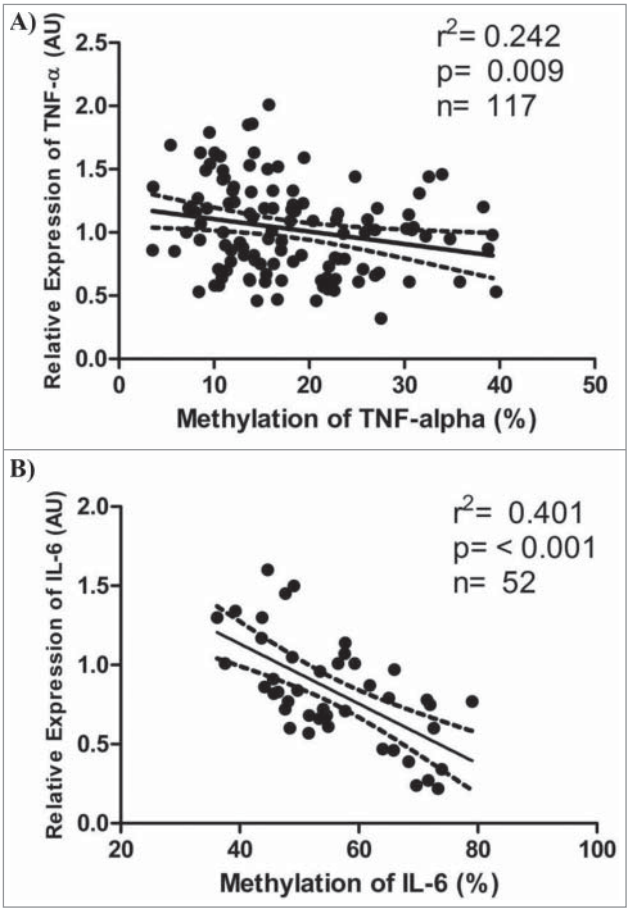

Figure 3. Associations between relative expression and methylation levels of *TNF-α* and *IL-6*. A) Pearson's correlation (r^2) between relative expression in arbitrary units (AU) and methylation levels (%) of *TNF-α*. B) Pearson's correlation between relative expression (AU) and methylation levels (%) of *IL-6* (only in men). The dotted lines represent the confidence rating limit (IC-95%).

and *LINE-1* were positively associated. A recent study of our group has shown that *LINE-1* methylation was a biomarker of weight loss in obese subjects.[6] In a recent randomized, crossover study of a 6-month weight loss intervention, over a 12-month period, changes in percentage body fat were positively associated with *LINE-1* methylation (β=0.19, p=0.001).[38] In that study, *LINE-1* methylation was statistically significantly elevated at 6 and 12 months compared to baseline. Moreover, analysis of rectal biopsies free of colorectal disease obtained from 185 individuals showed that higher waist and hip perimeters were associated significantly with lower methylation of *LINE-1*.[26] Interestingly the present study found that central obesity and fat mass percent were significant predictors for low *LINE-1* methylation levels, although no associations remained after adjusting for gender and smoking.

In the present research, *TNF-α* methylation analysis demonstrates a negative association with central adiposity. In this sense, a cross-sectional study with 40 normal-weight young women showed that those with higher truncal fat (≥52.3%) presented lower methylation of the *TNF-α* gene promoter than those with lower truncal adiposity.[9] In 2 other works, authors concluded that *TNF-α* methylation levels could be used as epigenetic biomarker concerning the response to a low-calorie diet.[8,46] Indeed, methylation profile could help to predict the susceptibility to weight loss as well as some obesity-related comorbidities, such as hypertension or type 2 diabetes.[47] These results are in line with the findings from the present work, suggesting an important relationship between epigenetics mechanisms and body composition.

Recent studies also reported a good correlation between leukocyte DNA methylation level and degree of inflammation, suggesting that cytosine methylation may represent a novel mechanism underlying the association between obesity, inflammation and disease risk.[48,49] In this context, our study observed that the methylation levels of both, *TNF-α* and *IL-6*, negatively correlated with the mRNA levels of both cytokines in white blood cells. However, it is important to note that a relationship between *IL-6* mRNA and plasma levels was not found, which can be explained because this cytokine is not only released to plasma by white blood cells, but also by muscle, adipose tissue and other organs. It is important to highlight that the present study was conducted in young, apparently healthy individuals, with no physiopathological conditions. Thus, it is possible to consider this negative association as a compensatory mechanism, with the purpose of restoring homeostatic balance.

This study presents some limitations that should be considered. Firstly, the dietary assessment software did not provide important information in order to discuss the data deeply. We only examined consumption of specific nutrients, which does not account for the combinations and interactions of multiple nutrients in the human diet. Moreover, random measurement error in ascertaining dietary intake with the food frequency questionnaire could have resulted in some misclassification of intake. Secondly, some individuals were not included in the present analyses owing to the lack of methylation data. However, there was no relevant difference in selected characteristics (except age and gender) between those with and without those data. To minimize the potential bias, all the models were adjusted for age, gender and smoking status when necessary. Thirdly, we did not account for the proportion of white blood cell subtypes from the buffy coat in the analyses. DNA methylation is tissue- and cell-specific, and it could depend on the cell type distribution. In this sense, there is some evidence that DNA methylation is inversely related to

the proportion of lymphocytes.[44] Finally, the cross-sectional design of this research does not allow determination of causality and the associations observed should be checked carefully. Therefore, type I/II errors cannot be ruled out, as well as the involvement of other mechanisms in the regulation of methylation status. Future studies are required to replicate and extend these findings to different populations.

As a conclusion, our findings contributes to the growing evidence that the health impacts of changes in dietary habits and other lifestyle factors may be mediated through epigenetic modifications. Moreover, our work provides preliminary evidence for the use of WBC DNA methylation biomarkers to monitor lifestyle interventions trials and identify early biomarkers of metabolic complications.

Conclusions

This study suggests that higher *LINE-1* and *TNF-α* methylation could be associated with some indicators of adiposity, especially body fat, waist girth and waist-to-hip ratio. In addition, high *LINE-1* methylation levels are directly associated with some dietary factors (calories, iron) but inversely with total fat mass. Moreover, high *LINE-1* methylation is associated with a healthier lifestyle (physical activity and not smoking) and might be an early indicator of resistance to win adiposity even when eating more food.

Authors' Contributions

Contributors JLM-R: field work, data collection, analysis, and writing of the manuscript; FIM and MLM: design, analysis, and editing of the manuscript; DMM: design, field work, and data collection; JB: project leader, scientific interpretation, financial management, and editing of the manuscript; JAM: design, field work, scientific interpretation, financial management, and editing of the manuscript. All authors read and approved the final manuscript.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We wish to thank the volunteers of this study and the excellent technical assistance of Ana Lorente and Caroline Müller. This study was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG-APQ-01609-10), CAPES Foundation (Ministry of Education of Brazil, process n° 6409-13-0), CIBERobn, MINECO (ref. AGL2013-45554-R) and the Health Department of the Government of Navarra (482009).

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7. GENERAL CONCLUSIONS

This work provided evidence that the quality of a Mediterranean-based nutritional intervention may lead to changes in the expression of some miRNAs (let-7b, and miR-155-3p) in WBC from patients with MetS. Specifically, low consumption of total fat and saturated fatty acids were associated with higher expression of let-7b after the nutritional intervention.

The *in vitro* study confirmed the pro- and anti- inflammatory roles of miR-155-3p and let-7b on THP-1 cells, respectively. However, depending of the differentiation state of cell and the type of fatty acids in the culture medium, these miRNAs may act in an unexpected way.

The methylation study suggested that higher global DNA (LINE-1) and TNF methylation may be associated with some indicators of adiposity. High LINE-1 methylation levels were directly associated with some dietary factors (calories, iron, riboflavin) but inversely with total fat mass. Moreover, high LINE-1 methylation was associated with a healthier lifestyle (physical activity and not smoking) and might be an early indicator of resistance to gain adiposity even when eating more food.

Finally, our data suggested that the modulation of the expression of miRNAs and DNA methylation through a nutritional approach might be a future alternative or adjunct to current pharmacologic therapy targeting endogenous miRNAs or determined genes. Furthermore, this study also expands the understanding of the role of fatty acids on the epigenetic regulation in immune cells.

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