

UNIVERSIDADE ESTADUAL PAULISTA
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**AVALIAÇÃO DO ESTRESSE OXIDATIVO E INGESTÃO
DIETÉTICA DE ANTIOXIDANTES EM INDIVÍDUOS
PORTADORES DE SÍNDROME METABÓLICA**

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Tese apresentada à Faculdade de
Medicina, Universidade Estadual
Paulista “Júlio de Mesquita Filho”,
Câmpus de Botucatu, para obtenção
do título de Doutora em Patologia.

Botucatu – SP
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Universidade Estadual Paulista
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Sumário

Síndrome Metabólica, Inflamação e Estresse Oxidativo	6
Defesa Antioxidante	8
Biomarcadores do estresse oxidativo	9
Ingestão dietética, estresse oxidativo e síndrome metabólica	8
Considerações Finais	9
Referências bibliográficas	13
Evaluation of the redox state in women with and without metabolic syndrome and the influence of patterns of dietary intake on body mass index and DNA damage	17
Abstract	18
Introduction	19
Material and methods	20
<i>Study population</i>	20
<i>Definition of metabolic syndrome</i>	20
<i>Assessment for components of metabolic syndrome</i>	21
<i>Evaluation of total antioxidant capacity (TAP)</i>	21
<i>Evaluation of malondialdehyde (MDA)</i>	22
<i>Extraction of lymphocytes</i>	22
<i>Comet assay</i>	22
<i>Plasma evaluation of carotenoids, retinol and α-tocopherol</i>	24
<i>Assessment of dietary intake</i>	24
<i>Statistical analysis</i>	24
Results	25
Discussion	28
Conclusion	33
References	33

Tabelas

Table 1. Anthropometric data, blood pressure and plasmatic biochemistry profile in G1, G2 and G3.....	25
Table 2. DNA damage and oxidative stress in G1, G2 and G3.	26
Table 3. Dietary intake of macronutrients in G1, G2 and G3.....	26
Table 4. Dietary intake of micronutrients in G1, G2 and G3.	27
Table 5: Plasma levels of carotenoids, α -tocopherol, uric acid and retinol in G1, G2 and G3.	27
Table 6. Final linear regression model relating DNA damage with consumption of macro- and micronutrients and plasmatic variants.	28
Table 7. Final linear regression model relating BMI with consumption of micronutrients and plasmatic variants.	28

Lista de siglas e abreviações

SM - síndrome metabólica

RI – resistência à insulina

NCEP - ATPIII – National Cholesterol Education Program – Adult Treatment Panel III

IDF – International Diabetes Federation

OMS – Organização Mundial da Saúde

DM – *diabetes melitus*

EO – estresse oxidativo

IL-1 – interleucina-1

IL-6 – interleucina-6

TNF- α – fator de necrose tumoral- α

MDA – malondialdeído

HNE – 4-hidroxi-nonenal

AGEs – produtos finais de glicação

ALEs – produtos finais de lipoxidação

TBA – ácido tiobarbitúrico

HPLC – cromatografia líquida de alta precisão

TAP – capacidade antioxidante total

AUC – área sobre a curva

AAPH – azobis-amidinopropane dihydrochloride

ENDO III – endonuclease III

FPG – fosfatidil-pirimidina glicosilase

MetS – metabolic syndrome

BMI – body mass index

CVD – cardiovascular disease

T2D – type 2 diabetes

WC – waist circumference

OS – oxidative stress

SBP – systolic blood pressure

DBP – diastolic blood pressure

TAP – total antioxidant capacity

HPLC – high pressure liquid chromatography

MDA – malondialdehyde

PUFAs – polyunsaturated fatty acids

SÍNDROME METABÓLICA, INFLAMAÇÃO E ESTRESSE OXIDATIVO

A síndrome metabólica (SM), também conhecida como síndrome X, síndrome da resistência à insulina (RI), ou síndrome plurimetabólica, é caracterizada pelo agrupamento de fatores de risco cardiovasculares como hipertensão arterial, resistência à insulina, obesidade central e dislipidemia aterogênica (LDL-colesterol alto, triglicerídeos altos e HDL-colesterol baixo) e apresenta elevada prevalência na população mundial ⁽¹⁾.

Para o diagnóstico da SM existem pelo menos 3 critérios empregando os cinco componentes: circunferência da cintura, pressão arterial, glicemia, trigliceridemia e HDL-colesterol. O *National Cholesterol Education Program – Adult Treatment Pannel III* (NCEP -ATP III) ⁽²⁾ utiliza pelo menos 3 componentes alterados. O *International Diabetes Federation* (IDF) ⁽³⁾ considera o perímetro abdominal mais 2 componentes alterados e a Organização Mundial da Saúde (OMS) ⁽⁴⁾ utiliza a relação cintura/quadril, a presença de *diabetes melitus* (DM) tipo 2 ou a resistência à insulina, a microalbuminúria, hipertensão arterial e trigliceridemia.

Observando esses componentes que classificam o indivíduo como portador de SM, pode-se notar que todos eles são complicações que comumente acometem indivíduos obesos, mostrando que existe uma ligação direta entre essas duas doenças ⁽⁵⁾. Em geral, essas duas doenças se iniciam com o aumento do tecido adiposo abdominal, o qual é metabolicamente mais ativo, contendo uma quantidade maior de macrófagos residentes comparado a outros depósitos de gordura ⁽⁶⁾. Essa condição favorece a inflamação e o estresse oxidativo (EO), que são fatores precursores de diversas complicações, mas principalmente de componentes que envolvem SM, como RI, hipertensão arterial e hiperlipidemia ⁽⁷⁾⁽⁸⁾.

A inflamação e o EO acontecem quando a oferta energética passa a exceder a capacidade de armazenamento dos adipócitos e, com isso, eles hipertrofiam ⁽⁹⁾. Essa hipertrofia implica em maior liberação de adipocinas, dentre elas, citocinas pró-inflamatórias, como interleucina-1 (IL-1), interleucina-6 (IL-6) e o fator de necrose tumoral-alfa (TNF- α), resultando em uma inflamação crônica de baixo grau, que se inicia no tecido adiposo e, com o tempo, atinge a circulação e outros órgãos ^(10, 11). Uma das primeiras consequências dessa inflamação é a RI, uma vez que o TNF- α impede a fosforilação do receptor do hormônio, interferindo em sua cascata de ação e impedindo sua função ⁽¹²⁾. A RI e o DM tipo 2 classicamente são acompanhados pela dislipidemia,

que é caracterizada pela hipertrigliceridemia , baixos níveis de HDL-colesterol e aumento de LDL-colesterol ⁽¹³⁾. A RI diminui a função do hormônio, levando a uma alteração na estocagem de lipídeos, que é um mecanismo insulino- dependente ⁽¹⁴⁾.

Outra causa da inflamação é o EO, que pode ser desencadeado pela necrose dos adipócitos. Quando a massa adiposa aumenta, a irrigação insuficiente pode levar à falta de oxigênio e, assim, à necrose celular. O processo de fagocitose para eliminação dessas células mortas resulta em maior infiltrado inflamatório e também em EO, pela liberação de radicais livres, como óxido nítrico e peróxido de hidrogênio ^(15, 16), condições que acarretam consequências no desenvolvimento dos componentes da SM ⁽¹⁷⁾.

O EO é classicamente definido como um evento resultante do desequilíbrio entre substâncias oxidantes e antioxidantes ^(18, 19), geradas em um cenário de reações de óxido-redução, onde a oxidação implica em perda de elétron e a redução, em ganho. Visto que a geração e a ação dessas substâncias dependem desse sistema de óxido-redução, autores têm usado o termo desequilíbrio do sistema redox para se referir ao EO ^(20, 21). Popularmente denominados radicais livres, os produtos oxidantes incluem espécies reativas de oxigênio e nitrogênio, que realizam a oxidação de lipídeos (lipoxidação) e glicose (glicação), substâncias que se encontram em excesso na obesidade, seja por um consumo excessivo de alimentos, o qual aumenta a quantidade de nutrientes energéticos na circulação, bem como pelo processamento desses nutrientes dentro das células ⁽²²⁾. Os produtos gerados na lipoxidação são o malondialdeído (MDA), glioxal, acroleína, 4-hidroxi-nonenal (HNE) e os gerados na glicação são o glioxal e o metil glioxal. Esses compostos se ligam ao grupamento amino dos aminoácidos, resultando em produtos finais de glicação (AGEs) e de lipoxidação (ALEs) ⁽²³⁾ que são altamente reativos e participam do desenvolvimento de outros componentes da SM.

Estudos clínicos em pacientes com hipertensão arterial demonstram que a pressão arterial sistólica e diastólica correlaciona-se positivamente com o biomarcadores do EO e negativamente com os níveis de antioxidantes ⁽²⁴⁻²⁶⁾. Esse fato é atribuído à disfunção endotelial causada pelas espécies reativas, EO, inflamação vascular, a qual realiza uma desestabilidade entre os produtos vasoconstritores e vasodilatadores. Isso é evidenciado por uma associação inversa entre fatores que desencadeiam a vasodilatação, níveis plasmáticos de MDA e associação positiva com antioxidantes ⁽²⁷⁾.

O EO desempenha um papel importante na patogênese da RI por desregular a liberação de adipocinas pelo tecido adiposo como TNF- α e IL-6 as quais podem desencadear inflamação, um mecanismo já descrito acima ⁽²⁸⁻³⁰⁾. Assim, pode-se observar que as complicações da obesidade são fatores que quando associados, determinam a SM e que a inflamação o EO são fatores diretamente relacionados e presentes nas duas patologias.

Defesa Antioxidante

O EO é controlado pelo sistema de defesa antioxidante endógeno, que inclui enzimas antioxidantes, como a superóxido dismutase, a catalase, glutathione peroxidase, glutathione reductase e compostos não enzimáticos como ferritina, transferrina, bilirrubina, ceruloplasmina, e albumina e os captadores de baixo peso molecular, como o ácido úrico e ácido lipoico ⁽³¹⁾. Existem, também, os antioxidantes exógenos, oriundos de frutas e hortaliças, dentre eles os hidrofílicos como vitamina C e os flavonoides e os lipofílicos, como a vitamina E os carotenoides. Os carotenoides são distribuídos em um grupo de pigmentos que dão cor amarelo-alaranjado às plantas, animais e microorganismos. Mais de 700 carotenoides foram descobertos, porém a luteína, zeaxantina, criptoxantina, alfa-caroteno, beta-caroteno e licopeno representam 95% dos carotenoides no plasma humano ⁽³²⁾.

Os antioxidantes são capazes de interceptar os radicais livres gerados pelo metabolismo celular ou por fontes exógenas por meio da doação de átomos de hidrogênio a estas moléculas, interrompendo a reação em cadeia, o que impede o ataque sobre os lipídeos, os aminoácidos das proteínas, a dupla ligação dos ácidos graxos poliinsaturados e as bases do DNA, evitando a formação de lesões e perda da integridade celular ⁽³³⁾. Outro papel dos antioxidantes é no mecanismo de proteção, o qual age no reparo das lesões causadas pelos radicais livres, um processo relacionado à remoção de danos da molécula de DNA e à reconstituição das membranas celulares danificadas ⁽³⁴⁾.

A literatura relata que uma dieta rica em frutas, legumes e grãos previne diversas doenças, entre elas as doenças cardiovasculares e o câncer ^(35, 36). Esse fato ocorre porque os antioxidantes exógenos e endógenos agem em sinergismo no combate aos radicais livres ⁽²⁴⁾. Entretanto é importante ressaltar que essa ingestão necessita ser de

maneira constante e regrada e que a ingestão de vitaminas em forma de suplemento pode resultar em efeito pró-oxidante, chamado estresse antioxidativo⁽³⁷⁾.

Biomarcadores do estresse oxidativo

As espécies reativas são muito instáveis e possuem uma meia vida muito curta, o que torna um grande desafio a avaliação precisa dessas espécies. Para isso, foram desenvolvidos métodos para medir marcadores de produtos que refletem o estado redox em amostras biológicas; são produtos de oxidação de lipídeos, DNA e proteínas⁽³⁸⁾. Dentre os mais comuns estão os produtos de peroxidação lipídica pelo fato dos ácidos graxos poliinsaturados (como fosfolipídeos e glicolipídios) e colesterol serem as moléculas mais vulneráveis às espécies reativas. Quando esses lipídeos são oxidados, ocorre a formação de dois produtos classicamente aferidos em amostras biológicas, o MDA e o isoprostrano^(39, 40).

O MDA é formado pela peroxidação de ácidos graxos poliinsaturados e pode interagir com proteínas. Ele pode ser detectado através do ácido tiobarbitúrico (TBA), por um método colorimétrico baseado na reação MDA e TBA, formando uma cor rosa, aferindo, assim, o MDA e todas as espécies que reagem com esse ácido⁽⁴¹⁾. O MDA, especificamente, pode ser avaliado pela cromatografia líquida de alta precisão (HPLC). A mesma reação entre MDA e TBA acontece, mas devido ao detector de fluorescência do aparelho, somente o MDA é identificado, tornando esse teste mais específico⁽⁴²⁾.

O isoprostano é um produto estável de peroxidação lipídica e pode ser aferido tanto nos tecidos como nos fluidos biológicos, incluindo urina, plasma e líquido. O nível desse composto no plasma e na urina correlaciona-se com os níveis de espécies reativas e EO em estudos experimentais em humanos⁽⁴³⁾. Contudo, em indivíduos saudáveis com risco de obesidade e hiperlipidemia seus níveis estão aumentados, sugerindo ser um bom marcador para riscos cardiovasculares⁽⁴⁰⁾.

Capacidade total antioxidante (TAP) também pode ser considerada um marcador de EO, uma vez que afere o estado da capacidade antioxidante em fluidos biológicos. Esse método obtém uma visão mais profunda sobre o envolvimento do EO em várias condições fisiopatológicas, mas também monitora a eficácia das intervenções antioxidantes⁽⁴⁴⁾. Nesse método, a capacidade antioxidante da amostra é quantificada comparando a área sobre a curva (AUC) relativa à cinética de oxidação do BODIPY (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-díaza-s-indacene), um composto

lipofílico fluorescente oxidável, frente ao iniciador de radical 2,20 -azobis-(2-amidinopropane) dihydrochloride (AAPH) em relação à oxidação de fosfatidilcolina usada como uma referência de matriz lipídica ⁽⁴⁵⁾.

Outra abordagem para aferir a capacidade antioxidante é medir os antioxidantes individualmente. No entanto, existem muitos e isso demandaria em tempo de dosagem e exigiria uma variedade de técnicas analíticas, instrumentos e procedimentos. Além do que esta abordagem também é pobre em informações sobre a possível sinergia e cooperação entre os antioxidantes hidrofílicos e lipofílicos ⁽⁴⁵⁾.

Proteínas e DNA também são moléculas altamente suscetíveis a modificações por alterações no estado redox ⁽⁴⁶⁾. A oxidação de proteínas acontece quando aminoácidos das proteínas (prolina, arginina, treonina, lisina, histidina e cisteína) se ligam a produtos de lipoxidação e glicoxidação, formando grupos carbonil. Essa reação chamada carbonilação pode ser irreversível e levar a alterações na sua função biológica. ⁽²³⁾ A detecção desses produtos tóxicos (carbonil) pode ser feita pelo espectrofotômetro de massa ⁽⁴⁷⁾.

Quanto à lesão do DNA, pode ser feito o teste do cometa, uma técnica de eletroforese celular em microgel, muito útil e largamente empregada para avaliação de danos e reparos de DNA em células individuais. Seu princípio básico é o da lise de membranas celulares, seguida pela indução da migração eletroforética do DNA liberado em matriz de agarose. Quando vista ao microscópio, a célula migrada adquire a forma aparente de um cometa, com cabeça, região nuclear e cauda, que contém fragmentos ou fitas de DNA que migraram na direção do ânodo. A análise dos cometas baseia-se no grau de fragmentação do DNA e sua migração pela microeletroforese ⁽⁴⁸⁾. Medidas como o comprimento total da cauda e a densidade de DNA fornecem dados indiretos sobre o estado do DNA da amostra. Para detectar os danos oxidativos utilizam-se as enzimas de reparo endonuclease III (ENDOIII) e a fosfatidil-pirimidina de DNA glicosilase (FPG), pelas quais podemos detectar a oxidação nas bases de purina e pirimidinas ⁽⁴⁹⁾, respectivamente.

Ingestão dietética, estresse oxidativo e síndrome metabólica

De acordo com o que foi descrito, percebe-se que, quando ocorre um desequilíbrio alimentar, tanto por uma grande oferta de nutrientes, como por uma baixa ingestão de antioxidantes, acarreta um quadro de EO, promovendo obesidade e SM ⁽²²⁾.

Corroborando esse fato, a literatura aponta que indivíduos portadores de SM e obesos possuem um alto consumo de gorduras e açúcares oriundos de alimentos industrializados com alto teor de sódio⁽⁵⁰⁻⁵²⁾ e têm uma baixa ingestão de antioxidantes.

Dietas com alto teor de antioxidantes como a conhecida dieta do Mediterrâneo, a qual é composta por óleo de oliva, frutas, vegetais, cereais, castanhas e uma quantidade menor de carnes vermelhas e alimentos ricos em açúcar, também são maneiras de controlar o EO e a inflamação^(53, 54). Pesquisadores mostraram que indivíduos portadores de SM e obesos retardaram e atenuaram as complicações como RI, hipertensão arterial e hiperlipidemia, quando tiveram uma intervenção e passaram a consumir esse tipo de dieta. Um dos mecanismos apontados para essa melhora foi a diminuição do EO e inflamação⁽⁵⁴⁻⁵⁸⁾.

Dentre esses estudos, Mitjavila e colaboradores⁽⁵⁹⁾ observaram a diminuição de alguns marcadores de EO após um ano de intervenção dietética. Nesse mesmo estudo, indivíduos com SM que consumiram a dieta do mediterrâneo foram comparados a um grupo que consumiu uma dieta apenas com níveis baixos de gordura e foi mostrado que a dieta mais rica em antioxidante foi a que mais resultou em melhora dos marcadores de EO e diminuiu os danos no DNA. Isso mostra a importância da qualidade e da regularidade da dieta e a eficácia dos antioxidantes na sua composição.

Contudo, além da investigação do hábito alimentar desses indivíduos, é de extrema importância examinar os indicadores de EO e inflamação, para que haja uma ferramenta a mais na conduta de tratamento dessas enfermidades.

Considerações Finais

De acordo com a abordagem dessa revisão, pode-se ressaltar o envolvimento da inflamação e, principalmente, do EO na patogênese da SM. Foi salientado que a obesidade pode ser um evento chave no desenvolvimento da síndrome; por isso, uma das estratégias de tratamento é controlar e atenuar o EO, para evitar o desenvolvimento de complicações que levam à SM.

O fator principal no desencadeamento dessas duas patologias é o desequilíbrio alimentar. Nas últimas décadas, houve um aumento na ingestão de açúcares e gorduras e, em paralelo, uma redução no consumo de frutas e vegetais que, por si só, promove o EO. Isso tem sido a causa essencial na crescente epidemia de doenças crônicas, que atingem países desenvolvidos e em desenvolvimento. Uma diminuição na ingestão

alimentar já seria um fator determinante para a diminuição do EO. Esse excesso de calorias, na forma de açúcares e gorduras ingeridas, combinado com um estilo de vida sedentário, obriga o corpo a gerenciar o excesso de energia que deve ser metabolizada. Uma das soluções é aumentar o armazenamento de energia na forma de gordura, levando à obesidade. Outros macronutrientes sofrem oxidação na mitocôndria, favorecendo um aumento da produção de radicais livres e o EO, que tem sido proposto como um mecanismo unificador, ligando ingestão excessiva de nutrientes à RI, à SM e ao diabetes. Portanto, observamos que, além dos aspectos quantitativos da ingestão de alimentos, é importante o equilíbrio alimentar, onde antioxidantes precisam ser introduzidos por terem um papel crucial na defesa antioxidante, que está prejudicada na SM.

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Evaluation of the redox state in women with and without metabolic syndrome and the influence of patterns of dietary intake on body mass index and DNA damage

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Keywords: Metabolic Syndrome, body mass index, comet assay, micronutrients, macronutrients, women

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Conflicts of Interest

The authors declare no conflict of interest.

Abstract: The aim of the study was to evaluate the redox state of women of reproductive age with and without metabolic syndrome (MetS) and the influence of food consumption on the body mass index (BMI) and DNA damage. Were selected 57 women attended in the Endocrinology Clinic of Botucatu Medical, who were divided according to the presence or absence of MetS and healthy women volunteers. The control group (G1, n=36), women with a BMI in the range of normal weight (18,5- 24.9 kg/m²); G2, n=21, group IMC \geq 25 kg/m² without MetS and G3, n=23, with a BMI \geq 25 kg/m² with MetS. Were evaluated: dietary intake of macronutrients and micronutrients; plasma total antioxidant capacity (TAP), malondialdehyde (MDA), carotenoids, retinol and α -tocopherol, and the comet assay in peripheral lymphocytes. The control group had lower values of MDA, DNA damage and oxidized purines than overweight women and obese women with MetS. Pyrimidines were greater in G2 and TAP was higher in G3 compared to G1. Dietary intake differed significantly among groups: G2 and G3 consumed more protein and less vitamin C than G1; G2 had increased sodium intake; and G3 ingested less calcium than G1 and lower iron. The concentrations of α -carotene were lower in G2 and G3, the G3 group showed lower values for cryptoxanthin and β -carotene and higher values for retinol. Uric acid was proportional increasing BMI (G3 > G2 > G1). In addition, a greater consumption of protein and sodium correlated with higher DNA damage, and polyunsaturated fat and α -carotene appeared to provide a protection from DNA damage. The vitamin D intake and plasma α -carotene levels had a negative correlation with BMI. The data conclude that worsened nutritional status influences in metabolic and oxidative stress, and DNA damage and the diet quality and BMI are the factors that most influenced changes in these parameters. **Keywords:** Metabolic Syndrome, body mass index, comet assay, micronutrients, macronutrients, women

Evaluation of the redox state in women with and without metabolic syndrome and the influence of patterns of dietary intake on body mass index and DNA damage

Introduction

Metabolic syndrome (MetS) is a complex entity including risk factors for cardiovascular disease (CVD) and type 2 diabetes (T2D) ⁽¹⁾. Typical features of MetS are central obesity with a high waist circumference (WC) and insulin resistance, factors which eventually lead to high insulin, glucose, and triglyceride levels in blood as well as increased blood pressure, increasing the risk of developing T2D and CVD ⁽²⁾. MetS is multifactorial but often associated with obesity, which is triggered by ⁽¹⁾ a diet of poor quality, characterized by a low intake of fruits and vegetables and a high consumption of fats, sugars and sodium, processed foods among other nutrients ⁽²⁾ accompanied by a sedentary lifestyle ⁽³⁾. This situation is relevant because this eating habit with the passage of time triggers a change in antioxidant defense leading to the formation of free radicals exacerbated in biological systems which could lead to a change in the redox system, triggering oxidative stress (OS). This condition causes membrane peroxidation, inflammatory or degenerative pathological processes which lead to various complications metabolic, as insulin resistance, T2D and CVD ⁽⁴⁾. Therefore it is of utmost importance to assess how much the quality of the diet is associated with OS and change in body composition. Thus, the aim of the study was to evaluate the redox state of women of reproductive age with and without MetS and the influence of food consumption on the BMI and DNA damage.

Material and methods

Study population

To compose the groups with and without MetS were evaluated all women of reproductive age attended the Endocrinology Clinic of Botucatu Medical School from February 2012 to February 2013 with a BMI (body mass index) $> 25 \text{ kg/m}^2$. Were excluded women who consumed more than 60 g/day alcohol, used antioxidant supplements during the sixty days prior to the study, with altered hematological parameters and albumin, with changed liver and kidney function, cancer, smokers, those who used statins, those with diabetes, with changes in thyroid function, menopausal and those who refused to participate in the study. Thus, we selected 57 women, who were divided according to the presence or absence of MetS which was defined according to International Diabetes Federation (IDF) definitions ⁽⁵⁾. The control group consisted of healthy women volunteers also of reproductive age with a BMI in the range of normal weight (18,5- 24.9 kg/m^2). Thus, they participated in the study 80 women, who were divided into 3 groups, control group (G1, n = 36); group $\text{IMC} \geq 25 \text{ kg / m}^2$ without MetS (G2, n = 21) and the group with a $\text{BMI} \geq 25 \text{ kg/m}^2$ with MetS (G3, n = 23).

Definition of metabolic syndrome

The diagnosis of MetS was made when at least three of the following criteria, proposed by the IDF ⁽⁵⁾ were present: $\text{WC} \geq 80 \text{ cm}$; fasting glucose $\geq 110 \text{ mg/dL}$; blood pressure $\geq 130/85 \text{ mmHg}$; triglyceride levels $\geq 150 \text{ mg/dL}$; HDL-cholesterol $< 50 \text{ mg/dL}$; or abnormal WC more two changed components above.

The study was approved by the Ethics Committee on Human Research of the Botucatu Medical School, UNESP (Protocol 3788-2011). All participants signed a consent form.

Assessment for components of metabolic syndrome

Body weight was measured using platform-type digital Filizola anthropometric scales, accurate to 0.1 kg, with the subject barefoot and in minimal attire ⁽⁶⁾. Height was determined using a mobile stadiometer with a precision of 0.5 cm ⁽⁶⁾. The BMI was calculated by dividing the current body weight expressed in kilograms by the square of height in meters. The WC was measured at the midpoint between the last rib and the iliac crest, following recommendations in the literature ⁽⁷⁾. The systolic (SBP) and diastolic (DBP) blood pressure were measured according to the procedures described by the VI Brazilian Guidelines on Hypertension ⁽⁸⁾. Plasma concentrations of fasting glucose, triglycerides, total cholesterol and fractions, uric acid were obtained from the records of the Clinical Hospital, Faculty of Medicine of Botucatu, UNESP. Records from no more than 30 days previously were used. In cases where there was no medical record from the previous 30 days, a new collection was made.

Evaluation of total antioxidant capacity (TAP)

Serum TAP was determined by the method reported by Aldini and collaborators ⁽⁹⁾ and validated by Beretta and collaborators ⁽¹⁰⁾ for application to high throughput studies to measure total antioxidant capacity in both the hydrophilic and lipophilic components of serum. This method measures the rate of oxidation of 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591), a lipid-soluble fluorescent probe, and uses the lipid-soluble radical initiator 2,29-azobis(4-methoxy-2,4-dimethylvaleronitrile). Oxidation is determined by monitoring the appearance of green fluorescence of the oxidation product of BODIPY (lex = 500 nm, lem = 520 nm) using a 1420 multilabel counter (Wallac Victor 2, Perkin Elmer Life Sciences, Boston, Massachusetts, USA). The results are expressed as TAP

values, which represent the percent inhibition of BODIPY oxidation in human serum with respect to that occurring in a control sample consisting of BODIPY 581/591 in phosphatidylcholine liposomes.

Evaluation of malondialdehyde (MDA)

Plasma MDA was measured by high pressure liquid chromatography (HPLC) with fluorometric detection ⁽¹¹⁾ with the data expressed as $\mu\text{mol/L}$.

Extraction of lymphocytes

To evaluate the levels of DNA damage (comet assay) peripheral blood lymphocytes. Blood samples (3 mL) were placed into tubes containing 3 mL of RPMI 1640 medium (Sigma- Aldrich) and then placed carefully on 3 mL Histopaque[®]1077(sigma-Aldrich). After centrifugation at 2500 rpm for 30 minutes at 10°C, the layer of lymphocytes was removed and mixed with 3 mL RPMI 1640 medium and centrifuged again at 1500 rpm for 15 minutes. After this procedure, the supernatant was discarded and lymphocytes were resuspended to be used for the evaluation of DNA damage by comet assay.

Comet assay

Determination of oxidative DNA damage into DNA The alkaline comet assay ⁽⁴⁾, modified with lesion-specific enzymes, was used to detect single- and double-strand breaks, labile sites, oxidized purines and pyrimidines ⁽¹²⁾. Briefly, 10 μL of the isolated lymphocyte suspension (2×10^4 cells) were embedded into 0.5% low melting point agarose (Sigma) and spread on agarose-precoated microscope slides. Slides were immersed overnight in freshly prepared cold lysing solution [2.5 M NaCl, 100 mM

ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 1% sodium salt N-lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% dimethyl sulfoxide added fresh] at 4°C. After lysing, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD, USA) and then incubated at 37°C for 45 min with 100 µL of endonuclease III (EndoIII, 1:1000; New England Biolabs Inc), 100 µL of formamidopyrimidine-DNA glycosylase (FPG, 1:1000; New England Biolabs Inc), 100 µL of EndoIII recognizes oxidized pyrimidines (SBs EndoIII), while FPG identifies oxidized purines (SBs FPG), or with enzyme buffer only. Buffer only is used to identify SBs. Subsequently, the cells were exposed to alkali buffer (1 mM EDTA and 300 mM NaOH, pH =13.4) at 4°C, for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted in the same solution at 4°C, for 30 min, at 25 V and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 50µL SYBR Green (1:10000; Invitrogen) and analysed in a fluorescence microscope at x 400 magnification, using an image analysis system (Comet Assay IV—Perceptive Instruments, Suffolk, UK). One hundred randomly selected cells (50 from each of two replicate slides) were evaluated from each sample and the mean of the tail intensity (%DNA Tail) was determined. Tail Intensity, according to Comet Assay IV—Perceptive instruments, is defined as ‘the sum of all intensity values in the tail region minus those which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity’.

Plasma evaluation of carotenoids, retinol and α -tocopherol

Carotenoids, retinol and α -tocopherol were measured in 100 μ L of plasma by reversed-phase HPLC (Waters Alliance 2695 Separation Module, Waters, Wilmington, MA, USA). The column used was C30 (Waters Alliance, YMC carotenoid: 4.6 x 150 mm; 3.0 μ m). The measurements were performed as previously described by Yeum and collaborators⁽¹³⁾.

Assessment of dietary intake

To determine the habitual diet of the participants, women recorded their dietary intake over three 24-hour periods on non-consecutive days. These data were collected in the period prior to blood collection. To calculate the dietary intake was used Dietpro software⁽¹⁴⁾.

Statistical analysis

Comparisons between groups were made using one-way ANOVA followed by Tukey's multiple comparison test for symmetrical data. Asymmetric data was analyzed using a generalized linear model with gamma distribution followed by the Wald adjusted multiple comparison test. Data are presented as means with standard deviations. A stepwise multiple linear regression model was used to assess which nutrients influenced BMI. BMI was a continuous response variable and intakes of nutrients were the explanatory variables. In all tests, the significance level was set at 5%. All analyses were performed using SAS for Windows, v. 9.3.

Results

Table 1 shows the anthropometric, blood pressure and biochemical profile of these women included in the study and the comparison between groups. It is observed that the G1 group is younger than the G2 and G3 which has a higher body weight, increased BMI, WC and higher SBP. The G3 has a higher DBP to G1 and G2. Weight, BMI, WC and SBP were significantly different among the groups, and were all higher in G3 than the other groups. Height was not different among groups and DBP was significantly greater in G3. With respect to biochemical determinations total cholesterol did not differ among groups; HDL-cholesterol was lower in G3 in comparison with the other groups; and LDL-cholesterol showed increased levels in G2 and G3 compared with G1. Triglycerides were higher in G2 and G3 than in G1. The glycemia variable also differed among the groups, being higher in G3.

Table 1. Anthropometric data, blood pressure and plasmatic biochemistry profile in G1, G2 and G3.

Variable	G1 (n=36)	G2 (n=21)	G3 (n=23)
Age (years)	27.08 ± 4.69 ^a	33.76 ± 8.54 ^b	35.13 ± 8.57 ^b
Weight (kg)	55.49 ± 5.01 ^a	75.25 ± 6.95 ^b	95.80 ± 21.04 ^c
Height (m)	1.62 ± 0.05 ^a	1.65 ± 0.07 ^a	1.63 ± 0.07 ^a
BMI (kg/m ²)	21.10 ± 1.67 ^a	27.69 ± 1.76 ^b	36.10 ± 7.34 ^c
WC (cm)	70.38 ± 5.47 ^a	89.17 ± 6.66 ^b	108.93 ± 17.27 ^c
SBP (mmHg)	109.17 ± 8.74 ^a	116.19 ± 9.73 ^b	126.09 ± 11.58 ^c
DBP (mmHg)	71.67 ± 7.75 ^a	76.19 ± 6.50 ^a	84.35 ± 7.12 ^b
Cholesterol (mg/dL)	184.56 ± 31.48 ^a	203.71 ± 30.90 ^a	198.04 ± 24.94 ^a
HDL-cholesterol (mg/dL)	68.08 ± 16.94 ^a	61.43 ± 15.00 ^a	46.00 ± 10.74 ^b
LDL-cholesterol (mg/dL)	98.64 ± 31.83 ^a	117.35 ± 36.27 ^{ab}	122.30 ± 26.06 ^b
Triglycerides (mg/dL)	88.39 ± 31.12 ^a	122.29 ± 63.34 ^b	148.70 ± 59.65 ^b
Glucose (mg/dL)	73.19 ± 4.71 ^a	78.95 ± 6.78 ^b	85.50 ± 7.66 ^c

G1= control group, G2= BMI ≥ 25 kg/m² without metabolic syndrome, G3= BMI ≥ 25 kg/m² with metabolic syndrome, BMI = body mass index, WC waist circumference, SBP = systolic blood pressure, DBP = diastolic blood pressure. Results are expressed as means and standard deviations. Means followed by different letter superscripts differ significantly among groups (ANOVA followed by Tukey's test at p = 0.05).

Oxidative stress biomarker and DNA damage

Table 2 shows OS and DNA damage, the TAP and MDA in groups G1, G2 and G3. These data indicate that G2 and G3 had greater DNA damage than G1; we found that oxidative damage to purines and pyrimidines oxidized was greater in G2, however

the G3 was higher than the control group. The TAP was higher in G3, but did not differ from G2. MDA was also higher in G3, and not different from G2.

Table 2. DNA damage and oxidative stress in G1, G2 and G3.

Variable	G1 (n=36)	G2 (n=21)	G3 (n=23)
DNA damage (%) **	49.92 ± 9.01 ^a	76.71 ± 13.33 ^b	76.09 ± 9.85 ^b
Oxidative damage to purines (%)**	60.82 ± 10.27 ^a	81.42 ± 11.28 ^b	76.72 ± 9.94 ^b
Oxidative damage to pyrimidines (%)**	54.16 ± 10.56 ^a	84.84 ± 4.65 ^b	71.75 ± 10.72 ^c
TAP *	37.76 ± 12.36 ^a	44.03 ± 14.42 ^{ab}	51.26 ± 10.07 ^b
MDA (umol/L)*	12.65 ± 8.42 ^a	34.47 ± 17.96 ^b	41.18 ± 20.0 ^b

G1= control group, G2= BMI ≥ 25 kg/m² without metabolic syndrome, G3= BMI ≥ 25 kg/m² with metabolic syndrome, TAP= total antioxidant capacity, MDA = malondialdehyde. Results are expressed as mean and standard deviation. Means followed by different letter superscripts differ at a significance level of p = 0.05. ** Generalized linear model with gamma distribution followed by Wald multiple comparison tests. * ANOVA followed by Tukey's multiple comparison tests.

Macronutrients intake

Dietary intake of macronutrients did not differ among groups, except for protein intake, which was greater in G3 (Table 3).

Table 3. Dietary intake of macronutrients in G1, G2 and G3.

Variable	G1 (n=36)	G2 (n=21)	G3 (n=23)
Carbohydrate (g)	206.74 ± 76.20 ^a	214.76 ± 80.74 ^a	207.60 ± 64.25 ^a
Cholesterol (mg)	176.54 ± 90.25 ^a	183.65 ± 76.00 ^a	194.75 ± 117.35 ^a
Fiber (g)	14.11 ± 6.67 ^a	12.20 ± 5.03 ^a	11.79 ± 3.84 ^a
Monounsaturated fat (g)	13.39 ± 6.64 ^a	14.31 ± 7.13 ^a	14.12 ± 6.46 ^a
Polyunsaturated fat (g)	9.89 ± 5.19 ^a	9.90 ± 9.05 ^a	8.89 ± 4.03 ^a
Saturated fat (g)	13.15 ± 7.17 ^a	14.34 ± 6.30 ^a	12.67 ± 6.03 ^a
Total fat (g)	59.36 ± 19.36 ^a	65.60 ± 30.70 ^a	56.98 ± 20.88 ^a
Protein (g)	70.72 ± 20.32 ^a	75.31 ± 20.91 ^{ab}	105.19 ± 133.14 ^b

G1= control group, G2= BMI ≥ 25 kg/m² without metabolic syndrome, G3= BMI ≥ 25 kg/m² with metabolic syndrome. Results are expressed as means with standard deviations. Means followed by the same letter do not differ significantly (ANOVA followed by Tukey's test at p = 0.05).

Micronutrients intake

The intake of certain micronutrients such as folate, phosphorus, magnesium, potassium, selenium, zinc, and vitamin A did not differ among groups (Table 4). However, the intake of vitamin C was reduced in G2 and G3 compared to G1; G3 had a lower intake of vitamin D; and vitamin E and calcium intake was lower in G3 compared to G2. Iron intake was lower in G3 than the other two groups (Table 4).

Table 4. Dietary intake of micronutrients in G1, G2 and G3.

Variable	G1 (n=36)	G2 (n=21)	G3 (n=23)
Calcium (mg)*	637.40 ± 198.70 ^{ab}	585.68 ± 347.74 ^a	441.93 ± 284.05 ^b
Iron (mg)**	37.90 ± 99.21 ^a	36.41 ± 112.13 ^b	10.97 ± 4.56 ^a
Folate (µg)**	151.69 ± 74.52 ^a	148.79 ± 65.24 ^a	133.98 ± 38.19 ^a
Phosphorus (mg)*	801.73 ± 243.94 ^a	803.30 ± 254.23 ^a	772.04 ± 172.78 ^a
Magnesium (mg)*	158.97 ± 79.51 ^a	213.44 ± 198.70 ^a	135.49 ± 35.30 ^a
Potassium (mg)*	1764.32 ± 634.93 ^a	2036.85 ± 1650.50 ^a	1565.00 ± 438.21 ^a
Selenium (µg)**	79.61 ± 82.75 ^a	81.24 ± 79.62 ^a	70.23 ± 29.17 ^a
Sodium (mg)**	1705.70 ± 666.84 ^a	2446.86 ± 1321.8 ^b	2130.33 ± 729.91 ^{ab}
Vitamin A (equiv. retinol)**	793.58 ± 401.58 ^a	648.93 ± 434.14 ^a	631.11 ± 486.15 ^a
Vitamin C (mg)**	148.35 ± 130.59 ^a	78.84 ± 47.14 ^b	79.75 ± 53.06 ^b
Vitamin D (µg)**	25.58 ± 49.87 ^a	3.62 ± 8.24 ^a	3.18 ± 5.55 ^b
Vitamin E (mg)**	76.29 ± 243.86 ^{ab}	41.64 ± 139.86 ^a	12.66 ± 6.33 ^b
Zinc (mg)**	10.23 ± 22.45 ^a	16.37 ± 34.35 ^a	17.28 ± 30.06 ^a

G1= control group, G2= BMI ≥ 25 kg/m² without metabolic syndrome, G3= BMI ≥ 25 kg/m² with metabolic syndrome. Results are expressed as means with standard deviations. * ANOVA followed by Tukey's multiple comparison test ** Generalized linear model with gamma distribution followed by Wald multiple comparison test. Means followed by different letter superscripts differ significantly at p = 0.05.

Antioxidants concentration in plasma

The amounts of antioxidants such as carotenoids, α -tocopherol, retinol and uric acid in plasma of three groups of women are shown in Table 6. There was no difference among groups in plasma concentrations of lutein, lycopene and α -tocopherol. G3 had lower plasma concentrations of cryptoxanthin and beta-carotene compared to G1 which had higher plasma concentrations of α -carotene. Uric acid concentrations increased across the groups, with lowest concentrations in G1 and highest concentrations in G3. Plasma retinol was higher in G3, differing only from G2 (Table 5).

Table 5: Plasma levels of carotenoids, α -tocopherol, uric acid and retinol in G1, G2 and G3.

Variable	G1 (n=36)	G2 (n=21)	G3 (n=23)
Lutein (µg/dL)	8.35 ± 6.13 ^a	5.13 ± 3.10 ^a	7.72 ± 6.54 ^a
Cryptoxanthin (µg/dL)	18.86 ± 18.42 ^a	10.15 ± 9.18 ^{ab}	7.86 ± 8.45 ^b
α -carotene (µg/dL)	5.75 ± 4.57 ^b	2.63 ± 1.49 ^a	2.76 ± 1.69 ^a
β -carotene (µg/dL)	12.24 ± 11.53 ^a	6.32 ± 3.99 ^{ab}	5.22 ± 3.36 ^b
Lycopene (µg/dL)	5.87 ± 6.94 ^a	6.09 ± 5.26 ^a	6.06 ± 5.86 ^a
α -tocopherol (µg/dL)	491.26 ± 254.46 ^a	461.93 ± 241.79 ^a	625.69 ± 255.47 ^a
Uric acid (mg/dL)	3.75 ± 0.77 ^a	4.39 ± 1.09 ^b	5.32 ± 1.14 ^c
Retinol (µg/dL)	80.20 ± 34.57 ^{ab}	68.40 ± 29.57 ^a	106.18 ± 76.86 ^b

G1= control group, G2= BMI ≥ 25 kg/m² without metabolic syndrome, G3= BMI ≥ 25 kg/m² with metabolic syndrome. Results are expressed as means and standard deviations. Means followed by different letter superscripts differ significantly among groups (generalized linear model with gamma distribution followed by Wald multiple comparison test at 5%).

Relation between DNA damage, consumption of macro- and micronutrients and plasmatic variants

There was a positive correlation between the consumption of sodium and protein and DNA damage (Table 6). Women who consumed the greatest amount of these nutrients had greater DNA damage. There was a negative correlation between polyunsaturated fat intake, plasma levels of α -carotene and DNA damage (Table 6).

Table 6. Final linear regression model relating DNA damage with consumption of macro- and micronutrients and plasmatic variants.

Variable	Estimate	EP	P – value
Intercept	57.67351	4.83736	< 0.0001
α - carotene ($\mu\text{g/dL}$)	-1.13616	0.45101	0.0139
Polyunsaturated fat (g)	-1.16142	0.31356	0.0004
Protein (g)	0.05391	0.02171	0.0153
Sodium (mg)	0.00898	0.00206	< 0.0001

Relation between BMI and plasmatic variants

There was a negative correlation between BMI and vitamin D intake and plasma α -carotene levels, and a positive correlation between BMI and plasma levels of retinol (Table 7).

Table 7. Final linear regression model relating BMI with consumption of micronutrients and plasmatic variants.

Variable	Estimate	EP	P – value
Intercept	26.18853	1.74965	< 0.0001
α - carotene ($\mu\text{g/dL}$)	-0.63645	0.20538	0.0027
Vitamin D (μg)	-0.05532	0.02072	0.0093
Retinol ($\mu\text{g/dL}$)	0.05065	0.01455	0.0008

Discussion

The regular consumption of fruit and vegetables has been reported as an important way to reduce the risk of chronic non-communicable diseases such as cancer and CVD^(15, 16).

Worldwide trends in dietary intake , particularly in young people, have resulted in changes in average nutritional status, most alarmingly, an increase in the occurrence of obesity and MetS ⁽¹⁷⁾ . This study examined women who were classified as BMI above the normal weight with MetS and compared them with normal BMI women. Some changes which are normally present in most obese and overweight people ^(18, 19) were observed. For example, glucose was higher in overweight and obese women compared to women of eutrophic woman. With regard to the lipid profile, we observed an increase in the fractions of LDL-cholesterol, decreased HDL-cholesterol and increased triglycerides in groups with higher BMI (G2 and G3) compared to the control group. That is, women with MetS and overweight women tend to have an increase in the lipid profile with decreased HDL-cholesterol ⁽²⁰⁾. This change in the lipid fraction increases the formation of MDA, as we observed. Women with MetS had increased lipid peroxidation, indicating the presence of OS. We observed an increase in TAP, in women with MetS, contrary to expectations ⁽²¹⁾. One of the justifications would be increased uric acid in this group compared with normal weight women (control group). Uric acid is involved with CVD ^(22, 23) and also accounts for > 50% of plasma antioxidant capacity ^(24, 25). These data are consistent with other studies that reported direct correlation between TAP and uric acid ⁽²⁶⁾. When the values of the total antioxidant capacity of each individual are normalized to values of uric acid, there is a decrease in the total antioxidant capacity in overweight individuals with MetS ⁽²⁷⁾. Thus, we suggest that the antioxidant capacity may not be a good marker of OS for individuals with increased levels of uric acid. We found oxidative DNA damage and lipid peroxidation increased in G2 and G3 compared with controls (G1) when purchased mainly in oxidized pyrimidines $G1 < G3 < G2$. Studies show that an increase in fat mass leads to increased OS and consequently to oxidative damage, and that MetS and T2D

generally aggravate OS and damage ^(28, 29). Karbownik-Lewinska and collaborators ⁽³⁰⁾ found that the concentration of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in peripheral blood lymphocytes in overweight and obese patients was an indicator of DNA damage. Further, there was a positive correlation with BMI, WC and triglycerides. These data are consistent with our study, where we observed the greatest DNA damage in women with higher BMI, WC and triglycerides.

We observed no differences in macronutrient intake among the groups, except that women with MetS (G3) had a higher protein intake than the control group (G1); however, consumption by G2 group did not differ from G1 or G3. The literature shows that subjects with MetS consume a large amount of protein, primarily in the form of meat ^(31, 32). We also found high sodium consumption in G2 and G3. Other studies that have analyzed the sodium intake in overweight and obese people observed the same result, confirming that this micronutrient is consumed in large quantities in this population ^(33, 34). This high consumption may be a factor in the development of hypertension, which is one of the features of MetS. We found that women of G2 and G3 ingested smaller amounts of vitamin E, D and calcium than women in G1. These data are consistent with several studies that have reported deficiency of these micronutrients in obese and overweight people ⁽³⁵⁻³⁷⁾.

In the evaluation of plasma carotenoids we observed that cryptoxanthin and β -carotene were lower in G3 than G1. The values of α -carotene were lower in G2 and G3 than in G1. This may be related to the greater use of these nutrients in a situation of OS since DNA damage, oxidative or not, was more intense in G2 and G3. This shows that the levels of α -carotene were directly related to protection from oxidative DNA damage. Higher plasma concentrations of α -carotene in G1 (5.75 mg/dL) reflects a reduction of approximately 60% in the risk of DNA damage compared to G2 and G3. In addition, we

observed in all groups that the higher the level of serum α -carotene, the lower the damage to DNA, but not the damage to purine and pyrimidine bases. Therefore, we conclude that this carotenoid also has a protective effect for non-oxidative damage. The literature shows that carotenoid antioxidants can prevent OS and DNA damage ^(38, 39). We also observed the same protection ratio to DNA damage when the consumption of polyunsaturated fats increases. The main sources of omega-3 fatty acids are cold water fish and seafood ⁽⁴⁰⁾ and omega-6 fatty acids are found in soy and sunflower oils and oilseeds ⁽⁴¹⁾. Their consumption has several advantages including helping to increase the levels of HDL-cholesterol and decrease LDL-cholesterol ⁽⁴¹⁾. They also prevent DNA damage ⁽³⁸⁾. The literature is sparse regarding the association of obese individuals, consumption of n-3 and n-6 polyunsaturated fatty acids (PUFAs), and DNA damage. It is also reported that n-3 PUFAs have a protective role in autoimmune diseases, T2D, rheumatoid arthritis and cancer, while n-6 PUFAs have a pro-inflammatory effect ⁽⁴²⁾. In addition, the same study ⁽⁴²⁾ showed that one of these two fatty acids had a positive effect on the prevention of DNA damage in an experimental study of colon cancer.

We also observed that the consumption of sodium and protein of the women studied had a positive correlation with DNA damage. One explanation would be that the consumption of processed products containing high concentrations of sodium and chemical additives that show carcinogenic potential and react with DNA ⁽⁴³⁾. Studies show that diets high in sodium may predispose individuals to the development of obesity or may be directly associated with the consumption of foods that lead to weight gain and complications such as hypertension ^(44, 45). Sodium intake was also positively correlated with OS in experimental study ⁽⁴⁶⁾. The mechanism that causes this effect is still being studied, but it has been suggested that a high-salt diet stimulates the formation of reactive species through the activation of NADPH oxidase ⁽⁴⁶⁾. With

respect to protein consumption, studies show that these can increase DNA damage by the presence of additives for their conservation (heterocyclic aromatic amines) ⁽⁴³⁾. Thus, a diet rich in fruits and vegetables and reduced in processed foods can protect oxidative DNA damage ⁽⁴⁾.

Other parameters were correlated with BMI in this study. Women who consumed less vitamin D and who had higher serum retinol concentration and low levels of α -carotene also had higher BMI. The literature reports that foods rich in vitamin D are primarily fish such as salmon and seafood ⁽⁴⁷⁾, of which little was consumed in our study population. Although we did not measure the serum concentration of vitamin D, it is widely known that obese postmenopausal women generally have reduced levels of vitamin D ⁽⁴⁸⁾ and this may be related to a lower intake. A complicating factor is that low levels of vitamin D may lead to the development of T2D, especially in obese and overweight people ⁽⁴⁹⁾. BMI was positively correlated with plasma levels of retinol in this study. This may be related to consumption of processed and fortified foods ⁽⁵⁰⁾ mainly milk and butter. This is corroborated by the negative relationship we found between BMI and the plasma concentration of carotenoids in G2 and G3 which are precursors of vitamin A and come from fruits and vegetables. Our results are consistent with those of plama and collaborators ⁽⁵¹⁾ who also found increased levels of carotenoids in obese women in contrast to reports in the literature that MetS decreased serum vitamin A, C and E due to expen diture triggered by OS in obese women ⁽⁵²⁾. Other studies have found adequate levels of these vitamins in this population ⁽⁵³⁾, therefore, there is still controversy in this regard.

Conclusion

We conclude that worsened nutritional status influences various parameters including metabolic and OS, and DNA damage. Diet quality (consumption of fruit and vegetables, intake of salt and fat) and BMI are the factors that most influenced changes in these parameters.

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Conflicts of Interest

The authors declare no conflict of interest.

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