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Influência do Padrão Alimentar e dos Polimorfismos dos Genes *GSTM1*, *GSTT1*, *GSTP1*, *CYP2E1*, *XRCC*1, *MTHFR* e *TS* sobre os níveis de danos oxidativos no DNA e de uracilas incorporadas ao DNA

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Dedicatória

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“Enquanto estiver vivo, sinta-se vivo.

Se sentir saudades do que fazia, volte a fazê-lo.

Não viva de fotografias amareladas...

Continue, quando todos esperam que desistas.

Não deixe que enferruje o ferro que existe em você.

Faça com que em vez de pena, tenham respeito por você.

Quando não conseguir correr através dos anos, trote.

Quando não conseguir trotar, caminhe.

Quando não conseguir caminhar, use uma bengala.

Mas nunca se detenha."

Madre Teresa de Calcutá

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RESUMO

Estima-se que 10% a 70% dos cânceres estejam relacionados a fatores alimentares como a ingestão aumentada de aditivos químicos, dietas ricas em gordura e baixo consumo de frutas e vegetais. Entretanto vários estudos moleculares epidemiológicos têm demonstrado que além de fatores ambientais, como a dieta, a carcinogênese pode ser modulada por genes envolvidos no biometabolismo de xenobióticos e no reparo de DNA. O presente estudo avaliou a possível influência do padrão alimentar e dos polimorfismos dos genes *GSTM1*, *GSTT1*, *GSTP1*, *CYP2E1*, *XRCC1*, *MTHFR* e *TS* sobre os níveis de danos oxidativos no DNA e de uracilas incorporadas ao DNA em dois grupos de indivíduos residentes em Botucatu com diferentes padrões alimentares. Grupo I: 49 indivíduos que possuem alimentação rica em produtos orgânicos, grãos integrais, frutas e vegetais e pobre em produtos industrializados. Grupo II: 56 indivíduos que adotam uma alimentação rica em produtos industrializados e pobre em frutas e vegetais. Ambos os grupos constituídos de voluntários não fumantes, não etilistas e não usuários de drogas. A quantificação do nível de danos oxidativos no DNA, uracilas incorporadas ao DNA e a eficiência do sistema reparo de DNA em linfócitos de sangue periférico, foi realizada por Prado et al. (em preparação), em estudo paralelo. Os polimorfismos dos genes *GSTM1, GSTT1, GSTP1, CYP2E1*, *XRCC1* e *MTHFR* e *TS* foram analisados pelas técnicas de PCR e PCR-RFLP Também foi realizada a análise dos níveis de luteína, criptoxantina, α-caroteno, β-caroteno, licopeno, retinol e α-tocoferol no soro, pela técnica de cromatografia líquida de alta pressão (HPLC). Com relação aos indivíduos do grupo I: a) portadores do genótipo *MTHFR C1298C* apresentaram maiores níveis de danos no DNA e b) portadores do genótipo TS *TSER*3R/3R apresentaram maiores níveis de purinas oxidadas. Com relação aos indivíduos do grupo II: a) portadores do genótipo

GSTT1 (-/-) apresentaram maiores níveis de purinas e pirimidinas oxidadas, uracilas incorporadas ao DNA e menor eficiência do sistema reparo de DNA; b) portadores do genótipo *CYP2E1 c1/c2* apresentaram maiores níveis de purinas e pirimidinas oxidadas, uracilas incorporadas ao DNA e menor eficiência do sistema reparo de DNA; c) portadores do genótipo *XRCC1 Arg399Arg* apresentaram maiores níveis de pirimidinas oxidadas e uracilas incorporadas ao DNA e menor eficiência do sistema de reparo do DNA; d) portadores do genótipo *MTHFR C1298C* apresentaram maiores níveis de quebras de fita simples e duplas de DNA e e) portadores do genótipo *TS TSER3R/R* apresentaram maiores níveis de purinas oxidadas e menor eficiência do sistema de reparo de DNA. Os indivíduos do grupo I apresentaram maiores níveis de todos os carotenóides e α-tocoferol do que os indivíduos do grupo II. Em conclusão, os polimorfismos de genes de metabolismo de xenobióticos, reparo de DNA e metabolismo de ácido fólico modulam os níveis de danos no DNA e a eficiência do sistema de reparo de DNA, principalmente em indivíduos com menor ingestão de micronutrientes.

REVISÃO BIBLIOGRÁFICA

I – INTRODUÇÃO

I.1 – Considerações Iniciais

Estima-se, que no mundo inteiro, por ano, cerca de dez milhões de pessoas recebam o diagnóstico de câncer e que mais de seis milhões morram em decorrência desta doença (Bingham e Riboli, 2004). O alto índice de neoplasias observado atualmente pode ser reflexo das mudanças ocorridas nos hábitos alimentares com o advento da era tecnológica. Vários aditivos químicos alimentares têm atividade carcinogênica comprovada, contudo, não só a ingestão aumentada desses produtos, mas, também, a diminuição do consumo de alimentos naturais são fatores que contribuem para o aparecimento das doenças da vida moderna. Muitos estudos têm demonstrado maior expectativa de vida e menor risco para o desenvolvimento de câncer e de doença isquêmica do coração em indivíduos que optam por dietas ricas em frutas e vegetais (Trichopolou *et al.*, 2003).

I.2 – Dieta e Câncer

O câncer mais comum no mundo (excluindo câncer de pele) é o câncer de pulmão (12,3% de todos os cânceres), mama (10,4%) e colorretal (9,4%). Entretanto, os vários tipos de cânceres apresentam incidências diferentes, com algumas regiões apresentando incidências até 100x maiores de determinados tipos de câncer como,

por exemplo, melanoma e câncer de nasofaringe (Bingham e Riboli, 2004). A incidência de câncer de pulmão, intestino, mama, próstata e bexiga é cerca de três vezes maior no nordeste da Europa quando comparada à incidência de alguns países africanos (Bingham e Riboli, 2004). Existem evidências que estas diferentes incidências de câncer estão altamente associadas a fatores ambientais e ao estilo de vida, com uma grande proporção desta variação relacionada à dieta (Bingham e Riboli, 2004).

Em 1981, Doll e Peto estimaram que nos EUA 32-35% dos casos de câncer estavam relacionados a fatores alimentares. Entretanto, outros estudos demonstraram que essa proporção pode variar de 10% para câncer de pulmão a 70% para câncer de intestino (Levi, 1999). Três constituintes/contaminantes da dieta tais como, álcool, aflatoxina e alimentos salgados, juntamente com a obesidade e a baixa ingestão de frutas e vegetais (La Vechia *et al.*, 2003; Bingham e Riboli, 2004), já foram claramente associados ao aumento da incidência de câncer em seres humanos (Montesano e Hall, 2001). Fergunson (2002) mostrou que aditivos químicos utilizados para conservação da carne (componentes N-nitrosos) e aminas heterocíclicas e hidrocarbonetos policíclicos aromáticos produzidos durante o preparo da carne em altas temperaturas, podem levar ao câncer de cólon e de próstata (Fergunson, 2002). Por outro lado, vários estudos epidemiológicos têm demonstrado que indivíduos que consomem grande quantidade de frutas e vegetais ricos em micronutrientes com propriedades antioxidantes, tais como vitamina C, vitamina E, carotenóides e flavonóides, apresentam menor incidência de câncer de pulmão, estômago, mama, cólon, faringe e fígado (Moller e Loft, 2002).

Vários micronutrientes atuam como cofatores enzimáticos nas reações de manutenção, reparo e metilação do DNA e no processo de apoptose (Ames e

Wakimoto, 2002). Variações na ingestão de micronutrientes podem estar relacionadas à eventual instabilidade genômica detectada pelo aumento na incidência de biomarcadores como mutação de ponto, quebra cromossômica ou ainda, por interferir na segregação cromossômica, expressão gênica, estresse oxidativo, necrose e apoptose (Fenech e Fergunson, 2001). Cerca de 40 micronutrientes, entre vitaminas, minerais essenciais e outros componentes são requeridos na dieta humana, em pequenas quantidades, para um metabolismo eficiente. Entretanto, não há consenso sobre o nível de micronutrientes necessários para prevenção de danos no DNA em seres humanos (Fergunson, 2002).

Dietas ricas em micronutrientes têm sido relacionadas à prevenção de danos oxidativos no DNA, otimização do processo de apoptose (Fergunson, 2002), e ao aumento da capacidade de reparo de danos no DNA. Collins *et al.* (2003) adicionando kiwi como suplemento alimentar durante três semanas a indivíduos saudáveis verificaram um aumento no nível de antioxidantes e maior eficiência do sistema de reparo do DNA, e sugeriram que estes efeitos poderiam diminuir o risco de desenvolvimento de câncer (Collins *et al.*, 2003).

A desnutrição causada por deficiência de nutrientes é principalmente um problema relacionado a condições associadas à pobreza, sendo um fator importante em países em desenvolvimento. Existe uma preocupação da Organização Mundial de Saúde não só com a desnutrição protéico-calórica, mas, também, com o estado micronutricional. A carência de determinados micronutrientes pode provocar uma série de doenças, desde a diminuição da imunidade, osteoporose, cegueira, bócio, anemia, diminuição da capacidade de aprendizado, letargia, retardo mental, e até a morte. Algumas doenças que provavelmente estão relacionadas à alimentação inadequada e que apresentam grande expressão mundial merecem destaque, como

por exemplo, o câncer, a osteoporose e outras doenças degenerativas (doenças cardiovasculares e doença de Alzheimer) (Fenech e Fergunson, 2001). De acordo com Ames (2001), as deficiências de micronutrientes como o ácido fólico, as vitaminas B12, B6, C e E, niacina, ferro e zinco podem mimetizar o efeito da radiação ou de agentes químicos na indução de danos ao DNA (Ames, 2001). A deficiência de ácido fólico em seres humanos é um importante fator associado ao desenvolvimento de câncer, e também tem sido relacionada à anemia megaloblástica, a defeitos de fechamento do tubo neural em neonatos e a doenças cardíacas (Duthie, 1999)

Sabe-se que não apenas os fatores ambientais estão relacionados à indução de danos e mutações no DNA, mas que existe uma suscetibilidade genética relacionada à maior ou menor ocorrência desses danos.

De acordo com o Conselho Norte-Americano de Pesquisa, os “marcadores de suscetibilidade” são definidos como diferenças biológicas, entre indivíduos ou populações, capazes de afetar a resposta do organismo a agentes ambientais. Entre os marcadores de suscetibilidade mais significativos, estão as diferenças genéticas na capacidade de reparo de danos no DNA, na dinâmica das reações do biometabolismo de agentes xenobióticos (Louro , 2002).

I.3 – Metabolismo de Xenobióticos

O sistema de metabolização xenobiótica humano compreende duas classes de enzimas: as de metabolismo oxidativo mediado ou de fase I e as enzimas conjugadas ou de fase II. O objetivo final de ambas as fases da metabolização xenobiótica é

aumentar a solubilidade em água dos compostos, facilitando assim sua excreção do organismo. Os aditivos químicos presentes no meio ambiente e na dieta necessitam de ativação metabólica para se tornarem metabólitos altamente reativos capazes de ligarem-se ao DNA e exibirem atividade carcinogênica. Esta ativação é feita pelas enzimas da fase I, que são principalmente enzimas da superfamília Citocromo P450. (Nebert, 1991; Perera, 1996; Shimada e Fujiikuriyama, 2004). Enzimas do Citocromo P450 são as principais enzimas na ativação metabólica dos hidrocarbonetos policíclicos aromáticos (PAHs), que são um dos principais carcinogênicos encontrados na carne grelhada a altas temperaturas.

Os aditivos químicos em sua maioria necessitam ser biotransformados para eliminarem seus efeitos carcinogênicos. (Taningher *et al*., 1999). As Glutationa S-transferases (GSTs) são enzimas de fase II que estão envolvidas nas reações de conjugação e detoxificação de vários xenobióticos, e agem então como enzimas inativadoras dos produtos da fase I, tornando os metabólitos resultantes das reações de fase I mais hidrofílicos e de fácil excreção (Nebert, 1991; Persson *et al*., 1995). Um polimorfismo genético que aumente a expressão das enzimas de fase I pode aumentar a quantidade de carcinogênicos reativos formados e, então, aumentar o risco de desenvolvimento de câncer (Dunning *et al*., 1999). Desta forma, os genótipos que conferem variações nos níveis de atividade enzimática das fases I e II estão associados à suscetibilidade individual ao desenvolvimento de câncer.

Recentemente, especial atenção tem sido direcionada na tentativa de elucidar as bases moleculares dos polimorfismos das enzimas envolvidas na ativação e detoxificação de agentes xenobióticos. Diversos investigadores têm estudado a interação entre os genótipos referentes a genes de biometabolismo e alguns tipos de alterações genéticas; como danos no DNA e micronúcleo, que há vários anos têm

sido usados como marcadores de exposição genotóxica e efeitos precoces de carcinógenos genotóxicos (Norppa, 2004).

I.3.1 Polimorfismos dos genes da Fase I

I.3.1.1 Genes da Super Família Citocromo P450 (CYPs)

As enzimas de fase I, que são codificadas pelos genes da super família do citocromo P-450 (CYP), representam a primeira linha de defesa contra compostos tóxicos e carcinogênicos. Essas enzimas catalisam reações de oxidação do substrato, resultando em aumento da hidrofilia que facilita a excreção da substância tóxica. Contudo, essas reações podem, também, converter pró-carcinógenos em formas eletrofílicas que reagem com o DNA (Miller e Miller, 1977; Guengerich, 1994). Por apresentar uma grande importância na ativação metabólica de pró-carcinógenos, muitos estudos têm objetivado estabelecer uma relação entre a distribuição de variantes polimórficas das diferentes enzimas CYPs e a susceptibilidade ao câncer. Esta superfamília compreende pelo menos cinco subfamílias, designadas de A a E pelo sistema de nomenclatura recomendada (Nebert *et al*., 1987).

I.3.1.1.1 *CYP1A1*

O gene *CYP1A1* está mapeado no cromossomo 15q22-24 (Hildebrand *et al*., 1985) e metaboliza hidrocarbonetos policíclicos aromáticos (PAH), aminas heterocíclicas

aromáticas e hidrocarbonetos aromáticos polihalogenados entre outros. Estes compostos têm afinidade pelo receptor do aril hidrocarbono, que por sua vez induz a ação da família CYP1, que os metaboliza, transformando-os em produtos carcinogênicos (Nebert e Dalton, 2006). Inicialmente foram descritos nove alelos polimorfos, dos quais quatro vêm sendo analisados quanto a uma possível associação à susceptibilidade ao câncer, que são: *CYP1A1*2A, *2B, *3* e **4*. O alelo denominado *CYP1A1*2A* apresenta uma citosina no lugar de uma timina na região 3' flanqueadora do gene *CYP1A1* (6235T para C), dando origem a um sítio de restrição *Msp*I (Kawajiri *et al.*, 1990). Este polimorfismo parece promover o aumento da expressão gênica (Arvanitis *et al.*, 2001), e possui três genótipos: *m1/m1*, *m2/m2* e *m1/m2* (Crofts *et al.*, 1993). Um estudo aponta um significante aumento do risco de câncer oral observado para indivíduos com genótipo homozigoto (*m2/m2*) (Cha *et al.*, 2007). Outros estudos não verificaram associação entre o *CYP1A1* e o risco de câncer de mama (Bailey *et al.*, 1996, Singh *et al.*, 2007). Em japoneses e em certas populações caucasóides, o risco aumentado de câncer de pulmão foi correlacionado positivamente com um ou ambos os polimorfismos do gene *CYP1 Msp*I e isoleucina – valina, este último devido a uma mutação no exon 7 (Nakachi *et al.*,1991; Kawajiri *et al.*, 1996; Xu *et al.*,1996) e diretamente associado ao hábito tabagista.

I.3.1.1.2 *CYP2E1*

A enzima CYP2E1 tem papel importante no metabolismo de compostos de baixo peso molecular, como a N-nitrosamina e benzeno, além da anilina, cloreto de vinil, uretano e etanol, transformando-os em metabólitos reativos que podem interagir com o DNA (Guengerich *et al.,* 1991). A N-nitrosamina é formada no trato digestivo pela

reação da amina com nitritos (Hecht *et al*., 1997), que estão presentes em produtos industrializados e carnes processadas, ou formados na flora do cólon (Suzuki *et al*., 1981, Bingham *et al*., 1996). Por isso, um alto consumo de produtos industrializados e processados pode levar a uma maior exposição à N-nitrosamina (Le Marchand *et al*., 2002). A CYP2E1 é predominantemente expressa no fígado, e estimulada por compostos químicos, hormônios ou condições metabólicas (Le Marchand *et al*., 1999), como obesidade, e pelo consumo de álcool (MacCarver *et al*., 1998). Existem dois polimorfismos funcionais de interesse do *CYP2E1*, o polimorfismo *RsaI* com substituição C-1054T (rs2031920) e a inserção 96- pb na região 3' flanqueadora (Kim *et al*., 1996, Le Marchand *et al*., 1999). De acordo com a nomenclatura convencional, o alelo selvagem *RsaI* (comumente chamado de alelo *c1*) e o alelo variante *c2* correspondem a *CYP2E1*5A* e *CYP2E1*5B*, respectivamente. O alelo de inserção é nomeado *CYP2E1*1D*, e o alelo de não inserção, *CYP2E1*1C*. Embora variações alélicas do gene *CYP2E1* estejam relacionadas ao aumento da transcrição do gene, não se conhece, ainda, o real efeito desses polimorfismos na atividade da enzima (Kim *et al*., 1994; Stephens *et al.,* 1994). Estudos *in vitro* indicaram um aumento da transcrição do gene *CYP2E1* associado ao alelo variante *c2* (Hayashi *et al*., 1991, Watanabe *et al*., 1994). Entretanto, alguns estudos em humanos mostraram que indivíduos com o alelo variante *c2* tiveram menor atividade da enzima (Le Marchand *et al*., 1999) e menor indução por ingestão crônica de álcool (Lucas *et al*., 1995). Por outro lado, outros estudos não observaram variação da atividade da enzima para os genótipos *RsaI* (Kim *et al*., 1996, Carriére *et al*., 1996, Powell *et al*., 1998). Em relação ao alelo inserção 96-pb, uma maior atividade de transcrição foi observada, e indivíduos com o alelo de inserção apresentaram um aumento na

atividade da enzima induzida por obesidade e alto consumo de álcool (MacCarver *et al*., 1998).

Estudos têm demonstrado associação entre a *CYP2E1* e maior risco de desenvolvimento de câncer, por exemplo indivíduos portadores do genótipo *c1/c1* apresentaram maior risco de desenvolvimento de câncer gástrico [(OR= 2.37, (IC) 95% 1.52-3.70] (Cai *et al*., 2005). Zhu *et al*., (2008) relataram que o genótipo *c1/c2* e *c2/c2* foi associado a maior nível de danos no DNA. Em estudo realizado por Le Marchand *et al*., (2002) foi constatado que indivíduos com o alelo 96-pb de inserção apresentam um risco aumentado em 60% de desenvolvimento de câncer colorretal. O mesmo estudo verificou que indivíduos com o variante de inserção e que estavam expostos a um maior nível de nitrosaminas devido a altos consumos de carne vermelha e carne processada tiveram um risco de câncer retal ainda maior (risco duas vezes maior para consumo de carne vermelha e três vezes maior para o consumo de carne processada). Morita *et al.* (2008) relataram que indivíduos com o alelo *c2* tiveram menor risco de adenomas de cólon proximal, e indivíduos com o alelo 96-pb de inserção foram associados com maior risco de desenvolvimento de grande adenomas (≥5 mm). Morita *et al* (2009) demonstraram que o alelo *c2* foi associado com menor risco de desenvolvimento de câncer colorretal enquanto indivíduos com um ou dois alelos 96-pb de inserção tiveram risco aumentado de desenvolvimento câncer colorretal. Além disso, indivíduos com dois alelos de inserção tiveram risco 2,28 vezes maior de desenvolvimento de câncer de cólon. Neste estudo, também foi verificada uma associação entre o consumo de álcool e câncer colorretal em indivíduos sem o alelo *c2* ou sem o alelo de inserção. Em relação ao consumo de carne, o risco de câncer de cólon foi maior em indivíduos com um ou dois alelos de inserção (Morita *et al*., 2009).

I.3.2 Polimorfismos dos genes da Fase II

I.3.2.1 Genes da família Glutationa S-transferases (GSTs)

As enzimas glutationas S-transferases (GSTs) possuem a função básica de detoxificação, mediando a conjugação de um grande número de compostos eletrofílicos com glutationa reduzida (GSH), numa reação metabólica de fase II (Pickett e Lu, 1989). Estas enzimas possuem a capacidade de detoxificar os metabólitos reativos dos PAHs, evitando que eles reajam com o DNA (Amorim *et al*., 2002), e também atuam na proteção contra produtos do estresse oxidativo (Hayes, 1995 citado por Egan *et al*., 2004). Além da detoxificação da fase II, as GSTs também desempenham papel importante na modulação da indução de outras enzimas e proteínas que atuam em outras funções celulares, como reparo de DNA (Mo *et al*, 2009).

As GSTs, em seres humanos, foram divididas em quatro classes alpha (α GSTA), mu (μ GSTM), pi (π GSTP) e theta (θ GSTT) (Mannervik , 1992).

I.3.2.1.1 *GSTM1*

A *GSTM1* está localizada no cromossomo 1p13.3, metaboliza uma classe de carcinogênicos, como substratos provenientes de PAH e benzo(alfa)pireno (Berhane *et al.*, 1994), e também metaboliza compostos formados pelo estresse oxidativo,

como hidroperóxidos e lipídios oxidados (Sorensen *et al*., 2007). O polimorfismo observado para o gene *GSTM1* é decorrente de deleção homo ou heterozigota dos alelos e tem sido associado ao aumento da suscetibilidade ao câncer, devido a deficiente detoxificação de compostos cancerígenos (Taningher, 1999). O genótipo homozigoto para os alelos nulos, representados por *GSTM1(-/-),* demonstra uma atividade nula da *GSTM1*, enquanto que os genótipos heterozigoto *(+/-)* e homozigoto *(+/+)* possuem maior atividade (Hirvonen *et al*., 1993). No Brasil, a *GSTM1 (-/-)* tem freqüência aproximada de 50% (Rossit, 2001), e tem sido relacionada ao aumento da incidência de câncer de pulmão e bexiga e ao aumento de aductos de DNA induzidos por hidrocarbonetos policíclicos aromáticos, que estão presentes na carne grelhada (Palli *et al*., 2003). Um estudo realizado no Brasil verificou um risco 4,7 vezes maior de desenvolvimento de leucemia mielóide aguda em indivíduos portadores do genótipo *GSTM1(-/-)* (Arruda e*t al.*, 2001). Outro estudo verificou um risco de desenvolvimento de câncer de próstata 2,69 vezes maior em indivíduos com o mesmo genótipo (Mittal *et al.*, 2006). Além disso, um trabalho de revisão concluiu que *GSTM1(-/-)* também está associado ao aumento do risco de câncer de próstata (Mo *et al.*, 2009). O mesmo genótipo foi associado a maior risco de desenvolvimento de câncer do trato aerodigestivo (OR= 2.5; IC 95% 1.3–4.7). O genótipo *GSTM1(-/-)* em associação com o genótipo *GSTT1* nulo leva a maior risco de desenvolvimento de câncer do trato aerodigestivo (OR 4.6; IC 95% 1.3–15.6) (Mo *et al.*, 2009). Em um estudo que avaliou a influência dos polimorfismos do gene *GSTM1* sobre os níveis de danos no DNA e aberrações cromossômicas induzidas por bleomicina em linfócitos de sangue periférico, foi verificado que sem tratamento com bleomicina não houve diferença significativa entre os genótipos *GSTM1*. (Kocaba¸s *et al*., 2000). Por outro lado, outros estudos sugeriram que a

GSTM1 pode proteger contra aberrações cromossômicas (van Poppel *et al*., 1992) e formação de aductos de DNA (Ketterer *et al*., 1992, Liu *et al*., 1991, Kato *et al*., 1995). Scarpato *et al.* (1997) encontraram aumento significativo na freqüência de aberrações cromossômicas em fumantes *GSTM1* nulo, quando comparados a fumantes *GSTM1* positivos, o mesmo ocorrendo para o gene *GSTT1*.

I.3.2.1.2 *GSTP1*

O *GSTP1* está localizada no cromossomo 11q18 (Moscow *et al*., 1988), e também tem sido relacionada a maior risco de desenvolvimento de câncer. Board *et al.* (1990) descreveram três diferentes alelos para a *GSTP1*: *GSTP1*A* (tipo selvagem), *GSTP1*B* e *GSTP1*C* (Board *et al*., 1990). A variação *GSTP1*B* apresenta uma transição de adenina para guanina na posição +313, códon 105 , exon 5, o que resulta na substituição do aminoácido isoleucina para valina. O alelo *GSTP1*C* possui duas transições, as mesmas observadas na *GSTP1*B* e uma segunda, observada na posição +314, códon 106, exon 6, mudando o aminoácido alanina para valina. A substituição Isoleucina por valina (códon 105) nos variantes *B* e *C* reduz a atividade catalítica da enzima (Pandya *et al*., 2000), que induz uma detoxificação menos eficiente de certos compostos mutagênicos e carcinogênicos, o que pode levar a aumento dos níveis de danos no DNA e mutações e aumentar o risco de desenvolvimento de câncer (Ali-Osman *et al*., 1997, Canalle *et al*., 2004).

A *GSTP1* metaboliza diversos compostos carcinogênicos, dentre eles o hidrocarbonetos policíclicos aromáticos, presentes em carnes excesivamente grelhadas e o benzo(alfa)pireno diolepóxido, que é um dos mais metabólitos

carcinogênicos derivados do tabaco (Hayes *et al*., 1995), tendo importância não somente na detoxificação de carcinógenos presentes em produtos industrializados, carnes grelhadas a altas temperaturas, como na detoxificação de carcinógenos inaláveis (Honma *et al*., 2008).

Um estudo realizado em 2002 associou o genótipo *GSTP1*B* ao risco aumentado de leucemia linfocitária aguda em crianças [OR = 1.5, 95% IC 1.1-2.0], enquanto que para o genótipo *GSTP1*C* não se observou esta associação (Krajinovic *et al.*, 2002). Além disso, o genótipo *GSTP1*B* em combinação com *GSTM1* nulo *(-/-)* foram associados a um maior aumento de leucemia linfocitária aguda (OR = 2.1; 95% IC-1.3-3.4) (Krajinovic *et al.*, 2002). Um estudo realizado no Brasil associou os genótipos *Isoleucina/Valina* e *Valina/Valina* do *GSTP1* em combinação com os genes *CYP1A1* e *CYP2E1* com risco aumentado de leucemia linfocitária aguda em crianças (Canalle *et al*., 2004). Um estudo com câncer colorretal não verificou associação desta doença com os genótipos *Isoleucina/Isoleucina*, *Isoleucina/Valina* e *Valina/Valina*, porém em combinação com os genótipos *GSTM1* nulo e *GSTT1* nulo, o *GSTP1 Isoleucina/Valina* ou *GSTP1 Valina/Valina* apresentaram risco aumentado de desenvolvimento de câncer colorretal (OR = 2.69, 95% IC: 1.02–7.11) (Ates *et al*., 2005). Por outro lado, a *GSTP1* não teve associação com risco de desenvolvimento de câncer de próstata (Mo *et al*., 2009). No Brasil este gene não teve associação com o risco de desenvolvimento de câncer de pulmão (Honma *et al*., 2008).

Os polimorfismos combinados *GSTM1/GSTP1* podem modular os níveis de aductos formados pela exposição a PAH em alguns tecidos humanos, tais como células sangüíneas mononucleares (Butkiewicz *et al.,* 2000). Contudo, outros fatores influenciam o nível de aductos de DNA, tais como a atividade de outras enzimas de

metabolismo (por ex. CYPs) e a eficiência do sistema reparo de DNA (Butkiewicz *et al.*, 2000).

I.3.2.1.3 *GSTT1*

O gene *GSTT1* está localizado no cromossomo 22q11.2 e apresenta polimorfismo de deleção assim como o gene *GSTM1*. O genótipo nulo para a *GSTT1*, a *GSTT1 (-/-)* apresenta atividade enzimática nula (Pemble *et al.*, 1994) e está relacionada com o aumento do risco de desenvolvimento de câncer devido à deficiente detoxificação de compostos carcinogênicos (Taningher, 1999). A *GSTT1* está envolvida no metabolismo de compostos menores, como monohalometano e óxido de etileno (Landi, 2000).

Diversos trabalhos tem relacionado o genótipo *GSTT1 (-/-)* com o aumento do risco de desenvolvimento de câncer. Sorensen *et al.*, (2004) demonstraram que indivíduos portadores do genótipo nulo *GSTT1 (-/-)* apresentaram um risco 2,4 vezes maior de desenvolvimento de câncer de pulmão. Entretanto, em outros estudos os mesmos autores não encontraram associação entre as GSTs e o risco de desenvolvimento de câncer de pulmão (Schneider *et al.*, 2004; Sorensen *et al.*, 2007). Soya *et al.* (2007) demonstraram que o gene *GSTT1 (-/-)* está associado significativamente com maior risco de desenvolvimento de câncer do trato aerodigestivo.

Outro estudo analisou a influência de polimorfismos da *GSTM1* e *GSTT1* sobre os níveis de danos oxidativos no DNA induzidos por água oxigenada em linfócitos humanos *in vitro*, e verificou que não houve diferença significativa nos níveis de danos oxidativos no DNA de indivíduos com genótipos nulos e positivos *GSTT1* e

GSTM1. Além disso, foi observado que a pré-incubação com quercetina, um flavonóide, oferece proteção contra danos oxidativos no DNA induzidos por água oxigenada, que foi mais acentuada em indivíduos *GSTT1 (+/+)* do que em indivíduos *GSTT1 (-/-),* sugerindo que a quercetina pode estimular a atividade de enzimas de metabolismo de fase II (Wilms *et al*., 2007[a]). Warwick *et al* (1997), demonstraram que indivíduos *GSTM1(-/-)* e *GSTT1(-/-)* apresentavam risco elevado de desenvolvimento de câncer relacionado ao hábito tabagista. Na população brasileira, foi detectado que aproximadamente 8% dos indivíduos apresentam o genótipo *GSTM1(-/-)/ GSTT1(-/-)* (Cabral , 1999; Hatagima , 1999).

I.4 Metabolismo do Ácido Fólico

O metabolismo do ácido fólico desempenha um papel importante no metabolismo do DNA, pois é responsável pela regulação da expressão gênica, síntese de nucleotídeos e reparo de DNA (Fenech, 2001). O metabolismo do ácido fólico está relacionado a enzima metilenotetrahidrofolato redutase (MTHFR), que cataliza a conversão da 5,10-metilenotetrahidrofolato (5,10-THF) em 5-metilenotetrahidrofolato (5-THF), a forma predominante do ácido fólico circulante. Este, por sua vez, fornece grupos metil para a remetilação da homocisteína em metionina (Bailey *et al*., 2002), que é um aminoácido essencial à síntese da S-adenosilmetionina (SAM), que é a principal doadora de grupos metil às reações de metilação, inclusive para a metilação do DNA (Selhub e Miller, 1992, Zingg e Jones, 1997), que atua na regulação da expressão gênica e na conformação da molécula de DNA (Duthie, 1999). Em condições de deficiência de acido fólico, a concentração de SAM é diminuída levando a hipometilação do DNA. Esta hipometilação pode acometer regiões do genoma como

os protoncogenes, favorecendo o risco de desenvolvimento de câncer (Yi *et al.*, 2000). Além disso, a deficiência do ácido fólico diminui a conversão de homocisteína em metionina causando um acúmulo de homocisteína no sangue. Este aminoácido é considerado um importante fator de risco para o câncer (Kark *et al.*, 1999) e pode gerar espécies reativas de oxigênio (ROS), levando a danos oxidativos no DNA (Oikawa *et al.*, 2003). Estudos demonstraram que o aumento da taxa de quebras cromossômicas está relacionada com a elevada concentração de homocisteína (Fenech *et al.*, 1997, Fenech *et al.*, 1998). A deficiência do ácido fólico também leva a uma menor concentração de 5,10-THF, que é utilizada para a síntese de purinas além de fornecer grupos metil para a enzima timilidato sintetase (TS), que converte a deoxiuridina monofosfato (dUMP) em deoxitimidina monofosfato (dTMP). Portanto, na deficiência de ácido fólico e consequentemente menor concentração de 5,10-THF, há menor síntese de purinas, e maior nível de incorporação de uracila ao DNA no lugar da timina (Eto e Krumdieck, 1986). Essa incorporação errônea da uracila pode ser tão intensa que as vias de reparo por excisão de base podem ser sobrecarregadas, levando a colapso do sistema reparo do DNA, com acúmulo de sítios apurínicos/apirimidínicos, quebras de fita simples e dupla o que pode levar a mutações pontuais e aberrações cromossômicas (Blount e Ames, 1995; Blount *et al.*, 1997; Ames, 2001; Fenech, 2001).

Polirmorfismos de genes do metabolismo do ácido fólico em combinação com o ácido fólico determinam as concentrações de SAM, homocisteína, 5,10-THF, 5-THF, e, portanto a estabilidade genômica.

I.4.1 Polimorfismos do gene *MTHFR*

O gene que codifica a enzima MTHFR foi mapeado no cromossomo 1, região 1p 36.3 e apresenta 11 éxons que variam de 102 a 432 pares de base em extensão. O polimorfismo *C667T* do gene *MTHFR* resulta na substituição de uma alanina por uma valina (Frosst *et al*., 1995). Indivíduos heterozigotos (*CT*) e homozigotos (*TT*) para este polimorfismo possuem 65% e 30% da atividade enzimática normal *in vitro*, respectivamente (Frosst *et al*., 1995, Crott *et al*., 2001).

Indivíduos homozigotos *TT* estão associados com maior concentração de homocisteína no plasma, devido a baixa atividade enzimática e com isso há remetilação insuficiente de homocisteína (Kluijtmans *et al*., 1997, Zittoun *et al*., 1998). Por este motivo, este genótipo também está associado ao maior risco de desenvolvimento de doenças cardiovasculares devido ao maior nível de homocisteína circulante (Sadeghian *et al*., 2006). Porém, diversos estudos associaram o genótipo *TT* combinado a adequada concentração de ácido fólico com menor risco de câncer colorretal (Ma *et al*., 1997, Chen *et al*., 1999) e leucemia linfocitária aguda (Skibola *et al*., 1999, Wiemels *et al*., 2001). Por outro lado, outros estudos associaram este genótipo ao maior risco de câncer endometrial (Esteller *et al*., 1997). Em condições de baixo consumo de ácido fólico, o genótipo *TT* do *MTHFR* foi associado ao maior risco de desenvolvimento de câncer gástrico (Miao *et al*., 2002). Os danos no DNA associados ao genótipo *TT* apresentam resultados contraditórios. Crott *et al* (2001) não encontraram efeito do polimorfismo *C677T* sobre danos cromossômicos *in vitro*. Devos et al (2008) verificaram que o genótipo *TT* diminuiu a incorporação de uracilas ao DNA em aproximadamente 34%.

Narayanan *et al.* (2004) não encontraram influência dos polimorfismos do *MTHFR* sobre os níveis de danos no DNA.

O polimorfismo *A1298C* do *MTHFR* resulta na substituição do glutamato por uma alanina e está associado à redução de 60% da atividade enzimática em homozigotos *CC* (van der Put *et al*., 1998, Wisberg *et al*., 1998). Estudos indicam um menor risco de leucemia linfocitária aguda em adultos e crianças com os genótipos heterozigotos (*AC*) e homozigotos (*CC*) (Skibola *et al*., 1999, Wiemels *et al*., 2001). Outros estudos demonstraram uma associação inversa entre o genótipo *CC* e câncer colorretal (Chen *et al*., 2002; Keku *et al*., 2002; Murtaugh *et al*., 2007). Estudos associaram a combinação entre os polimorfismos *C677T* e *A1298C* com o risco de desenvolvimento de câncer, por exemplo: indivíduos portadores dos genótipos *1298AA/677TT* ou *1298CC/677CC* apresentaram redução de 2,2 e 3,3 vezes, respectivamente do risco de desenvolvimento de leucemia linfocitária aguda infantil (Krajinovic *et al*., 2004).

I.4.2 Polimorfismos do gene *TS*

O gene timidilato sintetase, mapeado no cromossomo 18, é altamente polimórfico. Um polimorfismo genético da timidilato sintetase foi encontrado na seqüência na região promotora 5' não traduzida (*TSER*), que consiste em duas (*2R*) ou três repetições em tandem (*3R*) de 28-pb (Horie *et al*., 1995). O número de repetições em tandem determina a atividade da *TS*. Indivíduos homozigotos para três repetições (*3R*) possuem maior nível de RNAm comparados com homozigotos para duas repetições (*2R*) (Pullarkat *et al*., 2001). Estudos *in vitro* e *in vivo* demonstraram

maior atividade da *TS* associada ao alelo 3R (Horie *et al*., 1995, Horie *et al*., 1997, Pullarkat *et al*., 2001).

Outro polimorfismo identificado da *TS* é a *deleção/inserção* de 6-pb na região 3' não traduzida (*TS3'UTR* ou *1494del6*) (Ulrich *et al*., 2000). Este polimorfismo influencia a expressão e estabilidade de RNAm do *TS* (Ulrich *et al*., 2000, Chu e Dolnick, 2002, Mandola *et al*., 2004).

Estudos sobre a associação dos polimorfismos do *TS* com o risco de desenvolvimento de câncer apresentam resultados contraditórios. Chen *et al* (2003) demonstraram que o genótipo *2R/2R* estava associado ao menor risco de desenvolvimento de câncer colorretal (Chen *et al*., 2003). Ulrich *et al* (2002) associaram o genótipo *3R/3R* com maior risco de pólipos em indivíduos com consumo médio ou baixo de ácido fólico (Ulrich *et al*., 2002). Foi demonstrado também associação entre *2R/2R* e menor risco de desenvolvimento de câncer de cólon em homens (Ulrich *et al*., 2005). Por outro lado, outro estudo não encontrou associação do *TS* com risco de desenvolvimento de câncer de cabeça e pescoço (Zhang *et al*., 2004). Skibola *et al*. (2002) demonstraram risco aumentado de desenvolvimento de leucemia linfocitária aguda em indivíduos 2R/2R.

I.5 Sistema reparo de DNA

Vários estudos têm relatado a existência de grande variação inter-individual na capacidade de reparo do DNA (Vodicka *et al*., 2004). Indivíduos com menor capacidade de reparo de DNA apresentam maior risco de desenvolvimento de vários tipos de câncer (Vodicka *et al*., 2004). Polimorfismos de nucleotídeo único (SNPs)

em seqüências codificadoras ou reguladoras podem levar a alterações nas enzimas de reparo de DNA, modulando a suscetibilidade ao câncer (Vodicka *et al.*, 2004).

I.5.1 Polimorfismos do gene XRCC1

O gene de reparo *XRCC1* localizado no braço curto do cromossomo 19 (Mohrenweiser *et al.,* 1989) codifica a proteína XRCC1 envolvida no sistema de reparo de excisão de bases (BER), que forma um complexo com outras enzimas envolvidas no reparo de quebras de fita simples do DNA por excisão de bases: DNA ligase III, DNA polymerase β e polyadenosina difosfato (ADP) ribose] polimerase (PARP) (Abdel-Rahman *et al.*, 2000). A proteína XRCC1 tem papel importante em diversas etapas do processo de reparo de quebras de fita simples do DNA, otimizando o reparo de danos no DNA causados por ROS, agentes ionizantes e alquilantes (Abdel-Rahman *et al.*, 2000).

Já foram descritos e validados mais de sessenta polimorfismos do gene *XRCC1* (Huge et al., 2005), dos quais os mais relevantes ocorrem em três seqüências conservadas e resultam em substituições de aminoácidos (Shen *et al.*, 1998). Estes três polimorfismos foram detectados nos códons 194 (Arg→Gln), 280 (Arg→His) e 399 (Arg→Gln), numa freqüência alélica de 0,25, 0,08 e 0,25, respectivamente. Alguns estudos associaram estes polimorfismos a diferentes riscos de desenvolvimento de câncer e níveis de danos no DNA (Vodicka *et al.*, 2004). Um estudo mostrou que indivíduos com o alelo *399gln* tiveram um aumento do risco de danos cromossômicos, enquanto que indivíduos com o alelo 1*94trp* tiveram uma redução desses riscos (Skjelbred *et al.*, 2006[a]). Os mesmos autores demonstraram

aumento do risco de desenvolvimento câncer colorretal em indivíduos com o polimorfismo *280Hist* e menor risco em indivíduos com polimorfismo *399Gln* (Skjelbred *et al.*, 2006[b]). Outro estudo associou o alelo *399Gln* a maior frequência de micronúcleos em indivíduos fumantes (Mateuca *et al.*, 2008). Li *et al* (2009) avaliaram a associação dos polimorfismos do *XRCC1* com a evolução de lesões gástricas pré-cancerosas associadas à *H. pylori*, e verificaram que indivíduos portadores do genótipo *194Arg/Trp* + *194Trp/Trp* apresentaram maior chance de regressão das lesões gástricas, enquanto que indivíduos com o genótipo *399Arg/Gln* + *399Gln/Gln* tiveram uma diminuição da chance de regressão das lesões (Li *et al.*, 2009). Em estudo de meta-análise Kiyohara *et al* (2006) demonstraram associação do genótipo *399Gln/Gln* com maior risco de desenvolvimento de câncer de pulmão em asiáticos (Kiyohara *et al.*, 2006).

I.6 Carotenóides

Vários processos endógenos e exógenos geram ROS. Processos exógenos incluem poluição ambiental, radiação, vários compostos químicos de alimentos industrializados e carnes grelhadas a altas temperaturas, e processos endógenos incluem respiração mitocondrial, inflamação, biotransformação de compostos químicos e outros processos metabólicos (Loft *et al.*, 2008). O excesso de ROS pode levar ao estresse oxidativo, que pode causar danos oxidativos no DNA, que por sua vez, é um grande contribuinte para o desenvolvimento de câncer (Collins *et al.*, 1994; Watters *et al.*, 2008). Micronutrientes como carotenóides, retinol e vitamina E neutralizam os radicais livres (Krinsky *et al.*, 1998). Carotenóides são compostos pigmentados presentes em frutas e vegetais (Mangels *et al.*, 1993, Rao e Rao, 2007),

e 90% dos carotenóides da dieta humana são representados por luteína, criptoxantina, licopeno, α-caroteno e β-caroteno (Gerster, 1997). Como eles são neutralizadores de radicais livres, a dieta rica em carotenóides contribui para a prevenção de diversas doenças, como câncer (Ziegler, 1991, Rao e Rao, 2007), aterosclerose (D'Odorico *et al*., 2000), doenças oftálmicas (Krinsky e Johnson, 2005) e doenças cardiovasculares (Crews *et al*., 2005). Além de atuarem como antioxidantes, os carotenóides também atuam nas junções de comunicação (gap), resposta imunológica, regulação do crescimento celular, e modulam a expressão de genes de metabolismo de xenobióticos (Paiva e Russel, 1999, Rao e Rao, 2007). Com exceção do licopeno, os carotenóides são provitaminas A, ou seja, se há necessidade, se convertem em retinol (Ribaya *et al*., 2000, Sei, 2008). Retinol, ou vitamina A, possuem diversas funções atuando no crescimento, imunidade, visão e reprodução (Lasisi, 2008), além de agirem como antioxidantes em reações biológicas (Gelain e Moreira, 2008). O α-tocoferol ou vitamina E também é um antioxidante que neutraliza ROS e inibe a oxidação de LDL colesterol, o que contribui para a prevenção de aterosclerose e câncer (Wright *et al*., 2006), além de agir como regulador da expressão gênica, como molécula sinalizadora e como inibidor da atividade da proteína quinase C (Schneider, 2005).

Diversos estudos vêm associando a baixa concentração de micronutrientes no plasma com o maior risco de diversas doenças, e com isso vêm demonstrando a importância de uma dieta rica em frutas e vegetais para a prevenção de doenças, como o câncer.

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II – OBJETIVOS

Devido à importância dos fatores alimentares no processo carcinogênico, o presente projeto tem os seguintes objetivos:

1. Verificar a relação entre os polimorfismos dos genes de metabolismo *GSTM1, GSTT1, GSTP1, CYP1A1* e *CYP2E1* com o nível de danos oxidativos no DNA e incorporação de uracila no DNA e a eficiência do sistema reparo do DNA em linfócitos de sangue periférico de indivíduos com diferentes padrões alimentares.
2. Verificar a relação entre os polimorfismos do gene *XRCC1* com o nível de danos oxidativos no DNA e incorporação de uracila no DNA e a eficiência do sistema reparo do DNA em linfócitos de sangue periférico de indivíduos com diferentes padrões alimentares.
3. Verificar a relação entre os polimorfismos dos genes *MTHFR e TS* com a eficiência do sistema de reparo de DNA e o nível de danos oxidativos no DNA e incorporação de uracila no DNA e a eficiência do sistema reparo do DNA em linfócitos de sangue periférico de indivíduos com diferentes padrões alimentares.
4. Verificar a correlação entre o nível de nutrientes plasmáticos com o nível de danos oxidativos no DNA, com o nível de danos oxidativos no DNA e incorporação de uracila no DNA e a eficiência do sistema reparo do DNA em linfócitos de sangue periférico de indivíduos com diferentes padrões alimentares.

Manuscrito I

Trabalho elaborado segundo as normas da revista “The Journal of Nutrition”.

Influence of Diet on the serum concentration of carotenoids, retinol and α- tocopherol

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Abstract

Epidemiological studies have shown the importance of a diet rich in fruits and vegetables in the prevention of illnesses. Low plasma levels of micronutrients as carotenoids, retinol and α-tocopherol, with antioxidant properties, found in fruits and vegetables, are associated with increased risk some diseases, such as cancer, eye disease, cardiovascular disease, atherosclerosis and stroke. Therefore, is clear the importance of a continuous consumption of carotenoid rich foods in order to maintain high levels of serum carotenoids, retinol and α- tocopherol for the potential prevention of chronic diseases in individuals. Until now, the literature did not present studies which selected groups of volunteers with dietary patterns completely antagonists to compare them. The objective of this study was measure the serum levels of carotenoids, retinol and α-tocopherol of two groups of volunteers with antagonist dietary patterns. Group I : 49 naturalistic individuals with a great consume of organic fruits, vegetables and juice and poor consume of industrialized foods, and Group II: 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables. All micronutrients were measured by high-performance liquid chromatography (HPLC). The serum levels were higher in individuals of group I and lower and in few cases almost zero in individuals of group II, concluding that the type of dietary pattern determine the concentration of carotenoids, retinol and α-tocopherol on serum.

Introduction

Carotenoids are family of pigmented compound synthesized by plants and microorganisms and are responsible for the colors of the nature. They are present in fruits and vegetables [1,2], so that animals need to obtain them from diet [3]. Approximately 600 different carotenoids exist in nature [4]. Besides, only about 40 are present in human diet, and about 12 carotenoids can be found in human blood and tissues in measurable concentrations [5], and only 2 are found in retina and lens of the eye [6]. Approximately 90% of the carotenoids in the diet and human body is represented by lutein, cryptoxanthin, α-carotene, β-carotene, and lycopene [7]. Dietary carotenoids are considered to be beneficial in the prevention of many diseases, including cancer [2, 6, 8], eye diseases [6], atherosclerosis [9] , stroke [10], and cardiovascular disease [5]. They act as antioxidants to be efficient scavengers of various free radicals generated *in vitro* [11] and they have also been shown to protect low density lipoproteins (LDLs) against oxidation *in vitro* [12]. Recent studies are also describing that carotenoids may mediate their effects via other mechanisms such as gap junction communication, tumor-supressive activity, carcinogenesis, protection of DNA cell growth regulation, immune response, protect lipid against peroxidation, and as modulators of Phase I and II drug metabolizing enzymes modulating gene expression [2, 13, 14]. However, the supplementation of carotenoids is still unclear and controversy ; some recent studies have shown that supplementation of carotenoids in high concentrations may cause prooxidant effects [15]. Results from interventions studies involving β-carotene supplementation were conflicting, with increase, decrease and no effect of incidence of cancer [16]. One of them reported that β-carotene supplemented smokers were found to have increased lung cancer mortality when

compared to the group not receiving the β-carotene supplement [17]. This occured maybe due to the imbalaced administration of supplement. Antioxidants act in a combination to produce a protective together, and synergistic and antagonistic effects are very hard to predict [18,19].

Carotenoids are provitamins A, except lycopene, thus they can be changed into retinol during intestinal absorption [20] depending on vitamin A status [21]. Besides, no recommended dietary allowances for carotenoids exist. The provitamin A function is the only physiologic function of carotenoids clearly demonstrated in humans. Although they are not essencial to human survival, carotenoids have biological actions that is important to maintain health and the absence of them can not prevent serious diseases. [2,12].

Retinol or vitamin A is present in animal foods, represent the functional molecule in the retinoid compounds referred to as vitamin A, is important on vision cycle, growth, immunity, reproduction [22] and acts in biological systems as antioxidants [23]. Vitamin A supplementation can cause adverse effects [24], but can solve symptons of deficience in many countries [25]. Vitamin A deficiency usually results from malnutrition, but can also be due to abnormalities in intestinal absorption of retinol or carotenoids. Deficiency may cause night blindness (xeropthalmia), abnormal function of many epithelial cells, abnormal bone growth, and digestive problems [26]. Globally, more than 250 million children under the age of 5 years have vitamin A deficiency [27], and the prevalence exist in certain underdeveloped countries, such as Brazil [28].

Another important micronutrient is α-tocopherol, which is biologically and chemically the most active form of vitamin E in human body [29]. This micronutrient is also a fat-soluble antioxidant that protects lipids from peroxidation, is able to scavenge

mutagenic free redicals and inhibit the oxidation of LDL cholesterol, what contribute with the prevention of athlerosclerosis and carcinogenesis [30]. Moreover, it can acts as nonantioxidants also, as a regulator of gene expression, a signaling molecule [31] inhibitor of protein Kinase C activity (PKC) and smooth muscle cell proliferation [32], inhibitor of cellular proliferation, enhacement of immune responses, suppression of tumor angiogenesis and interference with sex-steroid signaling [30]. Epidemiological studies suggest that low concentrations of α-tocopherol are associated with increased risk for cardiovascular diseases [33]. Results of supplementation have shown decreased platelet adhesion and aggregation lipid peroxidation, and an anti-inflammatory effect [34]. Nevertheless, data reported that supplementation may cause a block of antioxidant properties [31]. α-Tocopherol is the most powerful lipid soluble antioxidant known, and could be combined with the individual carotenoids in an antioxidant vitamin index.

Epidemiological studies have shown the importance of a diet rich in fruits and vegetables in the prevention of illnesses, and low plasma concentrations of micronutrients with antioxidant properties, found in fruits and vegetables, are associated with increased risk for some diseases, as decribed above.

Based on these studies and evidences, the aim of this study was measure serum concentrations of carotenoids, retinol and α-tocopherol of two groups of volunteers with antagonist dietary patterns.

Materials and Methods

The present study was developed in Botucatu Medical School-UNESP, department of Internal Medicine and departament of Pathology, in Botucatu, State of São Paulo, Brazil, at period of 2005-2008. This study was approved by the local Ethical Committee. Informed consent to participate was obtained from all the volunteers.

Volunteers

We studied 105 healthy adult volunteers (average age 35,6 ± 11,4, ranging from 19 and 66 years), 52 men and 53 women. All of the volunteer were nonsmokers, were not abusing alcohol, and were not using supplementation, prescription or recreational drugs. The volunteers were divided in two groups: 1) 49 naturalistic individuals with a uncommon life style characterized by absence of stress, bucolic life and great consume of organic fruits, vegetables and juice and poor consume of industrialized foods (Group I); 2) 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables (Group II).

Dietary Assessment

For evaluation of the dietary pattern a Food-frequency Questionnaire adapted from Cardoso and Stocco [35] was applied, and was used for the classification of the groups. For calculating the estimated level of micronutrients ingested, a dietary record of three days was applied, and has provided data (data not shown), which were inserted in Avanutri program (AvaNutri Informática Ltda, RJ, Brazil). This procedure enabled us

to establish an estimate actual quantity of protective foods consumed daily by the population studied. To be classified in Group I, the individuals had to consume the percentile 75 of organic fruits and vegetables and below the percentile 25 of each industrialized products. The individuals of group II were those who consumed less than percentile 25 of organic fruits and vegetables and above the percentile 75 of industrialized products.

Laboratory analyses

Participants provided blood samples on the day of the baseline interview. Blood samples were centrifuged at 2600 rpm for 30 minutes and serum was separated and frozen at -80°C until analyzed for carotenoids, retinol and α-tocopherol. The micronutrients were measured using high-performance liquid chromatography (HPLC) as described by Yeum et al [36].

Statistic Analyses

As the data obtained were asymmetric distribution, was carried out a log, in order to meet normality distribution assumptions.

The statistical analysis of the data obtained consisted of applying the student´s T test to compare the differences of serum micronutrients concentrations between the groups, and the Tukey test to compare the genders differences of the groups. To establish the correlations between the micronutrients, and between juices, vegetables and fruits and micronutrients, the Pearson correlation was applied. The index of significance adopted was 5%. The statistical computer program used was SAS.

Results

The individuals of group I presented higher concentrations on serum of lutein, β-cryptoxanthin, α-carotene, β-carotene, lycopene, retinol and α-tocopherol than the individuals of group II (Figure 1). The variation between these groups was very high, with some carotenoids 10-fold higher in group than another.

Besides dietary intake, other factors as gender could be associated with carotenoid concentrations. We also investigated the possible different carotenoid concentrations between genders. We did not found difference of serum carotenoids concentrations between genders in both groups (Figure 2.)

The Pearson correlation between the micronutrients is on table 1. Fruit intake was correlated with vegetable (r=0.9214), juice (r=0.6657), β-cryptoxanthin (r=0.5614), lutein (r=0.5052), lycopene (r=0.5298), α-carotene (r=0.5595), β-carotene (r=0.5298), and α-tocopherol (r=0.2996). Vegetables intake was correlated with juice (r=0.6288), β-cryptoxanthin (r=0.599), lutein (r=0.5185), lycopene (r=0.5251), α-carotene (r=0.5904), β-carotene (r=0.5251), and α-tocopherol (r=0.2993). Juice intake was correlated with β-cryptoxanthin (r=0.3946), lutein (r=0.3017), lycopene (r=0.2927), α-carotene (r=0.3935), β-carotene (r=0.2927), and α-tocopherol (r=0.2418).

Serum concentrations of cryptoxanthin were correlated with lutein (r=0.4592), lycopene (r=0.6545), α-carotene (r=0.6485), β-carotene (r=0.6545), retinol (r=0.5246), and α-tocopherol (r=0.5355). The correlation was also found between lutein and lycopene (r=0.5657), α-carotene (0.5265), β-carotene (r=0.5657), retinol (r=0.3877), and α-tocopherol (r=0.5332), and between lycopene and α-carotene (r=0.5759) and a strong correlation between lycopene and β-carotene (r=1.0000). The correlations are shown on table 2.

Discussion

Epidemiological studies have shown the importance of a diet rich in fruits and vegetables in the prevention of illnesses. Low plasma concentrations of micronutrients with antioxidant properties are associated with increased risk for some diseases, such as cancer, eye disease and cardiovascular disease. This study compares the serum concentration of lutein, cryptoxanthin, lycopene, β-carotene, α-carotene, retinol and α-tocopherol of two groups of brazilian volunteers with antagonist diets, one group with 49 naturalistic individuals with a great consume of organic fruits, vegetables and juice and poor consume of industrialized foods (Group I), and another group with 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables (Group II).

The results of this study show a positive effect of a diet rich in fruits and vegetables and poor in chemical additives on serum concentrations of lutein, cryptoxanthin, α-carotene, β-carotene, lycopene, retinol and α-tocopherol. For all micronutrients, the serum concentrations were higher in group I and lower and in group II.

Several earlier studies have found an association between plasma carotenoid concentrations and intake of fruits and vegetables [37, 38, 39, 40, 41]. Lin YJ et al (2007), examined the plasma levels of carotenoids in young adults after a 4 weeks of dietary intervention composed of increased intakes of fruits and stir-fried vegetables from a Taiwanese mixed diet, and found that plasma carotenoids levels in subjects significantly increased for β-carotene, lycopene and β-cryptoxanthin, as in our study [42]. Others studies reported results similar to ours, such as Campbell et al (1994), that concluded that all carotenoids were related to total intake of fruit and vegetable among

99 participants from Minnesota in the US [43], and the European Prospective Investigation into Cancer and Nutrition (EPIC) study (2005), which found that carotenoid plasma levels are correlated to dietary fruit and vegetable intake [44], as Tucker et al (1999) demonstrated in 1999 [39]. However, Jansen et al (2004) reported that carotenoids were not able to distinguish between all quartiles of vegetable and fruit intake, maybe because of the measurement error in the intake data [45].

Our study found correlations between total fruit intake and carotenoids and α-tocopherol, except retinol. The same was found with vegetable and juice intake. Retinol did not have correlation with fruits, vegetables nor juices intake, because this micronutrient is found in foods with animal origin, which is eaten equally by both groups (data not shown).

This study did not use the supplementation of α-tocopherol, retinol, neither carotenoids; we only used diet to compared the differences of these micronutrients on serum levels between people with different dietary patterns, and could realize that even without supplementation, a diet rich in fruits and vegetables can maintain levels higher than those who do not have this type of diet, and show the importance of a continuous consumption of carotenoid, retinol and α-tocopherol rich foods in order to maintain at least the minimal levels on serum for the potential prevention of chronic diseases in individuals. Another study that also did not use the supplementation demonstrated that short-term intensive dietary intervention for 6 months on selected populations with increase fruit and vegetable intake raised plasma antioxidant concentrations of α-carotene, β-carotene, lutein, β-cryptoxanthin and ascorbic acid more in the intervention group than in controls [46]. The benefits of supplementation of carotenoids and retinol are still unclear. It appears that carotenoids can protect human body against some

diseases and promote health if is taken at dietary levels [14], due to an imbalance of synergistic effects of the nutrients that supplementation may cause [18].

Our study demonstrated that only dietary pattern was sufficient to correlate with serum concentrations of α-tocopherol, unlike some previous studies, which found significant association only when the diet is associated with supplements [47, 48, 49, 50]. However, studies with supplements concluded that high-dose vitamin E supplements may be associated with increased mortality [51]. Besides, studies observed that the supplementation of α-tocopherol was associated with lower γ-tocopherol concentration in serum [30, 52, 53]; what is not interesting, because this type of vitamin E is a powerful scavenger of reactive nitrogen oxide species and inhibitor of the cycloxygenase-2 enzyme, thus, it has anti-inflammatory properties [30,54] . On the other hand, other results of α-tocopherol supplementation have shown benefits such as decreased lipid peroxidation, decreases platelet adhesion and aggregation, and an anti-inflammatory effect [33, 55]. Thus, more studies with α-tocopherol are needed to clarify the mechanisms, and so far it is safer to consume α-tocopherol regularly from diet to assure the benefits.

Vitamin A deficiency is a public health problem in many regions of the world including Brazil. Data in the last 20 years indicate that the deficiency of this micronutrient ranges all country [56, 57, 58], especially in the southeast [58] and northeast [28, 58]. People in poor regions, generally have financials dificulties to buy foods from animal origin, as milk, meat, eggs, which are sources of retinol. Thus, the present study is important to elucidate the benefits of fruits and vegetables intake on serum levels carotenoids, because these micronutrients, present in fruits and vegetables, are bioconverted in retinol. In areas at risk of vitamin A deficiency, improvement in vitamin A status through dietary provitamin A carotenoid intake may be more

advantageous than periodic supplementation with preformed vitamin A (24). Therefore, it becomes easier to prevent vitamin A deficiencies, by promoting better eating habits, as increase fruits and vegetables intake, on our population. Morover, Brazil is the most generous solo in production of carotenoids of the world, because it has a great diversity of fruits and vegetables, which are not expensive in our country. In other words, they are acessible for the most of people.

The literature does not present studies which selected groups of volunteers with antagonist dietary patterns, as our study, probably because of the difficulty to find people with these extreme eating habits. In fact, most people in Brazil have a mixed diet, with both fruits and vegetables, and industrialized foods. Nevertheless, the individuals of group I live in a comunity in Botucatu neighbourhood, city where our study was realized, and they have an extreme healthy lifestyle, including organic fruits and vegetables intake daily, without chemical additives. In addition, the group II was also seletected in Botucatu city.

Finally, carotenoids, retinol and α-tocopherol were higher on serum concentrations of individuals with a diet rich in fruits and vegetables and poor in industrialized foods, and lower on serum concentrations in individuals with a diet poor in fruits and vegetables and rich in industrialized food, demonstrating the importance of healthy dietary patterns to maintain levels of micronutrients on serum to prevent vitamin deficiencies and chronic diseases.

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Legends

Table 1. Correlations between lutein, β-cryptoxanthin, lycopene, α-carotene, β-carotene, retinol, and α-tocopherol.

Table 2. Correlations between fruits, vegetables, juices, lutein, β-cryptoxanthin, lycopene, α-carotene, β-carotene, retinol, and α-tocopherol.

Figure 1. Levels of lutein, β-cryptoxanthin, α-carotene, β-carotene, lycopene, retinol and α-tocopherol on group I (n= 49), and group II (n=56). Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Student´s test was used to compare differences between groups. The individuals of group I presented higher serum carotenoids, retinol and α-tocopherol concentrations than the individuals of group II ($p < 0.013$).

Figure 2. Influence of gender on serum carotenoids, retinol and α- tocopherol concentration. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Student´s test was used to compare differences between groups. The carotenoids, retinol and α- tocopherol concentrations did not differed between the gender on both groups..

Table 1.

Micronutrients	Lutein	β-cryptoxanthin	Lycopene	α-carotene	β-carotene	Retinol	α-tocopherol
Lutein	1.000	0.646	0.600	0.682	0.589	0.752	0.684
β-cryptoxanthin	0.646	1.000	0.663	0.580	0.649	0.679	0.684
Lycopene	0.600	0.663	1.000	0.589	0.514	0.611	0.512
α-carotene	0.682	0.580	0.589	1.000	0.873	0.628	0.594
β-carotene	0.589	0.649	0.514	0.873	1.000	0.555	0.482
Retinol	0.752	0.679	0.611	0.628	0.555	1.000	0.831
α-tocopherol	0.684	0.653	0.512	0.594	0.482	0.831	1.000

Table 2.

Micronutrients and Fruits	Fruits	Vegetables	Juices
Lutein	0.5052	0.5185	0.3017
β-cryptoxanthin	0.5614	0.599	0.3946
Lycopene	0.5298	0.5251	0.2927
α-carotene	0.5595	0.5904	0.3935
β-carotene	0.5298	0.5251	0.2927
Retinol	0.1447	0.1397	0.0918
α-tocopherol	0.2996	0.2993	0.2418
Fruits	1.000	0.9214	0.6657

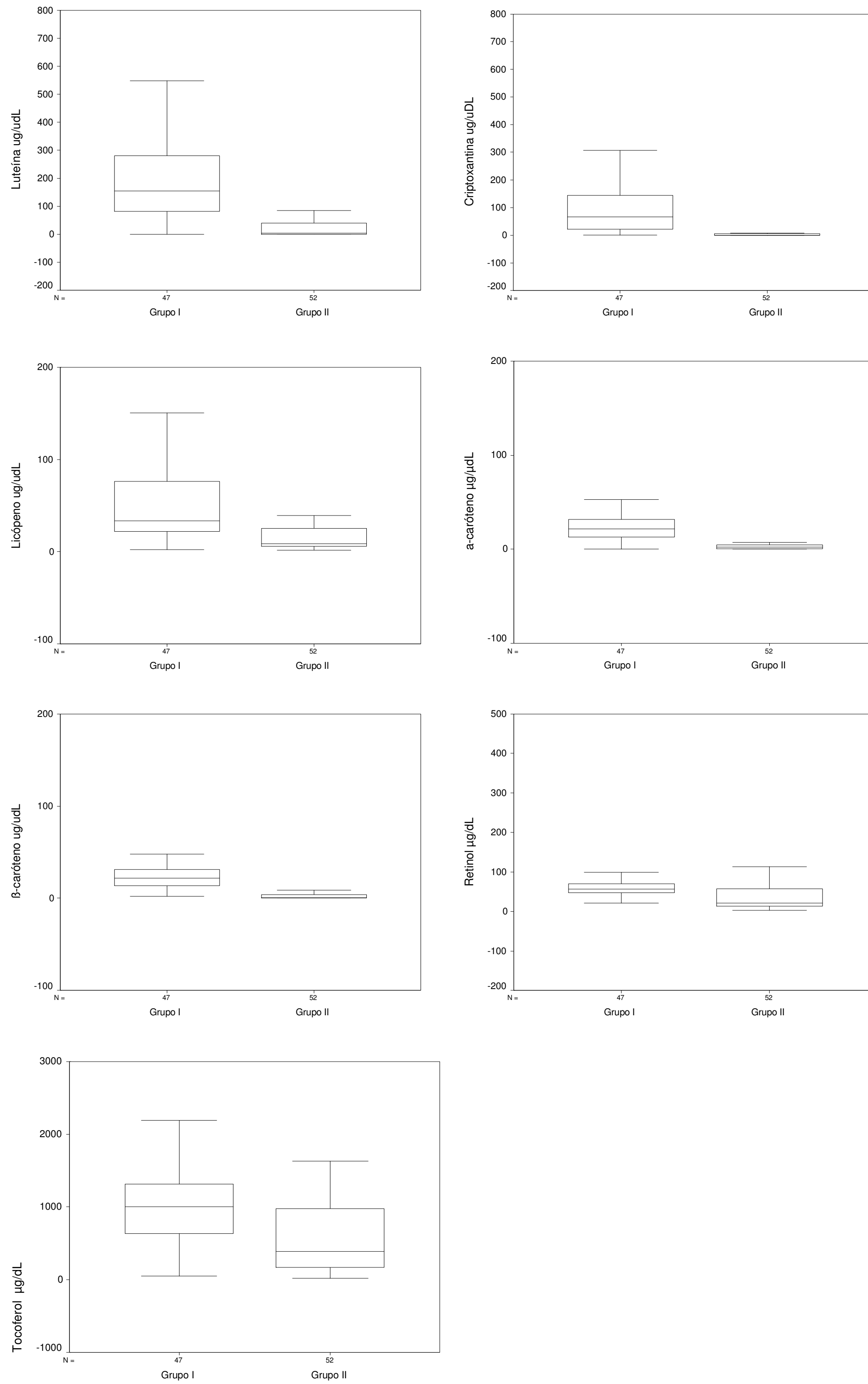

Figure 1.

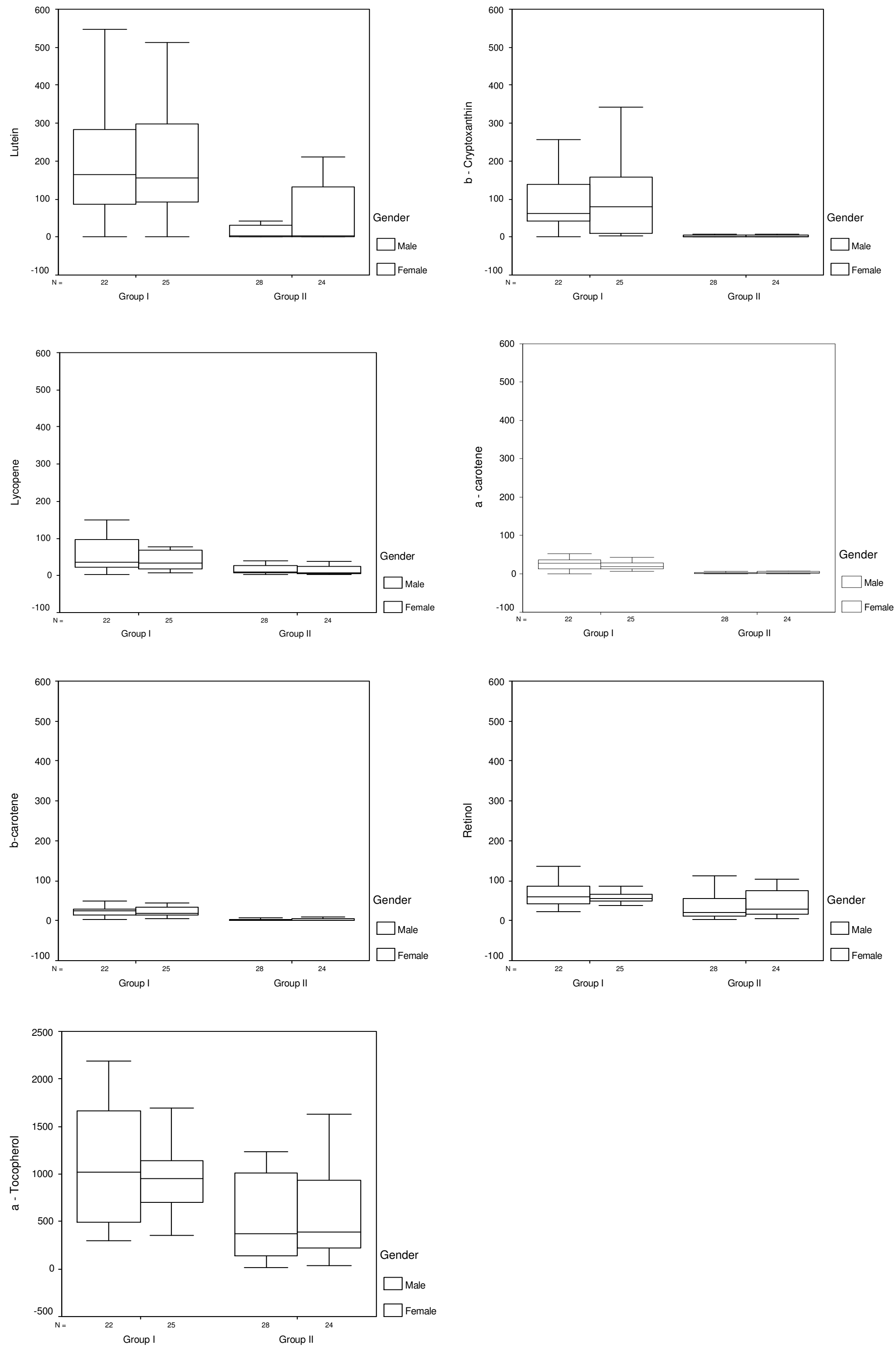

Figure 2.

Manuscrito II

Trabalho elaborado segundo as normas da revista “The Journal of Nutrition”.

Influence of diet and *194Trp* and the *399Gln* polymorphisms of the DNA repair gene *XRCC1* on the Oxidative DNA Damage, Misincorporation Uracil and DNA Repair Capability.

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(*Running Title*): Diet influence on DNA damage and repair

Key Words: Diet, oxidative DNA damage, uracil incorporation, DNA repair, comet assay

Abstract

Dietary factors can be related to fortuitous genomic instability, which can be preceded by oxidative damage and uracil incorporation in DNA which can trigger a carcinogenic process. Linked to diet, there is a genetic susceptibility to developing cancer. Polymorphic *XRCC1* in humans is involved in Base excision repair (BER) that repairs uracil and oxidative DNA damage. The aim of this study was to evaluate possible influence of *194Trp* and the *399Gln* polymorphisms of the DNA repair gene *XRCC1* and diet on level of oxidative DNA damage, misincorporation uracil and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets: Group I) 42 individuals with a diet rich in natural products such as whole grains, fruit, and vegetables, and low in processed products, and Group II) 42 individuals on a diet high in processed products and low in fruit and vegetables. PCR-RFLP was used to genotyping *194Trp* and the *399Gln* polymorphisms of *XRCC1*. None of the *194Trp* genotypes influenced the endpoints studied. Individuals carrying the *XRCC1 Arg399Arg* genotype presented higher levels of purine and pyrimidine damaged than individuals carrying the *XRCC1 Arg399Gln* genotype ($p < 0.05$), while in the group II individuals carrying the *XRCC1 Arg399Arg* genotype presented higher levels of pyrimidine damaged and uracil misincorporated on DNA than individuals carrying the *XRCC1 Arg399Gln* genotype ($P < 0.05$). Moreover, the individuals of group II carrying the *XRCC1 Arg399Arg* genotype presented lower DNA repair capability than individuals carrying the *XRCC1 Arg399Gln* genotype. In conclusion, our results demonstrated that *XRCC1 Arg399Arg* genotype is relationship with accumulative DNA damage and lower DNA repair capability in healthy individuals with antagonist diets.

1. Introduction

Diet has long been studied as a potentially important factor in the etiology of various cancers. The increased intake of many chemical food additives have already been proven to be carcinogenic [1], associated to decreased consumption of natural foods are factors that contributes to the emergence of modern-life diseases as the cancer [1].

The micronutrients can act as antioxidants, enzymatic cofactors or as part of the structure of proteins involved in oxidative DNA damage prevention, DNA synthesis and repair, maintenance methylation of DNA [2], and in the process of apoptosis [3]. Variations in the intake of micronutrients may be related to genomic instability detected by the increased incidence of biomarkers, such as point mutation and chromosomal breakage, or by interfering in chromosomal segregation, gene expression, oxidative stress, necrosis and apoptosis [4].

According to Ames [5], the deficiency of micronutrients, such folic acid in humans has been associated with megaloblastic anemia, defects of the neural tube closure in newborns, heart disease and cancer development, especially of in colon and rectum [6]. In conditions of folic-acid deficiency decreasing the availability of N5,N10-ethylenetetrahydrofolate, which is needed for the de novo synthesis of thymine, leads to the misincorporation of uracil into DNA that in excess not only generates point mutations, but can also cause single- and double-strand breaks, that could lead to the genomic instability and favours cancer [7,8].

Nevertheless, the development of cancer is related to an interaction between environmental factors such as diet and genetic factors. While several molecular epidemiology studies has been shown that polymorphisms of xenobiotic metabolizing enzymes may be a significant risk modifiers for environmentally- induced cancers,

polymorphisms of DNA repair genes could be another group of cancer genetic susceptibility. Several molecular epidemiological studies have investigated the individual capacity to repair DNA damage as a biomarker for cancer risk. In fact, interindividual variation in DNA repair indicate that individuals with a repair capacity of 65-80% of general population could present major risk development of cancer, since that DNA repair is a critical defense system maintaining the integrity of genome [9] Single nucleotide polymorphisms (SNPs) in codifier or regulator sequences cause changes in DNA repair enzymes, modulating susceptibility to cancer [9].

The X-ray repair cross-complementing Group 1 (*XRCC1*) gene located in the short arm of chromosome 19 is involved in Base excision repair (BER) that repairs uracil and oxidative DNA damage. Although no enzymatic activity has been attributed to XRCC1 protein, it is recruited within seconds to the sites of DNA strand breakage and acts as a scaffolding protein that directly interacts with OGG1, poly(ADP-ribose) polymerase (PARP), DNA ligase III, and DNA polymerase b, facilitating the repair of single base modifications, nonbulky adducts, oxidative DNA damage, alkylation adducts, and damage induced by ionizing radiation [9]. Recently it was shown that XRCC1 is phosphorylated at the BRCT I domain at Ser 371 by DNA dependent protein kinase in response to DNA damage [10]. More than 60 validated SNPs in the *XRCC1* gene were listed in the Ensembl database [11]. However the most extensively studied SNPs are *Arg194Trp* (*C26304T*) in exon 6, *Arg280His* (*G27466A*) in exon 9, and *Arg399Gln* (*G28152T*) in exon 10 [11]. The functional significance of these *XRCC1* variants has not been elucidated, but some of the polymorphisms may be associated with a reduced repair capacity and increased susceptibility to adverse health conditions, including cancer [11]. Several studies reported that the *XRCC1 399Gln* polymorphism was associated with increased levels of DNA damage in human cells exposed to various

mutagens [12] and as a risk factor for different types of cancer including head-neck, bladder, lung and glioma [13]. However, other studies reported that the *399Gln* polymorphism has no adverse effect on DNA repair or that this polymorphism is associated with lesser level of DNA damage [13]. In the same way the studies of *194Trp* reported conflicting results [14]. Several studies have found a relationship between the *XRCC1* polymorphisms and susceptibility to lung [15], bladder [16], gastric [17], and esophageal squamous cell carcinoma [18].

In the present study we evaluated the possible influence of *194Trp* and the *399Gln* polymorphisms of the DNA repair gene *XRCC1* and diet on level of oxidative DNA damage, misincorporation uracil and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets.

2. Material and Methods

2.1. Subject selection

This study was approved by the Ethical Committee for Human Research of the Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil. Informed consent was obtained from each volunteer.

A total of 105 healthy adult volunteers (average age 35,6 ± 11,4, ranging from 19 and 66 years), 52 men and 53 women was studied. All of the volunteer were nonsmokers, were not abusing alcohol, were not using prescription or recreational drugs and any vitamins and minerals supplementation. These volunteers were distributed in two groups: 1) Group I - 49 naturalistic individuals with a life style characterized by absence of stress, bucolic life and great consume of organic fruits, vegetables and juice

and poor consume of industrialized foods; 2) Group II - 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables.

For evaluation of the dietary pattern, calculating the estimated level of micronutrients ingested (data not shown) and for the classification the groups a Food-frequency Questionnaire adapted from Cardoso and Stocco [19] was applied. This quantitative questionnaire has provided data, which were analysed using the software AvaNutri (Avanutri Informática Ltda, RJ, Brazil). This procedure enabled us to establish an estimate of the quantity of protective foods consumed daily by the population studied. To be classified in Group I, the individuals had to consume the percentile 75 of organic fruits and vegetables and below the percentile 25 of each industrialized products. The individuals of group II were those who consumed less than percentile 25 of organic fruits and vegetables and above the percentile 75 of industrialized products.

Only were selected individuals that have adopted the respective diet pattern for at least 10 years.

2.2. Determination of Oxidative DNA Damage and level of uracil incorporate into DNA

Data on oxidative DNA damage, uracil misincorporation into DNA, and DNA repair were described and accomplished by Prado et al in a parallel study (in preparation). Briefly, The alkaline Comet assay [20], modified with lesion-specific enzymes was used to detect single and double strand breaks, labile sites (SBs), oxidised purines and pyrimidines and uracil [21]. All procedures were conducted in the dark to minimize spurious sources of DNA damage. Briefly, 10 µl of the isolated lymphocytes suspension [22] (≅ 2×10^4 cells) were embedded into 0.5% low melting point agarose

(Sigma) and spread on agarose-precoated microscope slides. Twelve slides were prepared for each sample. Slides were immersed overnight in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium salt N-Lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% DMSO added fresh) at 4^0C. After lysing, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD) and then three slides for each treatment were incubated at 37^oC for 45 min with 100 μL of endonuclaese III (Endo III - 1:1000; New England Biolabs Inc), 100 μL of formamidopyrimidine-DNA glycosylase (FPG - 1:1000; New England Biolabs Inc), 100 μL of uracil-DNA glycosylase (UDG - 1:1000; New England Biolabs Inc) or with enzyme buffer only. Endo III recognizes oxidised pyrimidines (SBs EndoIII), while FPG identifies oxidised purines (SBs FPG), especially 8-oxo-guanine and UDG recognizes uracil (SBs UDG). Enzyme buffer only is used to identified SBs. Subsequently the cells were exposed to alkali buffer (1mM EDTA and 300 mM NaOH, pH ≅ 13.4), at 4^oC, for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted in the same solution at 4^oC, for 30 min, at 25 V (1v/cm) and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 50 μL Syber Green (1:10000; Invitrogen), and analyzed in a fluorescence microscope at 400 x magnification, using an image analysis system (Comet Assay II – Perceptive Instruments, Suffolk, UK).

For each individual, one hundred randomly selected cells (50 from each of two replicate slides) for each treatment (FPG, Endo III, UDG or enzyme buffer only) were evaluated and the mean tail intensity (%DNA Tail) was determined, totaling 400 cells analyzed from each individual. Tail Intensity, according to Comet Assay II – Perceptive instruments, is defined as “the sum of all intensity values in the tail region less those

which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity".

To analyze the influence of dietary patterns and *194Trp* and the *399Gln* polymorphisms of the DNA repair gene *XRCC1* on the DNA repair capability, 200 µl of the freshly isolated lymphocytes were treated with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H_2O_2, these aliquots were incubated at 37 °C for 30 minutes, and DNA damage was checked again.

2.3. Analysis of *XRCC1* Gene Polymorphisms.

Genotypic analyses of the *XRCC1* Gene were evaluated by multiplex PCR-RFLP using primers for codon 399: XR1F (5'-TTGTGCTTTCTCTGTGTCCA-3') and *XR1R* (5'-TCCTCCAGCCTTTTCTGATA-3') and for codon 194: *XR2F* (5'-GCCCCGTCCCAGGTA-3') and *XR2R* (5'-AGCCCCAAGACCCTTTCATC- 3'), as described Abdel-Rahman *et al*. [9]. These primers generated a 491-bp product and a 615-bp product respectively, containing the polymorphic sites.

The PCR conditions consisted of an initial melting temperature of 94ºC (5 min) followed by 30 cycles of melting (94 ºC, 30 s), annealing (62 ºC, 1 min), extension (72ºC, 45 s), and a final extension step (72 ºC, 5 min). The PCR products were digested for 16 h at 37 ºC by *MspI* (New England Biolabs, Beverly, MA), which recognizes the wild-type Arg allele at codon 194 and the wild-type Arg allele at codon 399. The wild-type Arg allele for codon 194 is identifed by the presence of a band at 292 bp, while the mutant Trp allele is identified by the presence of a band at 313 bp (indicative of the absence of the MspI cutting site). For codon 399 The wild-type Arg allele is identified by the presence of two bands at 374 and 221-bp, while the mutant Gln allele is

identified by the presence of the uncut 615-bp band (indicative of the absence of the MspI cutting site). A 178 bp band, resulting from an additional invariant *MspI* cutting site in the 491 bp amplified fragment is always present and serves as an internal control for complete enzyme digestion.

All experiments included positive and negative controls for each polymorphism studied.

2.4. Statistical analysis

The data obtained in the Comet Assay were asymmetric distribution, thus, Log transformations were applied to DNA damage distributions to help meet normality distribution assumptions. To evaluate the influence of diet and *194Trp* and the *399Gln* polymorphisms of the DNA repair gene *XRCC1* on DNA damage the Tukey test was applied. The index of significance adopted was 5%.

3. Results

DNA damage, including single and double strand breaks, labile sites, oxidised purines and pyrimidines and misincorporated uracil on DNA, was detected by the alkaline Comet assay modified with lesion-specific enzymes, FPG, Endo III and UDG, respectively. The alkaline Comet assay measures SBs and AP sites. The enzyme-modified assay measures oxidative DNA damage as a combination of SBs, AP sites and oxidised bases – formamidopyrimidines and the oxidised purine 8-oxo-guanine when considering FPG (SBs FPG), or oxidised pyrimidine when considering endonuclease III (SBs Endo III). The enzyme-modified assay also measures misincorporated uracil on DNA when considering use of uracil-DNA glycosylase (SBs UDG).

Figure 1 summarizes the influence of *194Trp* polymorphisms of DNA repair gene *XRCC*1 on levels of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in each group. None of the *194Trp* genotypes influenced the baseline levels of SBs, purine and pyrimidine damaged, and misincorporation uracil on DNA, the levels of DNA damage induce with treatment of H_2O_2 (100 μM) and the DNA repair capability, in both groups.

In the group I Individuals carrying the *XRCC1 Arg399Arg* genotype presented higher levels of purine and pyrimidine damaged than individuals carrying the *XRCC1 Arg399Gln* genotype ($p < 0.05$) (Fig. 2), while in the group II individuals carrying the *XRCC1 Arg399Arg* genotype presented higher levels of pyrimidine damaged and uracil misincorporated on DNA than individuals carrying the *XRCC1 Arg399Gln* genotype ($P < 0.05$) (Fig. 2). Moreover, the individuals of group II carrying the *XRCC1 Arg399Arg* genotype presented lower DNA repair capability than individuals carrying the *XRCC1 Arg399Gln* genotype.

4. Discussion

Sporadic cancers result from gene-environment interactions where the environment includes endogenous and exogenous exposures [23,24] which include dietary exposures. Food mutagens could cause different types of DNA damage leading to nucleotide alterations and gross chromosomal aberrations. However, the effects of food mutagens in carcinogenesis can be modified by heritable traits, low-penetrant genes that affect mutagen exposure of DNA through metabolic activation and detoxification or cellular responses to DNA damage through DNA repair mechanisms or cell death [1]. In the last years several studies have explored the influence of SNPs on DNA repair genes and the interaction of these genotypes on genotoxic exposure biomarkers [25]. The determination of SNP is important aspect that may increase sensitivity and specificity of assays and identify effects and susceptible individuals and subgroups [26,27]. BER may be particularly important for the prevention of cancer because it repairs uracil and oxidative DNA damage. The *XRCC1* gene, involved in BER, encoded a protein that is involved in the efficient repair of DNA single-strand breaks formed by exposure to ionizing radiation and oxidative agents. In several studies, polymorphisms of *XRCC1* was been associated with higher levels of genotoxic damage and risk development of cancer [25].

Prado et al, in a parallel study of our laboratory, observed that dietary patterns influenced on level of oxidized purines and pyrimidines, which were higher in group II than in group I, and on levels of DNA damage induced by H_2O_2, which were lower in group I than in group II. However, the data presented a high heterogeneity suggesting the evolvement of other factors such DNA repair capability. Therefore, we have evaluated the influence of *194Trp* and the *399Gln* polymorphisms of the DNA repair

gene *XRCC1* and diet on level of oxidative DNA damage, uracil incorporation into DNA, and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets. The group I, with 49 individuals, has a great consume of organic fruits, vegetables, natural juices, and whole grains and poor consume of industrialized foods; and the group II, with 56 individuals, has a great ingestion of industrialized foods and poor ingestion of fruits, vegetables, natural juices, and whole grains.

Several epidemiologic studies have examine the effect of SNP in BER genes as factor for increase for cancer but few studies have investigated the relation between BER genotypes and biomarkers of genotoxicity. Recently Cornetta et al. (2006), using the comet assay for SBs assay, evaluated the relationship between polymorphisms in the BER genes include *194Trp* and the *399Gln* polymorphisms of the DNA repair gene *XRCC1* and the repair of DNA damage induced by *in vitro* X-ray irradiation in peripheral blood cells of healthy subjects and reported that *194Trp* genotypes did not influence the baseline levels of SBs and DNA repair capability. These data are according with findings reported for Godderis et al. [25] and Weng et al. [29], which also reported that *194Trp* genotypes did not influence the DNA damage level induced by styrene oxide, ethylene oxide and gamma-radiation or the level of baseline DNA damage in fresh peripheral blood leukocytes, respectively. These findings are according with our findings, which indicated that genotypes of *194Trp* polymorphism did not influence the baseline levels of SBs, purine and pyrimidine damaged, misincorporation uracil on DNA and the levels of DNA damage induce with treatment of H_2O_2 (100 μM) or the DNA repair capability, in both groups.

The presence of the *Gln399Gln* genotype has been associated with persistence of DNA damage, elevated formation of sister chromatid exchange (SCE) [30] and

baseline DNA damage in healthy individuals [29], probably because XRCC1 encompasses 2 BRCA C-terminal (BRCT) motifs with independent and important roles. The interaction of XRCC1 and ligase III is mediated by BRCT II domain, required in a cell cycle stage-specific pathway [31]. The central region (amino acids 315–403) named BRCT I is the most evolutionary conserved motif, but its precise function is not fully understood. It interacts with PARP-1 and PARP-2 and pol β. A requirement for PARP for efficient repair of SBs has been described [32,33]. Recently it was shown that XRCC1 is phosphorylated at the BRCT I domain at Ser 371 by DNA dependent protein kinase in response to DNA damage [34]. The most frequent *XRCC1* polymorphism (exon 10 codon 399, Arg to Gln) occurs in the interaction site with PARP (poly(ADP-ribose) polymerase). This may lead to a modification in repair activity, and XRCC1 Gln399 allele has been reported as a risk factor for different types of cancer [13]. However, in our study, the group I Individuals carrying the *XRCC1 Arg399Arg* genotype presented higher levels of purine and pyrimidine damaged than individuals carrying the *XRCC1 Arg399Gln* genotype, while in the group II individuals carrying the *XRCC1 Arg399Arg* genotype presented higher levels of pyrimidine damaged and uracil misincorporated on DNA than individuals with *XRCC1 Arg399Gln* genotype Moreover, the individuals of group II carrying the *XRCC1 Arg399Arg* genotype presented lower DNA repair capability than carrying the individuals with *XRCC1 Arg399Gln* genotype. These results are in according to results of Cornetta et al. [13] which reported lower DNA damage level in individuals carrying of *Gln399Gln* genotyp*e* than in individuals carrying of *Arg399Gln* or *Arg399Arg* genotype*.* These results were confirmed at 30 and 60 min of DNA repair. Gal et al. [35] have shown that *XRCC1 399Gln* allele was associated with a decreased risk of mortality in patients with oral cancer. Moreover, several studies have been reported that *XRCC1 399Gln* allele is

protective against the development of some types of cancer such as acute myeoloblastic leukaemia (t-AML) [36], nonmelanoma skin cancer [37] and bladder cancer [38].

To according with Cornetta et al. [13] the authors suggest that due to lower DNA repair capability induced by *XRCC1 399Gln* allele, damaged cells could accumulate a high DNA damage level and likely to be driven apoptosis. Consequently, cells with *XRCC1 399Gln* allele presented as the result a protection through elimination of potentially transformed cells, while the cells carrying *XRCC1 Arg399Arg* genotype can original clonal cancer.

The Comet assay is a test for detecting genotoxicity. Therefore, to assure the adequate genotoxicity analysis, in our study, the peripheral blood lymphocytes were lysed overnight, and underwent electrophoresis for 30 min. These procedures probably eliminated the majority of the apoptotic or necrotic cells. Moreover, the DNA clouds related to apoptosis/necrosis were excluded from quantitative analysis. We believe that these amendments assured the fully elimination of apoptotic cells of our analysis and can help to explain the higher levels of oxidative DNA damage, misincorportion uracil and lower DNA repair capability found in individuals carrying *XRCC1 Arg399Arg* genotype.

In conclusion, our results demonstrated that *XRCC1 Arg399Arg* genotype is relationship with accumulative DNA damage and lower DNA repair capability in healthy individuals with antagonist diets.

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Legends

Figure 1. Influence of *194Trp* polymorphisms of DNA repair gene *XRCC*1 on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare differences among genotypes in each group. None of the genotypes influenced the levels of DNA damage.

Figure 2. Influence of *399Gln* polymorphisms of DNA repair gene *XRCC*1 on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare differences among genotypes in each group. In the group I, individuals

carrying *XRCC1 Arg399Arg* genotype presented higher levels of purine and pyrimidine damaged than individuals carrying *XRCC1 Arg399Gln* genotype, while in the group II individuals carrying *XRCC1 Arg399Arg* genotype presented higher levels of pyrimidine damaged and uracil misincorporated to DNA than individuals carrying *XRCC1 Arg399Gln* genotype. Moreover, in the group II individuals carrying *XRCC1 Arg399Arg* genotype presented lower DNA repair capability than individuals carrying *XRCC1 Arg399Gln* genotype.

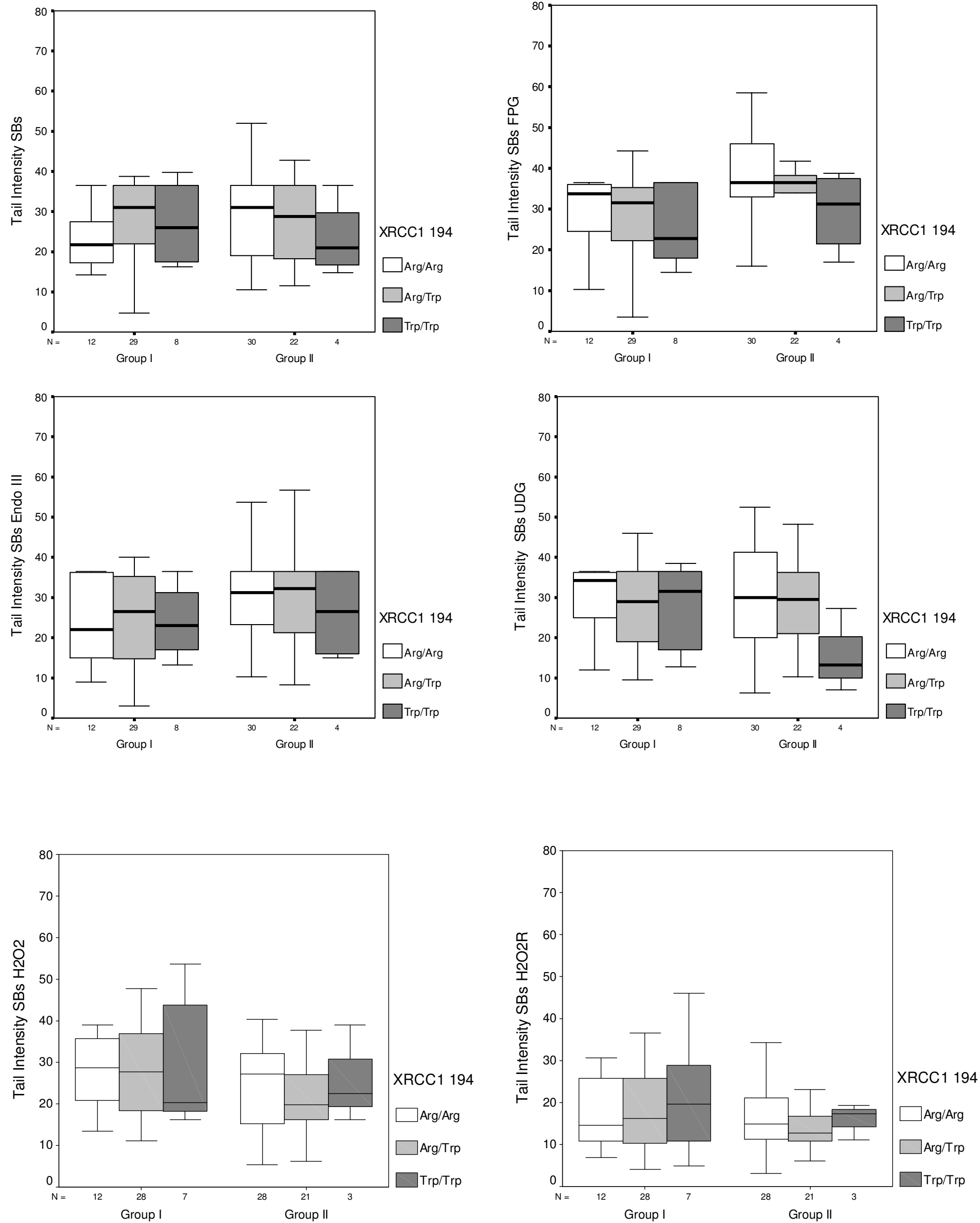

Figure 1.

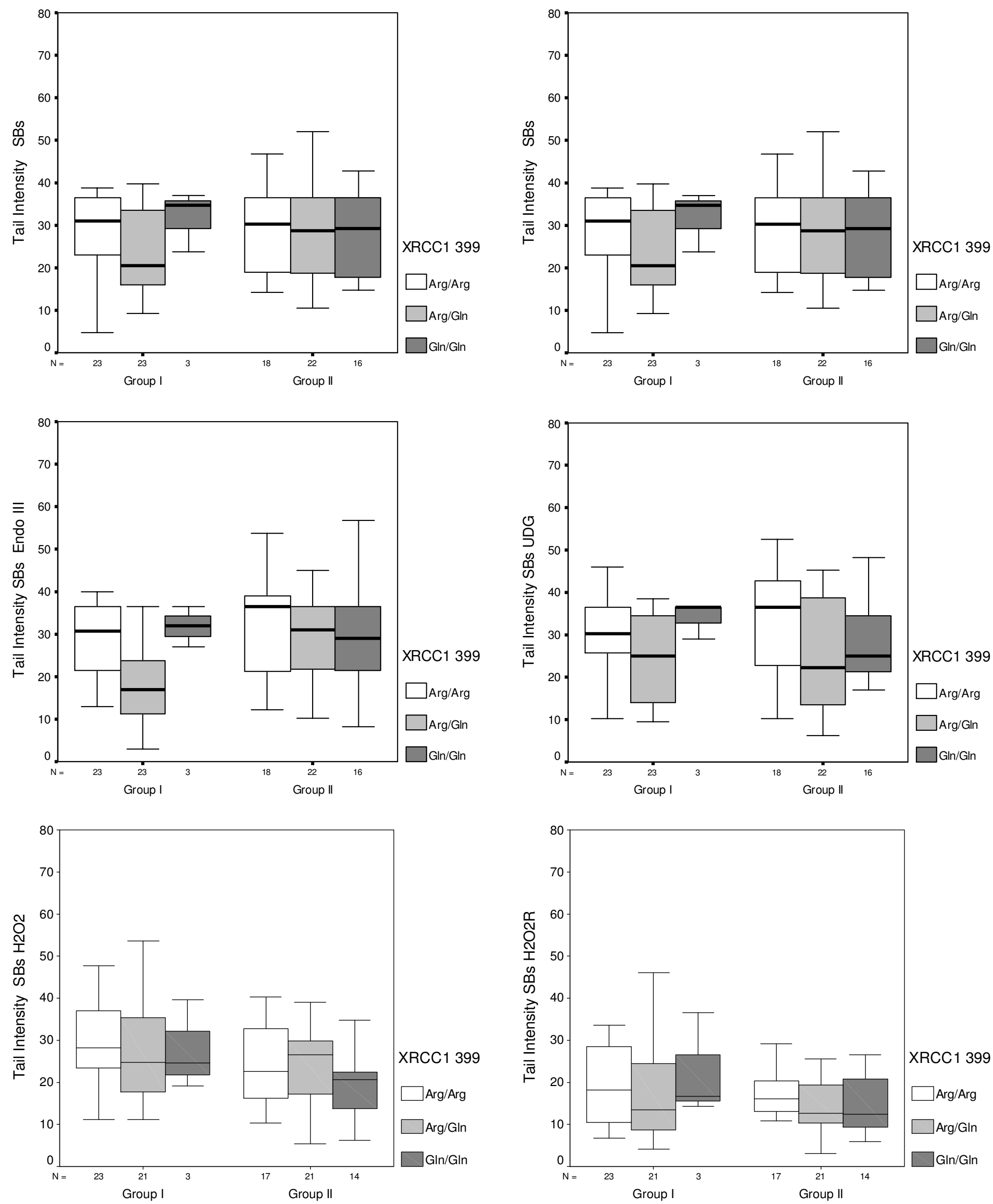

Figure 2.

Manuscrito III

Trabalho elaborado segundo as normas da revista “Mutation Research”.

Influence of diet and gene *GSTP1 A313G* polymorphism on oxidative DNA damage, misincorporation uracil, and system repair efficiency.

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Abstract

Dietary factors can be related to fortuitous genomic instability, which can be preceded by oxidative damage and uracil incorporation in DNA which can trigger a carcinogenic process. Linked to diet, there is a genetic susceptibility to developing cancer. Polymorphic *GSTP1* in humans is involved in the detoxification of various potentially toxic components found in foodstuffs. The aim of this study was to evaluate the possible influence of *GSTP1* gene *A313G* polymorphism on DNA oxidative damage levels in peripheral blood lymphocytes in two groups of individuals: Group I) 42 individuals with a diet rich in natural products such as whole grains, fruit, and vegetables, and low in processed products, and Group II) 42 individuals on a diet high in processed products and low in fruit and vegetables. PCR-RFLP was used for *GSTP1* genotyping. The individuals of group II carrying *GSTP1 G313G* genotype presented higher levels of purines oxidized when compared to individuals of group II carrying *GSTP1 A313A* or individuals of group II carrying. None of the *GSTP1 A313G* genotypes influenced the baseline levels of SBs, pyrimidine damaged, and misincorporation uracil on DNA, the levels of DNA damage induce with treatment of H_2O_2 (100 μM) and the DNA repair capability, in both groups.

In conclusion, the *GSTP1 A313G* polymorphisms could modulate the oxidative DNA damage level in healthy subjects that have a diet deficient in fruits and vegetables. We believe that further investigations are needed to clarify the mechanisms of *GSTP1 A313G* polymorphisms in combination with other genes and micronutrients status.

Keywords: diet, cancer, *GSTP1*, oxidative damage, micronutrients.

1. Introduction

It is estimated that in the world, per year, around 10 million people receive the diagnosis of cancer and more than six million die due this disease [1]. In 1981, Doll & Peto [2] published a study listing the main causes which can facilitate a carcinogenic process; diet was in first place with a 35% risk. Currently these can vary between 10% for lung cancer to 80% for colorectal cancer [1].

Fergunson [3] showed that chemical additives used in meat preservation (N-nitrous derivatives), and compounds such as heterocyclic amines and polycyclic aromatic hydrocarbonates produced during high temperature meat preparation can lead to the development of colon and prostate cancer.

According to Ames [4], deficiencies in micronutrients found in fruit and vegetables, such as folic acid, vitamins B12, B6, C, and E, niacin, iron, and zinc, as well as protein calorie undernourishment, can mimic the effect of radiation or chemical agents in inducing DNA damage. Folic acid deficiency can also lead to uracil incorporation in DNA instead of thymine [5], the main type of endogenous DNA damage [6], which can lead to genome instability. Also various micronutrients act as enzymatic cofactors in DNA maintenance, repair, and methylation reactions and apoptosis [7]. Therefore deficiencies in ingesting these micronutrients could lead to eventual genomic instability characterized by an increased frequency in point mutation or chromosome break biomarkers, or even interfere in chromosome segregation, gene expression, oxidative stress, necrosis, and apoptosis [8].

As well as these environmental factors, there is also a difference in individual genetic susceptibility to the risk of developing cancer [3], which could be linked to a higher or lower occurrence of DNA damage. According to the North American

Research Council, “susceptibility markers” are defined as biological differences between individuals or populations capable of affecting organism response to environmental agents. The most significant of these markers include genetic differences in the capacity to repair DNA damage and in biometabolism reaction dynamics to xenobiotic agents [9]. Currently the focus has been on trying to elucidate the molecular basis of the polymorphisms of enzymes involved in activating and detoxifying xenobiotic agents. There are basically two types of enzymes involved in chemical compound metabolism: Phase I codified by the cytochrome P450 (CYPs) gene super family, which through oxidation reactions can transform pro-carcinogens into carcinogenic substances capable of interacting with DNA [10,11], inducing different types of damage, and Phase II, such as glutathione s-transferases (GSTs) which act on the metabolism of certain toxic substances, making them more hydrophilic and therefore capable of being more easily excreted [12]. GSTs have a large array of substrates which can detoxify environmental carcinogenic agents found in foodstuffs, air, or medications [13].

GSTs in humans are polymorphic; an important gene being *GSTP1* which plays a fundamental role in protecting the organism against various types of cancer, because it is codified as an enzyme which acts in detoxifying polycyclic aromatic hydrocarbonates (PHA) originating from partially burnt organic material [13] and several chemical compounds found in processed foods. This enzyme has a single nucleotide polymorphism (SNP), which causes an isoleucine to valine substitution at position 313, making the enzyme less active favouring an accumulation of DNA damage [13]. This polymorphism is associated with an increased risk of developing bladder, testicular, prostate [14], pharyngeal, laryngeal, and lung cancer [15].

The objective of this study was to evaluate the influence of *GSTP1* gene *A313G* polymorphism on levels of DNA oxidative damage in peripheral blood lymphocytes in two groups of individuals with different dietary regimens.

2. Materials & Methods

2.1. Subject selection

This study was approved by the Ethical Committee for Human Research of the Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil. Informed consent was obtained from each volunteer.

A total of 105 healthy adult volunteers (average age 35,6 ± 11,4, ranging from 19 and 66 years), 52 men and 53 women were studied. All of the volunteer were nonsmokers, were not abusing alcohol, and were not using prescription or recreational drugs and any vitamins and minerals supplementation. These volunteers were distributed in two groups: 1) Group I - 49 naturalistic individuals with a life style characterized by absence of stress, bucolic life and great consume of organic fruits, vegetables and juice and poor consume of industrialized foods; 2) Group II - 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables.

2.2. Obtaining and evaluating Dietary Data

For evaluation of the dietary pattern, calculating the estimated level of micronutrients ingested (data not shown) and for the classification the groups a Food-frequency Questionnaire adapted from Cardoso and Stocco [16] was applied. This quantitative questionnaire has provided data, which were analysed using the software AvaNutri (Avanutri Informática Ltda, RJ, Brazil). This procedure enabled us to establish an estimate of the quantity of protective foods consumed daily by the population studied. To be classified in Group I, the individuals had to consume the percentile 75 of organic fruits and vegetables and below the percentile 25 of each

industrialized products. The individuals of group II were those who consumed less than percentile 25 of organic fruits and vegetables and above the percentile 75 of industrialized products.

Only were selected individuals that have adopted the respective diet pattern for at least 10 years.

2.3. Samples

Samples of peripheral blood (10ml) were collected: 2ml for detecting oxidative damage, DNA repair capability, and uracil levels incorporated in DNA; 4ml for DNA extraction to determine gene polymorphisms; and 4ml for a parallel study in which we analyzed plasma levels of different micronutrients.

2.4. Determining Oxidative Damage, Uracil Incorporation, and DNA Repair Capability

Data on oxidative DNA damage, uracil misincorporation into DNA, and DNA repair were described and accomplished by Prado et al in a parallel study (in preparation). Briefly, The alkaline Comet assay [17], modified with lesion-specific enzymes was used to detect single and double strand breaks, labile sites (SBs), oxidised purines and pyrimidines and uracil [18,19]. All procedures were conducted in the dark to minimize spurious sources of DNA damage. Briefly, 10 µl of the isolated lymphocytes suspension [20] ($\cong 2 \times 10^4$ cells) were embedded into 0.5% low melting point agarose (Sigma) and spread on agarose-precoated microscope slides. Twelve slides were prepared for each sample. Slides were immersed overnight in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium

salt N-Lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% DMSO added fresh) at 4^{0}C. After lysing, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD) and then three slides for each treatment were incubated at 37°C for 45 min with 100 μL of endonuclaese III (Endo III - 1:1000; New England Biolabs Inc), 100 μL of formamidopyrimidine-DNA glycosylase (FPG - 1:1000; New England Biolabs Inc), 100 μL of uracil-DNA glycosylase (UDG - 1:1000; New England Biolabs Inc) or with enzyme buffer only. Endo III recognizes oxidised pyrimidines (SBs EndoIII), while FPG identifies oxidised purines (SBs FPG), especially 8-oxo-guanine and UDG recognizes uracil (SBs UDG). Enzyme buffer only is used to identified SBs. Subsequently the cells were exposed to alkali buffer (1mM 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 (1v/cm) and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 50 μL Syber Green (1:10000; Invitrogen), and analyzed in a fluorescence microscope at 400 x magnification, using an image analysis system (Comet Assay II – Perceptive Instruments, Suffolk, UK).

For each individual, one hundred randomly selected cells (50 from each of two replicate slides) for each treatment (FPG, Endo III, UDG or enzyme buffer only) were evaluated and the mean tail intensity (%DNA Tail) was determined, totaling 400 cells analyzed from each individual. Tail Intensity, according to Comet Assay II – Perceptive instruments, is defined as “the sum of all intensity values in the tail region less those which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity”.

To analyze the influence of dietary patterns and *GSTP1 A313G* polymorphism on the DNA repair capability, 200 μl of the freshly isolated lymphocytes were treated

with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H_2O_2, these aliquots were incubated at 37 °C for 30 minutes, and DNA damage was checked again.

2.5. DNA Extraction

DNA was extracted using the Gene Catcher gDNA 3-10 ml Blood Kit (Invitrogen, Carlsbad, CA, USA), as per manufacturers recommendations.

2.6. Evaluation of Gene *GSTP1* Polymorphisms

GSTP1 genotyping was performed by PCR-RFLP [21] using *GSTP1-f*:GGCTCTATGGGAAGGACCAGCAGG and *GSTP1-r*:GCA CCTCCATCCAGAAACTGGCG primers.

The PCR reaction was performed using 25mM $MgCl_2$, 100mM dNTPs, 10µM of each primer, 1.5U/µl Taq DNA polymerase (Invitrogen), and 100ng/µL DNA, in a volume of 27µl. Cycling conditions for each amplification were: 94ºC for 30 seconds, followed by 94ºC for 1 minute (step 2), 59ºC for 1 minute, 72ºC for 2 minutes, and the process returned to step 2 and was repeated 39 times; this was followed by a final extension cycle of 72ºC for 10 minutes. Products with 445pb were obtained. A 15µl aliquot of PCR product was submitted to 1U BsmA I enzyme (Bio Labs-New England) and incubated at 55ºC for 12 hours. The resulting products were visualized in 2.5% agarose gel and stained with ethidium bromide. The wild IIe allele for codon 105 was identified by the presence of a Bsma I cleavage site. All experiments included positive and negative controls for each specific polymorphism.

2.7. Statistical Analysis

The data obtained in the Comet Assay were asymmetric distribution, thus, Log transformations were applied to DNA damage distributions to help meet normality distribution assumptions. To evaluate the influence of diet and *GSTP1 A313G* polymorphism on DNA damage the Tukey test was applied. The index of significance adopted was 5%.

3. Results

DNA damage, including single and double strand breaks, labile sites, oxidised purines and pyrimidines and misincorporated uracil on DNA, was detected by the alkaline Comet assay modified with lesion-specific enzymes, FPG, Endo III and UDG, respectively. The alkaline Comet assay measures SBs and AP sites. The enzyme-modified assay measures oxidative DNA damage as a combination of SBs, AP sites and oxidised bases – formamidopyrimidines and the oxidised purine 8-oxo-guanine when considering FPG (SBs FPG), or oxidised pyrimidine when considering endonuclease III (SBs Endo III). The enzyme-modified assay also measures misincorporated uracil on DNA when considering use of uracil-DNA glycosylase (SBs UDG).

Figure 1 summarizes the influence of *GSTP1 A313G* polymorphism on levels of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in each group. The individuals of group II carrying *GSTP1 G313G* genotype presented higher levels of purines oxidized when compared to individuals of group II carrying *GSTP1 A313A* or individuals of group II carrying. None of the *GSTP1 A313G* genotypes influenced the baseline levels of SBs, pyrimidine damaged, and misincorporation uracil on DNA, the levels of DNA damage induce with treatment of H_2O_2 (100 μM) and the DNA repair capability, in both groups.

4. Discussion

Sporadic cancers result from gene-environment interactions where the environment includes endogenous and exogenous exposures [22,23] which include dietary exposures. Food mutagens could cause different types of DNA damage leading to nucleotide alterations and gross chromosomal aberrations. However, the effects of food mutagens in carcinogenesis can be modified by heritable traits, low-penetrant genes that affect mutagen exposure of DNA through metabolic activation and detoxification or cellular responses to DNA damage through DNA repair mechanisms or cell death [25]. In the last years several studies have explored the influence of SNPs on DNA metabolism xenobiotic genes and the interaction of these genotypes on genotoxic exposure biomarkers [26]. The determination of SNP is important aspect that may increase sensitivity and specificity of assays and identify effects and susceptible individuals and subgroups [25,26].

Parallel studies performed in our laboratories have shown that individuals with a diet rich in natural products, such as vegetables and grain have lower levels purine and pyrimidine oxidized (Prado et al., in preparation). In our study, we observed that individuals of group II carrying *GSTP1 A313A* with a diet deficient in fruits and vegetables (group II) presented higher levels of purine oxidized. Several studies have been reported that diets rich in greens, fruit, and vegetables, adopted by Group I, can protect DNA from damage and exert a protector effect against cancer development [27]. The individuals of group II presented a diet poor in micronutrients and rich in chemical agents that are metabolized by phase I and II enzymes, and the balance between these phases could determine the level of reactive oxygen species (ROS) that could react with DNA induce oxidative DNA damage, especially in purine, that actually is a better

biomarker of oxidative stress exposition, as observed in this study. Since that the *GSTP1 A313G* polymorphism could the lead to inhibition of enzyme activity, we hypothesed that this deficiency enzymatic could allow a great interaction between ROS and DNA increasing the oxidative DNA damage level found in individual of group II. However, in our study, none of the *GSTP1 A313G* genotypes influenced the baseline levels of SBs, pyrimidine damaged, and misincorporation uracil on DNA, the levels of DNA damage induce with treatment of H_2O_2 (100 μM) and the DNA repair capability, in both groups. We believe that specific metabolism enzymes could be relationship with expression of another enzymes of the xenobiotic metabolism, as for example *GSTP1* induction is higher in individuals with *GSTM1**+ genotype than in those with *GSTM1*- genotype [28-29]. These possible interaction can help explain our results.

In conclusion, the *GSTP1 A313G* polymorphisms could modulate the oxidative DNA damage level in healthy subjects that have a diet deficient in fruits and vegetables. We believe that further investigations are needed to clarify the mechanisms of *GSTP1 A313G* polymorphisms in combination with other genes and micronutrients status.

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Legends

Figure 1. Influence of *GSTP1* gene polymorphisms on DNA damage (Tail Intensity) on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare differences among genotypes in each group. The individuals of group II carrying *GSTP1 G313G* genotype presented higher levels of purines oxidized when compared to individuals of group II carrying *GSTP1 A313A* or individuals of group II carrying *GSTP1 A313G* genotype.

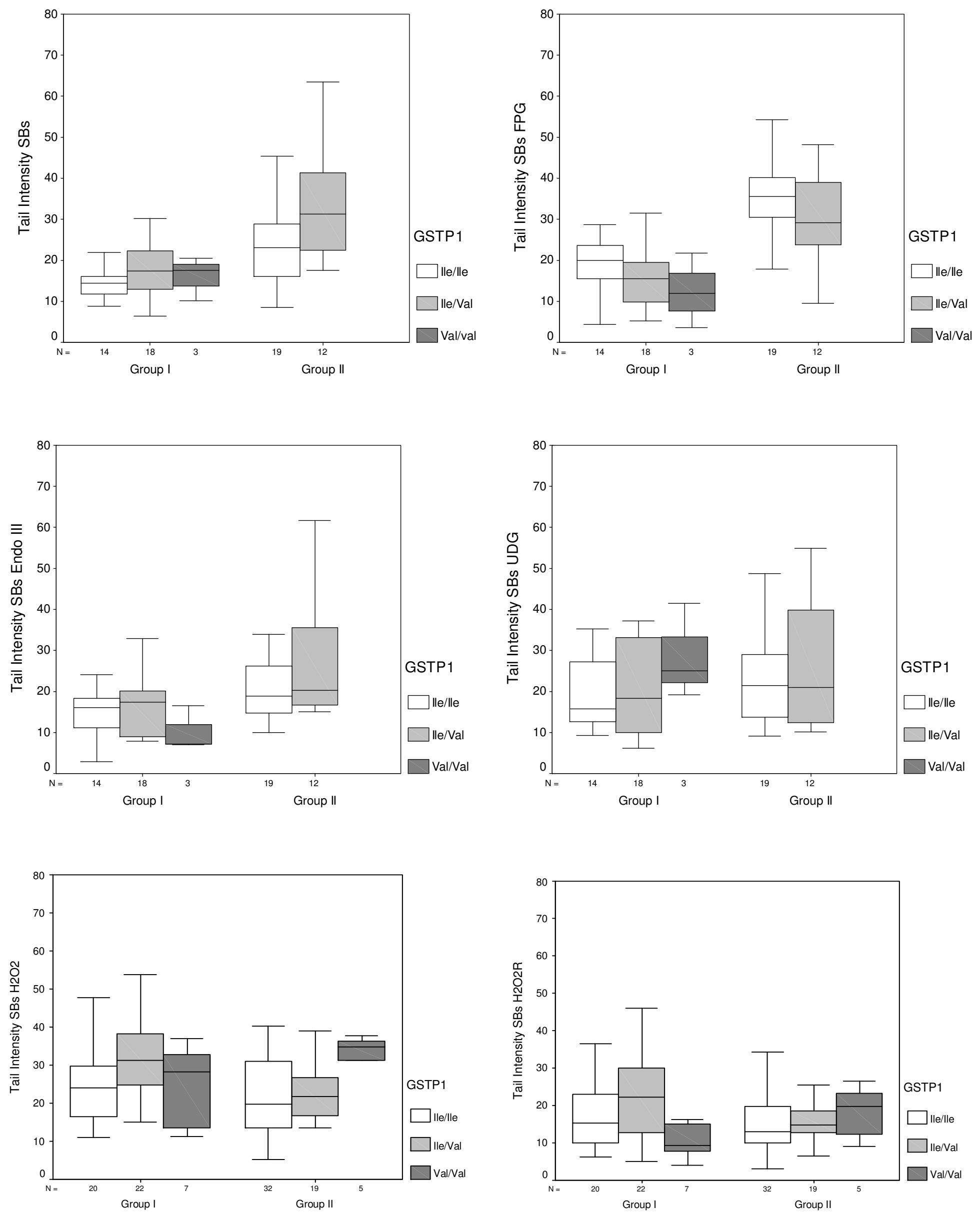

Figure 1.

Manuscrito IV

Trabalho elaborado segundo as normas da revista “The Journal of Nutrition”.

Influence of *A1298C* Methylenetetrahydrofolate Reductase polymorphism and Diet on oxidative DNA damage, uracil incorporation and the efficiency of DNA repair in Healthy Subjects

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Figure 1

Tables 2

Abstract

Epidemiological studies indicated the role for *A1298C* polymorphism of methylenetetrahydrofolate reductase (*MTHFR*) of folate and homocysteine metabolism in the etiology of several diseases, including cancer. We evaluated the influence of dietary pattern and *A1298C* polymorphism of *MTHFR* on oxidative DNA damage, uracil incorporation and efficiency of DNA repair in lymphocytes of two groups of health subjects with antagonists diets: one group with 49 individuals (GI) has a diet rich in organic fruits, vegetables and whole grains and poor in industrialized food, and another group with 56 individuals (GII) has a diet rich in industrialized food and poor in organic fruits, vegetables and whole grains. The *A1298C* polymorphism in the *MTHFR* gene was analyzed by PCR-RFLP and oxidative DNA damage, uracil incorporation and the efficiency of DNA repair were assessed by the Comet assay in a parallel study. Individuals carrying *1298CC* genotype presented higher level of oxidative DNA damage (strand breaks) on group II, but this polymorphism did not influence on uracil incorporation and the efficiency of DNA repair in both groups. We also observed that folate intake was correlated negatively with oxidized purines ($r = -0.213$, $p < 0.05$). In conclusion, the *A1298C* polymorphisms influenced on levels of DNA damage (strand breaks), and did not influence the misincorporation uracil and DNA repair capability. Moreover, we concluded that folate intake correlated negatively with oxidized purines. Further investigations is needed to clarify the mechanisms of *A1298C* polymorphisms in combination with others polymorphisms of *MTHFR*, micronutrients and homocysteine status.

1. Introduction

Several studies have demonstrated that dietary nutrients can protect against cancer [1]. Fruits and vegetables are sources of micronutrients, which in a proper concentration, are substrate and/or cofactors in the maintenance, repair, and methylation of DNA, and in the process of apoptosis [2]. Thus, dietary deficiency in fruits and vegetables may cause genomics instability [3,4], such as increased in point mutation, chromosomal breakage, or by interfering in the chromosome segregation, gene expression, oxidative stress, necrosis and apoptosis [3].

An important deficiency in micronutrient known to impair DNA metabolism and to increase the incidence of development of cancer, including cancer of the lung, breast and cervix [4], and colorectal cancer [5,6], is folate deficiency [7]. This vitamin is involved in a metabolizing enzyme, the methylenetetrahydrofolate (*MTHFR*), which is considered a key enzyme in the one-carbon metabolism, because it is responsible for the conversion of 5,10 methylenetetrahydrofolate to 5-methyleletetrahydrofolate, the primary circulating form of folate and a C donor for the vitamin B12-dependent remethylation of homocysteine to methionine [8]. Methionine is the immediate precursor of S-adenosylmethionine (SAM), the principal methyl donor for methylation reactions, including DNA methylation, which controls gene expression [9,10]. Folate deficiency may cause a depletion in SAM, leading to DNA hypomethylation [11] and, possible, proto-oncogene activation and transcription, and malignant transformation [12,13]. Moreover, folate deficiency also promotes an accumulation in the product of methyltransferase activity, S-adenosylhomocysteine [11], which is an independent risk for

cardiovascular disease [14; 15], cognitive impairment [16], and is considered to be an important risk factor for cancer, possible, because homocysteine causes a generation of reactive oxygen species (ROS), leading to oxidative DNA damage [17] .

Folate is also essential for DNA synthesis and repair. This micronutrient, in the form 5,10 methylenetetrahydrofolate, is a methyl donor for the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) [18,19]. Under conditions of folate depletion, the methylation of dUMP to dTMP is blocked, leading to an increase of deoxyuridine triphosphate, and inducing uracil misincorporation into DNA in place of thymine. The normal DNA repair removes the uracil, but if folate availability is continually limited, the repair cycle is impaired causing frequent point mutation, single and double-stranded DNA breaks, micronucleus formation and chromosome breakage, which are important risks for cancer [18,20]. In addition, not only folate plays an important role in DNA metabolism, but vitamin B12 also plays important roles in DNA synthesis and production of methionine from homocysteine for the maintenance of methylation patterns in DNA [10]. When vitamin B12 is limiting, the availability of 5,10-methylenetetrahidrofolate in the methylation of dUMP to dTMP for DNA synthesis and repair is reduced [13].Therefore, deficiencies of vitamin B12 also may cause genomic instability [7].

Cancer incidence may be influenced by some polymorphisms in *MTHFR* [21]. A common genetic polymorphism in the *MTHFR* is at base pair 1298 [22]. The 1298 A→C variant changes a glutamate to alanine [22,23,24] and is associated with reduced enzyme activity in homozygotes [22].

The *A1298C* was first associated with ovarian carcinoma [25], and studies have demonstrated that *A1298C* polymorphism of *MTHFR* is associated with a reduction risk of acute lymphoblastic leukemia in adults and children carrying the mutant allele in heterozygosity (*1298AC*) or homozygosity (*1298CC*) [26,27]. Reports also suggest an inverse association with colorectal cancer and homozygosity for A1298C variant [21]. Moreover, *A1298C* polymorphism was associated with risk of neural tube defects; however, more evidence is required to clarify if the risk of neural tube defects is associated with a reduced activity of the mutant *A1298C* of *MTHFR* [22,23,28]. Folate-responsive birth defects may involve multiple genetic polymorphisms that could alter folate metabolism, especially when folate and vitamin B12 are inadequate [29]. In addition, many types of cancers may also be involved in folate status and multiple genetic polymorphisms of *MTHFR*, such as *C677T* polymorphism, another polymorphism described of *MTHFR*, which is reported to be important in genomic stability [30]. It has been reported that individuals with the combined *1298AA/677TT* or *1298CC/677CC* genotype show a 2.5- and 3.3-fold respectively reduction of risk for childhood acute lymphoblastic leukemia [31]. Moreover, the combined heterozygosity for the *A1298C* and *C677T* was associated with reduced MTHFR activity, decreased plasma folate levels, and higher homocysteine levels [22].

The present study examined the influence of diet and *A1298C MTHFR* polymorphisms on level of oxidative DNA damage, uracil incorporation and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets.

2. Materials and Methods

The present study was developed in Botucatu Medical School-UNESP, Internal Medicine Department and Pathology Departament, in Botucatu, State of São Paulo, Brazil, at period of 2005-2008. This study was approved by the local Ethical Committee. Informed consent to participate was obtained from all the volunteers.

2.1. Volunteers

We studied 105 healthy adult volunteers (average age 35,6 ± 11,4, ranging from 19 and 66 years), 52 men and 53 women. All of the volunteer were nonsmokers, were not abusing alcohol, and were not using supplementation, prescription or recreational drugs. The volunteers were divided in two groups: 1) 49 naturalistic individuals with a great consume of organic fruits, vegetables and juice and poor consume of industrialized foods (Group I); 2) 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables (Group II).

2.2. Dietary Assessment

For evaluation of the dietary pattern a Food-frequency Questionnaire adapted from Cardoso and Stocco [32] was applied, and was used for the classification of the groups. For calculating the estimated level of micronutrients ingested, a dietary record of three days was applied, and has provided data, which were inserted in the program Avanutri (AvaNutri Informática Ltda, RJ, Brazil). This procedure enabled us to establish an estimate actual quantity of protective foods consumed daily by the population studied. To be classified in Group I, the individuals had to consume the

percentile 75 of organic fruits and vegetables and below the percentile 25 of each industrialized products. The individuals of group II were those who consumed less than percentile 25 of organic fruits and vegetables and above the percentile 75 of industrialized products.

2.3. *Laboratory analyses*

2.3.1. DNA extraction

Venous blood was obtained for genomic DNA and comet assay.

Genomic DNA was isolated from peripheral blood leucocytes using a commecially available kit (Invitrogen), according to the manufacture's instructions.

2.3.1. *MTHFR* genotype analysis

According to a previously described procedure [34] , genotyping for the *MTHFR* point polymorphism *A1298C* was performed by polymerase chain reaction amplification with the primers 5'–CTTCTACCTGAAGAGCAAGT-3' and 5'- CAT GTCCACAGCATGGAG -3' [32]. The amplified PCR fragment of 256 bp was digested with the restriction enzyme *MboII.* After eletroforesis through 8% polyacrylamide gel, the digestion products were visualized by staining ethidium bromide.

2.3.2. Determination of Oxidative DNA Damage and level of uracil incorporate into DNA

Data on oxidative DNA damage, uracil misincorporation into DNA, and DNA repair were described and accomplished by Prado et al in a parallel study (in preparation). Briefly, The alkaline Comet assay [35], modified with lesion-specific enzymes was used to detect single and double strand breaks, labile sites (SBs), oxidised

purines and pyrimidines and uracil [36]. All procedures were conducted in the dark to minimize spurious sources of DNA damage. Briefly, 10 µl of the isolated lymphocytes suspension [22] (≅ 2 x 10^4 cells) were embedded into 0.5% low melting point agarose (Sigma) and spread on agarose-precoated microscope slides. Twelve slides were prepared for each sample. Slides were immersed overnight in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium salt N-Lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% DMSO added fresh) at 4^0C. After lysing, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD) and then three slides for each treatment were incubated at 37°C for 45 min with 100 µL of endonuclaese III (Endo III - 1:1000; New England Biolabs Inc), 100 µL of formamidopyrimidine-DNA glycosylase (FPG - 1:1000; New England Biolabs Inc), 100 µL of uracil-DNA glycosylase (UDG - 1:1000; New England Biolabs Inc) or with enzyme buffer only. Endo III recognizes oxidised pyrimidines (SBs EndoIII), while FPG identifies oxidised purines (SBs FPG), especially 8-oxo-guanine and UDG recognizes uracil (SBs UDG). Enzyme buffer only is used to identified SBs. Subsequently the cells were exposed to alkali buffer (1mM 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 (1v/cm) and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, Ph 7.5), stained with 50 µL Syber Green (1:10000; Invitrogen), and analyzed in a fluorescence microscope at 400 x magnification, using an image analysis system (Comet Assay II – Perceptive Instruments, Suffolk, UK).

For each individual, one hundred randomly selected cells (50 from each of two replicate slides) for each treatment (FPG, Endo III, UDG or enzyme buffer only) were evaluated and the mean tail intensity (%DNA Tail) was determined, totaling 400 cells

analyzed from each individual. Tail Intensity, according to Comet Assay II – Perceptive instruments, is defined as "the sum of all intensity values in the tail region less those which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity".

To analyze the influence of dietary patterns and *MTHFR A1298C* on the DNA repair capability, 200 µl of the freshly isolated lymphocytes were treated with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H_2O_2, these aliquots were incubated at 37 °C for 30 minutes, and DNA damage was checked again.

2.4. Statistical Analysis

The data obtained in the Comet Assay were asymmetric distribution, thus, Log transformations were applied to DNA damage distributions to help meet normality distribution assumptions. The statistical analysis of the data obtained in the Comet Assay consisted of applying the t Student test to compare differences between the various types of DNA damage, and for evaluate the influence of *A1298C* polymorphism, the Tukey test was applied. The Pearson correlation was applied to correlate SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H2O2 and SBs H2O2R and the estimated consume of vitamin B12, folate, and Kcal and BMI. The index of significance adopted was 5%.

3. Results

The Table 1 summarizes the level of micronutrients estimated through a Dietary Record of Three Days and Recommended Dietary Allowances (RDA). The individuals of Group I presented higher levels of vitamin A, vitamin B2, vitamin B6, vitamin B12, vitamin C and folic acid than those in Group II. In addition, the individuals in Group II presented lower estimated intake level of vitamin A, vitamin B6, vitamin C and folic acid than the Recommended Dietary Allowances (RDA) (Table 1). These data were used in previous paper (Prado et al. in preparation).

The Pearson correlation between SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H2O2 and SBs H2O2R and the estimated consume of vitamin B12, folate, and Kcal and BMI are shown on Table 2. Vitamin B12, Energy intake (Kcal), folate and body mass index (BMI) did not have correlation with the biomarkers (Table 2). Folate was correlated negatively with oxidized purines ($r = -0.213$, $p < 0.05$) (Table 2). These data were used in previous paper (dos Santos et al. in preparation).

The individuals carrying *MTHFR 1298CC* genotype presented higher level of DNA damage (SBs) than individuals carrying *MTHFR 1298 AA* on group II (Figure 1).

4. Discussion

We have evaluated the influence of *A1298C* polymorphism of *MTHFR* and diet on level of oxidative DNA damage, uracil incorporation and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets. The group I, with 49 naturalistic individuals, has a great consume of organic fruits, vegetables, natural juices, and whole grains and poor consume of industrialized foods; and the group II, with 56 individuals, has a great ingestion of industrialized foods and poor ingestion of fruits, vegetables, natural juices, and whole grains. We verified that *1298CC* genotype influenced on level of oxidative DNA damage, with increased levels of oxidative DNA damage (strand breaks) on both groups, but did not influence on uracil misincorporation into DNA and DNA repair capability. Moreover, the results of the parallel study analyzing the effect of only diet presented that the same individuals of group I presented lower levels of oxidized purines and pyrimidines and lower level of DNA damage induced by H_2O_2 than the group II.

Reports showed that the mutant *1298CC* genotype has be found to have 60% of the 1298AA wild-type enzyme activity *in vitro* [22,23]. Some studies negatively associated this genotype with colorectal cancer [34, 35], and rectal cancer [36]. Although is reported that *1298CC* variant affects enzyme activity to a lesser degree than *677TT* variant [22], the reduced *MTHFR* activity due to this polymorphism may increase 5,10-methylenetetrahydrofolate, that is essential to conversion of dUMP to dTMP for DNA synthesis and repair [37]. More 5,10-methylenetetrahydrofolate levels could increase the availability of thymine, reduce the incorporation of the former base into DNA and consequently DNA damage, and promote DNA stability [13,38], what

can explain the protective effect of *1298CC* against colorectal cancer on these studies. However, such protection of this polymorphism occurred with adequate folate intake. Low folate increases uracil misincorporation during DNA synthesis [39], increased frequency of DNA strand breaks [18], and it has been suggest that the increased accumulation of such damaged DNA may promote cancer formation [20,40]. Besides, folate deficiency affects the availability of 5-methylenetetrahydrofolate, the primary circulating form of folate, for biological methylation [41]. In addition, the decrease in enzyme activity results in decreased folate and increased homocysteine [42], which enhances accumulation of DNA damage by inducing a methyl donor deficiency state, impairing DNA repair [18] and generating ROS [17]. Thus, folate is important to supply the decreased enzyme efficiency.

In our study, individuals of group II with *1298CC* polymorphism had higher levels of DNA damage, perhaps because the reduced *MTHFR* activity converts less 5,10-methylenetetrahydrofolate in 5-methylenetetrahydrofolate, impairing synthesis of purine and SAM, and therefore, impairing DNA methylation, which may enhance gene transcription and DNA strand break thereby promoting malignant transformation [43, 44]. Pogribny et al [43] in study with 344 Fischer rats reported that hipomethylation was associated to strand breaks in methyl-deficient rats. Indeed, a fact that was determinant to higher levels of oxidative DNA damage is that individuals of the group II has a diet poor in folate and other micronutrients, such as vitamin A, B6 and vitamin C, lower than the RDA. In addition, not only low folate status may cause DNA instability, but all micronutrients deficiency [2], that are present in fruits, vegetables and whole grains, and are antioxidants, acting as scavengers of various free radicals, which may damage DNA [45]. According to Ames (2001), deficiencies of micronutrients such as folic acid,

vitamins B12, B6, C and E, niacin, iron and zinc can mimic the effect of radiation or chemical agents in the induction of DNA damage [46].

Although studies reported that the *1298CC* of *MTHFR* did not alter the levels of homocysteine [22,23,47,48], Chango et al (2000) verified a trend to higher homocysteine levels in *1298CC* individuals [28], and it is possible that as in Chango's study, in our study, the individuals with *MTHFR 1298CC* could present decrease enzyme activity have leaded to a higher homocysteine concentrations, which also may have caused the increased in levels of DNA damage. However, whether the *1298A→C* affects homocysteine levels is not completely answered, because results of many studies are controversial [22,23,47,28,49].

Different of our study, several studies have reported a combined effect of *A1298C* and *C677T* polymorphism of *MTHFR*. A study investigating the same two polymorphisms of *MTHFR* with colorectal cancer reported lower risk among individuals with the *677TT* and *1298AA* genotype, and a non-significant higher risk among individuals with the *677CC* and *1298CC* genotype [50]. Another study found that among subjects with adequate folate (>400 μg), the combined *C677T* and *A1298C* variant genotypes were associated with reduced risk of microsatellite instability tumors [51]. However, the present study evaluated only *A1298C* polymorphims, which demonstrates the necessity of more studies to evaluate the role of this polymorphism alone or in combination with others polymorphisms of *MTHFR*.

To our knowledge, this is the first study that evaluated two healthy groups with antagonist dietary patterns. Although dietary intakes of both groups are completely different, the increased oxidative DNA damage was observed in individuals with *A1298CC* genotype of group II, even the group I had consumed folate and all micronutrients higher of recommended dietary allowances. Therefore, further larger

studies are needed to interpretation and clarify these results. It is noteworthy that all individuals selected are healthy, nonsmokers, do not drink alcohol, do not use supplementation and any types of drugs, to decrease the variability and not confound the results.

In conclusion, the *A1298C* polymorphisms influenced on levels of DNA damage (strand breaks), and did not influence the misincorporation uracil and DNA repair capability. Moreover, we concluded that folate intake correlated negatively with oxidized purines. Further investigations is needed to clarify the mechanisms of *A1298C* polymorphisms in combination with others polymorphisms of *MTHFR*, micronutrients and homocysteine status.

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Abreviation Footnote

MTHFR: methylenetetrahydrofolate reductase; dTMP: thymidine monophosphate; dUMP: deoxyuridine monophosphate.

Legends

Figure 1. Influence of *MTHFR A1298C* polymorphisms on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 μl of H_2O_2 (100 μM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare differences among genotypes in each group. The individuals carrying *MTHFR 1298CC* genotype presented higher level of oxidative DNA damage than individuals carrying *MTHFR 1298 AA* on group II.

Table 1. Level of micronutrients ingested by each group as estimated Dietary Record of Three Days and Recommended Dietary Allowances (RDA) These data were used in previous paper (Prado et al. in preparation).

Table 2. Correlation among the concentrations of micronutrients intake, kCal and BMI and the level of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H2O2 e SBs H2O2R in peripheral blood lymphocytes. These data were used in previous paper (dos Santos et al. in preparation).

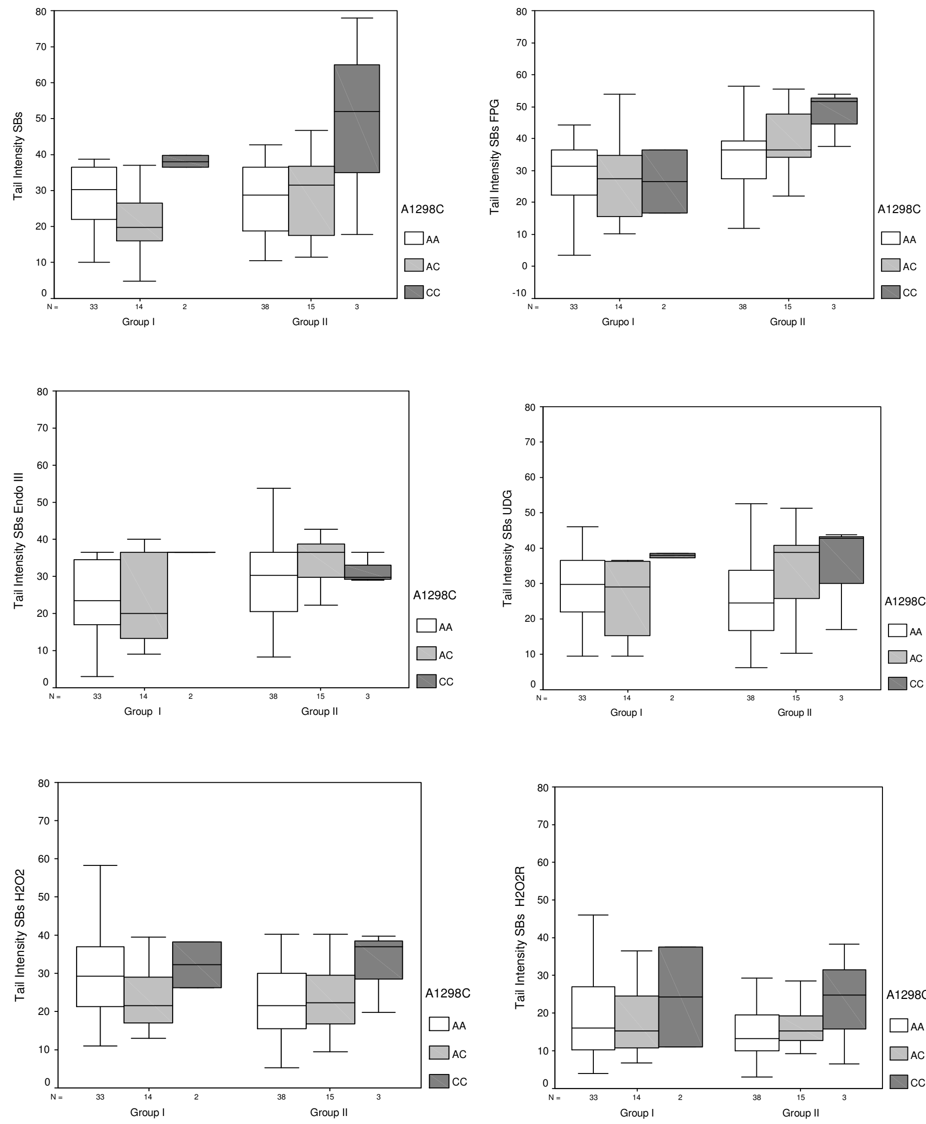

Figure 1.

Table 1.

Micronutrient	RDA	Group	Mean	SD	P
Vit. A (RE)	900	1	2593.8	1065	
		2	349.9	440,7	0.001
Vit. B2 (mg)	1.3	1	2.3	0.56	
		2	1.3	0.46	0.086
Vit. B6 (mg)	1.3	1	1.9	0,37	
		2	1.0	,42	0.036
Vit. B12 (mcg)	2.4	1	7.1	3,7	
		2	3.9	2,4	0.007
Vit. C (mg)	90	1	515.1	340	
		2	34.4	104,9	0.0001
Folic acid (mcg)	400	1	448.6	145,5	
		2	125.6	70,2	0.0001

1 RE = 1 mcg of retinol

Table 2.

	Tail Intensity SBs	Tail Intensity SBs FPG	Tail Intensity SBs ENDO III	Tail Intensity SBs UDG	Tail Intensity H2O2
Vit. B12 (mcg)					
r	-.021	-0.078	-0.074	0.087	-0.005
P	.848	0.482	0.501	0.432	0.966
Fol. (mcg)					
r	-.077	-0.213	-0.208	0.022	0.119
p	0.486	0.050	0.058	0.840	0.281
KCAL					
r	0.079	0.018	-0.031	0.015	-0.141
p	0.473	0.869	0.777	0.893	0.199
BMI					
r	0.093	-0.005	0.105	-0.011	0.224
p	0.401	0.966	0.345	0.920	0.041

Manuscrito V

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Influence of *C677T* Methylenetetrahydrofolate Reductase polymorphism and Diet on oxidative DNA damage, uracil incorporation and the efficiency of DNA repair in Healthy Subjects

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4126 words

1 Figure

2 Tables

Abstract

Epidemiological studies have indicated the role for *C677T* polymorphism of methylenetetrahydrofolate reductase (*MTHFR*) of folate and homocysteine metabolism in the etiology of several diseases, including cancer. We evaluated the influence of dietary pattern and *C677T* polymorphism of *MTHFR* on oxidative DNA damage, uracil incorporation and efficiency of DNA repair in lymphocytes of two groups of health subjects with antagonists diets: one group with 49 individuals (GI) has a diet rich in organic fruits, vegetables and whole grains and poor in industrialized food, and another group with 56 individuals (GII) has a diet rich in industrialized food and poor in organic fruits, vegetables and whole grains. The *C677T* polymorphism in the *MTHFR* gene was analyzed by PCR-RFLP and oxidative DNA damage, uracil incorporation and the efficiency of DNA repair were assessed by the Comet assay in a parallel study. The *C677T* polymorphism of *MTHFR* did not influence on oxidative DNA damage, uracil incorporation either efficiency of DNA repair in both groups. However, we observed that folate intake was correlated negatively with oxidized purines ($r = -0.213$, $p < 0.05$). In conclusion, the *C677T* polymorphisms did not influence on levels of oxidative DNA damage, uracil misincorporation into DNA either DNA repair capability. Moreover, we concluded that folate intake correlated negatively with oxidized purines. Further investigations is needed to clarify the mechanisms of *C677T* polymorphisms in combination with others polymorphisms of *MTHFR*, micronutrients and homocysteine status.

1. Introduction

Dietary factors have an important role in preventing cancer. High consumption of fruits and vegetables has been consistently related to a decreased incidence of cancer [1]. These foods are sources of antioxidants as vitamins and minerals, which, in a proper concentration, are substrate and/or cofactors in the metabolic pathways that regulate genomic stability [2]. Dietary deficiency in micronutrients required for DNA maintenance may impair activity of enzymes necessary for genomic stability [2,3] and may damage DNA to similar extents as significant exposure to known carcinogens such as ionizing radiation [3].

One of micronutrients deficiencies known to cause an imbalance on DNA metabolism is folate deficiency, which has been implicated in the development of several epithelial-cell cancers, including cancer of the lung, breast and cervix [4], and colorectal cancer [5,6]. This micronutrient has an essential role in DNA metabolism, due to its capacity to regulate gene expression, nucleotide synthesis and DNA repair; thus, a disruption in these functions may increase the risk of cancer [7].

The folate metabolism is involved by a key folate-metabolizing enzyme, the methylenetetrahydrofolate reductase (*MTHFR*), which catalyses the conversion of 5,10-methylenetetrahydrofolate into 5-methylenetetrahydrofolate, the primary circulating form of folate. This latter folic acid species provides methyl groups for the methionine synthase-mediated remethylation of homocysteine to methionine [8], which is the immediate precursor of S-adenosylmethionine, the principal methyl donor for methylation reactions, including DNA methylation [9,10]. Under conditions of folate deficiency, S-adenosylmethionine is depleted, and the product of methyltransferase

activity, S-adenosylhomocysteine, is elevated, leading to DNA hypomethylation and, possible, proto-oncogene activation [11].

Folate is also essential for the synthesis of purines and the pyrimidines nucleoside thymidine. 5,10-methylenetetrahydrofolate provides methyl groups for the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) [12,13]. If folate is low, dUMP may accumulate, inducing uracil misincorporation into DNA in place of thymine. This uracil is removed by DNA repair enzymes, but if folate is continually low, the repair cycle is impaired causing frequent point mutation, single and double-stranded DNA breaks, micronucleus formation and chromosome breakage, which are important risks for cancer [12,14].

In addition, vitamin B12 also plays important roles in DNA synthesis and production of methionine from homocysteine for the maintenance of methylation patterns in DNA [10]. When vitamin B12 is limiting, the availability of 5,10-methylenetetrahidrofolate in the methylation of dUMP to dTMP for DNA synthesis and repair is reduced [15]. Fenech et al [16] have shown that endogenous micronuclei frequency (as an indicator of chromosomal damage) is negatively associated with serum vitamin B12. Therefore, deficiencies of vitamin B12 may cause genomic instability [7].

A common genetic variation of *MTHFR* occurs when cytosine is replaced by thymine at bp 677, which yields a replacement of alanine with valine in the enzyme [17]. Individuals who are heterozygous (*CT*s) and homozygous (*TT*s) for this *MTHFR* polymorphism have as *in vitro* enzyme activity that is 65 and 30% of normal, respectively [17,18].

Homozygosity for this *677T* variant was shown to be associated with increased plasma homocysteine levels, which is thought to be due to inefficient recycling of homocysteine to methionine [19,20], particularly when folate status is low [21].

Homocysteine is considered to be an important risk factor for cancer. In a cohort study, death from cancer was significantly related to elevated homocysteine levels [22]. Studies have demonstrated that increased chromosome damage rate is correlated with elevated homocysteine level, even when there was not folate deficiency [16,23]. Moreover, homocysteine can generate reactive oxygen species (ROS) leading to oxidative DNA damage [24].

TT genotype is associated with an elevated risk for cardiovascular disease, which can be linked with homocysteine levels [25], and are reported to have higher risk for endometrial cancer [26]. However, studies reported that homozygosity for *677T* variant, associated with adequate folate status, is associated with lower risk of colorectal cancer [27,28] and acute lymphocytic leukemia [29,30]. The protector effect of this genotype may be due to impaired *MTHFR* activity, which causes an increase 5,10-methylenetetrahydrofolate concentration, then, more methyl groups are available for the conversion of dUMP to dTMP [15,18].

Therefore, the present study examined the influence of diet and *C677T MTHFR* polymorphisms on level of oxidative DNA damage, uracil incorporation and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets.

2. Materials and Methods

The present study was developed in Botucatu Medical School-UNESP, Internal Medicine Department and Pathology Departament, in Botucatu, State of São Paulo, Brazil, at period of 2005-2008. This study was approved by the local Ethical Committee. Informed consent to participate was obtained from all the volunteers.

2.1. Volunteers

We studied 105 healthy adult volunteers (average age 35,6 ± 11,4, ranging from 19 and 66 years), 52 men and 53 women. All of the volunteer were nonsmokers, were not abusing alcohol, and were not using supplementation, prescription or recreational drugs. The volunteers were divided in two groups: 1) 49 naturalistic individuals with a great consume of organic fruits, vegetables and juice and poor consume of industrialized foods (Group I); 2) 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables (Group II).

2.2. Dietary Assessment

For evaluation of the dietary pattern a Food-frequency Questionnaire adapted from Cardoso and Stocco [31] was applied, and was used for the classification of the groups. For calculating the estimated level of micronutrients ingested, a dietary record of three days was applied, and has provided data, which were inserted in the program Avanutri (AvaNutri Informática Ltda, RJ, Brazil). This procedure enabled us to

establish an estimate actual quantity of protective foods consumed daily by the population studied. To be classified in Group I, the individuals had to consume the percentile 75 of organic fruits and vegetables and below the percentile 25 of each industrialized products. The individuals of group II were those who consumed less than percentile 25 of organic fruits and vegetables and above the percentile 75 of industrialized products.

2.3. Laboratory analyses

2.3.1. DNA extraction

Venous blood was obtained for genomic DNA and comet assay.

Genomic DNA was isolated from peripheral blood leucocytes using a commecially available kit (Invitrogen), according to the manufacture's instructions.

2.3.1. MTHFR genotype analysis

According to a previously described procedure [18], genotyping for the *MTHFR* point polymorphism *C677T* was performed by polymerase chain reaction amplification with the primers 5'–TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3' [32]. The 198-pb product was obtained. Because the C to T transition at nucleotide 677 produces a *Hin*fI digestion site, the amplified product derived from the mutant gene was cleaved into 175-bp and 23-bp fragments by *Hin*fI, which leaves the wild-type gene unaffected. After eletroforesis through 8% polyacrylamide gel, the digestion products were visualized by staining ethidium bromide.

2.3.3. Determination of Oxidative DNA Damage and level of uracil incorporate into DNA

Data on oxidative DNA damage, uracil misincorporation into DNA, and DNA repair were described and accomplished by Prado et al in a parallel study (in preparation). Briefly, The alkaline Comet assay [33], modified with lesion-specific enzymes was used to detect single and double strand breaks, labile sites (SBs), oxidised purines and pyrimidines and uracil [34]. All procedures were conducted in the dark to minimize spurious sources of DNA damage. Briefly, 10 μl of the isolated lymphocytes suspension [35] (≅ 2 x 10^4 cells) were embedded into 0.5% low melting point agarose (Sigma) and spread on agarose-precoated microscope slides. Twelve slides were prepared for each sample. Slides were immersed overnight in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium salt N-Lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% DMSO added fresh) at 4^0C. After lysing, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD) and then three slides for each treatment were incubated at 37^oC for 45 min with 100 μL of endonuclaese III (Endo III - 1:1000; New England Biolabs Inc), 100 μL of formamidopyrimidine-DNA glycosylase (FPG - 1:1000; New England Biolabs Inc), 100 μL of uracil-DNA glycosylase (UDG - 1:1000; New England Biolabs Inc) or with enzyme buffer only. Endo III recognizes oxidised pyrimidines (SBs EndoIII), while FPG identifies oxidised purines (SBs FPG), especially 8-oxo-guanine and UDG recognizes uracil (SBs UDG). Enzyme buffer only is used to identified SBs. Subsequently the cells were exposed to alkali buffer (1mM EDTA and 300 mM NaOH, pH ≅ 13.4), at 4^oC, 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 (1v/cm) and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 50 μL Syber Green (1:10000; Invitrogen), and analyzed in a fluorescence microscope at 400 x magnification, using an image analysis system (Comet Assay II – Perceptive Instruments, Suffolk, UK).

For each individual, one hundred randomly selected cells (50 from each of two replicate slides) for each treatment (FPG, Endo III, UDG or enzyme buffer only) were evaluated and the mean tail intensity (%DNA Tail) was determined, totaling 400 cells analyzed from each individual. Tail Intensity, according to Comet Assay II – Perceptive instruments, is defined as "the sum of all intensity values in the tail region less those which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity".

To analyze the influence of dietary patterns and *MTHFR C677T* on the DNA repair capability, 200 μl of the freshly isolated lymphocytes were treated with 100 μl of H_2O_2 (100 μM), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H_2O_2, these aliquots were incubated at 37 °C for 30 minutes, and DNA damage was checked again.

2.4. Statistical Analyses

To evaluate the influence of *C677T* polymorphism was applied Turkey test. The data obtained in the Comet Assay were asymmetric distribution. Log transformations were applied to DNA damage distributions to help meet normality distribution assumptions. The statistical analysis of the data obtained in the Comet Assay consisted

of applying the t Student test to compare differences between the various types of DNA damage, and for influence in differences levels of DNA damage was applied Turkey test. The Pearson correlation was applied to correlate SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H2O2 and SBs H2O2R and the estimated consume of vitamin B12, folate, and Kcal and BMI. The index of significance adopted was 5%.

3. Results

The *C677T* polymorphism did not influence on level of oxidative DNA damage, uracil misincorporation into DNA and DNA repair capability on both groups (Figure 1).

The estimated concentrations of vitamin B12 and folate intake are shown on Table 1. Recommended dietary allowance (RDA), Body Mass Index (BMI) and Energy intake (Kcal) of groups are also shown on Table 1.

The Pearson correlation between SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H2O2 and SBs H2O2R and the estimated consume of vitamin B12, folate, and Kcal and BMI are shown on Table 2. Vitamin B12, Energy intake (Kcal) and folate (Table 2). Indeed, folate correlated negatively with oxidized purines ($r = -0.213$, $p < 0.05$)(Table 2). Body mass index (BMI) was correlated with level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice.

4. Discussion

We have evaluated the influence of *C677T* polymorphism of *MTHFR* and diet on level of oxidative DNA damage, uracil incorporation and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets. The group I, with 49 naturalistic individuals, has a lifestyle characterized by absence of stress, bucolic life and great consume of organic fruits, vegetables, natural juices, and whole grains and poor consume of industrialized foods, and the group II, with 56 individuals, with great ingestion of industrialized foods and poor ingestion of fruits, vegetables, natural juices, and whole grains. The *C677T* polymorphism did not influence on level of oxidative DNA damage, uracil misincorporation in DNA and DNA repair capability. The results of the parallel study analyzing the effect of diet presented that same individuals of group I present lower levels of oxidized purines and pyrimidines and lower level of DNA damage induced by H_2O_2. Moreover, folate intake correlated negatively with oxidized pyrimidines.

As described, MTHFR is an enzyme that reduces 5,10-methylenetetrahydrofolate into 5-methylenetetrahydrofolate, the principal circulating form of folate in serum, providing methyl groups for the synthesis of SAM, thus for DNA methylation. A variant of *MTHFR* occurs at pb 677 when cytosine is replaced by thymine. Individuals heterozygous (*CT*s) and homozygous (*TT*s) for this *MTHFR* polymorphism have as *in vitro* enzyme activity that is 65 and 30% of normal, respectively [18,19]. Although the wild-type *677CC* variant is the more active form, lower colon cancer risk [28, 29] and childhood and adult leukemia [30] was associated with *677TT* genotype. This can be explained by the reduced *MTHFR* activity, which

may increase 5,10-methylenetetrahydrofolate, that is essential to conversion of dUMP to dTMP for DNA synthesis and repair. More 5,10-methylenetetrahydrofolate levels could increase the availability of thymine, reduce the incorporation of the former base into DNA and consequently DNA damage, and promote DNA stability [15,19]. However, such protection of this polymorphism only occurs with adequate folate intake, since imbalanced diet affects the availability of 5-methylenetetrahydrofolate for biological methylation, balancing the beneficial effect of reduced uracil misincorporation [36]. In contrast, other studies reported that *677TT* genotype was associated with increased risk of cervical [37] and gastric dysplasia [38], and endometrial cancer [27]. In a Chinese population with dietary folate deficiency, for example, the *MTHFR 677TT* and *677CT* were associated with higher risk of gastric cancer [39]. In the present study, individuals of group I presented levels of vitamin B12 and folic acid significantly higher than individuals of group II, that presented levels of vitamin A, B6, vitamin C and folate lower than the recommended dietary allowance (RDA). Thus, because of the interaction gene-environment, it might be expected that in this study, the *C677T* of *MTHFR* polymorphism would influence on level of oxidative DNA damage, uracil misincorporation into DNA and DNA repair capability, measured on blood of peripheral lymphocytes on both groups. However, although the group II ingested lower micronutrients than the group I, the *C677T* polymorphism did not influence on both groups.

Nevertheless, it is noticed that individuals of our study were all healthy subjects, nonsmokers, were not users of drugs, not users of medication for at least 30 days, were not abusing alcohol, and the majority was young individuals, what can contribute to these results. Besides, another factor that may influence on these results is the presence of others polymorphisms in the *MTHFR* gene, which acts in a combination [38]. Others

studies found similar results as ours, as Narayanan et al (2004), that find that lymphocyte DNA stability biomarkers were similar for all *MTHFR C677T* or *A1298C* variants [41]. In another human study, all *MTHFR C677T* variants did not influence on DNA strand breakage, micronuclei frequency formation and chromatid exchange [42]. Moreover, a study *in vitro* found that *MTHFR C677T* genotype did not influence levels of chromosome damage measured by the cytokinesis block micronucleus assay [19]. In addition, another *in vitro* study reported that the *MTHFR C677T* polymorphism did not alter folate deficiency-induced uracil incorporation into DNA [40].

In contrast, a study reported that an increased DNA damage, measured by the micronucleus, were significant higher in subjects with the *677TT* genotype compared with the *677CC* or *677CT* genotypes [43]. However, individuals of this study have cardiovascular disease, what can confound the results [41]. This clarify the importance of a sample consisted in healthy subjects, non-smokers, non users of drugs, non users of medication for at least 30 days and non alcohol abusing, to decrease possible variability.

Although the genotype did not influence on DNA damage, uracil misincorporation into DNA either DNA repair capability, we observed that folate intake was correlated negatively with oxidized purines ($r = - 0.213$, $p < 0.05$). In fact, folate plays an important role in DNA metabolism, and folate deficiency not only leads to elevated DNA damage rate, but to an increased levels of homocysteine, which may cause oxidative DNA damage through generation of reactive oxygen species (ROS) [24].

We believe that this is the first study that evaluated two healthy groups with antagonist dietary patterns that adopted these eating habits for 10 years. Many studies used supplementation or special diets to analyze the effect of diet and genes polymorphisms on DNA stability and gene expression. Prado et al, in a parallel study of

our laboratory, observed that dietary patterns influenced on level of oxidized purines and pyrimidines, which were higher in group II than in group I, and on levels of DNA damage induced by H_2O_2, which were lower in group I than in group II. In the present study, we concluded that the *C677T* polymorphisms did not influence on levels of oxidative DNA damage, uracil misincorporation into DNA either DNA repair capability. Moreover, we concluded that folate intake correlated negatively with oxidized pyrimidines. Further investigations are needed to clarify the mechanisms of *C677T* polymorphisms in combination with others polymorphisms of *MTHFR*, micronutrients and homocysteine status.

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Abreviation Footnote

MTHFR: methylenetetrahydrofolate reductase; dTMP: thymidine monophosphate; dUMP: deoxyuridine monophosphate.

Legends

Table 1. Concentrations of vitamin B12, Folate and energy intake (Kcal) estimated by dietary record of three days, and Body Mass Index (BMI) on both groups.

Table 2. Correlation among the concentrations of micronutrients intake, kCal and BMI and the level of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H2O2 e SBs H2O2R in peripheral blood lymphocytes.

Figure 1. Influence of *MTHFR C677T* polymorphisms on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare differences among genotypes in each group. None of the genotypes influenced the levels of DNA damage.

Table 1.

Micronutrient	RDA	Group	Mean	Desvio Padrão	P
Vit. B12 (mcg)	2,4	1	7,0694	3,6629	
		2	3,9428	2,4299	0,007
Fol. (mcg)	400	1	448,594	145,454	
		2	125,594	70,213	0,0001
KcaL		1	2203,936	544,601	
		2	2201,318	447,206	0,232
BMI		1	24,5427	4,7468	
		2	24,5166	3,6638	0,136

Table 2.

	Tail Intensity SBs	Tail Intensity SBs FPG	Tail Intensity SBs ENDO III	Tail Intensity SBs UDG	Tail Intensity SBs H2O2	Tail Intensity SBs H2O2R
Vit. B12 (mcg)						
r	-.021	-0.078	-0.074	0.087	-0.005	-0.023
P	.848	0.482	0.501	0.432	0.966	0.835
Fol. (mcg)						
r	-.077	-0.213*	-0.208	0.022	0.119	0.136
p	0.486	0.050	0.058	0.840	0.281	0.216
KCAL						
r	0.079	0.018	-0.031	0.015	-0.141	-0.078
p	0.473	0.869	0.777	0.893	0.199	0.483
BMI						
r	0.093	-0.005	0.105	-0.011	0.224	0.122
p	0.401	0.966	0.345	0.920	0.041	0.271

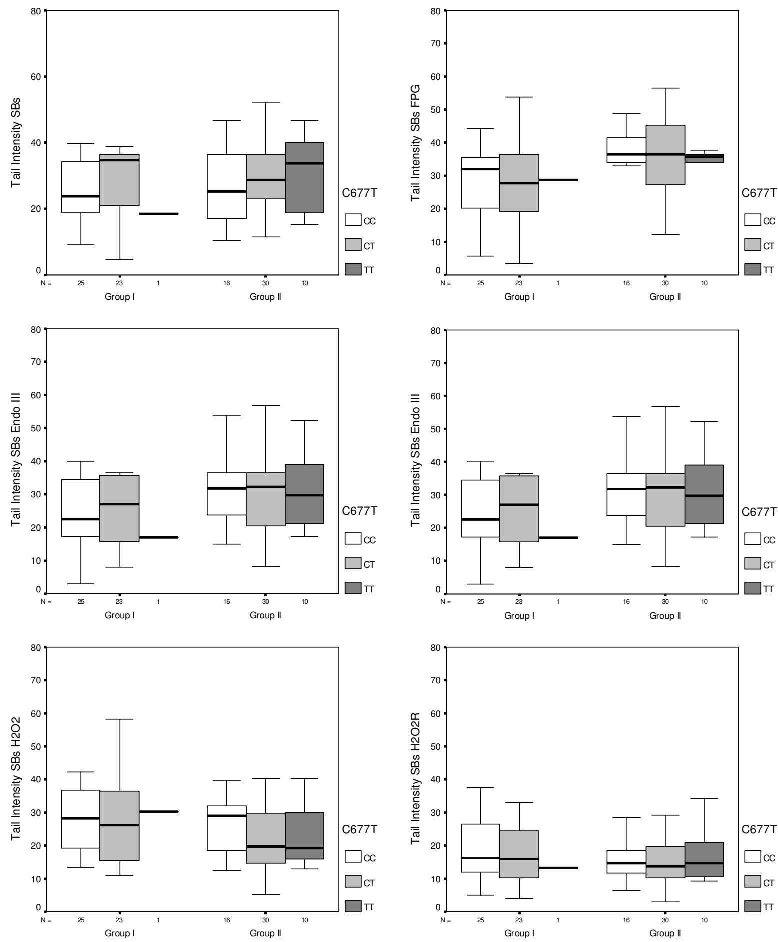

Figure 1.

Manuscrito VI

Trabalho elaborado segundo as normas da revista “The Journal of Nutrition”.

Influence of *TS3'UTR insertion/deletion* and *TSER 2R/3R* polymorphisms of Thymidylate Synthase and Diet on oxidative DNA damage, uracil incorporation and the efficiency of DNA repair in Healthy Subjects

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(*Running Title*): Influence of TS polymorphisms and diet on DNA damage.

Key Words: Diet, oxidative DNA damage, uracil incorporation, DNA repair, comet assay

Abstract

We evaluated the influence of dietary pattern and *TSER 2R/3R* polymorphism and *TS3UTR ins/del* polymorphism of *TS* on oxidative DNA damage, uracil incorporation and DNA repair capability in lymphocytes of two groups of health subjects with antagonists diets: one group with 49 individuals (group I) has a diet rich in organic fruits, vegetables and whole grains and poor in industrialized food, and another group with 56 individuals (group II) has a diet rich in industrialized food and poor in organic fruits, vegetables and whole grains. PCR and PCR-RFLP assays were used to identify the *TSER* and *TS3'UTR* polimorphisms and oxidative DNA damage, uracil incorporation and the DNA repair capability were assessed by the Comet assay in a parallel study. The individuals of group I carrying The *TS'3UTR ins/ins* presented higher levels of purine and pyrimidines oxidized than individuals carrying *TS'3UTR ins/del* ($p < 0.05$). The individuals of group I carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of pyrimidines oxidized when compared to *TSER 2R/2R*, while individuals do group II carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of SBs, purine and pyrimidines oxidized and misincorporated uracil into DNA and lower DNA repair capability when compared to individual carrying *TSER 2R/2R.*

In conclusion, the *TS* polymorphisms could modulate the DNA damage level and DNA repair capability even in healthy subjects that has a great consume of organic fruits, vegetables, natural juices, and whole grains (group I). However, this effect is more evident in healthy subjects that have a diet deficient in fruits and vegetables (group II). We believe that further investigations are needed to clarify the mechanisms of *TS* polymorphisms in combination with other genes and micronutrients status.

1. Introduction

Dietary factors have an important role in preventing cancer. High consumption of fruits and vegetables has been consistently related to a decreased incidence of cancer [1]. These foods are sources of antioxidants as vitamins and minerals, which, in a proper concentration, are substrate and/or cofactors in the metabolic pathways that regulate genomic stability [2]. Dietary deficiency in micronutrients required for DNA maintenance may impair activity of enzymes necessary for genomic stability [2,3] and may damage DNA to similar extents as significant exposure to known carcinogens such as ionizing radiation [3].

Folate deficiency is one of micronutrients deficiencies known to cause an imbalance on DNA metabolism, and it has been implicated in the development of several epithelial-cell cancers, including cancer of the lung, breast and cervix [4], and colorectal cancer [5,6]. The primary function of this micronutrient is to carrier single-carbon units. Folate-dependent reactions include the biosynthesis of thymidylate, purines, methionine, and glycine thus linking it to nucleotide synthesis and providing methyl groups [7]. Studies have shown associations between genetic polymorphisms in folate-metabolizing enzymes and carcinogenesis [8,9,10]. Mechanisms linking folate to carcinogenesis include an altered provision of S-adenosylmethionine (SAM) for methylation reactions, including DNA methylation, and changes in the availability of nucleotides, such as thymidylate, for DNA synthesis and repair affecting processes such as cell proliferation that are dependent on nucleic acids [11,12]. Low folate status cause a limiting levels of 5,10-methylenetetrahydrofolate, which will restrict the production of 5-methylenetetrahydrofolate, impeding the methylation of homocysteine to methionine, and, therefore, causing an elevated plasma homocysteine concentrations, which, in its turn, is considered to be an important risk factor for cancer, possible, because

homocysteine causes a generation of reactive oxygen species (ROS), leading to oxidative DNA damage [13]. In addition, not only folate plays an important role in DNA metabolism, but vitamin B12 also plays important roles in DNA synthesis and production of methionine from homocysteine for the maintenance of methylation patterns in DNA [14]. Therefore, deficiencies of vitamin B12 also may cause genomic instability [15].

Thymidilate synthase (*TS*) is a key enzyme of folate metabolism, and catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) [16]. Thus, *TS* plays a pivotal role in providing a nucleotide available for DNA synthesis and repair. Impairment of *TS* was found to be associated with chromosome damage and fragile site induction [17,18], which may cause individual susceptibility to cancer. Indeed, 5,10-methylenetetrahydrofolate is a common cofactor for the enzymes *TS* and 5,10-methylenetetrahydrofolate reductase (*MTHFR*) [19]. Moreover, *TS* is also known to be a target for chemotherapeutic drugs such as 5-fluorouracil, and *TS* mRNA and protein expression levels are prognostic indicators for several cancers [20,21,22].

A genetic polymorphism in the *TS* gene was found in the tandem repeat sequence in the 5'untranslated enhanced region (*TSER*), which consists of either 2 repeats (*2R*) or 3 repeats (*3R*) of 28-bp [23], although more rare alleles such as *4R*, *5R* and *9R* also exist [24,25]. The number of tandem repeats is known to affect *TS* activity levels; individuals homozygous for triple repeats (*TS 3R/3R*) was reported to have 3.6 times higher *TS* mRNA levels compared with those homozygous for the double repeat (*TS 2R/2R*) genotype [26]. *In vitro* and *in vivo* studies reported higher *TS* gene expression in association with the *3R* than with the *2R* [23,26,27]. Therefore, it has been

postulated that *TS* consumption of 5,10-methylenetetrahydrofolate should be greater in *3R/3R* homozygotes than in *2R/3R* heterozygotes or *2R/2R* homozygotes [19].

Since *TS* plays a pivotal role in providing a nucleotide available for DNA synthesis and repair, several studies have been associated *TSER* polymorphisms with increase and decrease in risk of some types of cancer, for example, Ulrich et al (2002) reported that *TS 3R/3R* genotype increased the risk of polyps among individuals with low and medium folate consumption [28]. In addition, studies reported that patients with *2R/2R* genotype had better response (decreased tumor burden) to 5-FU-based therapy [26,29] and longer survival after the treatment, compared with patients with *3R/3R* genotype [30].

A second *TS* polymorphism was identified: a *6-bp deletion/insertion* as *bp 1494* in the 3'-untranslated region of the *TS* gene (*TS3'UTR* or *1494del6*) [31], and is thought to influence *TS* mRNA expression and stability [31,32,33]. The association between the *TS3'UTR* polymorphism and the risk of cancer presented mixed results [28,34,35,36,37].

Therefore, the present study examined the influence of diet and *TSER* and *TS'3UTR* of *TS* polymorphisms on level of oxidative DNA damage, uracil incorporation and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets.

2. Materials and Methods

The present study was developed in Botucatu Medical School-UNESP, Internal Medicine Department and Pathology Departament, in Botucatu, State of São Paulo, Brazil, at period of 2005-2008. This study was approved by the local Ethical Committee. Informed consent to participate was obtained from all the volunteers.

2.1. Volunteers

We studied 105 healthy adult volunteers (average age 35,6 ± 11,4, ranging from 19 and 66 years), 52 men and 53 women. All of the volunteer were nonsmokers, were not abusing alcohol, and were not using supplementation, prescription or recreational drugs. The volunteers were divided in two groups: 1) 49 individuals with a great consume of organic fruits, vegetables and juice and poor consume of industrialized foods (Group I); 2) 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables (Group II).

2.2. Dietary Assessment

For evaluation of the dietary pattern a Food-frequency Questionnaire adapted from Cardoso and Stocco [38] was applied, and was used for the classification of the groups. For calculating the estimated level of micronutrients ingested, a dietary record of three days was applied, and has provided data, which were inserted in the program Avanutri (AvaNutri Informática Ltda, RJ, Brazil). This procedure enabled us to establish an estimate actual quantity of protective foods consumed daily by the population studied. To be classified in Group I, the individuals had to consume the percentile 75 of organic fruits and vegetables and below the percentile 25 of each industrialized products. The individuals of group II were those who consumed less than percentile 25 of organic fruits and vegetables and above the percentile 75 of industrialized products.

2.3. *Laboratory analyses*

2.3.1. DNA extraction

Venous blood was obtained for genomic DNA and comet assay. Genomic DNA was isolated from peripheral blood leucocytes using a commecially available kit (Invitrogen), according to the manufacture's instructions.

2.3.1. TS genotype analysis

Polymerase chain reaction (PCR) and PCR-based restriction fragment lenght polymorphism (PCR-RFLP) assays were used to identify the *TSER* and *TS3'UTR* polimorphisms, respectively. The primers of the TSER polymorphism were 5'-GTGGCTCCTGCGTTTCCCCC-3' (foward) [23] and 5'-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3'(reverse) [31], which generated 152 bp fragment for 6 bp deletion (i.e. 0 bp) or 158 bp for 6 bp insertion (i.e. 6 bp). The restriction enzyme DraI was used to distinguish the TS3'UTR polymorphism, in which the presence of the 6 bp insertion creates a DraI restriction site, and the expected fragment sizes were 88 and 70 bp. Both the expected fragments of these two polymorphisms were separated on 3% NuSieve 3 :1 agarose gel.

2.3.2. Determination of Oxidative DNA Damage and level of uracil incorporate into DNA

Data on oxidative DNA damage, uracil misincorporation into DNA, and DNA repair were described and accomplished by Prado et al in a parallel study (in preparation). Briefly, The alkaline Comet assay [39], modified with lesion-specific enzymes was used to detect single and double strand breaks, labile sites (SBs), oxidised purines and pyrimidines and uracil [40]. All procedures were conducted in the dark to minimize spurious sources of DNA damage. Briefly, 10 µl of the isolated lymphocytes suspension [41] (≅ 2 x 10^4 cells) were embedded into 0.5% low melting point agarose

(Sigma) and spread on agarose-precoated microscope slides. Twelve slides were prepared for each sample. Slides were immersed overnight in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium salt N-Lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% DMSO added fresh) at 4^0C. After lysing, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD) and then three slides for each treatment were incubated at 37^oC for 45 min with 100 μL of endonuclaese III (Endo III - 1:1000; New England Biolabs Inc), 100 μL of formamidopyrimidine-DNA glycosylase (FPG - 1:1000; New England Biolabs Inc), 100 μL of uracil-DNA glycosylase (UDG - 1:1000; New England Biolabs Inc) or with enzyme buffer only. Endo III recognizes oxidised pyrimidines (SBs EndoIII), while FPG identifies oxidised purines (SBs FPG), especially 8-oxo-guanine and UDG recognizes uracil (SBs UDG). Enzyme buffer only is used to identified SBs. Subsequently the cells were exposed to alkali buffer (1mM EDTA and 300 mM NaOH, pH ≅ 13.4), at 4^oC, for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted in the same solution at 4^oC, for 30 min, at 25 V (1v/cm) and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 50 μL Syber Green (1:10000; Invitrogen), and analyzed in a fluorescence microscope at 400 x magnification, using an image analysis system (Comet Assay II – Perceptive Instruments, Suffolk, UK).

For each individual, one hundred randomly selected cells (50 from each of two replicate slides) for each treatment (FPG, Endo III, UDG or enzyme buffer only) were evaluated and the mean tail intensity (%DNA Tail) was determined, totaling 400 cells analyzed from each individual. Tail Intensity, according to Comet Assay II – Perceptive instruments, is defined as “the sum of all intensity values in the tail region less those

which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity”.

To analyze the influence of dietary patterns and *TS3’UTR insertion/deletion* and *TSER 2R/3R* polymorphisms of Thymidylate Synthase on the DNA repair capability, 200 µl of the freshly isolated lymphocytes were treated with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H_2O_2, these aliquots were incubated at 37 °C for 30 minutes, and DNA damage was checked again.

2.4. Statistical Analysis

To evaluate the influence of *TS* polymorphisms, the Tukey test was applied. The data obtained in the Comet Assay were asymmetric distribution, thus, Log transformations were applied to DNA damage distributions to help meet normality distribution assumptions. The statistical analysis of the data obtained in the Comet Assay consisted of applying the t Student test to compare differences between the various types of DNA damage, and for influence in differences levels of DNA damage, the Tukey test was applied. The index of significance adopted was 5%.

3. Results

DNA damage, including single and double strand breaks, labile sites, oxidised purines and pyrimidines and misincorporated uracil on DNA, was detected by the alkaline Comet assay modified with lesion-specific enzymes, FPG, Endo III and UDG, respectively. The alkaline Comet assay measures SBs and AP sites. The enzyme-modified assay measures oxidative DNA damage as a combination of SBs, AP sites and oxidised bases – formamidopyrimidines and the oxidised purine 8-oxo-guanine when considering FPG (SBs FPG), or oxidised pyrimidine when considering endonuclease III (SBs Endo III). The enzyme-modified assay also measures misincorporated uracil on DNA when considering use of uracil-DNA glycosylase (SBs UDG).

The figure 1. summarizes the influence of *TS'3UTR* on levels of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in each group. The individuals of group I carrying The *TS'3UTR ins/ins* presented higher levels of purine and pyrimidines oxidized than individuals carrying *TS'3UTR ins/del* ($p < 0.05$).

The figure 2. summarizes the influence of *TSER* on levels of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in each group. The individuals of group I carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of pyrimidines oxidized when compared to *TSER 2R/2R*, while individuals do group II carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of SBs, purine and pyrimidines oxidized and misincorporated uracil into DNA and lower DNA repair capability when compared to individual carrying *TSER 2R/2R.*

4. Discussion

We have evaluated the influence of *TS'3UTR* and *TSER* polymorphisms of *TS* and diet on level of oxidative DNA damage, uracil incorporation into DNA, and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets. The group I, with 49 individuals, has a great consume of organic fruits, vegetables, natural juices, and whole grains and poor consume of industrialized foods; and the group II, with 56 individuals, has a great ingestion of industrialized foods and poor ingestion of fruits, vegetables, natural juices, and whole grains. Our findings indicated that the individuals of group I carrying The *TS'3UTR ins/ins* presented higher levels of purine and pyrimidines oxidized than individuals carrying *TS'3UTR ins/del*. Moreover, the individuals of group I carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of pyrimidines oxidized when compared to *TSER 2R/2R*, while individuals do group II carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of SBs, purine and pyrimidines oxidized and misincorporated uracil into DNA and lower DNA repair capability when compared to individual carrying *TSER 2R/2R. TS* catalyses the transformation of dUMP to dTMP in a reaction essential for pyrimidine biosynthesis and thus DNA synthesis and repair, and utilizes as cofactor 5,10-methylenetetrahydrofolate [19]. Another enzyme in folate metabolism, *MTHFR*, also utilizes 5,10-methylenetetrahydrofolate to generate 5-methylenetetrahydrofolate, which is the major circulating form of folate and a required cofactor for the remethylation of homocysteine to methionine [42]. Shi et al (2005) reported that the enhanced expression of 3R allele may increase the conversion of dUMP to dTMP, reducing the chance of uracil misincorporation into DNA, which could limit DNA double-strand breaks in rapidly proliferating tissues [37]. Several studies are according with our study, reported the association of the *3R* allele to increased risk of some

cancers. Chen et al (2003) found that *TS 2R/2R* genotype may be associated with reduced risk of colorectal cancer [34]. In a case-control study of 510 colorectal polyps and 604 polyp-free controls, Ulrich et al (2002) found that *3R/3R* genotype increase the risk of polyps among individuals with low and medium folate consumption [28]. In 2005, the same author reported that *TSER 2R/2R* was associated with a reduced risk of colon cancer among men [7]. Moreover, in a study of Trinh et al (2002), the *3R/3R* genotype was associated with reduced plasma folate and, among individuals with low dietary folate intake, with plasma elevated plasma homocysteine [43], which may increase DNA damage [13].

In contrast, Brown et al (2004) did not find an association between *TSER* polymorphisms and homocysteine concentrations, possible due to the different ethnic origins of the two populations [19]. Another study found no association between *TSER* polymorphisms and risk of squamous cell carcinoma of the head and neck [36], and a study involving lymphocyte leukemia reported that compared with *TSER 3R/3R*, *TSER 2R/2R* was associated with increased risk for adult lymphocyte leukemia [44]. Another study reported that individuals with *TSER 2R* allele had an increased risk for malignant lymphomas [45], suggesting that the effect of the *TSER* polymorphism and folate pathway on cancer may be specific to certain cancers or ethnic groups [37].

The *3R* genotype has been associated with increased *TS* protein concentrations and higher absolute enzyme activity *in vivo* [46], it has been postulated that *TS* consumption of 5,10-methylenetetrahydrofolate should be greater in *3R/3R* homozygotes than in *2R/3R* heterozygotes or *2R/2R* homozygotes [19]. Since *MTHFR* competes with *TS* for 5,10-methylenetetrahydrofolate, *TSER 3R* allele may decrease the levels of 5,10-methylenetetrahydrofolate available to *MTHFR*, thereby affecting 5-methylenetetrahydrofolate, thus, causing an increased in plasma homocysteine levels,

and consequently DNA damage. In our study individual carrying *TSER 3R* allele presented higher level of DNA damage, which also could be relationship with high plasma homocysteine levels. Moreover, the limiting levels of 5,10-methylenetetrahydrofolate could affect purine synthesis because it would be converted lower in 10-formyl-tetrahydrofolate, which is used to purine synthesis, impairing cell proliferation that are dependent on nucleic acids [47], and possible impairing DNA repair, as observed on *TSER 3R/3R* and *2R/3R* individuals of group II. Perhaps an adequate provision of 5,10-methylenetetrahydrofolate for purine synthesis is a protected pathway, mainly in the presence of low folate. The group I, characterized by a dietary pattern rich in micronutrients, carrying *3R/3R* and *2R/3R* genotypes presented only higher oxidized pyrimidines. However, individual of group II, characterized by a dietary intake poor in micronutrients, with *3R/3R* and *2R/3R* genotypes presented higher strand breaks, oxidized purines, uracil incorporated into DNA and lower DNA repair capability, suggesting that dietary patterns, especially folate intake, could interact to *TS* pathway; although the group I have presented higher pyrimidines for the *3R* genotype.

In our study, individuals of group I carrying *3'UTR ins/ins* genotype presents an increased oxidized purines and pyrimidines. The presence of the *del* allele has been reported to result in enhanced *TS* mRNA degradation *in vitro*. Moreover a study observed reduced *TS* mRNA expression in colorectal tumors of patients carrying *TS3'UTR del/del*; in other words, the *6 bp* deletion allele had an ~50% lower mRNA than did the *6 bp* insertion allele [33]. If *TS* has a lower activity conferred by del allele, this enzyme would utilize less 5,10-methylenetetrahydrofolate as cofactor for the conversion of dUMP to dTMP. However, more 5,10-methylenetetrahydrofolate would be available for provision of methyl groups for methylation reactions and for purine synthesis. An adequate provision of 5,10-methylenetetrahydrofolate for purine synthesis

is important to combat depurination, which is the most common form of spontaneous DNA damage, with approximately 10,000 depurination/cell/day [48,49]. Although this damage is repaired by apurinic endonucleases, abasic sites are present in cellular DNA, with 5-10,000 lesions/cell/day [49] and adequate supply of purine may be important to repair these DNA damage [28]. Why the *TS3'UTR ins/ins* influenced only on group I, who have high folate intake, is still unclear. But it might be an interaction between folate and *TS3'UTR ins/del* genotype.

Some studies are similar to ours. Hubner et al (2007), reported that individuals carrying *TS 3'UTR del/del* presented reduced colorectal adenoma risk compared to those carrying either *ins/del* or *ins/ins* genotypes [50]. Another study found that subjects with one or two *TS3'UTR 6 bp* insertion alleles had a nearly 1.5-fold greater risk of lung cancer than those without the *6 bp* insertion allele [37]. Zhang et al (2004) reported that *TS3'UTR del/del* was associated with decreased risk of squamous cell carcinoma of the head and neck [36]. In addition, Kealey et al (2005) verified the impact of *TS 3'UTR ins/del* polymorphism on folate and homocysteine levels and found that individuals who have the *TS 3'UTR del/del* genotype may be genetically predisposed to have a superior folate status, and the same genotype was associated with lower homocysteine concentrations [47]. As described, homocysteine can generate reactive oxygen species (ROS) leading to oxidative DNA damage [13]. Several studies have demonstrated that increased chromosome damage rate is correlated with elevated homocysteine level, even when there was not folate deficiency [51,52]. Thus, this study is in agreement with ours, since *TS3'UTR del/del* was associated with lower oxidized purines and pyrimidines. On the other hand, some studies did not find association between *TS3'UTR ins/del* polymorphism and cancer risk [28,34].

In relation to folate intake, was observed by Prado et al. (in preparation) in parallel study of our laboratory that folate intake was correlated negatively with oxidized purines ($r = -0.213$, $p < 0.05$). In fact, folate plays an important role in DNA metabolism, and folate deficiency not only leads to elevated DNA damage rate, but to an increased levels of homocysteine, which may cause oxidative DNA damage through generation of reactive oxygen species (ROS) [13].

We believe that this is the first study that evaluated two healthy groups with antagonist dietary patterns. Prado et al (in preparation) observed that dietary patterns influenced on level of oxidized purines and pyrimidines, which were higher in group II than in group I, and on levels of DNA damage induced by H_2O_2, which were lower in group I than in group II.

In conclusion, the *TS* polymorphisms could modulate the DNA damage level and DNA repair capability even in healthy subjects that has a great consume of organic fruits, vegetables, natural juices, and whole grains (group I). However, this effect is more evident in healthy subjects that have a diet deficient in fruits and vegetables (group II). We believe that further investigations are needed to clarify the mechanisms of *TS* polymorphisms in combination with other genes and micronutrients status.

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Legends

Figure 1. Influence of *TS3'UTR* polymorphisms of *TS* on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare differences among genotypes in each group. The individuals of group I carrying The *TS'3UTR ins/ins* presented higher levels of purine and pyrimidines oxidized than individuals carrying *TS'3UTR ins/del* ($p < 0.05$).

Figure 2. Influence of *TSER* polymorphisms of *TS* on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare

differences among genotypes in each group. The individuals of group I carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of pyrimidines oxidized when compared to *TSER 2R/2R*, while individuals do group II carrying *TSER 3R/3R* or *TSER 2R/3R* genotypes presented higher levels of SBs, purine and pyrimidines oxidized and misincorporated uracil into DNA and lower DNA repair capability when compared to individual carrying *TSER 2R/2R.*

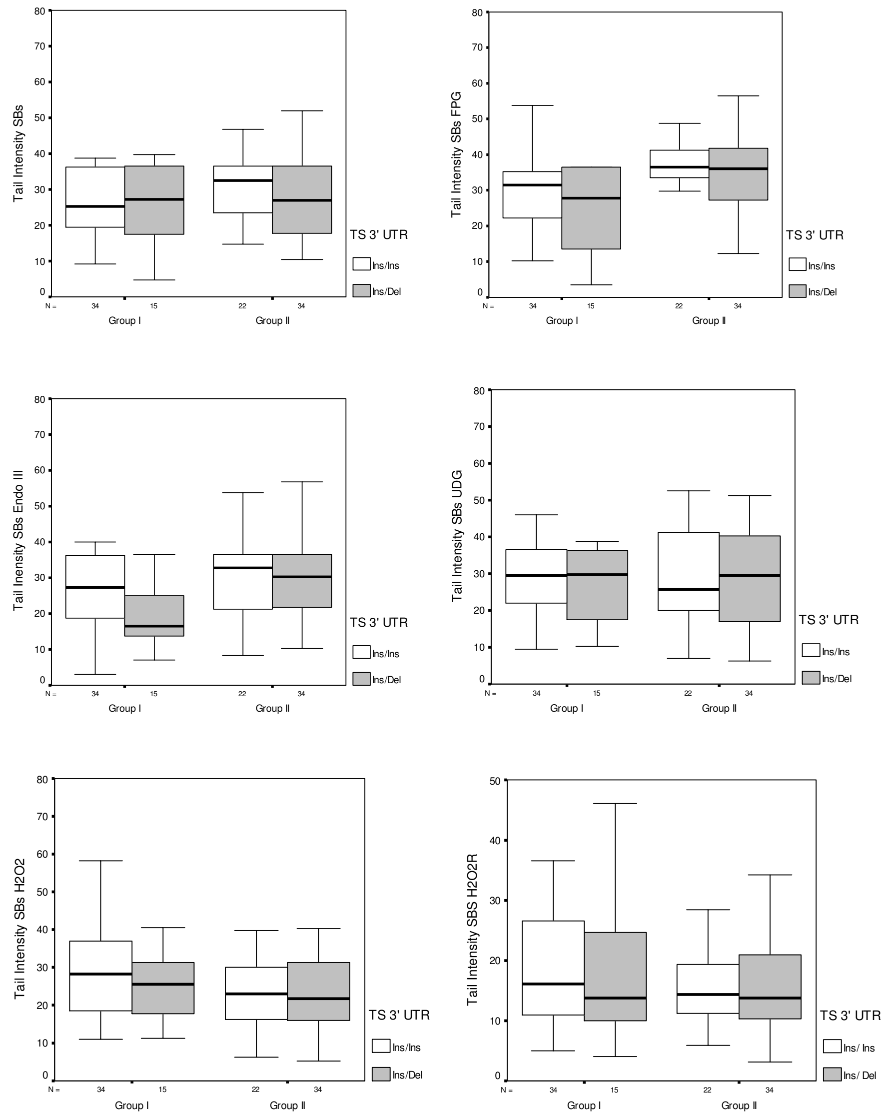

Figure 1.

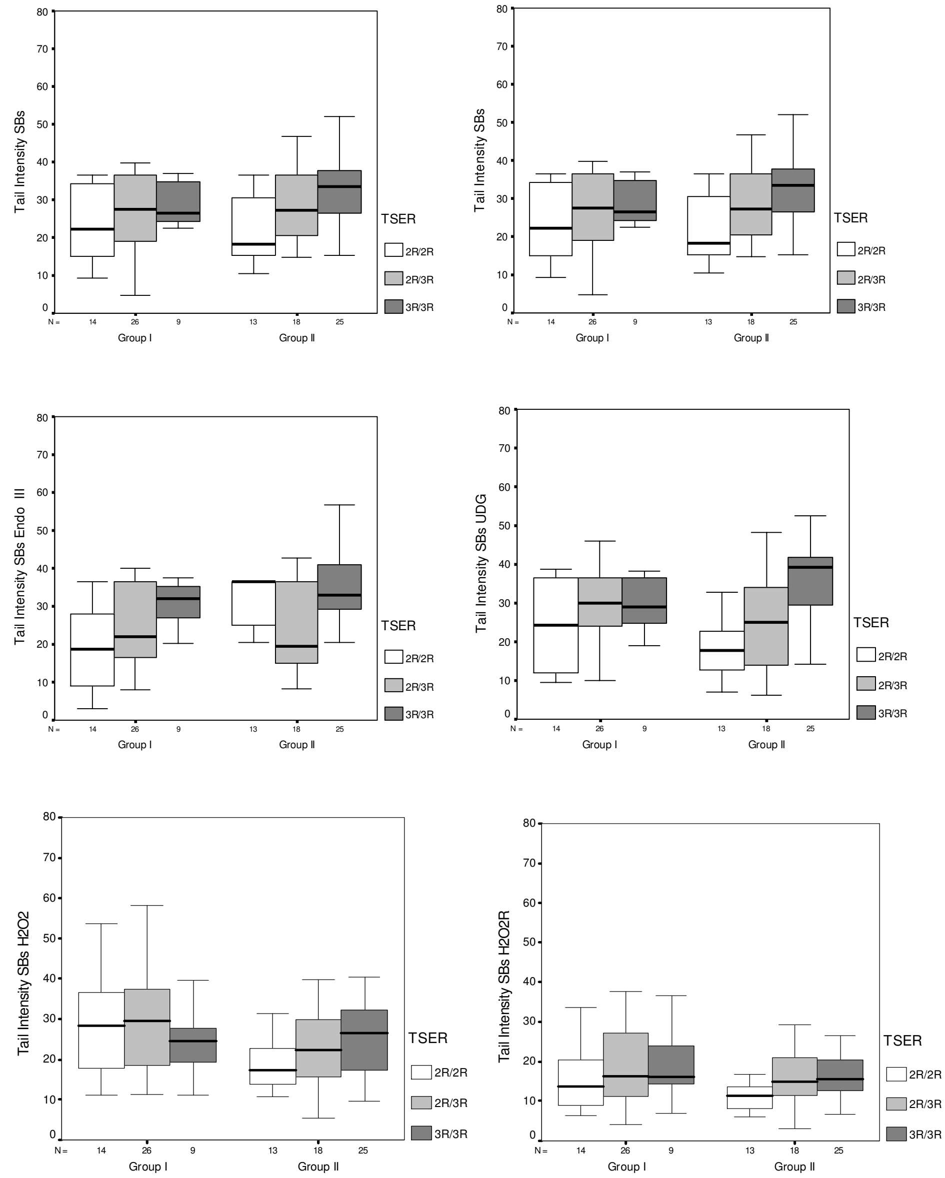

Figure 2.

Manuscrito VII

Trabalho elaborado segundo as normas da revista "The Journal of Nutrition".

Influence of diet and gene *GSTP1 A313G* polymorphism on oxidative DNA damage, misincorporation uracil, and system repair efficiency.

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Abstract

Dietary factors can be related to fortuitous genomic instability, which can be preceded by oxidative damage and uracil incorporation in DNA which can trigger a carcinogenic process. Linked to diet, there is a genetic susceptibility to developing cancer. Polymorphic *GSTP1* in humans is involved in the detoxification of various potentially toxic components found in foodstuffs. The aim of this study was to evaluate the possible influence of *GSTP1* gene *A313G* polymorphism on DNA oxidative damage levels in peripheral blood lymphocytes in two groups of individuals: Group I) 42 individuals with a diet rich in natural products such as whole grains, fruit, and vegetables, and low in processed products, and Group II) 42 individuals on a diet high in processed products and low in fruit and vegetables. PCR-RFLP was used for *GSTP1* genotyping. The individuals of group II carrying *GSTP1 G313G* genotype presented higher levels of purines oxidized when compared to individuals of group II carrying *GSTP1 A313A* or individuals of group II carrying. None of the *GSTP1 A313G* genotypes influenced the baseline levels of SBs, pyrimidine damaged, and misincorporation uracil on DNA, the levels of DNA damage induce with treatment of H_2O_2 (100 μM) and the DNA repair capability, in both groups.

In conclusion, the *GSTP1 A313G* polymorphisms could modulate the oxidative DNA damage level in healthy subjects that have a diet deficient in fruits and vegetables. We believe that further investigations are needed to clarify the mechanisms of *GSTP1 A313G* polymorphisms in combination with other genes and micronutrients status.

1. Introduction

It is estimated that in the world, per year, around 10 million people receive the diagnosis of cancer and more than six million die due this disease [1]. In 1981, Doll & Peto [2] published a study listing the main causes which can facilitate a carcinogenic process; diet was in first place with a 35% risk. Currently these can vary between 10% for lung cancer to 80% for colorectal cancer [1].

Fergunson [3] showed that chemical additives used in meat preservation (N-nitrous derivatives), and compounds such as heterocyclic amines and polycyclic aromatic hydrocarbonates produced during high temperature meat preparation can lead to the development of colon and prostate cancer.

According to Ames [4], deficiencies in micronutrients found in fruit and vegetables, such as folic acid, vitamins B12, B6, C, and E, niacin, iron, and zinc, as well as protein calorie undernourishment, can mimic the effect of radiation or chemical agents in inducing DNA damage. Folic acid deficiency can also lead to uracil incorporation in DNA instead of thymine [5], the main type of endogenous DNA damage [6], which can lead to genome instability. Also various micronutrients act as enzymatic cofactors in DNA maintenance, repair, and methylation reactions and apoptosis [7]. Therefore deficiencies in ingesting these micronutrients could lead to eventual genomic instability characterized by an increased frequency in point mutation or chromosome break biomarkers, or even interfere in chromosome segregation, gene expression, oxidative stress, necrosis, and apoptosis [8].

As well as these environmental factors, there is also a difference in individual genetic susceptibility to the risk of developing cancer [3], which could be linked to a higher or lower occurrence of DNA damage. According to the North American

Research Council, "susceptibility markers" are defined as biological differences between individuals or populations capable of affecting organism response to environmental agents. The most significant of these markers include genetic differences in the capacity to repair DNA damage and in biometabolism reaction dynamics to xenobiotic agents [9]. Currently the focus has been on trying to elucidate the molecular basis of the polymorphisms of enzymes involved in activating and detoxifying xenobiotic agents. There are basically two types of enzymes involved in chemical compound metabolism: Phase I codified by the cytochrome P450 (CYPs) gene super family, which through oxidation reactions can transform pro-carcinogens into carcinogenic substances capable of interacting with DNA [10,11], inducing different types of damage, and Phase II, such as glutathione s-transferases (GSTs) which act on the metabolism of certain toxic substances, making them more hydrophilic and therefore capable of being more easily excreted [12]. GSTs have a large array of substrates which can detoxify environmental carcinogenic agents found in foodstuffs, air, or medications [13].

GSTs in humans are polymorphic; an important gene being *GSTP1* which plays a fundamental role in protecting the organism against various types of cancer, because it is codified as an enzyme which acts in detoxifying polycyclic aromatic hydrocarbonates (PHA) originating from partially burnt organic material [13] and several chemical compounds found in processed foods. This enzyme has a single nucleotide polymorphism (SNP), which causes an isoleucine to valine substitution at position 313, making the enzyme less active favouring an accumulation of DNA damage [13]. This polymorphism is associated with an increased risk of developing bladder, testicular, prostate [14], pharyngeal, laryngeal, and lung cancer [15].

The objective of this study was to evaluate the influence of *GSTP1* gene *A313G* polymorphism on levels of DNA oxidative damage in peripheral blood lymphocytes in two groups of individuals with different dietary regimens.

2. Materials & Methods

2.1. Subject selection

This study was approved by the Ethical Committee for Human Research of the Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil. Informed consent was obtained from each volunteer.

A total of 105 healthy adult volunteers (average age 35,6 ± 11,4, ranging from 19 and 66 years), 52 men and 53 women were studied. All of the volunteer were nonsmokers, were not abusing alcohol, and were not using prescription or recreational drugs and any vitamins and minerals supplementation. These volunteers were distributed in two groups: 1) Group I - 49 naturalistic individuals with a life style characterized by absence of stress, bucolic life and great consume of organic fruits, vegetables and juice and poor consume of industrialized foods; 2) Group II - 56 individuals with great ingestion of industrialized foods and poor ingestion of fruits and vegetables.

2.2. Obtaining and evaluating Dietary Data

For evaluation of the dietary pattern, calculating the estimated level of micronutrients ingested (data not shown) and for the classification the groups a Food-frequency Questionnaire adapted from Cardoso and Stocco [16] was applied. This quantitative questionnaire has provided data, which were analysed using the software AvaNutri (Avanutri Informática Ltda, RJ, Brazil). This procedure enabled us to establish an estimate of the quantity of protective foods consumed daily by the population studied. To be classified in Group I, the individuals had to consume the percentile 75 of organic fruits and vegetables and below the percentile 25 of each

industrialized products. The individuals of group II were those who consumed less than percentile 25 of organic fruits and vegetables and above the percentile 75 of industrialized products.

Only were selected individuals that have adopted the respective diet pattern for at least 10 years.

2.3. Samples

Samples of peripheral blood (10ml) were collected: 2ml for detecting oxidative damage, DNA repair capability, and uracil levels incorporated in DNA; 4ml for DNA extraction to determine gene polymorphisms; and 4ml for a parallel study in which we analyzed plasma levels of different micronutrients.

2.4. Determining Oxidative Damage, Uracil Incorporation, and DNA Repair Capability

Data on oxidative DNA damage, uracil misincorporation into DNA, and DNA repair were described and accomplished by Prado et al in a parallel study (in preparation). Briefly, The alkaline Comet assay [17], modified with lesion-specific enzymes was used to detect single and double strand breaks, labile sites (SBs), oxidised purines and pyrimidines and uracil [18,19]. All procedures were conducted in the dark to minimize spurious sources of DNA damage. Briefly, 10 µl of the isolated lymphocytes suspension [20] (≅ 2 x 10^4 cells) were embedded into 0.5% low melting point agarose (Sigma) and spread on agarose-precoated microscope slides. Twelve slides were prepared for each sample. Slides were immersed overnight in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium salt N-Lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% DMSO added fresh)

at 4^0C. After lysing, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD) and then three slides for each treatment were incubated at 37^oC for 45 min with 100 μL of endonuclaese III (Endo III - 1:1000; New England Biolabs Inc), 100 μL of formamidopyrimidine-DNA glycosylase (FPG - 1:1000; New England Biolabs Inc), 100 μL of uracil-DNA glycosylase (UDG - 1:1000; New England Biolabs Inc) or with enzyme buffer only. Endo III recognizes oxidised pyrimidines (SBs EndoIII), while FPG identifies oxidised purines (SBs FPG), especially 8-oxo-guanine and UDG recognizes uracil (SBs UDG). Enzyme buffer only is used to identified SBs. Subsequently the cells were exposed to alkali buffer (1mM EDTA and 300 mM NaOH, pH ≅ 13.4), at 4^oC, for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted in the same solution at 4^oC, for 30 min, at 25 V (1v/cm) and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 50 μL Syber Green (1:10000; Invitrogen), and analyzed in a fluorescence microscope at 400 x magnification, using an image analysis system (Comet Assay II – Perceptive Instruments, Suffolk, UK).

For each individual, one hundred randomly selected cells (50 from each of two replicate slides) for each treatment (FPG, Endo III, UDG or enzyme buffer only) were evaluated and the mean tail intensity (%DNA Tail) was determined, totaling 400 cells analyzed from each individual. Tail Intensity, according to Comet Assay II – Perceptive instruments, is defined as "the sum of all intensity values in the tail region less those which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity".

To analyze the influence of dietary patterns and *GSTP1 A313G* polymorphism on the DNA repair capability, 200 μl of the freshly isolated lymphocytes were treated with 100 μl of H_2O_2 (100 μM), for 30 minutes, in ice, and analysis of the level of

damage was evaluated. Then, after two wash in PBS to remove of H_2O_2, these aliquots were incubated at 37 °C for 30 minutes, and DNA damage was checked again.

2.5. DNA Extraction

DNA was extracted using the Gene Catcher gDNA 3-10 ml Blood Kit (Invitrogen, Carlsbad, CA, USA), as per manufacturers recommendations.

2.6. Evaluation of Gene *GSTP1* Polymorphisms

GSTP1 genotyping was performed by PCR-RFLP [21] using *GSTP1-f*:GGCTCTATGGGAAGGACCAGCAGG and *GSTP1-r*:GCA CCTCCATCCAGAAACTGGCG primers.

The PCR reaction was performed using 25mM $MgCl_2$, 100mM dNTPs, 10µM of each primer, 1.5U/µl Taq DNA polymerase (Invitrogen), and 100ng/µL DNA, in a volume of 27µl. Cycling conditions for each amplification were: 94ºC for 30 seconds, followed by 94ºC for 1 minute (step 2), 59ºC for 1 minute, 72ºC for 2 minutes, and the process returned to step 2 and was repeated 39 times; this was followed by a final extension cycle of 72ºC for 10 minutes. Products with 445pb were obtained. A 15µl aliquot of PCR product was submitted to 1U BsmA I enzyme (Bio Labs-New England) and incubated at 55ºC for 12 hours. The resulting products were visualized in 2.5% agarose gel and stained with ethidium bromide. The wild IIe allele for codon 105 was identified by the presence of a Bsma I cleavage site. All experiments included positive and negative controls for each specific polymorphism.

2.7. Statistical Analysis

The data obtained in the Comet Assay were asymmetric distribution, thus, Log transformations were applied to DNA damage distributions to help meet normality distribution assumptions. To evaluate the influence of diet and *GSTP1 A313G* polymorphism on DNA damage the Tukey test was applied. The index of significance adopted was 5%.

2. Results

DNA damage, including single and double strand breaks, labile sites, oxidised purines and pyrimidines and misincorporated uracil on DNA, was detected by the alkaline Comet assay modified with lesion-specific enzymes, FPG, Endo III and UDG, respectively. The alkaline Comet assay measures SBs and AP sites. The enzyme-modified assay measures oxidative DNA damage as a combination of SBs, AP sites and oxidised bases – formamidopyrimidines and the oxidised purine 8-oxo-guanine when considering FPG (SBs FPG), or oxidised pyrimidine when considering endonuclease III (SBs Endo III). The enzyme-modified assay also measures misincorporated uracil on DNA when considering use of uracil-DNA glycosylase (SBs UDG).

Figure 1 summarizes the influence of *GSTP1 A313G* polymorphism on levels of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in each group. The individuals of group II carrying *GSTP1 G313G* genotype presented higher levels of purines oxidized when compared to individuals of group II carrying *GSTP1 A313A* or individuals of group II carrying. None of the *GSTP1 A313G* genotypes influenced the baseline levels of SBs, pyrimidine damaged, and misincorporation uracil on DNA, the levels of DNA damage induce with treatment of H_2O_2 (100 μM) and the DNA repair capability, in both groups.

4. Discussion

Sporadic cancers result from gene-environment interactions where the environment includes endogenous and exogenous exposures [22,23] which include dietary exposures. Food mutagens could cause different types of DNA damage leading to nucleotide alterations and gross chromosomal aberrations. However, the effects of food mutagens in carcinogenesis can be modified by heritable traits, low-penetrant genes that affect mutagen exposure of DNA through metabolic activation and detoxification or cellular responses to DNA damage through DNA repair mechanisms or cell death [25]. In the last years several studies have explored the influence of SNPs on DNA metbolism xenobiotic genes and the interaction of these genotypes on genotoxic exposure biomarkers [26]. The determination of SNP is important aspect that may increase sensitivity and specificity of assays and identify effects and susceptible individuals and subgroups [25,26].

Parallel studies performed in our laboratories have shown that individuals with a diet rich in natural products, such as vegetables and grain have lower levels purine and pyrimidine oxidized (Prado et al., in preparation). In our study, we observed that individuals of group II carrying *GSTP1 A313A* with a diet deficient in fruits and vegetables (group II) presented higher levels of purine oxidized. Several studies have been reported that diets rich in greens, fruit, and vegetables, adopted by Group I, can protect DNA from damage and exert a protector effect against cancer development [27]. The individuals of group II presented a diet poor in micronutrients and rich in chemical agents that are metabolized by phase I and II enzymes, and the balance between these phases could determine the level of reactive oxygen species (ROS) that could react with DNA induce oxidative DNA damage, especially in purine, that actually is a better

biomarker of oxidative stress exposition, as observed in this study. Since that the *GSTP1 A313G* polymorphism could the lead to inhibition of enzyme activity, we hypothesed that this deficiency enzymatic could allow a great interaction between ROS and DNA increasing the oxidative DNA damage level found in individual of group II. However, in our study, none of the *GSTP1 A313G* genotypes influenced the baseline levels of SBs, pyrimidine damaged, and misincorporation uracil on DNA, the levels of DNA damage induce with treatment of H_2O_2 (100 μM) and the DNA repair capability, in both groups. We believe that specific metabolism enzymes could be relationship with expression of another enzymes of the xenobiotic metabolism, as for example *GSTP1* induction is higher in individuals with *GSTM1**+ genotype than in those with *GSTM1*-genotype [28-29]. These possible interaction can help explain our results.

In conclusion, the *GSTP1 A313G* polymorphisms could modulate the oxidative DNA damage level in healthy subjects that have a diet deficient in fruits and vegetables. We believe that further investigations are needed to clarify the mechanisms of *GSTP1 A313G* polymorphisms in combination with other genes and micronutrients status.

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Legends

Figure 1. Influence of *GSTP1* gene polymorphisms on DNA damage (Tail Intensity) on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H_2O_2 and SBs H_2O_2R in peripheral blood lymphocytes from Group I (n=49) and Group II (n=56) individuals. SBs = single and double strand breaks and alkali labile sites; SBs FPG = SBs more FPG sensitive sites (purine oxidized); SBs Endo III = SBS more Endo III sensitive sites (pyrimidine oxidized), SBs UDG = SBs more misincorporated uracil sites, SBs H_2O_2 = level of DNA damage after treatment with 100 µl of H_2O_2 (100 µM), for 30 minutes, in ice and SBs H_2O_2R = level of DNA damage after incubation at 37 °C for 30 minutes. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. Tukey test was used to compare differences among genotypes in each group. The individuals of group II carrying *GSTP1 G313G* genotype presented higher levels of purines oxidized when compared to individuals of group II carrying *GSTP1 A313A* or individuals of group II carrying *GSTP1 A313G* genotype.

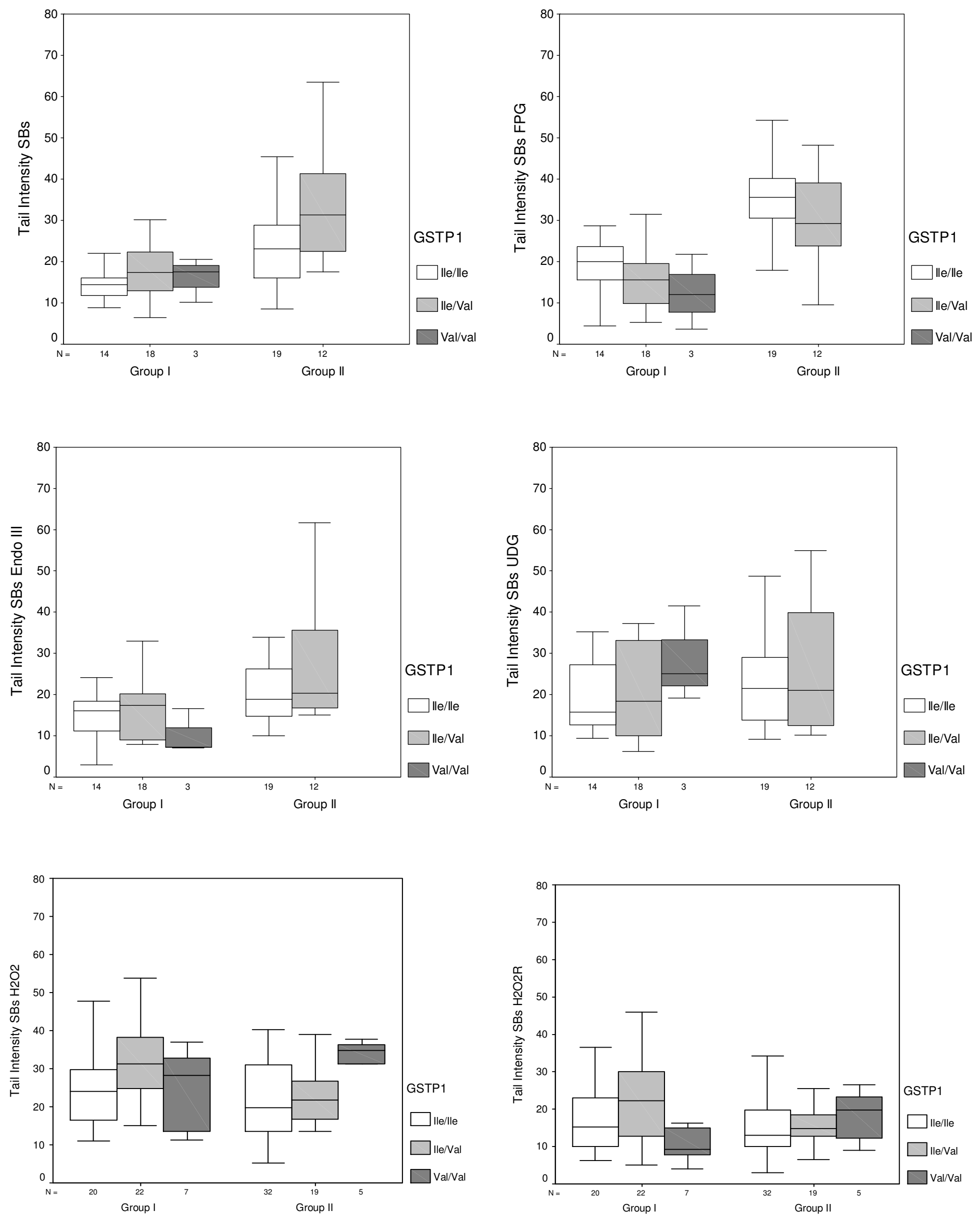

Figure 1.

Conclusões Gerais

Com relação aos indivíduos do grupo I, pode-se concluir que:

- Portadores do genótipo *MTHFR C1298C* apresentaram maiores níveis de danos no DNA;
- Portadores do genótipo TS *TSER*3R/3R apresentaram maiores níveis de purinas oxidadas;

Com relação aos indivíduos do grupo II, conclui-se que:

- Portadores do genótipo *GSTT1 (-/-)* apresentaram maiores níveis de purinas e pirimidinas oxidadas, uracilas incorporadas ao DNA e menor eficiência do sistema reparo de DNA;
- Portadores do genótipo *CYP2E1 c1/c2* apresentaram maiores níveis de purinas e pirimidinas oxidadas, uracilas incorporadas ao DNA e menor eficiência do sistema reparo de DNA;
- Portadores do genótipo *XRCC1 Arg399Arg* apresentaram maiores níveis de pirimidinas oxidadas e uracilas incorporadas ao DNA e menor eficiência do sistema de reparo do DNA;
- Portadores do genótipo *MTHFR C1298C* apresentaram maiores níveis de quebras de fita simples e duplas de DNA;
- Portadores do genótipo *TS TSER3R/R* apresentaram maiores níveis de purinas oxidadas e menor eficiência do sistema de reparo de DNA.

Com relação aos níveis de micronutrientes do soro, os indivíduos do grupo I apresentaram maiores níveis de todos os carotenóides e α-tocoferol do que os indivíduos do grupo II.

Em conclusão, os polimorfismos de genes de metabolismo de xenobióticos, reparo de DNA e metabolismo de ácido fólico modulam os níveis de danos no DNA e a eficiência do sistema de reparo de DNA, principalmente em indivíduos com menor ingestão de micronutrientes.

Anexos

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

EXPLICAÇÃO PARA O PACIENTE

A sociedade atual sofre uma alta taxa de doenças degenerativas crônicas, entre elas as doenças do coração, hipertensão, diabetes, doença de Alhzeimer, doença de Parkinson e principalmente o câncer. Essa situação é reflexo da mudança dos hábitos alimentares, que passaram a ser mais ricos em produtos industrializados e contendo inúmeros aditivos químicos e pobres em grãos integrais, frutas e vegetais. Grande parte desses aditivos químicos alimentares podem causar quebras no DNA e o acúmulo dessas quebras leva a vários tipos de doenças, inclusive o câncer. Os alimentos integrais, as frutas e os vegetais contêm grande quantidade de fibras, vitaminas e nutrientes que são essenciais para a manutenção de uma boa saúde.

Em virtude disto o(a) Sr.(a) está sendo convidado a participar de um estudo que vai investigar se a sua alimentação pode danificar ou pode proteger o seu DNA. Caso o(a) Sr.(a) aceite participar do estudo, serão coletados 10 ml do seu sangue, através do braço, com seringa e agulhas descartáveis e estéreis, para verificarmos as quebras do DNA do seu sangue. Além disso, será preenchido um questionário, no qual o(a) senhor(a) nos dará várias informações sobre os seus hábitos alimentares.

Como o(a) senhor(a) deve acompanhar pela televisão, a genética e a medicina têm evoluído muito e por isso eu gostaria de pedir seu consentimento para usar o seu material genético em estudos futuros, que contribuirão, mais ainda, para o entendimento das doenças degenerativas crônicas e do câncer.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

DEPARTAMENTO DE PATOLOGIA - FMB – BOTUCATU – SP

I. Identificação do paciente ou responsável legal

Nome:				
RG:		Sexo: ☐ M ☐ F	Código (não preencher) :	
Endereço:				
Cidade:				Bairro:
CEP:	Estado:	Telefone:		

II. DADOS SOBRE A PESQUISA CIENTÍFICA

1. Título do Protocolo de Pesquisa: "INFLUÊNCIA DO PADRÃO ALIMENTAR SOBRE OS NÍVEIS DE DANOS OXIDATIVOS NO DNA E DE MICRONÚCLEOS EM LINFÓCITOS"
2. Pesquisadores-Responsáveis: Dr. Marcelo Sady Plácido Ladeira e Dra. Daisy Maria Fávero Salvadori, Departamento de Patologia – Faculdade de Medicina de Botucatu, UNESP, Botucatu - SP.
3. Avaliação do Risco da Pesquisa: Sem risco

4 .Duração Prevista da Participação do Paciente : vinte minutos.

III. REGISTRO DAS EXPLICAÇÕES DO PESQUISADOR AO PACIENTE OU SEU REPRESENTANTE LEGAL SOBRE A PESQUISA, CONSIGNANDO:

1. Justificativa e Objetivos da pesquisa: Contribuir para o esclarecimento do papel dos padrões alimentares na manutenção da integridade do DNA

2. Procedimentos utilizados: Serão coletados: 10 ml de sangue periférico. Além disso, será preenchido um extenso questionário.

3. Desconfortos e riscos: Somente a picada para coleta de sangue. Não há riscos

4. Benefícios que poderão ser obtidos: Contribuição para o esclarecimento dos efeitos da alimentação sobre doenças degenerativas crônicas, em especial o câncer

5. Procedimentos vantajosos para o indivíduo: Avaliação do seu nível de danos no DNA.

IV - ESCLARECIMENTOS DADOS PELO PESQUISADOR SOBRE GARANTIAS DO SUJEITO DA PESQUISA:

1. Fui esclarecido sobre a garantia de ter acesso, a qualquer tempo, às informações sobre procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa, inclusive para dirimir eventuais dúvidas?
[ ] Sim [ ] Não
2. Fui esclarecido de que a segurança de minha identidade será preservada, mantendo-se todas informações em caráter confidencial?
[ ] Sim [ ] Não
3. Fui esclarecido sobre a disponibilidade de assistência no HCFMUNESP, por eventuais danos á saúde, decorrentes da pesquisa?
[ ] Sim [ ] Não
4. Fui esclarecido sobre a viabilidade de indenização por eventuais danos à saúde decorrentes da pesquisa?
[ ] Sim [ ] Não
5. Fui esclarecido que não receberei qualquer remuneração financeira por participar desta pesquisa. [ ] Sim [ ] Não

6. Fui informado que os médicos e pesquisadores que participam deste projeto de pesquisa estarão à minha disposição para esclarecimento de qualquer questão relacionado à pesquisa.
[ ] Sim [ ] Não

V. INFORMAÇÕES DE NOMES, ENDEREÇOS E TELEFONES DOS RESPONSÁVEIS PELO ACOMPANHAMENTO DA PESQUISA, PARA CONTATO EM CASO DE INTERCORRÊNCIAS CLÍNICAS E REAÇÕES ADVERSAS.

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Telefone: (14) 38116376

VI. CONSENTIMENTO PÓS-INFORMADO

Eu, ____ abaixo assinado, declaro que fui esclarecido sobre o objetivo do presente estudo, sobre eventuais desconfortos que poderei sofrer, assim como sobre os benefícios que podem resultar do estudo. Concordo, portanto, em participar, na qualidade de voluntário, do referido Projeto de Pesquisa, sob livre e espontânea vontade e permito a utilização do meu material genético em estudos futuros.

_____________________, _____ de ________________ de _______

____________________ ________________________

Paciente Pesquisador

Dados pessoais

Nome:

Idade:

Sexo:

Peso:

Altura:

Pratica atividade física:

Observações e história clínica:

- Inquérito proposto com os seguintes objetivos: (i) conhecer perfil dos hábitos alimentares; (ii) conhecer a freqüência e estimar a quantidade consumida de alimentos fonte de vitaminas interferentes no processo oxidativo/ prevenção do dano oxidativo; (iii) conhecer a freqüência e estimar a quantidade de alimentos fonte de carotenóides, retinol e vitamina E.

Registro Alimentar (1° dia)

Nome:	**Código:**

Refeição	Hora	Quantidade

Registro Alimentar (2° dia)

Nome:	**Código:**

Refeição	Hora	Quantidade

Registro Alimentar (3° dia)

Nome:	**Código:**

Refeição	Hora	Quantidade

Questionário de Frequência Alimentar

Data da entrevista ____/____/_____ Hora de Início: __________
Nome do entrevistador: ____
Nome do voluntário: ____ Sexo ( )F ( )M
Idade atual: _________ Data de nascimento: ____/____/______

1. Você mudou seus hábitos alimentares recentemente ou está fazendo dieta para emagrecer ou por qualquer outro motivo?
   (1) Não
   (2) Sim, para perda de peso
   (3) Sim, por orientação médica
   (4) Sim, para dieta vegetariana ou reduçao do consumo de carne
   (5) Sim, para redução de sal
   (6) Sim, para redução de colesterol
   (7) Sim, para ganho de peso
   Outro motivo: ______________________________

2. Você está tomando algo para suplementar sua dieta (vitaminas, minerais e outros produtos)?
   (1) Não (2) Sim, regularmente (3) Sim, mas não regularmente

3. Se a resposta da pergunta anterior for sim, por favor preencher o quadro abaixo:

Suplemento	Marca Comercial	Dose	Freqüência

4. As questões seguintes relacionam-se ao seu hábito alimentar usual no período de um ano. Para cada quadro abaixo responda, por favor, a freqüência que melhor descreva quantas vezes você costuma comer cada item e a respectiva unidade de tempo (se por dia, por semana, por mês ou no ano). Depois responda qual a sua porção individual usual em relação à porção média indicada. Escolha somente um círculo para cada coluna. Se você não come ou raramente come um determinado item, preencha o círcula da primeira coluna (N= nunca come). Não deixe itens em branco.

Grupo de alimentos	Com que freqüência você costuma comer?		Qual o tamanho de sua porção em relação à porção média?	
	Quantas vezes você come:	Unidade	Porção média (M)	Sua porção
Alimentos e preparações	Número de vezes: 1,2,3, etc. (N= nunca ou raramente come)	D= por dia S= por semana M= por mês A= por ano	Porção média de referência	P= menor que a porção média M= igual à porção média G= maior que a porção média E= bem maior que a porção média
Sopas e massas	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Sopas (de legumes, canja, creme, etc)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 concha média (150g)	P M G E O O O O
Salgados fritos (pastel, coxinha, risólis, bolinho)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade grande (80g)	P M G E O O O O
Salgados assados (esfiha, bauruzinho, torta)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 unidades ou 2 pedaços médios (140g)	P M G E O O O O
Macarrão com molho sem carne	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 prato raso (200g)	P M G E O O O O
Macarrão com molho com carne, lasanha, nhoque	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 escumadeira ou 1 pedaço pequeno (110g)	P M G E O O O O
Pizza, panqueca	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 fatias pequenas ou 2 unidades (180g)	P M G E O O O O
Polenta cozida ou frita	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa ou 2 fatias pequenas (70g)	P M G E O O O O
Cereais integrais	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Aveia, quinoa, linhaça, trigo	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 colheres de sopa (40g)	P M G E O O O O
arroz	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O	D S M A	2 escumadeiras médias (120g)	P M G E

	O	O O O O		O O O O
Pães e biscoitos	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Pão francês, pão de forma, pão doce, torrada	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	1 unidade ou 2 fatias (50g)	P ME G O O O O
Pão de forma integral	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	2 fatias (50g)	P ME G O O O O
Biscoito sem recheio	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	4 unidades (24g)	P ME G O O O O
Biscoito recheado, waffer, amanteigado	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	3 unidades (41g)	P ME G O O O O

Pães e biscoitos	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Bolo simples	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	1 fatia média (60g)	P ME G O O O O
Bolo recheado	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	1 fatia média (60g)	P ME G O O O O
Hortaliças/verduras e frutas verdes	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Alface	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	3 folhas médias (30g)	P ME G O O O O
Almeirão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	3 folhas médias (30g)	P ME G O O O O
Agrião	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	1 prato de sobremesa (38g)	P ME G O O O O
Brócolis	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	1 ramo ou 2 colheres de sopa (30g)	P ME G O O O O
Chicória crua	N 1 2 3 4 5 6 7 8 9 10	D S MA	1 prato de sobremesa (38g)	P ME G

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Chicória cozida	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (30g)	P M G E O O O O
Couve manteiga crua	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 prato de sobremesa (38g)	P M G E O O O O
Couve manteiga cozida	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (30g)	P M G E O O O O
Chuchu	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de sopa cheia (30g)	P M G E O O O O
Mostarda	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 folhas médias (30g)	P M G E O O O O
Rúcula	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 prato de sobremesa (38g)	P M G E O O O O
Espinafre	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (30g)	P M G E O O O O
Pepino	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de sopa cheia (30g)	P M G E O O O O
Pimentão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de sopa cheia (30g)	P M G E O O O O
Abobrinha	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de sopa cheia (30g)	P M G E O O O O
Quiabo	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de sopa cheia (30g)	P M G E O O O O
Abacate	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa cheia (90g)	P M G E O O O O

Azeitona	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	6 unidades (25g)	P ME G O O O O
Figo	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 unidade média (50g)	P ME G O O O O
Kiwi	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 unidade média (40g)	P ME G O O O O

Verduras/ Hortaliças e frutas verdes	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Uva	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	10 gomos (80g)	P ME G O O O O
Jiló	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	2 colheres de sopa (32g)	P ME G O O O O
Verduras/Hortaliças amarelo-alaranjados	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Abóbora	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 colher de sopa cheia (30g)	P ME G O O O O
Cenoura	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 colher de sopa (30g)	P ME G O O O O
Mamão formosa	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 fatia média (160g)	P ME G O O O O
Mamão papaya	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	½ unidade média (160g)	P ME G O O O O
Manga	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 unidade média (120g)	P ME G O O O O
Laranja	N 1 2 3 4 5 6 7 8 9	D S M	1 unidade média	P M G

	10 O O O O O O O O O O O	A O O O O	(180g)	E O O O O
Mexerica	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (180g)	P M G E O O O O
Tangerina	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (180g)	P M G E O O O O
Abacaxi	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 fatia grande (180g)	P M G E O O O O
Pêssego	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (150g)	P M G E O O O O
Ameixa	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (70g)	P M G E O O O O
Cajamanga	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (150g)	P M G E O O O O

Verduras/ Hortaliças e frutas vermelhas	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Tomate	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 fatias médias (40g)	P M G E O O O O
Molho de tomate caseiro/lata/caixa	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1,5 colher de sopa (25g)	P M G E O O O O
Extrato de tomate	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O	D S M A O O O	1,5 colher de sopa (25g)	P M G E O O O

	O O	O		O
Vinagrete	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	3 colheres de sopa (45g)	P ME G O O O O
Rabanete	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 colher de sopa cheia (30g)	P ME G O O O O
Acerola	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	10 unidades (50g)	P ME G O O O O
Caqui	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 unidade média (120g)	P ME G O O O O
Goiaba	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 unidade grande (225g)	P ME G O O O O
Pitanga	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	10 unidades (50g)	P ME G O O O O
Melancia	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 fatia média (150g)	P ME G O O O O
Morango	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	7 unidades médias (84g)	P ME G O O O O
Hortaliças/verduras vermelho-arroxeadas	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Beterraba	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 colher de sopa cheia (30g)	P ME G O O O O
Repolho	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	2 colheres de sopa (30g)	P ME G O O O O
Berinjela	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 colher de sopa cheia (30g)	P ME G O O O O
Uva	N 1 2 3 4 5 6 7 8 9 10	D SA M	10 gomos (70g)	P ME G

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Verduras/ Hortaliças e frutas branco-amareladas	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Couve-flor	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 ramo (30g)	P ME G O O O O
Acelga crua	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 prato de sobremesa (38g)	P ME G O O O O
Acelga cozida	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (30g)	P ME G O O O O
Repolho	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (30g)	P ME G O O O O
Melão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 fatia média (150g)	P ME G O O O O
Maçã	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (110g)	P ME G O O O O
Banana nanica	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (100g)	P ME G O O O O
Banana prata	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (86g)	P ME G O O O O
Banana maçã	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (75g)	P ME G O O O O
Goiaba	N 1 2 3 4 5 6 7 8 9	D S M	1 unidade grande	P M G

	10 O O O O O O O O O O O	A O O O O	(225g)	E O O O O
Pêra	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (110g)	P M G E O O O O
Jabuticaba	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	10 unidades (70g)	P M G E O O O O
Tubérculos	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Mandioca cozida	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 escumadeira cheia (90g)	P M G E O O O O
Mandioca frita	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de servir cheias (100g)	P M G E O O O O
Batata frita	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de servir cheias (100g)	P M G E O O O O
Batata cozida	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 escumadeira cheia (90g)	P M G E O O O O
Batata doce frita	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de servir cheias (100g)	P M G E O O O O
Batata doce cozida	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 escumadeira cheia (90g)	P M G E O O O O

Tubérculos	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Farinha de mandioca, farofa, cuscuz, tapioca	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 colheres de sopa (40g)	P M G E O O O O
Bebidas	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Suco de tomate	N 1 2 3 4 5 6 7 8 9 10	D S M A	½ copo americano (80ml)	P M G E

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Suco natural de laranja	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de limão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de goiaba	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de melancia	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de melão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de acerola	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de maracujá	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de abacaxi	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de uva	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de Pêssego	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de Cajú	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O
Suco natural de morango	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S MA O O O O	½ copo americano (80ml)	P ME G O O O O

Cerveja	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	2 latas (700ml)	P ME G O O O O
Vinho	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 taça pequena (50ml)	P ME G O O O O
Café ( ) c/ açúcar ( ) s/ açúcar	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	2 xícaras de café (90ml)	P ME G O O O O
Chá ( ) c/ açúcar ( ) s/ açúcar	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	2 xícaras de café (90ml)	P ME G O O O O
Refrigerantes	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 copo de requeijão (240ml)	P ME G O O O O
Suco industrializado	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 copo de requeijão (240ml)	P ME G O O O O
Leite e derivados	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Leite ( ) integral ( ) desnatado ( ) Semi-desn.	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	½ copo de requeijão (125ml)	P ME G O O O O

Leite e derivados	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Leite fermentado	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 pote pequeno (90ml)	P ME G O O O O
Iogurte ( ) integral ( ) desnat. ( ) semi-d.	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 unidade pequena (140g)	P ME G O O O O
Queijo amarelos	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1,5 fatias grossas (30g)	P ME G O O O O
Queijos brancos (ricota, minas)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D SA M O O O O	1 fatia média (30g)	P ME G O O O O
Requeijão	N 1 2 3 4 5 6 7 8 9 10	D SA M	3 pontas de faca (15g)	P ME G

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Manteiga( ) comum ( )light	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 pontas de faca (15g)	P M G E O O O O
Margarina( )comum ( )light	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 pontas de faca (15g)	P M G E O O O O
Leguminosas e ovos	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Amendoim	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 punhados (60g)	P M G E O O O O
Feijão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 concha média (86g)	P M G E O O O O
Lentilha	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (35g)	P M G E O O O O
Grão-de-bico	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (35g)	P M G E O O O O
Guandu	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (35g)	P M G E O O O O
Vagem	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de sopa cheia (30g)	P M G E O O O O
Soja	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (35g)	P M G E O O O O
Ervilha	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de servir (35g)	P M G E O O O O
Ovo (cozido, frito)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade (50g)	P M G E O O O O
Carnes	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Carne bovina	N 1 2 3 4 5 6 7 8 9	D S M	1 bife média ou 2	P M G

	10 O O O O O O O O O O O	A O O O O	pedaços (100g)	E O O O O
Hambúrguer, nuggets, almôdega	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade média (60g)	P M G E O O O O
Frango( ) com pele ( ) sem pele	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 pedaço ou 1 filé pequeno (60g)	P M G E O O O O
Carne suína	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 fatia média (100g)	P M G E O O O O
Vísceras	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Fígado, coração, língua, estômago, etc.	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 bife médio ou 2 pedaços (100g)	P M G E O O O O

Pescados, frutos do mar	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Bacalhau, cação, salmão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 filé pequeno ou 1 posta pequena (100g)	P M G E O O O O
Lambari, merluza, pintado	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 filé pequeno ou 1 posta pequena (100g)	P M G E O O O O
Sardinha, atum	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 filé pequeno ou 1 posta pequena (100g)	P M G E O O O O
Embutidos, processados, defumados	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Presunto	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 fatias médias (30g)	P M G E O O O O
Salame	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	4 fatias (30g)	P M G E O O O O
Mortadela	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O	D S M A O O O	2 fatias médias (30g)	P M G E O O O

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Lingüiça	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 gomo médio (60g)	P M G E O O O O
Peito de perú	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 fatias médias (30g)	P M G E O O O O
Salsicha	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 fatias médias (30g)	P M G E O O O O
Bacon	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 pedaços pequenos (40g)	P M G E O O O O
Carne seca, carne de sol	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 pedaços pequenos (40g)	P M G E O O O O
Oleaginosas	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Amêndoa	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (30g)	P M G E O O O O
Avelã	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (30g)	P M G E O O O O
Castanha	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (30g)	P M G E O O O O
Castanha-do-pará	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (30g)	P M G E O O O O
Noz	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (30g)	P M G E O O O O
Óleos	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Soja, girassol, canola, milho	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 fio (4ml)	P M G E O O O O
Azeite	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O	D S M A O O O	1 fio (4ml)	P M G E O O O

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Temperos naturais	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Salsa, cebolinha, tomilho, louro, orégano, alecrim, coentro, manjericão	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de sopa (3,8g)	P M G E O O O O
Doces e sobremesas	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Açúcar, mel, geléia	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	½ coher de sopa (6g)	P M G E O O O O
Chocolate, bombom, brigadeiro	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 barra pequena (25g)	P M G E O O O O
Achocolatado em pó (adicionado ao leite)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (25g)	P M G E O O O O
Sobremesas, doces, tortas e pudins	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 pedaço ou 1 fatia média (60g)	P M G E O O O O
Sorvetes cremosos	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 bola grande (70g)	P M G E O O O O
Doce de frutas (calda/barra)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher grande ou 2 unidades (70g)	P M G E O O O O
Outros	Quantas vezes você come	Unidade	Porção média (M)	Sua porção
Glutamato monossódico (ex: sazon, ajinomoto)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 pitada (0,35g)	P M G E O O O O
Adoçante	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 gotas	P M G E O O O O
Balas ou chicletes	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade	P M G E O O O O
Enlatados	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 colheres de sopa (60g)	P M G E

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Salgadinhos industrializados	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 pacote (30g)	P M G E O O O O
Maionese	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de chá (4g)	P M G E O O O O
Mostarda	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 colher de chá (4g)	P M G E O O O O
Shoyu	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 fio (5ml)	P M G E O O O O